Doktorego-tesia
Doctoral Thesis

Lide Totorikaguena Iturriaga

Sistema kannabinoidearen funtzioa ugaztunen obarioen fisiologian eta obozitoen heltze-prozesuan

The role of the cannabinoid system in ovarian physiology and oocyte maturation in mammals

Leioa, 2019

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

Doktorego-tesia
Doctoral Thesis

Sistema kannabinoidearen funtzioa ugaztunen obarioen fisiologian eta obozitoen heltze-prozesuan

The role of the cannabinoid system in ovarian physiology and oocyte maturation in mammals

Lide Totorikaguena Iturriaga

Leioa, 2019

Zuzendariak/Supervisors:
Ekaitz Agirregoitia, Naiara Agirregoitia

eman ta zabal zazu
Universidad del País Vasco
Euskal Herriko Unibertsitatea

MEDIKUNTZA ETA ERIZAINTZA FAKULTATEA
FACULTAD DE MEDICINA Y ENFERMERÍA

Euskal Herriko Unibertsitateak (EHU/UPV) diruz lagunduriko ikerketa. "Ikertzaileak prestatzeko doktoretza-aurreko beka" (PIF15/149) eta hiru hilabetetako egonaldia egiteko Bruselako Vritje Universiteit Brussel Unibertsitatean, Follicle Biology Unit (FOBI) ikerketa-taldeko Johan Smitz irakaslearen babespean.

Research funded by the University of the Basque Country (UPV/EHU). "Predoctoral fellowship for researchers" (PIF15/149) and a three-month stay at the Vritje Universiteit Brussel University in Brussels, under the supervision of Prof. Johan Smitz from the Research Unit Follicle Biology (FOBI).

Dabilen harriari goroldiorik ez.

Esker onak

Gogoan dut nola, masterra egitera nindoala, laborategian hanka bat jarri orduko nire tesi zuzendaria izango zenak bota zidan lehenengo galdera: *"gustuko al duzu kozinatzea? Zientzia egitea bizkotxo bat egitea bezala delako"*. Eta tesi hau bukatzear nengoela nik bizitakoa eta hausnartutakoa borobiltzen zuen esaldia irakurri nuen Interneteko iritzi-artikulu batean: *"zientzia kozinatzea bezalakoa da: bakoitzak bere ukitua ematen dio, etxean eginda, maitasunez eginda eta familia eta lagunekin partekatuta, gozoagoa da"*.

Bagara, hortaz, sinisten dugunok zientzia gozoagoa eta hobea egitea posiblea dela bizitzak eta pertsonak erdigunean jarriz. Eta zorionekoa ni, bide malkartsu eta zirraragarri honetan, zientzia egiteko bestelako moduetan sinisten duzuenokin topo egin dudalako. Zortea izan dudalako eskuzabaltasunaz, elkartasunaz, maitasunaz eta poztasunaz inguraturik egoteagatik. Baina baita ere, lanaz, konstantziaz, kemenaz, disziplinaz eta pazientziaz. Niretzat zientzialari erreferenteak direnak aurrez aurre izan ditudalako. Eskerrik asko Ekaitz, mundu honetan sartzeko aukera emateaz gain erakutsi eta eskaini didazun guztiagatik. Prozesu honen giltza izan zarelako, eztabaidarako eta hausnarketarako iturri amaigabea. Eskerrik asko Naiara eman didazun konfiantza eta babesagatik. Zientziak dituen arantzei aurre egiteko zure aholku zintzoengatik. Eskerrik asko gure taldeari, TALDE hitzak bere osotasunean esan nahi duena erakutsi didazue, kolektiboki lan eginez, txikitasunean handi baikara. Zer esanik ez, nire eredu zareten neska gazte zientzialari bikain, baina, batik bat, pertsona izugarriei. Mila esker, Maider, Iraia eta, batez ere, zuri, Itziar. Baina bereziki Estiri, izan ahalko nukeen bidaiderik bereziena izateagatik, porturik ikusten ez bagenuen ere, amorerik eman gabe arraunean jarraitu duzulako. Sasi guztien gainetik, biok elkarrekin. Plazer bat izan da zurekin ikasi eta partekatutako guztia.

Bruselako familiari, *bedankt, merci, grazas, gràcies, grazie, gracias*! Zientziaren prekarietateak erbesteratu zintuzten eta enpatiagatik, akaso, hartu ninduzuen hain gozo. Zorionekoa ni, beste behin, zuek bezalako emakumeak ezagutzeagatik.

Eskerrik asko, bide honetan zehar babestu eta animatu nauzuen lagunei.

Amaitzeko, eskerrik beroenak etxekoei. Emandako sostengu, maitasun eta indarragatik. Nik neure buruarengan sinesten ez nuenetan ere, eskua eman eta altxatzen laguntzeagatik. Amumei, Gernikako bonbardaketatik biziraun zuten baserritarrei. Zuek su motelean irakindakotik edan dugulako gara garena. Eta, Ibai, mila esker bihotzez, bizitzako abentura honetan gozoagoa baita binaka ibiltzea bertan murgilduta.

Aipatu ez baina bihotzean zaituztedanei. Murruen beste aldean egonda ere animoak ematen jarraitzen diguzuenoi.

"Pasatzen dugu bizitza urruneko adibideen bila miresgarriak diren pertsonen atzetik, eta azkenean konturatzen zara zure ondoan dagoena dela inoiz inon izan den jenderik onena. Jende xumea. Hala tokatutako lanetan energia guztia inbertitu ondoren, garagardo baten inguruan barre egiteko gai den jendea. Jende xumea. Defendatzen dituen gauzetan energia inbertitu ahala, bizitzari drama kentzen saiatzen dena" (O. Enbeita)

Gure hizkuntzan dugun esaera ederrenetarikoa da eta eskerrik asko.

Biba zuek!

Hitzaurrea

Esku artean duzun hau nazioarteko tesia dugu, doktore-tesia burutzeko ikerketak bi nazioetan egin baitira: Euskal Herriko Unibertsitatean (Leioako kanpuseko Medikuntza eta Erizaintza fakultatean) eta Flandriako Vritje Universiteit Brussel Unibertsitatean (Brusela hiriko Medikuntza eta Farmazia fakultatean). Beraz, tesi guztia bai euskaraz zein ingelesez idatzia izan da, bi atal izan ezik. Sarrerako atala euskara hutsean idatzi da eta emaitzen atala, aldiz, ingelesez. Izan ere, emaitzak 4 ataletan banatu dira eta bakoitzaren hasieran, sarrera txiki bat, egin diren esperimentuen materialak eta metodoak eta eztabaida laburki azalduta ageri dira.

Foreword

This is an international thesis that has been carried out over two countries: at the University of the Basque Country (Faculty of Medicine and Nursing, Leioa) and at the Vritje Universiteit Brussel University in Flanders (Faculty of Medicine and Pharmacy, Brussels). Therefore, the entire thesis has been written in both Basque and English languages, with the exception of two sections. The introduction, that has been written entirely in Basque and the results in English. In fact, the results have been divided into 4 sections and at the beginning of each one there is a brief introduction, materials and methods of the experiments carried out and the discussion.

Aurkibidea

Laburpena
Summary

Laburpena

Ugaztun emeen obozitoen heltze-prozesua ugalkortasunean garrantzia handia duen prozesua da, obulua ernaldua izateko beharrezkoa delako. Obozitoak obulutegiko folikuluetan sortzen dira eta lehenengo meiosiaren profasean daude geldituta, obulutegietan. Oraindik ez dira ondo ezagutzen zeintzuk diren meiosiaren berraktibazioa eragiten duten seinaleak baina hauei esker abiatuko da obulazioa non, gizakietan, hilero obozito bakarra obulutegitik irtengo den. Gero eta ebidentzia gehiago dago seinale horietako asko G proteinei loturiko hartzaileen (GPCR) menpe daudela eta horien aktibazioak edo inaktibazioak obozitoen heltze-prozesua modulatzen dituzten seinaleztapen ur jauziak pizten dituela. Gakoa da aurkitzea zeintzuk diren berpizkunde meiotikoa eragiten eta erregulatzen dituzten kanpo seinaleak, seinale horiek farmakoen bidez kontrolatuz heltze-prozesua ere kontrolatu genezakeelako. Horrela, ikusi da kannabinoideek eragiten duten seinaleztapena eta obozitoen heltze-prozesua hasteko gertatu behar dena oso antzekoa dela, beraz, prozesuan lagungarriak izan litezkeen estekatzaile interesgarriak ditugu.

Tesi honetan sistema kannabinoidearen funtzioa aztertu da ugaztun emeen folikulugenesian, obogenesian eta obozitoen heltze-prozesuan eta haren parte-hartzea baieztatzeaz gain, THC fitokannabinoideak obozitoen heltze-prozesua modulatzen duela ikusi da. Gainera, etorkizunerako kannabinoideen erabilera terapeutikoa aztertzen jarraitzea interesgarria litzateke, besteak beste, obozitoen *in vitro* heltze (IVM) medioetan osagai gisa erabilita. Nabarmendu behar da obozitoen *in vitro* heltzea askoz alternatiba onuragarriagoa, erosoagoa eta arrisku gutxiagokoa izango litzatekeela ugalkortasun klinika batera joan behar den edozein pazienterentzako, obarioen estimulaziorako beharrezkoak diren hormonak hartzea ekidingo litzatekeelako edo behintzat dosia murriztuko litzatekeelako. Are gehiago, hormonen estimulazioa kontraindikatua dagoen emakumeentzako haurdunaldia lortzeko aukera bakarra izan ahalko litzateke.

Ugalkortasuna, obozitoen heltzea, IVM, sistema kannabinoidea, THC fitokannabinoidea

Summary

Oocyte's maturation is a very important process in fertility. The oocyte meiotic maturation is a complex process whereby immature oocytes acquire the characteristics required for successful fertilization and embryogenesis. For that, immature oocytes arrested at the diplotene of prophase I (GV) must resume meiosis until the metaphase II of meiosis (MII). However, although all the mechanisms that leads for the reactivation of meiosis are not still known, there are known certain molecules that are able to modulate this process. In this sense, we were interested in the role that cannabinoids could have as IVM promoters because cannabinoid`s molecular pathway is similar to the one by which oocyte's meiosis resumption is activated.

That's why, the aim of this Doctoral Thesis was to study the function of the cannabinoid system in the process of folliculogenesis, ovogenesis and oocyte maturation.The results obtained in the following PhD thesis confirm its participation. Beyond that, we have seen that in fact, the phytocannabinoid (THC) modulates oocyte maturation. We have characterized the role of the phytocannabinoid THC in the *in vitro* maturation (IVM) process, due to its possible use in clinic is most feasible and reliable than any synthetic cannabinoid. The most important goal of IVM method is that could be the only hope or alternative for a not insignificant number of patients.

Fertility, oocyte maturation, IVM, cannabinoid system, THC phytocannabinoid

Laburduren zerrenda
List of abbreviations

Laburduren zerrenda

2n: Diploidea
2-AG: 2-arakidonoilglizerola
α- MEM: Alpha- medio esentzial minimoa
AC: Adenilato ziklasa
ACEA: Arakidonoil-2´-kloroetilamida
ACTB: Aktina zitoplasmikoa
AEA: N-arakidonoiletanolamina (anandamida)
AKT: B proteina kinasa
pAKT: B proteina kinasa fosforilatua
AMP: Adenosina 5´-monofosfatoa
ANOVA: Bariantzaren analisia
ART: Lagunduriko ugalketa teknikak
ATP: Adenosina 5´-trifosfatoa. Adenosine 5' Triphosphate
BMP15: Hezur-proteina morfogenetikoa 15
BSA: Behi-serum albumina.
Ca^{2+}: Kaltzioa
cAMP: AMP ziklikoa
CB1: 1 motako kannabinoide-hartzailea
CB2: 2 motako kannabinoide-hartzailea
CBD: Kannabidiola
CBN: Kannabinola
CC: Kumuluko zelulak
CDC25B: M faseko fosfatasa eragilea
CDK1: Ziklinaren menpeko kinasa
cDNA: DNA osagarria
cGMP: Guanosina monofosfato ziklikoa
Cnr1 $^{-/-}$: CB1 hartzailerik gabeko sagua
Cnr2 $^{-/-}$: CB2 hartzailerik gabeko sagua
Cnr1 $^{-/-}$/ Cnr2 $^{-/-}$: CB1 eta CB2 hartzailerik gabeko sagua
CNP: C-motako peptido natriuretikoa
COC: Kumulu-obozito konplexua
CREB: cAMPren erantzunerako elementuak kodetzen dituen DNA zatiari lotzen zaion transkripzio faktorea
CYCB: B ziklina
CYP11A1: Zitokromo P450, familia 11, subfamilia A, polipeptidoa 1
DMSO: Dimetil sulfoxidoa
DNA: Azido desoxirribonukleikoa
dNTP: Deoxirribonukleotido trifosfatoa
E2: Estrogenoa
eCG: Behorraren hormona gonadotropina korionikoa
ECL: Kimioluminiszentzia-areagotzailea
ECS: Sistema endokannabinoidea
EDTA: Azido diaminoetanotetraazetikoa
EGF: Epidermiseko hazkuntza-faktorea
EGFR: Epidermiseko hazkuntza-faktorearen hartzailea
ERK1/2: 1 eta 2 motako seinale extrazelularren kinasa erregulatzaileak
pERK1/2: Fosforilatutako 1 eta 2 motako seinale extrazelularren kinasa erregulatzaileak
FAAH: Gantz-azidoen amida hidrolasa
FBS: Behi fetuaren seruma
FF-MAS: Meiosia aktibatzen duen likido folikularreko esterola
FSH: Hormona folikulu-estimulatzailea
FSHR: Hormona folikulu-estimulatzailearen hartzailea
G_{α}: G proteina heterotrimerikoen alpha azpiunitatea
$G_{\beta\gamma}$: G proteina heterotrimerikoen beta eta gamma azpiunitateak
$G_{i\alpha}$: G proteina inhibitzailea
$G_{s\alpha}$: G proteina estimulatzailea
GAPDH: Glizeraldehido 3-fosfato Deshidrogenasa
GDF9: Hazkundearen desberdintzapen-faktorea 9
GDP: Guanosina 5'-difosfatoa
GnRH: Gonadotropinen jariapen-hormona

GPCR: G proteinei loturiko hartzailea
GTP: Guanosina 5'-trifosfatoa
GV: Besikula germinala
GVBD: Besikula germinalaren haustura
hCG: Giza gonadotropina korionikoa
hpf: Ernaldu osteko orduak
hRNA: RNA heterogeneoa
HTF: Giza tubuluko jariakina
HU-210: [(6aR,10aR)-3-(1,1-Dimetil-heptil)-6a,7,10,10a-tetrahidro-1-hidroxi-6,6 dimetil-6H-dibenzo[b,d]piran-9-metanola]
ICSI: Espermatozoide-injekzio intrazitoplasmatikoa
IP3: Inositol trifosfatoa
IP3R: Inositol trifosfatoaren hartzailea
IVF: *In vitro* ernalketa
IVM: Obozitoen *in vitro* heltzea/ Obozitoen *in vitro* heltze-prozesua
IZK: Immunozitokimika
JNK: Jun N-bukaera kinasa
JWH-015: (2-Metil-1-propil-1H-indol-3- il)-1-naftalenil-metanona
JWH-133: 3-(1,1-dimethylbutyl)-6aR,7,10,10aR-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran
KSOM: Potasioz osagarritutako sinplex metodo bidez optimizaturiko ingurunea
LH: Hormona luteinizatzailea
LHCGR: Hormona luteinizatzailearen hartzailea
MI: Metafase I
MII: Metafase II
MAGL: Monoazilglizerollipasa
MAP: Mitogenoz aktibaturiko proteina
MAPK: MAP kinasa
MPF: Heltzea sustatzeko faktorea
mRNA: RNA mezularia
n: Haploidea
NAPE: N-arakidonoilfosfatidiletanolamina
NAT: N-aziltransferasa
NPR2: 2-motako peptido natriuretikoaren hartzailea
P450scc: P450 Kolesterolaren alboko katearen zatiketa-entzima
PAGE: Poliakrilamidazko gel elektroforesia
PB: Korpuskulu polarra
PBS: Fosfato/gatz-indargetzailea
PCR: Polimerasaren kate-erreakzioa
PCOS: Obario polikistikoaren sindromea
PDE: Fosfodiesterasa
PFA: Paraformaldehidoa
PI3K: Fosfatidilinositol-3 kinasa
PKA: cAMParen menpeko proteina kinasa
PVA: polibinil alkohola
PVDF: Polibinilideno difluoroa
qRT-PCR: Polimerasaren kate-erreakzio kuantitatiboa denbora errealean
RAS: Ras proteinak (arratoiren sarkoma)
RNA: Azido erribonukleikoa
rRNA: RNA erribosomala
RT: Alderantzizko transkripzioa
RT-PCR: Alderantzizko transkripzio-polimerasaren kate-erreakzioa
SDS: Sodio dodezil sulfatoa
Ser: Serina
TAE: Tris/azetato indargetazailea
TBS: Tris/gatz-indargetzailea
TEMED: N,N,N,N'-tetrametiletilenodiamina
THC: Δ^9-tetrahidrokannabinola
Thr: Treonina
tRNA: Transferentziazko RNA
Tyr: Tirosina
WB: Western blot
WT: Wild-type, basatia
ZP: Mintz peluzidoa
ZP1: Mintz peluzidoa glikoproteina 1
ZP2: Mintz peluzidoa glikoproteina 2
ZP3: Mintz peluzidoa glikoproteina 3
ZP4: Mintz peluzidoa glikoproteina 4

List of abbreviations

2n: Diploid

2-AG: 2-Arachidonoylglycerol

α- MEM: alpha- Minimum essential medium

AC: Adenylyl cyclase

ACEA: Arachidonyl-2'-chloroethylamide

ACTB: Actin, cytoplasmic

AEA: N-arachidonoylethanolamine, anandamide

AKT: Protein quinase B

pAKT : phosphorated-AKT

AMP: Adenosine monophosphate

ANOVA: Analysis of variance

ART: Assisted reproductive techniques

ATP: Adenosine triphosphate

BMP15: Bone morphogenetic protein 15

BSA: Bovine Serum Albumin

Ca^{2+}: Calcium

cAMP: Cyclic adenosine-3', 5'- monophosphate

CB1: Cannabinoid receptor type 1

CB2: Cannabinoid receptor type 2

CBD: Cannabidiol

CBN: Cannabinol

CC: Cumulus cells

CDC25B: M-phase inducer phosphatase 2

CDK1: Cyclin dependent kinase 1

cDNA: Complementary Deoxyribonucleic acid

cGMP: Cyclic guanosine monophosphate

Cnr1 $^{-/-}$: Mice missing CB1 receptor

Cnr2 $^{-/-}$: Mice missing CB2 receptor

Cnr1 $^{-/-}$/Cnr2 $^{-/-}$: Mice missing CB1 and receptors

CNP: C-type natriuretic peptide

COC: Cumulus-oocyte complex

CREB: cAMP response element-binding

CYCB: Cyclin B

CYP11A1: Cytochrome P450, family 11, subfamily A, polypeptide 1

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dNTP: Deoxy-Nucleoside triphosphate

E2: Estrogen

eCG: Equine chorionic gonadotropin

ECL: Enhanced chemoluminiscence

ECS: Endocannabinoid system

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ERK1/2: Extracellular-signal-regulated kinase 1 and 2

pERK1/2: Phosphorilated extracellular-signal-regulated kinase 1 and 2

FAAH: Fatty acid amide hydrolase

FBS: Fetal bovine serum

FF-MAS: Follicle fluid meiosis activating sterol

FSH: Follicle-stimulating hormone

FSHR: Follicle-stimulating hormone receptor

G_{α} : Alpha subunit of the heterotrimeric G protein

$G_{\beta\gamma}$: Beta-gamma subunits of the heterotrimeric G protein

$G_{i\alpha}$: Gi-protein inhibitor

$G_{s\alpha}$: G stimulatory

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GDF9: Growth differentiation factor 9

GDP: Guanosine diphosphate

GnRH: Gonadotropin Releasing Hormone

GPCR: G protein coupled receptors

GTP: Guanosine Triphosphate

GV: Germinal vesicle

GVBD: Germinal vesicle breakdown

hCG: Human chorionic gonadotropin

hpf: Hours post fertilization

hRNA: Heterogeneous RNA

HTF: Human Tubal Fluid

HU-210: [(6aR,10aR)-3-(1,1-Dimethyl-heptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6 dimethyl-6H-dibenzo [b,d]pyran-9-methanol]

ICC: Immunocytochemistry

ICSI: Intracytoplasmic sperm injection

IP3: Inositol triphosphate

IP3R: Inositol triphosphate receptor

IVF: *In vitro* fertilization

IVM: *in vitro* maturation

JNK: Jun N-terminal kinase

JWH-015: 2-Methyl-1-propylindol-3-yl naphthalen-1-yl methanone

JWH-133: 3-(1,1-dimethylbutyl)-6aR,7,10,10aR-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran

KSOM : Potassium simplex optimization medium

LH: Luteinizing Hormone

LHCGR: Luteinizing Hormone/ choriogonadotropin receptor

MI: Metaphase I

MII: Metaphase II

MAP: Mitogen Activated Protein

MAPK: Mitogen Activated Protein Kinase

MGLL: Monoglyceride lipase

MPF: Maturation promoting factor

mRNA: Messenger ribonucleic acid

n: Haploid

NAPE: N-Acylphosphatidylethanolamine

NPR2: Natriuretic peptide receptor 2

P450scc: Cholesterol side-chain cleavage enzyme

PAGE: Polyacrylamide gel electrophoresis

PB: Polar body

PBS: Phosphate-buffered saline

PCOS: Polycystic ovary syndrome

PCR: Polymerase chain reaction

PDE: Phosphodiesterase

PFA: Paraformaldehide

PI3K: Phosphoinositide 3-kinase

PKA: Protein Kinase A

PVA: Polyvinyl alcohol

PVDF: Polyvinylidene Fluoride

qRT-PCR: Quantitative real time PCR

RAS: Rat Sarcoma

RNA: Ribonucleic Acid

rRNA: Ribosomal RNA

RT: Reverse Transcriptase

RT-PCR: Reverse Transcriptase- Polymerase Chain Reaction

SDS: Sodium Dodecyl Sulfate

Ser: Serine

TAE: Tris-acetate-EDTA

TBS: Tris-buffered saline

TEMED: N,N,N',N'-Tetramethylethylenediamine

THC: Δ^9-tetrahydrocannabinol

Thr: Threonine

tRNA: Transfer RNA

Tyr: Tyrosine

WB: Western blot

WT: Wild-type

ZP: Zona Pellucida

ZP1: Zona pellucida glycoprotein 1

ZP2: Zona pellucida glycoprotein 2

ZP3: Zona pellucida glycoprotein 3

ZP4: Zona pellucida glycoprotein 4

1

Sarrera
Introduction

1. Sarrera

1.1 Obozitoaren heltze-prozesua

Obozitoa enbrioiaren hazkuntzaren oinarria da, ernaldu berri den zelulabakarreko zigototik hasita, independenteki bizirauteko gai den erabat osatutako eta funtzionala den organismo zelulaniztuneraino. Hori horrela izanda, obozitoa oso zelula konplexua da. Obozitoaren hazkundea eta heltzea oso ondo koordinaturiko prozesuak dira eta, akatsak egonez gero, epe luzerako ondorioak izan ditzake ugalkortasunean eta, ondorioz, ondorengoen osasunean. Izan ere, heltze-prozesuak baldintzatzen du sortu behar den izaki berria *(Krisher, 2013)*. Hortaz, obozitoaren garapen gaitasuna funtsezkoa da enbrioiaren garapenerako. Obozitoak enbrioiari osagai kromosomikoaren erdia ematen dio baina bi gurasoen genomak ez dira simetrikoak eta ez diote ekarpen berdina egiten enbrioiari *(Keefe, 2015)*.

Obozitoak zelula oso espezializatuak dira eta espermatozoideekin batera prozesu meiotikoa burutzeko gai diren zelula bakarrak dira. Gainera, obozitoa ernaldua izateko eta enbrioiaren garapen goiztiarra aurrera eraman ahal izateko beharrezkoa da bai gameto emeak zein arrak gaitasun genetiko eta funtzional guztiak eskuratu izana *(Rahman eta lank., 2008)*. Ez hori bakarrik, obozitoa izaki berri bat sortzeko beste zelula baten DNA barneratzeko gain den zelula bakarra da *(Rahman eta lank., 2008)*.

Organismo baten ugalketa sexualaren lehen fasea gametogenesia da, obulutegietan eta testikuluetan dauden zelula germinaletatik gametoak eratzeko prozesua. Prozesu horri obogenesia deritzo emeetan eta espermatogenesia arretan eta zelula germinal haploideak (n) (obozitoak eta espermatozoideak) sortuko dira *(Rahman eta lank., 2008)*. Hortaz, beharrezkoa da ernalketaren mekanismoak ulertzeko obozitoan gertatzen diren aldaketak zehatz-mehatz jakitea, jatorri enbrionariotik hasita ernaltzeko gai den zelula oso espezializatua bihurtzeraino, II. metafaseko estadioan dagoen obozitoraino, alegia.

1.1.1 Folikulugenesia

Eme ugaztun guztien obozitoak fetuaren garapenean zehar sortzen dira. Horietako milaka obulutegian ageri dira eta bakoitza folikulu primordialen barruan dago. Folikulugenesia prozesu jarraitua da, obulazioarekin hasi eta folikuluaren endekapenarekin (atresia) amaitzen dena. Prozesu horretan folikulua garatu ez ezik, obozitoa hazten da, pikor-geruzako zelulak eta teka-zelulak ugaritzen dira, antroa izeneko barrunbea agertzen da eta esteroidogenesia hasten da *(Rimon-Dahari eta lank., 2016)*.

Obulutegietako oinarrizko unitatea folikulua da; obozito, pikor-geruzako zelulez eta teka zelulez osatuta dagoena. Izan ere, obulutegia garapen fase desberdinetan dauden folikuluen bilduma heterogeneo batez osatuta dago. Obulutegia, gonada femeninoa, zelula germinalen iturria da eta baita ere hormona sexual esteroideen hornitzaile nagusia *(McGee eta Hsueh, 2000)*.

Ugaztunetan, folikulugenesia obozitoa pikor-geruzako zelula deituriko geruza bakarreko zelula aitzindariekin elkartzean hasten da, folikulu primordialak sortzean, hain zuzen ere. Espeziearen arabera, jaio aurretik edo ostean gertatzen da *(McGee eta Hsueh, 2000)*. Esate baterako, behien, ardien eta gizakien kasuan, folikulu primordialak bizitza fetalean eratzen dira eta saguetan eta hamsterretan jaio eta handik gutxira *(Kurilo, 1981; McNatty eta lank., 1995; Fortune, 2003; Kezele eta Skinner, 2003; Wang eta Roy, 2007)*. Pikor-geruzako zelulaz inguraturik ez dauden obogoniak obulutegitik kanporatzen dira *(Motta eta lank., 1997)*.

Ugaztun emeetan, jaiotzean, folikulu primordialen barnean dauden obozitoak diplotenoko I. profasean gelditua daude, zelula folikular lauez inguratuta. Adinarekin eta hilekoaren ziklo (gizakietan) edota ziklo estral (gainontzeko ugaztun gehienetan) bakoitzaren agerpenarekin, folikuluen erreserba kantitatean eta kalitatean gutxitzen doa, ugaztunen ugalketa adina zehaztuz *(Broekmans eta lank., 2007)*. Esaterako, gizakietan, 500 obozito baino gutxiago obulatzen dira ugal-bizitzan zehar, baina jaiotzerakoan 300.000-400.000 obozito inguru daude *(Block, 1953; Forabosco eta lank., 1991; Hansen eta lank., 2008)*. Beraz, oro har, 1600 folikulu primarioetatik bakarrak beteko du folikulugenesi prozesua eta obulaziora iritsiko da. Sexualki heltzean (gizakietan nerabezaroan), folikuluak multzoka hazten dira eta garapen fase guztietatik pasatu beharko dira: folikulu primordiala, primarioa, sekundarioa, aurreantrala, antrala, dominatzailea eta obulatzailea *(Fair, 2003)*. Gizakien kasuan, prozesu osoa egiteko, folikulu primarioaren hasierako fasetik obulazio aurreko folikulura, 6 hilabete baino gehiago behar direla kalkulatzen da. Karraskarietan, folikulugenesiaren iraupena gizakian baino askoz ere laburragoa da, 30 egun ingurukoa. 28 egun behar dira folikulu primarioaren aktibaziotik antro folikularra daukan folikulu sekundariora heldu arte. Behin fase horretan, folikuluak hormona zirkulatzaileekiko sentikorrak dira eta hurrengo ziklo estralean eskuratuko dira, 2-3 egunen buruan obulazio aurreko folikuluetan (De Graaf-en folikulu ere deitua) bihurtuz *(McGee eta Hsueh 2000)*.

Egoera latentean dauden folikulu primordialak etengabe multzokatzen eta metatzen dira eta oraindik ez daude gonadotropina hormonaren menpe. Folikuluak garatzen doazen heinean eta fase antralera iristen direnean (obulazio aurreko etapa) gonadotropinen eraginaren menpe egongo dira. Antro folikularra deituriko barrunbea sortzen denean pikor-geruzako zelulak bi zelula motetan banatzen dira: muralak (esteroidogenesia bultzatzen dute) eta kumuluko zelulak (obozitoaren aldamenean daude) *(Rimon Dahari eta lank., 2016)*. Gonadotropinek antro folikularraren hazkundea bultzatzen dute eta, azkenean, obozitoaren eta *corona radiata*-ren (obozitoa inguratzen duen pikor-geruzako zelulez osaturiko geruza) artean dauden zelulen arteko loturen apurketa, AMP ziklikoaren (cAMP) kontzentrazioa jaisten delarik. Orduan hasiko da obulazioa *(Picton eta lank., 1998)*. Folikuluek ez badute garapenarekin jarraitzen endekatu egingo dira, hau da, atresia egoeran sartuko dira.

Ziklo bakoitzean garatzen den (folikulugenesia hasten duen) folikuluen kopurua erlatiboa da eta zenbait faktorek baldintzatuko dute: inaktibo dauden folikuluen gordailuaren tamainak, obulutegira heltzen den hormona folikulu estimulatzaile (FSH) kantitateak eta gonadotropinekiko folikuluek daukaten sentikortasunak. Aurretiaz aipatu bezala, folikuluen hazkundea prozesua jarraitua da eta ez da eteten egoera fisiologikoa edozein delarik ere. Beti gertatzen ez dena da De Graaf-en folikuluen eraketara

bideratzen duen heltze-prozesu osoa eta, beraz, ernaldua izateko gaitasuna duen obozitoaren eraketa *(Adashi eta lank., 1991)*.

- **Folikulugenesiaren faseak**

Ziklo bakoitzean, obulatzeko gaitasuna duten obozitoak sortuko dituzten folikuluak hormona gonadotropikoek zehaztuko dituzte. Hau da, teka zeluletan hormona luteinizatzailearen (LH) hartzaileek (LHCGR edo LHR) eta pikor-geruzako zeluletan FSH hormonarenak (FSHR) *(Espey, 1974)*. FSH maila jaistean hormona honen hartzaile kopuru txikiena duten folikuluak atresian sartuko dira. Hala ere, hormonen seinaleztapenaren inguruko zehaztasunak 1.1.2 atalean garatuko dira

Hauek dira, hurrenez hurren, folikulu motak **(1.1 irudia)**:

a. **Folikulu primordiala:** Atresia prozesutik biziraun duen obozito primarioa epitelioko zelula lauen geruza bakarraz (aurre-pikor geruzako zelulak) inguraturik dago. Obozitoa txikia eta heldugabea da eta inguratzen duten pikor-geruzako zelulen efektu inhibitzaileari esker I. meiosiko profasean etenda mantentzen da. Ugaztunetan, folikulu primordialak obulutegien garapenaren oinarrizko unitatea dira eta obozitoa ingurune kontrolatuan egotea bermatuko dute, odol-fluxutik hel daitezkeen kaltegarriak diren substantzietatik babestuz. Gizakietan, folikuluen garapena fetuaren 4. hilabetetik aurrera hasten da *(Baker, 1963)*.

b. **Folikulu primarioa:** folikulu primordialaren aktibazioa hastean folikulua helduz joango da folikulu primario bihurtu arte. Oraindik ez da ezagutzen zerk bultzatzen duen folikulu primordialetik primariorako aldaketa *(Moor eta lank., 1990)* nahiz eta jakina den erregulazioa obulutegi barnekoa dela eta gonadotropinekiko independentea *(Gosden eta Lee, 2010)*. Fase honetan, obozitoa bera hazten doa eta pikor-geruzako zelulak ugaritzen eta desberdintzen hasten dira jarduera mitotikoa oso altua dutelako, epitelio zelula lauak kubiko bihurtuz *(Fair, 2003; Fortune eta lank., 2000)*. Etapa honetan pikor-geruzako zelulek ez dute inongo efektu inhibitzailerik obozitoaren hazkuntzan baina bai meiosian *(Thibault eta lank.,1987)*. Folikulu primordialen hazkundearen aktibazioa hazkundearen desberdintzapen-faktorea 9 (GDF9) eta hezur-proteina morfogenetikoa 15 (BMP15) bezalako proteinei esker ematen da. GDF-9 hazkunde faktore β-eraldatzailearen (TGFβ) familiako proteina espezifikoa da, ugaztun guztien obozitoek ekoizten dutena *(Eppig, 2001)*. BMP-15 ere familia berdineko proteina da eta bi hauen arteko sinergia beharrezkoa da ugaztunen folikuluen eta obarioen hazkunde normala egoteko *(Eppig, 2001)*. Pikor-geruzako zelulen ugaritzea estimulatzen dute FSH hormonarekin batera edo gabe *(Eppig eta lank., 2002)*. Aipatu behar da zelula horiek jarduera mitotiko altua dutela eta, ondorioz, folikulua inguratzen duten zelulen geruzak ugarituz joango direla epitelio geruzatua sortuz eta folikuluaren tamaina handituz. Obozitoaren eta pikor-geruzako zelulen artean glikoproteinez osatutako geruza extrazelularra osatzen da, hots, mintz peluzidoa (ZP). Ia ornodun guztien obozitoak daude, gutxienez, glikoproteina geruza batez inguratuak eta leinu bakoitzean desberdin izendatzen da: koriona arrainetan, bilkin bitelinoa anfibioetan, bilkin peribitelinoa narrastietan eta hegaztietan eta mintz peluzidoa ugaztunetan *(Gupta, 2018)*. Mintz peluzidoa 3 glikoproteinek osatzen dute (ZP1, ZP2, ZP3 saguetan eta ZP4 gehigarria gizakietan) eta oso antolatua dagoen egitura dinamikoa da. Gainera, funtsezkoa da obogenesirako, ernalketarako eta

enbrioiaren garapen goiztiarrerako, obozitoa elikatu eta babesten baitu. Gainera, espermatozoideen elkartzean laguntzen du eta polispermia blokeatzen laguntzen du *(Gupta, 2018)*.

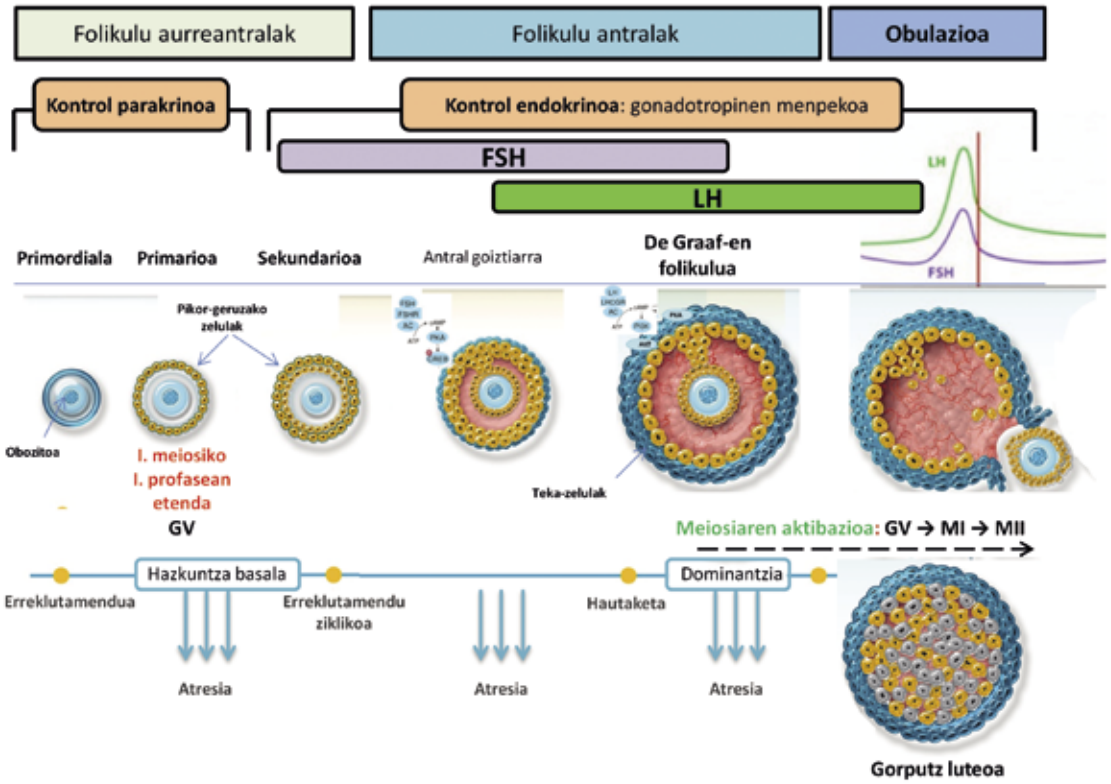
c. **Folikulu sekundarioa edo aurreantrala:** Pikor-geruzako zelulak ugarituz doaz FSH hormonaren eraginez eta zelula horien bi geruza edo gehiago eratzen dira tamainaz handitu den obozito primarioaren inguruan. Ondoren, obulutegiaren estromako zeluletatik teka zelulak gauzatzen dira eta, alde batetik, barrualdeko teka sortzen da eta, bestetik, baskularizazio handiko kanpoaldeko teka, geruza bien arten odol-hodiak eta linfozitoak agertuz *(Reynolds eta lank. 2002)*. Barrualdeko teka xafla basaletik hurbil dauden zelula epiteloideek osatzen dute eta LH hormonarako hartzaileak dituzte. Kanpoaldeko teka zelulak dira estromako zelulekin elkartzen direnak kanpoaldeko teka osatzeko eta kanpoko teka geruzari esker, folikuluak hazkunde handiagoa ahalbidetzen duten faktore endokrino sistemikoak askatzen dira eta zelula kanpoko espazioan fluxu folikularrez beteriko barrunbeak eratzen dira *(Hirshfield, 1991)*.

   Ostean, zelula folikularren artean likidoz betetako gune bat sortuko da, antro folikularra **(folikulu antral goiztiarra)**. Teka zeluletan, LHak esteroidogenesia bultzatzen du eta kolesteroletik abiatuta androgenoak ekoizten dira (batez ere, androstenediona). Horiek xafla basaletik zehar pikor-geruzako zeluletara barreiatzen dira eta estradiolean bihurtzen dira FSH hormonak estimulaturiko aromatasa entzimari esker *(Zlotkin eta lank., 1986)*. Aipatu behar da folikulu antralek FSH eta LH hormonen beharra dutela euren garapenerako eta obulaziorako. FSHa beharrezkoa da, batez ere, folikulu antralaren biziraupenerako, pikor-geruzako zelulen biziraupenerako, LH hartzaileen adierazpenerako eta estradiolaren ekoizpenerako. FSHa G proteinari loturiko bere hartzailera lotzen da eta adenil ziklasa (AC)/cAMP/proteina kinasa A (PKA) bidezidor klasikoa aktibatzen du. Horrela CREB (cAMP-response element-binding, ingelesez) transkripzio faktorea fosforilatzen da eta, ondorioz, aromatasa eta LHaren hartzailea aktibatzen dira. LHari esker, obulazioa eragingo duten seinaleak pizten dira (*Sanchez eta Smitz, 2012)*. FSHak PKAren beste bide independente batzuk ere aktibatzen ditu, esaterako, fosfoinositola 3-kinasa (PI3K) bidezko B proteina kinasa (AKT) *(Ulloa-Aguirre eta lank., 2007)*.

d. **Folikulu tertziarioa, obulazio aurrekoa edo De Graaf-en folikulua:** Folikulua garatzen eta antroa handitzen doan heinean pikor-geruzako zelulak zeharo diferentziatuta daude bi zelula funtzionaletan: alde batetik, pikor-geruzako zelula muraletan, zeinek folikuluaren horma estaltzen baitute eta xafla basalarekin kontaktuan dagoen epitelio geruzatua osatzen baitute. Bestetik, kumuluko pikor-geruzako zeluletan. Horiek, obozitoaren inguruan zelula zilindrikoen hainbat geruza osatzen dituzte, *corona radiata* barne *(Eppig, 2001)*. Azkenik, LHaren obulazioaren aurreko gailurrak seinale jakin batzuk aktibatuko ditu eta obulazioa piztuko da obozitoa obiduktuko finbrietarantz askatuz *(Baerwald, eta lank., 2012; Sanchez eta Smitz 2012)*. Folikuluak kumulu-obozito konplexua (COC) askatzeko gaitasuna lortzen du LHa edo giza gonadotropina korionikoa (hCG) dosi batekin estimulatua izatean. LHaren emendioak, obulazioa ez ezik, beste hainbat prozesu bultzatuko ditu: obozitoan meiosiaren berraktibazioa pizten da II. metafasera arte, kumuluko zelulak hedatu egiten dira eta folikuluaren horma hautsi egiten da COC obiduktura askatzeko. Bestalde, pikor-geruzako zelula muralek eta teka-zelulek luteinizazio prozesua hasiko dute. Pikor-geruzako zelula muralek eta

teka zelulak luteinizazioarekin hasiko dira eta, COC obulatu ondoren, pikor-geruzako zelulak eta teka zelulak progesteronak sortzen duen guruin endokrino baskularizatuan bereiziko dira: gorputz luteoan.

Laburbilduz, folikulugenesian, folikuluen hazkundea gertatzeaz gain, hormona esteroideak ekoiztu eta jariatzen dira. Hormona horiek zeregin autokrinoa daukate folikuluen garapenean eta endokrinoa *feedback*-mekanismoan, FSHaren eta LHaren ekoizpena eta askapena kontrolatzeko nerbio-sistema zentralean. Hortaz, ugaztunetan esteroidogenesia 2 zelula/2 gonadotropina modeloaren bidez azaltzen da. Modelo horretan, LHaren eraginez teka zeluletan androgenoak ekoizten dira kolesteroletik abiatuta eta, ondoren, FSHaren eraginez, estrogenoetan transformatzen dira pikor-geruzako zeluletan.



1.1 irudia. Folikulugenesiaren fase nagusien laburpena. Folikulugenesian folikulu primordialak aktibatzen dira, ondoren, folikulu primarioetan bihurtzeko. Pikor-geruzako zelulak ugaritzen doaz eta hainbat geruza eratzen dituzte obozitoaren inguruan. Etapa horretatik aurrera, teka zelulen geruza batek folikulua inguratzen du eta androgenoak ekoizten hasten dira, ondoren pikor-geruzako zeluletan estrogenoan bihurtzeko. Folikuluen hazkuntzaren lehenengo etapak folikulu sekundarioa eratu arte gonadotropinekiko independenteak dira. Hortik aurrera, hazkundea FSHaren estimulazioaren araberakoa da. Azkenik, obulazioa gertatzen da LHaren estimulazio-tontorrari esker.

Azpimarratu behar da ugaztun guztien kasuan folikulugenesia ez dela guztiz berdina baina saguen eta gizakiaren arteko oinarrizko ezberdintasunak, batez ere, tamaina eta denbora dira. Folikulugenesia gizakien kasuan askoz luzeagoa da, karraskarien faseekin alderatuta **(1.1 taula)**.

Giza folikulu baten hazkuntza-fase osoa 205 egunekoa edo hilekoaren zazpi zikloak baino askoz luzeagoa da, eta karraskarietan, ostera, 60 egunekoa. Hala ere, bi espezieetan folikulu goiztiarren hazkuntza oso luzea da. Bigarrenik, karraskarietan folikulugenesia jaio ondorengo prozesua da. Folikulu primordialak jaio eta hirugarren egunean eratzen dira eta folikuluen lehen olatua folikulu antraletaraino hurrengo hiru asteetan garatzen da.

Gizakietan, folikulu primordialak haurdunaldiaren erdialdetik hasi eta jaiotzera arte eratzen dira. Folikulu primordial batzuk biltegitik irten eta garatzen hasten dira. Behin hazkuntza-fasean sartu ondoren, hazten diren folikulu gehienak fase antralera igarotzen dira eta, ondorioz, atresiko bihurtzen dira nerabezarora iritsi arte *(Mcgee eta Hsueh, 2000)*. Hori dela eta, jaio ondoren, gutxi gorabehera, aurretiaz aipatu bezala, obulutegietan 300.000 folikulu primordial inguru dauden arren erreserba hori gutxituz doa nerabezarora arte, 200.000 izatera iristeraino *(McGee eta Hsueh, 2000)*.

Horrez gain, saguetan, besteak beste, folikulu primordialen erdia baino gehiago jaio eta 3-5 astera degeneratzen dira. Kontuan izan behar da, emeak heldutasun sexuala 6 asterekin eskuratzen duela (saguanduiaren eta ingurugiroko baldintzen arabera) eta ordurako obulutegi bakoitzean 104 obozito inguru daudela hazkunde etapa desberdinetan.

Gizakian, hilero obarioan dauden 15-20 folikulu primordial hasiko dira garapen-prozesuarekin baina soilik bat izango da gai guztiz heltzeko, De Graaf-en folikulua. Soilik folikulu mota hori apurtzean askatuko da obozitoa *corona radiata*-rekin batera obulazioa hasteko. Hortik aurrera, egoera normal batean, 400 obozito inguru obulatuko dira bizitza osoan zehar, bakoitza 28 egun inguruko zikloetan *(Lonergan eta Fair, 2016)*.

Beste ugaztun batzuetan, saguan adibidez, gauza bera gertatzen da baina zikloak motzagoak dira (gutxi gorabehera 5 egunekoak) . Gainera, folikulu antralen heltzea espontaneoki gertatzen da eta obulazioan folikulu eta obozitoen arteko erantzun koordinatua egon behar da. Saguetan, ziklo naturalean folikulu gutxi batzuek hasiko dute garapena FSHaren eraginez eta 8 eta 12 obozito askatuko dira obulazioan, bakoitza 2-3 orduko tartearekin (*Behringer eta lank., 2014)*.

1.1 taula. Folikuluen eta obozitoen batezbesteko diametroak folikulugenesian zehar saguan eta gizakian *(Griffin eta lank., 2006*)**.**

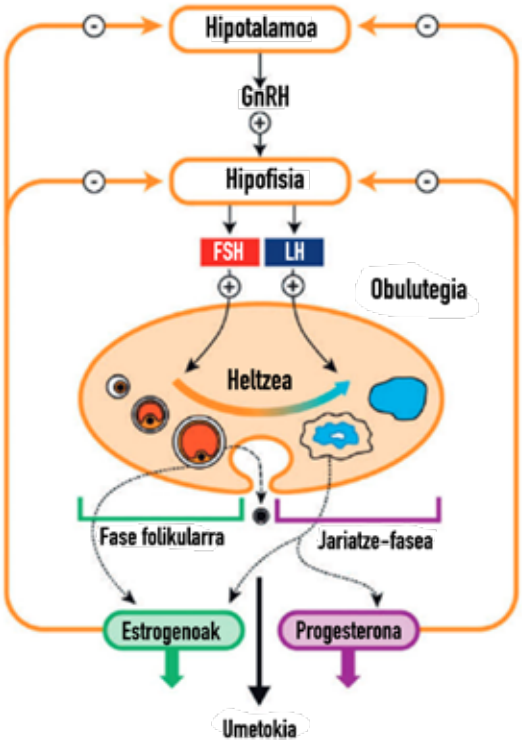
Folikulugenesiaren faseak	Sagua		Gizakia	
	Folikulua	Obozitoa	Folikulua	Obozitoa
Primordiala	17 µm	13 µm	44 µm	36 µm
Primarioa	52 µm	29 µm	70 µm	24 µm
Sekundarioa	104 µm	54 µm	114 µm	73 µm
Antral goiztiarra	248 µm	70 µm	889 µm	120 µm
De Graaf	424 µm	72 µm	18800 µm	120 µm

Hurrengo ataletan ikusiko dugun bezala, folikulugenesian zehar, obozitoak pixkanaka eta sekuentzialki lortuko ditu beharrezkoak diren makineria zitoplasmatikoak eta molekularrak garapen gaitasuna eskuratu eta enbrioiaren garapen goiztiarrari aurre egiteko *(Sirard eta lank., 1988; Gilchrist eta lank., 2008)*.

1.1.2 Hormonen seinaleztapena eta erregulazioa

Folikuluen eta obozitoen garapena ziklikoa da eta ugaztun emeen ugaltze-ahalmena ere ziklikoa eta aldizkakoa da. Ahalmen horri gizakietan hilekoa esaten zaio eta gainontzeko ugaztun gehienetan ziklo estrala. Gizakien kasuan, batez bestez, 11-13 urterekin hasi eta menopausia arte irauten du. Ugalkorra den urteen buruan, hilero aldaketa erritmikoak jasango ditu hormona sexualen jariaketan eta horrek dakartzan obulutegien eta sexu-organoen aldaketa fisikoetan. Ziklo bakoitzaren iraupena 28 egunekoa da, batez bestez. Hilekoak bi ondorio nagusi ditu. Alde batetik, gizakietan hilabete bakoitzean obulutegi batetik obozito bakarraren askapena gertatuko da eta, beraz, egoera normal batean zigoto bakarra eratuko da ziklo bakoitzean eta, beste alde batetik, endometrioa ernaldutako obozitoa txertatzeko prestatuko da. Bai bata zein bestea hormona desberdinen eraginpean gertatzen da. Ziklo sexual horiek hipotalamoak kontrolatzen ditu. Hipotalamoan sintetizatzen den gonadotropinen jariapen-hormonak (GnRH) hipofisiaren erdiko gingilean eragiten du eta, ondorioz, gonadotropinak isurtzen dira. Hormona horiek, hormona folikulu estimulatzaileak (FSH) eta hormona luteinizatzaileak (LH), erregulatzen dituzte obulutegietan eta endometrioan aldaketa ziklikoak *(Sadler, 2004)*.

Emeen sistema-hormonala hiru hormona multzoek osatzen dute eta hipotalamo-hipofisi-obario ardatz gisa ezagutzen da **(1.2 irudia)**:



1.2. irudia Obozitoen garapena hipotalamo-hipofisi-obario ardatzaren bidez FSH eta LH hormonen jariaketa *feedback* positibo edo negatibo batek kontrolatzen du, hilekoaren etaparen arabera *(Astiz, 2016)*.

a. Hipotalamoan sintetizatzen den gonadotropinen jariapen-hormona (GnRH).

b. Hormona adenohipofisiarioak edo gonadotropinak: hormona folikulu estimulatzailea (FSH) eta hormona luteinizatzailea (LH). Hipotalamoko GnRHaren eraginez jariatzen dira.

c. Obulutegiko hormonak: esteroideak (estrogenoak, progestagenoak) eta inhibina. Hormona adenohipofisarioei erantzunez jariatzen ditu obulutegiak.

1.1.2.1 Gonadotropinak

Emakumeen ugalketa zikloaren determinatzaile nagusiak dira FSHa eta LHa. Hormona horiek guztiek α azpi-multzo berdina eta hormona β kate espezifikoa daukate. Isoforma desberdinak deskribatu dira hainbat ugaztun espezietan, besteak beste, gizakian *(Wide eta Bakos, 1993)*, behian (*Cooke eta lank., 1997)* eta zaldian *(Matteri eta lank., 1986)* eta isoforma horien banaketa desberdina da nerabezaroan, hilekoaren zikloaren/ziklo estralaren eta sentsibilitatearen arabera. Espezieen arteko gonadotropinen aminoazidoen sekuentziaren homologia altua dela eta, gizakitik purifikatutako edo birkonbinatutako gonadotropinak (hCG) erabil daitezke saguaren folikuluen edo obozitoen heltzean *(Wolfenson eta lank., 2005)* edota behorraren hormona gonadotropina korionikoa (eCG) erabili daiteke hainbat espezietarako *in vivo* obarioen estimulaziorako protokoloetan, tartean, saguekin egindako esperimentuetarako.

Arestian aipatu bezala, gonadotropinak G proteinei loturiko mintz-hartzaile espezifikoetara lotzen dira: FSHa FSHRra eta LHa eta hCG LHCGRra. Orokorrean, bi hartzaile horiek Gs proteinei lotuak daude, zeinek adenilil ziklasak aktibatzen dituzten eta, ondorioz, AMP ziklikoaren (cAMP) kontzentrazioak emendatzen dira. FSHRak cAMP seinalea aktibatzen duen bitartean, LHCGRk bigarren mezulari gehigarriak erabiltzen ditu [(Ca^{2+} eta inositol trifosfatoa (IP3)] eta, beraz, efektu dibergenteak sortzen ditu. Hala ere, oraindik eztabaida gaia da zein puntutaraino bultzatzen dituzten seinaleztapen desberdinak eta ez erredundanteak *(Conti, 2002; Palermo, 2007)*.

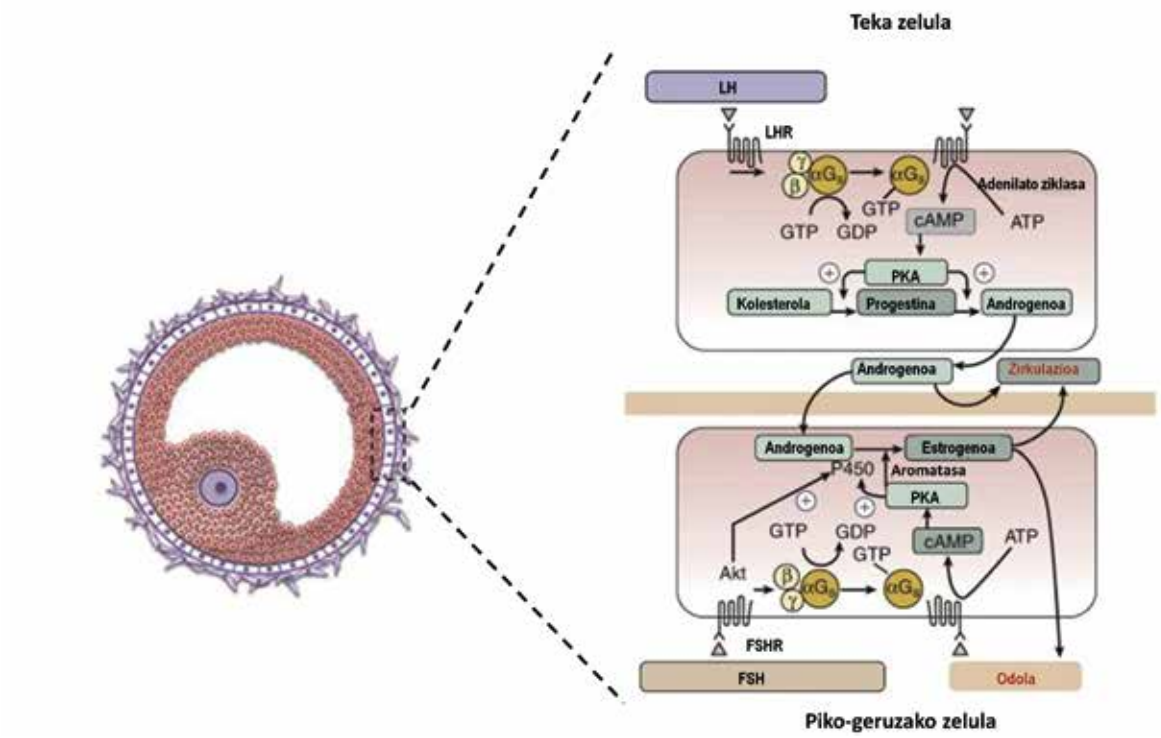
FSHak, batez ere, folikuluen hazkuntzan eragiten du, pikor-geruzako zelulen ugaritzea areagotuz. LHa asko emendatuko da folikulu dominatzailea heltzen denean eta obozitoaren heltze-prozesua, obulazioa eta gorputz luteoaren luteinizazioa induzituko ditu. LHa G proteinari loturiko bere hartzailera lotzean, hainbat bide metaboliko aktibatzen ditu, hala nola, AC/cAMP/PKA, PI3K/AKT eta RAS seinaleztapen ur jauziak, bakoitzak berebiziko garrantzia duelarik obulazioan *(Richards eta Pangas 2010b)*. Gizakian, enbrioiak hCG ekoizten du gorputz luteoaren degradazioa (luteolisia) saihesteko eta haurdunaldia babesteko *(Hirata eta lank., 2015)*.

1.1.1.2 Hormona esteroideak

Ugalketa-funtzioa garunean ekoiztutako gonadotropinen bidez erregulatua egoteaz gain, folikuluetatik eratorritako hormona esteroideen bitartez ere erregulatzen da. Kolesterola erabiltzen da substratu gisa eta P450 zitokromo-11 familia-A azpifamilia-1 polipeptidoak (CYP11A1) esteroidogenesiaren lehenengo

pausoa katalizatzen du pregnenolona ekoizteko. Ondoren, obarioak progestagenoak, androgenoak eta estrogenoak sintetizatuko ditu modu sekuentzialean *(Miller eta Auchus, 2011)*.

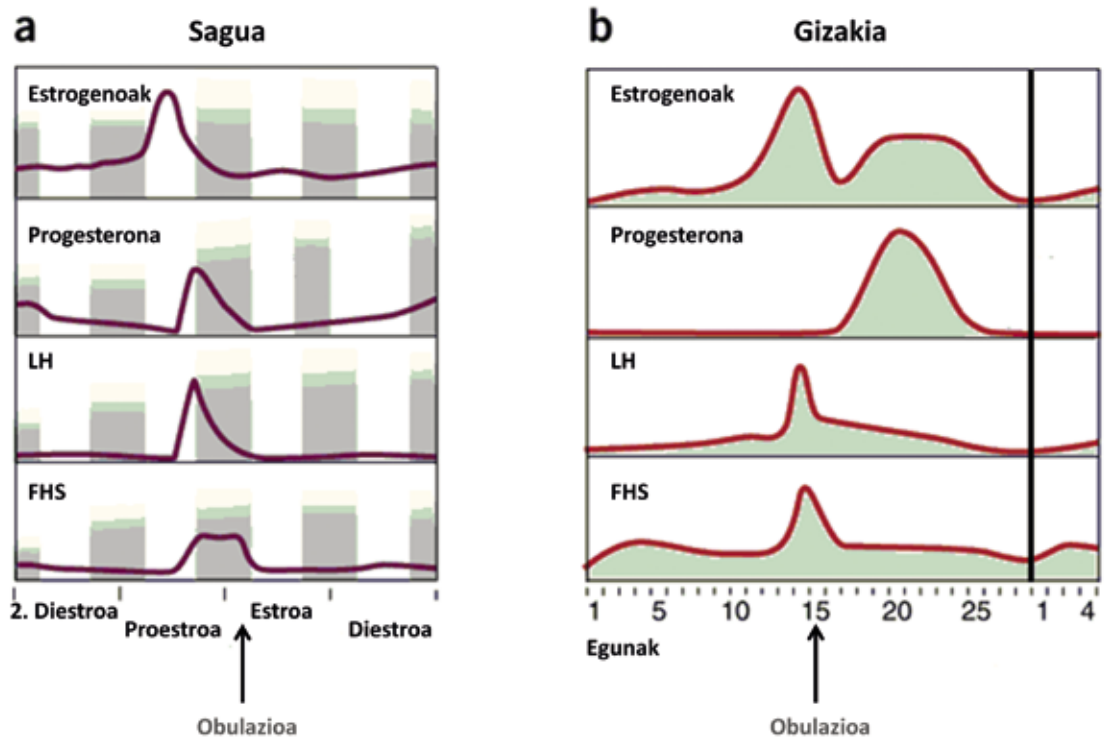
Aurretiaz 1.1.1 atalean aipatu bezala, folikulugenesian zehar gonadotropinek eta hormona esteroideek erregulatzen dituzten mekanismoak 2 zelula/2 gonadotropina teoriaren arabera azaltzen dira **(1.3 irudia).** LHak LHCGRaren bidez erregulatzen du teka zeluletan esteroideen ekoizpena. Folikulu aurreantralaren etapan, baskularizazio altuko teka zelulek progesterona eta androgenoak ekoizten dituzte odol-fluxutik etengabean askatzen den kolesteroletik. Pikor-geruzako zelulek, bestalde, FSHRa adierazten dute, eta hartzaile honek CYP11A1 estimulatzen du teka zelulek askatutako androgenoak estrogenoetan bihurtzeko aromatasa entzimaren bidez. De Graaf-en folikuluan edo obulazio aurreko folikuluan gertatzen den LHaren emendioa eta gero, teka zelulek pikor-geruzako zelulak androstenedionarekin hornitzen jarraitzen dituzte.



1.3 irudia. **Folikulugenesian eta esteroidogenesian zehar gertatzen diren prozesuak azaltzeko 2 zelula/2 hormona teoria irudikatzen duen sistema**. Estradiola da pikor-geruzako zelulek sortzen duten hormona esteroide gakoa. Hormona hori sintetizatzeko bi zelulen arteko komunikazioa ezinbestekoa da. Teka zelulek androgenoak (dehidroepiandrosterona (DHEA), androstendiola, androstendiona eta testosterona) ekoizten dituzte LH hormonari erantzunez eta, ondoren, pikor-geruzako zeluletara hedatzen dira eta hor estrogenoetan bihurtuko dira (estrona eta estradiola) FSHari erantzunez *(Strauss eta lank., 2014-tik moldatua).*

Ikusi da, aromatasaren bidez sexu-hormonak sortzeaz gain, hormonen menpeko minbizietan erabilgarria dela, aromatasaren inhibitzaileen bitartez estradiolaren mailak handitzea ekiditen delako. Baina endometrioko edo bularreko minbizia duten pazienteen kasuan, minbizi-zelulek estradiola behar dute garatzeko, beraz, tumore-zelulak hil egingo lirateke estradiolik gabe. Zenbat eta sexu-hormona horren maila handiagoak izan gorputzean, orduan eta errazagoa da minbizi-zelulak ugaltzea. Hori dela eta, *in*

vitro ernalketaren (IVF) tratamenduak sortzen duen folikuluen estimulazioa arriskutsua da gaixotasun horiek dituzten emakumeetan, estimulazio horrek estradiol-mailak ere handitzen baititu *(Mukhopadhyay eta lank., 2015)*.



1.4 irudia. **Estrogenoen, progesteronaren eta LH eta FSH hormonen ikuspegi orokor eskematikoa a) saguen ziklo estralean eta b) gizakien hilekoan zehar.** Estroan 4 etapa bereizten dira: proestroa, estroa, I. diestroa I eta II. diestroa. Estro zikloa bat dator hilekoaren fase folikularrarekin eta diestro fasea luteinizazio fasearekin. Karraskariaren endometrioa ez da isurtzen, baina hurrengo ziklorako berrantolatzen da *(Staley eta Scharfman, 2005)*.

Karraskarietan folikulu dominanteen hautaketa gizakien antzekoa da, eta FSHak, LHak, estrogenoek eta progesteronak erregulatzen dute **(1.4 irudia)**. Desberdintasunik handiena da saguetan poliobulazioa eragiten duen ziklo estral bakoitzean folikulu ugari nagusitzen direla 1.1.1 atalean ikusi bezala. Horri erantzuteko hainbat mekanismo proposatu dira: obozito bakarra obulatzen duten espezieak eta espezie poliobulatorioak bereizten dira, seguruenik, erantzun negatiboen seinaleen atarian edo folikulu bakoitzak jariatzen dituen *feedback* negatiboaren seinaleztapen mailan (estrogenoak eta inhibinak) eta espezie edo andui desberdinek FSHarekiko duen erantzun folikularrean bereizten dira *(McGee eta Hsueh, 2000)*.

1.1.2.3 Hormonen seinaleztapena obulazioan

Gizakietan obarioaren ziklo bakoitza hastean fase primarioan dauden (folikulu aurreantralak) 15-20 folikulu bitartean garatzen joango dira FSH hormonaren eraginez eta, saguetan, 8-12 inguru. Lehenengo atalean azaldu bezala, FSHa ez da beharrezkoa folikulu primordialen hazkundea bultzatzeko (folikulu

primarioetan bilakatu daitezen) baina, hormona agertu ezean, folikulu primarioak hil egiten dira atresiko bihurtuz. Egoera normalean, gizakietan, folikulu hauetatik bakarrak lortuko du erabateko heltzea eta obozito bakarra askatuko da. Gainontzekoak degeneratu eta folikulu atresiko bihurtuko dira *(Sadler, 2004)*. Pauso anitzeko prozesu hori LHaren aurre-igoerari erantzunez gertatzen da, hipotalamoaren, guruin hipofisarioaren eta obuluaren *feedback* sistemaren arabera sortzen baita. Lehen aipatu bezala, FSHak folikulu antralaren hazkundea eragiten du, estradiolaren ekoizpen altuarekin lotuta. Pikor-geruzako zelulak sortutako estradiol mailek GnRH pultsuak handitzen dituzte LHaren hazkundean eraginez *(Richards eta Pangas 2010b)*. Beraz, folikulu dominatzailearen aukeraketaren ostean etorriko da obulazioa. De Graaf-en folikulua LHaren tontorra eta 16-24 ordura askatuko da obozitoa inguratzen duen kumulu-obozito konplexuarekin (COC) batera.

1.1.2.4 Hormonen seinaleztapena gorputz luteoaren eraketan

Obulazioa amaitzen da obulutegiko folikulutik obiduktora askatzen denean obozitoa, espermatozoideak ernaldu ahal izateko. Behin obozitoa askatzen dela, zelula folikularren hondarrek, pikor-geruzako zelulak eta teka zelulak, diferentziazio-prozesuaren berprogramazioa hasiko dute gorputz luteoa eratzeko. Hori dela eta, FSH hormonak hasi duen adierazpen genikoa desaktibatzen da eta luteinizazio-prozesua kontrolatzen duten geneak aktibatuko dira *(Edson eta al. 2009; Richards eta Pangas 2010a, b)*. Gorputz luteoa ezinbestekoa da haurdunaldia bermatu eta mantentzeko, nagusiki progesteronaren jariaketaren bidez. Haurdunaldirik egon ez bada, gorputz luteoa degeneratu egiten da *(Rimon-Dahari 2016)*. Karraskarietan eta gizakietan, gorputz luteoak progesteronaz gain, androgenoak eta estrogenoak ekoizten ditu *(Desclin, 1970; Moore eta Persaud, 200)*.

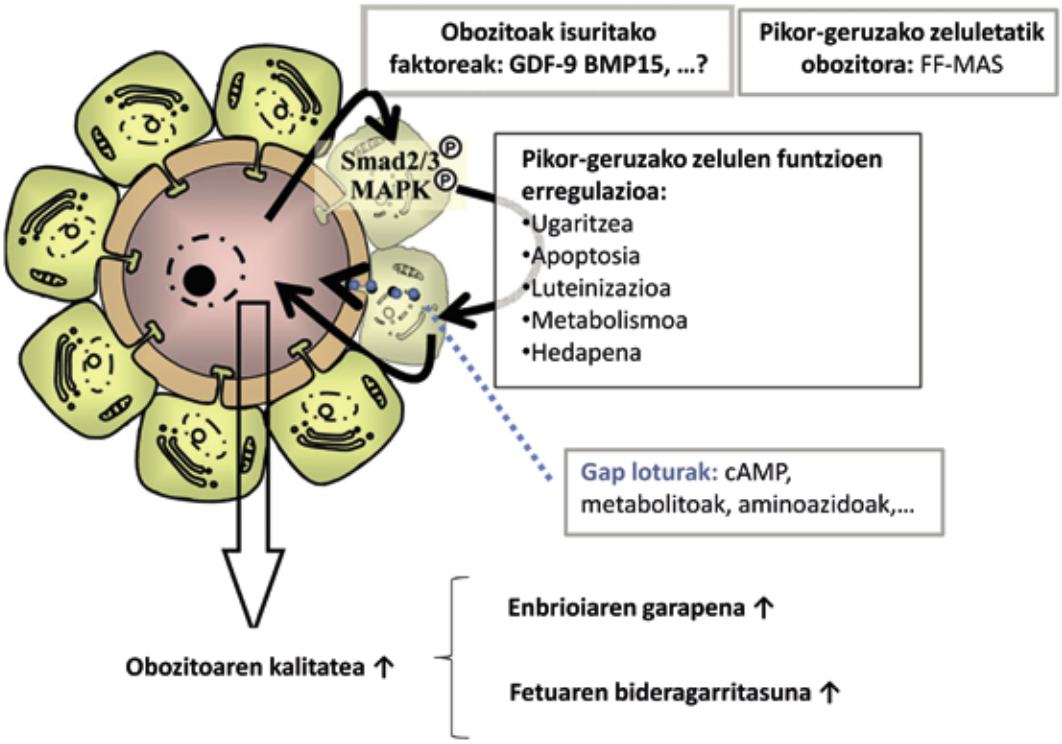
1.1.3 Obozitoaren eta kumulu-obozito konplexuaren (COC) arteko komunikazioa

Egiaztatu da folikulugenesia aurrera eramateko eta obozitoak garapen gaitasuna eskuratzeko beharrezkoa dela obozitoaren eta konpartimentu somatikoaren (pikor-geruzako zelulak eta teka zelulak) arteko koordinazioa. Zelula horien arteko komunikazioa funtsezkoa da obozitoaren hazkuntza eta bereizketa eragingo duten seinaleztapen-bide desberdinak arautzeko eta, ezinbestekoa, pisu molekular baxuko mantenugaien, metabolitoen eta molekulen ekarpenerako *(Eppig, 2001)*.

Gaur egun, argi dago obozitoaren eta pikor-geruzako zelulen arteko komunikazioa bi norabidekoa dela, pikor-geruzako zelulen zitoplasmaren luzapenek mintz peluzidoa zeharkatu eta oboplasamarekin konektatzen baitute Gap motako loturen bidez *(Gilula eta lank., 1978)*. Beraz, obozitoaren garapena, inguratzen duten pikor-geruzako zelulekin (kumuluko zelulak) duen loturaren araberako da. Alde batetik, obozitoak pikor-geruzako zelulen eta kumuluko zelulen funtzioak erregulatzen ditu (adibidez, glukosaren metabolismoa) *(Sugiura eta lank., 2005)*, apoptosia saihestu eta zelulen proliferazioa eta esteroidogenesia bultzatzen duten faktore espezifikoak (GDF-9 eta BMP15) jariatzen ditu. Gainera, estradiolaren ekoizpena mantenduz eta progesteronaren ekoizpena inhibituz *(Vanderhyden eta*

Macdonald 1998), folikuluen diferentziazioa sustatzen du fase aurreantraletik antralera eta kumuluko zelulen luteinizazioa ekiditen du *(Eppig 2001, Matzuk eta lank., 2002; Dragovic eta lank., 2005; Diaz eta lank., 2007a, 2007b.)*

Bestalde, pikor-geruzako zelulek nukleosidoez, aminoazidoez eta fosfolipidoez hornitzen dute obozitoa, mRNAren egonkortasuna eta balantze ionikoa mantentzeko *(Procházka eta lank., 1998; Hunter, 2000)*. Ez hori bakarrik, pikor-geruzako zelulen jarduera garrantzi handikoa da obozitoaren garapenerako, metabolito esentzialak eta seinale erregulatzaileak ematen dizkiolako. Gainera, obulazioa hasten da zuzenean teka zeluletan eta pikor-geruzako zeluletan eragiten duen LH hormonaren gorakadagatik, obozitoaren heltze-prozesua, folikuluaren apurketa eta gorputz luteoaren sorrera bultzatuz *(Russell eta Robker, 2007)* **(1.5 irudia)**.



1.5 irudia. Obozitoaren eta pikor-geruzako zelulen arteko bi norabideko komunikazioa. Komunikazio parakrinoa eta Gap loturen bidezko komunikazioa beharrezkoak dira folikulua eta obozitoa garatzeko (FF-MAS: meiosia aktibatzen duen likido folikularreko esterola, GDF-9: hazkundearen desberdintzapen-faktorea 9, BMP-15). Obozitoek GDF-9 jariatzean SMAD familiako kidea 2 edo 3 (SMAD 2/3) edo MAP kinasen menpeko proteina (MAPK) aktibatzen da pikor-geruzako zeluletan. Halaber, horiek pikor-geruzako zelulen funtzio eta gene-adierazpen ugari arautzen dituzte. Badirudi zelula-obozitoaren arteko bi norabideko komunikazioak obozitoaren kalitatea hobetzen duten obozitoko prozesu ezezagunak erregulatzen dituela *(Gilchrist eta lank., 2004-tik moldatua)*.

Horrez gain, folikuluetako zelula somatikoek obozitoak meiosian geldirik mantentzea ahalbidetzen dute eta seinale batzuk bidaltzen dituzte meiosia berraktibatzeko eta obozitoaren heltze-prozesua bukatzeko (metafase II-ra heltzeko) *(Eppig, 2001)*. cAMP kontzentrazio altuek meiosia inhibitzen dutenez, proposatu da cAMPren kontzentrazio altuak, obozitoak ekoiztu ez ezik, pikor-geruzako zelulen bidez lortzea. Izan ere, besteak beste, pikor-zeluletan agertzen diren C-motako peptido natriuretikoa (CNP)

eta 2 motako peptido natriuretikoaren hartzailea (NPR2) izeneko molekulei esker mantentzen dira cAMParen maila altuak 1.1.4.2 atalean azalduko dugun bezala *(Romero eta lank., 2016)*. CNP da NPR2 hartzailearen estekatzaile endogenoa eta pikor-geruzako zeluletan ekoizten da *(Zhang eta lank., 2010)*. Gainera, obozitoak jariatutako faktoreek eta estradiolak erregulatzen dute kumuluko zeluletan dagoen NPR2ren adierazpena. Horrek iradokitzen du obozitoak berak babesten duela meiosiaren geldialdia kumuluko zeluletan gakoak diren faktoreen adierazpena erregulatzean *(Zhang eta lank., 2011)*. Obozitoak kumuluko zelulen funtzioaren gain duen kontrolak iradokitzen du COCaren mikroingurune egokia izatea garrantzitsua dela obozitoak gaitasun meiotikoa eta garapen gaitasuna eskura ditzan eta ikerketa-talde ugarik hipotesi hau indartzeko lanean dihardu *(Assidi eta lank., 2011; Wathlet eta lank., 2012)*.

1.1.4 Obogenesia

Obulutegian dauden obozitoak enbrioiaren endodermotik eratortzen diren zelula germinal primordialetik sortzen dira. Ondoren, mesenteriorantz migratuko dute eta gandor genitaletan kanporatzen dira. Zelula horiek handiak dira eta nukleo biribila dute nukleolo bat edo gehiagorekin. Gainera, mitokondria txikiak, erretikulu endoplasmatikoa, polierribosomak, Golgi aparatua bezalako organuluak, mikrofilamenduak eta glukogeno partikula eta lipido tanta kopuru aldakorrak ageri dira zitoplasman *(Picton, 2001)*. Hasieran, zelula germinal primordialak obulutegira garraiatuko dira transferentzia pasiboaren bidez, enbrioiaren garapenean zehar gertatzen diren aldaketengatik. Behin obulutegi primordialera heltzean, zelula hauek mugikortasuna galtzen dute eta obogonietan (2n) bihurtzen dira. Obogoniak zatiketa mitotikoaren ondorioz banatzen hasiko dira kopuruz handitu arte. Azken horiek meiosian sartuko dira eta obozito primarioak sortuko dira, lehen zatiketa meiotikoko profasean sartuko direlarik. Ugaztun emeetan, bizitza fetalean zehar, obozitoen populazioa era bateratuan hasten da zatiketa meiotikoarekin.

Obozitoen heltze-prozesua obogenesiaren azken etapa da, guztiz garatua dagoen obozitoa (n) ernaldua izateko prestatzen denean. Ugaztunetan, obozitoek lehenengo aktibazio meiotikoa jasaten dute enbrioiaren garapenean zehar, eta, jaiotzean, I. profaseko diploteno fasean geldituta geratzen dira heldutasun sexuala lortu arte (gizakietan, nerabezaroan). Ugalketa ziklo bakoitzean zehar, LHaren kontzentrazioaren gorakadak meiosiaren berraktibazio eragiten du II. metafasera (MII) arte, hots, obozitoen heltze-prozesua bultzatzen du *(Liu eta lank., 2012)*. Ugaztunetan obozitoek meiosia obulutegian hasten dute oraindik etapa fetalean daudenean, baina helduen ugalketa-aparatuan ernaldu ondoren bakarrik amaitzen dute.

Obozitoaren heltze-prozesuan zehar zitoplasman, mintzean eta nukleoan aldaketa ugari gertatzen dira obozitoa ernalketarako ondo prestatuta egon dadin eta enbrioia garapenaren hasierako etapan mantendu ahal izateko.

Obozitoen heltzea obulazio aurreko gonadotropinek bultzatutako erantzun folikularrak eraginda hasten da. Horrek obozitoari ernalkuntzari eta enbrioiaren garapen goiztiarrari eusteko gaitasuna ematen dio. Heltze fase horretan, pikor-geruzako zelulek esteroideak ekoizten jarraitzen dituzte, baina progesterona nagusitzen hasten da. Gainera, azido hialuronikoa ekoizten dute, kumuluko zelulak hedatzea eta obozitoaren eta zelula horien arteko Gap loturen galera ahalbidetzen duena. Heltze nuklearraren eta zitoplasmatikoaren gaitasuna batera eskuratzen da *(Moor eta lank.,1998; Picton eta lank., 1998)*.

1.1.4.1 Obozitoaren garapen konpetentzia: zitoplasmaren heltzea

Aurretik aipatu dugun bezala, garapen-gaitasuna obozito batek ernaldu eta enbrioi batean bihurtzeko duen gaitasuna bezala definitzen da eta zitoplasmaren heldutasunaren menpe dago (heltze zitoplasmatikoa). Heltze zitoplasmatikoan zehar obozitoaren barruan eman beharreko aldaketa guztiak gertatzen dira, hala nola, mRNAren eta proteinen metaketa, organuluen eta zitoeskeletoaren berrantolaketa eta zelulen metabolismoan aldaketak. Beste era batera esanda, heltze nuklearrean meiosiaren berraktibazioa eta lehenengo korpuskulu polarraren askapena gertatzen bada, heltze zitoplasmatikoan, ezarpena baino lehen garapena burutzeko gaitasuna eskuratzen du obozitoak. *In vitro* heldutako obozitoen garapen gaitasuna *in vivo* heldutakoena baino askoz baxuagoa da, besteak beste, heltze zitoplasmatikoan akatsak egon direlako *(Xie eta lank., 2016)*.

Aipatu bezala, zenbait organuluk kopuruan eta antolaketan aldaketak izaten dituzte, hala nola, erribosomak eta mitokondriak mobilizatzen dira eta lekuz aldatzen dira, erretikulu endoplasmatikoa berrantolatzen da, pikor kortikalen sekrezioa eragiten da, enbrioiaren garapen goiztiarrerako esentzialak diren transkriptoen metaketa gertatzen da eta katioiak askatzeko gaitasuna eskuratzen da *(Ducibella eta Buetow, 1994; Eppig eta lank., 1998a; Stojkovic eta lank. 2001; Terasaki eta lank. 2001; Abbott eta lank., 2001; Ducibella eta lank., 2006)*.

Golgi aparatuak obozitoan daukan parte hartzeari buruz oso gutxi dakigu oraindik. Momentuz, argi dagoena da garapenean eta heltze-prozesuan zehar garrantzia duela eta beharrezkoa dela seinaleztapen desberdinetan parte hartzen duten proteinen adierazpenerako *(Ferreira eta lank., 2009)*. Pikor kortikalak Golgi aparatutik eratorritako organuluak dira eta obozitoetan soilik aurkitzen dira. Horiek, proteinez, egiturazko molekulez, entzimez eta glikosaminoglikanoez osatuta daude eta obozitoek polispermia ekiditeko erabiltzen dituzte *(Ferreira eta lank., 2009)*. Ugaztunen obozitoetan erreakzio kortikala eragiten duten seinaleztapen zelularrek inositol fosfato kaskada aktibatzea dute (PIP 2). Horrez gain, G proteinek erregulatzen duten espermatozoidearen eta obozitoaren arteko fusioak bigarren mailako mezulari batzuen ekoizpena eragiten du, besteak beste, inositol 1,4,5 trifosfatoarena (IP 3) eta diazilglizerolarena (DAG) *(Ferreira eta lank., 2009)*. Horrez gain, mintz peluzidoa eratzeko beharrezkoak diren glikoproteinen esportazioan parte hartzen du *(Picton eta lank., 1998)*.

Erretikulu endoplasmatikoaren funtzio ezagunen artean, proteinen tolestura eta degradazioa, lipidoen metabolismoa, nukleoaren banaketa, Ca^{2+} ioien gradientearen erregulazioa eta mintzen sintesia daude *(Lippincott-Schwartz eta lank., 2000)*. Kaltzioa pilatu eta askatzean, sistema honek seinaleztapen intrazelularrean funtzio garrantzitsua betetzen du. Kanal ionikoetara loturiko hartzaileek erregulatzen dute zitoplasman metatuta dagoen Ca^{2+}-aren askapena, esaterako, IP3aren eta rianodinaren hartzaileek. Biak daude erretikulu endoplasmatikoaren mintzean. Ernalketan zehar, obozitoaren aktibaziorako ezinbestekoa da Ca^{2+} askatzea IP3 eta bere hartzailearen bidez (IP3R) *(Ferreira eta lank., 2009)*. Gainera, obozitoaren heltze-prozesuan zehar erretikulu endoplasmatikoaren egitura aldaketak eta aldaketa biokimikoak erabakigarriak dira kaltzioaren erregulazio intrazelular egokiarentzako. Kaltzioaren askapenarekiko sentikortasuna obozitoaren heltze-prozesuaren ondoren emendatzen da, ernalketan espermatozoideak obozitoan sartzen direnean erretikulutik Ca^{2+} askatzen baita *(Ferreira eta lank. 2009)*.

Heltze zitoplasmatikoan, bestalde, kromosomek adierazitako mRNAn transkripzioa, biltegiratzea eta prozesaketa gertatzen da, proteinetan itzuliak izango direnak gero, erribosometan. mRNA horietatik eratorriak izango diren proteinek, heltze-prozesuan inplikatuta egoteaz gain, ernalketan, pronukleoen sorreran eta enbriogenesi goiztiarrean parte hartzen dute. Horrela, proteina horiek une egokian erabili arte gordetzen dira, enbrioiaren genoma aktibatu arte, alegia *(Ferreira eta lank., 2009)*.

Folikulugenesian zehar, RNA eta proteinen ekoizpenean ere aldaketak gertatzen dira. Hazkunde fasean, obozitoak hainbat RNA moten kantitate handiak sintetizatzen ditu: % 60-65 RNA erribosomala (rRNA), % 20-25 transferentzia RNA (tRNA) eta % 10-15 RNA heterogeneoa (hRNA). Azken horrek RNA mezularia (mRNA) barne hartzen du, obozitoan metatuko dena enbrioiaren garapenari laguntzeko *(Picton eta lank., 1998)*. Obozitoaren mRNAren transkripzioa eta metaketa folikulugenesian gertatzen da, baina meiosian zehar besikula germinala apurtzen denean gelditzen da *(Watson, 2007)*. Berehalako erabileraren eta erreserbako RNA mezulariaren arteko desberdintzapenak duen garrantzia dela eta, obozitoak degradazioen aurkako bi babes estrategia garatu ditu:

a. poliadenilazioa 5' muturrean, bi mRNA motetarako ohikoa den poli-A sekuentziarekin, baina luzera desberdinekoa dena *(Picton, eta lank. 1998; Bashirullah, eta lank. 2001)*.

b. 3' poliadenilazio laburra erreserbako RNA mezularian (kontserbatua dagoen AAUAAA segidarekin) *(Picton eta lank., 1998; Bashirullah eta lank., 2001)*. Aldatutako mRNA proteinekin ere lotzen da, degradazioetatik babesten duen konplexu erribonukleoproteiko bat eratuz eta obozitoaren zitoplasman gordetzea ahalbidetzen duena, heltze-prozesuan edo enbrioiaren garapenean beharrezkoa den arte *(Fulka eta lank., 1998)*.

Hala ere, heltze zitoplasmatikoarekin lotutako zelularen aldaketa ugarietatik, metabolismoa da kritikoenetarikoa. Izan ere, animalia heldugabeen obozitoek enbrioiaren garapenerako gaitasun baxua daukate eta jarduera metabolikoa eraldatuta daukate animalia helduen obozitoekin alderatuta.

Metatzen doazen produktuen berrantolaketa eta erabilera obozitoen heltze-prozesuaren funtsezko atala da. Desberdintzapen gertaera hauek ez daude heltze nuklearraren menpe; hala ere, ziklo meiotikoaren progresioa kontrolatzen dute eta obozitoaren garapen gaitasuna eskuratzeko beharrezkoak diren gertaera intrazelularrak erregulatzen dituzte. Oraindik, ez dago metodorik garapen gaitasuna eskuratu duten eta bidean geratu diren obozitoak bereizteko eta obozitoen heltze-prozesuarekin lotutako nukleoaren morfologia aldaketak erabiltzen dira horretarako *(Hyttel eta lank., 1997)*. Garapen gaitasun hori *in vitro* ernalkuntza eta enbrioien transferentzia-teknologiaren bidez soilik ebaluatu daiteke *(Sirard eta lank., 2003)*.

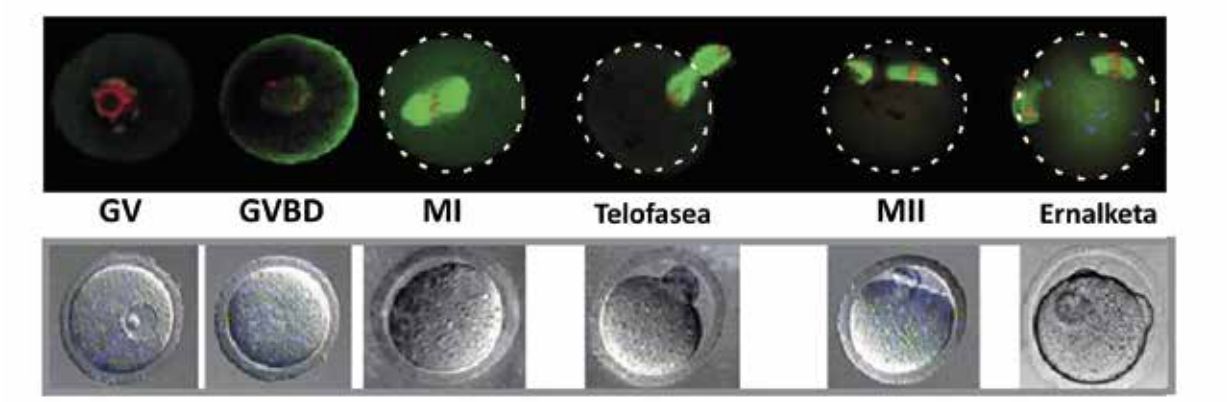
1.1.4.2 Obozitoaren garapen meiotikoa: nukleoaren heltzean

Obozitoen heltzea deritzo meiosiaren lehenengo blokeotik bigarren geldialdiko prozesuari eta ugaztun espezie gehienetan *in vivo* meiosiaren berraktibazioa edota nukleoaren heltzea obulazioaren aurreko

LHaren emendioak bultzatzen du *(Webb eta lank., 2002; Keefe eta lank., 2015)*. Aldiz, *in vitro*, meiosiaren berraktibazioa eta heltzea espontaneoki gertatzen dira obozitoa folikulutik banatzean *(Moor, 1988)*.

Aurreko atalean azaldu bezala, obozitoaren heltze-prozesuan zehar zitoplasman, mintzean eta nukleoan aldaketa ugari gertatzen dira, obozitoa ernalketarako ondo prestatuta egon dadin eta enbrioia garapenaren lehenengo etapan mantendu ahal izateko. Heltze nuklearraren kasuan, aldaketa nagusienen artean daude, besteak beste, besikula germinalaren (GV) haustura (GVBD), kromosomen kondentsazioa eta I. metafaseraino (MI) aurreratzea, lehenengo korpuskulu polarraren estrusioa eta bigarren zatiketa meiotikoaren metafasean (MII) gelditzea *(Picton eta lank., 1998)*. Azpimarratu behar da meiosia zelula germinalen (espermatogonia eta obogonia) bereizgarria den zelula-zatiketa mota dela. Bere helburua bikoitza da: kromosoma kopuru diploide (2n) bat kopuru haploide (n) batera murriztea eta informazio genetikoaren birkonbinazioa egitea *(Polanski eta lank., 2005)*.

Meiosiaren ezaugarri bereizgarriak dira elkarren segidako bi zatiketa zelular (I. eta II. meiosia) eta ziklo zelularreko bi geldialdi daudela: besikula germinalean (GV) I. profasean eta II. metafasean. Geldiarazte hauek hiru gertakari garrantzitsuren artean jazotzen dira: (1) fetuan, mitositik GVaren geldialdira, azido erretinoikoaren bidez erregulatua; (2) I. meiositik igarotzea eta (3) II. meiosiaren bukaera ernalketaren ondoren. Zatiketa meiotiko biak ziklinaren menpeko kinasaren (CDK1) jardueraren bidez erregulatuak daude *(Jones eta lank., 2008)*. Azken finean, ugalketan, gameto haploideak (n) sortu behar dira bikoiztutako genoma diploide (2n) batetik. Obozitoetan, genomaren erdia I. meiosiaren anafasean kanporatzen da, korpuskulu polarra deituriko garatu gabeko zelula txiki batera. Ondoren, geratzen den genoma erdia bigarren kospuskulu polar batera kanporatzen da II.meiosiaren anafasean *(Fabritius eta lank., 2011)* **(1.6 irudia)**.



1.6 irudia: Sagu obozitoaren heltze nuklearra. Goian: obozitoen inmunofluoreszentzia heltze-prozesuan [mikrotubuluak (berdez), kromatina (gorriz) eta espermatozoideak (urdinez)]. Behean: obozitoen morfologia heltze-prozesuan. **Laburdurak:** GV: besikula germinala, GVBD: besikula germinalaren haustura, MI: I. metafasea, MII: II. metafasea. *(Kubiak eta lank., 2008-tik moldatua).*

Hurrengo paragrafoetan zehatzago deskribatuko dira obozitoen heltze-nuklearrean zeharreko etapak eta bakoitzean gertatzen diren aldaketak.

Nukleoaren heltzeak hiru fase ditu:

1. **Fasea:**

Lehenengoa, jaiotza aurreko lehen blokeo edo geldiarazte meiotikoa da. Ugaztunetan, enbrioi emeen obulutegietan, obozitoak lehenengo meiosiaren profaseko fase diplotenean daude geldituta eta seinale zehatzak jasotzen dituzten arte ez dira berpizten bigarren fase meiotikora heltzeko. Obozito horiek hedugabeak dira eta besikula germinal bezala izendatzen dira (GV) *(Picton eta lank., 1998; Sagata, 1998)*. Obozito horiek G2 fase mitotikoaren baliokidea den etapan daude, DNA bikoiztuta baitago meiosiarekin hasi aurretik. I. profasean, kromosoma homologoen artean sinapsiak eratzen dira, tetradak ikusten dira eta kromatida homologoen artean elkar-gurutzamenduak gauzatzen dira. Horregatik, GV fasean dauden obozitoek DNA kantitate tetraploidea daukate 46 kromosoma bikoitz dituztelako. GV edo obozitoaren nukleoa, esferikoa da eta nukleolo handi batez osatua dago, barneragarria eta exozentrikoa. Besikula germinala obozito heldugabeetan eta garapen fasea etenda daukaten obozitoetan oboplasmaren erdialdean kokatzen da. Obozito osasuntsuetan GV posizio kortikal baterantz mugitzen da 1.6 irudian ikus daitekeen bezala *(Jones, 2008)*.

Etapa honetan, meiosia ingurune folikularreko faktore desberdinek eta pikor-geruzako zelulek inhibitzen dute.

Horren ostean, obulazioa baino ordu batzuk lehenago (gizakietan 12-24 h), LHaren estimulazioak meiosiaren berraktibazioa eragiten du eta besikula germinalaren haustura (germinal vesicle breakdown, ingelesez; GVBD) gertatzen da *(Picton eta lank., 1998; Smith, 2001)*. GVaren haustura hori da meiosiaren berraktibazioaren lehenengo adierazle mikroskopikoa.

2. **Fasea:**

Bigarren fasean, seinale espezifikoak jasotzean, meiosia berraktibatzen da I. metafaseraino **(MI)**, hormonen estimulazioaren ondoren *in vivo* eta ingurune folikularretik askatzean *in vitro*. I. metafasean dagoen obozitoak lehenengo meiosiko profase etapa bukatu du eta GVa eta haren nukleoloak desagertu dira.

Meiosiaren lehenengo berraktibazioan, kromosoma homologoak (kromatida bikoak eta kiasmetatik lotuta) banatzen dira, I. metafaseko ardatz mitotikoa eratzen da eta lehen gorputz polarra kanporatzen da. Aurreko etaparen aldean, honek ordu batzuk besterik ez du irauten. Hala ere, funtsezkoa da obozito konpetentea sortzeko *(Sun eta Nagai, 2003)*.

Mikroskopioan, MI faseko obozitoetan ez da korpuskulu polarra ez eta GVa ageri. Oboplasma homogeneoa dauka **(1.6 irudia)**.

3. **Fasea:**

Hirugarren fasea, obulazioa baino lehen gertatzen da lehenengo meiositik (MI) bigarren meiosiaren metafasera pasatuz (MII); eta orduan gertatzen da meiosiaren bigarren geldiaraztea. Bigarren meiosiaren

berraktibazioa espermatozoidearen bitartez (edota estimulu partenogenetikoen bitartez) gertatzen da eta espermatozoideak obozitoarekin kontaktua dutenean obozitoa guztiz heltzen da, haploidea (n) bihurtuz eta bigarren gorputz polarra askatuz *(Sun eta Nagai, 2003)*. MII estadioan dauden obozitoak jada helduak dira *(Jones, 2008)*.

Beraz, azkenean, obozitoaren heltze nuklearra plaka metafasikoaren periferiaranzko migrazioarekin bukatu behar da eta korpuskulu polar baten formako kromosomen kanporatzearekin. Horrela, kromosoma haploideen multzo batez eratutako obozitoa ernaltzeko prest dago *(Sathananthan eta lank., 2006)*.

- **Meiosiaren geldiaraztea:**

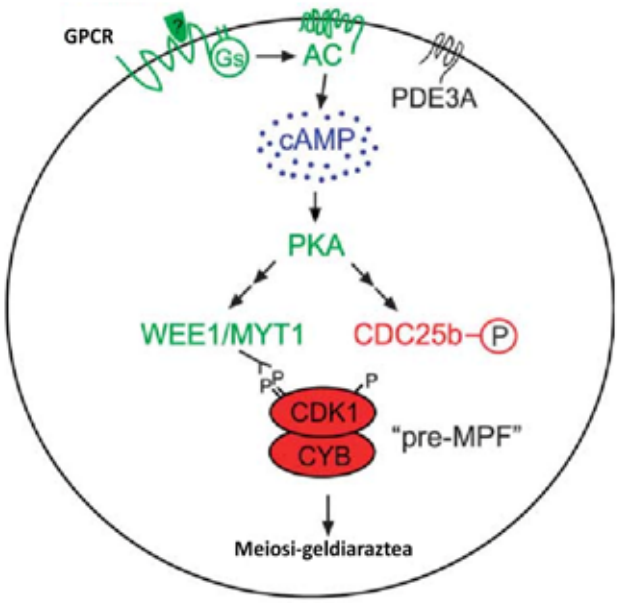
Meiosiaren geldiaraztea edota berraktibazioa folikulu antralen oboplasmako AMP ziklikoaren mailaren erregulazioak baldintzatzen du. cAMPren maila altuek meiosia inhibitzen dute eta AMP ziklikoaren maila baxuek, aldiz, berraktibatu *(Downs eta lank., 1989, Nogueira eta lank., 2003a, Thomas eta lank., 2004, Vanhoutte eta lank., 2008)*. Oraindik ez dira guztiz ezagutzen zeintzuk diren cAMPren mailak erregulatzen dituzten mekanismoak baina obozitoaren barruan cAMP mailak erregulatzeko prozesuen hipotesi ezberdinak planteatu dira:

i. cAMParen sarrera obozitoaren eta pikor-geruzako zelulen arteko Gap loturen bidez *(Thomas eta lank., 2004)*.
ii. Obozitoaren cAMParen sintesi endogenoa G proteinari lorturiko hartzaile baten bidez *(Vaccari eta lank., 2008)*.
    a. Adenilato ziklasaren bidez, ATP cAMP bihurtzen da *(Kalinowski eta lank., 2004)*.
    b. PKAren aktibazioaren bidez heltzea sustatzeko faktorea (*Maturation Promoting Factor* ingelesez, MPF) inaktibatzen da.
iii. Zelula somatikoek (teka zelulak, pikor-geruzako zelulak: muralak eta kumulukoak) Guanosina Monofosfato ziklikoz (cGMP) hornitzen dute obozitoa. cGMPk fosfodiesterasaren (PDE) jarduera inhibitzen du (cAMP degradatzen duen entzima) *(Tornell eta lank., 1991)*

Dena den, gero eta ebidentzia gehiago dago obozitoaren barneko cAMPren mailak MPFren jarduerak baldintzatzen duela. Animalia espezie ugaritan, MPF da heltze-prozesuaren erregulatzaile unibertsala eta eginkizun garrantzitsua betetzen du obozitoaren heltze-prozesuko etapetan. MPF ziklinaren menpeko kinasaren konposatu heterodimeroa da (CDK1 azpiunitate katalitikoaz eta B ziklina (B1, B2 eta B3) azpiunitate erregulatzaileaz osatua).

Folikulugenesian zehar, obozitoak CDK1 eta ziklina B azpiunitateak metatzen ditu aurre-MPF konplexu bezala eta azken hori inaktibo mantentzen da cAMP kontzentrazio altuetan. Meiosiaren berraktibazioan CDK1ak bere itu proteinen serina eta treoninaren hondar espezifikoak fosforilatzen ditu baina guztiz aktibo izateko B ziklinara lotu behar da *(Pan eta Li, 2019)*. CDK1 inaktibatzeko, aldiz, Thr14 eta Tyr15 fosforilatu behar dira. Laburbilduz, obozitoaren cAMP kontzentrazio altuek CDK1aren fosforilazioa eragiten dute Thr14n eta Tyr15-ean eta MPF inaktibo bihurtzen da *(Mehlmann, 2005)*.

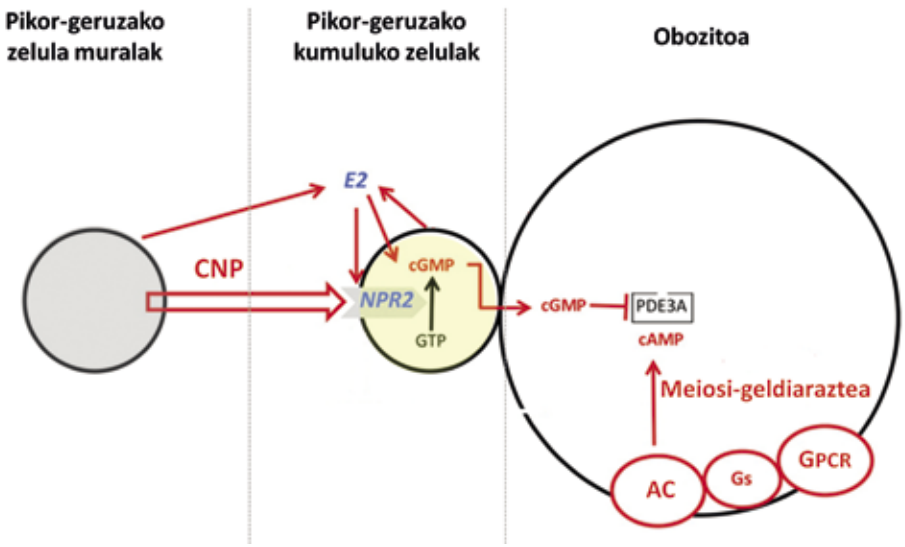
PKAren aktibazioaren bidez MPFa inaktibatzen da, zuzenean edo zeharka CDC25b fosforilatzen duelako (CDC25b-P). Zehaztugabeko pausu batzuen bidez, PKAk CDC25 fosfatasaren eta WEE1/MYT1 kinasaren jarduerak erregulatzen ditu. CDC25 fosfatasak CDK1 desfosforilatzen du eta WEE1/MYT1 kinasak, ordea, CDK1 fosforilatu egiten du. CDK1aren fosforilazioaren ondorioz, MPF inaktibo mantentzen da **(1.7 irudia)**. Adibidez, Cdc25b genea galdu duten saguek ezin dute MPF aktibatu eta, ondorioz, meiosia berraktibatzea ezinezkoa zaie *(Mehlmann, 2005)*.



1.7 irudia. Meiosiaren geldiaraztea bultzatzen duten seinaleztapen bidezidorrak. GPCRren aktibazioak, konstitutiboki edo ezagutzen ez den estekatzaile baten bidez, Gs aktibatzen du. Horrela, adenilato ziklasa (AC) estimulatzen da cAMP kontzentrazioak emendatuz. cAMPk proteina kinasa A (PKA) aktibatzen du, eta honek ziklo zelularren erregulazio-konplexua CDK1/B ziklina (CYB). CYB fosforilatzean (P) inaktibatu egingo da. Berdez dauden proteinak aktibatutako proteinak dira eta proteina gorriak, aldiz, inhibiturik daudenak meiosia berrabiatzeko aktibazio-bide klasikoan. *(Mehlmann 2005-etik moldatua)*.

Beraz, obozito barruan cAMPren kontzentrazioa bide desberdinetatik erregulatzen da. Alde batetik, ekoizpen aktiboaren bitartez (adibidez, adenilato ziklasaren bidez) eta, bestetik, hidrolisiaren bidez (fosfodiesterasen bitartez, PDE).

Azkenengo ikerketek baieztatu dute Guanosina Monofosfato ziklikoaren (cGMP) agerpenak ere obozitoan (Gap loturen bidez kumuluko zeluletatik bidalia) PDE3ren aktibazioa ekiditen duela eta, beraz, meiosiaren blokeoa mantentzen duela *(Norris eta lank., 2009)*. cAMP bezala, cGMP zelula eukariotoetan zein prokariotoetan agertzen den bigarren mezularia da, denbora luzean zehar bere zeregina ikertzaileen aldetik cAMParen itzalean egon den arren. cGMPa guadilil ziklasek ekoizten dute GTPtik abiatuz *(Turko IV eta lank., 1999)*. Orain arte, nahiko onartuta dago cGMPa kumuluko zeluletan ekoizten dela CNPak NPR2 aktibatu ondoren eta obozitora barreiatzen dela PDE3Ak cAMPan duen hidrolisi jarduna inhibitzeko **(1.8 irudia)**. Ondorioz, obozitoetan geldiarazte meiotikoa I. faseko diplotenoan mantentzen da (GV) (Pan eta Li, 2019). Are gehiago, CNP molekulak obozitoaren eta kumuluko zelulen arteko konexioak hobetzen ditu *(Zhang eta lank., 2010)*.



1.8 irudia. CNP proteinak induzituriko meiosiaren geldiaraztea kontrolatzen duten seinaleztapen bidezidorrak. cAMP maila altuak cGMPri esker mantentzen dira, azken honek, PDE inhibitzen duelako. cGMP pikor-geruzako zeluletan ekoizten da, zelula muraletatik jariatzen den CNPa kumuluko zeluletan dagoen NPR2 hartzailera lotzean, seguruenik, estradiolaren (E2) bidez. *(Zhang eta lank., 2010-etik moldatua)*..

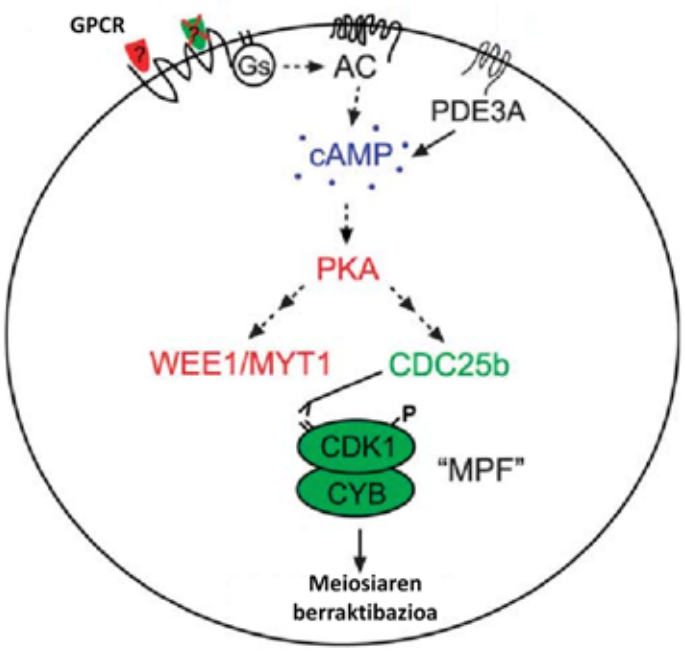
Arestian aipatutako aukera guztiak aztertu beharra dago zehazteko nola elkar eragiten duten folikuluko zelulek eta obozitoak cAMP maila altuak mantentzeko LHaren igoeraren aurretik.

- **Meiosiaren berraktibazioa:**

LHaren bidezko heltze meiotikoaren estimulazioa inguruko zelula somatikoen gainean duen ekintzaren bidez gertatzen da eta ez obozitoan, LHaren hartzaileak ez direlako ez obozitoan ez inguruko kumuluko zeluletan antzeman, kanpoaldeko pikor-geruzako zeluletan (muraletan) baizik. Hala ere, oraino ezezaguna da LHak zein mekanismoren bidez jarduten duen, eta horrela, pikor-geruzako zeluletan eraginez nola berraktibatzen duen meiosia obozitoan *(Mehlmann, 2005)*.

Badirudi, obozitoaren heltze-prozesuan zehar cAMP mailak goiz jaisten direnez, aukera gehiago dagoela meiosia induzitzen duen substantzia horrek cAMPren beherakada eman baino lehen jardutea *(Mehlmann, 2005)*, esaterako, G proteinari loturiko hartzaile baten bidez AC inaktibatuz edota PDE aktibatuz.

Gero eta indar handiagoa hartzen ari den hipotesiaren arabera, MPFaren fosforilazioak berrabiarazten du meiosia (Adhikari eta Liu 2014). Pre-MPF konplexua GVBDarekin batera aktibatzen da. Horretarako, CDK1 desfosforilatu behar da CYCBa lotzeko eta MPFa sortu dadin. Alde batetik, zenbait datuk baieztatzen dute folikuluaren barruan, PDE espezifikoek cAMParen mailak murrizten dituztela fosfoinositol 3-kinasa (PI3K) bidezidorraren aktibazioaren eraginez, AKT proteina fosforilatzen baitute eta azken honek PDE. PDEak cAMPa degradatzen du eta beherakada horrek PKA, WEE2 eta MYT1 kinasen desfosforilazioa eta inhibizioa dakar. CDC25Ba ere desfosforilatzen da, baina haren desfosforilazioak aktibatu egiten du. Aktiboa den CDC25Ba nukleora translokatzen da eta CDK1 aktibatzen du desfosforilazioaren bidez. Horri esker, CDK1 eta B ziklina lotuko dira MPF aktiboa sortzeko eta orduantxe berraktibatuko da meiosia *(Schindler, 2011)* **(1.9 irudia).**



1.9 irudia. **LHaren bidez eragindako obozitoen meiosiaren berraktibaziorako seinaleztapen bideak**. Berdez dauden proteinak aktibatutako proteinak dira eta proteina gorriak,aldiz, inhibiturik daudenak meiosia berrabiatzeko aktibazio-bide klasikoan (*Mehlmann, 2005*-etik moldatua).

Gainera, frogatu da bai LH hormonak eragindako meiosiaren berraktibazioa bai berraktibazio espontaneoa PDEaren bidezko AMP ziklikoaren hidrolisiaren menpekoa dela obozitoan. Bitarteko farmakologikoen bidez baieztatu zen obozitoan cAMParen kontzentrazio altuak mantentzean obozitoaren PDEaren inhibitzaile espezifikoak administratuz, meiosiaren berraktibazioa ekiditen zela nahiz eta hCGarekin eta epidermiseko hazkuntza-faktorearekin (EGF) estimulazio eragin zen (Shu eta lank., 2008).

Frogatua dago, baita ere, LH hormonak eragiten duen cAMPren beherakadak Gap loturen apurketa dakarrela eta Gap loturen komunikazioaren apurketak cAMPren hornidura gutxitzea eragiten duela oboplasman *(Sun eta Nagai, 2003)*. Proposatu da MAPK bidezidorrak eragiten duela Gap loturen fosforilazioa eta itxiera, cGMParen eta cAMParen hedapena mugatuz kumuluko pikor-geruzako zeluletatik obozitora eta, ondorioz, meiosia berraktibatuko litzateke *(Sun eta lank., 2009)*. Hala ere, azterketa esperimentalek baieztatu dute MAPK bidezidorraren inhibizioak Gap loturen ixtea eragozten duela, baina ez cGMPren kontzentrazioaren jaitsiera eta, beraz, ez duela meiosiaren berraktibazioa eteten *(Norris eta lank., 2008, 2009)*. Oro har, aurkikuntza horiek iradokitzen dute seinaleztapen bide gehiago aktibatzen direla LHak bultzatutako berraktibazio meiotikoan zehar.

Hala ere, oraindik ezezagunak direnez obozitoen heltze-prozesua pizten dituzten seinaleak, oso garrantzitsua da jakitea zeintzuk diren meiosia berrabiarazteko seinaleztapen ur-jauzi horiek pizten eta erregulatzen dituzten kanpoko seinaleak, farmakoen bidez kontrolatuz gero heltze-prozesua ere kontrolatu genezakeelako.

- **II. metafasean (MII) geldiaraztea**

Lehenengo zatiketa meiotikoa ez bezala, bigarren zatiketa mitosiaren antzekoa da: MPFaren jarduera azkar handitzen da eta ardatz mitotikoa arin eratzen da. Horrez gain, MII faseko kromosomak

kromosoma mitotikoen berdinak dira, hots, zinetokoro aktiboak dituzten kromatida ahizpak dira. Hala ere, ernalkuntza gertatu arte obozitoak luzaroan gelditzen dira MII-an etenda eta, bitartean, kromosomak ondo lerrokatuta daude metafase-plakan. Meiosiaren geldiarazte hori MPFaren eta MAPKaren jardueraren bidez mantentzen da. MAPKaren seinaleztapenak geldiarazte meiotikoan zehar ardatzaren egitura eta kromosomak lerrokatuta mantentzen ditu *(Jones, 2005)*.

Ernalketaren unean, espermatozoideak obozitoaren barruan sartzean, Ca^{2+} kontzentrazioak emendatzen dira eta horrek B ziklinaren degradazioa eta ziklo meiotikoaren amaiera bultzatzen du *(Brunet eta lank., 2005)*.

1.1.5 Heltze-prozesuko seinaleztapen-bide garrantzitsuenak

1.1.5.1 G proteinari loturiko hartzaileak (GPCR) obozitoen heltzean zehar

Obozitoaren heltze-prozesuan zehar MPFa aktibatuko duten eta bat egiten duten seinale transdukzio bide desberdinak aktibatzen dira *(Schmitt eta Nebreda, 2002)*. Iradoki da meiosiaren heltze-prozesua modulatuko lukeen G proteinari loturiko hartzaileren bat (GPCR) egotea. Obozitoetan G_s edo G_i bidezko seinaleztapena deskribatu da *(Richard eta lank., 2001; Shitsukawa eta lank., 2001; Horner eta lank., 2003; Mehlmann, 2005)* eta obozitoan adierazten diren 15 GPCR inguru identifikatu dira. Hala ere, oraindik ez da ezagutzen hartzaile gehienen funtzioa ez eta haien ligandoena zein den ere *(Mehlmann eta lank., 2004)*. Soilik GPR3 eta GPR12 barne hartzen dituzten hartzaileen talde bat aztertu da, biak G_s-ari lotuak, modu aktiboan egongo liratekeenak eta meiosiaren geldialdia mantenduko luketenak *(Mehlmann eta lank., 2004; Hinckley eta lank., 2005)*. Adibidez, *Xenopus* espeziearen obozitoetan progesteronaren hartzailea adenilato ziklasa inhibitzen duen G proteinei lotuta dagoela iradoki da *(Mehlmann eta lank., 2004; Hinckley eta lank., 2005)*.

Ugalketa funtzioa erregulatzen duten bi gonadotropina nagusien hartzaileak, FSHR eta LHR, GPCR familiaren zati dira *(Themmen eta Huhtaniemi 2000; Ascoli eta lank., 2002;)*. FSHRak eta LHRak seinaleztapena GPCR gehienak bezala erregulatzen dute, β arrestinen bitartez mintzaren banaketaren ondoren aktibatuz *(Ferguson eta lank., 1996; Lowther eta lank., 2011)*. LHaren tontorrak ere seinaleztapena aktibatzen du epidermiseko hazkuntza-faktorea (EGF) eta bere hartzailearen (EGFR) bidez *(Park eta lank., 2004; Su eta lank., 2010)*. EGFR sistemaren eta G proteinari loturiko hartzaileen arteko komunikazio estu hori beste zelula mota batzuetan ere ikusi da *(Gschwind eta lank., 2001)*.

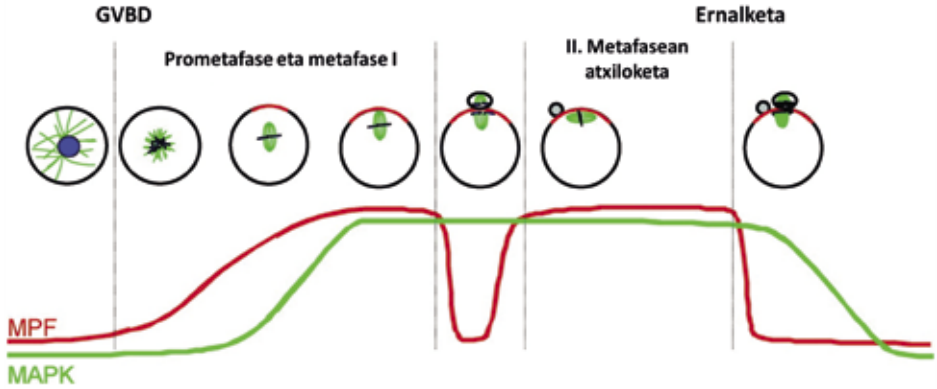
1.1.5.2 MAP kinasen (MAPK) bidezidorren seinaleztapena obozitoen heltze-prozesuan

Obozitoen heltze-prozesua ziklo zelularreko kinasek bultzatzen dute, batez ere, ziklo mitotikoaren erregulazioaz arduratzen direnak. MPFa eta mitogeno bidez aktibatutako proteina kinasak (MAPK) funtsezko molekulak dira meiosia erregulatzen dituzten bidezidorretan eta ardatz mitotikoaren eraketan

parte hartzen dute. Izan ere, haien aktibazioa GVaren hausturarekin batera gertatzen da. Heltze-prozesuan zehar haien jarduera emendatuz doa, jarduera-maximoa obozitoa heldua denean eskuratzen duten arte **(1.10 irudia)**.

Horrez gain, obozitoetan, LHRaren eta FSHRaren estimulazioarekin lotutako lehen gertakarietako bat da MAPK bideen aktibazioa *(Seger eta lank., 2001; Sela-Abramovich eta lank., 2005)*, beste GPCR mota batzuekin ikusi den bezala *(Ferguson eta lank., 1996)*.

MAPKak obozitoetan meiosiaren berraktibazioa bultzatzeaz gain ardatz mitotikoaren eraketan, MI-etik MII-rako trantsizioan eta MII-ko meiosiaren geldiaraztean parte hartzen du *(Liang eta lank., 2007)*. Gainera, esteroidogenesiaren erregulazioan, pikor-geruzako zelulen biziraupenean eta ugaritzean esku hartzen du *(Sasseville eta lank., 2010)*. Baina, batez ere, II. metafasearen antolaketa mantentzen laguntzen du eta, aipatu bezala, II. metafasean gehien adierazten den bidezidorretarikoa da. Horregatik proposatzen da MAPKaren bidezidorrak eginkizun garrantzitsua duela meiosiaren bigarren geldiaraztearen erregulazioan *(Wang eta lank., 2010)* **(1.10 irudia).**



1.10 irudia. MPFaren (gorriz) eta MAPKaren (berdez) jarduerak sagu-obozitoetan heltze-nuklearrean zehar.
Heltze-nuklearraren urrats desberdinak eskematizatu dira *(Brunet eta Maro, 2005)*.

MAPKaren seinaleztapenaren barruan hobekien ezaugarritu diren proteinak ERK1 eta ERK2 (*Extracellular signal-regulated kinases*, ingelesez) dira *(Zhang eta Liu, 2002)*. Bi ERK mota horiek obozitoen heltze-prozesuan zehar isolatu dira *(Duranthon eta Renard, 2001)* eta zeregin garrantzitsua daukate nukleoaren garapen gaitasuna eskuratzeko beharrezkoak diren seinaleztapen ur-jauzietan *(Sun eta Nagai, 2003)*. Horien aktibazioa tirosina (Tyr204/187) eta treonina (Thr202/185) hondarren fosforilazio bidez gertatzen da *(Crews eta Erikson, 1992; Roskoski, 2012)*.

ERK 1/2 seinaleztapen-molekula oso garrantzitsua da meiosian zehar, batez ere, ardatz mitotikoaren muntaduran eta mikrotubuluen antolaketan. Hain zuzen ere, ERKaren adierazpena ezabatuta duten (Erk1/2$^{-/-}$) saguen obozitoetan ez da meiosia berraktibatzen nahiz eta hCG hormona ziztatu zaien *(Fan eta lank. 2009)*. *In vitro* egindako lanetan, obozitoen heltze-mediora hormonak gehitzean ERKaren adierazpena emendatzen da MII etapan eta blastozistoen garapenean ere igoera adierazgarria ikusten da *(Song eta lank., 2018)*. Gainera, EGF hartzaileak ERK1/2aren aktibazioa bultzatzen du *(Fan eta*

lank., 2009; Yamashita eta lank., 2009). Guzti hori kontuan izanda, ERK1/2 obozitoen heltze-prozesuan ebaluatu beharreko markatzaile garrantzitsu gisa proposatzen da *(Conti eta lank., 2012)*.

1.1.5.3 PI3K/AKT bidezidorraren seinaleztapena obozitoen heltze-prozesuan

Fosfatidilinositol 3-kinasaren (PI3K) seinaleztapena funtsezko bidezidorra da zelulen proliferazioa, biziraupena, migrazioa eta metabolismoa erregulatzeko. Minbizian parte hartzen duen seinaleztapen-bide garrantzitsua ere bada *(Lee eta Chang, 2019)* baina, azken urteetan, genetikoki eraldatutako saguek eman duten informazioari esker, ezagutzera eman da PI3Kak ezinbesteko eginkizuna duela saguen obarioen obogenesian, folikulugenesian (folikulu primarioen aktibazioan eta biziraupenean), obulazioan eta kartzinogenesian *(Zheng eta lank., 2012)*.

PI3Kak inositol fosfolipidoen inositol eraztunean dagoen 3'-OH taldea fosforilatzen duten lipido kinasak dira. PI3Karen isoformak hiru klasetan banatu dira (I. klasea, II. klasea eta III. klasea), substratuen lehentasunetan eta sekuentzia-homologian oinarrituta. Funtzio fisiologikoei dagokienez, I. klaseko PI3Ka da ugaztunetan glukosaren homeostasia, zelulen migrazioa, hazkuntza eta ugaritzea erregulatzeko hobeto ezaugarritu dena *(Cantley, 2002; Engelman eta lank., 2006; Vanhaesebroeck eta lank., 2010)*. I. klaseko taldea, gainera, tirosina kinasa hartzaileen bidez aktibatua izan daiteke, hurrenez hurren, EGFR, GPCR, FSHR eta LHRen bidez *(Vanhaesebroeck eta lank., 2010)*.

FSHak cAMP/PKA eta PI3K bidezidorrak aktibatzen ditu eta, horren ondorioz, aromatasaren adierazpena areagotzen da (CYP19). Aromatasak androgenoak estradiolean bihurtzen ditu, FSHaren eragina areagotzen du folikuluetan eta obulutegiko folikuluaren garapena bultzatzen du *(De Souza, 2018)*.

PI3K bidezidorra osatzen duten osagaiak dira, besteak beste, B protein kinasa, AKT bezala ezagunagoa, 1 motako proteinaren menpeko kinasa (PDK1), FOXO transkripzio faktoreak, eta GSK-3α eta GSK-3β proteina kinasak *(Reddy eta lank., 2005; Liu eta lank., 2007)*. Frogatuta dago horiek guztiak obozito mailan daudela eta, ondorioz, heltze-prozesuaren mekanismoak ulertzeko bidezidor aztertuenetakoa izan da *(Liu eta lank. 2006)*.

AKTa serina/treonina familiako proteina kinasa bat da eta ugaztunen genoman 3 isoforma kontserbatu dira AKT1 (PKBα), AKT2 (PKBβ) eta AKT3 (PKBγ), bakoitzak berezko propietate eta funtzioak dituenak. AKTaren aktibazioa PI3K bidezidorraren seinaleztapenarekin gertatzen da eta bide horren oinarrizko molekula da *(Manning eta Toker, 2017)*. AKTa bi lekuetan fosforilatzen da: domeinu katalitikoan treonina hondarrean (T308) eta C-terminalean serina hondarrean (S473) eta seinale intrazelularrak itzultzen ditu fosforilatutako proteinen bidez *(Cecconi eta lank., 2010)*. AKTa oso molekula garrantzitsua da PI3K bidezidorrean eta 2 pausotan aktibatzen da:

1. PI3Kak fosfatidilinositol-3,4,5-trifosfatoa (PIP3) sintetizatzen du eta PIP3ak AKTa erakartzen du mintz zelularrerantz. Hor treonina 308 hondarrean fosforilatzen du partzialki aktibatzeko. Monofosforilatutako AKTa zitoplasmara askatzen da.

2. Zitoplasman AKTaren aktibazio maximorako rapamizinaren 2 konplexuak (mTOR) fosforilatu behar du serina 473 (S473) hondar hidrofoboan *(Manning eta Toker, 2017)*. Behin guztiz aktibatuta dagoenean, AKT proteinak seinaleztapen bidezidor asko erregulatzen ditu *(Manning eta Cantley, 2007)*.

AKTa folikuluen garapenarekin *(Cecconi eta lank., 2012)*, obozitoen heltze-prozesuan meiosiaren berraktibazioarekin, ardatz mitotikoaren eraketarekin *(Hoshino eta Sato, 2008; Kalous eta lank., 2009)* eta II. metafasean bigarren korpuskulu polarraren kanporatzearekin *(Cecconi, eta lank. 2010)* erlazionatu da. Horrez gain, AKTaren parte hartzea deskribatu da enbrioiaren garapenean eta ikusi da haren aktibazioak sagu blastozistoen eklosioa kaltetu dezakeela *(Riley, eta lank. 2005)*. Azkenik, AKTaren gabezia ugalkortasun arazoekin erlazionatu da saguetan *(Brown eta lank., 2010)* eta gizakien kasuan AKTaren erregulazioan egon diren akatsak ugalketa mailako gaixotasun batzuekin lotu dira, esate baterako, obario polikistikoa sindromearekin eta obulutegiko minbiziarekin *(Cecconi eta lank., 2012)*.

1.2 *In vitro* heltze-prozesuaren (ingelesez, *in vitro maturation*, IVM) teknika

IVM teknikaren bidez pazienteari, hormonekin estimulatu gabeko edo estimulazio txikia jaso duten obarioen folikulu antraletatik, COCak erauzten zaizkio eta hazkuntza-medioetan heltzen dira *(De Vos eta lank., 2016)*, ondoren, *in vitro* ernalketa (ingelesez, *in vitro fertilization*, IVF) edo espermatozoide-injekzio intrazitoplasmatikoa (ingelesez, *intracytoplasmic sperm injection*, ICSI) tekniken bidez haurdunaldia lortzen saiatzeko *(Barnes eta lank., 1995, 1996)*.

IVM teknika lehenengo aldiz Pincus eta Enzemann-ek (1934) proposatu zuten eta 1965.urtean Edwards izan zen, untxietan garatutako lan aitzindaria oinarritzat hartuta *(Pincus eta Enzmann, 1934)*, obozitoen *in vitro* heltzea zehatz-mehatz deskribatu zuen lehena. Espezie desberdinetako obozitoak heldu zituen laborategian, besteak beste, saguaren, ardiaren, behiaren, txerriaren, txakurraren, *Rhesus* espezieko tximinoaren eta gizakiaren obozitoak *(Chang, 1955; Edwards 1962)*, obulutegiak estimulatzeko hormona exogenoak administratu beharrik gabe. Hala ere, 1990. hamarkada hasierarte ez zen lehenengo haurra jaio IVM teknikaren bidez *(Cha eta lank., 1991)*. Ordutik hona, munduan 5.000 haur baino gehiago jaio dira *(Lu eta lank., 2018)*.

Ohiko IVF teknikan *in vivo* heldutako obozitoak erauzten dira, ostean, *in vitro* ernaltzeko eta azkenean, *in vitro* sortutako enbrioiak pazienteari transferitzen zaizkio *(Lonergan eta Fair, 2016)*. IVM teknikarekin, ordea, obozito heldugabeak erauzten dira *in vitro* heltzeko eta, ondoren, IVF teknikarekin jarraitzen da. IVM oso aukera erakargarria da, obozitoen eta enbroien kopurua emendatzeko erabiltzen den gonadotropinen bidezko obarioen estimulazioa gutxitzen edo ekiditen delako *(Lonergan eta Fair, 2016)*. Izan ere, obario polikistiko sindromea (PCOS) zeukaten emakumeentzako ugalketa-teknika bezala plazaratu zen, gonadotropinak induzituriko obarioen hiperestimulazio sindromea pairatzeko arriskua murrizten duelako *(Trounson eta lank., 1994)*. Beraz, obozitoen *in vitro* heltze-prozesua garatzeak abantaila garrantzitsuak ditu, haurdunaldia lortu nahi dutenentzat ez ezik, baita animalien ekoizpen sistemetarako ere.

Azken hamarkadan IVM teknikaren hobekuntza nabarmenak egon diren arren, IVMaren emaitzak ez dira beste ugalketa-teknikak bezain eraginkorrak eta oso aldakorrak dira klinika batetik bestera (heltze-tasari, enbrioi-tasari eta IVMtik eratorritako enbrioiak ezartzeko ahalmenari dagokionez) *(Sanchez eta lank., 2017)*. Horren arrazoia neurri batean izan liteke, alde batetik, ugalkortasun zentroen arteko IVM protokoloen eta ikuspuntu teknikoen heterogeneotasuna. Izan ere, IVM protokoloaren aldaketa nabarienetarikoa izan da obulazioa hCGarekin induzitzea (albaitaritzan erabiltzen den moduan) denbora labur batez gonadotropinekin estimulatu ondoren. Gonadotropina bidezko tratamendua, folikuluen garapena hobetzeko eta, horrela, obozitoen garapen gaitasuna emendatzeko egiten da *(Sanchez eta lank., 2017)*. Metodo berri honi "hCGk bultzaturiko IVM edo trukatutako IVF" deritzo *(De Vos eta lank., 2016)* eta obozitoen heltze-tasa hobetzen dela erakutsi duen arren (PCOS pazienteetan) jaiotza-tasa ez da emendatzen *(Sanchez eta lank., 2017)*. Gainera, gaur egun, klinikan 6-12 mm bitarteko folikuluak obulaziora bultzatzean obozitoen bilduma heterogeneoa eskuratzen da, hau da, MII, MI edota GV fasean

dauden obozitoak obulatzea eragiten da eta, horrek, asko zailtzen du laborategian, hurrengo pausoa den IVF teknikaren bitartez, enbrioiak lortzea. Eta, bestetik, folikulu antral goiztiarretatik lortzen diren obozito heldugabeen garapen gaitasuna oraindik baxua da *(Herta eta lank., 2018)*.

1.2.1 Heltze-ingurunea eta hazkuntza

Lagunduriko ugalketa-tekniken (ingelesez assisted reproductive techniques, ART) artean, obozitoen *in vitro* heltzea (IVM) da gutxien garatuta dagoena (urtean zikloen % 0,1) *(Kupka eta lank. 2016)*, baina bere ezarpenak aurrerapen kualitatiboa suposatuko luke.

Esan bezala, obozitoen *in vitro* heltzea *in vitro* ernalkuntza (IVF) baino lehen egiten den pausu bat da eta, teknika horretan, hormonekin estimulatu gabeko edo estimulazio txikia jaso duten obulutegietako folikulu antraletatik heldu gabeko obozitoak erauzten dira (besikula germinal fasean; GV), azkenik, hazkuntza-inguruneetan exogenoki heltzeko. Obozitoen heltzerako erabiltzen diren hazkuntza-inguruneak dituzten osagaiak dira gatzen disoluzio orekatuak, bikarbonato indargetzailea eta proteina- eta energia-iturriak. Heltze-ingurunean gehitzen diren ioi inorganikoak bide metabolikoetan eta seinaleztapen bidezidorretan parte hatzen dutenak dira, sodioa (Na^+), potasioa (K^+) eta kaltzioa (Ca^{2+}), hurrenez hurren *(Thompson, 1996)*. Indargetzaile bezala erabiltzen den bikarbonatoak (25 mM) pH 7.2 eta 7.4 bitartean mantentzen laguntzen du, pH fisiologikoaren berdina. Prozesu hori guztia kontrolaturiko baldintzetan egiten da, orokorrean, % 5eko CO_2-arekin. Normalean, energia-iturria pirubatoa izaten da baina proteinak eta aminoazidoak ere gehitzen dira. Azkenik FSHa, LHa, estradiola edota EGFa bezalako hormonak gehitzen dira LH hormonaren tontor aurreobulatorioa sorrarazten duen *in vivo* seinaleztapena simulatzeko eta modu horretan meiosiaren berraktibazioa bultzatzeko *(Sirard, eta lank., 1988)*. Osagai horiez gain, tenperatura faktore garrantzitsua da heltze-prozesuan, 37 ºC eta 39 ºC bitartekoa izanik ugaztun espeziearen arabera.

1.2.2 Balizko pazienteak eta onurak

Gaur gaurkoz, IVM teknika abeltzaintzan, zelula-ama enbrionarioen teknologietan, klonazioan eta animalia transgenikoen ekoizpenerako gehien erabiltzen den metodoa da *(Herta eta lank., 2018)*. Baina gizakietara bideratutako tratamenduetan ez dago hedatua, nahiz eta IVM metodoaren abantaila garrantzitsuena den hormonen estimulazioen ondorioz egon litezkeen albo-kalteak murriztuko liratekeela eta aukera bakarra izan daitekeela gonadotropina dosi altuak onartzen ez dituzten pazienteentzako, esate baterako:

- Obulutegietako sindrome polikistikoa (PCOS) daukatenen pazienteentzako *(Nader 2010; González-Ortega eta lank., 2019)*. Obario polikistikoa deituriko gaitza jasateko probabilitatea % 3tik % 7ra igo da azken urteotan eta paziente horientzako hormonen estimulazioa kontraindikatua dago *(Eilertsen, 2012)*. Gainera, obulutegietako hiperestimulazio sindromea izateko arrisku altua daukate. PCOS duten emakume antzuetan obozito heldugabeak *in vitro* heldu ondoren enbrioien transferentziaren

bidez haurdunaldi klinikoaren eta inplantazioaren tasak % 35-40 eta % 15-20 izatera iritsi dira, hurrenez hurren, *(Chian eta lank., 2013)*.

- Obulutegietako hiperestimulazio sindromea (OHSS) daukaten pazienteentzako. Hainbat ugalketa ziklo egitearen albo kalte ohikoena bihurtzen ari da OHSSa. IVF zikloen % 33an gertatzen da eta zikloen % 2a larria izan daiteke, gaixoaren bizia arriskuan jarriz *(Delvigne eta Rozenberg, 2002; Halupczok eta lank., 2015)*.

- Aurretiaz izan duten gaixotasun batengatik obozitoak edota obulutegien zati bat izoztu eta bitrifikazioa egin zaien pazienteentzako, estimulazio hormonala ez zelako gomendagarria edo ez zelako denbora nahikorik egon lagunduriko ugalketarako ziklo normal bat egiteko (ugal-adinean dauden pazienteak dira ohikoenak, minbiziaren aurka kimioterapia/radioterapiak jaso dituztenak) *(Nikseresht eta lank., 2015)*. Nabarmendu beharra dago azken hamarkadatan % 1,5 igo dela ugaltze-aldian dauden emakumeen minbizia izateko arriskua *(Rodriguez-Wallberg eta Oktay, 2014)*.

- Leuzemia izan duten pazienteentzako (haurren minbizi-kasu ohikoena da). Zelula ama hematopoietikoen transplanteetarako hautagaiak izan ohi dira eta kasuen artean % 70-90ak obulutegietan kalteak izaten ditu. Gainera horientzako kontraindikatuta dago obulutegiko ehunaren transplantea, minbizidun zelulak berriz ezartzeko arriskua dagoelako *(Grynberg eta lank., 2013)*.

- Obulazioan disfuntzioa edo obulaziorik ez duten (anobulazioa) pazienteentzako: hormonekin estimulatu arren obozito helduak lortzeko ezintasuna duten pazienteak *(Luciano eta lank., 2013)*.

- Obulutegiko erreserba gutxitua duten pazienteentzako: normalean zelula-germinalen kalitatea gutxitzen delako eta erabakigarria da haurdunaldia lortzeko; amatasun berantiarra da adibiderik ohikoena *(Lee eta lank., 2015)*.

- Azaldu ezineko antzutasuna diagnostikatu zaien pazienteentzako: ezin da baztertu antzutasunaren kausa ugalketa prozesua baldintzatzen duen obozitoen heltze okerra dela *(Coticchio, eta lank., 2015)*..

Horrez gain, badira antzutasun arazorik izan gabe lagunduriko ugalketen beharra dutenak eta kasu horietan ere IVM izango litzateke hormona jaso behar ez izateko aukerarik onena. Izan ere, obozitoak erazteko hormona dosia murriztu edo ekidingo litzake *(Söderström-Anttila eta lank., 2005)*. Horrela, ugalketa-tratamendu osoaren urrats arriskutsuena saihestuko litzateke, hain zuzen ere, hormonen administrazioak higadura handia dakar eta albo-kalte desatseginen agerpena maila desberdinetan *(Cousineau eta Domar 2007)*.

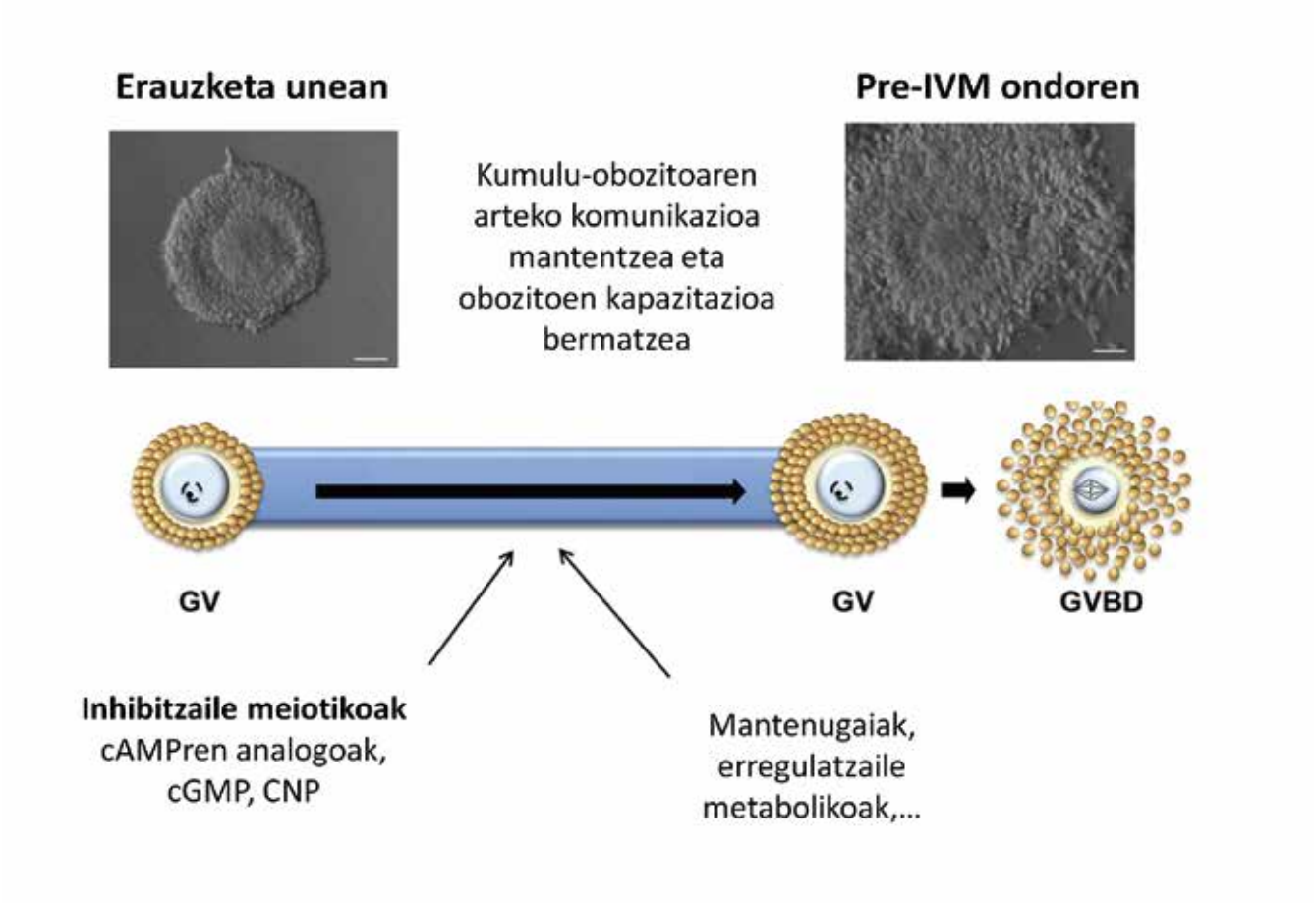
Amaitzeko, kontrolatutako obarioen estimulazio ekiditen denez, IVM prozedurarekin ez da beharrezkoa ultrasoinuen bidezko jarraipena eta, beraz, ez da ohiko *in vitro* ernalketa (IVF) bezain garestia *(Chang eta lank., 2014)*.

1.2.3 Pre-IVM fasea edo kapazitazio-fasea (CAPA-IVM)

IVM teknikaren erabilgarritasuna handitzeko asmoz, teknika horren beste erronka bat da diametro txikiagoko COC talde homogeneoagoak eskuratzea, folikuluen gehiegizko garapena ekiditeko. Heltze-prozesuko etapa bereko COCak eskuratzeko (GV fasekoak) beharrezkoa da gizakietan hCGaren administrazioa ekiditea (eta animaliekin egindako tratamendu esperimentaletan, behorraren gonadotropina korionikoa (eCG) ematea) *(Romero eta lank., 2016)*. Saguekin egiten diren ikerketa gehienetan eCG hormona erabiltzen da nahiko garatuta dauden folikuluetatik obozitoak eskuratzeko. Hala ere, honek *in vitro* heltze-eredua baldintzatu dezake obozitoaren garapen gaitasuna ere baldintzatzen delako. Hau da, obozitoaren garapen gaitasuna, ernaldua izateko gaitasuna eta enbrioi bideragarri baten bihurtzeko gaitasuna gradualki lortzen da folikulugenesian zehar. Prozesu hori obozitoaren hazkuntzarekin hasten da folikulugenesiaren lehenengo etapetan. Obozitoa bere amaierako tamainatik hurbil dagoenean, beste eraldaketa batzuk jasango ditu, hain ikusgarriak ez direnak baina zeharo garrantzitsuak izango direnak ugalketa-helburua lortzeko, garapen gaitasuna zehazteko, alegia. Folikuluak baldintzatzen dituen eraldaketa horiei deritze obozitoaren "kapazitazioa" eta garapen gaitasuna duten obozitoen proportzioa folikuluen tamainarekin handitzen da (horregatik erabiltzen da, adibidez, eCG) *(Mermillod eta lank., 1999)*. Kapazitazio-prozesuan obozitoak ezarpenaren aurreko garapenean laguntzen duen makineria zitoplasmatiko guztia eskuratzen du *(Herta eta lank., 2018)*. Hala ere, askotan, hazkuntza folikularraren eta obozitoaren garapen gaitasunaren arteko erlazioa ez da zorrotza izaten, obozito jakin batek gaitasun hori folikulugenesiaren edozein etapatan lor dezakeelako eta adibide ugari daude folikuluen tamainaren eta obozitoen konpetentziaren arteko desoreka gertatzen dela frogatzen dutenak *(Mermillod eta lank., 1999)*.

IVM teknikaren berrikuntza bide horretatik dator: hormonatu gabeko folikulu antral goiztiarretatik eratorritako obozito heldugabeek gaitasun meiotikoa eta garapen gaitasun osoa eskuratzea (*Romero eta lank., 2016)*. Horretarako, obozitoen heltze-prozesuan gertatzen den sinkronizazio eza gainditu beharra dago (heltze zitoplasmatikoaren eta nukleoaren heltzearen artean). Izan ere, folikulu antraletatik erauzten diren obozitoetan heltze nuklearra arinegi berraktibatzen da eta kapazitazio-prozesua asaldatzen da *(Herta eta lank,. 2018)*. Beraz, meiosiaren berraktibazio goiztiarra saihestu behar da, lehenago heltze nuklearraren eta zitoplasmatikoaren arteko sinkronizazioa hobetzeko *(Herta eta lank., 2018)*. Helburu hori lortzeko, *in vitro* heltzearen aurreko pauso luzeago bat gehitu da, pre-IVM (*in vitro* aurre heltze-prozesua) edo CAPA-IVM (kapazitazio-kultiboa). Etapa horretan, garapen gaitasuna eskuratzeko denbora nahikoa izateko helburuarekin, IVM egin aurretik meiosiaren geldiaraztea behartzen da eta cAMParen seinaleztapen bidezidorrak modulatzen dira cAMP maila altuak mantentzeko obozito barnean. cAMP maila altuak mantentzeko bideetariko bat PDEa inhibitzen duten konposatuak erabiltzea izan da, esaterako, CNPa *(Sanchez eta lank., 2019)* **(1.11 irudia)**. Konposatu hori, obozitoaren heltze-prozesuaren inhibitzaile naturala da eta konposatu honen potentziala obozitoen garapen gaitasuna hobetzeko eta, ondorioz, enbrioiaren kalitatea emendatzeko, frogatua izan da ugaztunetan *(Sanchez eta lank., 2017)*.

Aipatutako guztia dela eta, garrantzitsua da obozitoen heltze-prozesuan jarduten duten seinaleztapen bideak zeintzuk diren jakitea, horrek *in vitro* heltze-prozesua nola hobetu dezakeen jakiteko.



1.11 irudia. **Obozitoen kapazitazioa eta in vitro heltzearen aurreko etapa (Pre-IVM).** Obozito guztiak ez dira konpetenteak enbrioi batean bihurtzeko. COCak folikulutik erauztean bat-bateko meiosiaren berraktibazioa gertatzen da eta, ondorioz, aukera gehiago dago heltze nuklearraren eta zitoplasmatikoaren artean sinkronizazio arazoak egoteko. Irudi honetan, Pre-IVM kultibo estrategia posible bat irudikatu da obozitoen garapen gaitasuna eta IVM teknika hobetzeko xedearekin. Meiosiaren blokeoa eragile farmakologikoen bidez lor daiteke, obozitoak denbora nahikoa izan dezan heltze nuklearra eta heltze zitoplasmatikoa sinkronizatzeko. GVaren haustura (GVBD) inhibitzean edo atzeratzean heltze gaitasun handiagoa eta blastozisto kopuru altuagoa eskuratzen da. *(Herta eta lank., 2018-tik moldatua).*

1.3 Ernalketa eta enbrioiaren garapena

1.3.1 Ernalketa

Ernalketan oso desberdintzapen altuko bi zelula haploide (n), obozitoa eta espermatozoidea, elkartuko dira eta elkar aktibatu ondoren zigoto diploide bat (2n) eratuko da. Horretarako, beharrezkoa da bai obozitoak bai espermatozoideak zigoto bideragarria sortzea bermatuko duten aldaketak jasan behar izatea. Tesi honetan nahiz eta obozitoan zentratu garen laburki azalduko dugu ernalketa gertatzeko beharrezkoa den beste zelula germinalaren sorrera nolakoa den eta ernalketarainoko prozesuan jasaten dituen eraldaketak zeintzuk diren.

1.3.1.1 Espermatozoideen heltzea

Espermatogenesia bukatu eta gero, espermatozoideak, oso zelula espezializatuak izan arren, oraindik ez dira gai obozitoa ernaltzeko. Aldaketa morfologikoez gain, aldaketa fisiologiko eta biokimiko sakonak ere jasan behar izango dituzte epididimoan zeharreko garraioan. Aldaketa horiei guztiei espermatozoidearen heltze-prozesua deritze *(Yanagimachi, 1994)*. Eiakulazioan zehar, espermatozoideak likido seminalean dauden faktore inhibitzaileekin batzen dira, ondoren azalduko diren kapazitazioa eta erreakzio akrosomikoa deituriko prozesuak saihesteko *(Töpfer-Petersen eta lank., 2000)*. Espermatozoideen ernalketarako gaitasun osoa emeen ugaltze-aparatuan bukatzen da, kapazitazioarekin eta erreakzio akrosomikoarekin *(Sadler, 2004)*.

1.3.1.2 Espermatozoideen kapazitazioa

Espermatozoideek ernalketaren azkeneko pausoak aurrera eramateko emearen ugaltze aparatuan jasaten duten aldaketa multzoari kapazitazio deritzo *(Sadler, 2004)*.Espermatozoideek ernaltzeko gaitasuna lortu dezaten emearen ugaltze traktuarekin kontaktuan egon behar dira faktore inhibitzaileengandik askatzeko obozitoa ernaldua izan aurretik *(Töpfer-Petersen eta lank., 2000)*. *In vitro*, saguetan eta gizakietan, espermatozoideen kapazitazioa epe laburrean gerta daiteke medio nahiko sinple eta kimikoki definituan inkubatuta *(Yanagimachi, 1994)*.

1.3.1.3 Espermatozoideen akrosomaren erreakzioa eta ernalketa

Espermatozoideen kapazitazioa gertatu ondoren, akrosomaren (espermatozoidearen buruaren alde apikalean kokatzen den besikula) kanpo-mintza eta espermatozoidearen buruko mintz plasmatikoa fusionatzean dira *(Flesch eta Gadella, 2000)*. Horrek, lehenik eta behin, akrosoman dauden entzimak askatzea eragiten du, nagusiki, hialuronidasa eta akrosina, eta horrela espermatozoideak pikor-geruzako zelulak zeharkatu ahal izango ditu. Obozitoaren mintz plasmatikoa granulu kortikalen mintzarekin fusionatzean proteinen exozitozia ahalbidetzen du, askatzean polispermia blokeatzeaz arduratzen baitira *(Liu, 2011)*.

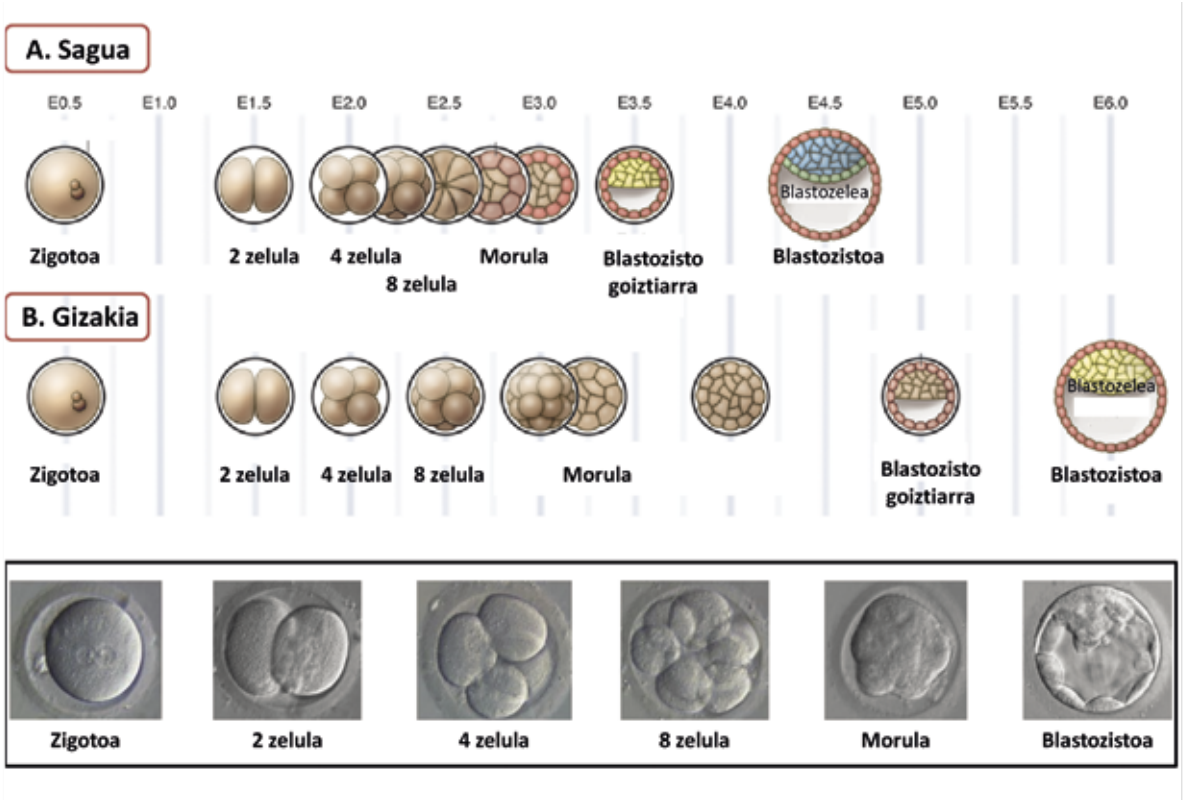
Ernalketa, gertaera bakar batez baino, prozesu multzo batzuez osatuta dago. Bere zentzurik zabalenean, espermatozoidea obozitoaren *corona radiata*-n barneratzen denean hasten da eta espermatozoidea obozitoan sartu ondoren emearen eta arraren kromosomak nahastean bukatzen da. Ernalketan obozitoaren metabolismoa aktibatzen da eta oso prozesu garrantzitsua da zigotoaren segmentaziorako eta enbrioiaren garapenerako *(Talmor-Cohen eta lank., 2002)*.

1.3.2 Enbrioiaren garapena

Obozito sekundario helduen eta espermatozoide bideragarrien arteko ernalketak pronukleo maskulino eta femeninoa duen enbrioi zelulabakarraren sorrera dakar (zigotoa ere deritzo). Ernalketaren ondoren, enbrioia morfologikoki desberdindutako hainbat etapatatik pasatzen da eta zelula kopuruaren arabera sailkatzen da **(1.12 irudia)**: zelula bakarreko enbrioiak zatiketa mitotiko sekuentzialak izaten ditu 2 zelula, 4 zelula, 8 zelula, 16 zelula, morula eta blastozisto etapetatik pasatzeko *(Assou eta lank., 2011)*.

Hala ere, teknologia "omikoen" aroan, oraindik ere, ernalkuntzaren inguruko mekanismoen ezagutza mugatua da, prozesu honetan morfologikoki eta funtzionalki desberdinak diren bi zelula (obozitoak eta espermatozoideak) batzen baitira organismo oso bat eratzeko. Are gehiago, zelula germinal horiek hurrengo belaunaldirako informazio genetikoa eta epigenetikoa igortzeaz arduratuko dira *(Canovas eta lank., 2017)*. Horregatik, ugaztunetan enbrioiaren garapenaren inguruan egindako ikerketek oso informazio garrantzitsua ematen digute etapa horietan gertatzen diren prozesu konplexuak ulertzeko, hala nola, garapen zinetikoa, enbrioiaren genoma aktibatzeko unea, genearen adierazpen patroia, kromosomen segregazioa eta aldaketa epigenetikoen patroiak *(Schultz, 2002; Santos eta Dean, 2004; Niakan eta lank., 2012; Gutierrez-Adan eta lank., 2015)*.

Urte askotan zehar egindako ikerketek enbrioiaren garapenaren eredu oso bat sortu dute saguan, erregulazio atal desberdinak integratzen dituena: morfologikoa, zelularra, transkripzionala, post-transkripzionala eta epigenetikoa. Hala ere, karraskariak ez ziren ugaztunetan egindako lehenengo ikerketek erakutsi zuten ugaztun espezieen artean nabarmen aldatzen direla bai denborak bai desberdintasun goiztiarreko gertakarien mekanismoak *(Oron eta Ivanova, 2012)*. Hori dela eta, laburki deskribatuko dugu enbrioiaren garapen goiztiarra saguetan eta gizakietan **1.12 irudian** ikusi daitekeen bezala.



1.12 irudia. Saguaren eta gizakiaren enbrioiraren garapeneko etapak ezarpenerarte. (A) Saguan, ernaldutako obozitoak hiru zatiketa jasango ditu, zortzi zelulako enbrioia sortuz. Hortik aurrera, zatiketa zelularrak bi zelula-populazio sortzen ditu, enbrioia barrualdea osatuko dutenak eta kanpoaldea osatuko dutenak. Blastozelea deituriko barrunbea enbrioiaren barruan 32 zelulako fasetik eratzen hasten da eta enbrioia hazten eta heltzen doa blastozistoaren faseraino. (B) Gizakiaren enbrioi goiztiarraren garapena (ezarpenaren aurrekoa) ere antzerakoa da, nahiz eta trinkotzea 8-16 zelularen fasean gertatzen den *(Cockburn eta Rossant, 2010)*.

1.3.2.1 Ezarpen aurreko enbrioiaren garapena

Ezarpen aurreko fasearen iraupena, zigototik (ernalketa unetik) blastozistora arte (ezarpen aurreko fasea), 4 egunekoa da saguetan *(Bedzhov eta lank., 2014)*, eta behia eta gizakia bezalako beste espezie batzuetan, berriz, 7 egun behar dira prozesua osatzeko *(Norwitz eta lank., 2001; Lonergan eta lank., 2016)*.

- **Zigotoa**

Lehenengo etapan, zigotoan, pronukleoa garatzen da, DNAren erreplikazioa eta sintesia gertatzen da eta RNAren transkripzioa hasten da. Azkenik, pronukleo femeninoak eta maskulinoak fusionatzen dira (singamia) zigotoa sortzeko *(Rahman eta lank., 2008)*.

Ondoren, zigotoak hiru zatiketa jasaten ditu. Momentu honetan gertatzen da aurre-ezarpenaren garapeneko gertakari garrantzitsuenetako bat: zigotoaren genomaren aktibazioa. Enbrioiaren

transkripzioaren aktibazioa obozitoak emandako RNAren degradazioarekin batera gertatzen da, bi zelulako enbrioiaren fasean amaitzen dena *(Oron eta Ivanova, 2012)*.

- **2 zelulako enbrioia, 4-8 zelulako enbrioia eta 8-16 zelulako enbrioia**

Hurrengo etapa, 2 zelularen etapa, bi zelula simetrikoren lehen zatiketa mitotikoari dagokio. Saguetan zatiketa hori garapeneko lehenengo egunean gertatzen da, gutxi gorabehera, ernalkuntzaren ondorengo 24 orduetan [*hours post fertilization* (hpf), ingelesez]; gizakietan, berriz, 25 hpf-tan ematen da. Prozesuak aurrera darrai eta zelulek simetrikoki zatitzen jarraituko dute. Saguetan, zatiketak 12 orduko periodoetan ematen dira; 36 ordutara 4-8 zeluletako enbrioia sortzen da eta 48 ordutara 8-16 zeluletakoa ikus daiteke. Gizakietan, aldiz, 4-8 zeluletako enbrioia 40 hpf-tara sortzen da eta 8-16 zeluletakoa 50 hpf-tara *(Rahman eta lank., 2008)*.

- **Morula: trinkotzea eta polarizazioa**

Saguetan behin zortzi zelulako enbrioia osatuta trinkotu egiten da, zelulen arteko loturak sendotzen direlako eta enbrioi goiztiarraren zelulak (blastomeroak) lautu egiten dira. Trinkotzean E-katerinina eta kateninen (alfa, beta eta delta) arteko proteina konplexuak eratzen dira. Zelulen atxikimendua handitzen den momentu berean, blastomeroak polarizatu egiten dira eta, beraz, kanpoko eskualdeak (alde apikala) barrurantz begira dauden eskualdeetatik (basolateralak) bereziten dira *(Oron eta Ivanova, 2012*). Enbrioiak masusta itxura hartzen duenez morula izenez ezagutzen da. Gizakietan 16 zelula izatean hasten da trinkotzea *(Rahman eta lank., 2008)*. Oraindik ez dago argi nola hasten diren trinkotzea eta polarizazioa baina prozesu biak funtsezkoak dira blastozistoen leinuak ondo bereizteko.

- **Blastozistoa: Barneko zelula masaren (*Inner cell mass*, ICM, ingelesez) eta trofoektodermoaren (TE) desberdintzapena eta blastozelearen eraketa**

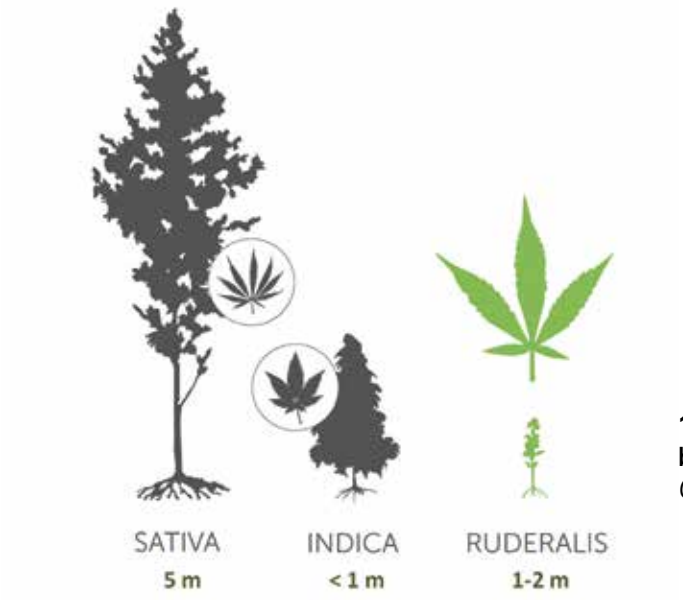
Enbrioia trinkotu eta polarizatu ondoren, kanpoko zelulak jariakina ponpatzen hasten dira enbrioiaren kanpoaldetik barrualdera eta jariakina pilatzen hasten da zelulen arteko espazio interzelularretan, pixkanaka-pixkanaka tamaina handitzen doan barrunbe bat eratzen delarik, blastozelea. Momentu horretatik aurrera enbrioia blastozistoaren fasean sartzen da. Blastozisto fasean bi zelula geruza desberdin sortzen dituzten bi zatiketa gehigarri gertatzen dira. Bi zelula-populazio horiek garapenerako helmugetan desberdintzen dira: kanpoko zelulek TE leinua eratuko dute, eta barneko zelulek, ICMa osatuko dute. ICMa da etorkizuneko enbrioiaren jatorria, eta ICMaren inguruan dagoen trofoektodermotik eratorriko da plazenta *(Oron eta Ivanova, 2012)*.

Blastozistoaren etapa berantiarrean enbrioia mintz peluzidotik ateratzen da eta prozesu hori eklosio izenez ezagutzen da. Momentu horretan, trofoektodermoko zelulak endometrioko epitelioari atxikitzeko gai dira eta enbrioiaren ezarpena gertatuko da *(Cockburn eta Rossant, 2010)*. Gizakiekin *in vitro* egiten diren esperimentuetan ezarpenaren aurreko enbrioiaren fase horretaraino heldu daiteke (14 eguneko enbrioia) (BOE-A-2006-9292).

1.4 Sistema kannabinoidea

1.4.1 Jatorria

Kalamu landarea *(Cannabis sativa L.)*, herri-mailan marihuana izenarekin ezaguna, *Cannabaceae* familiako landare loreduna da eta hainbat habitatetan eta altitudetan aurkitu daiteke, itsaso mailatik Himalaiako mendi alpinoetaraino. Badirudi jatorria hor bertan duela, Erdialdeko Asian, Himalaia mendikatean, nahiz eta 5000tik 6000 urte bitarteko antzinatasuna duenez zaila den espezie horren jatorria zehaztea *(ElSohly MA, 2017)*. Gaur egun, hiru barietate geografiko nagusi deskribatzen dira, *Cannabis sativa* (Europa eta Erdialdeko Asia), *Cannabis indica* (Hego Asia, Afrika eta Hego Amerika) eta *Cannabis ruderalis* (Erdialdeko Asia) eta, nahiz eta barietate bakoitzak osagai aktibo berberak dituen, horien osaera eta kantitatea alda daitezke (*ElSohly, 2017)* **(1.13 irudia)**.



1.13 irudia. Cannabis landarearen barietate nagusiak: *C. sativa, C. indica eta C. ruderalis*

Cannabis sativa-k oso historia aberatsa du, duela 4000 urte gutxienez, medikuntzan, helburu espiritualekin edota dibertimendurako erabilia baitzen *(Mechoulam, 1986)* eta lehenengo aipuak Kristo aurreko III. mendekoak dira. Txinan, K.a. 2600. urtean Nei Ching deituriko mediku batek gomendatzen zuen malariaren sintomak, idorreria, erreumak eragindako minak eta dismenorrea arintzeko *(Li, 1973)*. Kalamua janari-iturri eta ehungintzarako zuntz-iturri zaharrenetako bat da *(ElSohly, 2017)* eta aspaldidanik izan du oso harreman estua gizakiarekin. *(Mechoulam, 1986)*. Europan ere aspaldikoa da landare horren erabilera. Egipton eta mendebaldeko Asian sortu zen ehungintzarako kannabis landarearen kultiboa Europara Kristo aurretik 1000. urte inguruan sartu zen *(ElSohly, 2017)* eta, jada, VI. mendean kalamua landatzen zen. Mendebaldeko medikuntzara XIX. mende erdialdean sartu zen, Willian B. O'Shaughnessy mediku irlandarraren eskutik, Indian egon baitzen britainiarrei zerbitzatzen eta han ezagutu zituen landarea eta bere erabilera terapeutikoak. Gerora, O'Shaughnessy-k, 1843an, *"On the preparations of the Indian hemp, or gunjah"* lana argitaratu zuen *(Frankhauser, 2002)*. Garrantzia

handia izan zuen baita ere Moreu psikiatra frantsesak 1845ean argitaratu zuen liburuak, *"Du Hachisch et de l'Alienation Mentale: EtudesPsychologiques"* zeinetan kannabisaren eraginen deskribapen oso zehatzak egin zituen *(Frankhauser, 2002)*.

O'Shaughnessy-ren eta Moreau-ren lanek eragin handia izan zuten mendebaldeko medikuntzan, batez ere, aukera terapeutiko gutxi zegoelako amorrua, sukar beltza eta tetanosa bezalako gaixotasun infekziosoentzako. Kalamuaren erabilera medikoa Ingalaterra eta Frantziatik Europa osora hedatu zen eta ondoren Ipar Amerikara *(Frankhauser, 2002)*. 1860 urtean egin zen kalamuari buruzko lehenengo hitzaldi klinikoa Ohio Estatuko Medikuen Elkarteak antolatuta. XIX.mendearen bigarren erdialdean, 100 artikulu zientifiko baino gehiago argitaratu ziren Europan eta Estatu Batuetan kannabisaren balio terapeutikoari buruz. Gainera, XIX. eta XX. mendeen artean hainbat laborategik kannabisaren erauzkinak eta tinturak merkaturatu zituzten, hala nola, Merck (Alemania), Burroughs-Wellcome (Ingalaterra), Bristol Meyers Squibb (Estatu Batuak), Parke-Davis (Estatu Batuak) eta Eli Lilly (Estatu Batuak) konpainiek *(Frankhauser, 2002)*. Baina XX. mendeko lehen hamarkadetan, dosifikatzeko zituen zailtasunak eta konposatu berrien agerpenak, kannabis terapeutikoaren kontsumoaren gainbehera ekarri zuten. Horri gehitu behar zaizkio lege aldetik egon ziren murrizpenak *(Murray eta lank., 2007)*.

Hala ere, ikertzaile batzuek jarraitu zuten kannabinoideen ikerketarekin eta XIX. mende amaieran kannabinola (CBN) izan zen isolatu zen lehenengo landare-kannabinoidea (fitokannabinoidea), kannabisaren olio gorri baten estraktutik abiatuta. Horren egitura 1930. urtean argitu zen R.S. Cahn-eri esker eta, 1940ko hamarkadan, lortu zen sintesi kimikoa egitea Estatu Batuetako R. Adams-en eta Erresuma Batuko Lord Tood-en laborategietan *(Todd, 1946)*. Cannabidiola (CBD) izan zen eskuratu zen bigarren fitokannabinoidea Adams eta lankideen bidez, seguruenik azido kannabidiolikoarekin konbinatuta *(Pertwee, 2006)*. Garai berdintsuan, 1942an, Wollner-ek, Matchett-ek, Levine-ek eta Loewe-k tetrahidrokannabinola edo delta-9-tetrahidrokarbokannabinola (THC) erauzi zuten lehenengoz kannabisetik, hau ere Δ8- eta Δ9-THCarekin nahaste bezala *(Pertwee, 2006)*. Geroago, 1960ko hamarkadan, THC purifikatu eta egitura deskribatu zen *(Gaoni eta Mechoulan, 1964)*. Mechoulan-en laborategian lortu zen baita ere, 1965ean THCa eta CBDa sintetizatzea, lehen aldiz *(Pertwee, 2006)*.

Guzti horrek, kannabisaren osagai aktiboei buruzko ikerketak ugaritzen lagundu zuen *(Gaoni eta Mechoulam, 1964)* eta aro berria ireki zen kalamu landarea eta kannabinoideak izeneko haren konposatuak ikertzeko *(Pertwee, 2006)*.

1.4.2 Kannabinoideak

Kannabinoideak kalamu landareak (*Cannabis sativa* L.) sintetizatutako metabolito sekundarioen multzo gisa definitzen dira.

Cannabis sativa landarea konposatu kimiko ugarik osatzen dute. Identifikatutako 483 konposatuetako batzuk soilik kannabisaren landarean ageri dira, esate baterako, 60 kannabinoide baino gehiago;

aldiz, terpenoak, talde ugariena osatzen duten 140 mota inguru, landareen erresuman oso hedatuta daude. "Kannabinoide" terminoak orain arte *Cannabis sativa*-n soilik aurkitutako C21 konposatu terpenofenolikoen multzoa adierazten du *(Brenneisen, 2007)*.

1964. urtetik aurrera nagusitu zen kannabinoideen inguruko ikerketa, landare honen Δ^9-tetrahidrokannabinola (THC) *(Mechoulam eta Gaoni 1965)* osagai psikoaktibo ugariena aurkitu zenean. THC da efektu bioaktiboen arduradun nagusia eta kannabinoide ugariena, baina marihuanan dibenzopiranetik eratorritako 66 konposatu identifikatu dira *(ElSohly, 2002)*. Horietatik, garrantzitsuenetarikoak Δ^9-tetrahidrokannabibarina (THCren efektua antagonizatzen duena), kannabidiola (CBD, bigarren konposatu ugariena eta eragin psikoaktiborik gabea) eta kannabinola (CBN) dira *(Elsohly eta Slade, 2005)*.

1.4.3 Barne-sistema kannabinoidea

Kannabinoideen izaera lipofilikoagatik luzaroan pentsatu zen konposatu ez-hidrodisolbagarrien (anestesikoak, disolbatzaileak...) moduan jokatzen zutela, efektua mintz zelularren jariakortasunean eragindako aldaketengatik zela, alegia *(Lawrence eta Gill, 1975)*.

1980ko hamarkadaren erdialdean, bi aurkikuntza berritzaile egin ziren San Luis Unibertsitateko Allyn Howlett laborategian, kannabinoide-hartzaileak deskribatu ziren. Behaketa ugarik THCak hartzaile jakin batzuen bidez joka zezakeela iradokitzen zuten, Howlett-ek eta bere taldeak lehen aldiz hartzailea identifikatu zuten arte, 1 motako kannabinoide hartzailea (CB1, geroago CNR1 izendatuko dena) *(Howlett, 2005)*. Urte batzuk geroago, hartzailea karakterizatu zen eta bere kokapena, batez ere, nerbio-sistema zentralean deskribatu zen *(Devane eta lank., 1988; Matsuda eta lank., 1990)*. Ondoren, 2 motako kannabinoidea (CB2, geroago CNR2 deitua) identifikatu zen nagusiki maila-periferikoan eta sistema immunologikoan *(Munro eta lank., 1993)*.

Hartzaileen presentzia ezagututa, horiek aktibatzen dituzten barne-estekatzaileen bilaketa hasi zen, barne-kankabinoide edo endokannabinoide bezala ezagutzen direnak. Identifikatu zuten lehenengoa N-arakidonoiletanolamina izan zen, anandamida (AEA) bezala ere ezaguna (Devane eta lank., 1992). *"Ananda"* hitzetik dator, sanskritoz barruko zoriontasuna esan nahi duena eta estekatzaile horren efektu euforikoak deskribatzen ditu *(Fezza eta lank., 2014)*. Aurkitu zen bigarrena 2-arakidonoilglizerola (2-AG) izan zen *(Mechoulam eta lank., 1995)*. Bi konposatuak azido arakidonikotik eratorriak dira eta zelulak behar dituen unean bertan ekoizten dira. Bi konposatu horiek, CB1 zein CB2 hartzaileetara lotzeko gaitasuna dute baina hartzaile bakoitzarekiko dituzten afinitateak desberdinak dira **(1.2 taula)**. Endokannabinoide hauek metatu beharrean zelularen eskariaren arabera sintetizatzen dira eta, askatu ondoren, haien ekintza guneetatik ezabatzen dira zelularen andeakuntza prozesuen bidez.

Horrela, ondorioztatu zen sintesi eta andeakuntza entzimek ere parte hartzen dutela. Estimulu bati erantzunez, kaltzio zitoplasmikoaren metaketa gertatzen denean, endokannabinoideen sintesirako makineria aktibatuko litzateke, horrela, konposatu horien sorrera handituz. AEAren sintesia kaltzioaren menpeko bi entzimaren ekintza sekuentzialaren bidez gertatzen da, N-aziltransferasa (NAT) eta D

fosfolipasaren espezifikoa den N-arakidonoilfosfatanolamina (NAPE-PLD) (*Di Marzo eta lank., 1994; Maccarrone eta lank., 2015)*.

Aitzitik, diazilglizerol lipasa (DAGL) α/β da 2-AG sintesiaren arduraduna *(Maccarrone eta lank., 2015)*. Sortutakoan, (i) lipido haiek hartzaile jakin batzuetara lotu edo (ii) berriro bildu eta degradatu daitezke. Endokannabinoideen andeakuntza gehienbat bi entzimaren bidez egiten da: gantz-azidoen amida hidrolasa (FAAH) AEArentzat *(Giang eta Cravatt, 1997, Cravatt eta lank., 2001*) eta monoazilglizerolipasa (MGL), 2-AGarentzat *(Dinh eta lank., 2002)*.

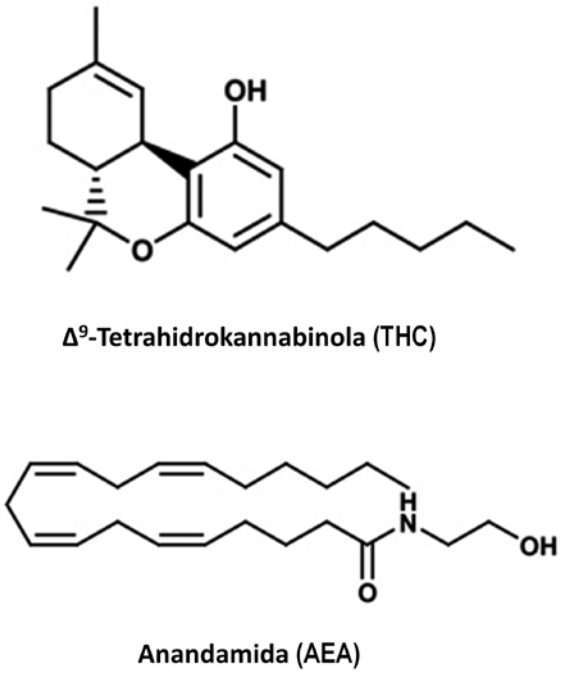
Modu honetan, barne-sistema kannabinoidea osatzen dute kannabinoide-hartzaileek, euren barne-estekatzaileek (endokannabinoideak) eta sintesi eta degradazio-entzimek. Laburbilduz, gaur egun, kannabinoide hitzak landarearen kannabinoideak biltzen ditu, fitokannabinoideak bezala ere ezagutzen direnak, baina endokannabinoideei buruz hitz egiten dugunean, animalien zelulek sor dezaketen kannabinoideei buruz ari gara. Gainera, bi talde horien analogo sintetikoak ere badaude.

Barne-estekatzaileak desberdintzen dituen ezaugarria da ez direla aldez aurretik sintetizatzen ez eta besikuletan gordetzen. Horregatik, jarraitzen da hauen inguruko ikerketa sakonak egiten, neurotransmisoreen jardueraren kontrolean duten funtzio garrantzitsua argitzeko helburuarekin, esate baterako, nerbioetan eta egitura periferikoetan *(Zou eta Kumar, 2018)*.

1.4.3.1 Δ⁹-tetrahidrokannabinol (THC) fitokannabinoidea

THC fitokannabinoide ohikoena da kalamuan eta landarean CBDaren alelo ko-dominante baten bidez ekoizten da *(de Meijer eta lank., 2003)*. THC fitokannabinoidea AEAren analogoa da **(1.14 irudia)** eta CB1 eta CB2 kannabinoide-hartzaileen agonista partziala da. THCak Ki ~ 80,3-32,2 nM afinitate-konstantea dauka, hurrenez hurren, bi hartzaileentzako **(1.2 taula)** *(Rhee eta lank., 1997)*. Eragin psikoaktiboa, analgesikoa, muskulu erlaxatzaile eta antiespasmodikoak ditu *(Pacher eta lank., 2006)*. Gainera, bronkodilatadorea da, antioxidatzaile neurobabeslea, ikterizia kolestatikoan agente antipruritikoa eta aspirina baino 20 aldiz eraginkorragoa hanturaren kontra eta hidrokortisona baino 2 aldiz indartsuagoa *(Russo, 2011)* .

THCak interes handia piztu du haren analogo sintetikoak (dronabinola eta nabilona) erabilera medikorako baimenduak izan direnetik *(Dinis-Oliveira, 2016)* Izan ere, fitokannabinoideen erabilera eta, THCarena kasu honetan, klinikan errazagoa eta fidakorragoa da beste edozein kannabinoide sintetikorekin alderatuta *(Wiley eta lank., 2014; Schreiber eta lank., 2019)*. Gaur egun, badaude merkatuan kannabinoideak dituzten baimendutako medikamentuak: Marinol®: THC sintetikodun kapsulak (dronabinola) sesamo-olioan disolbatuak. Sativex®: kannabis estandarizatuaren estraktua duen aerosola (THC: CBD 1:1 eta % 5 beste kannabinoideena). Cesamet®: THC sintetikoaren analogo baten kapsulak (nabilona). Sativex®-aren kasuan, esklerosi anizkoitza duten pazienteentzako erabiltzeko baimena daukate *(Feliú eta lank., 2015)*.



1.14 irudia. Δ9-tetrahidrokannabinolaren (THC) eta anandamidaren (AEA) egitura kimikoa. *(Sharir eta Abood, 2010)*

Nahiz eta kannabisaren erabilera terapeutikoaren edo aisialdiko erabileraren arteko eztabaidak erabat desberdinak izan, errealitatea da kannabisak gure gorputzean dituen eraginak ezagutzea ezinbestekoa dela, bere kontsumoaren balizko erabilera eta ondorioei aurre egiteko.

1.4.3.2 Kannabinoide-hartzaileak

Kannabinoide-hartzaileak hartzaile metabotropikoak dira eta mintz plasmatikoa zeharkatzen duten 7 domeinuz osatuta daude: N-muturra zelularen kanpoaldean kokatua dago eta C-muturra zelula barnean *(Howlett eta lank., 2002)*. G proteinei loturiko hartzaileen (GPCR) familiako hartzaileak dira eta nagusiki $G_{\alpha i/o}$ G proteina azpiunitateei lotuta daude *(Howlett eta lank. 2002)*.

Kannabinoide-hartzaileak alfa, beta eta gamma azpiunitateez osatuta daudenez, heterotrimerikoak deritze. Alfa azpiunitatea (G_{α}) da garrantzi handiena duena, GDP (Guanosina difosfatoa) eta GTP (Guanosina trifosfato) nukleotidoei lotzeko aukera du, bi egoera horien artean aldaketak eginez. Gainera, erabakigarria da GPCR bidezko zelula barruko seinaleak aktibatzeko. Beta-gamma dimeroaz ($G_{\beta\gamma}$) osatutako azpitaldea seinaleztapen bide zehatzagoak aktibatzeko gai da. Egoera basalean α azpiunitatea GDPra lotua dago eta βγ dimeroari hertsiki elkartua. G proteina horien aktibazioak αi eta βγ azpiunitateen banaketa dakar.

Kannabinoide-hartzaileak G proteina inhibitzaileekin (G_i) lotuta daude, batez ere, αi azpiunitateak AC inhibitzen du, eta cAMParen ekoizpena txikitzen da. Horren ondorioz, PKAaren aktibazioa ere murrizten da. Bestalde, βγ dimeroak seinaleztatze-bide desberdinak aktiba ditzake, hala nola, PI3K/AKT eta MAPK,

p38 MAPK eta Jun N-bukaera kinasa (JNK) *(Liu eta lank., 2000; Rueda eta lank., 2000; Herrera eta lank., 2005)*. Deskribatu da hartzaile horiek neurri txikiagoan ere lotu daitezkeela AC aktibatzen duten G proteinetara (G_s), esaterako, G_q eta G_{13}-rekin *(Bonhaus eta lank., 1998; Lauckner eta lank., 2005)*. Azken ikerketetan aurkitu da G_i azpiunitate klasikoez gain beste G azpiunitate batzuk estimulatzeko gai direla, hala nola, $G_{\alpha z}$, $G_{\alpha q/11}$ eta $G_{\alpha 12/13}$. Gainera, G proteinaren azpiunitatearen aktibazioaren patroi espezifikoa ezberdina da estekatzailearen arabera, hau da, THC edo ACEA (anandamidaren analogo sintetikoa) den ala ez *(Ibarra-Lecue, eta lank., 2015)*. Izan ere, konposatu kannabinoideek hartzaile bakoitzarekiko afinitate desberdinak dituzte **(1.2 taula)**.

1.2 taula. Estekatzaile kannabinoide ezagun batzuen afinitate-profilak *binding* esperimentu bidez lortuak *(Agirregoitia, 2008-tik moldatua)*.

Estekatzailea	Ki (nM)		Erreferentzia
	CB1	CB2	
Barne-kannabinoideak			
AEA	78	>500	*Pertwee, 2005a*
2-AG	58	145	*Pertwee, 2005a*
Kannabinoide sintetikoak			
ACEA	1,4	>2.000	*Howlett eta lank., 2002*
JWH-015	383	13,8	*Howlett eta lank., 2002*
JWH-033	666±77	320±127	*Selwood, 2009*
HU-210	0.061	0.52	*Pertwee, 2006*
Fitokannabinoideak			
Δ^9-THC	80,3	32,2	*Rhee eta lank., 1997*

Ikerketa farmakologiko goiztiarrek CB1 eta CB2 hartzaileen arteko ezberdintasunak antzeman dituzte bi hartzaileetara lotu daitezkeen agonisten arabera. Oso adibide garrantzitsua da THCaren erantzuna, zeina CB1 hartzaileentzat agonista partziala baita COS edo CHO zeluletan, baina CB2 hartzaileen kasuan, agonista partzial ahul edo antagonista gisa jarduten du adenil ziklasa jarduera inhibitzeko *(Bayewitch eta lank., 1996)*. Milurteko berrian sendagaien diseinua nabarmen aurreratu da CB1 edo CB2 hartzaileen artean hautatzeko aukera duten estekatzaile berrien garapenean. Anandamida eta 2-AG endokannabinoideek eta haien egiturazko analogoek beste hartzaileekin elkar eragiteko gaitasuna dute, modu horretan, zelula seinaleztapen sarea hedatuz *(Howlett, 2017)*.

Estekatzaile kannabinoideak hartzaileetara lotzeko modua garrantzi handia duen azterketa-eremua da, agonista eta antagonista kannabinoideen diseinua hobetzen lagun dezakeelako. Horrela, estekatzaileek kannabinoide-hartzaileekin nola elkar eragiten duten ezagututa estrategia farmakologiko desberdinak diseinatu daitezke.

- **1 motako kannabinoide-hartzailea (*CB1 edo CNR1)***

CB1 hartzailea oso kontserbatua agertzen da espezie batzuetan. Giza CB1 hartzaileak aminoazido bat gutxiago dauka N-terminalean, beste ugaztun espezieekin alderatuta (472 aminoazido vs 473 aminoazido), baina arratoien eta gizakiaren hartzaileak oso kontserbatuta daude. Esate baterako, arratoiaren eta gizakiaren CB1 hartzailearen aminoazidoen % 98an antzekotasuna dago. CB1 hartzailearen funtzioa oso ondo azter daiteke saguetan, izan ere, saguen eta gizakien artean ere oso ondo kontserbatzen da hartzailea: % 97ko antzekotasuna dago aminoazidoetan eta % 90koa nukleotidoetan *(Abood, 2005)*. Hala ere, gizakiaren CB1 hartzailearentzat bi isoforma identifikatu dira, hartzailearen N-terminaletan desberdinak direnak *(Ruehle, 2017)*.

CB1 hartzailea ugaria da nerbio-sistema zentralean, batez ere, kognizioan eta epe laburreko memorian eta funtzio-motorrean eta mugimenduan parte hartzen duten eskualdeetan *(Ghosh eta lank., 2018)*. Dentsitate handia du hipokanpoan, zerebeloan, amigdalan edo garun-enborrean. Garuneko zelula mota guztiek [neurona zentral eta periferikoen axoi terminalek *(Herkenham eta lank., 1990)*, astrozitoek *(Sanchez eta lank., 1998)*, oligodendrozitoek *(Molina-Holgado eta lank., 2002)* eta mikrogliek *(Cabral eta lank., 2001)*] CB1 hartzailea espresatzen dute. Neurona hauetan hainbat neurotransmisore askatzea inhibitzen dute, hala nola, azetilkolina, norepinefrina, dopamina, 5-hidroxitriptamina, glutamatoa eta GABA. Ehun periferikoetan ere agertzen da immunitate-sistemaren zeluletan, bihotzean, biriketan, hezur-muinean, umetokian, obarioetan, hestean *(Di Carlo eta Izzo, 2003)*, prostatan, testikuluetan eta espermatozoideetan *(Galiegue eta lank., 1995; Rossato eta lank. 2005; Gervasi eta lank., 2009; Agirregoitia eta lank., 2010)*, ehun adiposoan (*Cota eta lank., 2003)*, erretinan *(Buckley eta lank., 1997)*, gibelean *(Osei-Hyiaman eta lank., 2005)*, endotelio baskularrean *(Liu eta lank., 2000)* eta ehun gastrointestinalean, non funtzio garrantzitsua betetzen duen glukosaren metabolismoan, zelulen ugaltzean, hanturan eta apoptosian *(Ghosh eta lank., 2018)*.

CB1 hartzaileak funtsezko eginkizuna du hainbat funtzio fisiologikoren artean, hala nola, beldurrean, antsietatean, jateko gogoan, mina hautematean, etab. eta sistema endokannabinoidearen desorekak patologia ugari sor ditzake *(Ruehle, 2017)*. Horregatik, CB1 itu terapeutiko interesgarria da gaixotasun ugarientzako, besteak beste, hesteetako gaixotasunetarako, depresiorako, sindrome metabolikorako eta garun iskemiarako *(Ghosh eta lank., 2018)*.

- **2 motako kannabinoide-hartzailea (CB2 edo *CNR2*)**

CB2 hartzaileari buruz oraindik informazio gutxiago daukagu CB1 hartzailearekin alderatuta. CB2 hartzaileak CB1ak baino espezieen artean homologia gutxiago erakusten du; adibidez, gizakien eta saguaren CB2 hartzaileek aminoazidoen % 82 partekatzen dute *(Shire eta lank., 1996)*, eta saguak eta arratoiak % 93. Gizakien, arratoien eta saguen sekuentziak C-terminalean aldatzen dira: saguen sekuentzia 13 aminoazido motzagoa da, eta arratoien klona gizakiaren CB2 baino 50 aminoazido luzeagoa *(Brown eta lank., 2002; Howlett, 2017)*.

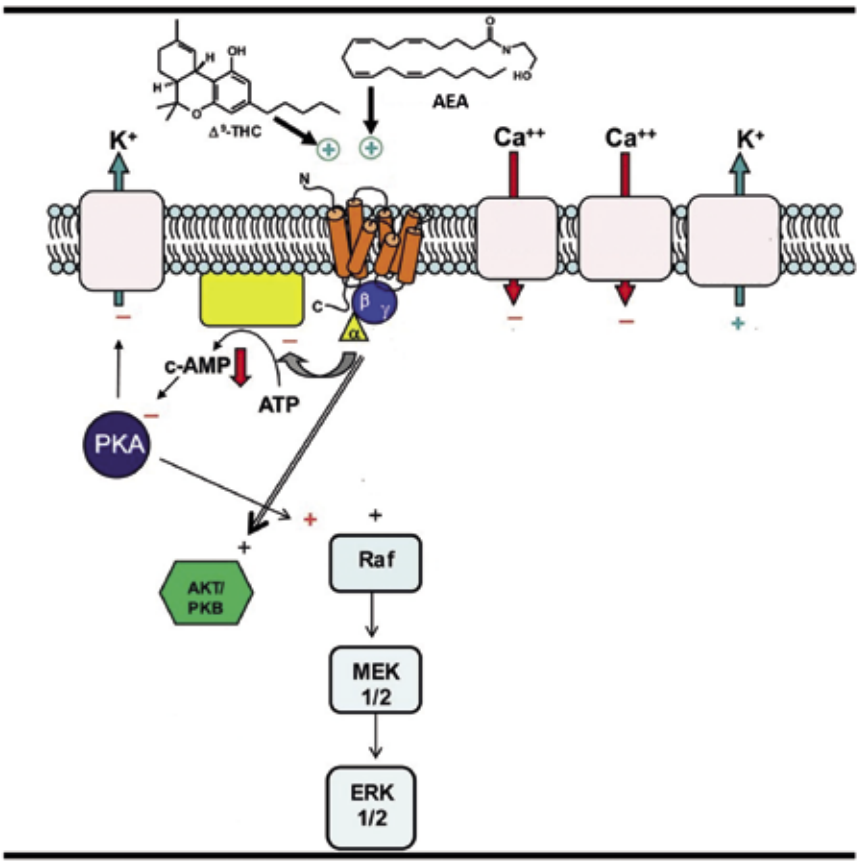
Nerbio-sistema zentralari dagokionez, CB2a zelula mikroglialetan *(Nuñez eta lank., 2004*), garunaren hainbat neurona-azpipopulaziotan *(Skaper eta lank., 1996; Ashton eta lank., 2006*) eta garun-enborrean espresatzen da *(Van Sickle eta lank., 2005; Ashton eta lank., 2006)*. Baita ere funtzio immunologikoa duten organo periferikoetan, makrofagoetan, barean, amigdaletan, timoan eta leukozitoetan eta baita biriketan, obulutegietan, obozitoetan , testikuletan eta espermatozoideetan ere *(Munro eta lank., 1993; Galiegue eta lank., 1995; Brown eta lank., 2002; El-Talatini eta lank., 2009a; Agirregoitia eta lank., 2010; Peralta eta lank., 2011*). Zelula immuneen aktibazioak, mikroglialak barne, CB2-aren espresio maila areagotzen du *(Kaminski eta lank., 1992; Croxford eta Yamamura, 2005)*.

1.4.4 Kannabinoide-hartzaileen bidezko seinaleztapena PI3K/AKT eta MAP kinasen (MAPK) bidezidorretan

MAPK familiak zelulaz kanpoko estimuluak erantzun zelularrean transmititzeaz arduratzen diren bideetan parte hartzen du. MAP kinasen familiak zelulen proliferazioa, desberdintzapena, mugikortasuna eta biziraupen-mailako funtzioak erregulatzen dituzte *(Roux eta Blenis, 2004)*. Familia horren barruan proteina aipagarrienetako bat MAPK da, zelulaz kanpoko seinaleen erregulazio kinasa izenaz ere ezagutzen dena (ERK1/2). Aurreko ikerketek erakutsi dute CB1 hartzailearen estimulazioari esker, ERK 1/2 G proteinen bidez fosforilatzen dela hainbat zelula motatan, hala nola, fibroblastoetan, astrozitoetan eta glioma zeluletan *(Bouaboula eta lank. 1995; Sánchez eta lank., 1998; Guzmán eta Sánchez 1999)*. Kannabinoide-hartzaileen bidezko ERK 1/2aren aktibazio-mekanismoetariko bat $G_{\beta\gamma}$ dimeroaren bidezkoa da. Horren ostean, ERK 1/2ak PI3K ere aktibatu dezake, bere forma aktiboan mintz fosfolipidoen fosforilazioaz arduratzen baita, AKT bildu eta aktibatzeko **(1.15 irudia)**. Hau da, PI3Karen seinaleztapenaren bide nagusia.

Kannabinoide-hartzaileen estimulatzaileen eta PI3K inhibitzaile farmakologikoen konbinazioa erabiltzen duten hainbat ikerketetan, PI3K bidearen parte hartzea frogatu da, AKTaren ondorengo aktibazioa kaltetuta baitago *(Galve-Roperh eta lank., 2002; Sánchez eta lank., 2003)*. Hala ere, PI3K/AKT bidearen aktibazioa ez da beti ERK 1/2 aktibatuta dagoenaren araberakoa eta zelula mota bakoitzerako espezifikoa izan daiteke. Adibidez, frogatu da CB2aren estimulazioak HL60 gizakiaren leuzemia promelokitikoko zelulen lerroan ERK 1/2aren fosforilazioa aktibatzen duela, baina ez PI3K/AKT bidea *(Kobayashi eta lank., 2001)*. Ostera, ERK 1/2 aktibazioa beharrezkoa da PI3K eta AKT aktibatzeko CB1 edo CB2 estimulatzean prostatako minbiziko PC-3 zeluletan *(Sánchez eta lank., 2003)*. Harrigarriki, Molina-Holgado eta lankideek frogatu dutenez, neurona kortikal primarioetan PI3K / AKT bidea aktibatzeak ez du ERK 1/2 aktibatzen *(Molina-Holgado eta lank., 2005)*.

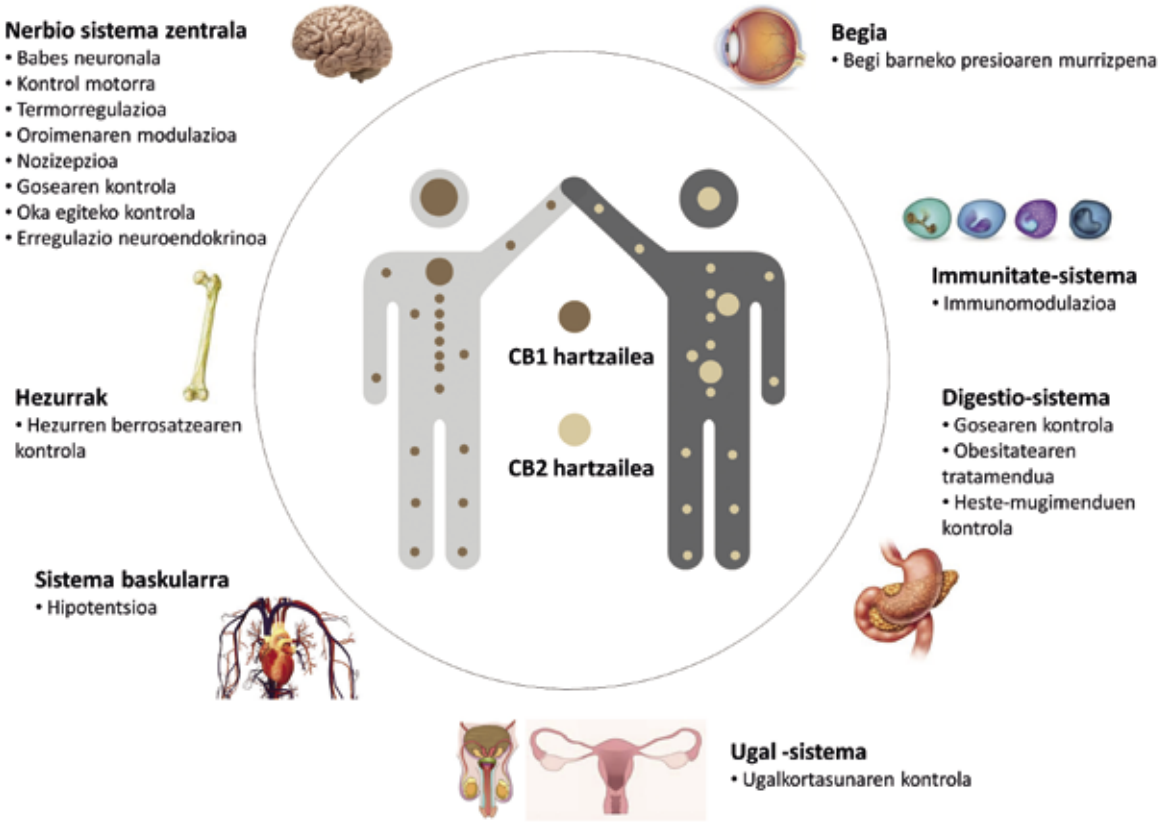
Beraz, bistakoa da PI3K/AKT bidea aktibatzea ez dela beti ERK 1/2 aktibazioak eragindakoa, ez eta ERK 1/2ren aktibazioa beti gertatzen dela kannabinoide-hartzaileen bidez. Kontuan izan behar da zelula motaren, CB1 edo CB2 hartzaileak edo bi hartzaileen estimulazioaren eta aktibazioak sortzen duten seinalearen erantzunean eragina izango dutela. Proposatu da CB1aren estimulazioak ERK 1/2 aktibazioaren bidez PKA aktibatzen duela *(Bouaboula eta lank., 1995)* eta hori garrantzia handiko da PKAk zelulen zikloaren berraktibazio prozesuetan parte hartzen duelako, obozitoetan meiosiaren berpizkundean, 1.1.5 atalean ikusi dugun bezala.



1.15 irudia. Kannabinoide-hartzaileen aktibazioa. Estekatzaile kannabinoidea mintz plasmatikoan barneratzen da eta albo difusioaz proteina heptahelikoidalera lotzen da. Irudian agertu ez arren, kannabinoide-hartzaileak ere glikosilatu daitezke N- muturrean *(Dhopeshwarkar eta Mackie, 2014-tik moldatua)*.

1.5 Sistema kannabinoidea ugalkortasunean

Kannabinoideen inguruko jakintza izugarri handitu da azken urteetan eta ikerketa horiek ondorioztatu dute sistema kannabinoidearen funtzio garrantzitsuenetakoa neuromodulazioa dela. Izan ere, CB1 G proteinei loturiko hartzailerik ugariena da nerbio-sistema zentralean. Hala ere, CB1 eta CB2 hartzaileen presentzia eta sistema endokannabinoidearen deskribapena eta ekintza-mekanismoak nerbio-sistema zentralean gertatzeaz gain, ehun periferikoetan ere deskribatu dira **(1.16 irudia)**. Aurkikuntza horiek sistema honek burutzen dituen funtzioei buruzko ikerketa sakona ekarri dute. Funtzio horietako asko kannabisaren aisialdirako erabileraren ondorioak aztertzetik zehaztu dira, beste batzuk, aldiz, animalia ereduetan eta *in vitro* sistemetan kannabinoideen administrazioak izan ditzakeen ondorioetatik zehaztu dira *(du Plessis eta lank., 2015; Brents, 2016; Correa eta lank., 2016)*.



1.16 irudia. Sistema endokannabinoidea sistema eta organo desberdinetan. Hartzaileen presentzia CB1 marroi ilunean eta CB2 marroi argian agertzen da *(Carracedo, 2006a-tik moldatua)*.

Sistema kannabinoidea inplikatua dagoen prozesu horietako bat ugalkortasuna da. Sistema hau gametogenesian, ernalketan, enbrioiaren ezarpenean, plazentazioan, haurdunaldian eta erditzean deskribatu da *(Battista eta lank., 2008; Maccarrone, 2009)* eta sistema kannabinoidean parte hartzen duen makinaria guztia ugal-aparatuko organo, ehun eta zeluletan dagoela frogatu da *(Galiegue eta lank., 1995; Sugiura eta lank., 1996; Brown eta lank., 2002; Schuel eta lank., 2002a; Ruiz-Llorente eta lank., 2003; Macarrone eta lank., 2003,2005,2007; Gye eta lank., 2005; Rossato eta lank., 2005; Sarfaraz eta lank., 2005; El-Talatini eta lank., 2009a; Bagavandoss eta Grimshaw, 2010; Peralta eta lank., 2011)* . Hori

horrela, sistema hau praktika klinikoan ugalketa potentziala aurreikusteko biomarkatzaile posible gisa proposatu da *(Sun eta Dey, 2012)*. Horretarako, animalia-eredu desberdinek emandakoa berrikusi dugu, baita ugalketa-patologien inguruan egin den ikerketa ere.

1.5.1 Sistema kannabinoidea emearen ugalkortasunean.

Emeari dagokionez, sistema endokannabinoidea hainbat ugaztun espezietako hipotalamo-hipofisi-obulutegiaren ardatzean *(Brents, 2016)*, likido folikularrean, obulutegietan, obozitoetan eta pikor-geruzako zeluletan deskribatu da *(Schuel eta lank., 2002a; El-Talatini eta lank., 2009a; Bagavandoss eta Grimshaw, 2010; Peralta eta lank., 2011; López-Cardona eta lank., 2016; López-Cardona eta lank., 2017)*.

1.5.1.1 Hipotalamo-hipofisi-obario (HPO) ardatza

Sistema endokannabinoidea etengabe kontrolatua dago hainbat estimulu fisiologikoren arabera, hala nola, hormona-mailaren arabera. Sistema endokannabinoidea GnRH bezalako hormonen ekoizpenaz arduratzen den hipotalamoaren guneetan antzeman da, zeinek hipotalamo-hipofisi-obario (HPO) ardatzaren bidez jarduten duten ugalketa-prozesuen hainbat alderdi kontrolatzeko. Oro har, sistema endokannabinoidearen efektoreek eragin handia dute ugalkortasunean, ugalketan eta funtzio endokrinoan, karraskariekin, primateekin eta gizakiekin egindako ikerketek frogatu duten moduan. Horrek kannabisak eta THCak ugalketaren fisiologiaren hainbat alderditan duten eragina azaldu dezake, besteak beste, hormonen askatzean HPO ardatzean *(Walker eta lank., 2019)*. Gonadotropinek, progesteronak eta estrogenoak, esaterako, AEA mailak (El-Talatini eta lank., 2009b) eta FAAH entzimaren adierazpena erregulatzen dituzte hilekoan zehar *(Bambang eta lank., 2010)*.

Orokorrean, sistema endokannabinoidea kanpotik modulatzen denean, kannabinoideek HPO ardatzaren erregulazioa asaldatzen dute, ugal-sistema kaltetuz (obulaziorik gabeko hilekoak, adibidez). Hala nola, GnRH hormonaren jariaketa murrizten dute (*Gammon eta lank., 2005)* eta hormona horrek gonadotropinen (FSHa eta LHa) askapena ekiditen du, gonaden funtzioa ezabatuz *(Brents, 2016)*. Gainera, ikusi da kannabinoideekin tratatutako animalietan, GnRHa exogenoki ematean LHaren jariaketa berrezartzen dela *(Ayalon eta lank., 1977; Tyrey, 1978; Smith eta lank., 1979)*.

Deskribatu da CB1 hartzaileak erregulatuko lukeela GnRHraen sintesia eta liberazioa *(Scorticati eta lank., 2004; Meccariello eta lank., 2008; Chianese eta lank., 2011)* nahiz eta iradoki den CB1 hartzailearen aktibazioak LHaren askapena ere modulatzen duela hipofisian *(Wenger eta lank., 2001)*. Horrez gain, THCaren efektu inhibitzaile zuzena frogatu da folikulugenesian FSHaren menpeko hainbat funtzioren interferentziagatik, estrogenoaren eta progesteronaren metaketa inhibituz eta LHaren hartzaileen emendioa eraginez *(Adashi eta lank., 1983)*.

Era berean, sistema endokannabinoidearen adierazpenean egon litezkeen kalteak antzutasunarekin erlazionatuta daude *(Brents, 2016)* jakina baita kannabinoide-hartzaileen gabeziak HPO ardatzeko hormonen askapena inhibitzen duela, besteak beste, GnRHa, FSHa, 17-β-estradiola (*Cacciola eta lank.,*

2013) eta LHarena (Wenger eta lank., 2001; Oláh eta lank., 2008). Gainera, CB1 hartzailea falta duten saguen ($Cnr1^{-/-}$) % 40k haurdunaldia galtzen du *(Wang eta lank., 2004)*.

Laburbilduz, sistema endokannabinoidea eta HPO ardatza erlazionatuta daudela baieztatu da nahiz eta hori kontrolatzen duten mekanismoak oraindik ez diren guztiz ezagutzen *(Brents, 2016)*.

1.5.1.2 Obulutegia: folikulugenesia eta obozitoen heltze-prozesua

Kannabinoideen eta ugalketa-sistemaren arteko lotura, lehenengoz, AEA fluxu folikularrean aurkikuntzari esker egin zen *(Schuel eta lank., 2002a)* eta, gainera, frogatu zuten bere kontzentrazioa aldatzen zihoala obulazioan eta haurdunaldi goiztiarrean zehar *(El-Talatini eta lank., 2009b)*. Horrez gain, bai AEA bai endokannabinoide hori metabolizatzen duten entzimak gizakiaren obulutegian lokalizatu ziren *(El-Talatini eta lank., 2009a)* eta, ondoren, sistema endokannabinoide osoa deskribatu zen giza *(El-Talatini eta lank., 2009a)* eta arratoien *(Bagavandoss eta Grimshaw, 2010)* obarioan. Azterketa immunohistokimikoei esker, kannabinoide hartzaileak identifikatu ziren folikulu primario, folikulu sekundario eta folikulu antralen pikor-geruzako zeluletan, bai gizakian *(El-Talatini eta lank., 2009a)* zein arratoietan *(Bagavandoss eta Grimshaw, 2010)*. Ikerketa guzti horiei esker, kannabinoide-hartzaileen eta degradazio-entzimen lokalizazioa deskribatu zen eta frogatu zen AEAaren kontzentrazioa aldakorra dela obarioaren zikloan zehar, kontzentrazioaren gorakada bat ikusten baita obulaziora arte. Beraz, horrek guztiak iradokitzen du endokannabinoideen seinaleztapenak folikulugenesia erregulatzen lagun lezakeela *(El-Talatini eta lank., 2009b; Bagavandoss eta Grimshaw , 2010)*.

Duela urte gutxi, sistema kannabinoidea gizakien *(Peralta eta lank., 2011)* behien *(Lopez-Cardona eta lank., 2016)* eta saguen obozitoetan espresatzen dela deskribatu da *(Lopez-Cardona eta lank., 2017)*. Horrez gain, baieztatu da gutxienez azkenengo bi espezieetan kannabinoide sintetikoak gai direla obozitoen heltze-prozesua modulatzeko *(Lopez-Cardona eta lank., 2016; Lopez-Cardona eta lank., 2017)*.

Sistema kannabinoidearen osagaien kontzentrazioen eta lokalizazioaren aldaketak "tonu kannabinoide" bat dagoela erakutsi du, izan ere, FAAHk erregulatzen dituen AEA maila intrafolikular altuek obulazioa ahalbidetzen dute baina ernaldutako obozitoa ezartzeko (<2 nM), hots, haurdunaldiaren arrakastarako, beharrezkoak dira maila baxuagoak *(Maccarrone, 2009)*.

Bestalde, kannabinoideak kanpotik hartzean sistema endokannabinoide horren seinaleztapena asaldatzen da, tximinoekin egindako ikerketa batean ikusi baita, THCaren kontsumo kronikoa obulazioaren atzerapenarekin erlazionatuta dagoela *(Asch eta lank., 1981)*.

1.5.1.3 Ernalketa eta enbrioiaren garapena

Ernalketa, seguruenik, sistema endokannabinoideak kontrolaturiko ugalketaren beste gertaera bat da. Bi gametoetan hartzaile- eta entzima-kannabinoideak egoteak *(El-Talatini eta lank., 2009a; Francavilla eta*

lank., 2009; Agirregoitia eta lank., 2010; Bagavandoss eta lank., 2010; Peralta eta lank., 2011; Bari eta lank., 2011; Lopez-Cardona eta lank., 2016; López-Cardona eta lank., 2017) aukera ematen digu pentsatzeko espermatozoideen elkarrekintza nolabait kannabioideen seinaleztapenarengatik araututa dagoela.

Esan bezala, AEAaren kontzentrazio-gradiente bat dago obiduktu proximalean (*Schuel eta lank. 2002a)* eta espermatozoideak AEAaren kontzentrazioaren murrizketaren eraginpean daudenean, kapazitazio-prozesua aktibatzen da. Sistema kannabinoideak arautzen dituen mekanismo posibleen barruan funtzio berri bat ezagutzeaz gain, gametoen arteko elkarreraginaren azterketa horiek gizonezko antzutasuna tratatzeko potentzial kliniko garrantzitsua dute.

Enbrioiaren garapenari dagokionez, ugaztunetan, sistema kannabinoidearen oreka funtsezkoa da haurdunaldi goiztiar arrakastatsua izateko. Etapa horretan, haurdunaldi bideragarria mantentzeko ezinbestekoa da enbrioiaren garapenaren eta ezarpenerako umetokiko harreraren sinkronizazioa. Sistema endokannabinoideak funtsezko rola betetzen du fase guztietan, ezarpen aurreko enbrioiaren garapenetik hasita enbrioiaren ezarpenera arte *(Sun eta Dey, 2012)*.

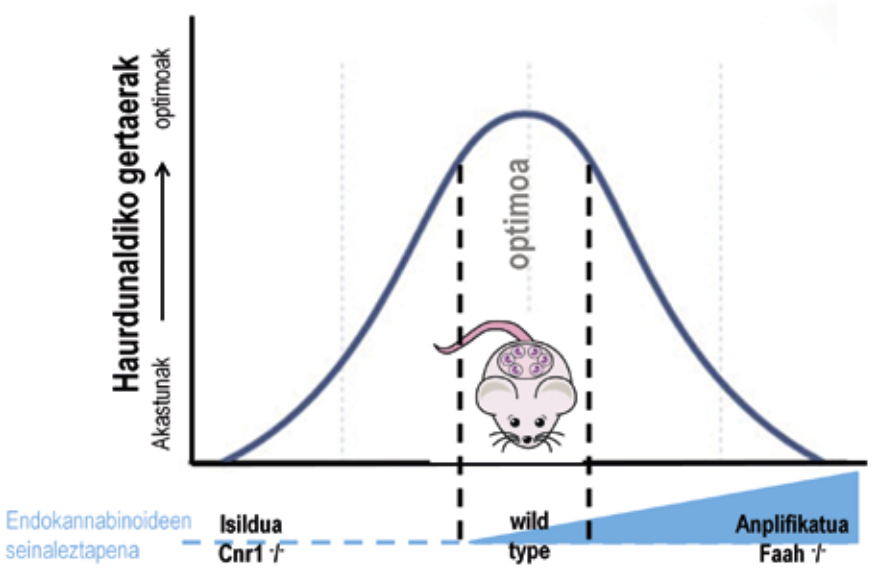
Enbrioiaren garapen goiztiarrean zehar kannabinoide hartzaileak, endokannabinoideak eta sintesi- eta degradazio-entzimak aurkitu izanak ikerketa lerroa zabaldu du. Ugaztunetan, AEAren sintesi eta degradazio-entzimak enbrioiaren garapenaren bi zelulen fasean adierazten dira (*Bambang eta lank., 2010; Taylor eta lank., 2010)*. Bestalde, saguetan deskribatu da CB1 hartzailea enbrioietan 2 zelulen etapa bukaeratik blastozistoaren etaparaino adierazten dela. Aitzitik, CB2a zelula 1eko fasetik blastozistoraino adierazten da *(Paria eta lank., 1995)*. Ezarpen aurreko fasean, CB1a, batez ere, trofoektodermoaren zeluletan agertzen da eta CB2a, aldiz, ICMan *(Paria, eta lank., 1995)*. Bi hartzaile horien presentziaz gain, FAAH ere ageri da 1 eta 2 zeluletako enbrioietan baina 4 eta 8 zelulen faseetan, ordea, ez da antzeman; nahiz eta blastozistoan berriro hauteman daitekeen (ezarpen aurreko 4. egunean) *(Paria eta lank., 1999).*

Beraz, egin diren azterketa sistematikoek ondorioztatzen dute CB1 eta CB2 hartzaileak modulatzen duen seinaleztapena erabakigarria dela garapen goiztiarrean. Saguetan, CB1 hartzailea bakarrik obiduktuan eta umetokian agertzen da eta, ostera, CB1 eta CB2 hartzaileak ezarpen aurreko enbrioian agertzen dira *(Bukiya, 2019)*. Gainera, CB1aren funtzioaren galerak nerbio adrenergikoko terminaletatik norepinefrina askatzea areagotzen du eta muskulu leunaren uzkurdura handitzen du enbrioiaren mugimendua kaltetuz. CB1 hartzaileen estimulazioak, ordea, obiduktuko muskulua erlaxatu eta enbrioiaren mugimendua bultzatzen du *(Bukiya, 2019)*. Are gehiago, gizakietan, haurdunaldi ektopikoa daukaten pertsonek CB1 hartzailearen eta FAAH entzimaren adierazpen ahulagoa dute obiduktuan eta Falopio tronpetan *(Horne eta lank. 2008; Gebeh eta lank. 2012)* . Eta gaixotasun larria da, Ipar Amerikan bakarrik, 1000 haurdunaldi bakoitzeko 14 kasutan gertatzen baita eta haurdunaldiaren lehen hiruhilekoan amaren heriotza kausa nagusia da *(Stulberg eta lank., 2014).*

Ezarpenaren aurreko enbrioiaren garapena erabakitzailea da blastozisto osoa eta osasuntsua lortzeko eta, horretarako, besteak beste, sistema endokannabinoidearen ondo zehaztutako seinaleztapena beharrezkoa da. Izan ere, ezarpenaren aurreko enbrioiaren garapenerako kaltegarriak dira kannabinoide sintetiko, fitokannabinoide zein endokannabinoide maila altuak egotea (AEA, 2-AG, THC eta WIN55212-2) *(Sun*

eta Dey, 2012). Genetikoki eraldatutako sagu-ereduetan egindako ikerketek erakutsi dute, baita ere, sistema endokannabinoidea oso zorrotz doituta egon behar dela ernalketan eta enbrioiaren garapenean haurdunaldia normaltasunez gerta dadin **(1.17 irudia)**. Beraz, endokannabinoideen seinaleztapenak hainbat akats eragingo ditu haurdunaldian zehar isilarazita edo emendatua badago *(Sun eta Dey, 2012*). Hori dela eta, sistema endokannabinoidearen adierazpenean egon daitezkeen asaldurak zuzenean erlazionatuta daude antzutasunarekin, haurdunaldi ektopikoarekin eta bat-bateko abortuarekin *(Brents, 2016)*

Aurreko ataletan deskribatutako datuek agerian uzten dute sistema endokannabinoidearen edozein osagairen aktibazioak edo eraldaketek eragina izan dezaketela ugalketaren fase desberdinen erregulazioan eta bizitza berri baten hasiera arriskuan jar dezaketela. Horregatik, kannabinoideen erabilera aztertzen hasi da, antzutasunaren diagnostikoan biomarkatzaile posible gisa edota praktika klinikoan tratatzeko itu terapeutiko posible gisa *(Di Blasio eta lank., 2013; Rapino eta lank., 2014).*



1.17 irudia. Endokannabinoideen seinaleztapena haurdunaldian zehar. Haurdunaldiko gertaeretan (enbrioiaren garapena, obiduktuan zeharreko enbrioiaren garraioa, blastozistoaren garapen gaitasuna eta ezarpena) endokannabinoideen seinaleztapena isilarazita edo emendatua badago hainbat akats eragiten ditu *(Sun eta Dey, 2012-tik moldatua)*.

2

Helburuak
Aims

2. Helburuak

Helburu nagusia:

Sistema kannabinoideak obarioaren fisiologian eta obozitoaren heltze-prozesuan duen parte hartzearen inguruan ezagutza handitzea.

1. helburua:

Obozitoen heltze-prozesua zorrotz koordinaturiko prozesua da, meiosiaren lehenengo geldialditik, besikula germinaleko (GV) fasean, II. metafaseko (MII) meiosiaren geldialdiraino. Heltze-prozesua folikulu barruan gertatzen da, obozitoen eta pikor-geruzako zelulen arteko komunikazioaren bidez. Jakina da anandamidaren (AEA) kontzentrazioaren aldaketak gertatzen direla obozitoaren heltze-prozesuan zehar. Hori dela eta, tesi honen lehenengo helburua da sistema kannabinoidea deskribatzea gizakien pikor-geruzako zeluletan obozitoen heltze-prozesuan zehar.

Helburu zehatzak:

1.1. Kannabinoide-hartzaileen (CB1 eta CB2) eta kannabinoideen degradazio-entzimen (FAAH eta MAGL) adierazpena deskribatzea gizakien pikor-geruzako zeluletan.

1.2. Pikor-geruzako zeluletan, FAAH eta MAGL degradazio-entzimek CB1 kannabinoide-hartzailearekiko duten banaketa diferentziala ikertzea obozitoaren heltze-prozesuan.

2. helburua:

Gaur egun badakigu gizakiaren likido folikularrean AEA presente dagoela eta obozitoaren heltze-prozesuan zehar kannabinoide-hartzaileen lekualdaketa gertatzen dela obozitoan. Hori dela eta, ezagutza handitzeko asmoz, tesiko bigarren helburua da AEA andeatzen duten entzimen (FAAH eta MAGL) presentzia eta banaketa aztertzea meiosiaren berraktibazioan zehar.

Helburu zehatzak:

2.1. AEA andeatzen duten FAAH eta MAGL degradazio-entzimen adierazpena eta banaketa ezaugarritzea giza obozitoetan heltze-prozesuan zehar.

2.2. Degradazio-entzima horiek giza obozitoetan CB1 hartzailearekiko duten harremana aztertzea meiosiaren berraktibazioan zehar.

3. helburua:

Sistema kannabinoidea ugalkortasuneko hainbat prozesutan inplikatuta dagoela ikusi da eta badirudi obozitoen heltze prozesuaren modulazioan funtzio garrantzitsua betetzen duela. Hemendik aurrera proposatuko den esperimentazioa, gaur gaurkoz, ezin denez gizakian egin, animalia eredu bezala sagua erabiliko da. Ildo horretatik, hirugarren helburua da Δ^9-tetrahidrokannabinol (THC) fitokannabinoideak obozitoaren heltze-prozesuaren modulatzaile gisa izan lezakeen papera aztertzea.

Helburu zehatzak:

3.1. Obozitoaren *in vitro* heltze-prozesua exogenoki THCaren bidez modulatu eta kannabinoide-hartzaileen (CB1 eta CB2) adierazpena eta lokalizazio-dinamika aztertzea.

3.2. Obozitoaren *in vitro* heltze-prozesua THCaren bidez modulatzea obozitoaren heltze-abiaduran duen eragina aztertzeko.

3.3. $Cnr1^{-/-}$, $Cnr2^{-/-}$ eta $Cnr1^{-/-}/Cnr2^{-/-}$ *knockout* sagu ereduak erabiliz, obozitoak THCaren presentzian heldu eta prozesu horretan garrantzitsuak diren PI3K/AKT eta MAPK bidezidorren AKT eta ERK1/2 proteinen fosforilazio-patroia aztertzea. Era beran, THCak zein kannabinoide-hartzaileren bidez lan egiten duen zehaztea.

3.4. Obozitoen heltze-prozesuan THC gehitzeak geroko ernalketan eta enbrioiaren garapenean duen eragina ebaluatzea.

4. helburua:

Modu sistemikoan kannabinoideekin egiten den tratamenduak sistema endokannabinoidearen erregulazioa asaldatzen du eta ugalketa-prozesuak kaltetzen dira. Berdina gertatzen da sistemak berezko gabeziak dituenean. Hala ere, asaldura horien zergatia ez da guztiz ezagutzen. Tesi honen laugarren eta azken helburua da kannabinoide-hartzaileen faltak emearen obarioan sortzen dituen eraginak aztertzea. Azkenik, THCa *in vitro* erabiliko da obozitoaren garapen konpetentzia eta konpetentzia meiotikoan duen eragina aztertzeko.

Helburu zehatzak:

4.1. $Cnr1^{-/-}$, $Cnr2^{-/-}$ eta $Cnr1^{-/-}/Cnr2^{-/-}$ *knockout* sagu ereduak erabiliz, kannabinoide-hartzaileen gabeziaren eragina ebaluatzea obarioen morfologian, folikulugenesian, obulazioan eta obozitoen heltzean.

4.2. THCaren bidezko IVM sistema eraginkorragoa diseinatzea hormonak eman gabeko sagu gazteen obozitoen garapen gaitasuna emendatzeko.

2. Aims of the study

The central aim:

To explore the involvement of the cannabinoid system in the physiology of the ovary and the oocyte maturation.

1. First aim:

Oocyte maturation is a highly coordinated process that includes the progression of the oocyte from the first meiotic blockage at the germinal vesicle (GV) stage to the second meiotic blockage at the metaphase II (MII) stage. This progression occurs inside the follicle due to a careful communication between oocytes and granulosa cells. It is known that the fluctuation of the anandamide concentration (AEA) occurs during the oocyte maturation, therefore, the first objective of this thesis is to describe the cannabinoid system, during the oocyte maturation process, in the human granulose cells.

Specific aims:

2.1. To describe the expression of cannabinoid receptors CB1 and CB2 and cannabinoid-degrading enzymes fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL) in human granulosa cells.

2.2. To investigate the differential distribution of cannabinoid-degrading enzymes FAAH and MGLL with respect to CB1 at various stages during the nuclear maturation of the oocyte.

2. Second aim:

In view of the presence of AEA in human follicular fluid and the re-localization of cannabinoid receptors observed in the oocyte during its maturation, cannabinoid signaling can be postulated to be involved in the regulation of oocyte meiosis. Therefore, in order to increase the knowledge, the second objective of the thesis is to study the presence of AEA degrading enzymes (FAAH and MGLL) during the reactivation of oocyte meiosis in human.

Specific aims:

2.1. To characterize, in detail, the expression and distribution of the two AEA-degrading enzymes FAAH and MGLL during the meiotic resumption of oocytes.

2.2. To explore the relationship of FAAH and MGLL with the CB1 receptor in human oocytes during the meiotic resumption of oocytes.

3. Third aim:

The cannabinoid system is involved in many fertility processes and appears to play an important role in modulating the oocyte maturation. Due to the fact that today we cannot continue with the experimentation on humans proposed in the following aims, we have used an animal model, mice. Being this the general content, with the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, the third aim is to characterize the role of the phytocannabinoid Δ^9-tetrahydrocannabinol (THC) in the *in vitro* maturation (IVM) process.

Specific aims:

2.1. To study whether the use of THC modulates oocyte maturation in a mouse model and to analyse the expression dynamics and location of the cannabinoid receptor (CB1 and CB2).

2.2. Examine whether the use of THC modulated oocyte meiotic velocity.

2.3. Generating knockout oocytes for CB1 and/or CB2 receptors ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$) to visualize the physiological effects of THC through the oocyte maturation acting via CB1 and/or CB2 and analysing the phosphorylation pattern of the AKT and ERK1/2 proteins.

2.4. To evaluate the impact of the addition of THC on oocyte maturation media in subsequent fertilization and embryonic development.

4. Fourth aim:

Generally, when the endocannabinoid system (ECS) is modulated by systemically administred cannabinoids, the cannabinoids alter HPO axis regulation, potentially leading to disruption of the reproductive system; but, in the same way, altered ECS expression is also associated with reduced fertility. However, the cause of these disorders is not fully known. The fourth and final objective of this thesis is to investigate the effects of cannabinoid receptor deficiency on the female's ovary. Finally, THC will be used *in vitro* to investigate the effect on oocyte development and meiotic competence.

Specific aims:

2.1. Using a genetic approach generating knockout mice for CB1 and/or CB2 receptors ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$) to study the effect of the lack of cannabinoid receptors in ovarian morphology, folliculogenesis, oocyte retrieval and oocyte maturation.

2.2. To design a more efficient IVM system using THC to improve the developmental competence of oocytes from unprimed juvenile mice.

3

Materialak eta metodoak
Materials and methods

3. Materialak eta metodoak

3.1 Materialak

3.1.1 Gailuak

Tesian zehar erabili ziren gailuak deskribatuko dira atal honetan.

3.1 taula. Teknika desberdinak egiteko erabilitako gailuen zerrenda.

Gailua	Etxe komertziala
Balantza	AE Adam
Berogailua	Kowell OP1-I
Biosegurtasun kabina Bio II A	Telstar
Centronic mikrozentrifuga	JP Selecta
CO_2 inkubadorea	Memmert
Chemidoc XRS	Bio-RAD
Elektroforesi kubeta (Western Blot)	Bio-RAD
Elektroforesi kubeta agarosa gelerako	Bio-RAD
Fluoreszentziazko mikroskopioa Axioskop	Zeiss
Fluoreszentziazko mikroskopio fokukidea Axio Observer, Apotome 2.0	Zeiss
Fluoreszentziazko mikroskopio fokukidea LSM800	Zeiss
Gasen erauzketarako beira-arasa	Indelab
Heraeus Fresco 21 zentrifuga	Thermo Scientific
Makler Kamera	Sefi Medical Institute
Mikroskopio estereoskopikoa SMZ 745	Nikon
Mikroskopio optikoa Olympus BX50	Olympus Optical
Mikroskopio optikoa Eclipse E200	Nikon
Nanodrop espektofotometroa ND-100	Thermo Scientific
pH-metroa Basic 20	Crison
Plaka berogailua	Labolan
Rack magnetikoa DynaMan 2	Thermo Fisher
Shandon Citadel 1000	Thermo Fisher
Shandon Finesse 325 mikrotomoa	Thermo Scientific
Termoziklatzailea T100 (PCR)	Bio-RAD
Termoziklatzailea StepOne (qPCR)	Thermo Fisher
Hematoxilina-eosia tindaketarako makina	Thermo Scientific
Parafina blokeak egiteko makina	Thermo Scientific™ Shandon™ Histocentre™
Parafina bainu histologikoa	JP Selecta Termofin-3000459
Plaka hozgarria	JP Selecta

3.1.2 Erreaktiboak

3.1.2.1 Agonista kannabinoideak

THC: delta-9-tetrahidrokannabinola, Δ^9-THC

Erreferentzia: 1972-08-3 THCpharm

Disolbagarria: **Dimetil sulfoxidoa (DMSO)**. Erreferentzia: Sigma

3.1.2.2 Beste erreaktibo batzuk

3.2 taula. Teknika desberdinak egiteko erabilitako erreaktiboen zerrenda.

Erreaktiboak	Etxe komertziala
DNA Taq Polimerasa	Sigma
Dynabeads mRNA Direct Micro Kit	Ambion
Etanola	Scharlau
Etidio Bromuroa	Sigma
Fluoromont G	Electron Microscopy Sciences (EMS)
Formaldehidoa	Electron Microscopy Sciences (EMS)
GoScript Reverse Transcription Kit	Promega
Hematoxilina	Richard-Allan Scientific
Hoechst 33342	Thermo Scientific
ImProm-II Reverse transcription System	Promega
Kloroformoa	Sigma
Nukleotidoen pisu molekularra	Nippon Genetics
Olio minerala (hazkuntza-medioetarako)	Sigma
Poli-L-lisina	Sigma
Proteinen pisu molekularra	Invitrogen
RNasy mRNA Purification Kit	Ambion
TaqMan Fast Universal PCR Master Mix	ThermoFisher
Tritoia X-100	Sigma
Trizol- Tri Reagent	Sigma
Vectamount	Vector Labs

3.3 taula. Esperimentu desberdinak egiteko erabilitako serum-en zerrenda.

Serumak	Etxe komertziala
Ahuntz seruma (*Normal Goat Serum,* NGS)	Abcam
Behi fetuaren seruma (*Fetal Bovine Serum,* FBS)	Biowest
Behi-serum albumina (*Bovine Serum Albumin*, BSA)	Sigma

3.1.2.3 Antigorputz primarioak eta sekundarioak

3.4 taula. **Antigorputz primarioen zerrenda**.

Antigorputz primarioa	Etxe komertziala	Ostalaria	Diluzioa	Erabilera
1 motako hartzaile kannabinoidea (CB1) (anti-CB1)	Frontier Institute	Untxia	1:1000	WB (Gizakia)
2 motako hartzaile kannabinoidea (anti-CB2)	Invitrogen	Untxia	1:200	WB
1 motako hartzaile kannabinoidea (CB1) (anti-CB2)	Santa Cruz Biotechnology	Ahuntza	1:200	IZK (Sagua)
1 motako hartzaile kannabinoidea (CB1)(anti-CB1)	Cayman Chemicals	Untxia	1:100	IZK (Sagua)
2 motako hartzaile kannabinoidea (CB2)(anti-CB2)	Cayman Chemicals,	Untxia	1:400	IZK (Sagua)
Anti-FAAH	Cayman	Untxia	1:200	WB
Anti-MGL	Cayman	Untxia	1:200	WB
Anti-FAAH	Cayman	Untxia	1:200	IZK (Gizakia)
Anti-MGL	Cayman	Untxia	1:200	IZK (Gizakia)
Phospho-Akt (Ser473) (D9E) XP®	Cell Signaling	Untxia	1:200	IZK
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling	Untxia	1:400	IZK
Anti-β-Actin	Sigma	Sagua	1:1000	WB

3.5 taula. Antigorputz sekundarioen zerrenda.

Antigorputz sekundarioa	Etxe komertziala	Ostalaria	Diluzioa	Erabilera	Inkubazioa denbora
Alexa Fluor 488 anti-rabbit	Molecular Probes	Ahuntza	1:2500	IZK	37 °C 2 h
Alexa Fluor 680 anti-rabbit	Invitrogen	Ahuntza	1:4000	IZK/WB	37 °C 2 h
Alexa Fluor 594 anti-goat IgG	Molecular Probes	Astoa	1:2500	IZK	37 °C 2 h

3.1.2.4 Hormonak

3.6 taula. Hormonen zerrenda.

Hormonak	Etxe komertziala
Behorraren hormona gonadotropina korionikoa (eCG)	Folligon
Giza gonadotropina korionikoa (hCG)	Veterin Corion

3.1.3 Soluzioak

Laborategian prestatu ziren soluzioak, honako hauek dira:

3.7 taula. Laborategian prestatutako soluzioen zerrenda.

Soluzioa	Prestaketa
Fosfato/gatz-indargetzailea (Phosphate buffered saline; PBS)	137 mM NaCl 2,7 mM KCl 1,5 mM KH_2PO_4 8,1 mM Na_2HPO_4 H_2O-a bolumena bete arte
Tris-HCl 10 mM pH-a:7,4	1000 ml H_2O 12,11 g Tris-Base HCl-aren pH-a 7,4-ra heldu arte
Tris-HCl / 1,5M / pH 8,8 Tris	1000 ml H_2O 121,14 g Tris-Base HCl-aren pH-a 8,8-ra heldu arte
Tris-HCl 0,5M pH:6,8	1000 ml H_2O 62,07 g Tris-Base HCl-aren pH-a 6,8-ra heldu arte
Homogeneizazio-soluzio indargetzailea	10 mM Tris-HCl, pH 7,4 % 1 (b/b) Proteasen inhibitzaileen koktela H_2O-a bolumena bete arte
Bradford erreaktiboa	0,117 mM Coomasie urdin distiratsua % 5 (b/b) Etanola % 15 (p/b) Azido ortofosforikoa H_2O bolumena bete arte Prestakina bi orduz nahasten da, irabiatuz. Disolbatu ez den koloratzailea erauzteko, disoluzioa birritan iragazten da iragaz-paperaz.

Soluzioa	Prestaketa
Sodio dodezil sulfatoa (SDS) - % 10	100 ml H_2O 10 g SDS
Proteinentzako karga-indargetzailea (Laemmli sample buffer) - 5X	% 10 (p/b) SDS % 25 (b/b) Tris-HCl 250 mM pH 6,8 % 0,025 (p/b) bromofenol urdina % 50 (b/b) glizerola % 5 β-merkaptoetanola H_2O-a bolumena bete arte Karga-indargetzaileak 5 aldiz diluitua geratu behar du laginean
Gel banatzailea	% 30 akrilamida / % 1 bisakrilamida: akrilamida, % 30etik nahi den %ra eramateko, diluitu egin behar da (adibidez, % 12rako, 2,5 aldiz diluitu). Tris-HCl 1 M pH 8,8: % 25 (bol) % 10 SDS: % 1 (bol) H_2O-a bolumena bete arte % 10 (p/b) Persulfato amonikoa: 50 µl TEMED: 10 µl
Gel kontzentratzailea	% 30 akrilamida / % 1 bisakrilamida: akrilamida % 4ra (7,5 aldiz diluitu) Tris-HCl 0.5 M pH 6.8: % 25 (bol) % 10 SDS: % 1 (bol) H_2O: bolumena bete arte % 10 (p/b) persulfato amonikoa: 25 µl TEMED: 5 µl
Elektroforesi-indargetzailea - 10X	250 mM Trizma-Base % 1 (p/b) SDS 2 M Glizina H_2O-a bolumena bete arte pH 8,3 doitua atera behar da, ez ukitu Erabiltzeko, H_2O-rekin10 aldiz diluitu behar da.
Transferentzia-indargetzailea - 10X	250 mM Trizma-Base % 0,25 (p/b) SDS 1,92 M Glizina H_2O bolumena bete arte pH 8,3 doitua atera behar da, ez ukitu Erabiltzeko, H_2O-rekin 10 aldiz diluitu behar da. Diluitzean, % 20 (b/b) metanola gehitu behar zaio..
Ponceau gorria	% 1 (p/b) *Ponceau* gorria % 5 (b/b) Azido azetiko glazial H_2O bolumena bete arte

Soluzioa	Prestaketa
Tris/gatz-indargetzailea (Tris buffered saline; TBS)	50 mM Tris-HCl pH 7,5 150 mM NaCl H_2O bolumena bete arte
Blotto soluzioa	TBS % 0,1 Tritoia X-100
Blokeo soluzioa	*Blotto* % 5 esne gaingabetua
Luminola (250 mM)	10 ml DMSO 0,44 g luminol
Azido p-kumarikoa (90 mM)	10 ml DMSO 0,15 g azido p-kumarikoa
Kimioluminiszentzia-areagotzailea (*Enhanced chemiluminesence*; ECL)	1,25 mM luminol 0,2 mM azido p-kumariko 0,1 M Tris-HCl pH: 8.5 H_2O bolumena bete arte Nahastura hori 4 °C-an eta iluntasunean gordetzen da erabilia izan arte. Erabiliko den egunean, iluntasunean gordeta dugun nahasturara % 0,3 (b/b) H_2O_2 (% 30) gehitu behar zaio..
Tris/azetato indargetazailea + EDTA (TAE)	0,4 M Tris-Base % 5,7 (b/b) Azido azetiko glaziala 10 mM EDTA pH 8
Agarosa-gela	% 1,5 agarosa 100 ml TAE 1X (DEPC) Irakiten jarri (1-2 min) % 1 etidio bromuro
PBS-PVA 0,1%	0,5 g PVA + 500 ml PBS 1X
Zitrato Trisodiko Bufferra 10 mM	1,47 g zitrato trisodikoa + 500 ml ur destilatua. pH 6

3.1.4 Inguruneak

3.1.4.1 Saguen obozitoen eta enbrioien manipulazio eta hazkuntzarako inguruneak

Jarraian, gametoen manipulaziorako, obozitoen *in vitro* heltzerako, *in vitro* ernalketarako eta *in vitro* enbrioien hazkuntzarako erabili ziren hazkuntza-inguruneen konposizioa deskribatzen da. Ingurune

guztiak laborategian prestatu ziren eta filtrazio bidez esterilizatu ziren (0,22 µm iragazkiak). Ingurune guztiak, M2 izan ezik, ondoko baldintza hauetan inkubatu ziren: CO_2 % 5 eta hezetasun-maila % 95. Esperimentuetan erabili baino 4-5 ordu lehenago sartu ziren inkubagailuan pHa orekatu zedin.

- **M2 ingurunea**

Inkubagailutik kanpo aldi luzeetan manipulatu beharreko prozesuetan erabili zen M2 ingurunea, eta baita laginen berreskurapenerako prozedura guztietarako, izan obulutegi, obozito zein arren epididimoak. 4 °C-an gorde zen, gehienez, 30 egunez. Plaka berogailuan 37 °C-an berotu zen erabili baino 30 minutu lehenago, pHa prestaketan egonkortu baitzen **(3.8 taula)**.

3.8 taula. M2 ingurunearen osaketa.

M2			
ERREAKTIBOAK			KANTITATEAK
Pausuak	Izena	ERREFERENTZIA	gr/mL
1	Hepes	Sigma H-4034	2,4845
2	Kaltzio kloruroa ($Cl_2Ca.2H_20$)	Sigma C-7902	0,1260
3	Sodio kloruroa (NaCl)	Sigma S-5886	2,7665
4	Potasio kloruroa (KCl)	Sigma P-5405	0,1780
5	Fosfato potasikoa (KH_2PO_4)	Sigma P-5655	0,0810
6	Magnesio sulfatoa($MgSO_4.7H_2O$)	Sigma M-1880	0,1465
7	Sodio bikarbonatoa ($NaHCO_3$)	Sigma S-8875	0,1745
8	Pirubato sodikoa	Sigma P-2256	0,0180
9	Glukosa anhidra	Sigma G-7021	0,5000
10	Sodio laktatoa % 60 syrup	Sigma L-7900	1,8850
11	Gorri fenola	Sigma P-5530	0,0050
12	Penizilina	Sigma P-4687	0,0312
13	Estreptomizina	Sigma S-9137	0,0250
14	Ondo disolbatu		
15	pH-a doitu Na OH-rekin (7,3-7,4)		
16	Osmolaritatea neurtu (285-287 mosmol)		
17	Albumina (*Bovine Serum Albumine*,BSA, ingelesez)	Sigma A-3311	4
18	Filtrazioz esterilizatu (0,22µm-ko filtroekin)		
19	Errotulatu (M2, data,...) bakarrik gomazko tapoiekin estali		
20	4 °C-an gorde, 30 egunez erabili		

- **Obozitoen *in vitro* heltze-ingurunea (ingelesez, *in vitro maturation* (IVM) ingurunea)**

Obozito heldugabeak heltzeko erabili zen ingurunea. Esperimentuaren egun berean, IVM prozesua hasi baino 5 ordu lehenago prestatu zen eta inkubagailuen gorde zen 37 °C-an egonkortzeko **(3.9 taula).**

3.9 taula. IVM ingurunearen osaketa.

	IVM ingurunea	
Erreaktiboak	**Etxe komertziala**	**Kantitateak**
TCM-199	Sigma / M4530	Bolumena bete arte
Gentamizina	Sigma / G1397	1:200
Behi fetuaren seruma % 10 (FSB)	Biowest / S181B	% 10 bol/bol
Epidermiseko hazkuntza-faktorea (EGF)	Sigma / E9644	10 ng/ml

- **HTF ingurunea**

In vitro ernalketak (IVF) egiteko erabili zen . HTF izenez egin zen ezaguna (*Human Tubal Fluid*) eta orekan dagoen gatz disoluzioa da, aminoazidorik gabea eta potasio ioiaren kontzentrazioa emearen ugal-aparatuan dagoen berdina da *(Quinn eta lank., 1985)*. 4 °C -an gorde zen , gehienez 30 egunez **(3.10 taula)**.

3.10 taula. HTF ingurunearen osaketa.

HTF			
ERREAKTIBOAK			**KANTITATEAK**
Ordena	**Izena**	**ERREF.**	**gr/mL**
1	Kaltzio kloruroa ($Cl_2Ca.2H_20$)	Sigma C-7902	0,1500
2	Sodio kloruroa (NaCl)	Sigma S-9625	2,96875
3	Potasio kloruroa (KCl)	Sigma P-5405	0,1780
4	Fosfato potasikoa (KH_2PO_4)	Sigma P-5655	0,02520
5	Magnesio sulfatoa($MgSO_4.7H_2O$)	Sigma M-1880	0,02460
6	Sodio laktatoa %60 syrup	Sigma L-7900	1,71000
7	Pirubato sodikoa	Sigma P-2256	0,01820
8	Glukosa anhidra	Sigma G-7021	0,2500
9	Sodio bikarbonatoa ($NaHCO_3$)	Sigma S-5761	1,0500
10	Penizilina	Sigma P-4687	0,03750
11	Estreptomizina	Sigma S-9137	0,02500
12	Gorri fenola	Sigma P-5530	0,00200
13	Ondo disolbatu		
14	pH-a ez da doitu behar		
15	Osmolaritatea neurtu (300 mosmol)		
16	Albumina (BSA)	Sigma A-3311	2
17	Filtrazioz esterilizatu (0,22µm-ko filtroekin)		
18	Errotulatu (HTF, data,...) bakarrik gomazko tapoiekin estali		
19	4 °C-an gorde, 30 egunez erabili		

- **KSOMaa ingurunea**

Sagu enbrioien hazkuntzarako KSMOaa ingurunea erabili zen. Behin prestatuta -20 °C-an gorde zen, gehienez 60 egunez. Esperimentua baino egun bat lehenago atera zen hozkailuan 4 °C-an desizoztu zedin **(3.11 taula)**.

3.11 taula. KSOMaa ingurunearen osaketa.

KSOMaa			
Sagu enbrioien hazkuntza-ingurunea			
ERREAKTIBOAK			KANTITATEAK
Ordena	Izena	ERREFERENTZIA	gr/mL
1	Kaltzio kloruroa ($Cl_2Ca.2H_20$)	Sigma C-7902	0,1257
2	Sodio kloruroa (NaCl)	Sigma S-5886	2,7759
3	Potasio kloruroa (KCl)	Sigma P-5405	0,0932
4	Fosfato potasikoa (KH_2PO_4)	Sigma P-5655	0,0238
5	Magnesio sulfatoa($MgSO_4.7H_2O$)	Sigma M-1880	0,0246
6	Sodio laktatoa %60 syrup	Sigma L-7900	0,715
7	Glukosa anhidra	Sigma G-7021	0,0180
8	Pirubato sodikoa	Sigma P-2256	0,011
9	Sodio bikarbonatoa ($NaHCO_3$)	Sigma S-5761	1,05
10	Glutamina	Sigma G-5763	0,0735
10	EDTA	Sigma E-6635	0,0019
11	Penizilina	Sigma P-4687	0,03145
12	Estreptomizina	Sigma S-9137	0,0250
13	Gorri fenola	Sigma P-5530	0,005
14	Aminoazido esentzialak 50X	Sigma B-6766	10 ml
15	Aminoazido ez-esentzialak 100X	Sigma M-7145	5 ml
16	Ondo disolbatu		
17	Ez da pH-a doitu behar % 5eko CO_2rekin doitzen da		
18	Osmolaritatea neurtu (250-255 mosmol)		
19	Albumina (BSA)	Sigma A-3311	0,5 mg/mL
20	Filtrazioz esterilizatu (0,22µm-ko filtroekin)		
21	Errotulatu (KSMO, data,...) bakarrik gomazko tapoiekin estali		
22	4 °C-an gorde, 15 egunez erabili		

- **Pre-IVM faserako α-MEM oinarrizko hazkuntza-ingurunea.**

Hormonekin estimulatu gabeko eta sexualki heldu gabeko (aurre puberrak) saguen obozitoak *in vitro* heldu aurretik heltze-zitoplasmatikoa eta nukleokoa sinkronizatzeko erabili zen ingurunea. Esperimentuaren egun berean, IVM prozesua hasi baino 5 ordu lehenago prestatu zen eta inkubagailuan gorde zen 37 °C-an egonkortzeko **(3.12 taula)**.

3.12 taula. Oinarrizko ingurunearen osaketa.

Pre-IVM etaparako oinarrizko hazkuntza-ingurunea (BM)		
Erreaktiboak	**Etxe komertziala**	**Kantitateak**
α-MEM (Minimum Essential Media)	Life Technologies	15,536 µl
Behi fetuaren seruma %10 (FBS)	Life Technologies	400 µl
Intsulina	SIGMA	16 µl (*stock* 5 µg/ml)
T-S mix (Apotransferrina+Selenioa)	SIGMA	16 µl (*stock* 5 µg/ml)
• Apo-Transferrina	SIGMA T-2036	5 µg/ml
• Sodio selenitoa	SIGMA S-9133	5 µg/ml
E2 (Estradiola)	SIGMA	16 µl (*stock* 10 µM)
Pre-IVM etaparako α-MEM oinarrizko hazkuntza-ingurunea		
Erreaktiboak	**Etxe komertziala**	**Kantitateak**
BM	-	7980 µl
CNP-22 (C-type Natriuretic Peptide-22)	Phoenix Pharmaceuticals,INC.	20 µl (10 µM)

- **IVM faserako α-MEM oinarrizko hazkuntza-ingurunea.**

Hormonekin estimulatu gabeko sagu aurre puberren obozito heldugabeak *in vitro* heltzeko erabili zen ingurunea. Esperimentuaren egun berean, *in vitro* heltze-prozesua (IVM) hasi baino 5 ordu lehenago prestatu zen eta inkubagailuen gorde zen 37 °C-an egonkortzeko **(3.13 taula)**.

3.13 taula. Pre-IVM ingurunearen osaketa.

α-MEM ingurunea // Pre-IVM		
Erreaktiboak	**Etxe komertziala**	**Kantitateak**
α-MEM	Life Technologies	9580 µl
Behi fetuaren seruma %10 (FBS)	Life Technologies	250 µl
Intsulina	SIGMA	10 µl (stock 5 µg/ml)
T-S mix	SIGMA T-2036/S-9133	10 µl (stock 5 µg/ml)
E2 (17-β-estradiol)	SIGMA	10 µl (stock 10 µM)
FSH	Merck-Serono	100 µl (2,5 mIU/ml)
Epidermiseko hazkuntza-faktorea (EGF)	R&D systems	40 µl

- **L-15 ingurunea.**

Hormonekin estimulatu gabeko sagu aurre puberren obozito heldugabeak biltzeko ingurunea. IBMX konposatuari esker GV egoeran blokeatuta geratzen dira obozitoak. Inkubagailutik kanpo aldi luzeetan manipulatu beharreko prozesuetan erabili zen L-15 ingurunea, eta baita laginen berreskurapenerako prozedura guztietarako, izan obulutegi zein obozito. 4 °C -an gorde zen , gehienez 30 egunez. Plaka berogailuan 37 °C-an berotu zen erabili baino 30 minutu lehenago **(3.14 taula)**.

3.14 taula. L-15 ingurunearen osaketa.

L-15		
Erreaktiboak	**Etxe komertziala**	**Kantitateak (50 ml)**
Leibovitz's L-15 Ingurunea, GlutaMAX™ gehigarriarekin	Life Technologies	45 ml
IBMX (3-isobutyl-1-methylxanthine)	SIGMA I5879	50 µl

- **M16 ingurunea**

Sagu aurre puberren enbrioien *in vitro* ernalketarako eta *in vitro* hazkuntzarako M16 ingurunea erabili zen. Esperimentua baino 12h arinago prestatzen zen eta inkubagailuan gordetzen zen egonkortzeko **(3.15 eta 3.16 taulak)**.

3.15 taula. M16 *in vitro* ernalketarako ingurunearen osaketa.

M16			
Sagu prepubereen enbrioien *in vitro* ernalketarako hazkuntza-ingurunea			
ERREAKTIBOAK			**KANTITATEAK**
Ordena	**Izena**	**ERREF.**	**5 ml**
1	M16	Sigma M-7292	4950 µl
2	NEAA (aminoazido ez-esentzialak)	Invitrogen 11140-035	50 µl
3	BSA (bovine serum albumine,	Sigma A-9647	0,130 gr (% 3)

3.16 taula. M-16 enbrioien *in vitro* hazkuntzarako ingurunearen osaketa.

M16			
Sagu prepubereen enbrioien *in vitro* hazkuntza-ingurunea			
ERREAKTIBOAK			**KANTITATEAK**
Ordena	**Izena**	**ERREF.**	**5 ml**
1	M16	Sigma M7292	4850 µl
2	NEAA (aminoazido ez-esentzialak)	Invitrogen 11140-035	50 µl
3	EAA (aminoazio esentzialak)	Invitrogen 11130-036	100 µl

3.2 Metodoak: gizakia

3.2.1 Teknikak

3.2.1.1 Giza obozitoen eta pikor-geruzako zelulen lorpena

Giza obozitoak eta pikor-geruzako zelulak Gurutzeta Unibertsitate Ospitaleko Giza Ugalkortasun Unitateko FIV/ICSI programako pazienteetatik eskuratu ziren. Lagin biologikoen eta giza laginen erabilera dela eta, ikerketa UPV/EHUren eta Osakidetzaren Ikerkuntza Klinikoaren Batzorde Etikoaren oniritziaz egin zen (Osakidetza, CEIC E07/54 kodea, 3.akta/2008). Pazienteek ere, onarpen sinatua eman zuten.

Obozitoak erauzteko hormonen bidezko obulutegien estimulazioa egin zitzaien pazienteei, ahalik eta folikulu gehien eskuratzeko asmoz. Obulutegien estimulazioa egiteko FSH hormona birkonbinantea eta LH hormona birkonbinantea erabili ziren. Tratamenduan zehar, kontrolak egin zitzaizkien obarioetako folikuluen kopurua eta tamaina zehazteko. Paziente horiei hCG (giza hormona gonadotropina-korionikoa) izeneko hormona injektatu eta 36 ordura erauzi zitzaizkien obozitoak. Behin obozito-kumulu konplexua (COC) identifikatuta, heldutasun-maila estimatu zen bolumena, dentsitatea eta inguratzen duten zelulen egoera aztertuz, eta lau mailatan sailkatu ziren: heldua (i), heldugabea (ii), erabat heldugabea(iii) eta/edo oso heldua (iv). Obozitoak 2 orduz inkubatu ziren *in vitro* ernalketa (IVF) medioan (FIV Medio, Medicult) 37 °C-an eta % 6 CO_2ko kontzentrazioarekin.

Bestalde, espermatozoide-injekzio intrazitoplasmatikoa (ingelesez, *intracytoplasmic sperm injection,* ICSI) teknikaren ondoren, obozitoei hialuronidasa 40 UI/l-ko kontzentrazioa (Hyadase; Medicult) erabiliz pikor-geruzako zelulak erauzi zitzaizkien. Pikor-geruzako zelulak inguratzen zuten obozitoaren nukleoaren heldutasun mailaren arabera sailkatu ziren [GV (besikula germinala), MI (I. metafasea) edo MII (II. metafasea)]. Azkenik, zentrifugatu ostean, hialuronidasa medioa eta zelulak 4 ºC-ra gorde ziren eta pikor-geruzako zelulen *pellet*-a -80 °C-ra izoztu zen gerora erabiltzeko.

3.2.1.2 Sistema kannabinoidearen espresio genikoaren analisia

Heltze-prozesuan zehar, kannabinoide sistema osatzen duten kannabinoide hartzaileen eta kannabinoideen degradazio-entzimen adierazpena ikusteko obozitoen eta pikor-geruzako zeluletan, polimerasaren kate-erreakzio kuantitatiboa denbora errealean (RT-qPCR) teknika maneiatu zen. Teknika horren bidez, adierazpen genikoa neurtzen da eta, horretarako, DNA zati jakin baten kopia asko lortzeko polimerasaren kate-erreakzioa (*Polymerase Chain Reaction*; PCR) erabiltzen da, oligonukleotido espezifikoak *(primer-ak)* erabiliz.

- **RNA: erauztea eta kontzentrazioa neurtzea**

Obozitoen RNA, pikor-geruzako zelulen RNA (fase bakoitzeko 10 obozitoetatik eskuratuak), garuneko kortexaren RNA (CB1a, FAAH eta MGLLarako kontrol positiboa) eta Jurkat zelulen RNA (CB2 hartzailerako kontrol positiboa) erauzi ziren Dynabeads® mRNA Purification Kit-arekin (Ambion) hornitzailearen argibideak jarraituz.

Isolamendu protokoloa mRNA gehienen 3' muturreko poliA hondakinen eta Dynabeads® azalerarekin kobalenteki loturiko oligo (dT) hondarren arteko base pareetan oinarritzen da. PoliA buztana ez duten beste RNA mota batzuek ez dute hibridatzen pikorrekin eta erraz deuseztatzen dira. RNA erribosomikoa, DNA, proteinak eta RNA molekula txikiak (esaterako, transferentziako RNA (tRNA), mikro RNA eta RNA nukleolar txikiak) ez dira pikorrekin lotzen eta baztertu egiten dira.

Lortutako RNA laginak 30 µl Tris-HCl soluzioarekin birsuspenditu ziren erretrotranskripzio protokoloarekin jarraitzeko.

RNAren kuantifikazioa NanoDrop®ND-1000 (Thermo Scientific) izeneko espektofotometroaren bidez neurtu zen. Laginaren RNAren kontzentrazioa eta absorbantzia neurtu ziren eta laginaren purutasuna zehazteko uhin-luzera ezberdinen arteko ratioa kalkulatu zen. Ur puruarekin RNA lagina 20 aldiz diluitu ondoren espektroekorketa egin zen 200-350 nm artean. Espektrofotometroak ohiko kurba sortu behar zuen.Kurba horretan, 280 nm-an (proteina) eta 260 nm-an (RNA) absorbantzia-puntu maximoak agertzen ziren. Gure RNA purutzat hartzeko 2 baliotik gertu egon beha ziren A260/280 eta A260/230 ratioak.

Bestalde, RNAren kalitatea neurtzeko, RNA % 1,5-2 agarosa-gel batean kargatzen zen. Lagineko RNA kalitate onekoa izateko, gelean korriaraztean, 28S eta 18S RNA erribosomikoen banda diskretuak eta 2:1 proportzioan agertzea gomendatzen zen.

- **Alderantzizko transkripzioa (RT)**

RNA mezulariaren azterketa egiteko DNA osagarria (cDNA) sintetizatu behar da alderantzizko transkriptasa baten bidez. cDNA eratzeko ImProm II Alderantzizko Transkripzio sistema (Promega) erabili zen.

0,2 ml-ko Eppendorf saio-hodi batean nahastu ziren 159 ng RNA, 2 µl azarezko hexamero eta ur purua (RNasa-rik gabe). Azarezko hexameroak RNAra lotzeko, nahasketa 10 minutuan 65 °C-an berotu zen, eta, ondoren, izotzean bost minutuz utzi ziren RNAren egitura sekundarioak desegiteko. Horren ostean, behin alderantzizko transkripzio mix-a gehituta lagina termozikladorean inkubatu zen 5 minutuz 25 °C-an, 60 minutuz 55 °C-an (cDNA eratzeko) eta 15 minutuz 75°C-an (RT inhibitzeko). Azkenean, izotzean mantendu zen. Lortutako cDNA -20 °C-an gorde zen erabilia izan arte.

- **Denbora errealean egindako PCR kuantitatiboa qRT-PCR *(Quantitative Real Time Polymerase chain Reaction)***

Lagin bateko mRNA guztien cDNA eratu eta gero, intereseko genearen sekuentzia-tartea anplifikatu zen 2 primer-ak erabiliz. Sortutako DNAren kopurua zehazteko zunda fluoreszentzia erabili zen.

qRT-PCR egiteko StepOne Real-Time PCR System (Applied Biosystems) 96 putzutako termoziklagailua, erabili zen, espezifikoki diseinatua CB1a (Hs01038522_s1), CB2a (Hs00361490_m1), FAAH (Hs01038660_m1) eta MGLLa (Hs00200752_m1) bereizteko.

Kontrol positiborako ehun desberdinak erabili ziren, esaterako, garuneko kortexa CB1a, FAAH eta MGLLarako eta Jurkat zelulak CB2 hartzailerako.

qPCRa burutzeko, Taq-polimerasa, *primer*-ak eta beharrezko erreaktibo guztiak etxe komertzialak azaldutako kontzentrazio zehatzetan gehitu ziren eta lagin bakoitza termozikladorean sartu zen. Ondoren, denbora-tarte, tenperatura eta ziklo egokiak ezarri ziren: lehenik eta behin atxikipen-etapa deiturikoa 95°C-an 20 segundoz, 40 zikloko etapa 95°C-an segundo batez eta 60°C-an 20 segundoz. Lagin bakoitzeko mRNA kantitatea analizatzeko $2^{-\Delta\Delta CT}$ metodoa erabili zen GAPDH (Applied Biosystems-ekoa 4333764-0906029) erreferentzia genea erabiliz. Non $-\Delta\Delta Ct = \Delta Ct$ (lagina) $- \Delta Ct$ (kalibratzailea), eta $\Delta Ct = Ct$ (itu-genea) $- Ct$ (erreferentzia-genea) diren.

Atari-zikloari Ct deritzogu *(cycle threshold)*. Anplifikazio-kurba konkretu batentzat fluoreszentziaren detekzio-ataria gainditzeko behar den ziklo kopuruak markatuko du Ct-ak. PCR-erreakzioaren ziklo bakoitzak PCR-produktua bi aldiz handiagoa dela adierazten digu. Hau da, ziklo baten desberdintasuna duten bi laginen artean, batak besteak baino bi aldiz material gehiago du.

3.2.1.3 Proteinen presentziaren identifikazioa

Intereseko proteinak identifikatzeko Western Blot edo Immunoblot teknikak erabiltzen dira, intereseko proteina antzematen duen antigorputz espezifikoak erabiliz. Proteinaren lokalizazioari buruzko informazioa emateaz gain, kuantifikazio erlatiboa ere jakin daiteke.

- **Proteinen erauzketa eta kuantifikazioa (*Bradford* metodoa)**

Pikor-geruzako zelulen proteinen erauzketarako, 10 obozitoetatik erauzitako pikor-geruzako zelulak multzokatu ziren (n=5).

Obozito eta pikor-geruzako zeluletan dagoen proteinaren kontzentrazioa *Bradford* metodoaren arabera kalkulatu zen *(Bradford, 1976)*, *Coomasie* koloratzaile urdin argitsuarentzako polipeptidoen afinitatean oinarrituta. Koloratzailea proteinei lotzean, *Coomasie* urdinaren gehienezko uhin–luzeraren aldaketa eragiten du, 465 nm-tik 595 nm-ra igarotzen baita. Absortzioaren hazkundea, 595 nm-tan neurtutakoa,

laginean dagoen proteinaren kontzentrazioarekiko proportzionala da zuzenean eta horren balioa xurgapen patroi estrapolazio bidez kalkulatu daiteke.

Lerro estandarra egiteko, BSA kontzentrazio ezagun batetik hasten da (1 mg/ml 10 mM Tris-HCl), bolumen desberdinetan banatzen dena (0 ml-tik 25 ml-ra) eta *Bradford* (Bio-Rad) erreaktiboarekin inkubatzen da 10 minutuz. 595 nm-tara neurtutako absorbantzia baloreak batzen dira eta grafikoki adierazten dira (y = ax + b) funtzio lineala lortzeko.

Laginak 190 µl *Bradfor*rarekin inkubatu ziren (200 µl-ko disoluzioan) 10 minutuz.

- **Western Blot (WB): Elektroforesia (SDS-PAGE), transferentzia eta immunodetekzioa**

Teknika hau hiru etapetan banatzen da. Lehenengoan, poliakrilamidazko gel elektroforesian (SDS-PAGE) proteinak eremu elektriko bati esker gelaren poroetan zehar barreiatzen dira eta poroen tamainaren eta proteinaren kargaren, neurriaren, pisu molekularraren, egituraren eta hidrofobizitatearen arabera banatzen dira. Ondoren, transferentzia deritzon etapan, gelean barreiaturiko proteinak nitrozelulosa, nylon edo PVDF (Polibinilideno difluoroa, ingelesez *polyvinylidene difluoride*) mintz baten azalera itsatsiko dira, elektroforesi bidez intereseko proteina aurkitu ahal izateko antigorputz espezifikoekin.

Western Blot teknikarekin hasi aurretik elektroforesirako akrilamidazko bi gel prestatu behar dira, gel banatzailea eta gel kontzentratzailea, besteak beste. Lauki formako bi kristal aurrez aurre elkartzen dira 0,75 mm-ko tartea utzita eta oinarria ixten da, tarte horretan gelaren zati banatzailea (beheko partean) eta kontzentratzailea (goiko partean) doazelako. Gel kontzentratzailea goiko aldean egotearen arrazoia proteinak gel banatzailera igaro baino lehen lerrokatu behar direla da. Gel banatzaileari esker, SDSagatik proteinak negatiboki kargaturik daudenez eta, ondoren, SDS, β-merkaptoetanol eta beroagatik desnaturalizatzen direnez, pisu molekularraren arabera banatzen doaz.

Bi gel mota hauek egiteko osagai desberdinak nahastu behar dira proportzio zehatz batzuetan. Lehenenik eta behin, gel banatzailea egiten da eta kristalen artean isurtzen da. Behin polimerizatuta dagoenean gel kontzentratzailea gehitzen da. Hauek dira, hurrenez hurren, gel bakoitza egiteko protokoloak **(3.17 eta 3.18 taulak)**:

3.17 taula. Western Blot teknikarako gel banatzailearen osagaiak.

Gel banatzailea	
Osagaiak	**% 12ko akrilamida**
H_20	Bolumena bete arte
Tris-HCl pH 8,8 1.5 M	3,75ml
Akrilamida %30-Bis %1	6 ml
SDS %10	150 µl
PSA %10	80 µl
TEMED	10 µl

3.18 taula. Western Blot teknikarako gel kontzentratzailearen osagaiak.

Gel kontzentratzailea	
Osagaiak	**% 4ko akrilamida**
H_20	Bolumena bete arte
Tris-HCl pH 6,8	0,9 ml
Akrilamida %30-Bis %1	635 µl
SDS %10	40 µl
PSA %10	20 µl
TEMED	5 µl

Gela polimerizatu eta gero elektroforesi-indargetzaileaz beteriko elektroforesi-euskarrian jartzen da.

Konparatu beharreko laginen proteina kontzentrazioa berdindu ostean, bai pikor-geruzako zelulena baita kontrol positiboa bezala erabili zen garun kortexarena ere (2 µg/ml), proteinentzako karga indargetzailearekin berreseki edo diluitu ziren (50 mmol/L Tris-HCl pH 6.8, % 2 SDS, % 10 glizerola, % 5 β-mercaptoetanola eta % 0.1 bromofenol urdina) eta 3-5 minutuz irakiten jarri ziren 95 °C-an. Jarraian, akrilamidazko gelaren kale bakoitzean pikor-geruzako zelulak eta garun kortexa kargatu ziren pisu molekular ezaguneko proteina markatzailearekin batera (Bio-Rad Laboratories). Elektroforesian zehar, 100 V-eko boltaian korriarazi ziren proteinak kontzentrazio-gela zeharkatu arte eta, hortik aurrera, korrontea 200 V-era aldatu zen gelaren bukaeraraino heldu arte eta proteinak tamainaren arabera banatzeko.

Elektroforesia amaitutakoan, proteinak nitrozelulosazko mintz batean transferitu ziren hotzean (ordu batez, 0,3A) transferentzia elektroforetikoa erabiliz (Bio-Rad Laboratories). Horretarako, transferentzia-*sandwich*-aren muntaiaren prestaketa egin zen honako ordena hau jarraituz polo positibotik negatibora: kuxina, *Whatman* paperak (x3), gela (zati kontzentratzailerik gabe), transferentzia-mintza (PVDF), *Whatman* paperak (x3) eta kuxina.

Hurrengo pausurako, PVDF mintza errekuperatu zen eta lotura ez-espezifikoak ekiditeko blokeo-soluzioan (esne gaingabetua % 5 TBST-rekin (10 mM Tris–HCl, pH 7.4, 140 mM NaCl)) inkubatu zen ordu betez giro tenperaturan. Garbiketa egin eta gero, gizakientzako antigorputz poliklonal primario espezifikoekin inkubatu zen gau osoan zehar 4 °C-an. Hurrengo egunean, *Blotto* soluzioarekin (TBS, % 0,1 Tritoi X-100 ,% 5 eko esne gaingabetuarekin) garbiketak egin ziren (5 minutuko 3 garbiketa) eta untxiaren IgG-aren kontrako antigorputz sekundario fluoreszentearekin inkubatu zen giro-tenperaturan. Lehortu ostean, *Odyssey* izpi infragorrien irudi sistema erabiliz errebelatu zen (system LICOR Biosciences).

3.2.1.4 Proteinen lokalizazioa

- **Immunozitokimika (IZK) pikor-geruzako zeluletan eta obozitoan**

Immunozitokimika teknikari esker zeluletan dauden proteinak lokalizatu ditzakegu. Immunodetekzio ez-zuzena antigorputz primario espezifikoa eta antigorputz sekundario markatua erabiliz egiten da eta antigorputzen marka fluorogenikoa bada, antigeno espezifikoak fluoreszentzia-mikroskopio bidez detektatu eta lokalizatuko dira.

Immunofluoreszentzia bidez hartzaile bakoitzaren kokalekua zehazteko, pikor-geruzako zelulak PBS + BSA (1µg/µl)-an eseki ziren. Mikroskopio-estalki baten gainean, Poli-L-lisina jarri zen pikor-geruzako zelulak euskarri horretan itsasteko. Zelulak finkatzeko, paraformaldehido % 3 (10 min) erabili zen. Pauso bakoitzaren bukaeran, estalkiak PBSarekin garbitu ziren (3 aldiz 5 minutuz). Finkatze-prozesua amaituta, zelulak PBS + % 10 (bol/bol) behi-umekiaren serumarekin + Tritoi X100-ekin blokeatu ziren 45 minutuz giro tenperaturan. Tindaketa immunofluoreszente ez-zuzena egiteko, untxi-antigorputz poliklonal primario espezifiko bakoitzarekin (**3.4 taulan** ikusten den bezala) inkubatu ziren gau osoan zehar 4 °C-an. Lotura bikoitzerako anti-CB1 ahutz antigorputz poliklonal primarioa erabili zen eta PBSarekin berriro garbiketak (3x 5 min) egin eta gero konposatu fluorogenikoz (Alexa Fluor 488) konjugatutako untxiaren IgGen kontrako ahuntz-antigorputz sekundarioarekin inkubatu zen iluntasunean, bi orduz eta 37 °C-an. Estalkien azkeneko garbiketa egin ondoren lagin guztietan nukleoak Hoechst 33342 koloratzailearekin tindatu ziren, amaitzeko, Fluoromont G-rekin G *(*EMS*)* muntatzeko porta baten gainean. Pikor-geruzako zelulak eta obozitoak mikroskopio fokukidearen bidez aztertu ziren argon ioi bidezko laserrarekin, alde batetik, 488 nm-an (urdina) kitzikatuz eta 505- 520 nm-an (berdea) jasoz eta, bestetik, 594 nm-an (berdea) kitzikatuz eta 617 nm-an (gorria) jasoz. Nukleoen markaketa argon ioi bidezko laserrarekin 405 nm-an (UV) kitzikatuz, 440 nm-an (urdina) jaso zen, baina, sekuentzialean, Alexa-488 eta Hoestch 33342-ren seinaleak nahas ez zitezen.

Bi kontrol negatibo egin ziren. Alde batetik, antigorputz bakoitzerako peptido blokeatzaile espezifikoak erabili ziren eta, bestetik, tratamendu batean antigorputz primarioarekin egindako inkubaketa ez zen egin.

Obozitoekin egindako immunofluoreszentziaren kasuan, meiosiaren fase bakoitzeko obozitoak multzokatu ziren (GV, MI eta MII). Aurreko atalean pikor-geruzako zelulekin egindako prozedura berdina jarraitu zen.

3.3 Metodoak: sagua

Tesi honetan zehar erabili diren animalia guztiak (saguak) kontrolaturiko tenperaturan mantendu dira (23 ± 1 °C), 14 h-ko argi-zikloan eta 10 h-ko iluntasunean eta ura eta janaria *ad libitum* (modu librean) eman zazkie. Saguekin egindako esperimentu guztiak Euskal Herriko Unibertsitateak (UPV/EHU, CEEA erreferentzia zenbakia: M20-2015-016-027-028-173) onartuak izan ziren. Animaliekin egindako esperimentuak, Europako legediaren arabera eta "Laborategiko animalien erabilera eta zaintza" gidaren jarraibideen arabera burutu ziren. Erabilitako animalia guztiak lepondoko lokadura bidez hil ziren sufrimendua ahalik eta gehien ekiditeko.

3.3.1 Genotipo basatidun animaliak (*Wild type*, WT)

Obozitoak eta espermatozoideak lortzeko B6D2F1 lerroko animalia hibridoak erabili ziren, C57BL6/J emeen eta DBA/2 arren (Envigo) gurutzaketatik sortuak. Animalien zaintza eta ekoizpena Euskal Herriko Unibertsitateko Animaliategi zerbitzu orokorraren (SGIker) gomendioak jarraituz egin ziren.

Lerro bereko sagu aurrepubereak erabili ziren obozito heldugabeekin egindako esperimentuentzako. Azken animalia horiek Bruselako *Vritje Universiteit Brussel* Unibertsitateko *Follicle Biology Unit* taldeko kideen adeitasunez lortuak dira.

3.3.2 CB1, CB2 eta KO bikoitzerako hartzaileentzako *knockout* animaliak

Tesi honetan, Cb1 ($Cnr1^{-/-}$) hartzailearen genea, Cb2 ($Cnr2^{-/-}$) hartzailearen genea edota bi hartzaileak ($Cnr1^{-/-}$/$Cnr2^{-/-}$) ezabatuak zituzten sagu ereduak erabili ziren obozitoak eta enbrioiak eskuratzeko, bai *in vivo* bai *in vitro* egindako esperimentuetan.

$Cnr1^{-/-}$ eta $Cnr2^{-/-}$ genotipoko sortze animaliak Madrilgo Unibertsitate Complutense-ko (UCM), Biokimika eta Biologia molekularreko saileko *Grupo de Señalización por Cannabinoides* taldeak utziak dira.

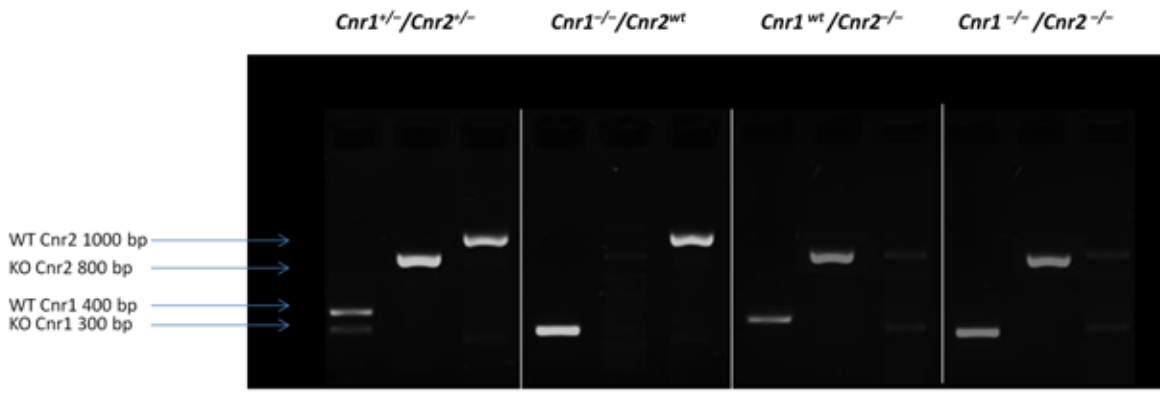
3.3.2.1 Hazkuntza

Animalien lerroen mantentzerako gurutzaketak egiteko 2 eme/ar-eko proportzioa erabili zen eta lortutako kumaldiari titia kendu zitzaion erditu ondorengo 21. egunean. Emeak eta arrak bereiziz banatu ziren.

3.3.2.2 Sagu-ehunaren DNAren lorpena genotipoa aztertzeko

Saguen buztan zati bat ebaki zen eta 35 µl–ko indargetzaile-soluzio batean inkubatu zen [Tris-HCl 10 mM, EDTA 100 mM, SDS % 0.5 (p/bol), pH 7.4] 20 mg/mL proteinasa Krekin batera (Sigma P-8044)

gau osoan zehar 55 °C-an. Hurrengo egunean, 200 µl ddH_20 gehitu ziren lagina diluitzeko eta astintze bizia egin ondoren 10.000 rpm-ra zentrifugatu zen 5 minutuz giro tenperaturan. Ondoren PCR konbentzionala egin zen aurreko soluzioaren 1 µl-rekin . **3.1 irudian** emaitzak ageri dira eta **3.19 taulan** erabilitako oligonukleotidoak.



3.1 irudia. **Saguen genotipoen azterketaren emaitzak PCR bidez.** Kontrolerako saguak: bi geneentzako heterozigosia erakusten dutenak *Cnr1*$^{+/-}$*/Cnr2*$^{+/-}$. Erabili diren sagu transgenikoen genotipoak: *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ edo *knockout* bikoitza *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$.

3.19 taula. Genotipoen azterketa egiteko erabili diren oligonukleotidoak. *Cnr1*arentzako 3 *primer*-ak batera joan daitezke PCRan, *Cnr2*arentzako 2 PCR egitea komeni da: 2 PCR: P1+P2 eta P1+P3.

Genea	*Primer*-a	Sekuentzia	Produktua (bp)	Tº eta zikloak
Cnr1	P1	5' GCTGTCTCTGGTCCTCTTAAA 3'	WT: 400	54 ºC, 35 ziklo
	P2	5' GGTGTCACCTCTGAAAACAGA 3'	CB1 KO: 300	
	P3	5' CCTACCCGGTAGAATTAGCTT 3'		
Cnr2	P1	5' AAATGCTTGATTGGTGTCAGCTCTC 3'	WT: 1100	54 ºC, 35 ziklo
	P2	5' GGCTCCTAGGTGGTTTTCACATCAGCCTCT 3'	CB2 KO: 850	
	P3	5' TAAAGCGCATGCTCCAGACTGCCTT 3'		

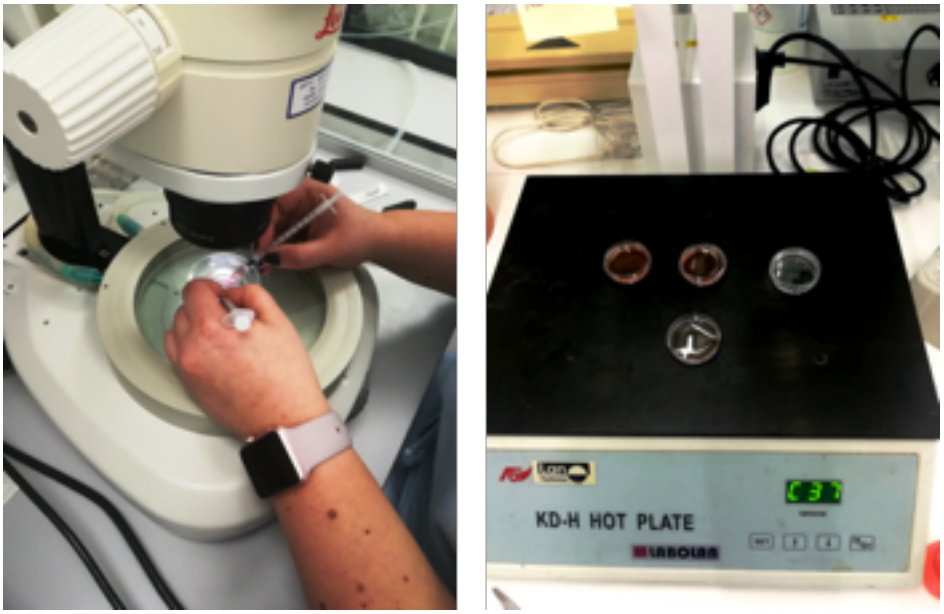
3.3.3 Sagu enbrioien *in vitro* ekoizpena

3.3.3.1 Kumulu-obozito konplexuen (COC) lorpena

8-10 asteko sagu emeen obozitoak erabili ziren eta umedun behorraren (eCG) hormona eta giza gonadotropina korionikoa (hCG) injektatu zitzaien obulatzeko. Serum fisiologikoan disolbatuta 5 UI kantitate erabili zen bi hormonentzako eta animalia bakoitzari 0,1 ml injektatu zitzaien.

Obozitoen *in vitro* madurazioa egin zen kasuetan bakarrik eCG hormona injektatu zitzaien. Enbrioien ekoizpenerako, ordea, *in vivo* heldutako obozitoak eskuratzeko bi hormonak injektatu ziren (eCG eta hCG) 48 orduko tartearekin.

COCak eskuratzeko, laburki azalduta, saguak lepondoko lokadura bidez hil ziren. Ondoren, abdomenaren behealdean mozketa txiki bat egin zitzaien diafragmaren hertzeetaraino zabaltzeko eta, modu horretan, organoak ikusi ahal izateko. Jarraian, obarioak erauzi ziren eta M2 medioa zeukaten plaketan jarri. Horren ostean, orratz baten bidez folikuluak ziztatu ziren barruko COCak jasotzeko **(3.2 irudia)**

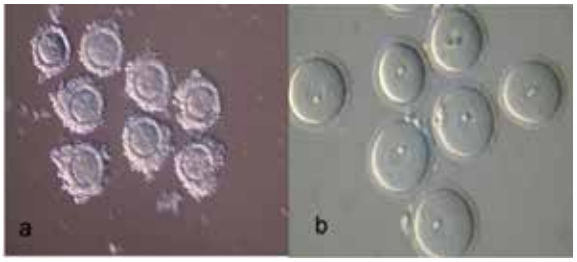


3.2 irudia. COC-en erauzketa

3.3.3.2 Sagu obozitoen *in vitro* heltzea (*in vitro maturation, IVM*, ingelesez)

(Martin-Coello eta lank., 2008)-ren protokoloari aldaketa batzuk eginez burutu zen **(3.4 irudia)**:

Obozitoen *in vitro* heltzerako, eCG eman eta 48 ordura saguak hil ziren lepondoko lokadura bidez. Esan bezala, ondoren, abdomenaren behealdean mozketa txiki bat egin zen diafragmaren hertzeetaraino zabaltzeko, modu horretan organoak ikusi ahal izateko. Jarraian, obarioak erauzi ziren eta M2 medioa zeukaten 35 mm-ko plaketan jarri ziren. Gantz guztia garbitu ondoren folikuluak ziztatu ziren 30-G kalibredun orratzarekin. GV fasean zeuden COCak aukeratu ziren, hain zuzen ere, besikula germinala eta zitoplasma uniformea zituztenak eta pikor-geruzako zelulez osatutako geruza ugariz guztiz inguraturik zeudenak **(3.3 irudia)**. Obulutegien erauzketatik aurrerako prozedura guztiak mikroskopio estereoskopikoa erabiliz egin ziren eta plaka berogailu batean 37 ºC-an.

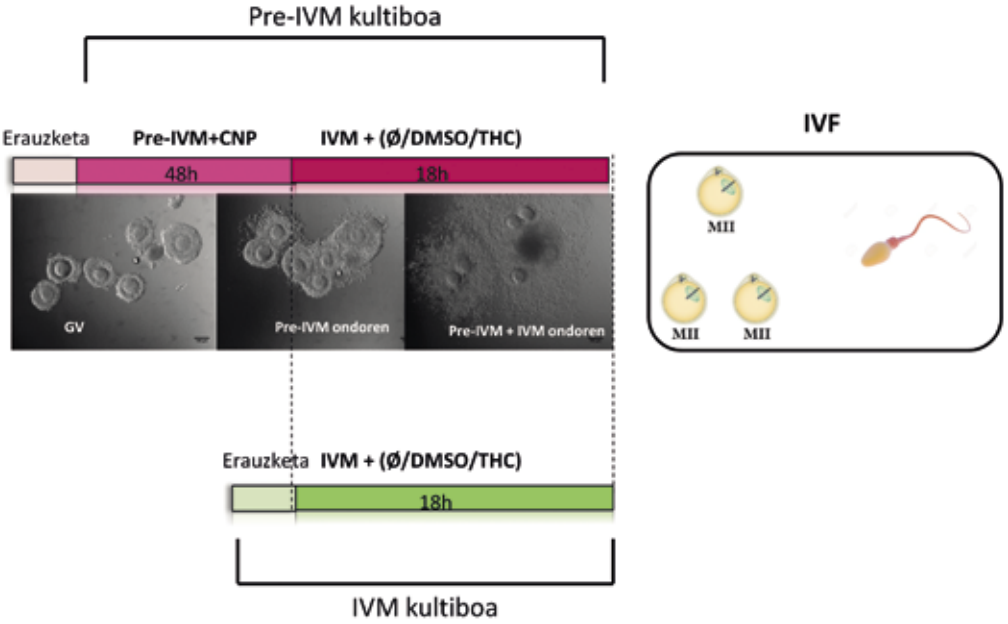


3.3 irudia. IVM teknikarako COCak hautatzeko irizpideak: a) COC egokiak kumuluko pikor-geruzako zelulez inguratuak eta b) egokiak ez diren COC, biluziak *(Nikseresht eta lank., 2017).*

Obarioen puntzioaren eta COCen aukeraketak ez zuen iraun 30 minutu baino gehiago horien kalitatean eraginik izan ez zezan. TCM-199 heltze-medioa erabili zen eta % 0.5-eko gentamizina (Sigma G-1272)), 0,01 mg/mL epidermiseko hazkuntza-faktorea (EGF; Sigma E-4127) eta % 10 (bol/bol) behi-umekiaren seruma (FBS) gehitu ziren medio horretara. Heltze-mediora pasatu aurretik aukeratutako COCak birritan garbitu ziren M2 medioan. Ostean, 20ko taldeetan inkubatu ziren 17 orduz, 500 µl heltze-medioa zeukaten 4 putzutako Nunc plaketan (Nunc, Roskilde) 37 °C-an, CO_2 % 5eko atmosfera kontrolatuan eta % 95eko hezetasunarekin.

3.3.3.3 Sexualki heldu gabeko saguen (aurre-pubereak) COCen erauzketa, kultiboa eta PRE-IVM heltze prozesua

19-21 eguneko WT (C57BL/6xCBA) leinuko sagu heldugabeen COCak eskuratu ziren. Sagu horiei ez zitzaien hormonarik injektatu. Saguak lepondoko lokadura bidez hil ziren eta, ondoren, aurretik deskribatu bezala obarioak erauzi zitzaizkien. Obulutegiak L-15 Leibovitz medioa (Invitrogen) eta IBMX % 0,001 zeukaten 35 mm plaketan jarri ziren. Gantz guztia garbitu ondoren obulutegiak ziztatu ziren 30-G kalibredun orratzarekin. GV fasean zeuden COC heldugabeak batu eta MEM medioan (% 2,5 FBS, 5 µg/ml intsulina, 5 lg/ml apo-transferrina eta 5 µg/ml sodio selenitoa) inkubatu ziren. Pre-IVM esperimentuak egiteko mediora CNP-22 (CNP) eta 17-β -estradiola (E2) gehitu ziren eta 48 orduz utzi ziren inkubatzen. Geroxeago, IVM esperimentuak egiteko, aldiz, MEM mediora epidermiseko hazkutza-faktorea (EGF) eta hormona folikulu estimulatzailea (FSH) gehitu ziren eta 17 orduz egon ziren kultiboan **(3.4 irudia)**.



3. 4 irudia. Hormonarik jaso gabeko sagu heldugabeen obozitoen *in vitro* heltzea: Pre-IVM eta IVM faseak. IVM teknikaren kasuan, obozitoak THCarekin edo THCrik gabe heldu ziren. (FOBI taldetik moldatua)

Horrez gain, bi kontrol egin ziren obozitoaren garapen gaitasuna ebaluatzeko: 1) IVM kontrola, hormonarik jaso gabeko 20 eguneko saguen obozitoak zuzenean inkubatu ziren heltze-medioan 18 orduz. 2) *In vivo* kontrola, non *in vivo* heldutako obozitoak eskuratu ziren, 2.5 IU eCGa eta 2.5 IU hCG hormona-dosiak jasotako 25-27 eguneko sagu helduetatik. Obozito guztiak esperma lagin berarekin ernaldu ziren eta enbrioien kultiboa baldintza berdinetan egin zen.

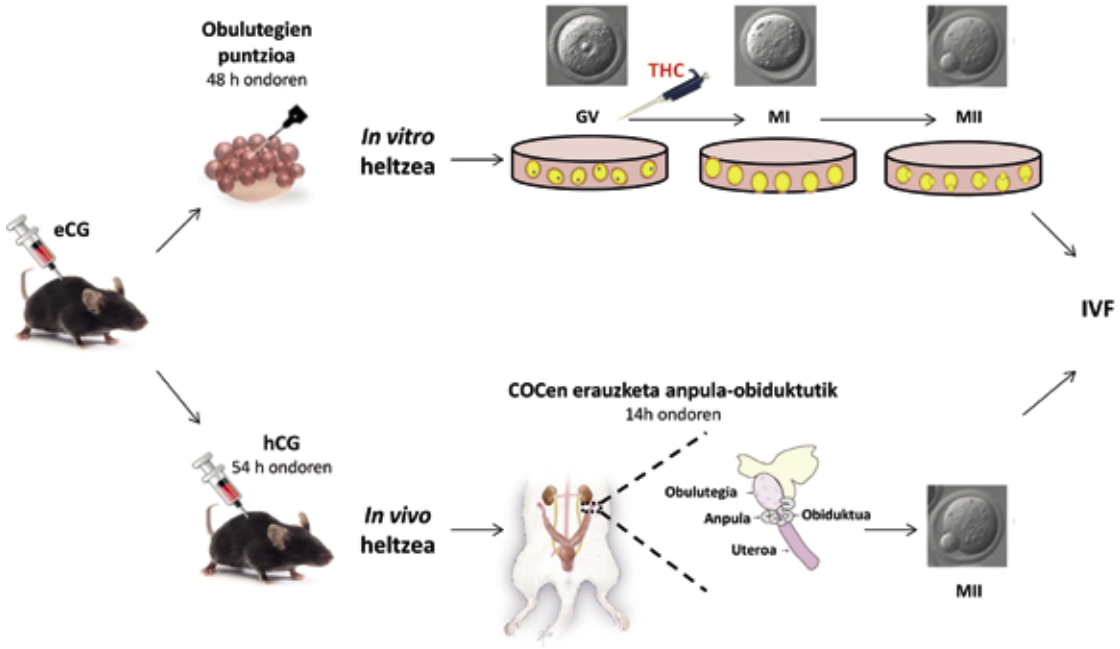
3.3.3.4 THC fitokannabinoidearekin inkubazioak

Heltzean zehar COCak THC fitokannabinoidearen dosi desberdinekin inkubatu ziren. *Stock*-eko soluzioak DMSO garraiatzailearekin prestatu ziren konposatu kannabinoideen izaera lipofilikoa dela eta. Dosi gorakorrak aukeratu ziren hartzaile kannabinoideen aktibazioan egon zitekeen eragina neurtzeko, besteak beste, 1 nM, 10 nM, 100 nM eta 1 µM. Kontrolerako COC talde bat konposatu kannbinoideentzako erabili zen DMSO kantitate berarekin inkubatu zen.

THC fitokannabinoidea eta THCa diluitzen den konposatua (DMSO, garraiatzailea) heltze-mediora gehitu ziren obozitoak jarri baino 5 minutu lehenago, farmako horien degradazioa ekiditeko. Heltze-prozesua amaitutakoan COC horiek *in vitro* ernalkuntza egiteko edota ondoren deskribaturik dauden esperimentuak egiteko erabili ziren.

3.3.3.5 Sagu obozitoen *in vivo* heltzea

In vivo heldutako obozitoak eskuratzeko sagu emeak lepondoko lokadura bidez hil ziren hCG eman eta 14 ordutara. Laparotomia bidezko "V" formako ebakia egin zen diafragmaren hertzeetaraino zabalduz, barrunbe peritonealeko organo guztiak agerian uzteko. Obiduktuak eskuratzeko mozketak egin ziren obulutegi eta infundibuluaren artean eta obiduktu-isatsaren eta uteroko adarraren zati kranealaren artean eta, ondoren, 35 mm-ko plaketan jarri ziren M2 medioarekin. #5 Pintzekin anpula ziztatu zen meiosia berrabiarazi zuten obozitoen multzoa berreskuratzeko, MI edo/eta MII fasean zeuden obozitoak, alegia **(3.5 irudia)**.



3.5. irudia. ***In vivo*** **eta** ***in vitro*** **heldutako obozitoak eskuratzeko teknikak** ***in vitro*** **ernalketa (IVF) egin aurretik.** *In vitro* heltzean (IVM) obozitoak TCHaren presentzian edo THCrik gabe heldu ziren.

3.3.4 *In vitro* ernalketa (IVF)

In vitro ernalketa Martín-Coello eta lankideen (2008) metodoa jarraituz egin zen. Horretarako, HTF (*Human Tubular Fluid,* ingelesez) medioa erabili zen 2 mg/ml BSA gehituta, bai esperma prestatzeko baita obozitoekin inkubatzeko ere.

3.3.4.1 Espermatozoideen prestaketa

Aurretiaz bakartutako eta 8-10 aste bitarteko B6D2F1 lerroko sagu arrak erabili ziren. Espermatozoideak ugalkortasun arazorik gabekoak zirela frogatutako arren epididimoetatik eskuratu ziren. *In vitro* heltze-prozesua egin eta 16 ordutara edota *in vivo* heltze-prozesuan hCG ziztatu eta 13 ordu ondoren sakrifikatu

ziren saguak. Lepondoko lokadura bidez hil eta gero, epididimoaren isatsa eta konduktu deferentearen zati bat erauzi zitzaizkien eta 35 mm-ko plaka batean jarri ziren. Material kirurgikoaren laguntzaz soberan zegoen gantz kantitate handiena garbitu eta konduktu deferentean dagoen odol-hodia kendu ostean, epididimoaren isatsa egun bat lehenagotik egonkortutako 500 µml-ko HTF tanta batean jarri zen. Aipatu behar da HTF tanta olio mineralez estali zela eta aurretiaz egonkortu zela medioaren pHa orekatua egon zedin.

3.3.4.2 Espermatozoideen kapazitazioa

HTF tanta horretan, mikroskopio esteroidoskopikopean semena lortzeko pintza batzuen laguntzarekin isatsaren oinarrian presionatu zen. Jarraian, plaka inkubagailuan gorde zen espermatozoideak ordu betez inkubatzeko 37 ºC-an, % 95eko hezetasunean eta % 5eko CO_2 kontzentrazioarekin, horrela, espermatozoideen sakabanatzea errazteko. 10 minututara Makler-kamararen bidez kontzentrazioa kuantifikatu zen eta ernalketa orduan ml-ko miloi bat espermatozoideko kontzentrazioa izateko hartu beharreko bolumena kalkulatu zen.

3.3.4.3 Gametoen inkubazioa

In vitro zein *in vivo* superobulazioaren bidez heldu ziren obozitoak HTF medioan bi aldiz garbitu ziren 4 putzutako Nunc plaketan jarri aurretik. Putzu bakoitzean 500 µl HTF gehitu ziren eta jarraian olio mineralez estali. Azkenik, 1 x 10^6 espermatozoideak/ml dosia gehitu zen. Gametoak 5 orduz inkubatu ziren 37 °C-tan, % 95eko hezetasunean eta % 5eko CO_2 kontzentrazioarekin.

3.3.5 Enbrioien *in vitro* hazkuntza

Ernalketarako beharrezko denbora bete eta gero ustezko zigotoak berreskuratu ziren eta HTF medioa zeukaten 35 mm-ko plaketara pasatu ziren, bertan, pipetaren laguntzaz pikor-geruzako zelulak eta zona peluzido-an itsatsitako espermatozoideak kanporatzeko. Amaitzeko, zigotoak birritan garbitu ziren HTF medioan, 25 µl-ko KSOMaa medioarekin egindako tantetan jarri aurretik. Berriz ere, medio hori olio mineralez estali zen eta enbrioiak blastozisto fasera heldu arte kultibatu ziren inkubagailuan.

Zatiketa-tasa 1go egunean ebaluatu zen, ernalketa egin eta 24 ordura, eta azkenik, blastozisto-tasaren analisia 4. egunean (96-100 ernalketa ondorengo orduak, ingelesez *hours post fertilization*, hpf) egin zen.

3.3.5.1 Ezarpenaren aurreko enbrioiaren garapenaren ebaluazioa

24 orduko kultiboaren ondoren, zatitu ez ziren zelula horiek Hoechst 33342 (Thermo Scientific) bidez tindatu ziren eta fluoreszentziako mikroskopioarekin (Axioskop, Zeiss) ebaluatu ziren. Era berean, 2 zeluletan zatitu ziren enbrioi-tasak aztertu ziren eta baita *in vitro* kultiboko hurrengo etapetako enbrioi-tasak ere: 4-8 zelula, morula eta blastozistoa.

3.3.6 Obozitoen eta pikor-geruzako zelulen immunofluoreszentzia

Inmunofluoresentzia teknikaren bidez zeluletan proteina/antigeno espezifiko baten kokapena, adierazpena eta lokalizazioa aztertu daitezke. Hain zuzen ere, intereseko proteina antigeno-antigorputz elkarrekintzaren bitartez detektatzen da, fluoreszentea den antigorputza erabiltzean honek igorritako fluoreszentzia mikroskopioan analizatuz.

CB1 eta CB2 hartzaileen presentzia aztertu zen sagu obozitoetan THC fitokannabinoidearekin *in vitro* heldu ondoren. Fase-meiotiko desberdinetako obozitoak ebaluatzeko, bai *in vitro* bai *in vivo* heldutakoak, 20 obozitoko lagina (n=20) eskuratu zen eta, horretarako, genotipo desberdin bakoitzeko WT (C57BL/6xCBA) edo $Cnr1^{-/-}$, $Cnr2^{-/-}$, eta $Cnr1^{-/-}/Cnr2^{-/-}$ anduieko 3 eme erabili ziren 3 erreplika independenteetan.

AKT eta ERK1/2 proteinen fosforilazio-patroia analizatu zen THC fitokannabinoidearen presentzian edo absentzian *in vitro* heldutako obozitoetan. Obozitoak heltze-prozesuan zehar denbora-tarte desberdinetan hartu ziren, 0 h, 10 min, 30 min eta 1 h, THCaren hasierako erantzuna aztertzeko, eta 17 h-tara, ikusteko fosforilazio-patroian antzemandako aldaketak heltze-prozesu osoan zehar mantentzen ziren.

Obozitoak PBS + % 1eko polibinil alkoholarekin (PBS-PVA) (Sigma P-8136) garbitu ziren eta ostean, % 4 paraformaldehidoarekin 10 minutuz fixatu ziren giro tenperaturan (Panreac). Ondoren, obozitoak 3 aldiz garbitu ziren PBSarekin eta 45 minutuz iragazkortu ziren giro tenperaturan % 10 (bol/bol) behi-umekiaren serumarekin (FBS) eta % 1 tritoi X-100 detergentearekin (zelulak iragazkortu eta zelula barneko antigenoak markatu ahal izateko). Serumaren kontzentrazio altuaren bitartez antigorputzak ehunarekiko duen espezifikotasun eza blokeatu zen. Iragazkortasunaren ondoren, tindaketa immunofluoreszente ez-zuzena egiteko, obozitoak PBS-PVAan diluitutako untxi-antigorputz poliklonal primario espezifiko bakoitzarekin + % 5 FBS inkubatu ziren gau osoan zehar 4 °C-an. Antigorputz ezberdinentzako kontzentrazioak **3.4** eta **3.5 tauletan** ageri dira.

Antigorputz primarioarekin inkubazio-aldia bete ondoren, obozitoak PBSarekin 3 aldiz garbitu ziren eta PBS-PVAn eta % 5eko FBSan diluitutako konposatu flourogenikoz konjokatutako untxiaren IgG-en kontrako ahuntz-antigorputz sekundarioarekin inkubatu ziren bi orduz giro tenperaturan. Alexa Fluor® 488 antigorputz sekundarioa erabili zen. Geroxeago, PBS-PVArekin 3 garbiketa egin ondoren nukleoak Hoechst 33342 5µg/mL markatzailearekin tindatu ziren 10 minutuz giro tenperaturan obozito bakoitzaren heltze-fasea errazago zehazteko. Behin obozitoak azkenengoz garbituta, mikro-tantetan estalki borobilen gainean jarri ziren kontu handiz eta buruz behera muntatu ziren porta baten gainean Fluoromont (EMS) muntai medioaren laguntzaz. Zelulak, mikroskopio fokukidearen bidez analizatu ziren Euskal Herriko Unibertsitateko (UPV/EHU) SGIker mikroskopia zerbitzu orokorrean. Irudiak Image J softwarea erabiliz aztertu ziren.

Esperimentu guztietan kontrol negatiboak egin ziren antigorputz primarioa kenduta.

3.3.7 Fluoreszentziazko mikroskopioa eta mikroskopio fokukidea

Obozioten nukleoa aztertzeko eta, era horretan, meiosiaren faseak zehazteko, fluoreszentziazko mikroskopioa erabili zen *(Gutiérrez-Adán eta Pintado, 2000)*. Nukleoen tindaketarako erabili zen Hoechst 33342 markatzailea argon ioi bidezko laserrarekin 405 nm-an (UV) kitzikatuz eta 440 nm-an (urdina) jasoz ikusi zen.

Proteinen presentzia eta kokapena analizatzeko ApoTome.2 (Zeiss) eta LSM 800 (Zeiss) mikroskopio fokukideak erabili ziren. Markaketa detektatzeko argon ioi bidezko laserra erabili zen 488 nm-an (urdina) kitzikatuz eta 505-520 nm-an (berdea) jasoz baina era sekuentzialean, Alexa-488 eta Hoestch 33342 markatzailearen seinaleak ez nahasteko. 2 µm-ko xafla optikoak moztu ziren obozitoaren irudi tridimentsionala egiteko. 20X handipena erabili zen 1.5x eko zoom-arekin.

3.3.8 Obozitoen apoptosiaren azterketa

Zelula apoptotikoak detektatzeko TUNEL (Terminal Deoxynucleotidil Transferase-mediated dUTP Nick end-Labeling) teknika erabili zen.

In situ cell death detection Kit, TMR red (ROCHE) kit-a erabili zen eta fabrikatzailearen argibideak jarraitu ziren. Proba honen bidez DNAn egon litezkeen apurketak deoxinukleotidil transferasa edo TdT terminalaren bidez identifikatu daitezke eta entzima batek bigarren markatzaile batekin (fluorokromoa) lotuta dauden dUTPak katalizatuko ditu. 60 µl erreakzio erabili zen guztira, Label Soluzioko 55 µl+ 5 µl entzima.

Kontroleko obozitoak eta DMSOarekin eta THCarekin heldutako obozitoak fabrikatzailearen argibideen arabera prozesatu ziren. Bukatzeko, nukleoak tindatzeko Hoechst 33342 markatzailea erabili zen.

3.3.9 Obulutegien morfologiaren eta folikulugenesiaren ebaluazioa

3.3.9.1 Obulutegien bilketa eta histologia

WT eta Cnr1$^{-/-}$, Cnr2$^{-/-}$, eta Cnr1$^{-/-}$/Cnr2$^{-/-}$ sagu emeak 3.3.3.2 atalean deskribatzen den bezala hil ziren, horietariko batzuei eCG ziztatu eta 48 ordura. Obulutegiak osorik erauzi ziren azterketa histologikoa egiteko eta M2 medioan jarrita soberan zeukaten gantza kendu zitzaien. PBSan garbitu ondoren *Bouin* soluzioan murgildu ziren (% 1 azido pikrikoa (pisu/bol), % 10 formaldehidoa (bol/bol) eta % 5 azido

azetikoa (pisu/bol)) eta 4-5 orduz utzi ziren. Denbora hori pasatuta % 70eko etanolean garbitu ziren eta obulutegiak kutxatiletan sartu ondoren % 50eko etanolera pasatu ziren parafina blokeak egiteko (**3.19 taula)**. Alkoholean deshidratatu eta gero xilolean tratatu ziren parafina histologikora pasatu aurretik.

Parafina solidifikatuzko blokeak 8 µm-ko lodieran moztu ziren mikrotomoarekin eta zatiak portetan jarri ziren.

3.19 Taula. Parafina blokeak egin aurretiko ehunaren tratamendua.

Parafinan murgilketa protokoloa	
Alkohola % 50	1 h 30 min
Alkohola % 70	1 h 30 min
Alkohola % 96	1 h 30 min
Alkohola % 96	1 h 30 min
Alkohola % 100	1 h 30 min
Alkohola % 100	1 h 30 min
Alkohola % 100	1 h 30 min
Metilo benzoatoa	1 h
Metilo benzoatoa	1 h 45 min
Zitrosola	45 min
Parafina	2 h 30 min
Parafina	2 h 30 min

3.3.9.2 Hematoxilina-eosina tindaketa

Obulutegi zatiekin zeuden portei parafina kentzeko xilolean murgildu ziren eta, ondoren, kontzentrazio beherakorreko etanoletatik (% 100, % 96, % 70, % 50) pasatu ziren. Hurrengo taulan agertzen den protokoloa jarraiki hematoxilina-eosina tindaketa egin zen folikuluen kontaketa egiteko. Azpimarratu behar da hematoxilina-eosina tindaketa, gehienetan, modu automatikoan egin zela makina tindatzailea erabiliz.

3.20 Taula. Ehunak hematoxilina-eosinarekin tindatzeko protokoloa.

Hematoxilina-eosia tindaketa protokoloa	
Zitrosola	10 min
Zitrosola	10 min
Zitrosola	5 min
Alkohola % 100	10 seg
Alkohola % 96	10 seg
Alkohola % 96	10 seg
Alkohola % 70	10 seg
Alkohola % 50	10 seg
Ura	5 min
Harris Hematoxilina	45 min
Ura	2 min

Hematoxilina-eosia tindaketa protokoloa	
Ur klorhidrikoa	3 seg
Ura	5 min
Eosina Y	25 seg
Ura	5 min
Alkohola % 50	10 seg
Alkohola % 70	10 seg
Alkohola % 96	10 seg
Alkohola % 100	10 seg
Alkohola % 100	10 seg
Alkohola % 100: Zitrosola 1:1	10 seg
Zitrosola	5 min
Zitrosola	5 min
Zitrosola	5 min

Portak prestaketa histologikoentzako espezifikoa den erretxina batekin muntatu ziren.

Mozketa histologikoak fixatzeko erabili ziren portak aldez aurretik polilisinatu egin ziren: 24 orduz % 70eko etanola eta HCl 5 ml/l soluzioan eduki ondoren garbitu, ondo sikatu eta Poli-L-lisinan (% 0,1) murgildu ziren 10 minutuz. Azkenik, lehortzen utzi ziren.

3.3.9.3 Obulutegien azterketa eta folikuluen kontaketa

Folikuluen kopurua estimatzeko porta bakoitzean zeuden mozketa histologikoen argazkiak atera ziren. Irudi guztiak kamera digital batera konektaturiko mikroskopio optiko batekin atera ziren (Olympus XC50). Analisia bost mozketero egin zen.

Obulutegien azalera FIJI softwarea erabiliz neurtu zen eta, aldiz, bolumena kalkulatu zen azalera, mozketaren lodiera eta mozketa kopurua biderkatuz Tilly J-ak (2003) deskribatu bezala.

Folikuluen kopuruaren zenbaketa eta sailkapena bi pertsonek egin zuten, subjektibotasunak ahalik eta gutxien eragiteko. Folikuluak 4 taldetan sailkatu ziren, besteak beste, folikulu primordialak (obozitoa inguratzen duten pikor-geruzako zelula lauen geruza bakarra), folikulu primarioak (obozitoa inguratzen duten pikor-geruzako zelula kubiko geruza bakarra), folikulu sekundarioak (obozitoa inguratzen duten pikor-geruzako zelula kuboide geruza) eta De Graaf-en folikuluak edo antralak (antroa deituriko likidoz beteriko barrunbea ageri da obozito barruan) *(Silva eta lank., 2004)*.

Obulutegi bakoitzaren bolumena neurtu eta (mozketaren azalera x mozketaren lodiera x mozketa kopurua) folikuluen zenbaketa egin ostean folikuluen kopurua ezarri zen folikuluak/obario-ehun mm3 kalkulua eginda. Horren ondoren, genotipo bakoitzean, obulutegi bakoitzean zeuden folikulu desberdinen ehunekoak kalkulatu ziren.

3.4 Erabilitako programak

3.4.1 Irudiak

- (Olympus XC50).
- Adobe Photoshop CS3: (Adobe) Irudien doikuntza
- ImageJ (National Institutes of Health) Dohaineko software irudien analisi eta neurketetarako (azalera) eta *blots*-en dentsitometria neurtzeko.

3.4.2 Bestelakoak

- Graphpad
- SPSS Statistics (v.22)
- Excell

3.5 Datuen analisi estatistikoak

Emaitzen analisi estatistikoa aurreko atalean aipatutako softwareekin egin zen, esaterako, IBM SPSS Statistics (v.22)eta GraphPAD Prism-arekin (v. 5.01).

Konparazio anizkoitzak behar zituzten esperimentuen froga estatistikoak, bide bakarreko bariantza ANOVA analisi sinplearen bitartez egin ziren, eta jarraian Tukey edo Bonferroni-ren *post hoc* frogak egin ziren. Desberdintasunak estatistikoki adierazgarriak kontsideratu ziren $p < 0,05$ edo $p < 0,01$ izanik. Bi talde desberdinen batez bestekoen erkaketetarako, desberdintasunak *Student* testatekin egin ziren eta emaitzak estatistikoki esanguratsuak kontsideratu ziren $*p < 0,05$.

4

Emaitzak
Results

4.1 Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation

4.1 Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation

Ekaitz Agirregoitia [a,b], Inés Ibarra-Lecue [c], **Lide Totorikaguena** [a], Rosario Mendoza [c,d], Antonia Expósito [b,d], Roberto Matorras [c,d], Leyre Urigüen [b,c,e], and Naiara Agirregoitia [a,b].

[a]Department of Physiology, Faculty of Medicine and Dentistry, University of the Basque Country, Leioa; [b]BioCruces Health Research Institute, Barakaldo; [c]Department of Pharmacology, Faculty of Medicine and Dentistry, University of the Basque Country, Leioa; [d]Human Reproduction Unity, Department of Obstetrics and Gynecology, Cruces University Hospital, University of the Basque Country, Barakaldo; and [e]Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Madrid, Spain

Abstract

Objective: To describe the expression of cannabinoid receptors CB1 and CB2 and cannabinoid-degrading enzymes fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL) in human granulosa cells and to investigate their differential distribution with respect to CB1 at various stages during the nuclear maturation of the oocyte.

Design: Analysis of granulosa cells from germinal vesicle (GV), metaphase I (MI), and MII oocytes by quantitative reverse transcriptase—polymerase chain reaction, Western blot, and indirect immunofluorescence assays.

Setting: Academic research laboratory.

Patient(s): Patients from the Human Reproduction Unit of Cruces University Hospital undergoing intracytoplasmic sperm injection.

Intervention(s): We analyzed the granulosa cells of 300 oocytes from 53 patients. The oocyte maturation stages were 75 at GV stage, 51 at MI, and 174 at MII.

Main Outcome Measure(s): The mRNA and protein expression of CB1, CB2, FAAH, and MGLL and localization in granulosa cells at each oocyte maturation stage.

Result(s): CB1, FAAH, and MGLL are present in human granulosa cells during oocyte maturation, but the presence of CB2 receptor is not entirely clear in those cells. CB1 and FAAH were detected in the periphery of the granulosa cells from the GV to the MII oocytes, and they colocalized in some portions of the cell membrane. On the other hand, MGLL immunostaining was more homogeneous across the cell and overlapped with CB1 only weakly.

Conclusion(s): The presence of the cannabinoid system in granulosa cells suggests a possible

role of this system in the nuclear maturation of the oocyte. (Fertil Steril_ 2015;104:753–60. ©2015 by American Society for Reproductive Medicine.

Key Words: *Cannabinoid, granulosa cells, oocyte maturation*

Introduction

Oocyte maturation is a highly coordinated process that includes the progression of the oocyte from the first meiotic blockage at the germinal vesicle (GV) stage to the second meiotic blockage at the metaphase II (MII) stage. This progression occurs inside the follicle owing to a careful communication between oocytes and granulosa cells. These granulose cells are somatic cells that surround the oocyte, but, when the follicle antrum is formed, they separate into mural granulosa cells forming the follicle inner wall and into cumulus granulose cells, which are closely communicated with each other and with the oocyte through gap junctions (*Sanchez and Smitz, 2012)*.

Oocyte maturation can also be carried out *in vitro* as long as the oocyte is cultured without removing the surrounding granulosa cells. This cumulus-oocyte complex (COC) is the structure that is ovulated *in vivo*. For oocyte maturation bidirectional communication is necessary, via gap junctions and via paracrine signaling, between the oocyte and granulose cells *(Albertini et al., 2001)*. In this sense, many substances from the COC have been implicated in the regulation of the oocyte maturation; some of these act once they are translocated by the gap junction, and others act on receptors present on the cumulus cells and/or the oocyte *(Gilchrist et al., 2004)*.

Among all these substances, the cannabinoid compounds have been highlighted in some studies. Endocannabinoids are fatty acid derivatives that exert their effects by bindin to membrane Gi/o protein-coupled receptors CB1 and CB2. Anandamide (AEA) and 2 arachiconoylglycerol (2-AG) are the better characterized endocannabinoids. AEA and 2-AG are degraded by fatty acid amide hydrolase (FAAH), which is found on the internal membranes of cells, although 2-AG is degraded by monoglyceride lipase (MGLL) as well. Several studies have been published concerning the role of cannabinoids in the female reproductive system *(de Miguel et al., 1998; Wenger et al., 1999; Wenger et al., 2001)*, and the cannabinoid receptors and degradation enzymes have been found in various parts of the mammalian femalereproductive system as uterus, oviduct *(Das et al., 1995; Paria et al., 2001; Dennedy et al., 2004; Wang et al., 2004)*, preimplantation embryos, and placenta *(Park et al., 2003; Helliwell et al., 2004; Habayeb et al., 2008)*.

In regard to the oocyte maturation, it is known that AEA is present in the human follicular fluid *(Schuel et al., 2002a)* and that its concentration in follicles with mature oocytes is higher than in follicles with immature oocytes *(El-Talatini et al., 2009a)*. The localization of the cannabinoid receptors and enzymes in rat *(Bagavandoss and Grimshaw, 2010)* and human ovary led to the hypothesis that AEA plays a role in folliculogenesis, preovulatory follicle maturation, oocyte maturity, and/or ovulation *(El-Talatini et al., 2009a)*. The hypothesis about oocyte maturation was reinforced with the localization of cannabinoid receptors *(Peralta et al., 2011)* and cannabinoid-degrading enzymes (authors' unpublished data) during the nuclear maturation of human oocytes, where each protein is relocated during that maturation.

Taking into account the necessary bidirectional communication between the oocyte and the granulosa cells, our study aimed to characterize in depth, using a variety of experimental methods, the differential

expression of the two cannabinoid receptors and the two degrading enzymes in human granulosa cells that surround oocytes at each stage of meiotic resumption.

Material and methods

Granulosa Cells Collection

Granulosa cells were obtained from 53 patients (ages 25–40 years; mean, 36.7 ± 2.6) undergoing intracytoplasmic sperm injection (ICSI) at the Human Reproduction Unit of the Cruces University Hospital. Ethical approval was obtained from the Clinical Research Ethical Committee of the Basque Health System (Osakidetza, CEIC reference no.r E07/54, 3/ 2008). Informed consent was obtained from all patients. We analyzed the granulosa cells of 300 oocytes from 53 patients. Of the 300 oocytes studied, 75 were at the GV stage, 51 at MI, and 174 at MII. The main ICSI indications were male factor (79.9%) and failure of IUI. The main female-associated conditions were endometriosis (9.8%) and tubal factor (11.2%). Patients had no history of cannabinoid drug consumption.

The ovarian stimulation protocol has been published elsewhere *(Matorras et al., 2002; Matorras et al., 2004)*. Briefly, it consists of down-regulation with GnRH agonist and triptorelin acetate on a long protocol or with GnRH antagonist and the cetrorelix protocol, ovarian stimulation with recombinant FSH and highly purified urinary menopausal gonadotropins or recombinant LH, and ovulation being triggered with 250 µg recombinant hCG. Oocyte retrieval was performed 35–37 hours after hCG administration. Follicles were aspirated with a negative pressure of 115–120 mm Hg with a single lumen 18-gauge oocyte pick-up needle (K-OPS-6035-RWH-B-ET; Cook) under transvaginal ultrasound guidance. Follicular fluids were observed at low magnification (x 40–100) under the stereomicroscope at 37 ºC. The COCs were incubated for 2 hours in IVF Medium (IVF Medium, Medicult, Origio) at 37 ºC and 6% CO_2 in air. Immediately before micromanipulation for the ICSI procedure, oocytes were denuded from the cumulus oophorus one by one in 30-µL droplets by a brief exposure to 40 IU/L hyaluronidase solution (Hyadase; Medicult, Origio), followed by mechanical removal of the corona radiate cells with the use of plastic pipettes of defined diameters (denuding pipette; Cook). The droplets containing the cumulus and granulosa cells after oocyte denuding were recovered in Eppendorf tubes, taking into account the nuclear maturation stage of the oocyte for separation into granulosa cells from GV, MI, or MII, and were centrifugated at 800 g. Finally, the medium containing the hyaluronidase solution was removed and the cells were placed at 4 ºC for early use or in the freezer at -80 ºC for later study.

Reverse Transcription

RNA from granulosa cells (obtained from 10 oocytes of each stage; n = 3), cerebral cortex (positive control for CB1, FAAH, and MGLL) and Jurkat cells (positive control for CB2), were isolated with the Dynabeads mRNA Purification Kit (Ambion). The procedure for obtaining the cDNA was performed with ImProm-II Reverse Transcription System (Promega). Briefly, about 150 ng of RNA and random

primers were heated at 65 ºC for 10 minutes and chilled on ice for 5 minutes. Then, once the reverse transcription mix was added, it was annealed at 25 ºC for 5 minutes. A firststrand synthesis reaction was carried out at 55 ºC for 60 minutes, and the reverse transcriptase was inactivated at 70 ºC for 15 minutes. Real-time Quantitative Polymerase Chain Reaction (PCR) Analysis Quantitative PCR was performed in three replicates with the StepOne thermocycler using a TaqMan assay (Applied Biosystems) specifically designed for recognizing CB1 (Hs01038522_s1), CB2 (Hs00361490_m1), FAAH (Hs01038660_m1), and MGLL (Hs00200752_m1). All the primers used were functional because we detected mRNA amplification when we used different tissues as positive controls (human cerebral cortex for CB1, FAAH, and MGLL and Jurkat cells for CB2). In addition, the controls performed in the absence of reverse transcriptase or in the absence of template were negative (data not shown).

The thermal profile for this PCR consisted of a "holding stage" of 20 seconds at 95 ºC and 40 cycles with two steps: 1 second at 95 ºC and 20 seconds at 60 ºC. We used GAPDH (4333764-0906029) as the endogenous control gene. The amount of CB1, CB2, FAAH, and MGLL mRNA in each sample was determined by the $2^{-\Delta\Delta CT}$ method with GAPDH as the reference gene. The average ΔCt of the cortex samples was used as the calibrator.

SDS/PAGE and Immunoblotting

Granulosa cells from 10 oocytes were collected and pooled (n= 5) in sodium dodecyl sulfate (SDS) sample buffer (50 mmol/ L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue). Human cerebral cortex membranes were prepared as described elsewhere *(Uriguen et al, 2009)*. Jurkat cells were purchased from Merck Millipore (Molsheim). Gel electrophoresis was carried out in SDS polyacrylamide gels, composed of 5% stacking (0.5 M Tris-HCl, pH 6.8, 10% SDS) and 12% resolving (1.5 M Tris-HCl, pH 8.8, 10% SDS), using a miniprotean system with molecular weight standards (Bio-Rad Laboratories). Proteins were then transferred to nitrocellulose membranes (1 hour, 0.3 A) using an electrophoretic transfer system (Bio-Rad Laboratories). The nonspecific binding sites in the membranes were blocked for 1.5 hours with Tris-buffered saline with Tween (TBST; 10 mM Tris- HCl, pH 7.4, 140 mM NaCl) containing 5% skimmed milk.

The membranes were incubated with a primary rabbit polyclonal anti-CB1 receptor antibody (1:1,000; Frontier Institute), anti-CB2 receptor antibody (1:200), anti-FAAH antibody (1:200), and anti-MGL antibody (1:200; Cayman Chemicals), overnight at 4 ºC and all in TBST, 5% skimmed milk and 0.1% Tween buffer. Membranes were incubated with fluorescent antirabbit IgG secondary antibody (Alexa 680 Rabbit, Invitrogen) at a dilution of 1:4,000. They were then dried and imaged using the Odyssey infrared imaging system (LI-COR Biosciences). Integrated intensities were analyzed using Excel (Microsoft Corp.) and Prism (GraphPad Software) software.

Immunofluorescence

Granulosa cells were suspended in phosphate buffered saline (PBS) + bovine serum albumin (BSA; 1 µg/µL), smeared onto a slide coated with poly-L-lysine, and fixed with 3% paraformaldehyde for 10 minutes. Then the slides were washed 3 times in PBS and incubated for 45 minutes in PBS/10% (v/v) bovine fetal serum and 1% Triton x 100 at room temperature. For indirect immunofluorescence staining, slides were incubated with primary rabbit polyclonal anti-CB1, anti-CB2, anti-FAAH, and anti-MGL antisera at a dilution of 1:200 overnight at 4 ºC. For double staining, primary goat polyclonal anti-CB1 (Santa Cruz Biotechnology) was used. Slides were then washed in PBS + BSA (1 µg/µL) 3 times, incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes) for 2 hours at 37 ºC in the dark (1:2,500), washed in PBS + BSA (1 µg/µL) 3 times (in all cases we stained the nuclei with Hoechst 33342 during the second wash), assembled with Fluoromount G (EMS), and finally examined by confocal microscopy. Two negative controls were performed using a specific blocking peptide for each antibody and by omitting the primary antibody before addition of the secondary antibody.

Statistics

Results shown represent mean ± SEM. Statistical analysis was performed by analysis of variance with a post hoc analysis by Bonferroni's multiple comparison test (GraphPad Prism® 5).

Results

Quantitative Reverse Transcriptase (RT-) PCR Analysis of CB1, CB2, FAAH, and MGLL mRNA in Human Granulosa Cells during Nuclear Maturation of Oocytes

We detected the presence of CB1, FAAH, and MGLL but not CB2 receptor transcripts in human granulose cells using quantitative RT-PCR. However, when we analyzed the relative amount of each mRNA, we did not find significant differences between granulosa cells of oocytes in GV, MI, or MII **(Fig. 4.1)**.

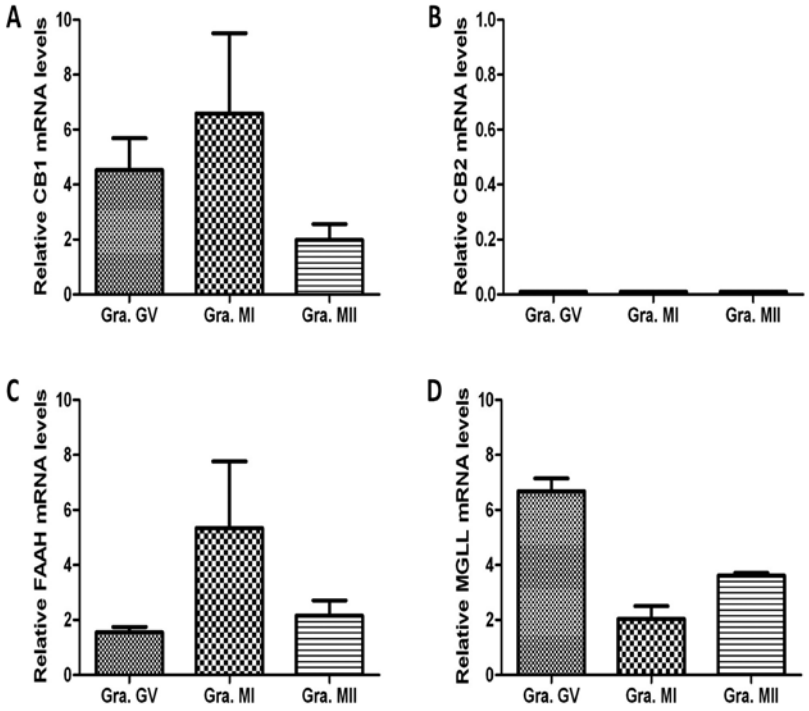


Figure 4.1 mRNA expression of CB1, CB2, FAAH and MGLL in human granulosa cells (Gra.) come from GV, MI and MII oocytes using specific primers. The relative concentrations of proteins mRNA were determined by quantitative RT-PCR. Cycle threshold values were normalized to GAPDH and the average ΔCt of the cortex sample (for CB1, FAAH and MGLL) and Jurkat cells (for CB2) were used for calibration. Each value is the mean (± SEM) of 3 replicates.

Immunocytochemical Localization CB1, CB2, FAAH, and MGLL in Human Granulosa Cells during Nuclear Maturation of Oocytes

Immunofluorescence analysis revealed that CB1 receptor was present in the human granulosa cells and that its localization was predominantly in the periphery of the cells **(Fig. 4.2)**. When we evaluated whether the localization of CB1 receptor changed if the granulosa cells came from GV, MI, or MII oocytes, we did not observe any change in the localization **(Fig. 4.2)**. With regard to CB2, this receptor showed a very weak immunostaining and although it is difficult to ensure that it was a specific staining, it appears to be a more peripheral staining than that within the granulosa cells from all studied maturation stages **(Fig. 4.2)**. On the other hand, the FAAH-degrading enzyme's immunostaining pattern did not change regardless of whether the granulosa cells came from a GV, MI, or MII oocyte, but, in this case, its localization was present over the entire granulosa cell **(Fig. 4.2)**. Finally, similar results were found for the MGLL, although the staining was weaker than for the FAAH **(Fig. 4.2)**. When the antibodies were blocked with the specific peptide before addition to the granulosa cells, no specific fluorescence was observed **(Fig. 4.2)**.

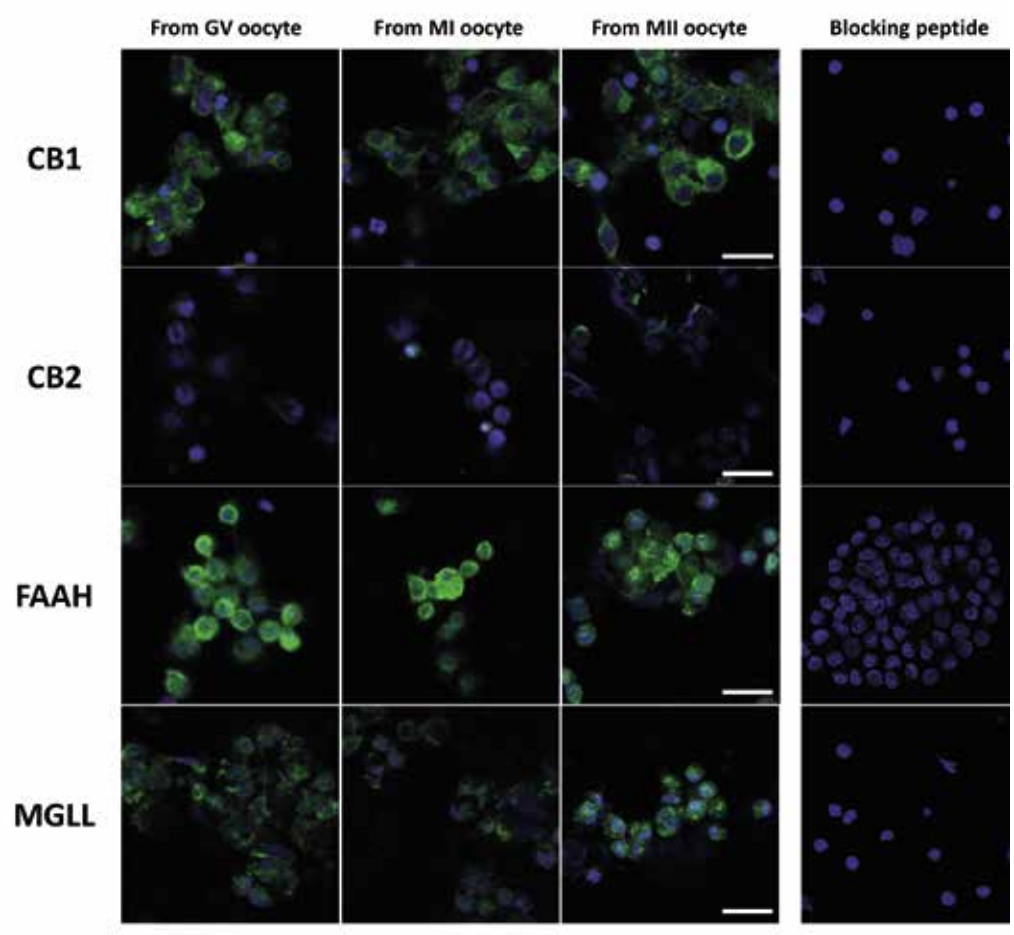


Figure 4.2 Immunofluorescence analysis of CB1, CB2, FAAH and MGLL in human granulosa cells come from GV, MI and MII oocytes. The distribution of cannabinoid receptors and cannabinoid-degrading enzymes is shown in green. A negative control consisting of preadsorption of primary antibody with each specific blocking peptide is shown. Hoechst-labeled DNA is shown in blue. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 10 μm.

Immunoidentification of CB1, CB2, FAAH, and MGLL Proteins in Human Granulosa Cells

To confirm the presence/absence of studied proteins, **Figure 4.3** shows representative Western blots using human granulose cells, human prefrontal cerebral cortex gray matter, and Jurkat cells. The anti-CB1 receptor polyclonal antiserum labeled a major band at ~60 kDa in granulosa cells and cortex protein extracts. The anti-CB2 receptor polyclonal antiserum recognized a major band at ~40 kDa in granulose cells and in Jurkat cells. The anti-FAAH polyclonal antibody labeled a major band at about ~60 kDa in granulosa cells and cortex protein extracts. Finally, the anti-MGLL polyclonal antibody labeled two major bands around ~35 kDa in granulose cells and in cortex.

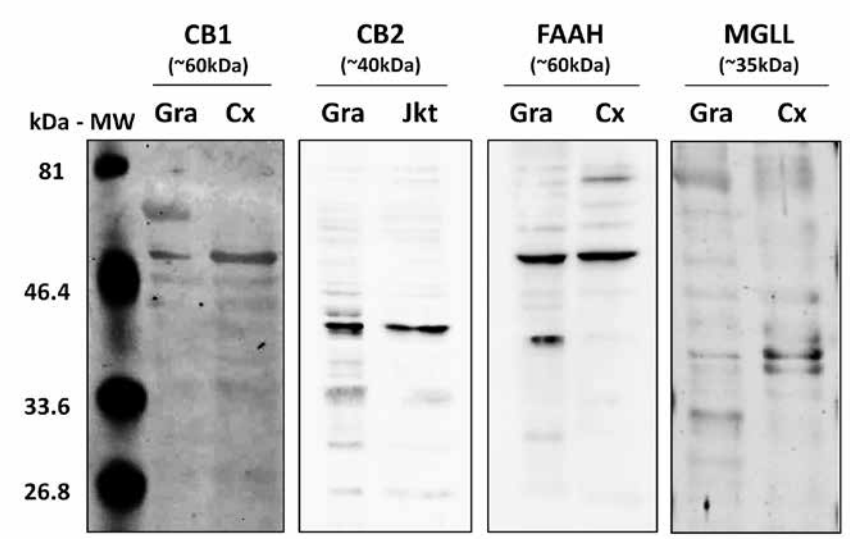


Figure 4.3 Western blotting analysis of CB1 and CB2 receptor, FAAH and MGLL in human granulosa cells (Gra), gray matter from the human prefrontal cerebral cortex (Cx) and jurkat cells (Jur) using a rabbit antiserum against the CB1, CB2, FAAH and MGLL. Molecular weights (MW-kDa) are indicated on the left. n=3; representative western blots are shown.

Immunocytochemical Localization of CB2, FAAH, and MGLL Compared with that of the CB1 Receptor in Human Granulosa Cells during Nuclear Maturation of Oocytes

As we observed that there was no change in the location of the studied proteins in human granulosa cells during nuclear maturation of oocytes, we analyzed in depth the localization of each protein and the colocalization of those proteins in comparison with CB1 receptor since this was the most abundant (and maybe the unique) cannabinoid receptor in these cells. For this purpose, we used granulosa cells coming from different stages, and the results were the same for all stages. For that reason, only representative data are shown **(Fig. 4.4)**.

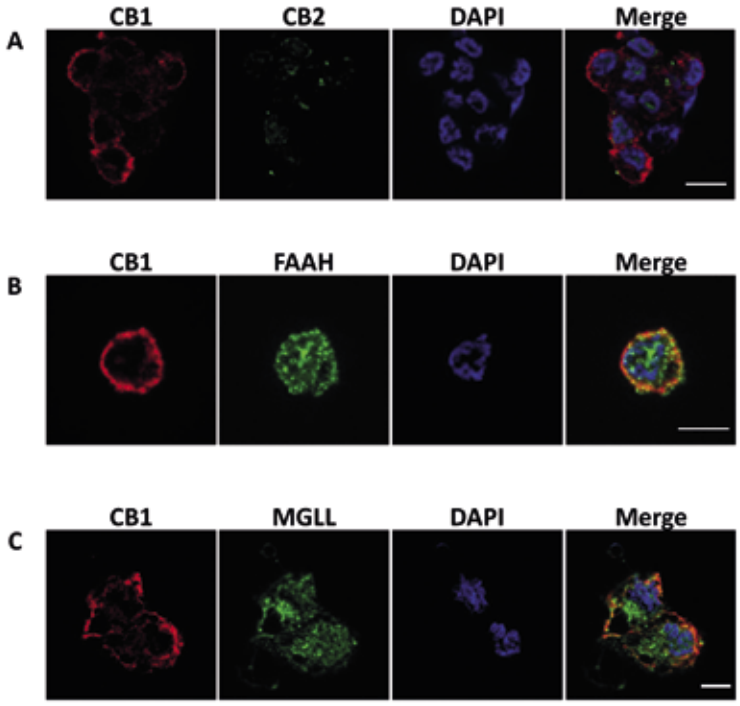


Figure 4.4 Immunofluorescence analysis of CB2, FAAH and MGLL compared to CB1cannabinoid receptor in human granulosa cells. The distributions of CB2, FAAH and MGLL are shown in green and the distributions of CB1 is shown in. Their overlap are shown in yellow. Hoechst-labeled DNA is shown in blue. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 10 μm.

We confirmed that CB1 receptor was localized in the periphery of granulosa cells **(Fig. 4.4A, 4.4B, and 4.4C)**, unquestionably in the cell membrane. In the same way, we again found a very weak presence of the CB2 receptor in those cells **(Fig. 4.4A)**. With regard to FAAH, this degrading enzyme was present in both the cytoplasm and the plasma membrane of granulose cells, in fact, FAAH colocalized with the CB1 receptor at the cell membrane **(Fig. 4.4B)**. Finally, MGLL showed a weak colocalization with the CB1 receptor in the periphery of the cell, but its localization was predominantly in the cytoplasm of the granulosa cells **(Fig. 4.4C)**.

Discussion

The role of the endocannabinoid system has been studied in mammalian reproductive events, such as spermatogenesis, sperm motility, fertilization, embryo oviductal transport, preimplantation embryo development, implantation, and postimplantation embryonic growth *(Wang et al., 2006b; Taylor et al., 2007; Sun and Dey, 2012)*. Even so, the role of this system in the ovary and oocyte events has rarely been analyzed *(El-Talatini et al., 2009a; Bagavandoss and Grimshaw, 2010; Peralta et al., 2011)*. The aim of the present study was to gather further data on the presence and localization of cannabinoid receptors CB1 and CB2 as well as cannabinoid-degrading enzymes FAAH and MGLL in granulosa cells during the resumption of meiosis of the human oocyte to determine possible role of the cannabinoid system in this process. Western blot analysis revealed the presence of CB1, CB2, FAAH, and MGLL proteins in human granulosa cells. With regard to CB1, the band of about 60 kDa (positive control: gray matter from the human prefrontal cerebral cortex) accords in size with previous reports *(Rossato et al., 2005; De Jesus et al., 2006)*. In the case of CB2, the bands of approximately 40 kDa (positive control: Jurkat cells) correspond to its theoretical molecular mass *(Filppula et al., 2004)* and are in agreement with previous reports *(Zhang et al., 2007; Agirregoitia et al., 2010)*. We have to note that we needed to load a great amount of sample to see a positive mark in granulosa cells. On the other hand, the band around 60 kDa observed in human granulosa cells using an anti-FAAH antibody, which also appeared in the human cerebral cortex, has been described in some studies using rat brain *(Bisogno et al., 1998)* and human sperm *(Francavilla et al., 2009)* and bladder *(Bakali et al., 2013)*. Finally, the two major bands observed around 35 kDa in granulosa cells and in the cortex are near the theoretical molecular weight for MGLL around 33.3 kDa *(Dinh et al., 2002)*. However, we cannot exclude that the band around 33 kDa shown in granulosa cells **(Fig. 4.3)** corresponds to MGLL too.

Two previous works have described the presence of cannabinoid system components in the granulosa cells using immunohistochemical assays in ovarian sections. Thus, with regard to granulosa cells, in rat ovaries it has been described in the presence of CB1 receptor in the plasma membrane of granulosa cells of antral follicles *(Bagavandoss and Grimshaw, 2010)*, and the same result has been observed in human antral follicles as well *(El-Talatini et al., 2019a)*. In our study we have gone a step further, and we have seen that CB1 is still present in the plasma membrane of granulose cells from ovulated oocytes (GV) and that it is maintained there at least until oocyte and granulosa cells are separated after oocyte maturation (MII). Moreover, we have detected that the mRNA of CB1 is present in granulosa cells during the nuclear maturation of the oocyte, but we cannot confirm whether the expression pattern observed during the maturation changed significantly. Even so, we have to point out that the mRNA expression pattern of both cannabinoid receptor CB1 and cannabinoid-degrading enzyme FAAH in granulose cells during the nuclear maturation of the oocyte is the same, although we cannot corroborate this expression pattern at the protein level.

The presence of CB2 receptor in granulosa cells is controversial because this receptor is not present in granulosa cells of rat antral follicles *(Bagavandoss and Grimshaw, 2010)*, but its signal has been described in the antral follicles of human ovary, although more weakly than that of CB1 *(El-Talatini et al., 2019a)*. In that sense, we immunodetected CB2 signal by Western blot (loading a great amount of granulose

cells); we did not find the mRNA of CB2 in granulose cells, and the immunocytochemical assays showed a weak staining of CB2. Taking into account all of these data, we are not able to discard the presence of CB2 in granulosa cells, but we can affirm that its presence would be more residual and much lower than CB1 receptor.

On the other hand, the absence of FAAH described in rat granulosa cells from antral follicles *(Bagavandoss and Grimshaw, 2010)* and the weak staining observed in human granulosa cells from secondary and antral follicles *(El-Talatini et al., 2019a)* are not consistent with our observations. Our study was carried out in granulosa cells from ovulated oocytes and the presence of FAAH has been corroborated using immunocytochemistry, Western blot, and PCR. For this reason, we can conclude that the nondetection of this enzyme in granulosa cells from antral follicles described in other studies could be due to a relatively low level of FAAH expression rather than its absence. Even so, it could also be possible that FAAH would start its expression once the ovulation has occurred. But this hypothesis should be checked because we have not performed experiments with granulose cells from non stimulated follicles.

Finally, to the best of our knowledge, this is the first time that MGLL has been found to be localized in the granulosa cells. Although we are not able to confirm whether the expression pattern observed during oocyte maturation changed significantly the mRNA expression pattern of MGLL seem to be just the contrary compared with CB1 and FAAH. In our study we used granulosa cells isolated from follicles maturated *in vivo* after hormone stimulation of the patient. For this reason, the recovered oocytes had already been stimulated and only the final nuclear maturation steps were carried out *in vitro* during 2 hours. This could be the reason that we did not see significant differences in the protein or mRNA pattern between granulosa cells from GV, MI, or MII oocytes.

The regulation of "AEA tone" by FAAH has been proposed as an important event in the oviduct during early pregnancy *(Wang et al., 2006a)*, as well as in blastocyst activation *(Wang et al., 2003)* and implantation *(Paria et al., 1999)*, but no hypotheses have been proposed in relation to such a process in oocyte maturation. Even so, it has been described that AEA concentration in human follicular fluid rises during oocyte maturation *(El-Talatini et al., 2019a)* and that the concentration is even higher in the oviduct than in follicular fluid *(Gebeh et al., 2012)*.

In humans, one oocyte completes its growth each month and is ovulated in response to a midcycle surge of LH, and in response to this LH surge, the oocyte resumes meiosis *(Hunt and Hassold, 2008)*. Therefore, the oocyte and its granulosa cells find increasing concentrations of AEA while they mature and so the presence of the cannabinoid system in the granulosa cells leads to the hypothesis that the resumption of meiosis in the oviduct, where AEA concentration is higher than it is in follicular fluid, could be modulated, inter alia, by the cannabinoid system. In contrast, the elevated levels of cyclic AMP (cAMP) produced by adenylyl cyclase are crucial in maintaining oocytes under meiotic arrest, and the source of cAMP has been suggested to be the product of the influx of cAMP through gap junction communication from the cumulus cells to the oocyte *(Sanchez and Smitz, 2012)*. Taking all these data into account, we hypothesize that the stimulation of CB1 cannabinoid receptor by its agonist in granulosa cells, first in the follicle and then in the oviduct, could activate a number of signal transduction pathways via the Gi/o

family of G proteins, leading to the inhibition of adenylyl cyclase and the consequent inhibition of cAMP production *(Wang et al., 2003)*. Moreover, although the most abundant protein in the plasma membrane of granulosa cells was CB1, FAAH colocalized with it. This overlapping pattern in the expression of CB1 and FAAH has been reported in other cell types, such as neurons, suggesting that the endocannabinoid system might be responsible for an autofeedback control of neurotransmitter release *(Bouskila et al., 2012)*.

Finally, Schuel and Burkman *(Schuel and Burkman, 2005)* observed that sea urchin eggs might release AEA after activation by the fertilizing sperm and this released AEA might then react with cannabinoid receptors in sperm to block acrosome reaction, thereby helping to prevent polyspermy. So as granulosa cells from ovulated oocytes have the enzymatic machinery to degrade AEA, this fact led us to hypothesize that granulosa cells could regulate the AEA tone near the microenvironment where the fecundation takes place to modulate the sperm physiology, as Schuel and Burkman proposed.

It will be interesting to analyze what the role of the cannabinoid system in granulosa cells is in relation to the oocyte where CB1, CB2, FAAH, and MGLL relocate during the nuclear maturation *(Peralta et al., 2011; author's unpublished data)*. To date, the presence of 2-AG has only been described in the uterus *(Wang et al., 2007),* but it remains unknown whether it is present in the ovary or the oviduct, a question that merits investigation.

In conclusion, we report for the first time the presence of the cannabinoid system in granulosa cells from GV, MI, and MII oocytes. This could suggest that AEA, which is present in follicular and oviductal fluids *(Schuel et al., 2002a; El-Talatini et al., 2009a)*, also has a role during the final nuclear maturation of the oocyte. It should be noted that the granulosa cells used in this study were obtained in the context of an IVF/ICSI program and hence may not reflect the real status of oocyte maturation during spontaneous natural ovarian cycles. Further studies are needed to investigate the cannabinoid receptors in unstimulated cycles.

4.2 Dynamic of expression and localization of cannabinoid-degrading enzymes FAAH and MGLL in relation to CB1 during meiotic maturation of human oocytes

4.2 Dynamic of expression and localization of cannabinoid-degrading enzymes FAAH and MGLL in relation to CB1 during meiotic maturation of human oocytes

Ekaitz Agirregoitia[1], **Lide Totorikaguena[1]**, Antonia Expósito[2], Rosario Mendoza[2], Roberto Matorras[2], Naiara Agirregoitia[1]

[1]Department of Physiology, Faculty of Medicine and Dentistry,University of the Basque Country, Barrio Sarriena s/n,Leioa 48940, Bizkaia

[2]Human Reproduction Unit, Cruces University Hospital, Universityof the Basque Country, Plaza de Cruces s/n,Barakaldo 48903, Bizkaia

Abstract

The endogenous cannabinoid system has been characterized in some female reproductive organs but little is known about the expression and localization pattern of cannabinoid-degrading enzymes in relation to the CB1 cannabinoid receptor in human oocytes. In this study, we focus on the investigation of the presence and differential distribution of fatty acid amide hydrolase (FAAH) and monoglyceridelipase (MGLL) in relation to CB1 during the maturation of human oocytes. We used a total of 290 human oocytes not suitable for *in vitro* fertilization/intracytoplasmic sperm injection (ICSI): germinal-vesicle (GV) and metaphase-I (MI) stages and metaphase-II (MII) oocytes that had not developed into an embryo after ICSI. Cannabinoid-degrading enzymes and the cannabinoid CB1 receptor were present in human oocytes. Specifically, FAAH was detected in the periphery of the oocyte from the GV to MI stage and co-localized withCB1. Later, by the MII stage, FAAH was spread within theoocyte, whereas MGLL immune staining was homogeneous across the oocyte at all stages of maturation and only overlapped with CB1 at the GV stage. This coordinated redistribution of cannabinoid system proteins suggests a role for this system in the maturation of the female gamete.

Keywords: *Meiosis. Resumption. Enzyme. Maturation. Gamete*

This work was supported by a grant from the University of the Basque Country (GIU14/26).

Introduction

Oocyte meiotic maturation is the process by which immature oocytes become fertilizable eggs. Fully grown oocytes are arrested at the first meiotic prophase, in the germinal vesicle (GV) stage and, once they become meiotically competent, resume meiosis upon receiving specific ovulatory signals. Meiotic maturation continues with the transition from meiosis I to meiosis II until oocytes are then arrested at metaphase of meiosis II (MII); an oocyte does not complete meiosis II until it is fertilized by sperm *(Schindler, 2011)*.

The molecular mechanisms responsible for oocyte maturation are not fully understood and several different molecules have been implicated as modulators of the process. Among these molecules, the role of the cannabinoid compounds has been highlighted by some authors *(Sun and Dey, 2012)*. Endocannabinoids are fatty acid derivatives that exert their effects by binding to membrane Gi/o protein-coupled receptors CB1 and CB2. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids. Both are degraded by fatty acid amide hydrolase (FAAH), which is an integral membrane protein with its hydrolytic site facing the lipid bilayer, although 2-AG is also degraded by monoacylglycerol lipase (MGLL), which, in contrast, is a cytosolic enzyme. The distributions of FAAHand MGLL overlap in the central nervous system, but they are not identical. For example, in some cerebral areas, MGLL is localized presynaptically, whereas FAAH is localized postsynaptically. Nevertheless, one proposal is that, together, endocannabinoid-metabolizing enzymes can complement the distribution of cannabinoid receptors in the brain and that this provides multiple opportunities for local regulation of endocannabinoid tone *(Yazulla, 2008)*.

Several papers have been published on the role of cannabinoids in the female reproductive system *(de Miguel et al., 1998; Wenger et al., 1999, 2001)* and both cannabinoid receptors and degradation enzymes have been found in various parts of the mammalian female reproductive system, namely, the uterus and oviduct (*Das et al., 1995; Paria et al., 2001; Dennedy et al., 2004; Wang et al., 2004)* preimplantation embryos and the placenta *(Park et al., 2003; Helliwell et al., 2004; Habayeb et al., 2008)*. With regard to oocyte maturation, AEA is known to be present in human follicular fluid *(Schuel et al., 2002a)* and its concentration is higher in follicles with mature oocytes than in those with immature oocytes *(El-Talatini et al., 2009a)*. The localization of the cannabinoid receptors and degrading enzymes in rat *(Bagavandoss and Grimshaw, 2010)* and human ovary has led to the hypothesis that AEA plays a role in folliculogenesis, preovulatory follicle maturation oocyte maturity, and ovulation (*El-Talatini et al. 2009a)*. Finally, the hypothesis with respect to the role of the cannabinoid system in oocyte maturation has been reinforced by findings concerning the localization of cannabinoid receptors during the nuclear maturation of human oocytes, in which each receptor is re-localized *(Peralta et al., 2011)*.

In view of the presence of AEA in human follicular fluid and the re-localization of cannabinoid receptors observed in the oocyte during its maturation, cannabinoid signaling can be postulated to be involved in the regulation of oocyte meiosis *(Peralta et al., 2011)*. Therefore, to shed further light on this issue,

our aim is to characterize, in detail and to compare the expression and distribution of the two AEA-degrading enzymes, FAAH and MGLL and further to explore their relationship with the CB1 receptor in human oocytes during the meiotic resumption of oocytes obtained during *in vitro* fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) cycles.

Materials and methods

Oocyte collection

Human oocytes were obtained from 90 patients (aged 25-40 years; mean 35.02 ± 3.23 years) undergoing IVF/ICSI at the Human Reproduction Unit of Cruces University Hospital. Ethical approval was obtained from the Clinical Research Ethics Committee of the Basque Health System (Osakidetza, CEIC reference number E07/54, 3/2008). Informed consent was obtained from all patients. Overall, 290 oocytes from these patients were analyzed. The main indications for IVF/ICSI were: male factor (62%), failure of intrauterine insemination (7%) and idiopathic infertility (10%). The main associated female conditions were endometriosis (9.5%) and tubal factor (11.5%). Patients had no previous history of cannabinoid drug consumption.

All the oocytes used for this study, were those discarded from treatment cycles. The majority of oocytes corresponded to immature oocytes (GV and MI) or to MII oocytes from IVF/ICSI procedures in which fertilization failed. Additionally, there were 8 cases (44 oocytes) in which IVF/ICSI could not be attempted because of failure to obtain a sperm sample, since at that time oocyte vitrification was not available in our laboratory. After removing the corona cumulus cells, they were stored and transported in minimal media in micro-centrifuge tubes at 4ºC to the laboratory for processing. The zona pellucida of oocytes used for RT-PCR was removed by incubating oocytes into a Tyrode's acidic solution at 37°C for 30 seconds in order to remove possible attached granulose cells.

Our IVF cycle management has been described previously (Matorras et al., 2009). Briefly, it consists of long down-regulation with the gonadotropin-releasing hormone agonist triptorelin (Decapeptyl, Ipsen Pharma, Spain) or the GnRH antagonist cetrorelix (Cetrotide, Merck Serono, Spain). Then, ovarian stimulation was carried out with recombinant FSH (Gonal F, Merck Serono, Spain) and highly purified urinary menopausal gonadotropins (Menopur, Ferring, Spain) or recombinant LH, ovulation being triggered with 250 mg recombinant hCG (Ovitrelle, Merck Serono, Spain). Transvaginal ultrasound-guided oocyte retrieval was scheduled 36 h after hCG injection.

All IVF/ICSI procedures and assessments were performed by members of the research group. Oocyte retrieval was performed 35-37 hours after hCG administration. Follicles were aspirated with a negative pressure of 115–120 mm Hg with a single lumen 18-gauge oocyte pick-up needle (K-OPS-6035-RWH-B-ET; Cook, Spain) under transvaginal ultrasound guidance. Follicular fluids were examined at low

magnification (40X to 100X) under the stereomicroscope at 37ºC. The oocyte–cumulus-complex were incubated for 2 h in IVF Medium (IVF Medium, Origio, Denmark) at 37ºC and in 6% CO2 in air. Immediately prior to micromanipulation for the ICSI procedure, oocytes were denuded of the cumulus oophorus by a brief exposure to 40 IU/L hyaluronidase solution (Hyadase; Origio, Denmark, Sweden), followed by mechanical removal of the corona radiate cells with the use of plastic pipettes of (denuding pipette; Cook, Spain). Each oocyte was examined under the microscope to assess its maturation stage and integrity. MII oocytes were defined by the absence of the germinal vesicle and the presence of an extruded polar body. Metaphase II oocytes were microinjected and placed in the culture incubator following the standard IVF culture protocol. Oocyte fertilization assessment involved careful analysis of the two pronuclei and the nucleoli within them, during a single examination 16-18 h after the insemination procedure. Normally fertilized and non-fertilized oocytes were then placed in the incubator for a 24-hour culture period until embryo selection for transfer. The immature oocytes (at MI and GV stages) and the non-fertilized MII oocytes were placed in microcentrifuge tubes with a minimal amount of Flushing Medium (Origio, Denmark, Sweden) and stored in the fridge at 4ºC or in the freezer at -80ºC until use for the present study.

Of the 290 studied oocytes, 59 were at the GV, 65 at the MI and 166 at the MII stage. MII oocytes were collected on day 1 after IVF/ICSI, when fertilization failed, i.e., they were unfertilized. GV and MI oocytes were collected the day of oocyte pick up in ICSI cases. During the period of study, the fertilization rate of MII oocytes was 64% in our ICSI program.

RT-PCR analysis

Total RNA from oocytes (3 samples of ~30 oocytes, from each maturation stage: GV, MI and MII) and cerebral cortex was extracted by using the Dynabeads mRNA-DIRECT Kit (Ambion, Austin) following the manufacturer's instructions. Immediately after extraction, total RNA was kept frozen at −80 °C until further use. cDNA was obtained with the ImProm-II Reverse Transcription System (Promega, Madison). Briefly, a mixture of about 150 ng RNA and random primers was heated at 65 °C for 10 min and chilled on ice for 5 min. Then, after addition of the reverse transcription mix, it was annealed at 25 °C for 5 min. First strand synthesis was carried out at 55 °C for 60 min and the reverse transcriptase was inactivated at 70 °C for 15 min. Primers used for PCR were as follows: human FAAH, 5'-AAGCAACATACCCCATGCTC-3'and 5'-GTTTTGCGGTACACCTCGAT-3' (276-bp product; *Storr et al., 2009*); human MGLL, 5'-CAAGGCCCTCATCTTTGTGT-3' and 5'-ACGTGGAAGTCAGACACTAC-3' (162-bp product; *Ludanyi et al., 2008*). Human ACTB (β-actin) primers, 5'-TCCCTGGAGAAGAGCTACGA-3' and 5'-ATCTGCTGGAAGGTGGACAG-3' (362-bp product; *Agirregoitia et al., 2010*) were used as internal controls. All primer pairs were located on different exons to avoid amplification of genomic DNA. PCR was performed under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The mixture was electrophoretically separated on a 2% agarose gel

Immunofluorescence

After assessment of the meiotic stage, oocytes were suspended in phosphate-buffered saline plus bovine serum albumin (PBS+BSA; 1µg/µl) and smeared onto a slide coated with poly-L-lysine, in order to localize the target protein immuno-cytochemically. They were then fixed with 3% paraformaldehyde for 10 min followed by incubation in Triton X100 (0.1%) for 10 min to permeabilize the oocytes. Subsequently, slides were washed three times in PBS and incubated for 20 min in PBS/10% (v/v) bovine fetal serum at room temperature. For indirect immunofluorescence staining, slides were incubated with rabbit polyclonal FAAH and MGLL antibodies (Cayman Chemicals) at a dilution of 1:200 overnight at 4 °C. For double-staining, in addition to the anti-FAAH or anti-MGLL antibodies, we used goat polyclonal CB1 antibody (Santa Cruz Biotechnology) at a dilution of 1:50. Slides were then washed in PBS+BSA (1µg/µl) three times, incubated with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 donkey anti-goat IgG secondary antibodies (Molecular Probes) for 2 h at 37 °C in the dark and washed in PBS+BSA (1µg/µl) three times with staining of the nuclei with Hoechst 33342 during the second wash to facilitate assessment of the maturational stage of each oocyte. Finally, oocytes were mounted with Fluoromount G (EMS) and examined by confocal microscopy. Images were acquired with an Olympus Fluoview FV500 confocal microscope by using a 40× lens (UPLAPO; NA: 0.85). Each fluorochrome was sequentially acquired in order to avoid overlapping of fluorescent emission spectra. Two negative controls were performed by using specific blocking peptides for each antibody (Cayman Chemicals) and by omitting the primary antibody before addition of the secondary antibody to set the background acquisition parameters.

Results

RT-PCR analysis of CB1 and cannabinoid-degrading enzyme mRNA in human oocytes

We detected the presence of CB1 and FAAH but not MGLL degrading-enzyme transcripts in human oocytes by using RT-PCR. The expected 276-bp fragment for FAAH was detected during all stages of oocyte maturation and in samples of gray matter of human prefrontal cerebral cortex (positive control; **Fig. 4.5a)**. The 162-bp fragment corresponding to MGLL was detected in human cortex (positive control) but not in oocytes at any stage of maturation **(Fig. 4.5b)**. The housekeeping gene ACTB was detected in all tissues **(Fig. 4.5c)**.

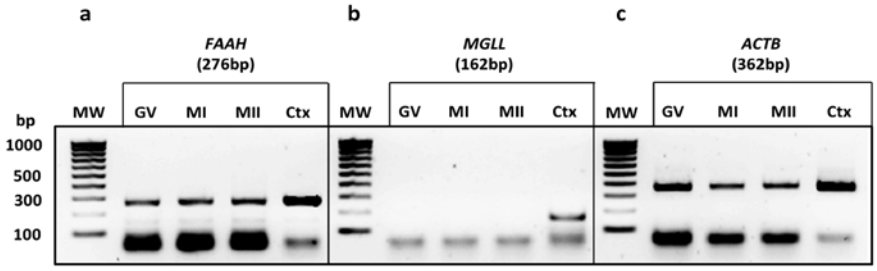


Figure 4.5 Ethidium bromide-stained 2% agarose electrophoresis gel of the RT-PCR products for CB1, FAAH, MGLL and β-actin (ACTB) in the human oocyte at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages and gray matter from the human prefrontal cerebral cortex (Ctx). CB1: amplified fragment using primers specific for the human cannabinoid receptor 1 (402-bp band). FAAH: amplified fragment using primers specific for the human cannabinoid-degrading enzyme FAAH (276-bp band). MGLL: amplified fragment using primers specific for the human cannabinoid-degrading enzyme MGLL (162-bp band). ACTB was used as an internal control (362-bp band). Molecular weights (MW; bp) are indicated on the left. n = 3; a representative RT-PCR experiment is shown.

Immunocytochemical localization of cannabinoid-degrading enzymes FAAH and MGLL in human oocytes

Immunofluorescence analysis revealed that both FAAH and MGLL were present in the human oocytes. However, the localization of these enzymes showed variations through the stages of the resumption of meiosis in human oocytes. On the one hand, FAAH localization in the oocyte was peripheral at the GV **(Fig. 4.6a)** and MI **(Fig. 4.6b)** stages, whereas at the MII **(Fig. 4.6c)** stage, it was found peripherally but also appeared homogeneously over the entire oocyte (z-stack images in **Supplementary Figs.1, 3**). On the other hand, MGLL immu-nostaining was found homogeneously in oocytes from the GV to MII stages (**Fig. 4.6e-g**; see also z-stacks images in **Supplementary Figs.2, 4**). When the anti-FAAH and anti-MGLL receptor antibodies were blocked with the specific peptide before addition to the oocyte, no specific fluorescence was observed **(Fig. 4.6d, h)**.

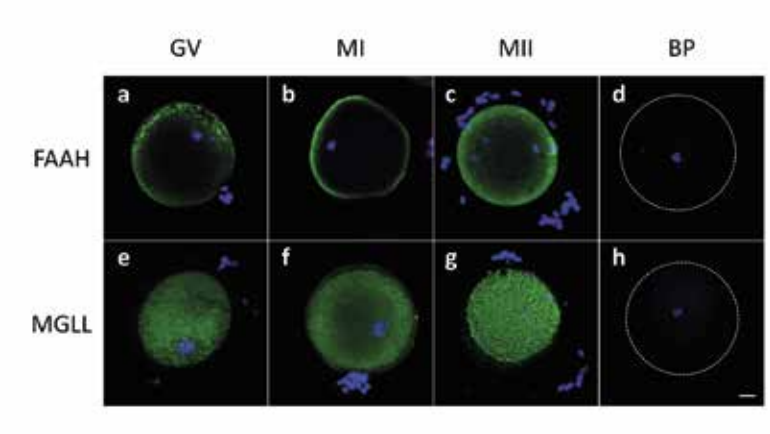


Figure 4.6. Immunofluorescence analysis of cannabinoid-degrading enzymes in human oocytes. The distributions of FAAH and MGLL are shown in green at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. A phase contrast images are shown of the negative control, consisting of pre-adsorption of the primary antiserum with the specific blocking peptide (BP). Hoechst-labeled DNA is shown in blue. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 20 μm.

Immunocytochemical localization of FAAH and MGLL compared with that of CB1 receptor during maturation of human oocytes

In the GV **(Fig. 4.7c)** and MI **(Fig. 4.7f)** stages, FAAH colocalized with the CB1 receptor at the periphery of the oocyte, although the CB1 receptor was also observed in the cytoplasm at MI (**Fig. 4.7d, f**). At the MII stage, the localization of FAAH had changed **(Fig. 4.7h)**: the enzyme appeared homogeneously over the entire oocyte, whereas all the CB1 immunostainig appeared at the periphery at MII (**Fig. 4.7g**). At MII, FAAH colocalized with CB1 to a lesser extent than at previous stages (**Fig. 4.7i**; see also z-stack images in **Supplementary Fig. 1**). With regard to MGLL, this enzyme showed a weak co-localization with the CB1 receptor at the GV **(Fig.4c)** stage, but generally, in other stages, the localization of CB1and MGLL was different: peripheral for CB1 **(Fig. 4.8d, g)** and cytoplasmic for MGLL (**Fig. 4.8e, h**; see also z-stack images in **Supplementary Fig.2**).

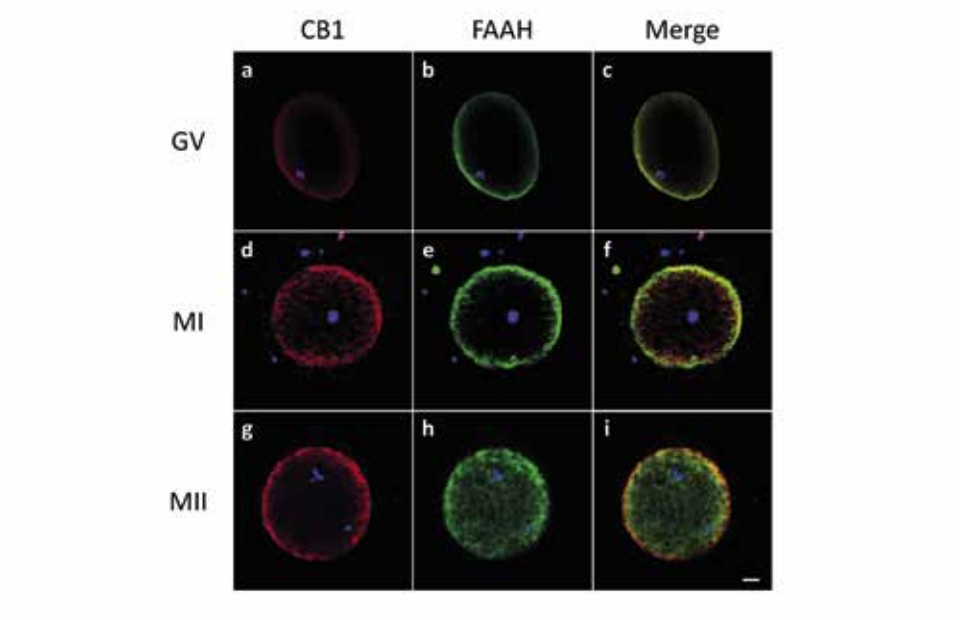


Figure 4.7 Immunofluorescence analysis of cannabinoid-degrading enzymes compared to CB1 cannabinoid receptor in human oocytes. The distributions of FAAH, CB1 and their overlap are shown in green, red and yellow respectively at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 20 μm.

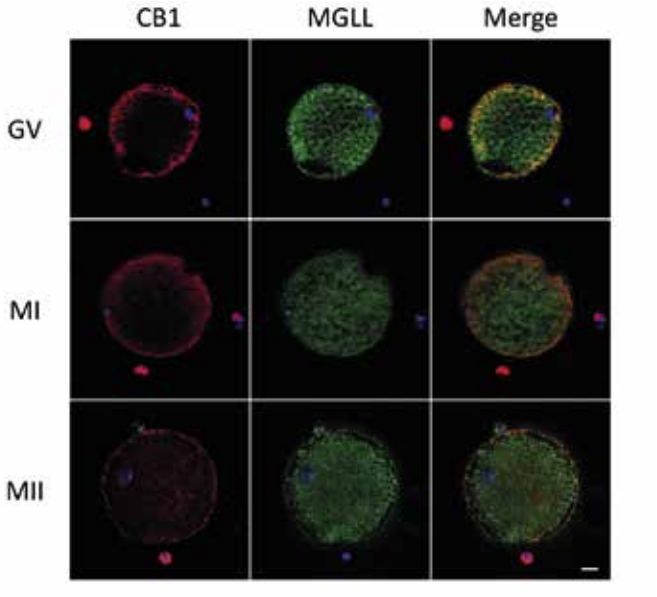


Figure 4.8 Immunofluorescence analysis of cannabinoid-degrading enzymes compared to CB1 cannabinoid receptor in human oocytes. The distribution of MGLL, CB1 and their overlap are shown in green, red and yellow respectively at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 20 μm.

Discussion

The role of the endocannabinoid system has been studied in various mammalian reproductive processes, such as spermatogenesis, sperm motility, fertilization, embryo oviductal transport, preimplantation embryo development, implantation, and post-implantation embryonic growth *(Wang et al., 2006a, 2006b; Taylor et al., 2007; Sun and Dey, 2012)*. Even so, the part played by this system in the ovary and in oocyte events has rarely been analyzed *(El-Talatini et al., 2009a; Bagavandoss and Grimshaw, 2010; Peralta et al., 2011)*. The aim of the present study was to gather further data on the presence and localization of cannabinoid-degrading enzymes in relation to the CB1 cannabinoid receptor during the resumption of meiosis inhuman oocytes in order to form a basis for explaining a possible role of the cannabinoid system in this process.

Our immunofluorescence analysis of human oocytes has confirmed the presence of cannabinoid-degrading enzymes FAAH and MGLL in these cells. As FAAH is a membrane-bound protein *(Deutsch et al., 2002)*, its observed localization in the oocyte periphery indicates that it is localized to the oolema. On the contrary, MGLL is a cytosolic serine hydrolase *(Dinh et al., 2002)* and this could explain the homogeneous localization of MGLL over the entire oocyte observed in our experiments.

Several authors have described the presence of cannabinoid system components in the oocyte. In rat ovaries, FAAH and the CB2 receptor have been found in both preantral and antral follicle oocytes, whereas the CB1 receptor is not present *(Bagavandoss and Grimshaw 2010)*. In human ovaries, however, only FAAH has been found localized to the theca cells of secondary and tertiary follicles, whereas CB1 immunostaining appears in oocytes from primordial to secondary follicles but not in tertiary follicles and, in contrast, intense CB2 staining has been observed in oocytes during all stages of follicle maturation *(El-Talatini et al., 2009a)*. Finally, in a study focused on human oocytes separated from follicles, the presence of both CB1 and CB2 has been corroborated during the resumption of oocyte meiosis from the GV to MII stages *(Peralta et al., 2011)*. Whether the differences in the localization of CB1, CB2 and FAAH during the follicle and/or oocyte maturation observed in the aforementioned three studies reflect differences between species or are also attributable to differences in methodology *(Bagavandoss and Grimshaw, 2010; Peralta et al., 2011)* is unclear. In any case, taken together, all the evidence to date leads us to believe that the previous non-detection of FAAH in human oocytes *(El-Talatini et al., 2009a)* is attributable to a relatively low level of expression of FAAH rather than its absence. Three statements uphold this view. First, FAAH has previously been detected in rat oocytes *(Bagavandoss and Grimshaw, 2010)* and has again been detected in our present study by using only the human oocyte separated from follicles. Second, the staining observed specifically in the plasma membrane in the present study was achieved by using a specific anti-FAAH antibody and verified with a blocking peptide. Finally, we detected, for the first time, the presence of FAAH mRNA in human oocytes. Further, the observed progression of the localization of the CB1 receptor in the present study is similar to that observed in a previous study of our group with a different antibody *(Peralta et al., 2011)* and as such, strengthens the evidence for its presence and re-localization pattern during the resumption of oocyte meiosis. In contrast, we were unable to compare our

MGLL localization with the results of other studies, since, to the best of our knowledge, this is the first time that MGLL has been localized to the oocyte.

The CB1 and FAAH immunostaining patterns changed during the various stages of meiosis resumption, as occurs with other proteins *(Kume et al., 1997; Agirregoitia et al., 2012)* and also with the CB2 receptor in mouse spermatogenesis *(Grimaldi et al., 2009)* and in human oocyte maturation *(Peralta et al., 2011)*. In contrast, MGLL maintained its cytoplasmic location throughout all the observed stages. Moreover, CB1 and FAAH co-localized in the periphery at the GV and MI stages but they overlapped less at MII. An overlapping pattern in the expression of CB1 and FAAH has been reported in other cell types, such as neurons, suggesting that the endocannabinoid system is responsible for an auto-feedback control of neurotransmitter release *(Bouskila et al., 2012)*. In meiotic progression, CB1 shows some internalization at MI but is then re-localized to the periphery at MII, whereas FAAH is internalized at the MII stage. This differing re-localization suggests a possible coordinated redistribution of CB1 and FAAH during the resumption of oocyte meiosis in order to regulate local endocannabinoid tone, as has been pro-posed for other cell types *(Yazulla, 2008)*. This is plausible since, in mammals, all of the qualitative and quantitative changes in protein synthesis occurring during oogenesis take place at the time of the resumption of meiosis *(Picton et al., 1998)*. Additionally, *Ji et al. (1997)* observed modifications in the protein pattern of human oocyte membranes during preovulatory maturation. More interesting still is our study in which we detected CB1 and FAAH in granulosa cells from GV to MII oocytes and their co-localization in some portions of the cell membrane. Moreover, MGLL immunostaining was more homogeneous across the cell and only overlapped with CB1 weakly *(Agirregoitia et al., 2015)*.

The reason for CB1 and FAAH re-localization during meiosis has not been studied at all. Even so, according to the classical theory of G-protein-coupled receptor (GPCR) functionality, these receptors have to reach the cell surface to act and, after agonist binding, GPCRs undergo a rapid "desensitization" and the ligand-receptor complex is internalized prior to being recycled back to the cell surface or being degraded *(Cahill et al., 2007)*. Therefore, taking into account our data for the CB1 receptor and FAAH-degrading enzyme, we can retain our previous hypothesis that CB1 is activated at the GV stage (when CB1 is localized at the plasma membrane) and subsequently internalized when the oocyte reaches the MI stage (when CB1 is also found within the cytoplasm; *Peralta et al., 2011*). Thus, as FAAH co-localizes with CB1 at the GV and MI stages, it could participate in the degradation of the cannabinoid that is bound to CB1 when the cannabinoid is internalized together with CB1 at MI. Additionally, at least at the GV and MI stages, FAAH might degrade free cannabinoids, which would enter the active site via the membrane *(McKinney and Cravatt, 2005)*. Then, according to our results, the receptor could be recycled, being mostly localized at the plasma membrane once again when the oocyte is blocked at the MII stage but FAAH cannot act at the periphery of oocyte because of its internalization. The presence of MGLL in the cytoplasm of oocytes might help FAAH in the degradation of cannabinoids *(Yazulla, 2008)*. Nevertheless, the co-localized MGLL and CB1 observed at GV might have a coordinated function, as has also been described in axon terminals of some rat cerebral areas *(Gulyas et al., 2004)*.

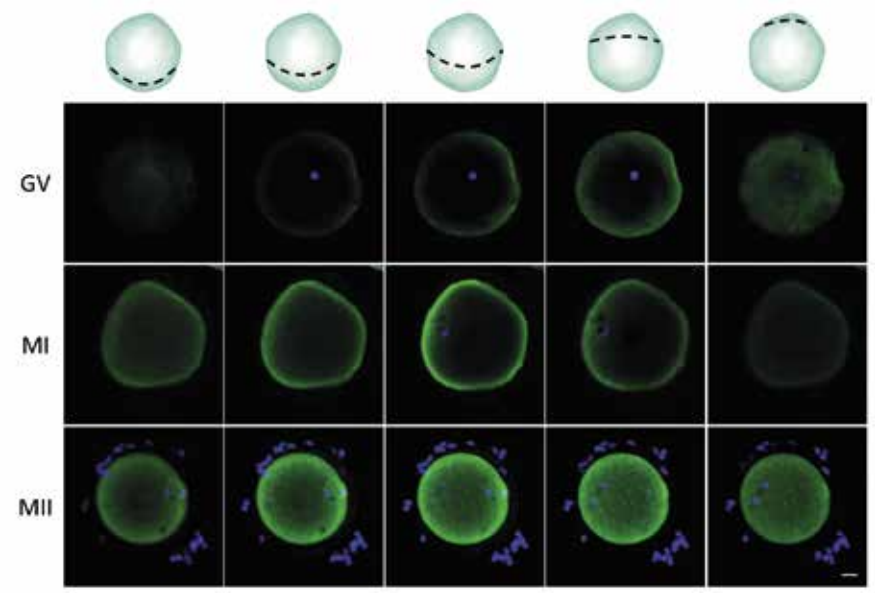
The regulation of "AEA tone" by FAAH has been pro-posed as an important event in the oviduct during early pregnancy *(Wang et al., 2006a, 2006b)*, in blastocyst activation *(Wang et al., 2003)* and during

implantation *(Paria et al., 1999),* but no hypotheses have been proposed in relation to such a process in oocyte maturation. Even so, the AEA concentration in human follicular fluid has been reported to rise during oocyte maturation *(El-Talatini et al., 2009a)* and this concentration is even higher in the oviduct *(Gebeh et al., 2012)*. In humans, on average, one oocyte completes growth each month and is ovulated in response to a mid-cycle surge of LH and in response to this LH surge, the oocyte resumes meiosis *(Hunt and Hassold, 2008)*. Hence, the resumption of meiosis in oocytes occurring in the oviduct, where the AEA concentra-tion is higher than in follicular fluid and the relocation of CB1 and FAAH during nuclear maturation lead to the hypothesis that signaling involving AEA, CB1 and FAAH in the oocyte is involved in the resumption of meiosis. To date, the presence of 2-AG has only been described in the uterus of the female reproductive tract *(Wang et al., 2007)* but whether it is present in the ovary or the oviduct is unknown; this merits investigation to elucidate the role of MGLL during the resumption of meiosis. In addition, the presence of CB1, FAAH and MGLL in granulosa cells suggests that the endocannabinoids act not only over the oocyte but also over the granulosa cells *(Agirregoitia et al., 2015)*.

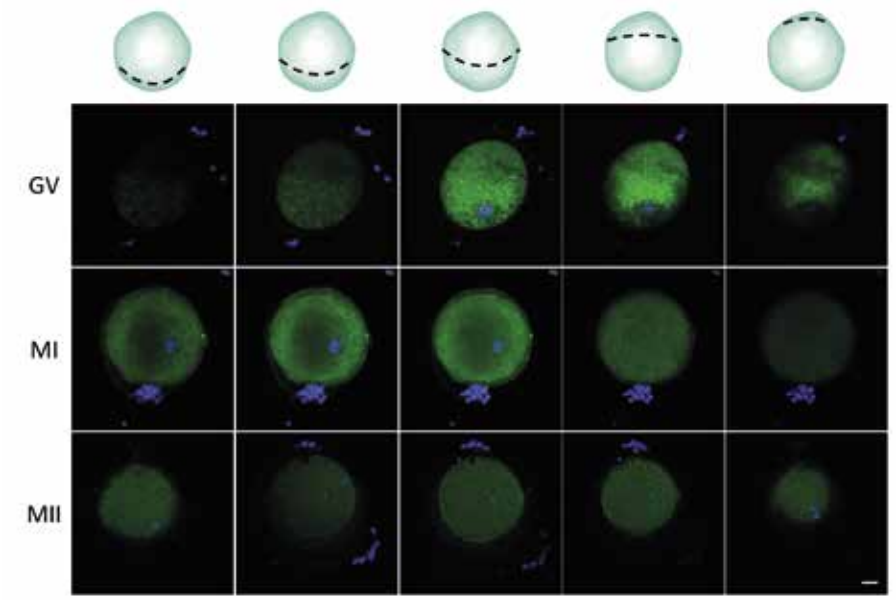
In the present study, we found FAAH but not MGLL mRNA in mature oocytes, although the proteins of both receptors are present during all stages of meiotic resumption. MGLL mRNA might be absent because its transcription becomes silent after the resumption of meiosis until the 4- to 8-cell stage *(Gosden and Lee, 2010)* and because the mRNA is selectivity degraded during oocyte maturation *(Stitzel and Seydoux, 2007)*. Moreover, the timing and pattern of RNA synthesis in the oocyte is known not necessarily to coincide with translation into an active protein *(Picton et al., 1998)*. Importantly, we do not consider that our observations concerning the behavior of the compounds of the cannabinoid system studied in this work can be attributed to an artifact associated with fertilization failure, since our results from unfertilized oocytes and non-sperm-exposed oocytes are not significantly different *(***Supplementary Fig. 5)**.

In conclusion, our study reports, for the first time, the presence of the cannabinoid-degrading enzymes FAAH and MGLL during the meiotic resumption in human oocytes. As CB1 and CB2 are present in oocytes *(Peralta et al., 2011)* and as AEA is present in follicular and oviductal fluids *(Schuel et al. 2002a; El-Talatini et al., 2009a)*, our results suggest that oocytes can regulate the "AEA tone" near the microenvironment in which the communication between oocyte and granulosa cells takes place. Further studies are needed to investigate the role of the cannabinoid system during the nuclear maturation of the oocyte, because our results might have an impact on the design of culture media for assisted reproductive technology and treatments, especially for *in vitro* maturation.

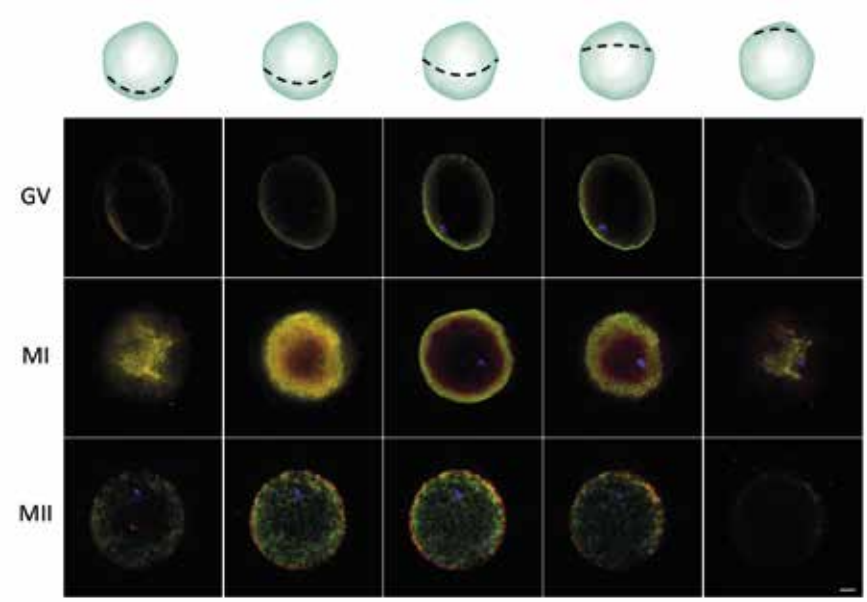
Supplementary figures



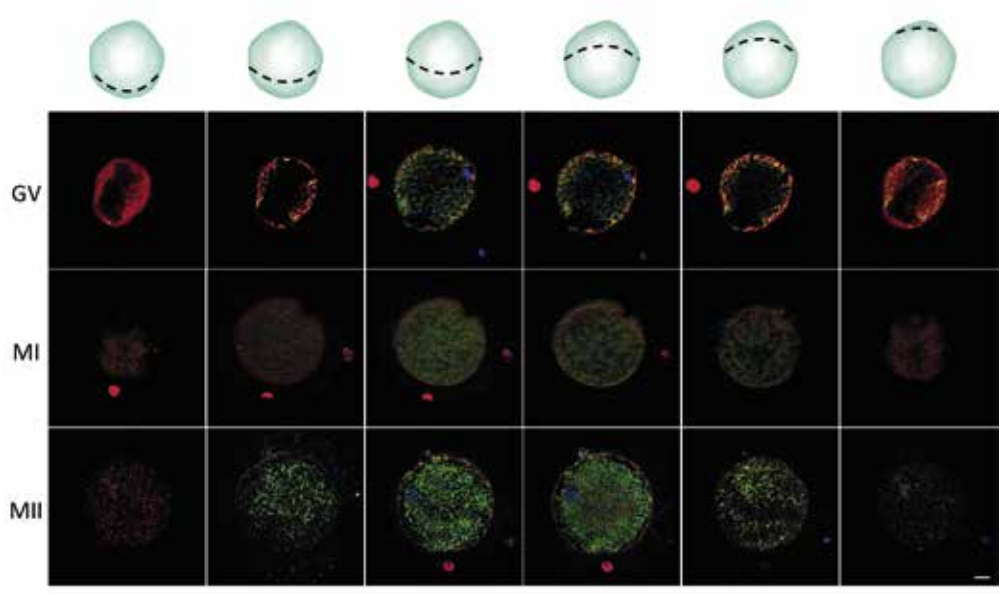
Supplementary figure 1. Immunofluorescence analysis of Z-stack images of cannabinoid-degrading enzyme FAAH in human oocytes. The distribution of FAAH is shown in green at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. Hoechst-labeled DNA is shown in blue. The scale bar represents 20 μm.



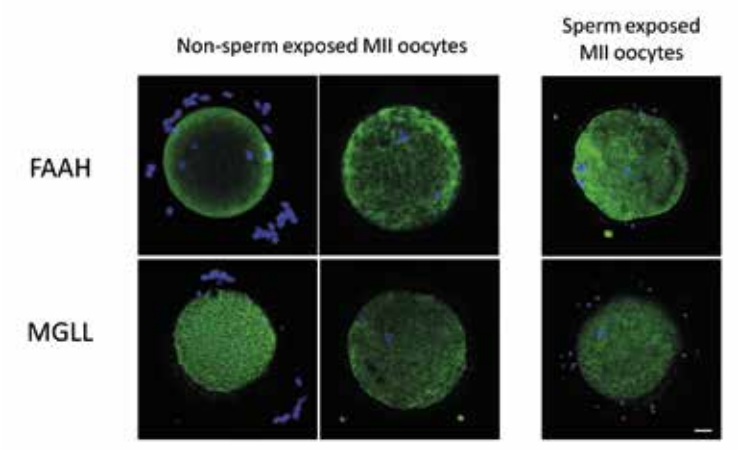
Supplementary figure 2. Immunofluorescence analysis of Z-stack images of cannabinoid-degrading enzyme MGLL in human oocytes. The distribution of MGLL is shown in green at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. Hoechst-labeled DNA is shown in blue. The scale bar represents 20 μm.



Supplementary figure 3. **Immunofluorescence analysis of Z-stack images of cannabinoid-degrading enzymes compared to CB1cannabinoid receptor in human oocytes.** The distributions of FAAH, CB1 and their overlap are shown in green, red and yellow respectively at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. The scale bar represents 20 μm.



Supplementary figure 4. Immunofluorescence analysis of Z-stack images of cannabinoid-degrading enzymes compared to CB1cannabinoid receptor in human oocytes. The distributions of MGLL, CB1 and their overlap are shown in green, red and yellow respectively at germinal vesicle (GV), metaphase I (MI) and metaphase II (MII) stages. The scale bar represents 20 μm.



Supplementary figure 5. Immunofluorescence analysis of cannabinoid-degrading enzymes in human oocytes to verify the similar distributions in non-sperm exposed MII oocytes and in sperm exposed but unfertilized MII oocytes. The distributions of FAAH and MGLL are shown in green. Hoechst-labeled DNA is shown in blue. The scale bar represents 20 μm.

4.3 Tetrahydrocannabinol Modulates *in Vitro* Maturation of Oocytes and Improves the Blastocyst Rates after *in Vitro* Fertilization

4.3 Tetrahydrocannabinol Modulates in Vitro Maturation of Oocytes and Improves the Blastocyst Rates after in Vitro Fertilization

Lide Totorikaguena[a], Estibaliz Olabarrieta[a], Angela Patricia López-Cardona[b,c], Naiara Agirregoitia[a], Ekaitz Agirregoitia[a].

[a]Department of Physiology, Faculty of Medicine and Nursing, UPV/EHU, Leioa, Spain, [b]Department of Animal Reproduction, INIA, Madrid, Spain, [c]G.I. Biogénesis, Universidad de Antioquia, Medellín, Colombia

Key Words

Oocyte • Maturation • THC

Abstract

Background/Aims: Among the assisted reproductive techniques, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone to mature exogenously in culture media supplemented with different molecules to promote maturation. In this sense, we are interested in the role that cannabinoids could have as IVM promoters because cannabinoid's molecular pathway is similar to the one by which oocyte's meiosis resumption is activated. With the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC) in the IVM process.

Methods: By immunocytochemistry, we detected the location pattern of cannabinoid receptor type 1 (CB1) and type 2 (CB2) during oocyte maturation in presence or absence of THC, as well as, the staining pattern of p-AKT and p-ERK. We used a genetic/ pharmacological approach generating knockout oocytes for CB1 and/or CB2 and they were incubated with THC during the oocyte maturation to visualize the physiological effects of THC, observing the rate of blastocyst achieved by oocyte.

Results: This study confirms that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation.

Conclusion: Our data suggest that THC may be useful IVM supplements in clinic as is more feasible and reliable than any synthetic cannabinoid.

Introduction

For years now, growing fertility problems and current changes in family patterns have resulted in a wider use of assisted reproduction techniques (ART) among couples trying to get pregnant *(Jose-Miller et al., 2007; Gonzalez and Morgado, 2008; SEF, 2016)*. Even so, the success of ARTs is not yet complete and the research work continues to improve these techniques. Among the ARTs, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone. The oocytes are then exogenously matured in culture media supplemented with different molecules to promote maturation *(Barnes et al., 1995, 1996)*.

The most important goal of IVM method is that could be the only hope or alternative for a high number of patients unable to tolerate high doses of gonadotropins, such as patients with polycystic ovary syndrome (PCOS) or ovarian hyperstimulation syndrome (OHSS) *(Nader, 2010; González-Ortega et al., 2019)*. In general, clinical pregnancy and implantation rates per embryo transfer have reached 35%–40% and 15%–20%, respectively, in infertile women with PCOS after IVM of immature oocytes *(Chian et al., 2013)*. Further candidates for oocyte IVM are patients whose ovarian tissue was frozen due to a pre-existing disease such as cancer, in whom hormone stimulation was not recommended or there was insufficient time to undergo a normal *in vitro* fertilization (IVF) cycle *(Nikseresht et al., 2015)*. In addition, by avoiding controlled ovarian stimulation, the IVM procedure eliminates the need for frequent ultrasound monitoring and is thus less costly than conventional IVF *(Chang et al., 2014)*.

The complexity lies in that the oocyte meiotic maturation is a complex process whereby immature oocytes acquire the characteristics required for successful fertilization and embryogenesis. For that, immature oocytes arrested at the diplotene of prophase I (designed as germinal vesicles -GV-) must resume meiosis until the metaphase II of meiosis (MII) *(Coticchio et al., 2015)*.

However, although some mechanisms that lead the reactivation of meiosis are still to be explained, it is known that the oocyte maturation involves the activation of various signal transduction pathways that converge to activate maturation-promoting factor. Among others, there are some evidence that heterotrimeric G-proteins, which inhibit adenylate cyclase, can interact with both PI3K and MAPK to promoting the oocyte maturation *(Schmitt and Nebreda, 2002)*. In this sense, we are interested in the role that cannabinoids could have as IVM promoters. On the one hand, the molecular cascade that cannabinoids exerts when they activate the G-protein coupled cannabinoid receptors (inhibition of adenylate cyclase, reducing cAMP, and activation of PI3K/Akt and MAPK patways) is very similar to the one that occurs during oocyte meiotic resumption *(Schindler, 2011; Dalton and Howlett, 2012*). On the other hand, some studies described the presence of the cannabinoid system in human *(Reich et al., 1982; Paria et al., 1995; Peralta et al., 2011; Agirregoitia et al., 2015; Agirregoitia et al., 2016)*, bovine *(López-Cardona et al., 2016) and murine (López-Cardona et al., 2017)* oocytes, and, at least in the two last species, synthetic cannabinoids were able to modulate the oocyte maturation *(López-Cardona et al., 2016, 2017)*.

Being this the general content, with the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC). The THC is called phytocannabinoid because it is a compound of the plant Cannabis sativa, and it has attracted particular attention since its synthetic analogs dronabinol and nabilone are licensed for medicinal use *(Dinis-Oliveira, 2016)*. Even so, the use of phytocannabinoids as THC in clinic is more feasible and reliable than any synthetic cannabinoid *(Wiley et al., 2014; Schreiber et al., 2019)*.

The aim of this study is to characterize the role of the phytocannabinoid THC in the IVM process. To that end, we used a genetic/pharmacological approach generating knockout oocytes for CB1 and/or CB2 receptors. They were modulated pharmacologically during the oocyte maturation to visualize the physiological effects of THC. Our results support the notion that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation.

Materials and methods

Experimental animals

The adult wild-type, Cnr1$^{-/-}$ (Marsicano et al., 2002), Cnr2$^{-/-}$ (Buckley et al., 2000) and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice used in this study were kept in an animal house under controlled conditions of temperature (22 ± 1°C) and photoperiod (light/dark cycle 14 h:10 h). Animals were given free access to water and food. All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027- 028-173) and were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation.

Isolation and *in vitro* maturation of cumulus–oocyte complexes

Female 8 to 10 week old WT (C57BL/6xCBA) or KO on a C57BL/6N background (Cnr1$^{-/-}$, Cnr2$^{-/-}$, and Cnr1$^{-/-}$/Cnr2$^{-/-}$) mice were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon, Intervet), and ovaries were collected 46 to 48 h later. The ovaries were cleaned of any connective tissue and placed in handling medium M2 supplemented with 4 mg/ml bovine serum albumin fraction V. Antral follicles were punctured with 30-gauge needles, and immature cumulus–oocyte complexes (COCs) were collected in handling medium. Only COCs with 3 compact cumulus cells were used. COCs were matured for 17 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 37 °C under an atmosphere of 5% CO_2 in air with maximum humidity.

IVM supplementation with cannabinoid agonist THC

The THC stock solutions were prepared in DMSO. During maturation (17 h), COCs were incubated with different doses of THC (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) to evaluate the effects of activation of cannabinoid receptor by this agonist. Once obtained the most efficient concentration for oocyte maturation, to see the phosphorylation pattern of ERK and AKT proteins and the effect on embryo development, the COCs were incubated with 10^{-7} M (100nM) of THC. COCs containing the same amount of DMSO were used as an incubation control.

Meiotic progression of mice oocytes undergoing IVM with THC

To determinate the impact of THC on germinal vesicle breakdown (GVBD), COCs from experimental group and control with DMSO at 2, 4, 6, 8, 12, 17 h of IVM were used as described previously *(Khatir et al., 1998)*. In sum, COCs were partially denuded in 0, 1% of hyaluronidase (Sigma H3506) and fixed in 4% paraformaldehyde (Panreac) for 20 min. Then, were washed twice in PBS and incubated in PBS containing Hoescht 33342 (0.01 mg/ml) for 15 min. Oocytes were then placed in glass slides and squashed with coverslip in order to visualize the nuclear stage under microscopy (Zeiss Axioskop).

Immunofluorescence

For these experiments, 3 females per genotype were used in 3 replicate trials. Once meiotic stages were established, to immunocytochemically localize the CB1 and CB2, 20 WT oocytes per stage were treated as previously described *(Peralta et al., 2001)*. Briefly, *in vitro* matured oocytes in presence or absence of THC were washed in PBS supplemented with 1% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde *(Panreac)* for 10 min at room temperature. The oocytes were then permeabilized by incubation in PBS with 10% (v/v) FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, oocytes were incubated overnight at 4 °C in PBS containing 1% PVA, 5% normal FCS serum, and 1:100 rabbit polyclonal anti-cannabinoid CB1 (Cayman Chemicals, Ann Arbor, MI, USA) and 1:400 rabbit polyclonal anticannabinoid CB2 (Cayman Chemicals). After incubation, oocytes were washed twice in PBS containing 1% PVA and incubated in PBS supplemented with 1% PVA, 5% FCS serum, and 1:500 goat polyclonal secondary antibody Alexa Fluor 488 (Molecular Probes, Eugene) for 2 h at room temperature. Next, the oocytes were washed 3 times in PBS–1% PVA. In all cases, nuclei were stained with Hoechst 33342 (0.01 mg/ml) during the second wash to facilitate the determination of the maturation stage of each oocyte *(Gutiérrez-Adán and Pintado, 2000)*. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS) and examined by confocal microscopy (Fluoview FV500, Olympus). Negative controls were prepared in the same way omitting the primary antibody before addition of the secondary antibody.

To monitor the activation of pERK1/2 and pAKT signaling during oocyte maturation in the presence of cannabinoids, an average number of 20 oocytes was collected at 0 h, 10 min, 30 min and 1 h to evaluate the initial response, and at 17 h as the optimal time for maturation. Rabbit polyclonal phospho-ERK1/2 (Cell

Signaling Technology) primary antibody was used at a 1:400 dilution and Rabbit phospho- Akt (Ser473) (Cell Signaling Technology) 1:200 primary antibody was used at a 1:400 dilution. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS) and examined by confocal microscopy (LSM 800, Zeiss). Negative controls were performed in the same way, except for omission of the primary antibody before secondary antibody addition.

Isolation and *in vivo* maturation of COCs

MII oocytes were collected from 8- to 10-week-old oviducts of female WT (C57BL/6xCBA) or KO ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$) superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon), followed 48 h later by 7.5 IU of human chorionic gonadotropin (Veterin Corion). Briefly, at 14 h after human chorionic gonadotropin administration, oviducts were removed from superovulated female mice and placed in a Petri dish containing M2 at 37 °C. After washing, collected oviducts were placed in fresh M2 medium, and COCs were released from the ampulla with the aid of Dumont #5 forceps and washed in new M2 medium until fertilization.

In vitro fertilization

Sperm from C57BL/6xCBA male mice aged 6 to 24 wk and of proven fertility were incubated for 1 h in human tubal fluid (HTF) medium under 5% CO_2 at 37 °C for capacitation. After *in vitro* or *in vivo* maturation, COCs were transferred to a 500 µl equilibrated HTF drop and overlaid with mineral oil and a 1×10^6 concentration of spermatozoa. All *in vitro* fertilization experiments were repeated 9 times using 3 females per genotype.

Rates of oocyte nuclear maturation and fertilization

To assess whether adding THC affected rates of oocyte nucleus maturation and fertilization, the presence of the first polar body and pronuclear formation were identified respectively. At 24 h after fertilization, all presumptive zygotes that had not divided into 2 cells were fixed in 4% paraformaldehyde for 10 min and then stained with Hoechst 33342 (0.01 mg/ml) for observation with a immunofluorescence microscope (Zeiss Axioskop) under UV light.

In vitro culture of embryos

Five hours after *in vitro* fertilization, 10-25 presumptive zygotes from oocytes of female WT (C57BL/6xCBA) or KO ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$) were washed in HTF medium and cultured in 20-µl drops of equilibrated culture medium KSOMaa overlaid with mineral oil at 37 °C under an atmosphere of 5% CO_2 in air with maximum humidity. Embryos were cultured for 5 d, and cleavage rates were assessed on d 1 (24 h after fertilization) and blastocysts on d 4 (96–100 h after fertilization).

TUNEL analysis

Apoptosis was determined by in situ DNA 3 end labelling of histological sections using a non-radioactive labelling method [Terminal deoxynucleotidyl transferasemediated dUTP nick-end labelling assay (TUNEL) from detection Kit, TUNEL POD (ROCHE). The control oocytes, the vehicle (DMSO) group and THC group were processed as per the manufacturer's instructions. The apoptotic cells appear in fluorescent green whereas the nucleus was marked in blue with Hoechst 33342.

Statistical analysis

All statistical tests were performed by using Graphpad software (GraphPad Software). The mean and standard error of the mean of cleavage rates, blastocyst yields, the percentage change of blastocyst rate between the 4 genotypes were compared by 1-way ANOVA followed by multiple pairwise comparisons by using the Tukey's post hoc test in most data. Paired t-test was performed to compare the difference between treatments (without and with THC). Values of $P < 0.05$ were considered significant.

Statement of Ethics

All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation.

Results

CB1 receptor location-pattern during the resumption of meiosis and nuclear maturation of oocyte in presence or absence of THC

To analyse if the THC has any effect on the relocation of CB1 during the oocyte maturation, we incubated the immature oocytes with increasing concentrations of THC during 17 h and we collected the oocytes at 10 min, 30 min, 1 h and 17 h to observe the CB1 location-pattern.

As it is known, in immature oocytes, CB1 is homogeneously localized over the GV and the receptor is relocated to the periphery of the oocyte after GVBD, when meiosis resume, which happens after more than one hour of maturation (Control of **Fig. 4.9**). However, the presence in the incubation media of any tested concentration of THC (from 10^{-9} M to 10^{-6} M) accelerated the relocation of CB1receptor from GV to the periphery of oocytes. Thus, the staining of CB1 in GV disappeared before 1 h of incubation (quicker than in control) and, specifically, the concentration of 10^{-7} M of THC led to accelerate that relocation before 30 min **(Fig. 4.9)**.

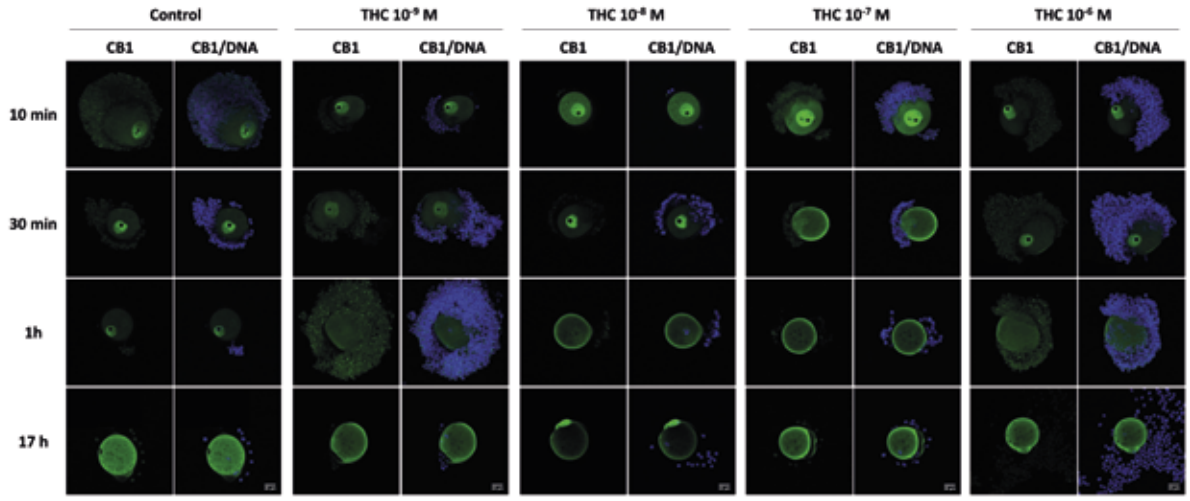


Figure 4.9 Immunolocalization of CB1 during the maturation of mouse oocytes. Immature COCs were cultured *in vitro* in absence of THC and in presence of THC at 1 nM (10^{-9} M), 10 nM (10^{-8} M), 100 nM (10^{-7} M) and 1 µM (10^{-6} M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had the same staining pattern; representative photomicrographs are shown. Scale bars, 25 µm.

CB2 receptor location-pattern during the resumption of meiosis and nuclear maturation in presence or absence of THC

To continue understanding the role of THC in oocyte maturation and after determining its effect on CB1 receptor relocation, we also examined the effect of THC on location of CB2 receptor. We performed the same experiment explained previously for CB1. CB2 showed a homogeneous distribution-pattern in the whole oocyte with more staining intensity in the periphery of the oocyte at all tested times both in absence or in presence of THC **(Fig. 4.10)**.

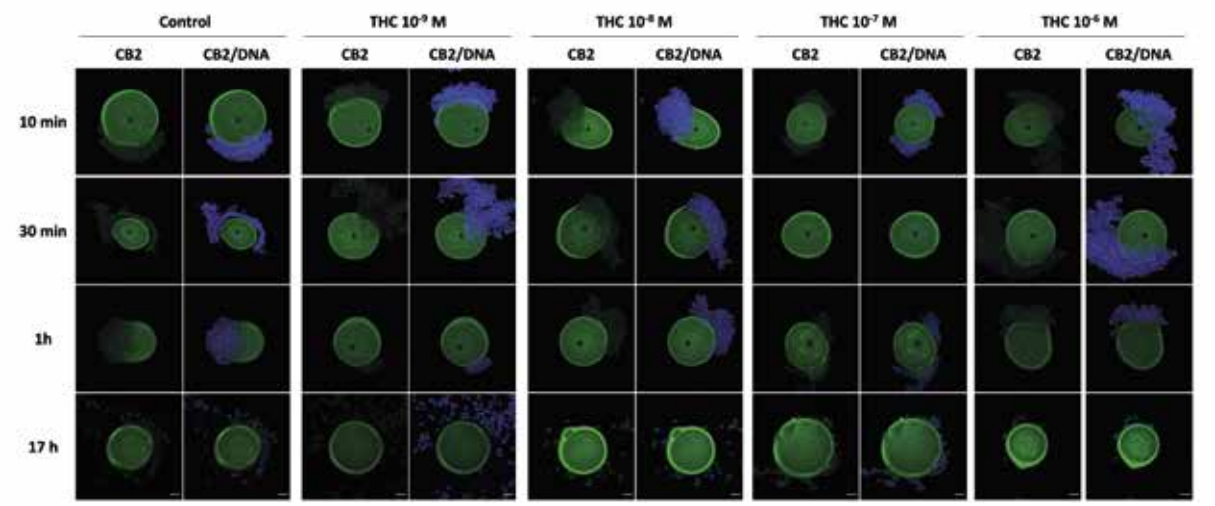


Figure 4.10. **Immunolocalization of CB2 during the maturation of mouse oocytes**. Immature COCs were cultured *in vitro* in absence of THC and in presence of THC at 1 nM (10^{-9} M), 10 nM (10^{-8} M), 100 nM (10^{-7} M) and 1 μM (10^{-6} M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 μm.

Meiotic progression of mice oocyte's exposed to THC

To determinate if THC has also some effect on oocyte nuclear maturation we fixed oocytes after 0, 2, 4, 6, 8 and 12 h of IVM where media was supplemented with 10^{-7} M of THC or vehicle (DMSO). Oocytes nuclear stage was classified in germinal vesicle (GV), germinal vesicle break down (GVBD), pro-metaphase I (PMI), metaphase I (MI) and metaphase II (MII). At 0h all the oocytes were in GV stage **(Fig. 4.11A)** and after 2 h of IVM a significant higher percentage of PMI was detected when we used THC compared with the oocytes maturated in presence of the vehicle **(Fig. 4.11B)**. After 4 h of IVM the majority of the oocytes were at PMI, but no differences were observed among treatments. Also after 8 h of IVM, all groups reached MI **(Fig. 4.11C)** at the same time. After 12 h of maturation a higher percentage of MII stage was found in THC experimental groups compared with control **(Fig. 4.11D)** but that difference was not significant. Finally, after 17 hours, all the oocytes of both treatments arrived at MII (data not shown).

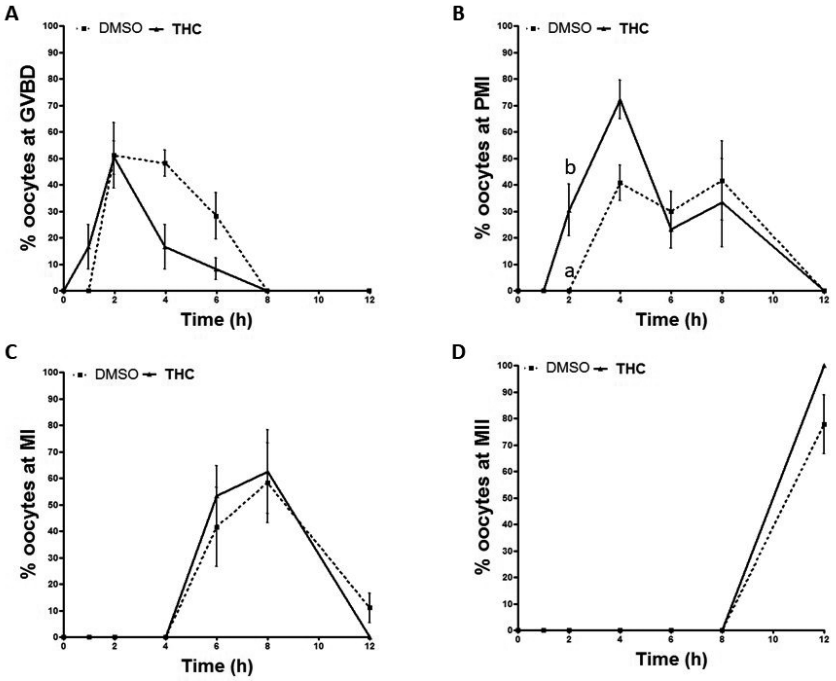


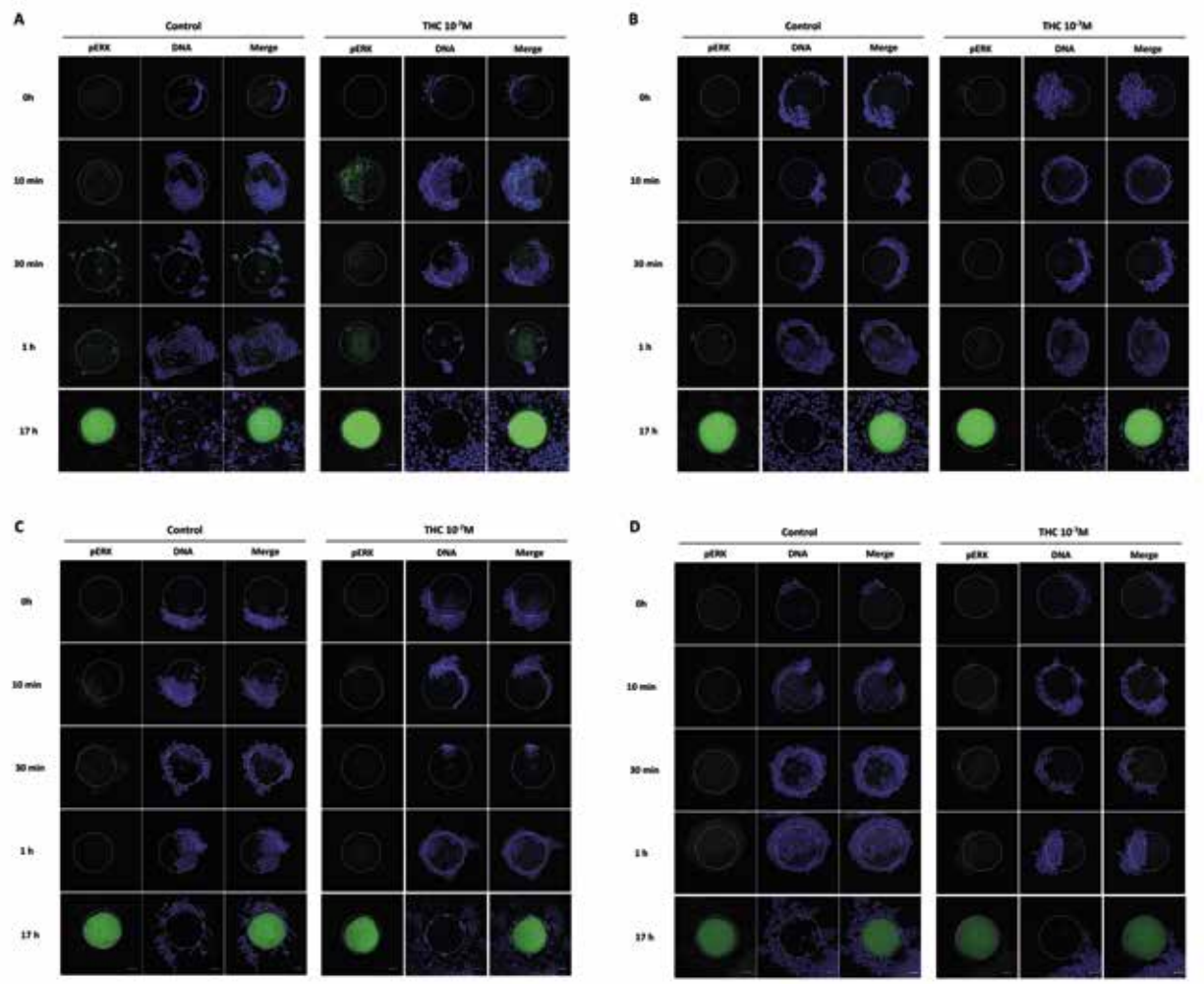
Figure 4.11. Changes in nuclear maturation of oocytes. Results are expressed as percentage of oocytes at each stage of maturation at each point: (A) germinal vesicle break down, GVBD; (B) pro-metaphase I stage, PMI; (C) metaphase I stage, MI; (D) metaphase II stage, MII. n = 5 independent experiments of 15 oocytes per treatment. Significant differences between treatments are indicated with different letters; $p < 0.05$ in all cases.

Phosphorylation pattern of ERK1/2 during the resumption of meiosis and nuclear maturation in presence or absence of THC

As signaling via MAPKs plays a role in the oocyte maturation and that pathway is commonly modulated by cannabinoids, we used the cannabinoid agonist THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of ERK1/2 compared with oocytes maturated in presence of the vehicle (DMSO). According to the results obtained in the relocation of cannabinoid receptors during the maturation of oocytes, for these experiments we used the concentration of THC that generated the most changes regarding the relocation of the receptors in comparison to the control (10^{-7} M).

In immature oocytes, ERK1/2 was dephosphorylated but, after 17 h of incubation (the time required for mouse oocyte maturation), ERK1/2 was phosphorylated in oocytes. At 10 min, ERK1/2 appeared phosphorylated only in the granulose cells of oocytes incubated with THC. We did not observe the phosphorylation of granulose cells of control oocytes until 30 min. However, at that time, the COCs incubated with THC had already dephosphorylated its granulose cells but, instead, we could observe the presence of phosphorylated ERK1/2 inside the oocyte. At 1 h of incubation, the oocytes incubated with THC showed more intense phosphorylated ERK1/2 compared with the control and, at that time, the granulose cells of control COCs had already been dephosphorylated **(Fig. 4.12A)**.

When we used oocytes from knockout mice where the cannabinoids receptors were absent ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$), we did not observe difference in the phosphorylation pattern of ERK1/2 between the oocytes matured in the presence or absence of THC **(Fig. 4.12B, 4.12C** and **4.12D)**.



.

Figure 4.12. Phosphorylation of ERK by THC during oocyte maturation. Immature GV oocytes from (A) wild type mice, (B) $Cnr1^{-/-}$ mice, (C) $Cnr2^{-/-}$ mice and (D) $Cnr1^{-/-}/Cnr2^{-/-}$ mice were cultured *in vitro* in presence of 10^{-7} M of THC or in absence of it (control) for 17 h and the phosphorylation status of ERK (pERK) was observed at 0, 10 min, 30 min, 1 h and 17 h. pERK is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

Phosphorylation pattern of AKT during the resumption of meiosis and nuclear maturation in presence or absence of THC

The pathways mediated by AKT are also key in the oocyte maturation and that kinase is also modulated by cannabinoids, so, we used THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of AKT compared with oocytes maturated in presence of the vehicle (DMSO).

We performed the same experiments as previously explained for pERK1/2. In this case, we observed few differences in the phosphorylation pattern of AKT in response to 10 and 30 min of treatment with THC, where the AKT was phosphorylated in granulose cells while it was undetectable in granulose cells exposed to vehicle. From that moment, the pattern of phosphorylation observed in the COCs treated with THC or vehicle was similar **(Fig. 4.13A)**.

When we used oocytes from knockout mice where the cannabinoids receptors were absent ($Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$), we did not observe difference in the phosphorylation pattern of AKT between the oocytes matured in the presence or absence of THC **(Fig. 4.13B, 4.13C** and **4.13D)**.

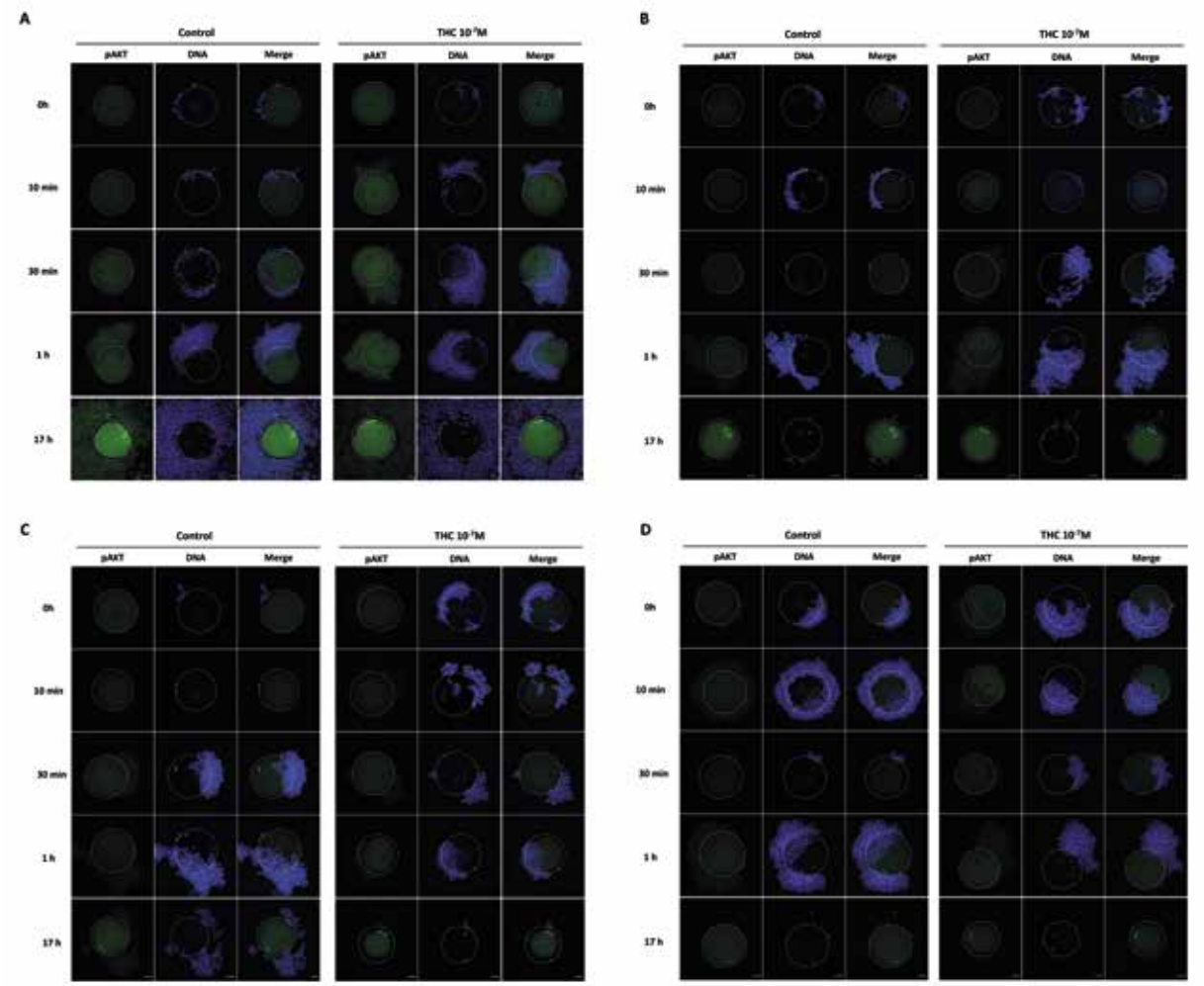


Figure 4.13. Phosphorylation of AKT by THC during oocyte maturation. Immature GV oocytes from from (A) wild type mice, (B) $Cnr1^{-/-}$ mice, (C) $Cnr2^{-/-}$ mice and (D) $Cnr1^{-/-}/Cnr2^{-/-}$ mice were cultured *in vitro* in presence of 10^{-7} M of THC or in absence of it (control) for 17 h and the phosphorylation status of AKT (pAKT) was observed at 0, 10 min, 30 min, 1 h and 17 h. pAKT is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

Apoptosis analysis in oocytes exposed to THC during maturation

To check if the presence of THC, during the 17 hours it was in contact with the COCs, was harmful, the detection of apoptotic oocytes or granulosa cells was performed using the in situ TUNEL analysis. We did not observe any apoptotic cell in the COCs treated with THC during maturation or in those treated only with the vehicle (DMSO) **(Fig. 4.14)**.

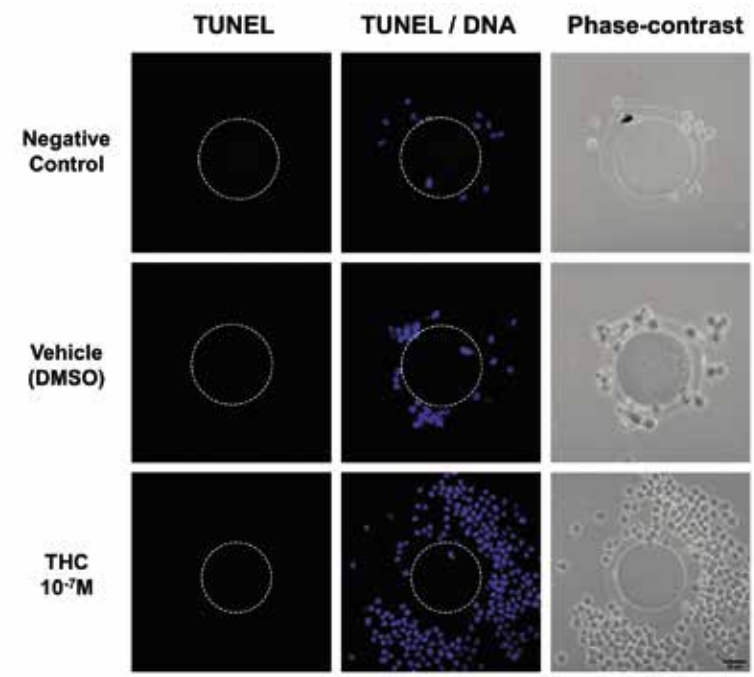


Figure 4.14 Analysis of apoptotic cells, by TUNEL, in COCs treated with vehicle (DMSO) or THC. Apoptotic cells showed in green and DNA, stained by Hoechst 3342, in blue. Representative photomicrographs are shown

Blastocyst rate produced from oocytes matured in presence or absence of THC

Our next objective was to test whether exposure of COCs to THC during oocyte maturation would affect the maturation observing the fertilization and/or subsequent embryo development rate. For that purpose, we cultured, *in vitro*, immature COCs during 17 h (the average time to mature mice oocytes) in absence or presence of 10^{-7} M of THC and, then, we performed the *in vitro* fertilization (IVF).

The incubation with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes. However, THC led to a significant improvement in blastocysts rate since twice as many embryos were produced when we used a 10^{-7} M concentration of THC, compared to vehicle **(Fig. 4.15A)**.

To be able to know if the observed action of THC was carried out by CB1 and/or CB2 receptor, we also performed the IVM with COCs from knockout mice $Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$, followed

by the subsequent IVF done using sperm from wild type male mice. That way, we were able to verify that the effect observed on *in vitro* embryo development were effectively attributable to the absence of the cannabinoid receptor during maturation and not to the *in vitro* fertilization (IVF) or embryo culture processes.

We did not observe any improvement on blastocyst rate between the oocytes treated with THC or with vehicle for any genotype **(Fig. 4.15B, C** and **D)**. In that sense, taking into account the percentage change of blastocyst rate between control and treatment for each genotype, we observed that the only genotype where incubation of oocytes with THC is beneficial for an increase in the number of blastocysts is the wild type **(Fig. 4.16).**

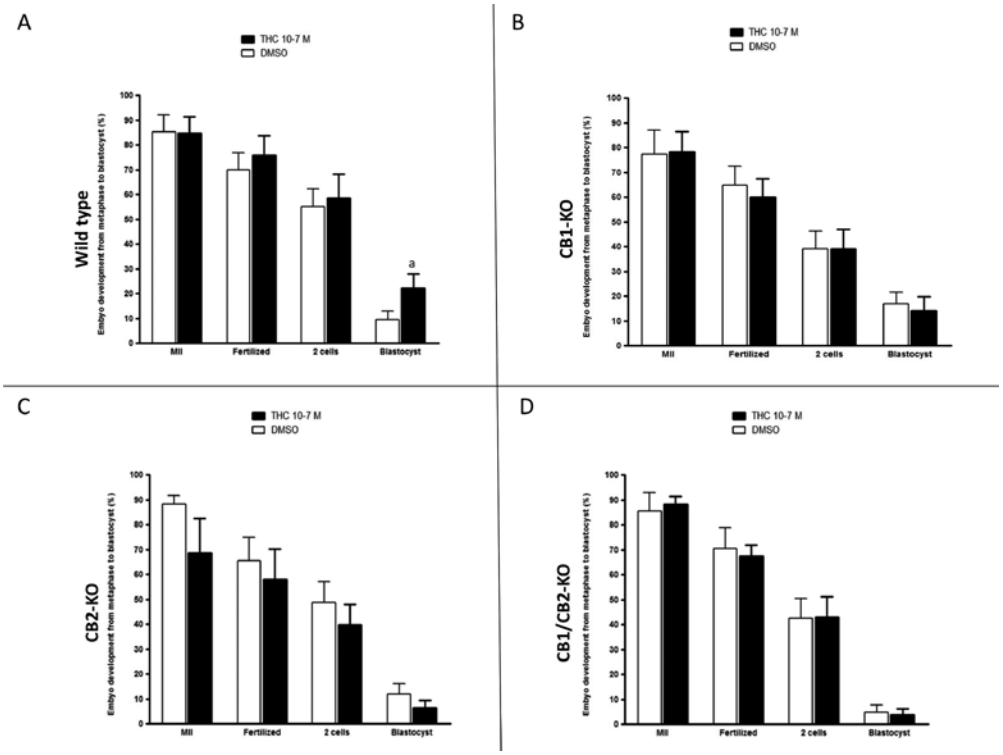


Figure 4.15. Response to THC 10−7 M during oocyte maturation (17 h) measured as rates of metaphase II oocytes (MII), fertilized oocytes, 2 cell-stage embryos and blastocysts after IVF. The figure shown the embryo development observed for oocytes incubated with vehicle (DMSO) (white) and incubated with THC 10^{-7} M (black). The sperm always came from WT mice and the oocytes came from A) wild type (WT) mice, B) $Cnr1^{-/-}$ mice, C) $Cnr2^{-/-}$ mice and D) $Cnr1^{-/-}$/ $Cnr2^{-/-}$ mice. Results are the mean of % ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p < 0.005$ in all cases.

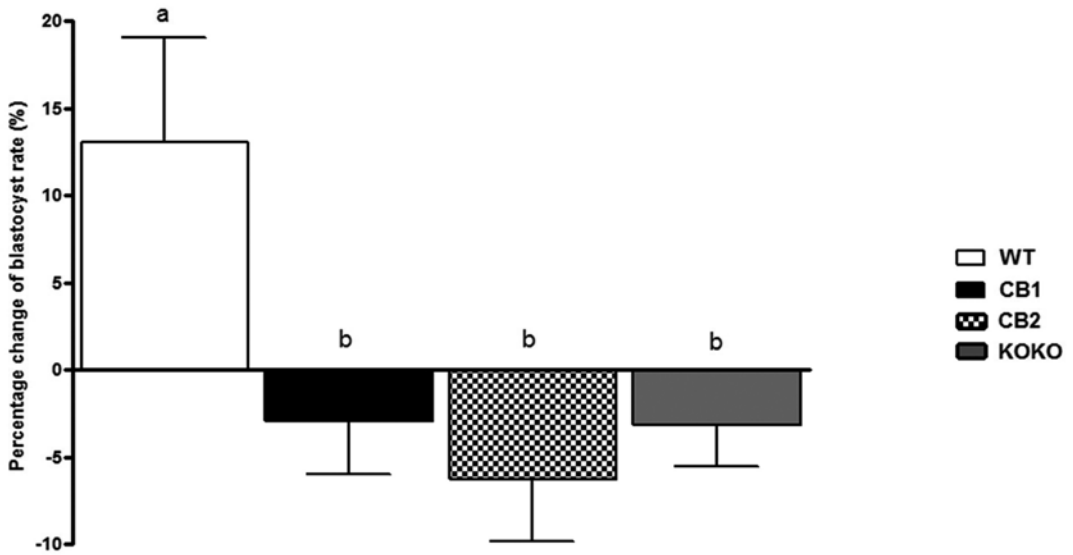


Figure 4.16. Percentage change of blastocysts rate between control and treatment. Blastocysts rate changes using oocytes from wild type (WT) mice (white), $Cnr1^{-/-}$ mice (black), $Cnr2^{-/-}$ mice (dotted) and $Cnr1^{-/-}/Cnr2^{-/-}$ mice (grey). Results are the mean ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p < 0.05$ in all cases

Discussion

Through systematic series of genetic and pharmacologic experiments, we examined whether the use of THC acting via CB1 and/or CB2 modulated oocyte maturation in a mouse model, and we tried to elucidate the mechanisms of such actions. The choice of using the phytocannabinoid THC has to do with the controversy generated by the use of synthetic cannabinoids *(Wiley et al., 2014; Schreiber et al., 2019)*, which is why it seems that the use of THC in the clinic could be more feasible and reliable. Our observations in wild type, Cnr1$^{-/-}$, Cnr2$^{-/-}$, and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice indicated that, when present during IVM of oocytes, the THC accelerated the relocation of CB1 receptor in oocytes. Moreover, the incubation of oocytes with THC during the IVM produced modulations in ERK1/2 and AKT phosphorylation and increased blastocyst rates achieved. Those effects seem to be achieved through both cannabinoid receptors studied (CB1 and CB2).

The first evidence that linked the endocannabinoid system and the oocyte maturation was that the endocannabinoid AEA (N-arachidonoylethanolamide) was present in human follicular fluid *(Coy et al., 2008)* and its concentration rose during oocyte maturation *(El-Talatini et al., 2009a,2009b)*. In previous works, it was detected the Cnr1 mRNA transcript and protein of CB1 receptor in oocytes during *in vitro* and *in vivo* maturation *(Peralta et al., 2011; López-Cardona et al., 2016, 2017)*. Furthermore, CB1 receptor could be an indicator of oocyte nuclear maturation, because it was clearly localized over GV oocyte and it moves towards the periphery as it changes to the stage MII *(Peralta et al., 2011; López-Cardona et al., 2016, 2017)*. In the present work we saw how the presence of THC in the maturation media accelerated that relocation of CB1 receptor from GV to the periphery. That difference in relocation velocity is interesting because, in oocytes matured *in vivo*, CB1 was localized peripherally sooner than in oocytes matured *in vitro (López-Cardona et al., 2017)* and, as is well established, the *in vitro* maturation process is delayed in comparison with *in vivo* maturation *(Chian et al., 2004)*. The presence of THC in the maturation media also accelerated the oocyte nuclear maturation of immature oocytes between 1 and 2 hours of incubation, similar times observed in the aforementioned acceleration of the relocation of CB1 receptor. The meiosis resumption also began earlier (around 2 h) when THC was present in the IVM media of bovine oocytes (*López-Cardona et al., 2016)*.

Although CB2 did not show any relocation difference during the IVM, we detect a peripheral location of this receptor did not previously found. So, as according to the classic theory of GPCR functionality, to exert its actions, a receptor needs to reach the cell surface *(Cahill et al., 2007)*, both CB1 and CB2 could have any involvement in oocyte maturation. Actually, there are data supporting the notion that GPCR-Gαi (such as CB1 and CB2) is a meiotic maturation inducer *(Mehlmann, 2005*).

To know if the changes observed during the maturation of oocytes incubated with THC were observed in other processes of IVM, we analysed the phosphorylation-pattern of ERK1/2 and AKT, since those essential kinases regulate the oocyte meiosis progression *(Sanchez and Smitz, 2012)*. Interestingly, the presence of THC during the oocyte maturation accelerated the phosphorylation-pattern of ERK1/2 and, to a lesser extent, the phosphorylation-pattern of AKT. Previously, it was described that the selective CB2

receptor agonist, JWH133, induced the ERK1/2 and AKT phosphorylation cascade in spermatogonia and their progression toward meiosis *(Grimaldi et al., 2009; Giacomo et al., 2016)*. In addition, in the previous functional experiments done with cows showed how the presence of THC during the IVM led to accelerate the phosphorylation pattern of ERK1/2 and AKT kinases *(López-Cardona et al., 2016)*. These results are in accordance with the fact that the activation (phosphorylation) of AKT stimulates the meiosis resumption, it is involved in the MI/MII transition and it regulates polar body emission and spindle organization *(Tomek and Smiljakovic, 2005; Kalous et al., 2006, 2009; Cecconi et al., 2012)*. In the same way, the prompt activation of ERK1/2 induces premature chromosome condensation and meiosis resumption as well as pronucleus breakdown *(Fissore et al., 1996)*. It is interesting to highlight that the activation of CB2 by a treatment with JWH133 induced and accelerated the meiosis progression in fetal oocytes of mice, although they observed how also decreased the pool of primordial and primary follicles *(De Domenico et al., 2017)*. Even so our treatment with THC was *in vitro* and it only lasted 17 hours, and, that could be why we do not observe apoptosis in oocytes or granulosa cells as observed by De Domenico and co-workers (2017) after systemic treatment with JWH133.

Finally, our last objective was to test whether all that changes described regarding the exposure of COCs to THC during oocyte maturation would affect the fertilization and/or subsequent embryo development rate in positive or negative manner. Although the incubation with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes, the THC led to an improvement in blastocysts rate (2 times more number of embryos). That improvement in the amount of blastocysts achieved had not been observed in previous experiments performed with THC in bovine oocytes *(López-Cardona et al., 2016)*, although it was observed using a synthetic agonist for CB1 in mice oocytes *(López-Cardona et al., 2017)*. In addition, the experiments carried out by knockout mice for CB1 and/ or CB2 receptors, confirmed that both receptors are involved in the modulation of oocyte maturation by THC since, when one of the two receptors is absent, the THC is not able to generate improvements in the ratio of blastocysts compared to the control.

4.4 The endocannabinoid system modulates the physiology of the ovary and its activation can improve the maturation of the oocytes *in vitro*

4.4 The endocannabinoid system modulates the physiology of the ovary and its activation can improve the maturation of the oocytes *in vitro*

Lide Totorikaguena[1], Estibaliz Olabarrieta[1], Francesca Lolicato[2], Jon Romero-Aguirregomezcorta[1], Johan Smitz[2], Naiara Agirregoitia[1] and Ekaitz Agirregoitia[1]

[1]Department of Physiology, Faculty of Medicine and Nursing, UPV/EHU, Leioa, Bizkaia

[2]Laboratory of Follicular Biology (FOBI), UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium.

Correspondence:

Ekaitz Agirregoitia, Dept. of Physiology, Faculty of Medicine and Nursing, UPV/EHU, Leioa, Bizkaia. Email: e.agirregoitia@ehu.eus

Running title: Cannabinoids on folliculogenesis

Key words: *Cannabinoid receptors, ovary, oocyte, maturation, THC*

Introduction

Fitocannabinoids are lipophilic compounds derived from the Cannabis sativa L. Research on cannabinoids began in the 1960s, when cannabis Δ^9-tetrahydrocannabinol (THC) was purified and described *(Mechoulam and Gaoni, 1967)*. THC is the most abundant cannabinoid in the plant and the primary responsible for bioactive effects in human *(ElSohly and Slade, 2005)*. Thanks to these early studies, an endogenous cannabinoid system was described in animals that consists of cannabinoid receptors (CB1 and CB2), their internal ligands (endocannabinoids) and the synthesis and degradation enzymes *(Correa et al., 2016)*.

The endocannabinoid system (ECS) has been identified in the regulation of female and male reproductive events and the ECS has been involved in reproduction processes such as, gametogenesis, fertilization, preimplantation embryo development, implantation and postimplantation embryonic growth *(Walker et al., 2019)*. Regarding the female, the ECS has been described in hypothalamus-pituitary-ovary (HPO) axis *(Brents, 2016)*, as well as, follicular fluid, oocytes and granulosa cells from various species like mice, rats, cows and humans *(Schuel et al., 2002a; El-Talatini et al., 2009a; Bagavandoss and Grimshaw, 2010; Peralta et al., 2011; Agirregoitia et al., 2015, 2016; López-Cardona et al., 2016, 2017)*.

Generally, when the ECS is exogenously modulated, the cannabinoids alter HPO axis regulation, potentially leading to disruption of the reproductive system generating, among others, anovulatory menstrual cycles; but, in the same way, altered ECS expression is also associated with reduced fertility *(Brents, 2016)*. In that sense, it is known that the lack of cannabinoid receptors inhibits the hormone release in HPO axis *(Wenger et al., 2001; Oláh et al., 2008; Cacciola et al., 2013)*, although it is not known what happen in the ovary of knock out animals for cannabinoid receptors. It seems that the absence of CB1 in oocytes causes embryo development failure *(López-Cardona et al., 2017)*.

Therefore, if both a cannabinoid overexposure and a cannabinoid under-exposure could be harmful to the oocyte physiology, it is not surprising that there are evidences where endocannabinoid signaling could regulate human follicle maturation and development *(Schuel et al., 2002; El-Talatini et al., 2009b)*. In fact, in mice and cows, the cannabinoids are able to improve the *in vitro* oocyte maturation (IVM) *(López-Cardona et al., 2016, 2017; Totorikaguena et al., 2019)*. Even so, the positive effect of cannabinoids on IVM could be improved since, in the studies mentioned above, the oocytes used derived from the largest cohort of follicles induced with exogenous equine chorionic gonadotropin (eCG), and that process may affect the *in vitro* model because the oocytes would begin at an advanced stage of development *(Romero et al., 2016)*. Nowadays, the best rate of maturation developmental potential was achieved using a previous "pre-maturation" step during which meiotic arrest is imposed via modulation of the cAMP signaling pathway using the "natural oocyte maturation inhibitor (C-type Natriuretic Peptide-22,CNP)" *(Zhang et al., 2010; Romero et al., 2016)*.

In summary, as oocyte meiotic maturation is an important process whereby immature oocytes acquire the characteristics required for successful fertilization and embryogenesis *(Lonergan and Fair, 2016),*

the aim of this work is double. On the one hand, to continue understanding the role of ECS in oocyte physiology, using a genetic approach generating knockout mice for CB1 and/or CB2 receptors, we are going to study the effect of the lack of cannabinoid receptors in ovarian morphology, folliculogenesis, oocyte retrieval and oocyte maturation. On the other hand, as the effectiveness of the IVM technique still does not generate the results of the *in vivo* maturation of oocytes, using immature oocyte cumulus complexes from unprimed juvenile mice, and performing a "pre-maturation" step, we are going to evaluate the use of THC in the oocyte's developmental competence.

Materials and methods

Experimental animals

Wild-type (WT), Cnr1$^{-/-}$ *(Marsicano et al., 2002)*, Cnr2$^{-/-}$ *(Buckley et al., 2000)*, and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice used in this study were kept in an animal house under controlled conditions of temperature (22 ± 1°C) and photoperiod (light/dark cycle 14 h:10 h). Animals were given free access to water and food. All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation.

Animals used for the study with immature oocytes from unprimed juvenile mice were C57BL/6J x CBA/ca. These animals were housed and bred following the national legislation and with the consent of the ethical committee of the Vrije Universiteit Brussel (Project numbers: 09-216-1 and 14-216-1).

Ovary collection and histology

Ovaries from female 8 to 10-week-old WT (C57BL/6 x DBA) or KO on a C57BL/6N background (Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$) mice were collected and some of them were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon, Intervet). In this way, twenty four ovaries (n= 24) were transferred to M2 medium at 37 ºC and cleaned of any connective tissue. We used some of them to measure the total volume and the area using the image J software. For that, the whole ovary was selected and its digital image was captured. All images were examined and captured using an Olympus BX50 optical microscope (Olympus Optical Co.) connected to a digital color camera (Olympus XC50).

The ovaries were mixed with Bouin's solution for 2-4 h and then, the ovaries were washed in 70% ethanol. After that, the ovaries were parafined in blocks. They were dehydrated in alcohol, clarified using xylol, embedded in histological paraffin, and the blocks were sectioned at 8 μm with a retraction microtome

(Shandon AS 325). Finally, the slides were stained with hematoxylin-eosin every fifth section and analyzed (48 μm between analyzed sections).

Follicle counting

As it has been described previously, the number of follicles was estimated by determining the mean total number of follicles per section after sampling every fifth section and section thickness for 8 μm *(Tilly, 2003)*.The slides were examined and captured using and Olympus BX50 optical microscope with 40x enlargement, by which the follicles and other structures of the ovary were observed. After that, all areas of the fragment were photographed using a digital colour camera (Olympus XC50) coupled to the objective of the light microscope to assessment the structures of interest.

Follicles were classified as primordial (a single layer of flattened granulose cells surrounding the oocyte); primary follicles (a single layer of cubic-shaped granulose cells surrounding the oocyte); secondary follicles (a single layer of cuboidal-shaped granulose cells surrounding the oocyte), and antral or preovulatory (a fluid-filled cavity inside the oocyte) *(Silva et al., 2004)*.

The total volume of each ovary was measured (section area x section thickness x number of sections), and the follicle count is stated as follicles per millimetre cubed of ovarian tissue *(Aiken et al., 2013)*.

Isolation and *in vitro* maturation of Cumulus–Oocyte Complexes to determinate meiotic progression

Female 8 to 10 week old WT, $Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mice were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin, and ovaries were collected 46 to 48 h later. The ovaries were cleaned of any connective tissue and placed in handling medium M2 supplemented with 4 mg/ml bovine serum albumin fraction V. Antral follicles were punctured with 30-gauge needles, and immature cumulus cell–oocyte complexes (COCs) were collected in handling medium. COCs were matured for 17 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity.

In order to determine the impact of the lack of cannabinoids receptors in mice oocyte maturation, COCs at 0, 1, 2, 4, 8,12 and 17 h of IVM (n = 30 per time point and group in three independent replicates) were used as described previously *(Khatir et al., 1998)*. Briefly, COCs were partially denuded by vortexing during 3 min in 0.1% of hyaluronidase (Sigma H3506) and fixed in 4% paraformaldehyde (Panreac) for 20 min. Then were washed twice in PBS and incubated in PBS containing 10 μg/mL Hoechst 33342 for 15 min. Oocytes were then placed in glass slides and squashed with coverslip for observation with an immunofluorescence microscope (Zeiss Axioskop) under UV light.

Collection of Cumulus-Oocyte Complexes from Small Antral Follicles from Unprimed Mice and Pre-Ovulatory Cumulus-Oocyte Complexes from Large Antral Follicles

Compact COCs of the first wave of follicular development were collected from small antral follicles of prepubertal mice (19–21 days old) without prior gonadotropin administration. For the collection of pre-ovulatory COCs (controls), compact COCs were collected by puncturing large antral follicles of prepubertal female mice (25–27 days old) following 48 h of priming with 2.5 IU eCG. Collection medium consisted of Leibovitz L-15 containing 10% heat-inactivated FCS, (all from Life Technologies), and supplemented with 200 μM 3-isobutyl-1-methylxanthine (Sigma) to prevent meiosis reinitiation during the period of collection and preculture handling *(Romero et al., 2016)*.

Isolation and culture of prepubertal mice COCs (Pre-*In vitro* maturation)

COCs from prepubertal female mice to 19–21 days old WT (C57BL/6xCBA) without prior gonadotropin administration were collected. Basal culture medium for the culture of COCs (Pre-IVM and IVM phases) consisted of a-MEM, 2.5% FCS (both from Life Technologies), 5 μg/ml insulin, 5 μg/ml apo-transferrin, and 5 μg/ml sodium selenite (all from Sigma). For Pre-IVM experiments, we cultured, *in vitro*, COCs from small antral follicles of unprimed female mice aged 19–20 days old for 48 h in presence of 25 nM CNP-22 (CNP; Phoenix Europe) and 10 μM 17-β-estradiol (E2; Sigma). For IVM experiments, recombinant epidermal growth factor was used as ovulatory stimuli, recombinant follicle stimulating hormone (FSH; Merck-Serono) was added and COCs were directly incubated in the medium of IVM in the presence or absence of THC 10^{-7} M for 18 h.

Additionally, two conditions were evaluated for oocyte developmental capacity: 1) an IVM control, in which COCs from small antral follicles of unprimed mice aged 20 days old were directly *in vitro* matured for 18 h and, 2) an *in vivo* control, where *in vivo* grown and matured oocytes were obtained from female aged 25–27 days old primed for 48 h with 2.5 IU eCG, followed by 14 h with 2.5 IU hCG (Chorulon; Intervet). These oocytes were inseminated with the same sperm sample and oocytes/embryos cultured under the exact same conditions as the IVM oocytes.

Incubation with Tetrahydrocannabinol (THC)

The THC stock solutions were prepared in DMSO. During maturation (17 h), COCs were incubated with 100 nM THC to evaluate the effects of activation of cannabinoid receptor by this agonist. The control group was performed using only the same amount of DMSO.

Evaluation of Meiotic progression

COCs from prepubertal female mice to 19–21 days old WT (C57BL/6xCBA) without prior gonadotropin administration were collected. Basal culture medium for the culture of COCs (Pre-IVM and IVM phases) consisted of a-MEM, 2.5% FCS (both from Life Technologies), 5 μg/ml insulin, 5 μg/ml apo-transferrin, and 5 μg/ml sodium selenite (all from Sigma). For Pre-IVM experiments, we cultured, *in vitro*, COCs from small antral follicles of unprimed female mice aged 19–20 days old for 48 h in presence of 25 nM CNP-22 (CNP; Phoenix Europe) and 10 μM 17-β-estradiol (E2; Sigma). For IVM experiments, recombinant epidermal growth factor was used as ovulatory stimuli, recombinant follicle stimulating hormone (FSH; Merck-Serono) was added and COCs were directly incubated in the medium of IVM in the presence or absence of THC 10^{-7} M for 18 h.

Additionally, two conditions were evaluated for oocyte developmental capacity: 1) an IVM control, in which COCs from small antral follicles of unprimed mice aged 20 days old were directly *in vitro* matured for 18 h and, 2) an *in vivo* control, where *in vivo* grown and matured oocytes were obtained from female aged 25–27 days old primed for 48 h with 2.5 IU eCG, followed by 14 h with 2.5 IU hCG (Chorulon; Intervet). These oocytes were inseminated with the same sperm sample and oocytes/embryos cultured under the exact same conditions as the IVM oocytes.

In vitro fertilization

COCs were collected after oocyte maturation and washed once in IVF medium. COCs were transferred to 200 μl equilibrated IVF medium (M16 - Sigma; NEAA - Thermo Fisher; BSA - Sigma) and overlaid with mineral oil and a 2×10^6 spermatozoa/ml concentration of spermatozoa obtained from CBAB6F1 male donor aged 6 to 12 weeks. After 1 h of co-incubation at 37 ºC, 5% CO_2, 5% O_2, and 100% humidity, presumptive zygotes were denuded, washed twice, and cultured in groups of 10–15 zygotes in 30 μl of embryo culture medium overlaid with oil for embryo culture (Sigma) at 37 ºC in 5% CO_2, 5% O_2, and 100% humidity. Cleavage (2- cell) rate was scored 24 h after IVF. On Day 5, blastocyst development and hatching were recorded. In total, 60–70 COCs (from four independent replicates) were assessed per condition.

Rates of oocyte nuclear maturation and fertilization

To assess whether adding THC fitocannabinoid also affected rates of fertilization, the presence of pronuclear formation was identified. At 24 h after *in vitro* fertilization, all presumptive zygotes that had not divided into 2 cells were fixed in 4% paraformaldehyde for 10 min and then stained with Hoechst 33342 (0.01 mg/ml) for observation with an immunofluorescence microscope (Zeiss Axioskop) under UV light.

Statistical analysis

All statistical tests were performed by using Microsoft Excel software and Graphpad software (GraphPad Software).

All the results were indicated as the mean ± S.E.M. Differences in ovary volume, ovary area, oocyte meiotic resumption, oocyte diameter and rates of fertilization and blastocyst formation between the 4 genotypes were compared by one-way ANOVA, followed by a Bonferroni`s Multiple Comparison test. Values of $P < 0.05$ were considered significant.

Results

Effect of the absence of CB1 and CB2 receptors on the size of mouse ovary

To study the role of CB1 and CB2 cannabinoid receptors in mouse folliculogenesis, firstly, we observed the macroscopic morphology of mice ovary and we measured the size of ovaries from Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mutant genotypes, as well as, the ovaries from wild type (WT) mice.

On one hand, comparing the volume of ovaries from different mice genotypes, ovaries from Cnr1$^{-/-}$ mice were clearly smaller and irregular than WT and, although this difference did not become significant, when both receptors, CB1 and CB2, were disrupted, the difference in ovary volume was significantly notable **(Fig. 4.17A).** On the other hand, we also measured ovarian volume after administration of 5 IU eCG. After that, the volume of WT ovary was significantly higher than the rest of studied genotypes **(Fig. 4.17B)**, in fact, although the ovary volume increased regardless the mouse genotypes after the administration of eCG, that increase only was significant in WT mice **(Fig. 4.17C)**.

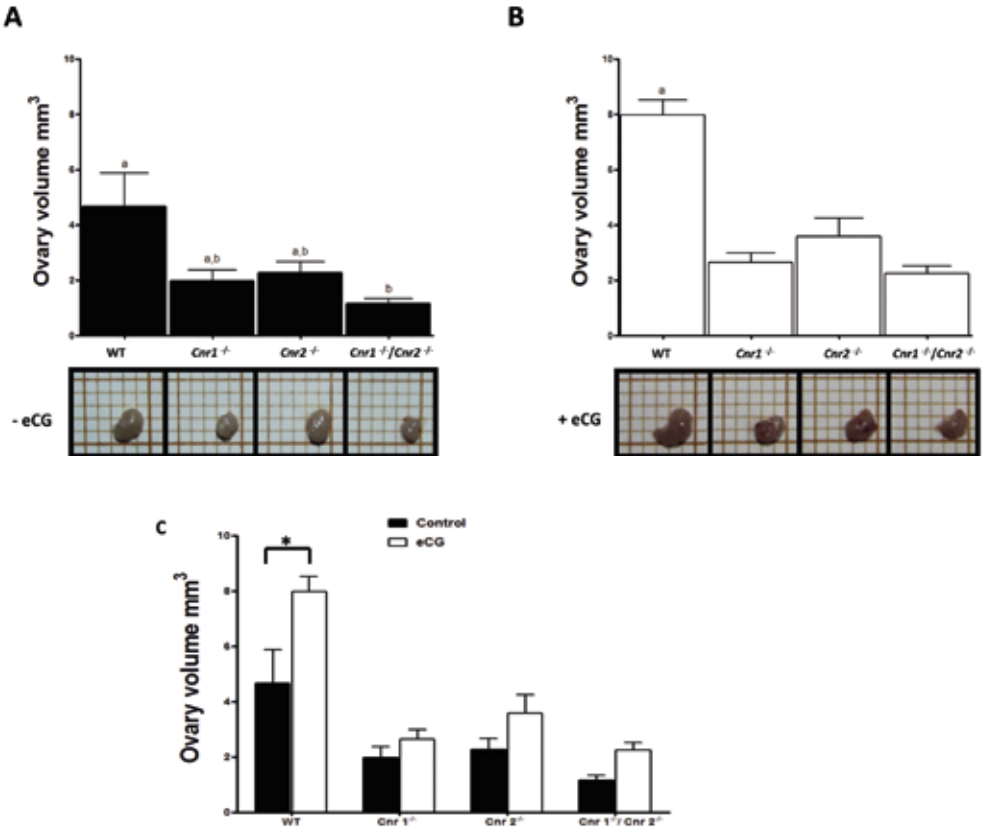


Figure 4.17 Effects of cannabinoid receptors' lack in ovarian morphology and volume. Representative photographs and the measurement of the volume of ovaries of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice (A) before and (B) after eCG administration. (C) Ovary volume comparison between eCG treated and not treated mice. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters or an asterisk (*) indicate significant differences between groups; p<0.05 in all cases.

To continue analysing the impact of the lack of cannabinoid receptors on the ovaries, we analysed histological sections of the ovaries and we measured the area of each ovary. Maintaining the trend of the previous results, the lack of cannabinoid receptors affected the size of the ovary, being smaller when the CB1 receptor was missing but increasing this difference, until significant, when both receptors, CB1 and CB2, were missing in comparison with WT mice **(Fig. 4.18A)**. Again, after the administration of eCG, the differences in the section of the ovary between genotypes grew, being significantly smaller the area when the CB1 receptor was missing and even smaller when CB1 and CB2 were missing **(Fig. 4.18B)**.

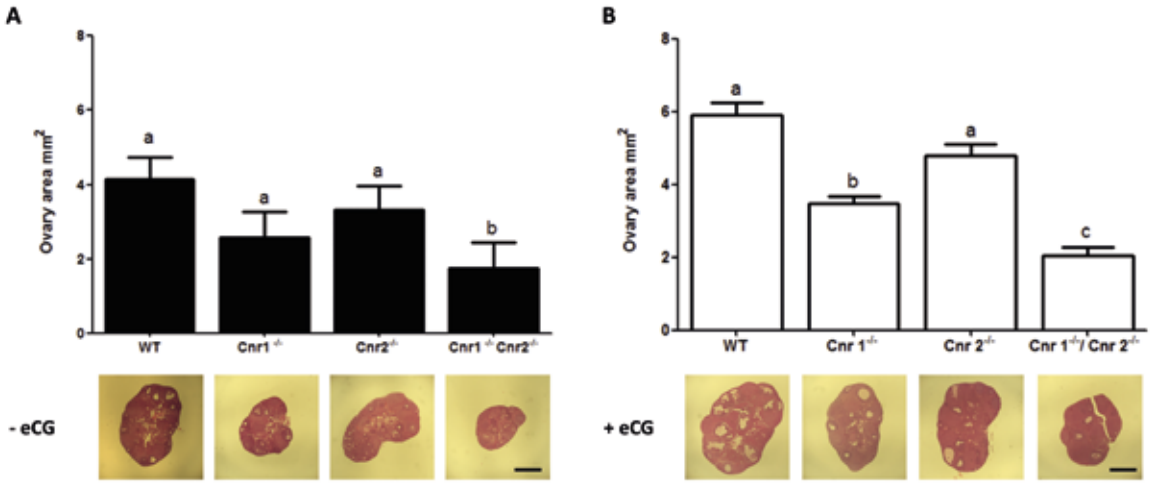


Figure 4.18 Effects of cannabinoid receptors' lack in ovarian size. Representative photographs of histological cross-section and the measurement of the area of ovaries of wild type (WT), $Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}Cnr2^{-/-}$ mice (A) before and (B) after eCG administration Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases. Scale bar: 500 µm

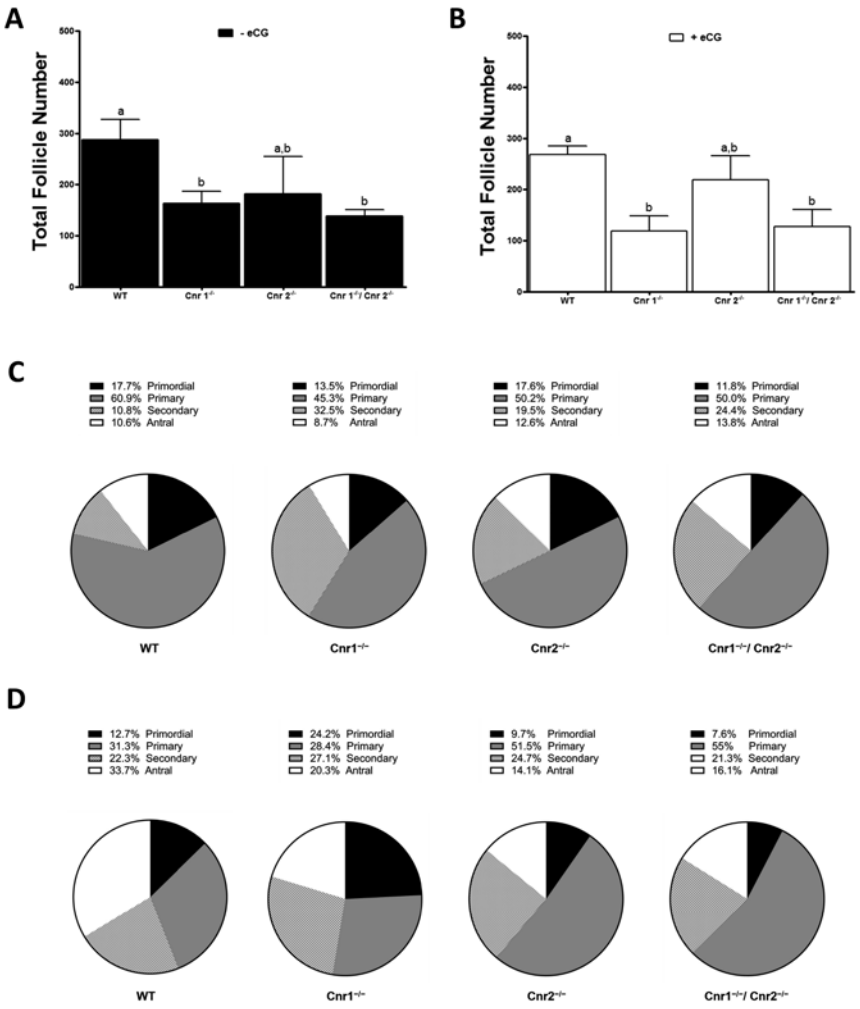
Effect of the absence of CB1 and CB2 receptors on the number of mouse follicles

Ovarian morphology was assessed by histological examination of the different growing follicles using hematoxylin and eosin staining. The number of total follicles varied between the different genotypes, being smaller in those mice ovaries where the CB1 receptor or both receptors, CB1 and CB2, were absent **(Fig. 4.19A)**. Furthermore, total number of follicles 48 hours after eCG treatment maintained the same pattern **(Fig. 4.19B)**.

In order to study in deep the development of those follicles, the initial (primordial and primary follicles) and advanced (secondary and antral follicles) growth follicles were counted. Although all genotypes showed a greater amount of primary follicles, the wild type mice showed a higher number of primary follicles (60.9%) than the knock out genotypes (around 45-50%) **(Fig. 4.19C)**. In fact, the KO mice, especially the $Cnr1^{-/-}$ (32.4%) and $Cnr1^{-/-}Cnr2^{-/-}$ (24.4%), had a higher number of secondary follicles than WT mice (10.8%) **(Fig. 4.19C)**. The $Cnr1^{-/-}$ (13.5%) and $Cnr1^{-/-}Cnr2^{-/-}$ (11.8%) genotypes also showed a lower number of primordial follicles than the rest of genotypes (around 17%) **(Fig. 4.19C)**. Finally, we did not find much difference in the number of antral follicles between genotypes **(Fig. 4.19C)**. After the ovarian stimulation (48 h post-eCG injection), wild type mice showed a higher number of

antral follicles (33.7%) in comparison with the other genotypes **(Fig. 4.19D)**. In addition, the mutant mice for cannabinoid receptors showed more follicles in early stages (primordial and primary) than WT mice **(Fig. 4.19D)**.

To further elucidate the effect of the cannabinoid receptor absence in the quantity of oocytes, we counted the number of oocytes after puncturing the ovary stimulated with eCG derived from $Cnr1^{-/-}$, $Cnr2^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mutant genotypes, as well as, the ovaries from WT mice. Considering that oocyte quality is predetermined by the appearance of oocytes under a microscope, we classified the oocyte as compact, denuded or expanded according to the appearance of their cumulus cells and the degree of expansion of the mural cells *(Hinrichs, 2010a; González-Fernández et al., 2018)*. We extracted less amount of oocytes on $Cnr1^{-/-}$ and $Cnr1^{-/-/}Cnr2^{-/-}$ ovaries, although the difference was only significant when both receptors, CB1 and CB2, were absent **(Fig. 4.19E)**. We obtained the highest number of expanded oocytes from the WT mice, being significantly high than $Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mice. The amount of expanded oocytes obtained from $Cnr2^{-/-}$ genotype was also significantly higher than those obtained from double-KO mice **(Fig. 4.19E**).



E

	Compact oocytes/ovary	Denuded oocytes/ovary	Expanded oocytes/ovary	Total No. of oocytes/ovary
WT	3.6 ± 0.6 ^{a}	3.1 ± 0.4	10.8 ± 1.2 ^{a}	15.9 ± 2 ^{a}
Cnr 1$^{-/-}$	4.7 ± 0.9 ^{ab}	3.3 ± 0.5	7.4 ± 1 ^{b}	12.7 ± 1.4 ^{ab}
Cnr 2$^{-/-}$	4.2 ± 0.2 ^{ab}	2.6 ± 0.3	9.7 ± 0.9 ^{ab}	16.9 ± 0.7 ^{a}
Cnr 1$^{-/-}$/Cnr 2$^{-/-}$	1.5 ± 0.1	2 ± 0.1	5.5 ± 0.3 ^{c}	10.2 ± 0.1 ^{b}

Figure 4.19 Effects of cannabinoid receptors' lack in ovarian folliculogenesis. (A and B) Total number of follicles per ovary of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice (A) before and (B) after eCG administration. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases. (C and D) Percentage of primordial, primary, secondary and antral ovarian follicles per ovary of wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice (C) before and (D) after eCG administration. (E) The number of oocytes achieved after the punctured antral follicles of eCG stimulated wild type (WT), Cnr1$^{-/-}$, Cnr2$^{-/-}$ and Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice. Results are the means ± S.E.M. of 6 independent experiments. The different combinations of letters indicate significant differences between groups; $p<0.05$ in all cases

Effect of the absence of CB1 and CB2 receptors on the velocity of meiotic progression of mice oocytes

We next examined whether the absence of cannabinoid receptors would have any impact on meiotic progression of mice oocytes. We fixed oocytes after 0, 1, 2, 4, 8, 12 and 17 h of IVM. Oocytes nuclear stage was classified in germinal vesicle (GV), germinal vesicle break down (GVBD), pro-metaphase I (PMI), metaphase I (MI) and metaphase II (MII). As it can be seen in **Fig. 4.20A**, at 0 h all the oocytes were at GV stage and after 1 h of IVM, we observed oocytes from mutant mice at GVBD, while all the WT oocytes remained at the GV phase **(Fig. 4.20B)**. At 2 h and 4 h of IVM, the KO-genotypes continued to be more advanced than WT oocytes (**Fig. 4.20C** and **4.20D**) but, at 4 h, although the only oocytes that had not reached PMI were those of the WT genotype, the oocytes without the two types of receptors (CB1 and CB2) had the highest amount of oocytes at GV **(Fig. 4.20D)**. Thereafter, at 8h and 12h, the oocytes of WT and $Cnr2^{-/-}$ genotypes accelerated their maturation process in comparison with the genotypes lacking the CB1 receptor ($Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$), in fact, at 12 h, almost all the oocytes of WT and $Cnr2^{-/-}$ have reached the MII (**Fig. 4.20E** and **4.20F**). Finally, at 17h of maturation, most oocytes of $Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$genotypes had also been able to reach MII stage **(Fig 4.20G)**.

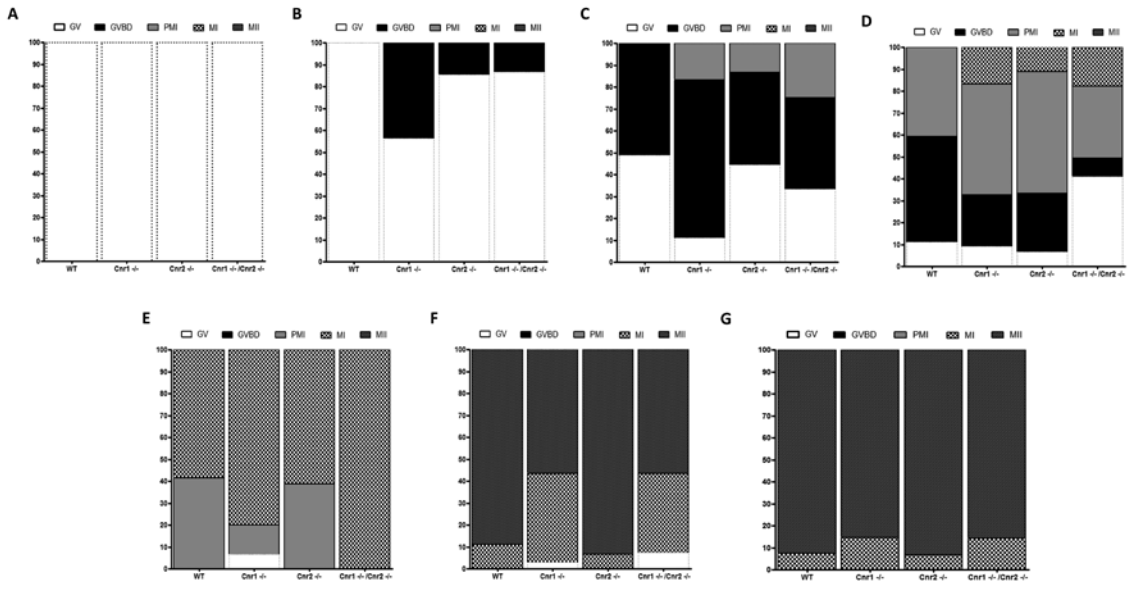


Figure 4.20 Changes in nuclear status of oocytes throughout maturation. Results are expressed as percentage of oocytes at each stage of maturation at each time point: (A) 0 h (B) 1 h (C) 2 h (D) 4 h (E) 8 h (F) 12 h and (G) 17 h. Five stages are shown in different colours: germinal vesicle (GV), germinal vesicle breakdown (GVBD), pro-metaphase I (PMI), metaphase I (MI) and metaphase II (MII).

Developmental capacity of oocytes recovered from unprimed ovaries following prolonged pre-IVM and IVM

Experiments were set up to evaluate developmental competence of oocytes undergoing the Pre-IVM called step, followed by an IVM culture period in absence or presence of 10^{-7} M of THC.

Following the Pre-IVM culture period, the oocytes from almost all culture conditions showed a high rate of meiotic resumption compared to those who did not mature with the previous stage **(Fig. 4.21A)**. Even so, only the cumulus cell-oocyte complexes (COCs) incubated in Pre-IVM medium for 48 h followed by maturation with THC 10^{-7} M had a significant high occurrence of PB oocytes (69.2%) in comparison to control IVM media ($P < 0.05$). In addition, those COCs incubated directly in IVM media with THC 10^{-7} M exceeded the percentage of PB (60.5%) reached in the control of the Pre-IVM (57.5%).

After measuring the diameter of the mature oocytes, the same pattern as the previous results was maintained **(Fig. 4.21B)**, although COCs obtained after Pre-IVM culture reached a larger mean of diameter than COCs cultured directly in IVM medium, only those COCs that were matured with THC 10^{-7} M had a significantly larger diameter than the COCs cultured in IVM medium. In fact, those COCs incubated with THC got the largest diameter. Finally, is interesting to note that when COCs were incubated with THC directly in IVM medium, the diameter of the COCs did not differ from the diameter found in the Pre-IVM conditions.

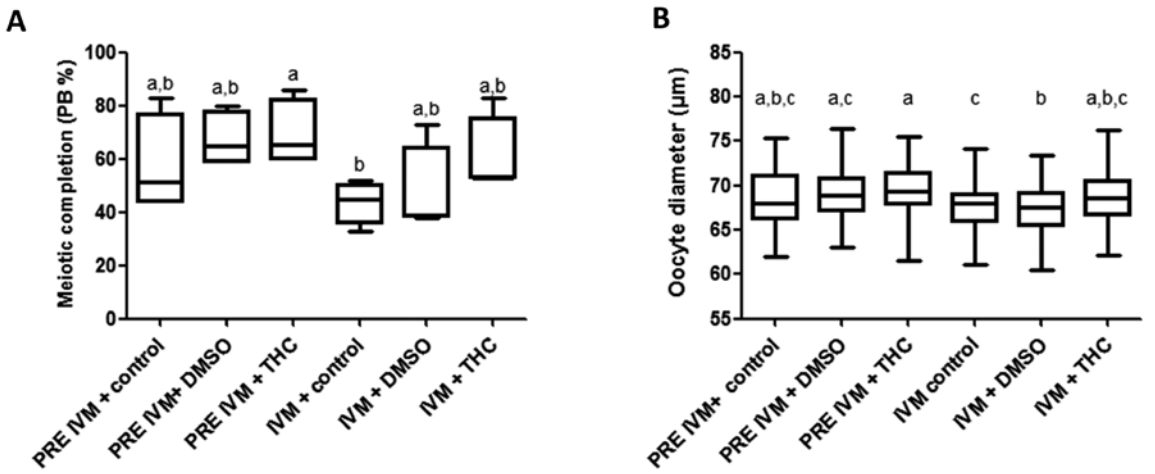


Figure 4.21 Evaluation of THC in meiotic maturation : meiotic completion up to (A) PB extrusion and (B) diameter of MII oocytes. Oocytes were collected from unprimed mice following Pre-IVM step in the presence of CNP conditions or without doing the Pre-IVM step. Then, the IVM was performed in presence or in absence of THC 10^{-7} M. Results are the mean ± S.E.M. of 4 independent experiments. At least 60 MII oocytes/treatment were measured. Significant differences between treatments are indicated with different letters; $p < 0.05$ in all cases.

Evaluation of the developmental competence of prepuberal mice oocytes

The last objective was to evaluate the developmental competence of oocytes undergoing Pre-IVM followed by an IVM culture period in presence of THC 10^{-7} M, observing the fertilization and the subsequent embryo development rate. Following IVM, the oocytes were *in vitro* fertilized, and embryos were cultured up to Day 5.

There were no significant differences in fertilization rate (two-cell) between the different culture and treatments (**Fig. 4.22A** and **4.22B**). Nevertheless, although the blastocyst rates of Pre-IVM and then matured in presence of THC 10^{-7} M was higher than controls and *in vivo* matured oocytes, that difference was not significant **(Fig. 4.22C)**. However, in the unique medium where we obtained blastocysts from COCs cultured directly in IVM medium (without Pre-IVM step) was where the COCs were matured in the presence of THC **(Fig. 4.22D)**.

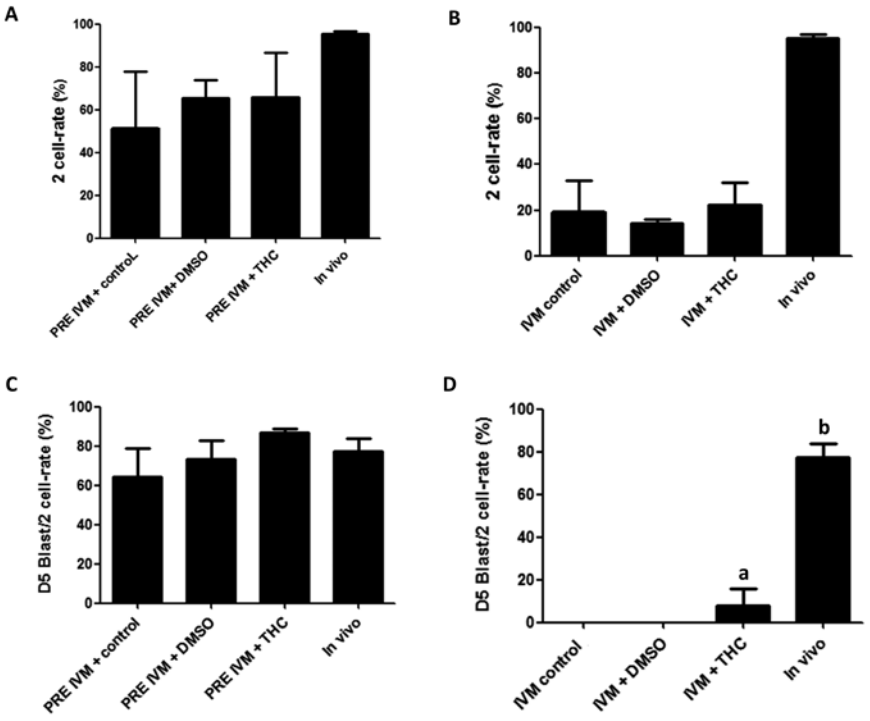


Figure 4.22 Evaluation of THC in developmental competence of oocytes collected from unprimed prepuberal mice. Oocytes were collected from unprimed mice (A and C) following Pre-IVM step in the presence of CNP conditions or (B and D) without doing the Pre-IVM step. Then, the IVM was performed in presence or in absence of THC 10^{-7} M. Finally, the oocytes were *in vitro* fertilized and embryos were cultured for up to 5 days. Evaluation parameters were (A and B) 2-cell rate and (C and D) blastocyst formation on Day 5 related to 2-cell rate (D5 Blast/2-cell). Results are the mean ± S.E.M. of 4 independent experiments. Differences between treatments are indicated with different letters; $p < 0.05$ in all cases.

Discussion

The results of the present study indicate that the lack of cannabinoid receptors affects the oocyte development, from the morphology of the ovary to the development of follicles and the maturation of oocytes. Even so, the use of THC in the medium during the maturation of wild type oocytes *in vitro* improves the maturation process, especially if the oocytes have passed a "pre-maturation" stage prior to *in vitro* maturation (IVM).

Endocannabinoid system (ECS) is present in the hypothalamus, pituitary *(Gammon et al., 2005)* and ovary *(Galiègue et al,. 1995; El-Talatini et al., 2009a)* and, therefore, the negative effects of cannabinoids (CBs) described in reproduction may come from their action at different levels of the hypothalamic-pituitary-ovary (HPO) axis. The most accepted hypothesis is that exogenous CBs exert untoward effects on reproduction by reducing GnRH secretion *(Gammon et al., 2005)*, preventing this hormone from stimulating the release of gonadotropins [follicle stimulating hormone (FH) and luteinizing hormone (LH)] and suppressing gonadal function *(Brents, 2016)*. This idea is supported by the observation that the peripheral administration of exogenous GnRH restores LH secretion in CB-treated animals *(Ayalon et al., 1977; Tyrey, 1978; Smith et al., 1979)*. It has been described that it would be the CB1 receptor which regulates GnRH synthesis and release *(Scorticati et al., 2004; Meccariello et al., 2008; Chianese et al., 2011)*, although, it has also been postulated that the activation of CB1 modulates the LH release at pituitary level *(Wenger et al., 2001)*. In addition, it has been demonstrated a direct inhibitory effect of THC on folliculogenesis due to the interference with several FSH-dependent functions, inhibiting the accumulation of estrogens and progesterone as well as inhibiting the increase of LH receptors *(Adashi et al., 1983)*. In summary, it is known that the regulation of the ECS and HPO axis are linked, although the mechanisms underlying this link it is not fully known *(Brents, 2016)*.

Another observation that reinforces the idea of the well-regulated ECS is required for the optimal function of HPO axis is that, not only cannabinoid treatment but also the lack of cannabinoid receptors causes a decrease in the levels of GnRH, FSH and 17-β-estradiol *(Cacciola et al., 2013)*, as well as, LH *(Wenger et al., 2001; Oláh et al., 2008)*. In addition, it is postulated that around the 40% of $Cnr1^{-/-}$ mice show pregnancy loss *(Wang et al., 2004)*.

After the analysis of the experiments carried out in this work, we have understood the effects on the ovary, folliculogenesis and maturation of the oocytes due to the lack of cannabinoid receptors. Thus, mice without CB1 or CB2 cannabinoid receptors generated smaller ovaries, which was accentuated when none of the receptors was present. This observation was reinforced by data that showed that CB-KO mice had fewer ovarian follicles compared to WT mice. Our observations indicate that the absence of cannabinoid receptors worsens the functional life span of the ovaries due to that life span is determined by the number of oocytes in the ovary, in fact, the infertility is characterised by a gradual decrease in follicle quantity and quality *(Shi et al., 2016)*.

Ovarian stimulation is widely used to improve the efficiency of oocyte production *(Takeo et al., 2019)*, but it is interesting to note how the ovarian stimulation with eCG did not generate an ovarian size growth as pronounced as in WT mice when cannabinoid receptors were absent. That fact probably was due to the less effect that eCG generated in CB-KO mice, since mutant mice for cannabinoid receptors showed less competent follicles than WT mice (the majority of the follicles in CB-KO mice are primordial and primary) after eCG administration. Following this observation, when we analysed the type of oocytes achieved from antral follicles punctured with needles, although the total amount of oocytes only was significantly less in $Cnr1^{-/-}/Cnr2^{-/-}$ mice, both $Cnr1^{-/-}$ and $Cnr1^{-/-}/Cnr2^{-/-}$ mice achieved a smaller number of expanded oocytes than WT mice. This fact is important because the meiotic competence is different between the different types of oocytes, since it has been demonstrated in equine species that only 21% of compact oocytes mature while 71% of expanded ones reach the MII stage *(Hinrichs, 2010b)*.

Finally, although almost all the extracted oocytes at GV stage reached the MII stage in all studied genotypes, we observed an evident acceleration in the first steps of maturation of the oocytes without cannabinoid receptors, more or less, until reaching the stage of MI, because from MI the maturation was decelerated, especially in oocytes without CB1. It is accepted that the failures in the acquisition of nuclear and cytoplasmic maturation (i.e. condensed chromatin configuration, transcriptionally silencing) compromise the obtaining of oocyte development capacity and reduces the successful fertilization and subsequent embryonic development *(Coticchio et al., 2015; Sánchez et al., 2017)*. Even so, it is difficult to know only by observing the velocity of maturation if an acceleration or a deceleration will lead to some improvement or some failure in maturation. For example, a systemic treatment with a CB2 agonist accelerates meiotic progression of fetal oocytes, but decreasing the pool of primordial and primary follicles, negatively affecting the ovarian reserve in the offspring *(De Domenico et al., 2017)*. The acceleration in the first steps of nuclear maturation also occurs when the bovine or mice WT oocytes are incubated *in vitro* with CB agonists during the IVM *(López-Cardona et al., 2016)*, or deceleration when mice oocytes are incubated with CB antagonists *(Cecconi et al., 2019)*. Even so, in the case of CB agonists, up to 50% more blastocysts are achieved compared to the control *(López-Cardona et al., 2016)*. However, these oocytes matured in the presence of cannabinoids also arrive earlier at MII stage *(López-Cardona et al., 2016)*, unlike the KO oocytes used in the present work that slowed maturation after reaching MI. The biggest difference is that, as we just said, while incubating with cannabinoids during IVM improves the achievement of embryos, when CB1-KO oocytes are used to perform IVF, 40-60% less blastocysts are achieved compared to WT oocytes *(López-Cardona et al., 2017)*. Therefore, it seems that the maturation pattern observed in CB-KO oocytes is not the most appropriate.

Considering all the data exposed so far, it appears that the alteration of the systemic ECS affects the stages prior to the oocyte maturation, but, in the same way, the cannabinoids are able to improve the oocytes IVM. Thus, as one of the biggest challenges is to develop systems to improve the developmental competence of oocytes and to adapt culture conditions to the stage-dependent oocyte needs *(Sánchez et al., 2017)*, our last objective was to test if the exposure to THC could improve oocyte competence acquisition from small antral follicles from juvenile unprimed mice without eCG stimulation. The intention was to try to improve the synchronization of meiotic and cytoplasmic maturation in antral oocytes arrested at the immature GV-stage introducing a pre-maturation step during which meiotic

arrest was imposed via CNP, a "natural oocyte maturation inhibitor" *(Romero et al., 2016)* and then, to perform the IVM in presence of THC. Thus, we observed that those oocytes reached a significantly higher polar body rate and a larger diameter when, after a Pre-IVM step for 48 h, were maturated with THC. In addition, the best result on blastocysts rate was achieved when THC was used after a Pre-IVM step. In fact, when the Pre-IVM step was not used, blastocysts were only achieved in the medium with THC.

In conclusion, we have shown that the lack of cannabinoid signalling causes damages to the ovarian function. In addition, since studies to date report a damage to reproduction due to a systemic overexposure to cannabinoids, our study raises the idea that, at least, the *in vitro* use of cannabinoids in the oocyte maturation process could yield positive results.

5

Eztabaida orokorra
General discussion

5. Eztabaida orokorra

Gaur egun, gero eta gehiago dira ugalketa tekniken laguntza behar dutenak haurdun geratu ahal izateko, bai ugalkortasunarekin lotutako arazoak emendatu direlako (haurdun geratu nahi duten bikoteen % 15-20) *(Siristatidis eta lank., 2018)* baita familia ereduak aldatu direlako ere *(Jose-miller eta lank. 2007; Gonzalez eta Morgado 2008)*. Lagunduriko ugalketa tekniken artean, badago berebiziko garrantzia duen bat obozitoa ernaldua izateko: obozitoen *in vitro* heltzea (ingelesez, *in vitro maturation*, IVM). IVM teknikan, pazienteari, hormonekin estimulatu gabeko obarioetatik edo estimulazio txikia jaso duten obarioetatik, heldu gabeko obozitoak erauzten zaizkio eta hazkuntza-ingurunean heltzen dira, ondoren, *in vitro* ernalketa (ingelesez, *in vitro fertilization*, IVF) edo espermatozoide-injekzio intrazitoplasmatikoa (ingelesez, *intracytoplasmic sperm injection*, ICSI) tekniken bidez haurdunaldia lortzen saiatzeko *(Barnes eta lank., 1995, 1996)*. IVM teknika Pincus eta Enzmann-ek (1934) proposatu zuten 1934an. Hala ere, 1991 urtera arte ez zen IVM bidezko lehenengo giza haurdunaldia deskribatu *(Cha et al., 1991)*. Trounson eta lankideek obario polikistiko sindromea (ingelesez, *Polycystic ovary syndrome*, PCOS) zeukaten emakumeentzako ugalketa-teknika bezala plazaratu zuten, gonadotropinak induzituriko obarioen hiperestimulazio sindromea pairatzeko arriskua murrizten duelako *(Trounson eta lank., 1994)*. Kontrolaturiko obarioen hiperestimulazioa oso erabilia da ahalik eta obozito heldu gehien ekoizteko *in vitro* ernalketarako tratamenduan eta enbrioien transferentziaren arrakasta-tasa hobetzeko *(Lu eta lank., 2018)*. 1990ko hamarkadan IVM teknikaren bidezko lehenengo jaiotzaz geroztik *(Cha eta lank., 1991)* 5.000 haur baino gehiago jaio dira munduan teknika hori erabiliz *(Lu eta lank., 2018)*.

Animalia ereduetan, adibidez, txerrietan *(Hirao eta lank., 1994)* eta behietan *(Van den Hurk eta lank., 2000; Hirao eta lank., 2013; Dieci eta lank., 2016)*, IVM bihurtu da abereen hazkuntzarako metodo arrakastatsuena, eta baita enbrioien zelula amen teknologian, klonazioan eta animalia transgenikoen ekoizpenean ere *(Herta eta lank., 2018)*. IVM teknikak kostu baxuagoak eta albo-ondorio gutxiago dituen arren, gizakiei bideraturiko lagunduriko ugalkortasunean gutxi erabiltzen den teknika da oraindik (ziklo guztien % 1 baino gutxiagotan) *(Lu eta lank., 2018)*, alde batetik, bestelako tekniken emaitzen erabateko arrakasta-mailara heldu ez delako eta, bestetik, haren inguruan dagoen ezjakintasunagatik. Hala ere, teknika horren ezarpenak aurrerapen kualitatiboa suposatuko luke. Ugalkortasuna babesteko eman den jakintzaren eta garapenaren emendioak etorkizun eta bide berriak zabaldu dizkio IVMaren esparruari. Izan ere, balizko paziente kopurua asko handitu da *(Delvigne eta Rozenberg 2002; Grynberg eta lank., 2013; Luciano eta lank., 2013; Coticchio, eta lank., 2015; Halupczok, eta lank., 2015; Lee eta lank., 2015Nikseresht M eta lank., 2015)*.

Azken urteetan folikulugenesia, obogenesia eta obozitoen heltze-prozesua erregulatzen dituzten mekanismo biologikoen ezagutza handituz joan da eta jakintza horrek aplikazio klinikorako bidea erraztu du *(Herta eta lank., 2018)*. Ugaztun emeen obozitoen heltze-prozesua ugalkortasunean garrantzia handia duen prozesua da, obulua ernaldua izateko ezinbestekoa delako. Baina, jakina den horretaz gain, gero eta ebidentzia gehiago dago obozitoaren kalitateak zeharo eragiten duela ernalketan eta enbrioien garapen egokian. Esaterako, azken urteetan gora egin duen gurasotasun berantiarrak zelula germinalen kalitatean eragina du eta, horrek, haurdunaldi arrunta izateko arazoak dakartzala erakutsi du *(Munné eta lank., 1995;*

Dailey eta lank., 1996; Munné eta lank., 2007). Obozitoen kalitatearen determinatzaile nagusienetariko bat obozitoaren beraren heltze-prozesua da nahiz eta, oraindik, gutxi dakigun obozitoaren kalitateari buruz eta haren kalitatea egokia izateko parte hartzen duen makinaria guztiaren inguruan *(Gilchrist eta lank., 2008)*.

Ez da arraroa gure ikerketa taldeak IVM teknika hobetzeko intentzioarekin, sistema kannabinoideak eduki lezakeen parte hartzeari erreparatzea. Izan ere, sistema kannabinoidea ugalkortasunean inplikatuta dagoela baieztatu da: gametogenesian, ernalketan, enbrioiaren ezarpenean, plazentazioan, haurdunaldian eta erditzean *(Battista, eta lank. 2008, Maccarrone, 2009)*. Gainera, sistema kannabinoidean parte hartzen duen makinaria guztia ugal-aparatuko organo, ehun eta zeluletan dagoela frogatu da *(Brents, 2016; Schuel eta lank., 2002a; El-Talatini eta lank., 2009a,b; Bagavandoss eta lank., 2010; Peralta eta lank., 2011; López-Cardona eta lank., 2016; López-Cardona eta lank., 2017)*. Baina, guretzat garrantzitsuena dena eta gure ikerketa-lerro honen hasiera suposatu zuena izan zen, ikusi dela kannabinoideek eragiten duten seinaleztapena eta obozitoen heltze-prozesua hasteko gertatu behar den seinaleztapena oso antzekoak direla. Esan bezala, hortaz oharturik, 2010ko hamarkada honetan, gure ikerketa-taldea prozesuan lagungarriak izan zitezkeen estekatzaile interesgarrien bila hasi zen. Izan ere, gure lan-taldeak, saguen *(López-Cardona eta lank., 2017)* behien *(López-Cardona eta lank., 2016)* eta gizakien *(Peralta eta lank., 2011)* obozitoen meiosiaren berraktibazioan CB1 eta CB2 kannabinoide-hartzaileak identifikatu ditu, bai RNA mezulari mailan bai proteina-mailan ere.

Tesi honetako emaitzen lehenengo bi kapituluak gure ikerketa-taldeko lehenengo lanen artean barne bildu ditzakegu. Alde batetik, giza obozitoetan kannabinoideentzako degradazio-entzimak, FAAH eta MAGLa, espresatzen zirela deskribatu genuen. Are gehiago, obozitoaren heltze-prozesuko etapa bakoitzean (GV, MI eta MII) CB1a, FAAH eta MAGLa proteinen lokalizazio denborala aldatzen joaten dela erakutsi genuen. Beste alde batetik, giza pikor-geruzako zeluletan kannabinoide-hartzaileak, CB1a eta CB2a, eta endokannabinoideen andeatzea katalizatzen duten bi entzimak, FAAH eta MGLLa, lokalizatu genituen obozitoaren heltze-prozesuan zehar. Aurkikuntza hori oso interesgarria izan zen, izan ere, obulazioan, pikor-geruzako kumuluko zelulak-obozito konplexua (ingelesez, *cumulus-oocyte complex*, COC) askatzen da eta obozitoaren heltze-prozesuan oso garrantzitsua da obozitoaren eta pikor-geruzako zelulen arteko bi norabideko komunikazioa. Hau da, pikor-geruzako zelulek ere obozitoaren heltze-prozesuan parte hartzen duten mekanismoak erregulatzen dituzte *(Albertini eta lank., 2001)*. Jakina denez anandamidaren (AEA) kontzentrazioa altuagoa dela obozito helduak dituen folikuluen likidoan heldu gabeko obozitoak dituzten folikuluetan baino *(El-Talatini eta lank., 2009b)*, gure lehenengo emaitza horien ondorioz, endokannabinoideen seinaleztapenak folikulugenesia, obulazioa edota obozitoen heltze-prozesua modulatu lezakeela pentsatzera eraman gintuen, hipotesi modura, behinik behin.

Deskribatu da meiosiaren berraktibazioa zein geldialdia cAMParen kontzentrazioak baldintzatzen duela eta cAMParen kontzentrazioaren aldaketa hori, hormonen estimulazioaz gain, G proteinen familia bidez aktibaturiko seinaleztapen bideek eragin lezaketela. Frogatu da obozitoan lokalizatzen den GPR3 hartzaileak meiosiaren geldiaraztea mantendu lezakeela *(Mehlmann eta lank., 2004)* eta horrek indartu egin du G proteinei loturiko hartzaileen funtzioa heltze-prozesuaren baitan *(Mehlmann eta lank., 2005)*. $G_{\alpha i}$ azpiunitateak AC inaktibatzeko pizten duen bidezidorra eta, ondorioz, cAMPa murrizten dela,

ondo zehaztuta dago *(Simonds, 1999)* eta saguan eta arratoian $G_{\alpha i}$-k inaktibatzen dituen AC isoforma desberdinak deskribatu dira *(Hanoune eta Defer, 2001)*. Hori guztia kontuan izanda, gure hipotesiari forma ematen jarraitzeko, pentsatu genuen agian GPCR inhibitzaileak, eta hortaz, CB1 edo/eta CB2 hartzaileak, meiosia berraktibatu lezaketela heltze-prozesuan, izan ere, onartua da kannabinoide-hartzaileak AC inaktibatzean cAMPa murrizten dutela *(Francis eta lank., 2002)*; obozitoek behar duten seinalea meiosia berraktibatzeko *(Mehlmann, 2005)*, alegia.

Beraz, kannabinoide-hartzaileek meiosiaren berraktibazioan parte hartuko balute, posiblea litzateke exogenoki kannabinoideen bidez meiosia berraktibatu eta *in vitro* heltze-prozesua modulatzea?

Bada, behiekin eta saguekin egindako esperimentuetan ikusi da kannabinoide sintetikoak erabiliz gure hipotesi hori baieztatzen dela *(López-Cardona eta lank., 2016; López-Cardona eta lank., 2017)*. Hain zuzen ere, HU-210 heltze-mediora gehitzean, obozitoaren heltze-prozesuan garrantzitsuak diren eta meiosia erregulatzen duten AKT eta ERK1/2 kinasen fosforilazio patroietan aldaketak antzeman ziren *(López-Cardona eta lank., 2016; López-Cardona eta lank., 2017)*, batez ere, CB1 hartzailearen bidez *(López-Cardona eta lank., 2017)*. Gainera, behietan enbrioiaren kalitatearekin erlazionaturiko geneen adierazpena emendatu zen *(López-Cardona eta lank., 2016)*.

Ikusirik kannabinoideak obozitoaren heltze-prozesua modulatzeko gai direla, THC fitokannabinoideak IVM prozesuan duen papera karakterizatzea izan zen hurrengo pausoa. Izan ere, THCak interes handia piztu du haren analogo sintetikoak (dronabinol eta nabilona) erabilera medikorako baimenduak izan direnetik *(Dinis-Oliveira, 2016)*. Hala ere, fitokannabinoideen erabilera, kasu honetan THCarena, beste edozein kannabinoide sintetikorekin alderatuta errazagoa eta fidakorragoa da klinikara eramateko *(Wiley eta lank., 2014; Kowal eta lank., 2016; Schreiber eta lank., 2019)*. Baina nola justifikatu daitezke THCak eragiten dituen albo-ondorioak, batez ere, antsietatea, paranoia, memoria eta lokomozioaren narriadura *(Hill eta lank., 2017a)*? Sintoma kaltegarriak, adibidez, psikotikoak, bakanak izan arren, batez ere, THC dosi altuen aurrean ager daitezke (40-300 μg/kg). Prozesu horietako asko kortex aurrefrontalaren jardueraren araberakoak dira, erabakiak hartzeko prozesuekin eta kontrol kognitiboarekin lotutako jarduera neuronala koordinatzen duen burmuinaren atala *(Gilman eta lank., 2019)*. Baina obozitoen *in vitro* heltzerako THCarekin egindako tratamenduan, esan bezala, exogenoki gehitzen den THCa obozitoarekin bakarrik dago kontaktuan, eta soilik 17 orduz (heltze-prozesuak saguetan irauten duen denbora). THC erabiltzeko modu segurua izan zitekeela baieztatu genuen ez baigenuen apoptosirik behatu THCarekin trataturiko obozitoetan ez eta pikor-geruzako zeluletan ere, de Domenico eta lankideek (2017) antzeman bezala kannabinoide sintetiko batekin (JWH-133) egindako tratamendu sistemikoaren ostean.

Ondo ezarria dago *in vitro* heltze-prozesua atzeratu egiten dela *in vivo* prozesuarekin alderatuta *(Chian eta lank. 2004)*, baina kannabinoideen bidez obozitoak exogenoki heltzean behietan meiosiaren berraktibazioa azkarrago gertatzen da GVaren haustura aurreratzen delako *(López-Cardona eta lank., 2016)*. Guk ere, THCa erabiliz *in vitro* heltze-prozesuan, CB1 hartzaileak periferiarantz azkarrago migratzen duela baieztatu dugu. Izan ere, CB1 hartzailearen mugimendua obozitoaren nukleotik, GV fasean, periferiara antzeman da behian *(López-Cardona eta lank. 2016)* eta saguan (*López-Cardona eta*

lank. 2017, gure lan honetan). Beraz, G proteinei loturiko hartzaileen teoriari jarraiki, hau da, GPCRek zelularen gainazala eskuratu behar dutela lan egin ahal izateko *(Cahill eta lank. 2007)*, baliteke CB1 hartzailea aktibo bilakatzea obozitoen heltze-prozesuan zehar. Gainera, lokalizazio dinamika hainbat animalia eredutan mantentzeak iradoki liezaguke sistema endokannabinoidea, CB1 hartzailearen bidez, ondo kontserbaturiko mekanismoa izan daitekeela ugaztunen obozitoen heltze-prozesuan. Hala ere, nahiz eta CB2 hartzaileak ez duen lokalizazio denboralaren dinamikan desberdintasunik erakutsi, azpimarratu behar dugu, gure lan honetan, CB2a lehenengo aldiz lokalizatu dugula periferian. Aurretiaz aipaturiko GPCRen teoria klasikoaren arabera, baliteke bai CB1a bai CB2a aktibo egotea obozitoen heltze-prozesuan. Zenbait datuk hipotesi hori indartuko lukete, izan ere, badirudi GPCR-$G_{\alpha i}$ –ek (CB1 eta CB2 hartzaileek duten alfa subunitate nagusia) meiosiaren berraktibazioa bultzatzen dutela *(Mehlmann, 2005)*.

Bestalde, aipatu bezala, obozitoaren heltze-prozesuan hainbat seinaleztapen bidezidor daude inplikatuak, besteak beste, MPFaren aktibazioa bultzatzen duten PI3K/AKT eta MAPK bidezidorrak *(Schmitt eta Nebreda 2002, Cecconi eta lank. 2012; Conti eta lank., 2012)*. Alde batetik, mielinaren 1 transkripzio faktorea (MYT1) MPF konplexuaren kinasa inhibitzailerik handiena, AKT proteinaren jardueraren bidez inhibitzen da, meiosiaren berraktibazioa estimulatuz *(Okumura eta lank., 2002)*. Saguen eta arratoien obozitoetan behatu da AKTaren jarduera blokeatzerakoan cdc25-ren jarduera ere murrizten dela eta, ondorioz, meiosiaren berraktibazioa atzeratu *(Kalous eta lank., 2009)*. Aldiz, obozitoak kannabinoideen presentzian heltzerakoan AKTaren fosforilazioa aurreratzen da behien pikor-geruzako zeluletan eta obozitoan *(López-Cardona eta lank., 2016)* eta guk ere, THCa erabiltzean, emaitza berdina behatu dugu saguetan. Horrek ere, obozitoen *in vitro* heltze-prozesuko kannabinoideen rol azeleratzaile bat iradokiko luke. Beste alde batetik, MAPK bidezidorrari dagokionez, gaur egun, IVM protokolo batzuetan EGF hartzailearen bidezko seinaleztapen bidezidorrak erabiltzen dira meiosia berraktibatzeko eta frogatu da hartzaile horren bitartez ERK1/2a aktibatzen dela, obozitoaren eta pikor-geruzako zelulen arteko Gap loturak apurtuz *(Su eta lank., 2002; Fan eta lank., 2009)*. Horrek guztiak cAMParen beherakada eragiten du, kumuluko pikor-geruzako zelulak hedatzen dira eta, beraz, meiosiaren aktibazioa hasten da *(Gonzalez-Robayna eta lank., 2000; Wayne eta lank., 2007)*. ERK1/2 proteinak paper garrantzitsua dauka obozitoen heltze-prozesuan, beharrezkoa baita mikrotubuluen antolaketan, ardatz mitotikoaren muntaduran eta ernalketa ondorengo pronukleoen eraketarako *(Fan eta Sun, 2004)*. Gure emaitzek erakusten dute ERKren aktibazio-maila gorena obozitoa heldua denean (MII) ematen dela eta kannabinoideek EGFa eta beste heltze-promotore batzuen antzerako efektuak *(Conti eta lank., 2012)* erakusten dituztela. Izan ere, THC fitokannabinoidearen presentzian heldu ondoren, proteina horren fosforilazio-patroia aldatu egiten da, aktibazio hori pikor-geruzako zeluletan zein obozitoan azkartuz, López-Cardona eta lankideek (2016) behietan kannabinoide sintetikoekin frogatu bezala.

Orokorrean, barne-sistema kannabinoidea exogenoki modulatzean, hipotalamo-hipofisi-obario ardatzaren erregulazioa eraldatzen da eta, ondorioz, ugalketa-sisteman kalteak sortzen dira *(Gammon eta lank., 2005; Brents, 2016)*. Baina, era berean, barne-sistema kannabinoidearen adierazpen asaldatua ugalkortasun murriztuarekin erlazionatu da *(Brents, 2016)*. Alde batetik, frogatu da kannabinoide-hartzaileen faltak HPO ardatzeko hormonen askapena inhibitzen duela *(Wenger eta lank., 2001; Oláh eta lank., 2008)*. Bestaldetik, deskribatu da CB1 hartzailea obozitoetan ez egoteak enbrioiaren garapenenean

akatsak eragiten dituela *(López-Cardona eta lank., 2017)*. Gainera, publikatu da Cnr1$^{-/-}$ genotipoko saguen % 40 inguruk haurdunaldiaren galera erakusten duela *(Wang eta lank., 2004)*. Hala ere, orain arte ez zen ezagutzen zer gertatzen zen obarioen morfologian, folikulugenesian eta obozitoen heltze-prozesuan kannabinoideen seinaleztapena ezabatzen zenean, eta hori izan da gure esperimentuek ekarri duten berrikuntza. Zentzu horretan, esan dezakegu aurretik geneukan susmoa baieztatu dugula, izan ere, gure emaitzek iradokitzen dute CB1 hartzailearen gabeziak efektu kaltegarriak dituela obarioaren morfologian (kontrolarekin alderatuz azalera eta bolumen txikiagoko obulutegiak dituzte), folikulugenesian (folikulu gutxiago dituzte kontrolarekin alderatuz eta eCG tratamenduaren aurrean ez dute ondo erantzuten), obulatzen duten obozitoen kalitatean (obozito hedatuen kopuru baxuagoa erakusten baitute) eta meiosiaren progresioan (obozitoaren nukleoaren heltzean sinkronizazio falta nabari da, besikula germinalaren apurketa aurreratzen da baina MII fasera beranduago heltzen dira). Are gehiago, CB2 hartzailearen galerak aipatu berri ditugun prozesu horietan akats hain esanguratsuak sortzen ez dituen arren, kannabinoide-hartzaile biak ez egoteak areagotu egiten du kaltea, batez ere, obulutegien tamaina txikiagoak eta folikulu kopuru gutxiago aurkituz; CB2 hartzaileak ere garrantzia duela erakutsiz. Ez hori bakarrik, eCG hormonaren eraginkortasuna ere kaltetua ikusi da KO animalietan. Ondorioz, badirudi kannabinoide-hartzaileen faltak obulutegien bizitza funtzionala eta kalitatea okertzen duela. Hain zuzen ere, antzutasuna obulutegien folikuluen kantitatearen eta kalitatearen beherakadaren ondorioa da *(Shi eta lank., 2016)* eta obozitoen garapen gaitasuna folikuluen tamainagatik eta kalitateagatik baldintzatua egon daiteke *(Otoi eta lank., 1997)*.

Hortaz, orain arte azaldutako aurrekari guztiek eta gure emaitzek iradoki badute sistema kannabinoidearen parte-hartzea obozitoen heltze-prozesuan, ez litzateke harritzekoa pentsatzea kannabinoideen bidezko modulazio exogenoa lagungarria izan litekeela, esaterako, *in vitro* heltzean. Hori dela eta, heltze-prozesuan THCaren eragina baieztatzeko (heldutako obozito horien garapen gaitasunean eta garapen meiotikoan, hain zuzen), THCaren presentzian heldutako obozitoak *in vitro* ernaldu genituen eta lortutako blastozistorainoko enbrioi-tasa aztertu genuen. Eta esperimentu horiek erakutsi zituzten emaitzarik interesgarrienak izan dira, aurkeztu dugun bezala, blastozisto kopuru bikoitza eskuratu genuela THCrik gabe heldutako obozitoekin alderatuta, nahiz eta ernalketa-tasari dagokionez ez genuen desberdintasunik antzeman. Gainera, THCaren eragina bai CB1 eta baita CB2 hartzaileen bidez gertatzen dela ondorioztatu genuen, hartzaile horietariko bat edo biak falta zirenean THC ez zelako gai izan blastozistoen tasa emendatzeko kontrolarekin alderatuta. Emaitza horiek bat datoz aurretik kannabinoide sintetikoekin egindako lanekin *(López-Cardona eta lank., 2017)*, THCaren balioa indartuz obozitoen heltze-prozesua hobetzeko.

IVM prozeduraren ahulguneetariko bat da *in vivo* heldutako obozitoekin alderatuz, *in vitro* obozitoen heldutasun-maila eta blastozistoen tasa baxuagoak direla nahiz eta ernalketa-tasak antzerakoan diren *(Lu eta lank., 2018)*. Eta badirudi arazoaren muina heltze zitoplasmatikoan egotea. Izan ere, nahiz eta nukleo mailan heldu diren obozitoak ernalduak izan daitezkeen, baliteke ondorengo garapenean arazoak izatea obozitoen heltze-prozesu osorako beharrezkoak diren faktore zitoplasmatikoetan gabeziak egon direlako *(Herta eta lank., 2018)*. Hau da, garapen gaitasuna edo kapazitazioa bukatzen ez duten obozitoak ez dira gai izango garapen egokia aurrera eramateko nahiz eta meiosia berraktibatzeko eta ernaltzeko gai izan. Adibidez, *in vitro* egiten den heltze-prozesuan gerta liteke organuluek ez amaitzea egin beharreko

ibilbidea eta horrek heltze-prozesu osoa baldintzatuko du *(Sun eta lank., 2001)*. Osatu gabeko heltze zitoplasmatikoaren eraginez, ernalketan akatsak gertatzen dira, hala nola, polispermia eta pronukloen eraketaren sinkronizazio eza *(Mattioli eta lank., 1988; Moor eta lank., 1990)*.

In vitro heldutako eta ernaldutako obozitoen garapen eskasaren arazoari aurre egiteko, laborategietan hainbat inhibitzaile (fisiologiko eta kimiko) erabili dira meiosiaren berraktibazioa inhibitzeko eta garatzen ari den folikuluaren barruan gertatzen dena imitatzen saiatzeko, obozitoari denbora emanez transkriptoak metatzeko eta haren kalitate zitoplasmikoa hobetzeko. Posiblea da obozitoak meiosiaren geldiaraztean mantentzea cAMParen degradazioa ekiditen duten fosfodiesterasaren inhibitzaileekin (PDE fosfodiesterasaren inhibitzaileekin) *(Sirard, 2001)*. GVa etapa oso garrantzitsua da transkripzio eta itzulpen prozesuetarako, ez bakarrik GVBD fase espezifikoko molekulentzat *(Khatir eta lank., 1998)*, baizik eta enbrioiaren garapenean garrantzitsuak diren proteina eta transkriptoen sintesi eta biltegirako. Datu klinikoek adierazten dute IVM sistemen barruan erronka handiena dela 2 eta 8 mm bitarteko folikuluetan obozitoaren nukleoaren eta zitoplasmaren heltzea sinkronizatzea meiosiaren berraktibazioa bultzatu aurretik. Folikulu antral ertainetatik eratorritako obozitoetan nukleoaren heltzearen berraktibazio "goiztiarra" gertatzen da, eta horrek, eten egiten du ezarpenaren aurreko enbrioiaren garapena bermatzen duen "kapazitazio" prozesua (makineria zitoplasmatikoaren lorpena) (*Hyttel eta lank., 1997, Dieleman eta lank., 2002; Gilchrist eta Thompson, 2007)*.

Datu horiek guztiak kontuan izanda, hurrengo belaunaldiko IVM sistemaren helburuak izango dira (1) lehenik eta behin fosfodiesterasa inhibitzaileak edo C-motako peptiko natriuretikoa (CNP) erabiliz bat-bateko meiosiaren berraktibazioa ekiditea, (2) obozitoen cAMP maila artifizialki emendatzea eta/edo, azkenik, (3) meiosiaren berraktibazioa eragitea. IVM sistema sofistikatuago horiek normalean MII obozitoen kopurua handitzea eta blastozistoen tasa hobetzea eragin nahi dute *(Herta eta lank., 2018)*.

Romero eta lankideek (2016) finkatu zuten Pre-IVM protokoloa moldatuz, IVM pausuan THCa gehitu genuen. Hori eginez, ikusi dugu THCaren presentziak sexualki garatu gabeko saguen obozitoen garapen gaitasuna emendatzen duela. CNParen bidez obozitoaren meiosia geldiarazterakoan (nukleoaren eta zitoplasmaren heltzea sinkronizatzeko) eta, ondoren, THCaren bidez meiosia berraktibatzerakoan; alde batetik, eskuratutako obozitoek diametro handiagoak erakutsi zituzten, *in vivo* eskuratzen diren balioetatik oso gertu *(Griffin eta lank., 2006)* eta, bestetik, korpuskulu polarren tasa altuagoak lortu zituzten. Bi faktore horiek garapen gaitasunarekin zuzenean lotuta daude, izan ere, nukleoaren heltzea, besteak beste, MII fasean eskuratzen den korpuskulu polarraren agerpenarekin egiaztatu daiteke, eta heltze zitoplasmatikoa, aldiz, obozitoaren diametroarekin *(Lonergan eta lank., 1994; Romero eta lank., 2016)*. Folikulu txikiagoen obozitoak, nahiz eta heldutasun nuklearra mantentzeko gai izan, zitoplasmatikoki heldugabeak direla frogatu da *(Barnes eta lank., 1991; Arlotto eta lank., 1996)*, obozitoaren garapen gaitasunaren eta obozitoaren tamainaren artean erlazio zuzena dagoelako *(Otoi eta lank., 1997)*. Azkeneko esperimentuetan, berriro ere, obozitoen garapen gaitasuna enbrioien ekoizpen-tasari erreparatuta ebaluatu genuen, eta gure hipotesia baieztatu zen, hau da, enbrioi-tasa altuagoa eskuratu zen IVM protokolo berritzailearen bidez.

Laburbilduz, tesi honetan jasotako emaitza guztiak aztertuta, ondorioztatu dezakegu sistema kannabinoideak folikulugenesia, obogenesia eta obozitoen heltze-prozesua modulatzen duela *in vivo*. Gainera, THC fitokannabinoideak obozitoaren garapen gaitasuna hobetu dezake *in vitro* eta, obozitoen heltze-prozesua exogenoki modulatuz THCarekin, emaitza positiboak eman ditzake IVF eta geroko enbrioi-tasari erreparatuz.

Tesi honetan egindako aurkikuntzak, beraz, erabilgarriak izan daitezke obozitoaren *in vitro* heltze-prozesua hobeto ulertzeaz gain, IVM teknikaren eraginkortasuna emendatzeko eta antzutasuna tratatzeko itu terapeutiko berrien identifikaziorako. Izan ere, THC fitokannabinoidea IVM inguruneetan osagai gisa erabiltzeak abantaila gehiago eskainiko lituzke klinikara bideratzerakoan. Azkenik, nabarmentzekoa da IVM teknikak ugalkortasun klinikara jo behar duen emakumeen bizi-kalitatea hobetuko lukeela askoz aukera onuragarriagoa, erosoagoa eta arrisku gutxiagokoa izango litzatekeelako eta, zenbait kasutan, haurdunaldia lortzeko aukera bakarra.

Hala ere, eta bukatzeko, klinikara heltzeko ziurtatu behar da THCa gehituta egindako IVM teknikaren bidez jaiotzen diren belaunaldi berriek garapen normal bat izango dutela.

Baliteke, gainera, THCa gehituta bakarrik ez lortzea guztiz IVM teknikaren arrakasta. Nolanahi ere, pauso bat izan litzateke fitokannabinoide hori gehituta "formula berri bat" topatzeko IVM ingurune eraginkor bat sortzerako bidean.

6

Ondorioak
Conclusions

6. Ondorioak

1. Sistema kannabinoidea gizakiaren obozitoetan espresatzen da. Obozitoen heltze-prozesuan zehar MAGLak kokapen zitoplasmatikoa mantentzen duen bitartean, CB1aren eta FAAHren kokapena aldatzen doa zelularen periferia eta zitoplasmaren artean. Kokapen aldaketa horiek meiosiaren berraktibazioarekin lotutako mintz-proteinen funtzioren bat iradoki dezake.

2. Gizakiaren pikor-geruzako zeluletan CB1 eta CB2 hartzaileak eta FAAH eta MAGL degradazio-entzimak espresatzen dira obozitoen heltze-prozesuan zehar, nahiz eta CB2aren presentzia oso mugatua egon. FAAHk eta MAGLak kokapen zitoplasmatikoa dute, baina FAAH ere CB1arekin kolokalizatzen da pikor-geruzako zelulen mintzean. Horrek, heltze-prozesuan zehar, AEA bezalako endokannabinoideen kontzentrazioa kontrolatzeko pikor-geruzako zelulek daukaten auto-*feedback* mekanismoa adierazi dezake.

3. Kannabinoide-hartzaileen gabeziak saguen obozitoaren garapen gaitasunean eragiten du, izan ere, obarioaren morfologian, folikuluen garapenean, obulazioan eta obozitoen heltze-prozesuan asaldurak eragiten ditu. Horrez gain, eCG hormonaren eraginkortasuna murrizten da kannabinoide-hartzaileak falta direnean. Beraz, sistema kannabinoidea emearen ugalketa-prozesuetarako bereziki garrantzitsua dela iradoki daiteke.

4. THC fitokannabinoidea obozitoen heltze-mediora gehitzean, CB1 hartzailearen lokalizazioaren denborazko dinamika aldatu egiten da saguen obozitoen *in vitro* heltze-prozesuan, nukleoaren inguruan kokatzetik periferiara azkarrago migratuz. Aldiz, mediora THC gehitu arren, CB2 hartzaileak kokapen nagusiki periferikoa mantentzen du. Hortaz, obozitoen heltze-prozesuan zehar THCak CB1aren bidez eragin dezakeela erakusten du eta, gainera, CB1 hartzailea obozitoaren heltze-nuklearraren abiaduraren adierazle egokia izan daiteke.

5. Heltze-mediora gehitutako THCaren eraginez, *in vitro* heldutako obozitoen meiosiaren berraktibazioa azkartzen da. Horrek adierazi dezake THCak obozitoen heltze-prozesuan parte hartze duela meiosiaren berraktibazioa modulatuz.

6. THCak obozitoen heltze-prozesuan garrantzitsuak diren seinaleztapen-bidezidorrak aktibatzen ditu CB1 eta CB2 hartzaileen bidez, izan ere, THCaren presentzian *in vitro* heldutako sagu obozitoetan, PI3K/AKT eta MAPK bidezidorretan inplikatuta dauden AKT eta ERK1/2 proteinen fosforilazio-patroian aldaketak sortzen ditu soilik WT saguetan.

7. THCaren presentzian heldutako obozitoetatik eratorritako enbrioi-tasa altuagoa da eta, badirudi, THCak hobetze-eragin hori gauzatzeko bi hartzaile kannabinoideak, CB1a eta CB2a, presente egon behar direla obozitoetan.

8. Garapen konpetentzia lortzeko beharrezkoa den Pre-IVM protokoloa moldatuz, hau da, ondoren IVM protokolora THCa gehituz, THCaren presentziak sexualki garatu gabeko saguen obozitoen garapen gaitasuna eta enbrioi-tasa emendatzen du. Ondorioz, badirudi THC fitokannabinoideak obozitoaren garapen gaitasuna hobetu dezakeela eta obozitoen heltze-prozesuan THCa *in vitro* erabiltzeak emaitza positiboak eman ditzakeela.

6. Conclusions

1. The cannabinoid system is present in human oocytes. MGLL maintains its cytoplasmic location throughout all the observed stage whereas CB1 and FAAH change their location during oocyte maturation between cellular periphery and cytoplasm. These changes in location may suggest a role for membrane proteins associated with meiotic resumption.

2. Cannabinoid receptors CB1 and CB2 and cannabinoid-degrading enzymes FAAH and MGLL are present in human granulosa cells during oocyte nuclear maturation although the presence of CB2 receptor is more residual than the other compounds. FAAH and MGLL have a cytoplasmic location but CB1 and FAAH co-localized in the membrane of granulosa cells, suggesting that they could have an autofeedback mechanism to control the concentration of endocannabinoids like AEA during the oocyte maturation.

3. The lack of cannabinoid signaling causes damages to the oocyte developmental competence. In fact, affects the oocyte development, from the morphology of the ovary to the development of follicles and the maturation of oocytes. Furthermore, the absence of cannabinoid receptors worsens the efficiency of eCG. Therefore, the endocannabinoid system would be an important modulator of female reproduction.

4. When present during IVM of oocytes, the THC accelerates the relocation of CB1 receptor in oocytes from germinal vesicle to the periphery. Nevertheless, CB2 do not show any relocation difference during the IVM in the presence of THC. CB1 receptor could be an indicator of oocyte nuclear maturation velocity and THC could modulate oocyte maturation through CB1.

5. The presence of THC during the oocyte maturation accelerate the meiotic resumption suggesting that THC could modulate oocyte maturation as meiotic inducer.

6. THC activates the PI3K/AKT and MAPK signaling pathways that are important in the oocyte maturation process through CB1 and CB2 receptors because the presence of THC during the *in vitro* oocyte maturation changes the phosphorylation-pattern of AKT and ERK1/2 only in wild type mice.

7. THC exposure during IVM led to an improvement in the blastocysts rate and it seems that both CB1 and CB2 receptors are required in the oocyte for THC to exert this potentiating effect.

8. Adapting a "pre-maturation" step following IVM in presence of THC improves the quality of oocytes from unprimed prepubertal mice as evidenced by a higher embryo rate. As a result, the *in vitro* use of THC in the oocyte maturation process could yield positive results.

7

Bibliografia
References

7. Bibliografia / References

A

Abbott AL, Fissore RA and Ducibella T. 2001. Identification of a translocation deficiency in cortical granule secretion in preovulatory mouse oocytes. *Biol Reprod.* 65(6):1640-7.

Abood ME. 2005. Molecular biology of cannabinoid receptors. *Handb Exp Pharmacol.* (168):81-115.

Adashi EY, Jones PB and Hsueh AJ. 1983. Direct antigonadal activity of cannabinoids: suppression of rat granulosa cell functions. *Am J Physiol.* 244(2):E177-85.

Adashi EY, Resnick CE, Hurwitz A, Ricciarelli E, Hernandez ER and Rosenfeld RG. 1991. Ovarian granulosa cell-derived insulin-like growth factor binding proteins: modulatory role of follicle-stimulating hormone. *Endocrinology.* 128(2):754-60.

Adhikari D, and K Liu. 2014. The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Mol Cell Endocrinol.* 382: 480-487.

Agirregoitia E. 2008.Opioide-hartzaileak (δ, κ, μ) eta kannabinoide-hartzaileak (CB1, CB2) giza espermatozoideetan espresatzen dira eta haien mugikortasunean eragiten dute. *Doctoral Thesis.*

Agirregoitia E, Carracedo A, Subiran N, Valdivia A, Agirregoitia N, Peralta L, et al. 2010. The CB(2) cannabinoid receptor regulates human sperm cell motility. *Fertil Steril.* 93:1378–87.

Aiken CE, Tarry-Adkins JL and Ozanne SE. 2013. Suboptimal nutrition in utero causes DNA damage and accelerated aging of the female reproductive tract. *FASEB J* .27(10):3959–65.

Albertini DF, Combelles CM, Benecchi E and Carabatsos MJ. 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 121:647–53.

Arlotto T, Schwartz JL, First NL and Leibfried-Rutledge ML. 1996. Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. *Theriogenology.* 45(5):943-56.

Asch RH, Smith CG, Siler-Khodr TM and Pauerstein CJ. 1981. Effects of delta 9-tetrahydrocannabinol during the follicular phase of the rhesus monkey (macaca mulatta). J Clin *Endocrinol Metab.* (1):50-5.

Ascoli M, F Fanelli, and DL Segaloff. 2002. The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocrine reviews.* 23: 141-174.

Ashton JC, Friberg D, Darlington CL and Smith PF. 2006. Expression of the cannabinoid CB2 receptor in the rat cerebellum: an immunohistochemical study. *Neurosci Lett*.396(2):113-6.

Assidi M, Montag M, Van der Ven K and Sirard MA. 2011. Biomarkers of human oocyte developmental competence expressed in cumulus cells before ICSI: a preliminary study. J Assist *Reprod Genet.* 28(2):173-88. doi: 10.1007/s10815-010-9491-7.

Assou S, Boumela I, Haouzi D, Anahory T, Dechaud H, De Vos J and Hamamah S. 2011. Dynamic changes in gene expression during human early embryo development: from fundamental aspects to clinical applications. *Hum Reprod Update.* 17(2):272-90. doi: 10.1093/humupd/dmq036. Review.

Astiz X. 2016. Laguntza bidezko ugalketa minbizia duten emakumeetan. *Elhuyar,*324.

Ayalon D, Nir I, Cordova T, Bauminger S, Puder M, Naor Z, Kashi R, Zor U, Harell A and Lindner HR. 1977. Acute effect of delta1-tetrahydrocannabinol on the hypothalamo-pituitary-ovarian axis in the rat. *Neuroendocrinology.* 23(1):31-42.

B

Baerwald AR, GP Adams, and RA Pierson. 2012. Ovarian antral folliculogenesis during the human menstrual cycle: a review. *Hum Reprod Update.* 18. 73-91.

Bagavandoss P and Grimshaw S. 2010.Temporal and spatial distribution of the cannabinoid receptors (CB1, CB2) and fatty acid amide hydroxylase in the rat ovary. *Anat Rec (Hoboken)*. 293:1425–32.

B**akali E Elliott RA, Taylor AH, Willets J, Konje JC and Tincello DG.** 2013. Distribution and function of the endocannabinoid system in the rat and human bladder. *Int Urogynecol J.* 24:855–63.

Baker T. 1963.A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond [Biol]*. 158:417–433.

Bambang KN, Karasu T, Gebeh A, Taylor AH, Marczylo TH, Lam P, Willets JM and Konje JC. 2010. From fertilization to implantation in mammalian pregnancy-modulation of early human reproduction by the endocannabinoid system. *Pharmaceuticals (Basel).* 3:2910–2929.

Bari M, Battista N, Pirazzi V and Maccarrone M. 2011. The manifold ac-tions of endocannabinoids on female and male reproductive events. *Front Biosci (Landmark Ed).* 16:498–516.

Barnes FL, Looney CR and Westhusin ME. 1991. Embryo cloning in cattle: The current state of technology. *Embryo Transfer Newsletter.* 6: 1-6.

Barnes FL, Crombie A, Gardner, DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C and Trounson AO. 1995. Blastocyst development and birth after in-vitro maturation of human primary oocytes,intracytoplasmic sperm injection and assisted hatching. *Hum Reprod.* 10:3243–3247.

Barnes FL, Kausche A, Tiglias J, Wood C, Wilton L and Trounson A. 1996. Production of embryos from in vitro matured primary human oocytes. *Fertil Steril.* 65:1151–1156.

Bashirullah A, RL Cooperstock, and HD Lipshitz. 2001. Spatial and temporal control of RNA stability. *Proceedings of the National Academy of Sciences*. 98: 7025-7028.

Battista N, Rapino C, Di Tommaso M, Bari M, Pasquariello N and Maccarrone M. 2008. Regulation of male fertility by the endocannabinoid system. *Mol Cell Endocrinol.* 286: S17-S23

Bayewitch M, Rhee MH, Avidor-Reiss T, Breuer A, Mechoulam R and Vogel Z. 1996. (-)-Delta9-tetrahydrocannabinol antagonizes the peripheral cannabinoid receptor-mediated inhibition of adenylyl cyclase. *J Biol Chem.* 271(17):9902-5.

Bedzhov I, Graham SJ, Leung CY, and Zernicka-Goetz M. 2014. Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo. Philosophical Transactions of the Royal Society of London B: *Biological Sciences.* 369: 20130538.

Behringer R. 2014. *Manipulating the mouse embryo: a laboratory manual.* Cold Spring Harbor Laboratory Pr

Bisogno T, Katayama K, Melck D, Ueda N, De Petrocellis L, Yamamoto S, et al. 1998. Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells—implications for cell proliferation and differentiation. *Eur J Biochem.* 254:634–42.

Block E. 1953. A quantitative morphological investigation of the follicular system in newborn female infants. *Cells Tissues Organs.* 17: 201-206.

BOE núm. 126, de 27/05/2006. Ley 14/2006, de 26 de mayo, sobre técnicas de reproducción humana asistida. *BOE*-A-2006-9292

Bonhaus DW, Chang LK, Kwan J and Martin GR. 1998. Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: Evidence for agonistspecific trafficking of intracellular responses. *J Pharmacol Exp Ther.* 287(3):884-888.

Bouaboula, M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P. 1995. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J.* 312 (Pt 2): 637-641.

Bouskila J, Burke MW, Zabouri N, Casanova C, Ptito M and Bouchard JF. 2012. Expression and localization of the cannabinoid receptor type 1 and the enzyme fatty acid amide hydrolase in the retina of vervet monkeys. *Neuroscience.* 202:117–30.

Bradford MM.1976. Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing Principle of Protein-Dye Binding. *Analytical Biochemistry.* 72(1-2):248-254.

Brenneisen R. 2007. Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. Marijuana and the Cannabinoids. *Humana Press*. 17-49.

Brents LK. 2016. Marijuana, the Endocannabinoid System and the Female Reproductive System. Yale J *Biol Med.* 89(2):175-91.

Broekmans FJ, EA Knauff, ER te Velde, NS Macklon, and BC Fauser. 2007 Female reproductive ageing: current knowledge and future trends. *Trends in Endocrinology & Metabolism.* 18: 58-65.

Brown SM, Wager-Miller J and Mackie K. 2002. Cloning and molecular characterization of the rat CB2 cannabinoid receptor. *Biochim Biophys Acta.* 1576(3):255-264.

Brown C, LaRocca J, Pietruska J, Ota M, Anderson L, Smith SD, Weston P, Rasoulpour T and Hixon ML. 2010. Subfertility caused by altered follicular development and oocyte growth in female mice lacking PKBalpha/Akt1. *Biol Reprod.* 82: 246-256.

Brunet S and Maro B. 2005. Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space. *Reproduction.* 130(6):801-11. Review.

Buckley N, Hansson S, Harta G and Mezey E. 1997. Expression of the CB 1 and CB 2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience* 82: 1131-1149.

Buckley NE, McCoy KL, Mezey É, Bonner T, Zimmer A, Felder CC, et al. 2000. Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. *Eur J Pharmacol.* 396(2–3):141–9.

Bukiya AN. 2019. Physiology of the Endocannabinoid System During Development. *Adv Exp Med Biol.* 1162:13-37. doi: 10.1007/978-3-030-21737-2_2.

C

Cabral GA, Harmon KN and Carlisle S J. 2001. Cannabinoid-mediated inhibition of inducible nitric oxide production by rat microglial cells: Evidence for CB1 receptor participation. *Adv Exp Med Biol.* 493:207-214.

Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, Pierantoni R and Cobellis G. 2013. Low 17beta-Estradiol Levels in Cnr1 Knock-Out Mice Affect Spermatid Chromatin Remodeling by Interfering with Chromatin Reorganization. *Biol Reprod.*88(6):152–152.

Cahill CM, Holdridge SV, and Morinville A. 2007. Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. *Trends Pharmacol Sci* 28: 23-31.

Cantley LC. 2002. The phosphoinositide 3-kinase pathway. *Science.* 296: 1655–1657.

Canovas S, Ross PJ, Kelsey G and Coy P. 2017. DNA Methylation in Embryo Development: Epigenetic Impact of ART (Assisted Reproductive Technologies). *Bioessays.* 39(11). doi: 10.1002/bies.201700106.

Carracedo A. 2006a. Acción antitumoral de los cannabinoides: implicación de p8 y la vía de estrés de retículo endoplasmático. *Doctoral Thesis*.

Cecconi S, Rossi G, Santilli A, Stefano LD, Hoshino Y, Sato E, Palmerini MG and Macchiarelli G. 2010. Akt expression in mouse oocytes matured in vivo and in vitro. *Reprod Biomed Online.* 20: 35-41.

Cecconi S, Mauro A, Cellini V and Patacchiola F. 2012. The role of Akt signalling in the mammalian ovary. *Int J Dev Biol.* 56: 809-817.

Cecconi S, Rossi G, Oddi S, Di Nisio V and Maccarrone M. 2019. Role of Major Endocannabinoid-Binding Receptors during Mouse Oocyte Maturation. *Int J Mol Sci.* 20(12).

Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY and Yoon TK. 1991. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril.* 55(1):109-13.

Chang M. 1955. The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in the fallopian tubes. *Journal of Experimental Zoology.* 128: 379-405.

Chang EM, Song HS, Lee DR, Lee WS and Yoon TK. 2014. In vitro maturation of human oocytes: Its role in infertility treatment and new possibilities. *Clin Exp Reprod Med.* 41:41–46.

Chian RC, Buckett WM and Tan SL. 2004. In-vitro maturation of human oocytes. *Reprod Biomed Online*. 8: 148-166.

Chian RC, Uzelac PS and Nargund G. 2013. In vitro maturation of human immature oocytes for fertility preservation. *Fertil Steril.* 99:1173–1181.

Chianese R, Ciaramella V, Fasano S, Pierantoni R and Meccariello R. 2011. Anandamide modulates the expression of GnRH-II and GnRHRs in frog, Rana esculenta, diencephalon. *Gen Comp Endocrinol.* 173(3):389–95.

Cockburn K and Rossant, J. 2010. Making the blastocyst: lessons from the mouse. *J clin invest.* 120:995-1003

Conti M. 2002. Specificity of the cyclic adenosine 3',5'-monophosphate signal in granulosa cell function. *Biol Reprod.* 67(6):1653-61. Review.

Conti M, Hsieh M, Zamah AM, and Oh JS. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Mol Cell Endocrinol*. 356: 65-73.

Cooke DJ, Crowe MA and Roche JF.1997. Circulating FSH isoform patterns during recurrent increases in FSH throughout the oestrous cycle of heifers. *J Reprod Fertil.*110(2):339-45.

Correa F, Wolfson ML, Valchi P, Aisemberg J and Franchi AM. 2016. Endocannabinoid system and pregnancy. *Reproduction.* 152(6):R191-R200

Cota D, Marsicano G and Tschöp M, Grübler Y, Flachskamm C, Schubert M, Auer D, Yassouridis A, Thöne-Reineke C and Ortmann S. 2003. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *The Journal of clinical investigation.* 112: 423-431.

Coticchio G, Dal Canto M, Mignini Renzini M, Guglielmo MC, Brambillasca F, Turchi D, Novara PV, and Fadini R. 2015. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Hum Reprod Update*. 21: 427-454.

Cousineau TM, and Domar AD. 2007. Psychological impact of infertility. Best *Pract Res Clin Obstet Gynaecol.* 21: 293-308.

Coy P, Grullon L, Canovas S, Romar R, Matas C and Aviles M. 2008. Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. *Reproduction.* 135:19–27.

Cravatt BF, Demarest K, Patricelli M P, Bracey M H, Giang D K, Martin BR and Lichtman AH. 2001. Supersensitivity to anandamide and enhanced **en**dogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc Natl Acad Sci USA*. 98(16):9371-9376.

Crews CM and Erikson RL. 1992. Purification of a murine protein-tyrosine/threonine kinase that phosphorylates and activates the Erk-1 gene product: relationship to the fission yeast byr1 gene product. *Proc Natl Acad Sci U S A*. 89(17):8205-9.

Croxford JL and Yamamura T. 2005. Cannabinoids and the immune system: potential for the treatment of inflammatory diseases?. *J Neuroimmunol.* 166(1-2):3-18.

D

Dailey T, Dale B, Cohen J and Munné S. 1996. Association between nondisjunction and maternal age in meiosis-II human oocytes. *Am J Hum Genet.* 59:176–84.

Dalton GD and Howlett AC. 2012. Cannabinoid CB 1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/ threonine kinases to activate ERK in neuronal cells. *Br J Pharmacol.* 165:2497–2511.

Das SK, Paria BC, Chakraborty I and Dey SK. 1995. Cannabinoid ligand-receptor signaling in the mouse uterus. *Proc Natl Acad Sci USA*. 92:4332–6.

De Domenico, E, Todaro F, Rossi G, Dolci S, Geremia R, Rossi P, et al. 2017. Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads and impairs ovarian reserve. *Cell Death Dis.* 8(10)

De Jesus, ML, Salles J, Meana JJ and Callado LF. 2006. Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. *Neuroscience.* 140:635–43.

De Meijer EP, Bagatta M, Carboni A, Crucitti P, Moliterni VM, Ranalli P, Mandolino G. 2003. The inheritance of chemical phenotype in Cannabis sativa *L. Genetics.* (1):335-46.

De Miguel R, Romero J, Munoz RM, Garcia-Gil L, Gonzalez S, Villanua MA, et al. 1998. Effects of cannabinoids on prolactin and gonadotrophin secretion: involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. *Biochem Pharmacol.* 56:1331–8.

De Souza DK, Salles LP, Camargo R, Gulart LVM, Costa E Silva S, de Lima BD, Torres FAG, Rosa E and Silva AAM. 2018. Effects of PI3K and FSH on steroidogenesis, viability and embryo development of the cumulus-oocyte complex after in vitro culture. *Zygote.* 26(1):50-61. doi: 10.1017/S0967199417000703

De Vos M, Smitz J, Thompson JG and Gilchrist RB. 2016. The definition of IVM is clear-variations need defining. *Hum Reprod.* 31(11):2411-2415.

Devane WA, Dysarz Fr, Johnson MR, Melvin LS, and Howlett AC. 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol.* 34: 605-613.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R. 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science.* 258: 1946-1949.

Delvigne A and Rozenberg S. 2002. Epidemiology and prevention of ovarian hyperstimulation syndrome (OHSS): a review. *Hum Reprod Update.* 8: 559-577.

Dennedy MC, Friel AM, Houlihan DD, Broderick VM, Smith T and Morrison JJ. 2004. Cannabinoids and the human uterus during pregnancy. *Am J Obstet Gynecol.* 190:2–9. discussion 3A.

Desclin JC Jr. 1970. Experimental morphological verification of the secretion of androgens by the corpus luteum in mice. *Ann Endocrinol (Paris)*.31(4):742-6.

Deutsch DG, Ueda N, Yamamoto S. 2002. The fatty acid amidehydrolase (FAAH). *Prostaglandins Leukot Essent Fatty Acids*. 66:201–210.

Dhopeshwarkar A and Mackie K. 2014. CB2 Cannabinoid receptors as a therapeutic target-what does the future hold?. *Mol Pharmacol.* 86(4):430-7. doi: 10.1124/mol.114.094649.

Di Blasio AM, Vignali M, and Gentilini D. 2013. The endocannabinoid pathway and the female reproductive organs. *Journal of molecular endocrinology*. 50 R1-R9.

Di Carlo G and Izzo AA. 2003. Cannabinoids for gastrointestinal diseases: potential therapeutic applications. *Expert Opin Inv Drug.* 12(1):39-49.

Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, and Piomelli D. 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*. 372(6507): 686-91.

Diaz FJ, Wigglesworth K, Eppig JJ. 2007a. Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Dev Biol.* 305(1):300-11.

Diaz FJ, Wigglesworth K, Eppig JJ. 2007b. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J Cell Sci.* 120(Pt 8):1330-40.

Dieci C Lodde V, Labreque R, Dufort I, Tessaro I, Sirard M-A and Luciano AM. 2016. Differences in cumulus cell gene expression indicate the benefit of a pre-maturation step to improve in-vitro bovine embryo production. *MHR: Basic Science of Reproductive Medicine*. 22: 882–897. (https://doi.org/10.1093/molehr/gaw055).

Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM and Vos PL. 2002. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of pre-implantation embryos. Theriogenology. 57(1):5-20. Review.

Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, et al. 2002. Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc Natl Acad Sci U S A*. 99:10819 24.

Dinis-Oliveira RJ. 2016. Metabolomics of Δ9-tetrahydrocannabinol: implications in toxicity. *Drug Metab Rev.* 48(1):80-7. doi: 10.3109/03602532.2015.1137307.

Downs SM, Daniel SA, Bornslaeger EA, Hoppe PC and Eppig JJ. 1989.Maintenance of meiotic arrest in mouse oocytes by purines:modulation of cAMP levels an d cAMP phosphodiesterase activity. *Gamete research.* 23: 323-34.

Dragovic RA, Ritter LJ, Schulz SJ, Amato F, Armstrong DT and Gilchrist RB. 2005. Role of oocyte-secreted growth differentiation factor 9 in the regulation of mouse cumulus expansion. *Endocrinology.* 146(6):2798-806.

Du Plessis SS, Agarwal A and Syriac A. 2015. Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility. *J Assist Reprod Genet.* 32(11):1575-88. doi: 10.1007/s10815-015-0553-8.

Ducibella T and Buetow J. 1994. Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Devel biology.* 165: 95-104.

Ducibella T, Schultz RM and Ozil JP. 2006. Role of calcium signals in early development. *Semin Cell Dev Biol.* 17:324-32

Duranthon V and J Renard. 2001. The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. *Theriogenology.* 55: 1277-1289.

E

Edson MA, Nagaraja AK and Matzuk MM. 2009. The mammalian ovary from genesis to revelation. *Endocr Rev.* 30:624–712. doi:10.1210/er.2009-0012.

Edwards RG 1962. Meiosis in ovarian oocytes of adult mammals. *Nature.* 196: 446–450.

Eilertsen TB, Vanky E and Carlsen SM. 2012. Increased prevalence of diabetes and polycystic ovary syndrome in women with a history of preterm birth: a case-control study. *BJOG.* 119(3):266-75. doi: 10.1111/j.1471-0528.2011.03206.x.

El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC and Konje JC. 2009a. Localisation and function of the endocannabinoid system in the human ovary. *PLoS One.* 4:e4579.

El-Talatini MR, Taylor AH, and Konje JC. 2009b. Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation. *Hum Reprod.* 24: 1989-1998.

ElSohly MA. 2002. Chemical constituents of cannabis. *Haworth Press, New York*.

Elsohly MA and Slade D. 2005. Chemical constituents of marijuana: the complex mixture of natural cannabinoids. *Life Sci.* 78(5):539-48.

ElSohly MA, Radwan MM, Gul W, Chandra S and Galal A. 2017. Phytochemistry of Cannabis sativa L. *Prog Chem Org Nat Prod.* 103:1-36. doi: 10.1007/978-3-319-45541-9_1.

Engelman J A, Luo, J and Cantley LC. 2006. The evolution of phosphatidylinositol 3- kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* 7: 606–619.

Eppig, JJ, O'Brien MJ, Pendola FL and WatanabeS. 1998a. Factors affecting the developmental competence of mouse oocytes grown in vitro:follicle-stimulating hormone and insulin. *Biol reprod*. 59: 1445-53.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction.* 122: 829-838.

Eppig JJ, K Wigglesworth, and FL Pendola. 2002. The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proceedings of the National Academy of Sciences.* 99: 2890-2894.

Espey LL. 1974. Ovarian proteolytic enzymes and ovulation. *Biol Reprod.* (2):216-35.

F

Fabritius AS, Ellefson ML and McNally FJ. 2011. Nuclear and spindle positioning during oocyte meiosis. *Curr Opin Cell Biol.* 23(1):78-84. doi: 10.1016/j.ceb.2010.07.008

Fair T. 2003. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci.* 78: 203-216.

Fan HY and Sun QY. 2004. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod.* 70(3):535-47.

Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, and Richards JS. 2009 MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science.* 324: 938-941.

Feliú A, Moreno-Martet M, Mecha M, Carrillo-Salinas F, Lago E, Fernández-Ruiz J, and Guaza C. 2015. A Sativex®-like combination of phytocannabinoids as a disease-modifying therapy in a viral model of multiple sclerosis. *British journal of pharmacology.* 172: 3579-3595.

Ferguson SS, WE Downey, A-M Colapietro, LS Barak, L Ménard, and MG Caron. 1996. Role of β-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science*. 271: 363-366.

Ferreira E, A Vireque, P Adona, F Meirelles, R Ferriani, and P Navarro. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology.* 71: 836-848.

Fezza F, Bari M, Florio R, Talamonti E, Feole M and Maccarrone M. 2014. Endocannabinoids, related compounds and their metabolic routes. *Molecules.* 19(11):17078–106.

Filppula S, Yaddanapudi S, Mercier R, Xu W, Pavlopoulos S and Makriyannis A. 2004. Purification and mass spectroscopic analysis of human CB2 cannabinoid receptor expressed in the baculovirus system. *J Pept Res.* 64:225–36.

Fissore RA, He CL and Vande Woude GF. 1996. Potential Role of Mitogen-Activated Protein Kinase during Meiosis Resumption in Bovine Oocytes. *Biol Reprod.* 55:1261–1270.

Flesch FM and Gadella BM. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim Biophys Acta.* 1469(3):197-235.

Forabosco A, C Sforza, A De Pol, L Vizzotto, L Marzona, and VF Ferrario .1991. Morphometric study of the human neonatal ovary. *The Anatomical Record.* 231: 201-208.

Fortune JE, Cushman RA, Wahl CM and Kito S. 2000. The primordial to primary follicle transition. *Mol Cell Endocrinol.* 163(1-2):53-60. Review.

Fortune JE. 2003. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Anim Reprod Sci.*78(3-4):135-63. Review.

Francavilla F, Battista N, Barbonetti A, Vassallo MR, Rapino C, Antonangelo C, et al. 2009. Characterization of the endocannabinoid system in human spermatozoa and involvement of transient receptor potential vanilloid 1 receptor in their fertilizing ability. *Endocrinology.* 150:4692–700.

Francis S, Poteet-Smith C, Busch J, Richie-Jannetta R and Corbin J. 2002. Mechanisms of autoinhibition in cyclic nucleotide-dependent protein kinases. *Frontiers in bioscience: a journal and virtual library.* 7: d580-592.

Frankhauser M. 2002. History of cannabis in Western medicine: New York: *The Haworth Integrative Healing Press.*

Fulka J, N First, and R Moor. 1998. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Mol Hum Reprod.* 4: 41-49.

G

Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, Bouaboula M, Shire D, Le Fur G and Casellas P. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem.* 232: 54-61.

Galve-Roperh I, Rueda D, del Pulgar TG, Velasco G, and Guzmán M. 2002. Mechanism of extracellular signal-regulated kinase activation by the CB1 cannabinoid receptor. *Mol Pharmacol.* 62: 1385-1392.

Gammon CM, Freeman GM Jr, Xie W, Petersen SL and Wetsel WC. 2005. Regulation of gonadotropin-releasing hormone secretion by cannabinoids. *Endocrinology.* 146(10):4491-9.

Gaoni Y and Mechoulam R. 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. *J Am Chem Soc.* 86: 1646-1647.

Gebeh AK, Willets JM, Marczylo EL, Taylor AH and Konje JC. 2012. Ectopic pregnancy is associated with high anandamide levels and aberrant expression of FAAH and CB1 in fallopian tubes. *J Clin Endocrinol Metab.* 97:2827–35.

Gervasi MG, Rapanelli M, Ribeiro ML, Farina M, Billi S, Franchi AM and Perez Martinez S. 2009. The endocannabinoid system in bull sperm and bovine oviductal epithelium: role of anandamide in sperm-oviduct interaction. *Reproduction.* 137: 403-414.

Ghosh S, Reuveni I, Zidan S, Lamprecht R and Barkai E. 2018. Learning-induced modulation of the effect of endocannabinoids on inhibitory synaptic transmission. *J Neurophysiol.* 119(2):752-760. doi: 10.1152/jn.00623.2017.

Giacomo D Di, De Domenico E, Sette C, Geremia R and Grimaldi P. 2016. Type 2 cannabinoid receptor contributes to the physiological regulation of spermatogenesis. *FASEB J.* 30:1453–1463.

Giang DK and Cravatt BF. 1997. Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc Natl Acad Sci USA*. 94(6):2238-2242.

Gilchrist RB, Ritter LJ and Armstrong DT. 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci.* 82– 83:431–46.

Gilchrist RB and Thompson JG. 2007. Oocyte maturation: emerging concepts and technologies to improve developmental potential in vitro. *Theriogenology.* 67(1):6-15.

Gilchrist RB, Lane M and Thompson JG. 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod.* 14:159–177.

Gilman JM, Yücel MA, Pachas GN, Potter K, Levar N, Broos H, Manghis EM, Schuster RM and Evins AE. 2019. Delta-9-tetrahydrocannabinol intoxication is associated with increased prefrontal activation as assessed with functional near-infrared spectroscopy: A report of a potential biomarker of intoxication. *Neuroimage.*197:575-585. doi: 10.1016/j.neuroimage.2019.05.012.

Gilula NB, ML Epstein, and WH Beers. 1978. Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. *J Cell Biol.* 78: 58-75.

Gonzalez M and Morgado B. 2008. Maternidad a solas por eleccion: primera aproximacion. *Anu Psicol.* 39:119–126.

González-Fernández L, Sánchez-Calabuig MJ, Alves MG, Oliveira PF, Macedo S, Gutiérrez-Adán A, et al. 2018. Expanded equine cumulus-oocyte complexes exhibit higher meiotic competence and lower glucose consumption than compact cumulus-oocyte complexes. *Reprod Fertil Dev.* 30(2):297–306.

González-Ortega C, Piña-Aguilar RE, Cancino-Villarreal P, Pérez-Peña E and Gutiérrez-Gutiérrez AM. 2019. Naturalcycle in vitro fertilization (IVF) combined with in vitro maturation in infertile patients with polycystic ovarian syndrome (PCOS) requiring IVF. *Taiwan J Obstet Gynecol.* 58:192–195.

Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, and Richards JS. 2000. Follicle-Stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-lnduced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. *Mol Endocrinol.* 14: 1283-1300.

Gosden R and Lee B. 2010. Portrait of an oocyte: our obscure origin. *J Clin Invest.* 120:973–983. doi:10.1172/JCI41294.

Griffin J, Emery BR, Huang I, Peterson CM and Carrell DT. 2006. Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *J Exp Clin Assist Reprod.* 3:2. doi: 10.1186/1743-1050-3-2.

Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T,Geremia R, De Petrocellis L and Di Marzo V. 2009. The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. *Proc Natl Acad Sci U S A*. 106:11131–11136

Grynberg M, El Hachem H, de Bantel A, Benard J, le Parco S and Fanchin R. 2013. In vitro maturation of oocytes: uncommon indications. *Fertil Steril.* 99(5):1182-8. doi: 10.1016/j.fertnstert.2013.01.090.

Gschwind A, Zwick E, Prenzel N, Leserer M and Ullrich A. 2001. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene.* 20(13):1594-600.

Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F and Freund TF. 2004. Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. *Eur J Neurosci.* 20:441–458

Gupta SK. 2018. The Human Egg's Zona Pellucida. *Curr Top Dev Biol.* 130:379-411. doi: 10.1016/bs.ctdb.2018.01.001.

Gutiérrez-Adán, A and Pintado B. 2000. Effect of flanking matrix attachment regions on the expression of microinjected transgenes during preimplantation development of mouse embryos. *Transgenic Res.* 9(2):81-9.

Gutierrez-Adan, A, White CR, Van Soom A, and Mann MR. 2015. Why we should not select the faster embryo: lessons from mice and cattle. *Reproduction, Fertility and Development.* 27: 765-775.

Guzmán M and Sánchez C. 1999. Effects of cannabinoids on energy metabolism. *Life sciences.* 65: 657-664.

Gye MC, Kang HH and Kang H J. 2005. Expression of cannabinoid receptor 1 in mouse testes. *Arch Androl.* 51(3):247-255.

H

Habayeb OM, Taylor AH, Bell SC, Taylor DJ and Konje JC. 2008. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. *Endocrinology.* 149:5052–60.

Halupczok, J, Kluba-Szyszka A, Bidzińska-Speichert B, and Knychalski B. 2015 Ovarian Hyperstimulation Caused by Gonadotroph Pituitary Adenoma–Review. Advances in clinical and experimental medicine: official organ *Wroclaw Medical University.* 24: 695.

Hanoune J, and Defer N. 2001. Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol.* 41: 145-174.

Hansen KR, NS Knowlton, AC Thyer, JS Charleston, MR Soules, and NA Klein. 2008. A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Hum Reprod.* 23: 699-708.

Helliwell RJ, Chamley LW, Blake-Palmer K, Mitchell MD, Wu J, Kearn CS, et al. 2004. Characterization of the endocannabinoid system in early human pregnancy. *J Clin Endocrinol Metab.* 89:5168–74.

Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, Decosta BR and Rice KC. 1990. Cannabinoid Receptor Localization in Brain. *Proc Natl Acad Sci USA.* 87(5):1932-1936.

Herrera B, Carracedo A, Diez-Zaera M, Guzman M and Velasco G. 2005. P38 MAPK is involved in CB2 receptor-induced apoptosis of human leukaemia cells. *FEBS Lett.* 579(22):5084-5088.

Herta AC, Lolicato F and Smitz JEJ. 2018. In vitro follicle culture in the context of IVF. *Reproduction.* 156(1):F59-F73. doi: 10.1530/REP-18-0173.

Hill KP, Palastro MD, Johnson B and Ditre JW. 2017a. Cannabis and Pain: A Clinical Review. *Cannabis Cannabinoid Res.* 2(1):96-104. doi: 10.1089/can.2017.0017.

Hinckley M, S Vaccari, K Horner, R Chen, and M Conti. 2005. The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. *Dev Biol.* 287: 249-261.

Hinrichs K. 2010. In Vitro Production of Equine Embryos: State of the Art. *Reprod Domest Anim.* 45(SUPPL. 2):3–8.

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M and Kato S. 1994. In vitro growth and maturation of pig oocytes. *Journal of Reproduction and Fertility.* 100: 333–339. (https://doi.org/10.1530/jrf.0.1000333).

Hirao Y, Naruse K, Kaneda M, Somfai T, Iga K, Shimizu M, Akagi S, Cao F, Kono T, Nagai T et al. 2013. Production of fertile offspring from oocytes grown in vitro by nuclear transfer in cattle. *Biology of Reproduction.* 89: 57. (https://doi.org/10.1095/biolreprod.113.109439).

Hirata R, Hojo T, Sano M, Hayashi N and Okuda K. 2015. Potential role of hCG in apoptosis of human luteinized granulosa cells. *J Reprod Dev.* 61(1):67-73. doi: 10.1262/jrd.2014-115

Hirshfield AN. 1991. Development of follicles in the mammalian ovary. *Int Rev Cytol.* 124:43-101.

Horne AW, Phillips III JA, Kane N, Lourenco PC, McDonald SE, Williams AR, Simon C, Dey SK, and Critchley HO. 2008. CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy. *PLoS One.* 3 e3969.

Horner K, G Livera, M Hinckley, K Trinh, D Storm, and M Conti. 2003. Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Developmental biology*. 258: 385-396.

Hoshino Y and Sato E. 2008 Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. *Dev Biol.* 314: 215-223.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R and Pertwee RG. 2002. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev.* 54(2):161-202.

Howlett AC. 2005. Cannabinoid receptor signaling. Handb *Exp Pharmacol.* (168):53-79. Review.

Howlett AC and Abood ME. 2017. CB1 and CB2 Receptor Pharmacology. *Adv Pharmacol.* 80:169-206. doi: 10.1016/bs.apha.2017.03.007.

Hunt PA and Hassold TJ. 2008. Human female meiosis: what makes a good egg go bad?. *Trends Genet.* 24:86–93.

Hunter M. 2000 Oocyte maturation and ovum quality in pigs. *Reviews of reproduction.* 5: 122-130.

Hyttel P, Fair T, Callesen H and Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology.* 7(1): 23-32.

I

Ibarra-Lecue I, Díez-Alarcia R, López-Cardona ÁP, Callado LF, Agirregoitia E, and Urigüen L. 2015. Evaluation of the functional selectivity of cannabinoid receptors signaling in mouse brain cortex 7th European Workshop on Cannabinoid Research and IACM 8th Conference on Cannabinoids in Medicine, pp. 54. *Sestri Levante, Italy* 17-19.

J

Ji YZ, Bomsel M, Jouannet P and Wolf JP. 1997. Modifications of the human oocyte plasma membrane protein pattern during preovulatory maturation. *Mol Reprod Dev.* 47:120–126

Jones KT. 2005. Mammalian egg activation: from Ca^{2+} spiking to cell cycle progression. *Reproduction.* 130(6):813-23.

Jones GM, Cram DS, Song B, Magli MC, Gianaroli L, Lacham-Kaplan O, Findlay JK, Jenkin G and Trounson AO. 2008. Gene expression profiling of human oocytes following in vivo or in vitro maturation. *Hum Reprod.* 23(5):1138-44. doi: 10.1093/humrep/den085.

Jose-Miller AB, Boyden JW and Frey KA. 2007. Infertility. *Am Fam Physician.* 75:849-856.

K

Kalinowski RR, CH Berlot, TL Jones, LF Ross, LA Jaffe, and LM Mehlmann. 2004. Maintenance of meiotic prophase arrest in vertebrate oocytes by a G s protein-mediated pathway. *Dev Biol.* 267: 1-13.

Kalous J, Solc P, Baran V, Kubelka M, Schultz RM and Motlik J. 2006. PKB/AKT is involved in resumption of meiosis in mouse oocytes. *Biol Cell.* 98:111–123.

Kalous J, Kubelka M, Šolc P, Šušor A and Motlík J. 2009. AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes. *Reproduction.* 138:645–654.

Kaminski NE, Abood ME, Kessler FK, Martin BR and Schatz AR. 1992. Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol Pharmacol.* 42(5):736-42.

Khatir H, Lonergan P and Mermillod P. 1998. Kinetics of nuclear maturation and protein profiles of oocytes from prepubertal and adult cattle during in vitro maturation. *Theriogenology* 50(6):917–29.

Keefe D, Kumar M and Kalmbach K. 2015. Oocyte competency is the key to embryo potential. *Fertil Steril.* 103(2):317-22. doi: 10.1016/j.fertnstert.2014.12.115. Review.

Kezele P and Skinner MK. 2003. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. *Endocrinology.* 144(8):3329-37.

Kobayashi Y, Arai S, Waku K and Sugiura T. 2001. Activation by 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, of p42/44 mitogen-activated protein kinase in HL-60 cells. *Journal of biochemistry.* 129: 665-669.

Kowal MA, Hazekamp A and Grotenhermen F. 2016. Review on clinical studies with cannabis and cannabinoids 2010-2014. *Cannabinoids.* 11: 1515.

Krisher RL. 2013. Oocyte physiology and development in domestic animals. *Wiley-Blackwell.*

Kubiak JZ, Ciemerych MA, Hupalowska A, Sikora-Polaczek M and Polanski Z. 2008. On the transition from the meiotic to mitotic cell cycle during early mouse development. *Int J Dev Biol.* 52(2-3):201-17. doi: 10.1387/ijdb.072337jk. Review.

Kume S, Yamamoto A, Inoue T, Muto A, Okano H and Mikoshiba K. 1997. Developmental expression of the inositol 1,4,5-trisphosphate receptor and structural changes in the endoplasmic reticulum during oogenesis and meiotic maturation of Xenopus laevis. *Dev Biol* 182:228–23 9.

Kupka M, D'Hooghe T, Ferraretti A, de Mouzon J, Erb K, Castilla J, Calhaz-Jorge C, De Geyter C, Goossens V, and EI-M Consortium. 2016. Assisted reproductive technology in Europe, 2011: results generated from European registers by ESHRE. *Hum Reprod.* 31: 233-248.

Kurilo LF. 1981. Oogenesis in antenatal development in man. *Hum Genet.* 57(1):86-92.

L

Lauckner JE, Hille B and Mackie K. 2005.The cannabinoid agonist WIN55,212-2 increases intracellular calcium via CB1 receptor coupling to G(q/11) G proteins. *Proc Natl Acad Sci USA.* 102(52):19144-19149.

Lawrence D and Gill E. 1975. The effects of Δ1-tetrahydrocannabinol and other cannabinoids on spin-labeled liposomes and their relationship to mechanisms of general anesthesia. *Mol Pharmacol.* 11: 595-602.

Lee HJ, Barad DH, Kushnir VA, Shohat-Tal A, Lazzaroni-Tealdi E, Wu Y-G, and Gleicher N. 2015. Rescue in vitro maturation (IVM) of immature oocytes in stimulated cycles in women with low functional ovarian reserve (LFOR). *Endocrine.* 1-7.

Lee HN and Chang EM. 2019. Primordial follicle activation as new treatment for primary ovarian insufficiency. *Clin Exp Reprod Med.* 46(2):43-49. doi: 10.5653/cerm.2019.46.2.43.

Li HL. 1973. An archaeological and historical account of cannabis in China. *Economic Botany.* 28: 437-448.

Liang CG, YQ Su, HY Fan, H Schatten, and QY Sun. 2007. Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. *Mol Endocrinol.* 21: 2037-2055.

Lippincott-Schwartz J, Roberts TH and Hirshberg K. 2000. Secretory protein trafficking and organelle dynamics in living cells. *Annu Rev Cell Dev Biol.* 16:557–89.

Liu J, Gao B, Mirshahi F, Sanyal AJ, Khanolkar AD, Makriyannis A and Kunos G. 2000. Functional CB1 cannabinoid receptors in human vascular endothelial cells. *Biochem J.* 346:835-840.

Liu K, Rajareddy S, Liu L, Jagarlamudi K, Boman K, Selstam G and Reddy P. 2006 Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer. *Dev Biol.* 299: 1-11.

Liu L, Rajareddy S, Reddy P, Jagarlamudi K, Du C, Shen Y, Guo Y, Boman K, Lundin E, Ottander U, Selstam G and Liu K. 2007. Phosphorylation and inactivation of glycogen synthase kinase-3 by soluble kit ligand in mouse oocytes during early follicular development. *Journal of molecular endocrinology.* 38: 137-146.

Liu M. 2011. The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology.* 9: 149.

Liu L, Kong N, Xia G and Zhang M. 2012. Molecular control of oocyte meiotic arrest and resumption. *Reprod Fertil Dev.* 25(3):463-71. doi: 10.1071/RD12310.

Lonergan P, Monaghan P, Rizos D, Boland MP and Gordon I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. *Mol Reprod Dev.* 37: 48-53.

Lonergan P and Fair T. 2016. Maturation of Oocytes in Vitro. *Annu Rev Anim Biosci.* 4:255-68. doi: 10.1146/annurev-animal-022114-110822.

Lonergan P, Fair T, Forde N and Rizos D. 2016. Embryo development in dairy cattle. *Theriogenology.* 86(1):270-7. doi: 10.1016/j.theriogenology.2016.04.040.

López-Cardona AP, Sánchez-Calabuig MJ, Beltran-Breña P, Agirregoitia N, Rizos D, Agirregoitia E and Gutierrez-Adán A. 2016. Exocannabinoids effect on in vitro bovine oocyte maturation via activation of AKT and ERK1/2. *Reproduction.* 152:603–612.

López-Cardona AP, Pérez-Cerezales S, Fernández-González R, Laguna-Barraza R, Pericuesta E, Agirregoitia N, Gutiérrez-Adán A and Agirregoitia E. 2017. CB1cannabinoid receptor drives oocyte maturation and embryo development via PI3K/Akt and MAPK pathways. *FASEB J.* 31:3372–3382.

Lowther KM, VO Nikolaev, and LM Mehlmann. 2011. Endocytosis in the mouse oocyte and its contribution to cAMP signaling during meiotic arrest. *Reproduction.* 141: 737-747.

Lu C, Zhang Y, Zheng X, Song X, Yang R, Yan J, Feng H and Qiao J. 2018. Current perspectives on in vitro maturation and its effects on oocyte genetic and epigenetic profiles. *Sci China Life Sci.* 61(6):633-643. doi: 10.1007/s11427-017-9280-4.

Luciano AA, Lanzone A and Goverde AJ. 2013. Management of female infertility from hormonal causes. *Int J Gynaecol Obstet.* 123 Suppl 2:S9-17. doi: 10.1016/j.ijgo.2013.09.007.

Ludanyi A, Eross L, Czirjak S, Vajda J, Halasz P, Watanabe M, PalkovitsM, Magloczky Z, Freund TF and Katona I. 2008. Down regulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. *J Neurosci* 28:2976–2990.

M

Maccarrone M, Cecconi S, Rossi G, Battista N, Pauselli R and Finazzi-Agro A. 2003. Anandamide activity and degradation are regulated by early postnatal aging and follicle-stimulating hormone in mouse *Sertoli cells. Endocrinology.* 144(1):20-28.

Maccarrone M, Barboni B, Paradisi A, Bernabo N, Gasperi V, Pistilli MG, Fezza F, Lucidi P and Mattioli M. 2005. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. *J Cell Sci.* 118(Pt 19):4393-4404.

Maccarrone M. 2007. CB(2) receptors in reproduction. *Br J Pharmacol.*

Maccarrone M. 2009. Endocannabinoids: friends and foes of reproduction. *Progress in lipid research.* 48: 344-354.

Maccarrone M, Bab I, Bíró T, Cabral GA, Dey SK, Di Marzo V, Konje JC, Kunos G, Mechoulam R, and Pacher P .2015. Endocannabinoid signaling at the periphery: 50 years after THC. *Trends Pharmacol Sci.* 36: 277-296.

Manning BD and Cantley LC. 2007. AKT/PKB signaling: navigating downstream. *Cell.* 129: 1261-1274.

Manning BD and Toker A. 2017. AKT/PKB Signaling: Navigating the Network. *Cell.* 169(3):381-405. doi: 10.1016/j.cell.2017.04.001.

Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, et al. 2002. The endogenous cannabinoid system controls extinction of aversive memories. *Nature.* 418(6897):530–4.

Martin-Coello J, Gonzalez R, Crespo C, Gomendio M, and Roldan E. 2008. Superovulation and in vitro oocyte maturation in three species of mice (Mus musculus, Mus spretus and Mus spicilegus). *Theriogenology.* 70: 1004-1013.

Matorras R, Urquijo E, Mendoza R, Corcostegui B, Exposito A and Rodriguez- Escudero FJ. 2002. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Hum Reprod.* 17: 1762–6.

Matorras R, Mendoza R, Exposito A and Rodriguez-Escudero FJ. 2004.Influence of the time interval between embryo catheter loading and discharging on the success of IVF. *Hum Reprod.* 19:2027–30.

Matorras R, Prieto B, Exposito A, Mendoza R, Crisol L, Herranz P and Burgues S. 2009. Mid-follicular LH supplementation in women aged 35–39 years undergoing ICSI cycles: a randomized controlled study. *Reprod Biomed Online.* 19:879–887.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, and Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature.* 346: 561-564.

Matteri RL, Papkoff H, Ng DA, Swedlow JR and Chang YS.1986. Isolation and characterization of three forms of luteinizing hormone from the pituitary gland of the horse. *Biol Reprod.* 34(3):571-8.

Mattioli M, Galeati G, and Seren E. 1988. Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronucleus formation. *Gamete Res.* 20(2):177-83.

Matzuk MM, Burns KH, Viveiros MM and Eppig JJ. 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science.* 296(5576):2178-80. Review.

McGee EA and Hsueh AJ. 2000. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev.* 21(2):200-14.

McKinney MK and Cravatt BF. 2005. Structure and function of fatty acidamide hydrolase. *Annu Rev Biochem* 74:411–432.

McNatty KP, Smith P, Hudson NL, Heath DA, Tisdall DJ, O WS and Braw-Tal R.1995. Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *J Reprod Fertil Suppl.* 49:123-35. Review.

Meccariello R, Franzoni MF, Chianese R, Cottone E, Scarpa D, Donna D, et al. 2008. Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian Rana esculenta. *Endocrinology.* 149(5):2149–58.

Mechoulam R and Gaoni Y. 1967. The absolute configuration of delta-1-tetrahydrocannabinol, the major active constituent of hashish. *Tetrahedron Lett* (12):1109–11.

Mechoulam R. 1986. The pharmacohistory of Cannabis sativa. Cannabinoids as therapeutic agents. ed Mechoulam R (*CRC Press, Boca Raton*).

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR, and Compton DR. 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochemical pharmacology.* 50: 83-90.

Mehlmann LM, Y Saeki, S Tanaka, TJ Brennan, AV Evsikov, FL Pendola, BB Knowles, JJ Eppig, and LA Jaffe. 2004. The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science.* 306: 1947-1950.

Mehlmann LM. 2005. Stops and starts in mammalian oocytes: recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction.* 130: 791-799.

Mermillod P, Oussaid B and Cognié Y. 1999. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J Reprod Fertil Suppl.* 54:449-60.

Miller WL and Auchus RJ. 2011. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev.* 32(1):81-151. doi: 10.1210/er.2010-0013. Review.

Molina-Holgado E, Vela. JM., Arevalo-Martin A, Almazan G, Molina-Holgado F, Borrell J and Guaza C. 2002. Cannabinoids promote oligodendrocyte progenitor survival: Involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. *J Neurosci.* 22(22):9742-9753.

Molina-Holgado F, Pinteaux E, Heenan L, Moore JD, Rothwell NJ and RM Gibson. 2005. Neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical neurons are mediated by phosphatidylinositol 3-kinase/AKT signaling. *Molecular and Cellular Neuroscience*. 28: 189-194.

Moor RM.1988. Regulation of the meiotic cycle in oocytes of domestic mammals. *Ann N Y Acad Sci*. 541:248-58.

Moor RM, Mattioli M, Ding J and Nagai T. 1990. Maturation of pig oocytes in vivo and in vitro. *J Reprod Fertil Suppl.* 40:197-210.

Moor RM, Dai Y, Lee C and Fulka J Jr. 1998. Oocyte maturation and embryonic failure. *Hum Reprod Update.* 4(3):223-36.

Moore KL, Persaud TVL. 2008. *Embriología Básica.* Elsevier.

Motta PM, Nottola SA and Makabe S. 1997. Natural history of the female germ cell from its origin to full maturation through prenatal ovarian development. *Eur J Obstet Gynecol Reprod Biol.* 75(1):5-10. Review.

Mukhopadhyay KD, Liu Z, Bandyopadhyay A, Kirma NB, Tekmal RR, Wang S and Sun LZ. 2015. Aromatase expression increases the survival and malignancy of estrogen receptor positive breast cancer cells. *PLoS One*. 10(4):e0121136. doi: 10.1371/journal.pone.0121136.

Munné S, Alikani M, Tomkin G, Grifo J and Cohen J.1995. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril.* 64:382–91.

Munné S, Chen S, Colls P, Garrisi J, Zheng X, Cekleniak N, et al. 2007. Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos. *Reprod Biomed Online.* 14:628–34

Munro S, Thomas KL, and Abu-Shaar M. 1993. Molecular characterization of a peripheral receptor for cannabinoids. *Nature.* 365: 61-65.

Murray RM, Morrison PD, Henquet C, and Di Forti M. 2007. Cannabis, the mind and society: the hash realities. *Nature Reviews Neuroscience.* 8: 885-895.

N

Nader S. 2010. Infertility and pregnancy in women with polycystic ovary syndrome. *Minerva Endocrinol.* 35: 211-225.

Niakan KK, Han J, Pedersen RA, Simon C and Pera RAR. 2012. Human pre-implantation embryo development. *Development.* 139: 829-841.

Nikseresht M, Toori MA, Rasti T, Kashani IR, and Mahmoudi R. 2015 The Nuclear Maturation and Embryo Development of Mice Germinal Vesicle Oocytes with and without Cumulus Cell after Vitrification. *J Clin Diagn Res.* 9: AF01-04.

Nikseresht M, Toori MA, Rahimi HR, Fallahzadeh AR, Kahshani IR, Hashemi SF, Bahrami S and Mahmoudi R. 2017. Effect of Antioxidants (β-mercaptoethanol and Cysteamine) on Assisted Reproductive Technology In vitro. *J Clin Diagn Res.* 11(2):BC10-BC14. doi: 10.7860/JCDR/2017/21778.9298.

Nogueira D, Albano C, Adriaenssens T, Cortvrindt R, Bourgain C, Devroey P and Smitz J. 2003a. Human oocytes reversibly arrested in prophase I by phosphodiesterase type 3 inhibitor in vitro. *Biology of reproduction.* 69: 1042-52.

Norris RP, Freudzon M, Mehlmann L M, Cowan AE, Simon AM, Paul DL, Lampe PD and Jaffe LA. 2008. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexion 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. *Development.* 135: 3229-38.

Norris RP, Ratzan WJ, Freudzon M, Mehlmann L M, Krall J, Movsesian MA, Wang H, KE H, Nikolaev VO and Jaffe LA. 2009. Cyclic GMP from the surrounding somatic cells regulated cyclic AMP and meiosis in the mouse oocyte. *Development.* 136: 1869.78.

Norwitz ER, Schust DJ and Fisher SJ. 2001. Implantation and the survival of early pregnancy. *New England Journal of Medicine.* 345: 1400-1408.

Núñez E, Benito C, Pazos MR, Barbachano A, Fajardo O, González S, Tolón RM and Romero J. 2004. Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. *Synapse.* 53(4):208-13.

O

Okumura E, Fukuhara T, Yoshida H, Hanada Si S, Kozutsumi R, Mori M, Tachibana K, and Kishimoto T . 2002. Akt inhibits Myt1 in the signalling pathway that leads to meiotic G2/M-phase transition. *Nat Cell Biol* 4: 111-116.

Oláh M, Milloh H and Wenger T. 2008. The role of endocannabinoids in the regulation of luteinizing hormone and prolactin release. Differences between the effects of AEA and 2AG. *Mol Cell Endocrinol.* 286(1-2 SUPPL. 1).

Oron E and Ivanova N. 2012. Cell fate regulation in early mammalian development. *Phys Biol.* 9(4):045002. doi: 10.1088/1478-3975/9/4/045002.

Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Bátkai S, Harvey-White J, Mackie K, Offertáler L, and L Wang. 2005. Endocannabinoid activation at hepatic CB 1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *The Journal of clinical investigation*. 115: 1298-1305.

Otoi T, Yamamoto K, Koyama N, Tachikawa S and Suzuki T. 1997. Bovine oocyte diameter in relation to developmental competence. *Theriogenology.*48(5):769-74.

P

Pacher P, Bátkai S, and Kunos G. 2006. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev.* 58: 389-462.

Palermo R. 2007. Differential actions of FSH and LH during folliculogenesis. *Reprod Biomed Online.* (3):326-37. Review.

Pan B and Li J. 2019. The art of oocyte meiotic arrest regulation. *Reprod Biol Endocrinol.* 17(1):8. doi: 10.1186/s12958-018-0445-8. Review.

Paria BC, Das SK and Dey SK. 1995. The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. *Proc Natl Acad Sci U S A*. 92: 9460-9464.

Paria BC, Zhao X, Wang J, Das SK and Dey SK. 1999. Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. *Biol Reprod.* 60:1151–7.

Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, et al. 2001. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. *J Biol Chem.* 276:20523-8.

Park B, Gibbons HM, Mitchell MD and Glass M. 2003. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. *Placenta.* 24:990–5.

Park J-Y, Y-Q Su, M Ariga, E Law, S-LC Jin, and M Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science.* 303: 682-684.

Peralta L, Agirregoitia E, Mendoza R, Exposito A, Casis L, Matorras R, and Agirregoitia N. 2011. Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. *Reprod Biomed Online.* 23:372–9.

Pertwee RG. 2005a. Cannabinoids. Handbook of Experimental Pharmacology. *Springer,* Berlin Heidelberg.

Pertwee RG. 2006. Cannabinoid pharmacology: the first 66 years. *Br J Pharmacol.* 147 Suppl 1:S163-71.

Picton H, D Briggs, and R Gosden. 1998. The molecular basis of oocyte growth and development. *Mol Cell Endocrinol.* 145: 27-37.

Picton HM. 2001. Activation of follicle development: the primordial follicle. *Theriogenology.* 55(6):1193-210. Review.

Pincus G and Enzmann EV. 1934. Can Mammalian Eggs Undergo Normal Development in Vitro?. *Proc Natl Acad Sci U S A*. 20(2):121-2

Polanski Z, Hoffmann S and Tsurumi C. 2005. Oocyte nucleus controls progression through meiotic maturation. *Dev Biol.* 281(2):184-95.

Procházka, R, E Nagyov, G Brem, K Schellander, and J Motlik. 1998. Secretion of cumulus expansion-enabling factor (CEEF) in porcine follicles. *Mol Reprod Dev.* 49: 141-149.

Q

Quinn, P, Kerin JF, and Warnes GM. 1985. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril.* 44: 493-498.

R

Rahman A.N.M.A. ,. Abdullah R.B and W.E. Wan-Khadijah. 2008. Gametogenesis, Fertilization and Early Embryogenesis in Mammals with Special Reference to Goat: A Review. *Journal of Biological Sciences.* 8 (7): 1115-1128. 10.3923/jbs.2008.1115.1128.

Rapino C, Battista N, Bari M and Maccarrone M. 2014. Endocannabinoids as biomarkers of human reproduction. *Hum Reprod Update* 20 501-516.

Reddy P, Shen L, Ren C, Boman K, Lundin E, Ottander U, Lindgren P, Liu YX, Sun QY and Liu K. 2005. Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development. *Dev Biol.* 281: 160-170.

Reich R, Laufer N, Lewysohn O, Cordova T, Ayalon D and Tsafriri A. 1982. In Vitro Effects of Cannabinoids on Follicular Function in the Rat1. *Biol Reprod.* 27:223–231

Reynolds LP, AT Grazul-Bilska, and DA Redmer. 2002. Angiogenesis in the female reproductive organs: pathological implications. *International journal of experimental pathology.* 83: 151-164.

Rhee MH, Vogel Z, Barg J, Bayewitch M, Levy R, Hanus L, Breuer A and Mechoulam R. 1997. Cannabinol derivatives: binding to cannabinoid receptors and inhibition of adenylylcyclase. *J Med Chem.* 40(20):3228-33.

Richard FJ, A Tsafriri, and M Conti. 2001. Role of phosphodiesterase type 3A in rat oocyte maturation. *Biol Reprod.* 65: 1444-1451.

Richards JS and Pangas SA. 2010a. New insights into ovarian function. *Handb Exp Pharmacol.*(198):3-27. doi: 10.1007/978-3-642-02062-9_1. Review

Richards JS and Pangas SA. 2010b. The ovary: basic biology and clinical implications. *J Clin Invest.* 120(4):963-72. doi: 10.1172/JCI41350. Review.

Riley JK, Carayannopoulos MO, Wyman AH, Chi M, Ratajczak CK and Moley KH. 2005. The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo. *Dev Biol.* 284: 377-386.

Rimon-Dahari N, Yerushalmi-Heinemann L, Alyagor L and Dekel N. 2016. Ovarian Folliculogenesis. *Results Probl Cell Differ.* 58:167-90. doi: 10.1007/978-3-319-31973-5_7. Review.

Rodriguez-Wallberg KA and Oktay K. 2014. Fertility preservation during cancer treatment: clinical guidelines. *Cancer Manag Res.* 6:105-17. doi: 10.2147/CMAR.S32380.

Romero S, Sánchez F, Lolicato F, Van Ranst H and Smitz J. 2016. Immature Oocytes from Unprimed Juvenile Mice Become a Valuable Source for Embryo Production When Using C-Type Natriuretic Peptide as Essential Component of Culture Medium. *Biol Reprod.* 95(3):64.

Roskoski R Jr. 2012. ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol Res.* 66(2):105-43. doi: 10.1016/j.phrs.2012.04.005

Rossant J and Tam PT. 2002. Mouse Development: Patterning, Morphogenesis and Organogenesis. *Gulf Professional Publishing*

Rossato M, Ion Popa F, Ferigo M, Clari G and Foresta C. 2005. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. *J Clin Endocrinol Metab.* 90:984–91.

Roux PP and Blenis J. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiology and molecular biology reviews.* 68: 320-344.

Rueda D, Galve-Roperh I, Haro A and Guzman M. 2000. The CB1 cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol Pharmacol.* 58(4):814-820.

Ruehle S, Wager-Miller J, Straiker A, Farnsworth J, Murphy MN, Loch S, Monory K, Mackie K and Lutz B. 2017. Discovery and characterization of two novel CB1 receptor splice variants with modified N-termini in mouse. *J Neurochem.* 142(4):521-533. doi: 10.1111/jnc.14099.

Ruiz-Llorente L, Sanchez MG, Carmena M J, Prieto JC, Sanchez-Chapado M, Izquierdo A and Diaz-Laviada I. 2003. Expression of functionally active cannabinoid receptor CB1 in the human prostate gland. *Prostate.* 54(2):95-102.

Russell DL and Robker RL. 2007. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update.* 13(3):289-312.

Russo EB. 2011. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br J Pharmacol.* 163(7):1344-64. doi: 10.1111/j.1476-5381.2011.01238.x. Review.

S

Sadler T. 2004. *Embriologia Medica, Lagman.* ed. Madrid: Panamericana.

Sagata N .1998. Introduction: meiotic maturation and arrest in animal oocytes. pp. 535-537: *Elsevier.*

Sanchez C, Galve-Roperh I, Rueda D and Guzman M. 1998, Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Delta(9)-tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. *Mol Pharmacol.* 54(5):834-843.

Sánchez G, Ruiz-Llorente L and Sánchez AM. 2003. Activation of phosphoinositide 3-kinase/PKB pathway by CB 1 and CB 2 cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. *Cellular signalling.* 15: 851-859.

Sanchez F and Smitz J. 2012. Molecular control of oogenesis. *Biochim Biophys Acta.* 1822:1896–912.

Sánchez F, Lolicato F, Romero S, De Vos M, Van Ranst H, Verheyen G, Anckaert E and Smitz JEJ. 2017. An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield. *Hum Reprod.* 32(10):2056-2068. doi: 10.1093/humrep/dex262.

Sanchez F, Le AH, Ho VNA, Romero S, Van Ranst H, De Vos M, Gilchrist RB, Ho TM, Vuong LN and Smitz J. 2019. Biphasic in vitro maturation (CAPA-IVM) specifically improves the developmental capacity of oocytes from small antral follicles. *J Assist Reprod Genet.* 36(10):2135-2144. doi: 10.1007/s10815-019-01551-5.

Santos F and W Dean. 2004. Epigenetic reprogramming during early development in mammals. *Reproduction.* 127: 643-651.

Sarfaraz S, Afaq F, Adhami VM and Mukhtar H. 2005. Cannabinoid receptor as a novel target for the treatment of prostate cancer. *Cancer Res.* 65(5):1635-1641.

Sasseville M, LJ Ritter, TM Nguyen, F Liu, DG Mottershead, DL Russell, and RB Gilchrist. 2010. Growth differentiation factor 9 signaling requires ERK1/2 activity in mouse granulosa and cumulus cells. *J Cell Sci.* 123: 3166-3176.

Sathananthan AH, K Selvaraj, ML Girijashankar, V Ganesh, P Selvaraj, and AO Trounson. 2006. From oogonia to mature oocytes: inactivation of the maternal centrosome in humans. *Microscopy research and technique.* 69: 396-407.

Schindler K. 2011. Protein kinases and protein phosphatases that regulate meiotic maturation in mouse oocytes. *Results Probl Cell Differ*. 53: 309-341.

Schmitt A, and AR Nebreda. 2002. Signalling pathways in oocyte meiotic maturation. *J Cell Sci.* 115: 2457-2459.

Schreiber S, Bader M, Lenchinski T, Meningher I, Rubovitch V, Katz Y, Cohen E, Gabet Y, Rotenberg M, Wolf EU and Pick CG. 2019. Functional effects of synthetic cannabinoids versus Δ9-THC in mice on body temperature, nociceptive threshold, anxiety, cognition, locomotor/exploratory parameters and depression. *Addict Biol.* 24:414-425.

Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, et al. 2002a. N-acylethanolamines in human reproductive fluids. *Chem Phys Lipids.* 121:211–27.

Schuel H and Burkman LJ. 2005. A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. *Biol Reprod.* 73:1078–86.

Schultz RM. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum Reprod Update.* 8: 323-331.

Scorticati C, Ferná Ndez-Solari J, De Laurentiis A, Mohn C, Prestifilippo JP, Lasaga M, et al. 2004. The inhibitory effect of anandamide on luteinizing hormone-releasing hormone secretion is reversed by estrogen. *Proc Natl Acad Sci.* 101(32):11891–6. Available from: www.pnas.orgcgidoi10.1073pnas.0404366101

Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF 3rd and Amsterdam A. 2001. The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *Journal of Biological Chemistry.* 276: 13957-13964.

Sela-Abramovich S, E Chorev, D Galiani, and N Dekel. 2005. Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology.* 146: 1236-1244.

Selwood D. 2009. The Cannabinoid Receptors. *Humana Press*, Totowa

Sharir H and Abood ME. 2010. Pharmacological characterization of GPR55, a putative cannabinoid receptor. *Pharmacol Ther.* 126(3):301-13. doi: 10.1016/j.pharmthera.2010.02.004

Shi L, Zhang J, Lai Z, Tian Y, Fang L, Wu M, et al. 2016. Long-term moderate oxidative stress decreased ovarian reproductive function by reducing follicle quality and progesterone production. *PLoS One.* 11(9):1–18. http://dx.doi.org/10.1371/journal.pone.0162194

Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessegue B, Bonnin-Cabanne O., LeFur G, Caput D and Ferrara P. 1996. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. *Bba-Gene Struct Expr.* 1307(2):132-136.

Shitsukawa K, C Andersen, F Richard, A Horner, A Wiersma, M Van Duin, and M Conti. 2001. Cloning and characterization of the cyclic guanosine monophosphate-inhibited phosphodiesterase PDE3A expressed in mouse oocyte. *Biol Reprod.* 65: 188-196.

Shu YM, Zeng HT, Ren Z, Zhuang GL, Liang XY, Shen HW, Yao SZ, Ke PQ and Wang NN. 2008. Effects of cilostamide and forskolin on the meiotic resumption and embryonic development of immature human oocytes. *Human reproduction.* 23: 504-13.

Silva JRV, Van Den Hurk R, De Matos MHT, Dos Santos RR, Pessoa C, De Moraes MO and de Figueiredo JR. 2004. Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. *Theriogenology.* 61(9):1691–704.

Simonds WF. 1999. G protein regulation of adenylate cyclase. *Trends Pharmacol Sci.* 20: 66-73.

Sirard M, J Parrish, C Ware, M Leibfried-Rutledge, and N First. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. *Biol Reprod.* 39: 546-552.

Sirard MA. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology.* 55(6), 1241–1254. doi:10.1016/s0093-691x(01)00480-0

Sirard MA, Dufort I, Coenen K, Tremblay K, Massicotte L and Robert C. 2003. The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals. *Reprod Suppl.* 61:117-29. Review.

Siristatidis CS, Maheshwari A, Vaidakis D and Bhattacharya S. 2018. In vitro maturation in subfertile women with polycystic ovarian syndrome undergoing assisted reproduction. *Cochrane Database Syst Rev.* 11:CD006606. doi: 10.1002/14651858.CD006606.pub4.

Skaper SD, Buriani A, DalToso R, Petrelli L, Romanello S, Facci L and Leon A. 1996. The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons. *Proc Natl Acad Sci USA.* 93(9):3984-3989.

Smith CG, Besch NF, Smith RG and Besch PK. 1979. Effect of Tetrahydrocannabinol on the Hypothalamic-Pituitary Axis in the Ovariectomized Rhesus Monkey. *Fertil Steril.* 31(3):335–9.

Smith GD. 2001. In vitro maturation of oocytes. Current women's health reports. 1: 143-151.

Sociedad Española de Fertilidad (SEF). Registro Nacional de Actividad 2016-Registro SEF. Informe estadístico de Técnicas de Reproducción Asistida 2016. URL: https://www.registrosef.com/

Söderström-Anttila V, Mäkinen S, Tuuri T and Suikkari AM. 2005. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. *Hum Reprod.* 20: 1534-1540.

Song BS, Jeong PS, Lee JH, Lee MH, Yang HJ, Choi SA, Lee HY, Yoon SB, Park YH, Jeong KJ, Kim YH, Jin YB, Kim JS, Sim BW, Huh JW, Lee SR, Koo DB, Chang KT and Kim SU. 2018. The effects of kinase modulation on in vitro maturation according to different cumulus-oocyte complex morphologies. *PLoS One.* 13(10):e0205495. doi: 10.1371/journal.pone.0205495.

Staley K and Scharfman H. 2005. A woman's prerogative. *Nat Neurosci.* 8(6):697-9. 10.1038/nn0605-697.

Stitzel ML and Seydoux G. 2007. Regulation of the oocyte-to-zygote transition. *Science.* 316:407–408

Stojkovic, M, SA Machado, P Stojkovic, V Zakhartchenko, P Hutzler, PB Gonçalves, and E Wolf. 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod.* 64: 904-909.

Storr M, Emmerdinger D, Diegelmann J, Yuce B, Pfennig S, OchsenkuhnT, Goke B, Lohse P and Brand S. 2009. The role of fatty acid hydrolasegene variants in inflammatory bowel disease. *Aliment PharmacolTher.* 29:542–551Cell Tissue Res.

Straus III JF, Modi B and McAllister JM. 2014. Defects in Ovarian Steroid Hormone Biosynthesis. *Cellular Endocrinology in Health and Disease.* Chapter 8: 285-309.

Stulberg DB, Cain LR, Dahlquist I and Lauderdale DS. 2014. Ectopic pregnancy rates and racial disparities in the Medicaid population, 2004-2008. *Fertil Steril.* 102(6):1671-6. doi: 10.1016/j.fertnstert.2014.08.031.

Su YQ, Wigglesworth K, Pendola FL, O'Brien MJ and Eppig JJ. 2002. Mitogen-activated protein kinase activity in cumulus cells is essential for gonadotropin-induced oocyte meiotic resumption and cumulus expansion in the mouse. *Endocrinology.* 143, 2221-32.

Su YQ, K Sugiura, Q Li, K Wigglesworth, MM Matzuk, and JJ Eppig. 2010. Mouse oocytes enable LH-induced maturation of the cumulus-oocyte complex via promoting EGF receptor-dependent signaling. *Mol Endocrinol.* 24: 1230-1239.

Sugiura T, Kondo S, Sukagawa A, Tonegawa T, Nakane S, Yamashita A and Waku K. 1996. Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through N-acylphosphatidylethanolamine pathway in testis: Involvement of Ca^{2+}- dependent transacylase and phosphodiesterase activities. *Biochem Bioph Res Co.* 221(1):206-206.

Sugiura K, Pendola FL and Eppig JJ. 2005. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol.* 279(1):20-30.

Sun QY, Lai L, Bonk A, Prather RS and Schatten H. 2001. Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: effects of gonadotropins, cumulus cells, follicular size, and protein synthesis inhibition. *Mol Reprod Dev.* 59(2):192-8.

Sun QY, and T Nagai. 2003. Molecular mechanisms underlying pig oocyte maturation and fertilization. *Journal of Reproduction and Development.* 49: 347-359.

Sun QY, Miao Y-L and Schatten H. 2009. Towards a new understanding on the regulation of mammalian oocyte meiosis resumption. *Cell cycle.* 8: 2741-7.

Sun X and Dey SK. 2012. Endocannabinoid signaling in female reproduction. *ACS Chem Neurosci.* 3:349–55.

T

Takeo T, Mukunoki A and Nakagata N. 2019. Ovulation of juvenile, mature, and aged female C57BL/6 mice following coadministration of inhibin antiserum and equine chorionic gonadotropin. *Theriogenology.* 135:1–6. Available from: doi.org/10.1016/j.theriogenology.2019.05.028

Talmor-Cohen A, Eliyahu E and Shalgi R. 2002. Signalling in mammalian egg activation: role of protein kinases. *Mol Cell Endocrinol.* 187(1-2):145-9

Taylor AH, Ang C, Bell SC and Konje JC. 2007. The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. *Hum Reprod Update.* 13:501–13.

Taylor AH, Amoako AA, Bambang K, Karasu T, Gebeh A, Lam PM, Marzcylo TH and Konje JC. 2010. Endocannabinoids and pregnancy. *Clin Chim Acta.* 411:921–930.

Terasaki M, LL Runft, and AR Hand. 2001. Changes in organization of the endoplasmic reticulum duringxenopus oocyte maturation and activation. *Molecular Biology of the Cell.* 12: 1103-1116.

Themmen AP, and IT Huhtaniemi. 2000. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocrine reviews.* 21: 551-583.

Thibault C, Szöllösi D and Gérard M. 1987. Mammalian oocyte maturation. *Reprod Nutr Dev.* 27(5):865-96. Review.

Thomas RE, Armstrong DT and Gilchrist RB. 2004. Bovine cumulus cell-oocyte gap junctional communication during in vitro maturation in response to manipulation of cell-specific cyclic adenosine 3',5'-monophosphate levels. *Biology of reproduction.* 70: 548-5

Thompson JG. 1996. Defining the requirements for bovine embryo culture. *Theriogenology.* 45: 27-40.

Tilly JL. 2003. Ovarian follicle counts - Not as simple as 1, 2, 3. *Reprod Biol Endocrinol.* 1:1–4.

Todd AR. 1946. Hashish. *Experientia.* 2: 55–60. Doi: 10.1007/bf02163886

Tomek W and Smiljakovic T. 2005. Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes. *Reproduction.* 130:423–430.

Töpfer-Petersen E, Petrounkina AM and Ekhlasi-Hundrieser M. 2000. Oocyte–sperm interactions. *Anim Reprod Sci.* 60: 653-662.

Tornell J, Billing, H and Hillensjo, T. 1991. Regulation of oocyte maturation by changes in ovarian levels of cyclic nucleotides. *Hum reproduction.* 6: 411-22.

Trounson A, Wood C and Kausche A. 1994. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertility and Sterility.* 62: 353–362. doi.org/10.1016/S0015-0282(16)56891-5

Turko IV, Ballard SA, Francis SH and Corbin JD.1999. Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (type 5) by sildenafil and related compounds. *Mol Pharmacol.* 56:124–30.

Tyrey L. 1978. Δ-9-Tetrahydrocannabinol Suppression of Episodic Luteinizing Hormone Secretion in the Ovariectomized Rat*. *Endocrinology.* 102(6):1808–14.

U

Ulloa-Aguirre A, Zariñán T, Pasapera AM, Casas-González P and Dias JA. 2007. Multiple facets of follicle-stimulating hormone receptor function. *Endocrine.* 32:251–263. doi:10.1007/s12020-008-9041-6

Uriguen L, Garcia-Fuster MJ, Callado LF, Morentin B, La Harpe R, Casado V, et al. 2009. Immunodensity and mRNA expression of A2A adenosine, D2 dopamine and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. *Psychopharmacology* (Berl). 206:313–24.

V

Vaccari S, Horner K, Melhmann,LM and Conti M. 2008. Generation of mouse oocytes defective in cAMP synthesis and degradation:endogenous cyclic AMP is essential for meiotic arrest. *Dev.Biol.* 316: 124-34.

Van den Hurk R, Abir R, Telfer EE and Bevers MM. 2000. Primate and bovine immature oocytes and follicles as sources of fertilizable oocytes. *Human Reproduction Update* 6: 457–74. (https://doi.org/10.1093/ humupd/6.5.457).

Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, Stella N, Makriyannis A, Piomelli D, Davison JS, Marnett LJ, Di Marzo V, Pittman QJ, Patel KD and Sharkey KA. 2005. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science.* 310(5746):329-32.

Vanderhyden BC, and EA Macdonald.1998. Mouse oocytes regulate granulosa cell steroidogenesis throughout follicular development. *Biol Reprod* 59 1296-1301.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M and Bilanges B. 2010 The emerging mechanisms of isoform-specific PI3K signalling. *Nature Reviews Molecular Cell Biology.* 11: 329-341.

Vanhoutte L, Nogueira D, Gerris J, Dhont M and De Sutter P. 2008. Effect of temporary nuclear arrest by phosphodiesterase 3-inhibitor on morphological and functional aspects of in vitro matured mouse oocytes. *Molecular reproduction and development.* 75: 1021-30.

W

Walker OS, Holloway AC and Raha S. 2019. The role of the endocannabinoid system in female reproductive tissues. *J Ovarian Res.* 12(1):3. doi: 10.1186/s13048-018-0478-9.

Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL and Dey SK. 2003. Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. *Proc Natl Acad Sci U S A*. 100: 14914–9.

Wang H, Guo Y, Wang D, Kingsley PJ, Marnett LJ, Das SK, et al. 2004. Aberrant cannabinoid signaling impairs oviductal transport of embryos. *Nat Med.* 10:1074–80.

Wang H, Xie H and Dey SK. 2006a. Endocannabinoid signaling directs periimplantation events. *AAPS* J. 8:E425–32.

Wang H, Dey SK and Maccarrone M. 2006b. Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr Rev.* 27:427–48.

Wang C and Roy SK. 2007. Development of primordial follicles in the hamster: role of estradiol-17beta. *Endocrinology.* 148(4):1707-16.

Wang H, Xie H, Sun X, Kingsley PJ, Marnett LJ, Cravatt BF, et al. 2007. Differential regulation of endocannabinoid synthesis and degradation in the uterus during embryo implantation. *Prostaglandins Other Lipid Mediat.* 83:62–74.

Wang S, Z Kou, Z Jing, Y Zhang, X Guo, M Dong, I Wilmut, and S Gao. 2010. Proteome of mouse oocytes at different developmental stages. *Proceedings of the National Academy of Sciences.* 107: 17639-17644.

Wathlet S, Adriaenssens T, Segers I, Verheyen G, Janssens R, Coucke W, Devroey P and Smitz J. 2012. New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression. *Fertil Steril.* 98(2):432-9.e1-4. doi: 10.1016/j.fertnstert.2012.05.007.

Watson AJ. 2007. Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence. *J Anim Sci.* 85(13 Suppl):E1-3.

Wayne CM, Fan HY, Cheng X, and Richards JS. 2007. Follicle-stimulating hormone induces multiple signaling cascades: evidence that activation of Rous sarcoma oncogene, RAS, and the epidermal growth factor receptor are critical for granulosa cell differentiation. *Mol Endocrinol.* 21 1940-1957.

Webb RJ, F Marshall, K Swann, and J Carroll. 2002. Follicle-stimulating hormone induces a gap junction-dependent dynamic change in [cAMP] and protein kinase a in mammalian oocytes. *Dev Biol.* 246: 441-454.

Wenger T, Toth BE, Juaneda C, Leonardelli J and Tramu G. 1999. The effects of cannabinoids on the regulation of reproduction. *Life Sci.* 65:695–701.

Wenger T, Ledent C, Csernus V and Gerendai I. 2001. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. *Biochem Biophys Res Commun.* 284:363–8.

Wide L and Bakos O. 1993. More basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular or luteal phase. *J Clin Endocrinol Metab.* 76(4):885-9.

Wiley JL, Marusich JA and Huffman JW. 2014. Moving around the molecule. Relationship between chemical structure and in vivo activity of synthetic cannabinoids. *Life Sci.* 97:55–63.

Wolfenson C1, Groisman J, Couto AS, Hedenfalk M, Cortvrindt RG, Smitz JE and Jespersen S. 2005. Batch-to-batch consistency of human-derived gonadotrophin preparations compared with recombinant preparations. *Reprod Biomed Online.* 10(4):442-54.

X

Xie HL, Wang YB, Jiao GZ, Kong DL, Li Q, Li H, Zheng LL and Tan JH. 2016. Effects of glucose metabolism during in vitro maturation on cytoplasmic maturation of mouse oocytes. *Sci Rep.* 6:20764. doi: 10.1038/srep20764.

Y

Yamashita Y, Hishinuma M, and Shimada M. 2009. Activation of PKA, p38 MAPK and ERK1/2 by gonadotropins in cumulus cells is critical for induction of EGF-like factor and TACE/ADAM17 gene expression during in vitro maturation of porcine COCs. *Journal of ovarian research.* 2 1.

Yanagimachi R. 1994. Fertility of mammalian spermatozoa: its development and relativity. *Zygote.* 2: 371-372.

Yazulla S. 2008. Endocannabinoids in the retina: from marijuana to neuroprotection.. *Prog Retin Eye Res.* 27(5):501-26. doi: 10.1016/j.preteyeres.2008.07.002.

Z

Zhang W and Liu HT. 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell research.* 12: 9-18.

Zhang R, Kim TK, Qiao ZH, Cai J, Pierce WM Jr and Song ZH. 2007. Biochemical and mass spectrometric characterization of the human CB2 cannabinoid receptor expressed in Pichia pastoris—importance of correct processing of the N-terminus. *Protein Expr Purif.* 55:225-35.

Zhang M, Su YQ, Sugiura K, Xia G and Eppig JJ. 2010. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science.* 330(6002):366-9. doi: 10.1126/science.1193573.

Zhang M, Su YQ, Sugiura K, Wigglesworth K, Xia G and Eppig JJ. 2011. Estradiol promotes and maintains cumulus cell expression of natriuretic peptide receptor 2 (NPR2) and meiotic arrest in mouse oocytes in vitro. *Endocrinology.* 152(11):4377-85. doi: 10.1210/en.2011-1118.

Zheng W, Nagaraju G, Liu Z, and Liu K. 2012. Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary. *Mol Cell Endocrinol.* 356: 24-30.

Zlotkin T, Farkash Y and Orly J.1986. Cell-specific expression of immunoreactive cholesterol side-chain cleavage cytochrome P-450 during follicular development in the rat ovary. *Endocrinology.* 119(6):2809-20.

Zou S and Kumar U. 2018. Cannabinoid Receptors and the Endocannabinoid System: Signaling and Function in the Central Nervous System. *Int J Mol Sci.* 19(3). pii: E833. doi: 10.3390/ijms19030833.

.

8

Argitalpenak
Publications

8. **Argitalpenak /** Publications

- **Lide Totorikaguena**, Estibaliz Olabarrieta, Francesca Lolicato, Jon Romero Aguirregomezcorta, Johan Smitz, Naiara Agirregoitia and Ekaitz Agirregoitia. The endocannabinoid system modulates the physiology of the ovary and its activation can improve the maturation of the oocytes in vitro. *Under revision*.

- **Lide Totorikaguena**, Estibaliz Olabarrieta, Ángela-Patricia López-Cardona, Naiara Agirregoitia, Ekaitz Agirregoitia. Tetrahydrocannabinol Modulates in Vitro Maturation of Oocytes and Improves the Blastocyst Rates after *in Vitro* Fertilization. *Cell Physiol Biochem*. 2019;53(3):439-452. doi: 10.33594/000000149.

- Ekaitz Agirregoitia, **Lide Totorikaguena**, Antonia Expósito, Rosario Mendoza, Roberto Matorras, Naiara Agirregoitia. Expression and localization of cannabinoid-degrading enzymes during the maturation of human oocytes Cell Tissue Res. 2016 Aug;365(2):393-401.

- Ekaitz Agirregoitia, Inés Ibarra-Lecue, **Lide Totorikaguena**, Rosario Mendoza, Antonia Expósito, Roberto Matorras, leyre Urigüen, Naiara Agirregoitia. Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation. *Fertility and Sterility* 2015. F and S20361.

8.1 Publications not included in the PhD thesis

- Asier Valdivia, Lorea Cortés, Maider Beitia, **Lide Totorikaguena**, Naiara Agirregoitia, Beatriz Corcostegui, Roberto Matorras, Jon Irazusta, Ekaitz Agirregoitia. Role of Angiotensin-(1-7) via Mas receptor in human sperm motility and acrosome reaction. Reproduction. *Under revision*.

- Estibaliz Olabarrieta, **Lide Totorikaguena**, Jon Romero-Aguirregomezcorta, Naiara Agirregoitia, Ekaitz Agirregoitia. Mu opioid receptor expression and localisation in murine spermatozoa and its role in fertilisation *in vitro*. *Reproduction, Fertility and Development*. 2019. Nov 13. doi: 10.1071/RD19176.

- Estibaliz Olabarrieta, **Lide Totorikaguena**, Naiara Agirregoitia, Ekaitz Agirregoitia . Implication of mu opioid receptor in the *in vitro* maturation of oocytes and its effects on subsequent fertilization and embryo development in mice. *Mol Reprod Dev.* 2019 Sep;86(9):1236-1244. doi: 10.1002/mrd.23248.

- **Lide Totorikaguena**, Esti Olabarrieta, Roberto Matorras, Edurne Alonso, Ekaitz Agirregoitia, Naiara Agirregoitia. Mu opioid receptor in the human endometrium: dynamics of its expression and localization during the menstrual cycle. *Fertil Steril*. 2017 Apr; 107(4):1070-1077

Eranskinak
Appendixes

Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation

Ekaitz Agirregoitia, Ph.D.,[a,b] Inés Ibarra-Lecue, B.S.,[c] Lide Totorikaguena, B.S.,[a] Rosario Mendoza, B.S.,[c,d] Antonia Expósito, Ph.D.,[b,d] Roberto Matorras, M.D., Ph.D.,[c,d] Leyre Urigüen, Ph.D.,[b,c,e] and Naiara Agirregoitia, Ph.D.[a,b]

[a] Department of Physiology, Faculty of Medicine and Dentistry, University of the Basque Country, Leioa; [b] BioCruces Health Research Institute, Barakaldo; [c] Department of Pharmacology, Faculty of Medicine and Dentistry, University of the Basque Country, Leioa; [d] Human Reproduction Unity, Department of Obstetrics and Gynecology, Cruces University Hospital, University of the Basque Country, Barakaldo; and [e] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Madrid, Spain

Objective: To describe the expression of cannabinoid receptors CB1 and CB2 and cannabinoid-degrading enzymes fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL) in human granulosa cells and to investigate their differential distribution with respect to CB1 at various stages during the nuclear maturation of the oocyte.
Design: Analysis of granulosa cells from germinal vesicle (GV), metaphase I (MI), and MII oocytes by quantitative reverse transcriptase–polymerase chain reaction, Western blot, and indirect immunofluorescence assays.
Setting: Academic research laboratory.
Patient(s): Patients from the Human Reproduction Unit of Cruces University Hospital undergoing intracytoplasmic sperm injection.
Intervention(s): We analyzed the granulosa cells of 300 oocytes from 53 patients. The oocyte maturation stages were 75 at GV stage, 51 at MI, and 174 at MII.
Main Outcome Measure(s): The mRNA and protein expression of CB1, CB2, FAAH, and MGLL and localization in granulosa cells at each oocyte maturation stage.
Result(s): CB1, FAAH, and MGLL are present in human granulosa cells during oocyte maturation, but the presence of CB2 receptor is not entirely clear in those cells. CB1 and FAAH were detected in the periphery of the granulosa cells from the GV to the MII oocytes, and they colocalized in some portions of the cell membrane. On the other hand, MGLL immunostaining was more homogeneous across the cell and overlapped with CB1 only weakly.
Conclusion(s): The presence of the cannabinoid system in granulosa cells suggests a possible role of this system in the nuclear maturation of the oocyte. (Fertil Steril® 2015;104:753–60. ©2015 by American Society for Reproductive Medicine.)
Key Words: Cannabinoid, granulosa cells, oocyte maturation

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/agirregoitiae-cannabinoid-system-granulosa-cells/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Oocyte maturation is a highly coordinated process that includes the progression of the oocyte from the first meiotic blockage at the germinal vesicle (GV) stage to the second meiotic blockage at the metaphase II (MII) stage. This progression occurs inside the follicle owing to a careful communication between oocytes and granulosa cells. These granulosa cells are somatic cells that surround the oocyte, but, when the follicle antrum is formed, they separate into mural granulosa cells forming the follicle inner wall and into cumulus granulosa cells, which are closely communicated with each other and with the oocyte through gap junctions (1).

Received May 15, 2015; revised June 9, 2015; accepted June 13, 2015; published online July 3, 2015.
E.A. has nothing to disclose. I.I.-L. has nothing to disclose. L.T. has nothing to disclose. R. Mendoza has nothing to disclose. A.E. has nothing to disclose. R. Matorras has nothing to disclose. L.U. has nothing to disclose. N.A. has nothing to disclose.
Supported in part by a grant from the University of the Basque Country (no. GIU14/26).
Reprint requests: Ekaitz Agirregoitia, Ph.D., Department of Physiology, Faculty of Medicine and Dentistry, University of the Basque Country, P.O. Box 699, E-48080 Bilbao, Bizkaia, Spain (E-mail: e.agirregoitia@ehu.es).

Fertility and Sterility® Vol. 104, No. 3, September 2015 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2015.06.013

Oocyte maturation can also be carried out in vitro as long as the oocyte is cultured without removing the surrounding granulosa cells. This cumulus-oocyte complex (COC) is the structure that is ovulated in vivo. For oocyte maturation bidirectional communication is necessary, via gap junctions and via paracrine signaling, between the oocyte and granulosa cells (2). In this sense, many substances from the COC have been implicated in the regulation of the oocyte maturation; some of these act once they are translocated by the gap junction, and others act on receptors present on the cumulus cells and/or the oocyte (3).

Among all these substances, the cannabinoid compounds have been highlighted in some studies. Endocannabinoids are fatty acid derivatives that exert their effects by binding to membrane Gi/o protein-coupled receptors CB1 and CB2. Anandamide (AEA) and 2-arachiconoylglycerol (2-AG) are the better characterized endocannabinoids. AEA and 2-AG are degraded by fatty acid amide hydrolase (FAAH), which is found on the internal membranes of cells, although 2-AG is degraded by monoglyceride lipase (MGLL) as well. Several studies have been published concerning the role of cannabinoids in the female reproductive system (4–6), and the cannabinoid receptors and degradation enzymes have been found in various parts of the mammalian femalereproductive system as uterus, oviduct (7–10), preimplantation embryos, and placenta (11–13).

In regard to the oocyte maturation, it is known that AEA is present in the human follicular fluid (14) and that its concentration in follicles with mature oocytes is higher than in follicles with immature oocytes (15). The localization of the cannabinoid receptors and enzymes in rat (16) and human ovary led to the hypothesis that AEA plays a role in folliculogenesis, preovulatory follicle maturation, oocyte maturity, and/or ovulation (15). The hypothesis about oocyte maturation was reinforced with the localization of cannabinoid receptors (17) and cannabinoid-degrading enzymes (authors' unpublished data) during the nuclear maturation of human oocytes, where each protein is relocated during that maturation.

Taking into account the necessary bidirectional communication between the oocyte and the granulosa cells, our study aimed to characterize in depth, using a variety of experimental methods, the differential expression of the two cannabinoid receptors and the two degrading enzymes in human granulosa cells that surround oocytes at each stage of meiotic resumption.

MATERIAL AND METHODS

Granulosa Cells Collection

Granulosa cells were obtained from 53 patients (ages 25–40 years; mean, 36.7 ± 2.6) undergoing intracytoplasmic sperm injection (ICSI) at the Human Reproduction Unit of the Cruces University Hospital. Ethical approval was obtained from the Clinical Research Ethical Committee of the Basque Health System (Osakidetza, CEIC reference no.r E07/54, 3/2008). Informed consent was obtained from all patients.

We analyzed the granulosa cells of 300 oocytes from 53 patients. Of the 300 oocytes studied, 75 were at the GV stage, 51 at MI, and 174 at MII. The main ICSI indications were male factor (79.9%) and failure of IUI. The main female-associated conditions were endometriosis (9.8%) and tubal factor (11.2%). Patients had no history of cannabinoid drug consumption.

The ovarian stimulation protocol has been published elsewhere (18, 19). Briefly, it consists of down-regulation with GnRH agonist and triptorelin acetate on a long protocol or with GnRH antagonist and the cetrorelix protocol, ovarian stimulation with recombinant FSH and highly purified urinary menopausal gonadotropins or recombinant LH, and ovulation being triggered with 250 μg recombinant hCG.

Oocyte retrieval was performed 35–37 hours after hCG administration. Follicles were aspirated with a negative pressure of 115–120 mmHg with a single lumen 18-gauge oocyte pick-up needle (K-OPS-6035-RWH-B-ET; Cook) under transvaginal ultrasound guidance. Follicular fluids were observed at low magnification (×40–100) under the stereomicroscope at 37°C. The COCs were incubated for 2 hours in IVF Medium (IVF Medium, Medicult, Origio) at 37°C and 6% CO_2 in air. Immediately before micromanipulation for the ICSI procedure, oocytes were denuded from the cumulus oophorus one by one in 30-μL droplets by a brief exposure to 40 IU/L hyaluronidase solution (Hyadase; Medicult, Origio), followed by mechanical removal of the corona radiate cells with the use of plastic pipettes of defined diameters (denuding pipette; Cook). The droplets containing the cumulus and granulosa cells after oocyte denuding were recovered in Eppendorf tubes, taking into account the nuclear maturation stage of the oocyte for separation into granulosa cells from GV, MI, or MII, and were centrifugated at 800 *g*. Finally, the medium containing the hyaluronidase solution was removed and the cells were placed at 4°C for early use or in the freezer at −80°C for later study.

Reverse Transcription

RNA from granulosa cells (obtained from 10 oocytes of each stage; n = 3), cerebral cortex (positive control for CB1, FAAH, and MGLL) and Jurkat cells (positive control for CB2), were isolated with the Dynabeads mRNA Purification Kit (Ambion). The procedure for obtaining the cDNA was performed with ImProm-II Reverse Transcription System (Promega). Briefly, about 150 ng of RNA and random primers were heated at 65°C for 10 minutes and chilled on ice for 5 minutes. Then, once the reverse transcription mix was added, it was annealed at 25°C for 5 minutes. A first-strand synthesis reaction was carried out at 55°C for 60 minutes, and the reverse transcriptase was inactivated at 70°C for 15 minutes.

Real-time Quantitative Polymerase Chain Reaction (PCR) Analysis

Quantitative PCR was performed in three replicates with the StepOne thermocycler using a TaqMan assay (Applied

Biosystems) specifically designed for recognizing CB1 (Hs01038522_s1), CB2 (Hs00361490_m1), FAAH (Hs01038660_m1), and MGLL (Hs00200752_m1). All the primers used were functional because we detected mRNA amplification when we used different tissues as positive controls (human cerebral cortex for CB1, FAAH, and MGLL and Jurkat cells for CB2). In addition, the controls performed in the absence of reverse transcriptase or in the absence of template were negative (data not shown).

The thermal profile for this PCR consisted of a "holding stage" of 20 seconds at 95°C and 40 cycles with two steps: 1 second at 95°C and 20 seconds at 60°C. We used GAPDH (4333764-0906029) as the endogenous control gene. The amount of CB1, CB2, FAAH, and MGLL mRNA in each sample was determined by the $2^{-\Delta\Delta CT}$ method with GAPDH as the reference gene. The average ΔCt of the cortex samples was used as the calibrator.

SDS/PAGE and Immunoblotting

Granulosa cells from 10 oocytes were collected and pooled (n = 5) in sodium dodecyl sulfate (SDS) sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue). Human cerebral cortex membranes were prepared as described elsewhere (20). Jurkat cells were purchased from Merck Millipore (Molsheim). Gel electrophoresis was carried out in SDS polyacrylamide gels, composed of 5% stacking (0.5 M Tris-HCl, pH 6.8, 10% SDS) and 12% resolving (1.5 M Tris-HCl, pH 8.8, 10% SDS), using a miniprotean system with molecular weight standards (Bio-Rad Laboratories). Proteins were then transferred to nitrocellulose membranes (1 hour, 0.3 A) using an electrophoretic transfer system (Bio-Rad Laboratories). The nonspecific binding sites in the membranes were blocked for 1.5 hours with Tris-buffered saline with Tween (TBST; 10 mM Tris-HCl, pH 7.4, 140 mM NaCl) containing 5% skimmed milk. The membranes were incubated with a primary rabbit polyclonal anti-CB1 receptor antibody (1:1,000; Frontier Institute), anti-CB2 receptor antibody (1:200), anti-FAAH antibody (1:200), and anti-MGL antibody (1:200; Cayman Chemicals), overnight at 4°C and all in TBST, 5% skimmed milk and 0.1% Tween buffer. Membranes were incubated with fluorescent antirabbit IgG secondary antibody (Alexa 680 Rabbit, Invitrogen) at a dilution of 1:4,000. They were then dried and imaged using the Odyssey infrared imaging system (LI-COR Biosciences). Integrated intensities were analyzed using Excel (Microsoft Corp.) and Prism (GraphPad Software) software.

Immunofluorescence

Granulosa cells were suspended in phosphate buffered saline (PBS) + bovine serum albumin (BSA; 1 μg/μL), smeared onto a slide coated with poly-L-lysine, and fixed with 3% paraformaldehyde for 10 minutes. Then the slides were washed 3 times in PBS and incubated for 45 minutes in PBS/10% (v/v) bovine fetal serum and 1% Triton ×100 at room temperature. For indirect immunofluorescence staining, slides were incubated with primary rabbit polyclonal anti-CB1, anti-CB2, anti-FAAH, and anti-MGL antisera at a dilution of 1:200 overnight at 4°C. For double staining, primary goat polyclonal anti-CB1 (Santa Cruz Biotechnology) was used. Slides were then washed in PBS + BSA (1 μg/μL) 3 times, incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes) for 2 hours at 37°C in the dark (1:2,500), washed in PBS + BSA (1 μg/μL) 3 times (in all cases we stained the nuclei with Hoechst 33342 during the second wash), assembled with Fluoromount G (EMS), and finally examined by confocal microscopy. Two negative controls were performed using a specific blocking peptide for each antibody and by omitting the primary antibody before addition of the secondary antibody.

Statistics

Results shown represent mean ± SEM. Statistical analysis was performed by analysis of variance with a post hoc analysis by Bonferroni's multiple comparison test (GraphPad Prism® 5).

RESULTS

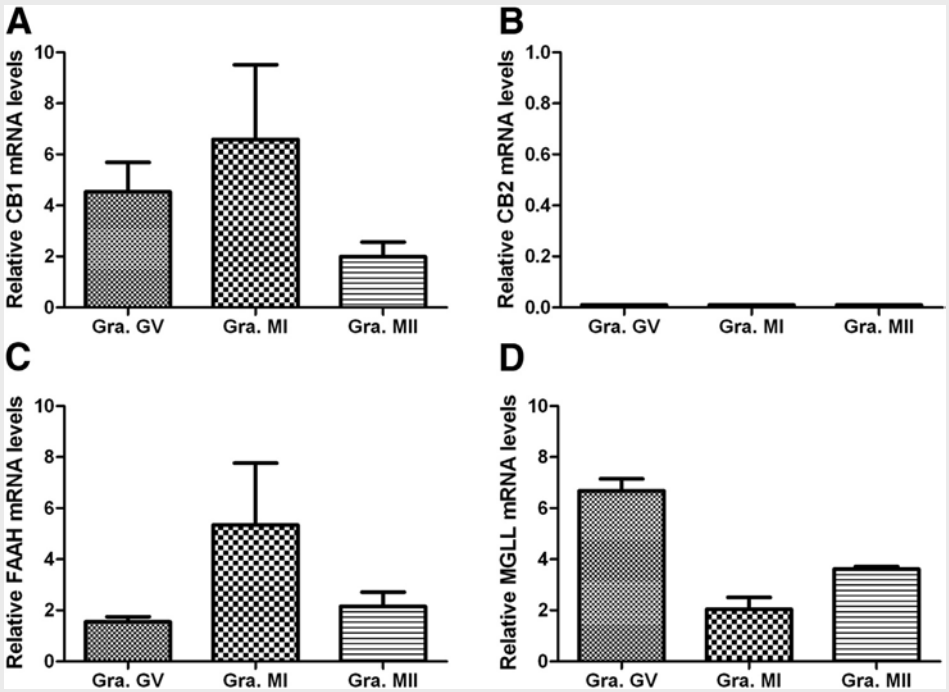
Quantitative Reverse Transcriptase (RT-) PCR Analysis of CB1, CB2, FAAH, and MGLL mRNA in Human Granulosa Cells during Nuclear Maturation of Oocytes

We detected the presence of CB1, FAAH, and MGLL but not CB2 receptor transcripts in human granulosa cells using quantitative RT-PCR. However, when we analyzed the relative amount of each mRNA, we did not find significant differences between granulosa cells of oocytes in GV, MI, or MII (Fig. 1).

Immunocytochemical Localization CB1, CB2, FAAH, and MGLL in Human Granulosa Cells during Nuclear Maturation of Oocytes

Immunofluorescence analysis revealed that CB1 receptor was present in the human granulosa cells and that its localization was predominantly in the periphery of the cells (Fig. 2). When we evaluated whether the localization of CB1 receptor changed if the granulosa cells came from GV, MI, or MII oocytes, we did not observe any change in the localization (Fig. 2). With regard to CB2, this receptor showed a very weak immunostaining and although it is difficult to ensure that it was a specific staining, it appears to be a more peripheral staining than that within the granulosa cells from all studied maturation stages (Fig. 2). On the other hand, the FAAH-degrading enzyme's immunostaining pattern did not change regardless of whether the granulosa cells came from a GV, MI, or MII oocyte, but, in this case, its localization was present over the entire granulosa cell (Fig. 2). Finally, similar results were found for the MGLL, although the staining was weaker than for the FAAH (Fig. 2). When the antibodies were blocked with the specific peptide before addition to the granulosa cells, no specific fluorescence was observed (Fig. 2).

FIGURE 1



The mRNA expression of **(A)** CB1, **(B)** CB2, **(C)** FAAH, and **(D)** MGLL in human granulosa cells (Gra) from GV, MI, and MII oocytes using specific primers. The relative concentrations of protein mRNA were determined by quantitative RT-PCR. Cycle threshold values were normalized to GAPDH, and the average ΔCt of the cortex sample (for CB1, FAAH, and MGLL) and Jurkat cells (for CB2) were used for calibration. Each value is the mean (±SEM) of three replicates.

Agirregoitia. Cannabinoid system in human granulosa cells. Fertil Steril 2015.

Immunoidentification of CB1, CB2, FAAH, and MGLL Proteins in Human Granulosa Cells

To confirm the presence/absence of studied proteins, Figure 3 shows representative Western blots using human granulosa cells, human prefrontal cerebral cortex gray matter, and Jurkat cells. The anti-CB1 receptor polyclonal antiserum labeled a major band at ≈60 kDa in granulosa cells and cortex protein extracts. The anti-CB2 receptor polyclonal antiserum recognized a major band at ≈40 kDa in granulosa cells and in Jurkat cells. The anti-FAAH polyclonal antibody labeled a major band at about ≈60 kDa in granulosa cells and cortex protein extracts. Finally, the anti-MGLL polyclonal antibody labeled two major bands around ≈35 kDa in granulosa cells and in cortex.

Immunocytochemical Localization of CB2, FAAH, and MGLL Compared with that of the CB1 Receptor in Human Granulosa Cells during Nuclear Maturation of Oocytes

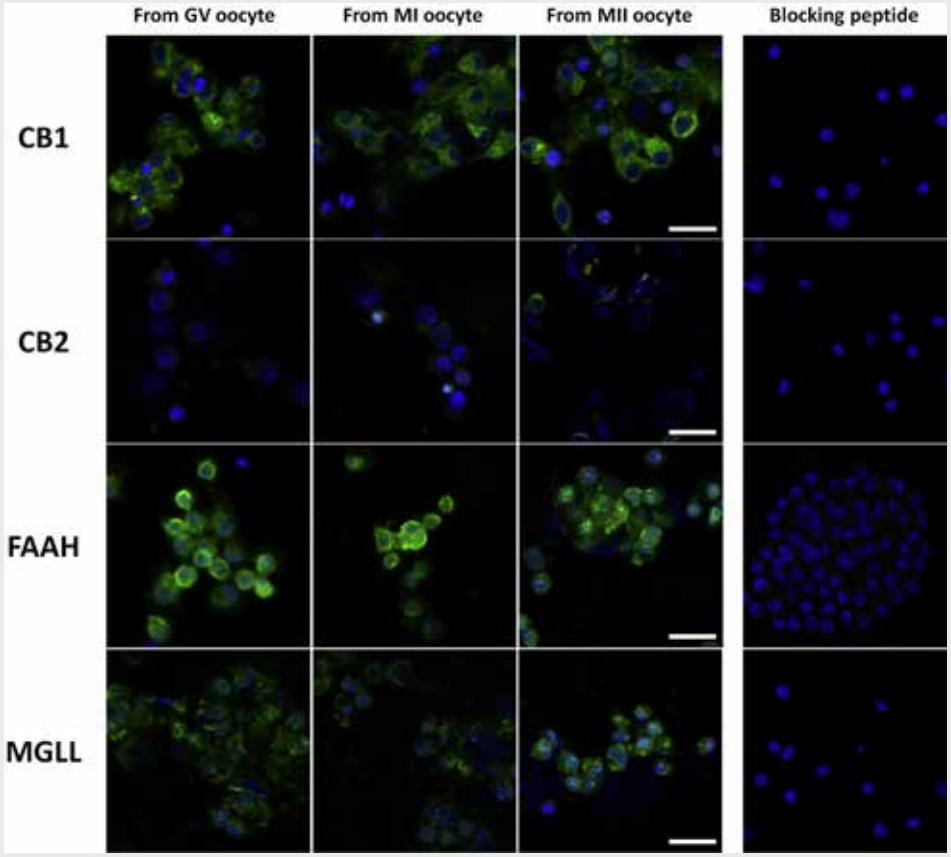
As we observed that there was no change in the location of the studied proteins in human granulosa cells during nuclear maturation of oocytes, we analyzed in depth the localization of each protein and the colocalization of those proteins in comparison with CB1 receptor since this was the most abundant (and maybe the unique) cannabinoid receptor in these cells. For this purpose, we used granulosa cells coming from different stages, and the results were the same for all stages. For that reason, only representative data are shown (Fig. 4).

We confirmed that CB1 receptor was localized in the periphery of granulosa cells (Fig. 4A, 4B, and 4C), unquestionably in the cell membrane. In the same way, we again found a very weak presence of the CB2 receptor in those cells (Fig. 4A). With regard to FAAH, this degrading enzyme was present in both the cytoplasm and the plasma membrane of granulosa cells, in fact, FAAH colocalized with the CB1 receptor at the cell membrane (Fig. 4B). Finally, MGLL showed a weak colocalization with the CB1 receptor in the periphery of the cell, but its localization was predominantly in the cytoplasm of the granulosa cells (Fig. 4C).

DISCUSSION

The role of the endocannabinoid system has been studied in mammalian reproductive events, such as spermatogenesis, sperm motility, fertilization, embryo oviductal transport, preimplantation embryo development, implantation, and postimplantation embryonic growth (21–23). Even so, the role of this system in the ovary and oocyte events has rarely been analyzed (15–17). The aim of the present study was to gather further data on the presence and localization of cannabinoid receptors CB1 and CB2 as well as cannabinoid-degrading enzymes FAAH and MGLL in granulosa cells during the

FIGURE 2



Immunofluorescence analysis of CB1, CB2, FAAH, and MGLL in human granulosa cells from GV, MI, and MII oocytes. The distribution of cannabinoid receptors and cannabinoid-degrading enzymes is shown in *green*. A negative control consisting of preadsorption of primary antibody with each specific blocking peptide is shown. Hoechst-labeled DNA is shown in *blue*. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 10 μm.

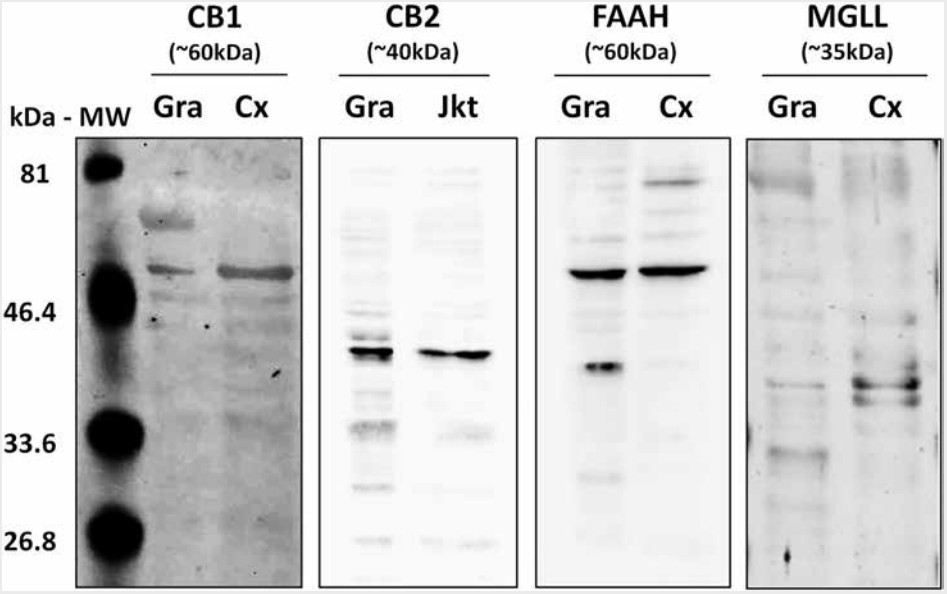
Agirregoitia. Cannabinoid system in human granulosa cells. Fertil Steril 2015.

resumption of meiosis of the human oocyte to determine possible role of the cannabinoid system in this process.

Western blot analysis revealed the presence of CB1, CB2, FAAH, and MGLL proteins in human granulosa cells. With regard to CB1, the band of about 60 kDa (positive control: *gray* matter from the human prefrontal cerebral cortex) accords in size with previous reports (24, 25). In the case of CB2, the bands of approximately 40 kDa (positive control: Jurkat cells) correspond to its theoretical molecular mass (26) and are in agreement with previous reports (27, 28). We have to note that we needed to load a great amount of sample to see a positive mark in granulosa cells. On the other hand, the band around 60 kDa observed in human granulosa cells using an anti-FAAH antibody, which also appeared in the human cerebral cortex, has been described in some studies using rat brain (29) and human sperm (30) and bladder (31). Finally, the two major bands observed around 35 kDa in granulosa cells and in the cortex are near the theoretical molecular weight for MGLL around 33.3 kDa (32). However, we cannot exclude that the band around 33 kDa shown in granulosa cells (Fig. 3) corresponds to MGLL too.

Two previous works have described the presence of cannabinoid system components in the granulosa cells using immunohistochemical assays in ovarian sections. Thus, with regard to granulosa cells, in rat ovaries it has been described in the presence of CB1 receptor in the plasma membrane of granulosa cells of antral follicles (16), and the same result has been observed in human antral follicles as well (15). In our study we have gone a step further, and we have seen that CB1 is still present in the plasma membrane of granulosa cells from ovulated oocytes (GV) and that it is maintained there at least until oocyte and granulosa cells are separated after oocyte maturation (MII). Moreover, we have detected that the mRNA of CB1 is present in granulosa cells during the nuclear maturation of the oocyte, but we cannot confirm whether the expression pattern observed during the maturation changed significantly. Even so, we have to point out that the mRNA expression pattern of both cannabinoid receptor CB1 and cannabinoid-degrading enzyme FAAH in granulosa cells during the nuclear maturation of the oocyte is the same, although we cannot corroborate this expression pattern at the protein level.

FIGURE 3



Western blotting analysis of CB1 and CB2 receptor, FAAH, and MGLL in human granulosa cells (Gra), gray matter from the human prefrontal cerebral cortex (Cx), and Jurkat cells (Jkt) using a rabbit antiserum against CB1, CB2, FAAH, and MGLL. Molecular weights (MW-kDa) are indicated on the left; n = 3; representative Western blots are shown.

Agirregoitia. Cannabinoid system in human granulosa cells. Fertil Steril 2015.

The presence of CB2 receptor in granulosa cells is controversial because this receptor is not present in granulosa cells of rat antral follicles (16), but its signal has been described in the antral follicles of human ovary, although more weakly than that of CB1 (15). In that sense, we immunodetected CB2 signal by Western blot (loading a great amount of granulosa cells); we did not find the mRNA of CB2 in granulosa cells, and the immunocytochemical assays showed a weak staining of CB2. Taking into account all of these data, we are not able to discard the presence of CB2 in granulosa cells, but we can affirm that its presence would be more residual and much lower than CB1 receptor.

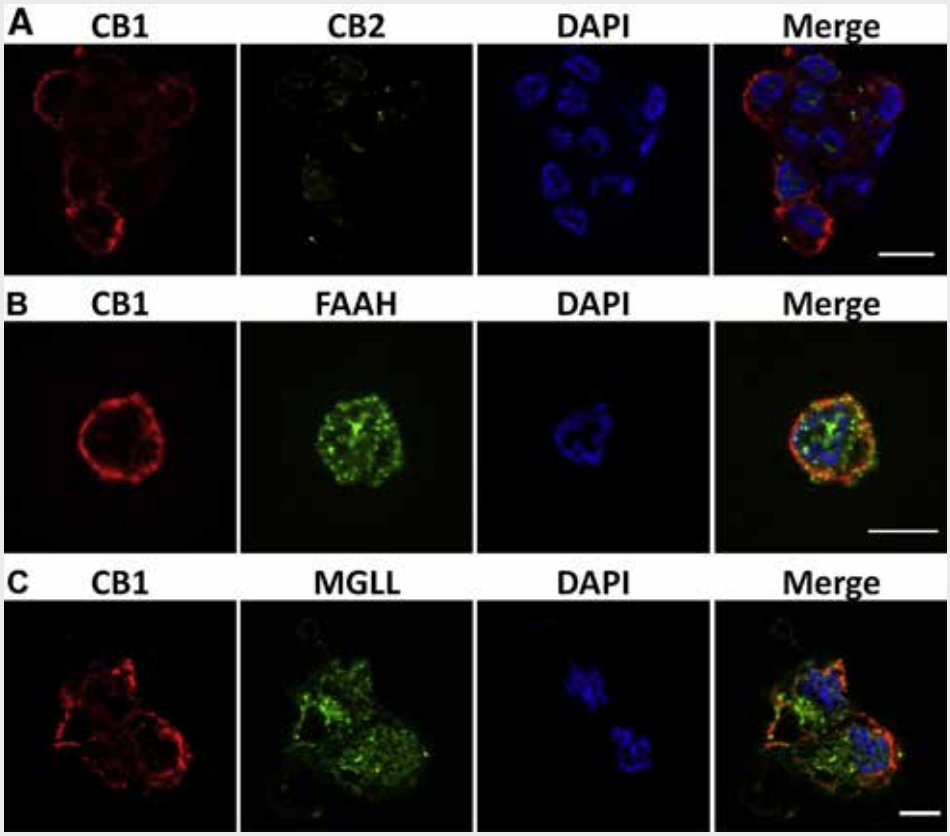
On the other hand, the absence of FAAH described in rat granulosa cells from antral follicles (16) and the weak staining observed in human granulosa cells from secondary and antral follicles (15) are not consistent with our observations. Our study was carried out in granulosa cells from ovulated oocytes and the presence of FAAH has been corroborated using immunocytochemistry, Western blot, and PCR. For this reason, we can conclude that the nondetection of this enzyme in granulosa cells from antral follicles described in other studies could be due to a relatively low level of FAAH expression rather than its absence. Even so, it could also be possible that FAAH would start its expression once the ovulation has occurred. But this hypothesis should be checked because we have not performed experiments with granulosa cells from nonstimulated follicles.

Finally, to the best of our knowledge, this is the first time that MGLL has been found to be localized in the granulosa cells. Although we are not able to confirm whether the expression pattern observed during oocyte maturation changed significantly, the mRNA expression pattern of MGLL seem to be just the contrary compared with CB1 and FAAH.

In our study we used granulosa cells isolated from follicles maturated in vivo after hormone stimulation of the patient. For this reason, the recovered oocytes had already been stimulated and only the final nuclear maturation steps were carried out in vitro during 2 hours. This could be the reason that we did not see significant differences in the protein or mRNA pattern between granulosa cells from GV, MI, or MII oocytes.

The regulation of "AEA tone" by FAAH has been proposed as an important event in the oviduct during early pregnancy (33), as well as in blastocyst activation (34) and implantation (35), but no hypotheses have been proposed in relation to such a process in oocyte maturation. Even so, it has been described that AEA concentration in human follicular fluid rises during oocyte maturation (15) and that the concentration is even higher in the oviduct than in follicular fluid (36). In humans, one oocyte completes its growth each month and is ovulated in response to a midcycle surge of LH, and in response to this LH surge, the oocyte resumes meiosis (37). Therefore, the oocyte and its granulosa cells find increasing concentrations of AEA while they mature and so the presence of the cannabinoid system in the granulosa cells leads to the hypothesis that the resumption of meiosis in the oviduct, where AEA concentration is higher than it is in follicular fluid, could be modulated, inter alia, by the cannabinoid system. In contrast, the elevated levels of cyclic AMP (camp) produced by adenylyl cyclase are crucial in maintaining oocytes under meiotic arrest, and the source of cAMP has been suggested

FIGURE 4



Immunofluorescence analysis of **(A)** CB2, **(B)** FAAH, and **(C)** MGLL compared with CB1 cannabinoid receptor in human granulosa cells. The distributions of CB2, FAAH, and MGLL are shown in *green*, and the distribution of CB1 is shown in red. Their overlap is shown in *yellow*. Hoechst-labeled DNA is shown in *blue*. n = 15 per stage. Representative photomicrographs are shown. The scale bar represents 10 μm.

Agirregoitia. Cannabinoid system in human granulosa cells. Fertil Steril 2015.

to be the product of the influx of cAMP through gap junction communication from the cumulus cells to the oocyte (1). Taking all these data into account, we hypothesize that the stimulation of CB1 cannabinoid receptor by its agonist in granulosa cells, first in the follicle and then in the oviduct, could activate a number of signal transduction pathways via the Gi/o family of G proteins, leading to the inhibition of adenylyl cyclase and the consequent inhibition of cAMP production (34). Moreover, although the most abundant protein in the plasma membrane of granulosa cells was CB1, FAAH colocalized with it. This overlapping pattern in the expression of CB1 and FAAH has been reported in other cell types, such as neurons, suggesting that the endocannabinoid system might be responsible for an autofeedback control of neurotransmitter release (38). Finally, Schuel and Burkman (39) observed that sea urchin eggs might release AEA after activation by the fertilizing sperm and this released AEA might then react with cannabinoid receptors in sperm to block acrosome reaction, thereby helping to prevent polyspermy. So as granulosa cells from ovulated oocytes have the enzymatic machinery to degrade AEA, this fact led us to hypothesize that granulosa cells could regulate the AEA tone near the microenvironment where the fecundation takes place to modulate the sperm physiology, as Schuel and Burkman proposed.

It will be interesting to analyze what the role of the cannabinoid system in granulosa cells is in relation to the oocyte where CB1, CB2, FAAH, and MGLL relocate during the nuclear maturation (17; author's unpublished data). To date, the presence of 2-AG has only been described in the uterus (40), but it remains unknown whether it is present in the ovary or the oviduct, a question that merits investigation.

In conclusion, we report for the first time the presence of the cannabinoid system in granulosa cells from GV, MI, and MII oocytes. This could suggest that AEA, which is present in follicular and oviductal fluids (14, 15), also has a role during the final nuclear maturation of the oocyte. It should be noted that the granulosa cells used in this study were obtained in the context of an IVF/ICSI program and hence may not reflect the real status of oocyte maturation during spontaneous natural ovarian cycles. Further studies are needed to investigate the cannabinoid receptors in unstimulated cycles.

Acknowledgments: The authors thank SGiker (University of the Basque Country) for personal and technical support and particularly Ricardo Andrade for his assistance with the confocal microscopy analysis.

REFERENCES

1. Sanchez F, Smitz J. Molecular control of oogenesis. Biochim Biophys Acta 2012;1822:1896–912.
2. Albertini DF, Combelles CM, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction 2001; 121:647–53.
3. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci 2004;82–83:431–46.
4. de Miguel R, Romero J, Munoz RM, Garcia-Gil L, Gonzalez S, Villanua MA, et al. Effects of cannabinoids on prolactin and gonadotrophin secretion: involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. Biochem Pharmacol 1998;56:1331–8.
5. Wenger T, Toth BE, Juaneda C, Leonardelli J, Tramu G. The effects of cannabinoids on the regulation of reproduction. Life Sci 1999;65:695–701.
6. Wenger T, Ledent C, Csernus V, Gerendai I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem Biophys Res Commun 2001;284:363–8.
7. Das SK, Paria BC, Chakraborty I, Dey SK. Cannabinoid ligand-receptor signaling in the mouse uterus. Proc Natl Acad Sci U S A 1995;92:4332–6.
8. Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, et al. Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 2001;276:20523–8.
9. Wang H, Guo Y, Wang D, Kingsley PJ, Marnett LJ, Das SK, et al. Aberrant cannabinoid signaling impairs oviductal transport of embryos. Nat Med 2004;10:1074–80.
10. Dennedy MC, Friel AM, Houlihan DD, Broderick VM, Smith T, Morrison JJ. Cannabinoids and the human uterus during pregnancy. Am J Obstet Gynecol 2004;190:2–9. discussion 3A.
11. Park B, Gibbons HM, Mitchell MD, Glass M. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta 2003;24:990–5.
12. Helliwell RJ, Chamley LW, Blake-Palmer K, Mitchell MD, Wu J, Kearn CS, et al. Characterization of the endocannabinoid system in early human pregnancy. J Clin Endocrinol Metab 2004;89:5168–74.
13. Habayeb OM, Taylor AH, Bell SC, Taylor DJ, Konje JC. Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 2008;149:5052–60.
14. Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, et al. N-acylethanolamines in human reproductive fluids. Chem Phys Lipids 2002; 121:211–27.
15. El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC, Konje JC. Localisation and function of the endocannabinoid system in the human ovary. PLoS One 2009;4:e4579.
16. Bagavandoss P, Grimshaw S. Temporal and spatial distribution of the cannabinoid receptors (CB1, CB2) and fatty acid amide hydroxylase in the rat ovary. Anat Rec (Hoboken) 2010;293:1425–32.
17. Peralta L, Agirregoitia E, Mendoza R, Exposito A, Casis L, Matorras R, et al. Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. Reprod Biomed Online 2011;23:372–9.
18. Matorras R, Urquijo E, Mendoza R, Corcostegui B, Exposito A, Rodriguez-Escudero FJ. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. Hum Reprod 2002;17: 1762–6.
19. Matorras R, Mendoza R, Exposito A, Rodriguez-Escudero FJ. Influence of the time interval between embryo catheter loading and discharging on the success of IVF. Hum Reprod 2004;19:2027–30.
20. Uriguen L, Garcia-Fuster MJ, Callado LF, Morentin B, La Harpe R, Casado V, et al. Immunodensity and mRNA expression of A2A adenosine, D2 dopamine, and CB1 cannabinoid receptors in postmortem frontal cortex of subjects with schizophrenia: effect of antipsychotic treatment. Psychopharmacology (Berl) 2009;206:313–24.
21. Wang H, Dey SK, Maccarrone M. Jekyll and hyde: two faces of cannabinoid signaling in male and female fertility. Endocr Rev 2006;27:427–48.
22. Taylor AH, Ang C, Bell SC, Konje JC. The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. Hum Reprod Update 2007;13:501–13.
23. Sun X, Dey SK. Endocannabinoid signaling in female reproduction. ACS Chem Neurosci 2012;3:349–55.
24. Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. J Clin Endocrinol Metab 2005; 90:984–91.
25. De Jesus ML, Salles J, Meana JJ, Callado LF. Characterization of CB1 cannabinoid receptor immunoreactivity in postmortem human brain homogenates. Neuroscience 2006;140:635–43.
26. Filppula S, Yaddanapudi S, Mercier R, Xu W, Pavlopoulos S, Makriyannis A. Purification and mass spectroscopic analysis of human CB2 cannabinoid receptor expressed in the baculovirus system. J Pept Res 2004;64:225–36.
27. Zhang R, Kim TK, Qiao ZH, Cai J, Pierce WM Jr, Song ZH. Biochemical and mass spectrometric characterization of the human CB2 cannabinoid receptor expressed in Pichia pastoris—importance of correct processing of the N-terminus. Protein Expr Purif 2007;55:225–35.
28. Agirregoitia E, Carracedo A, Subiran N, Valdivia A, Agirregoitia N, Peralta L, et al. The CB(2) cannabinoid receptor regulates human sperm cell motility. Fertil Steril 2010;93:1378–87.
29. Bisogno T, Katayama K, Melck D, Ueda N, De Petrocellis L, Yamamoto S, et al. Biosynthesis and degradation of bioactive fatty acid amides in human breast cancer and rat pheochromocytoma cells—implications for cell proliferation and differentiation. Eur J Biochem 1998;254:634–42.
30. Francavilla F, Battista N, Barbonetti A, Vassallo MR, Rapino C, Antonangelo C, et al. Characterization of the endocannabinoid system in human spermatozoa and involvement of transient receptor potential vanilloid 1 receptor in their fertilizing ability. Endocrinology 2009;150:4692–700.
31. Bakali E, Elliott RA, Taylor AH, Willets J, Konje JC, Tincello DG. Distribution and function of the endocannabinoid system in the rat and human bladder. Int Urogynecol J 2013;24:855–63.
32. Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 2002;99:10819–24.
33. Wang H, Xie H, Dey SK. Endocannabinoid signaling directs periimplantation events. AAPS J 2006;8:E425–32.
34. Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL, Dey SK. Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. Proc Natl Acad Sci U S A 2003;100: 14914–9.
35. Paria BC, Zhao X, Wang J, Das SK, Dey SK. Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. Biol Reprod 1999;60:1151–7.
36. Gebeh AK, Willets JM, Marczylo EL, Taylor AH, Konje JC. Ectopic pregnancy is associated with high anandamide levels and aberrant expression of FAAH and CB1 in fallopian tubes. J Clin Endocrinol Metab 2012;97:2827–35.
37. Hunt PA, Hassold TJ. Human female meiosis: what makes a good egg go bad? Trends Genet 2008;24:86–93.
38. Bouskila J, Burke MW, Zabouri N, Casanova C, Ptito M, Bouchard JF. Expression and localization of the cannabinoid receptor type 1 and the enzyme fatty acid amide hydrolase in the retina of vervet monkeys. Neuroscience 2012;202:117–30.
39. Schuel H, Burkman LJ. A tale of two cells: endocannabinoid-signaling regulates functions of neurons and sperm. Biol Reprod 2005;73:1078–86.
40. Wang H, Xie H, Sun X, Kingsley PJ, Marnett LJ, Cravatt BF, et al. Differential regulation of endocannabinoid synthesis and degradation in the uterus during embryo implantation. Prostaglandins Other Lipid Mediat 2007;83:62–74.

Cell Tissue Res (2016) 365:393–401
DOI 10.1007/s00441-016-2381-2

REGULAR ARTICLE

Dynamic of expression and localization of cannabinoid-degrading enzymes FAAH and MGLL in relation to CB1 during meiotic maturation of human oocytes

Ekaitz Agirregoitia[1] · Lide Totorikaguena[1] · Antonia Expósito[2] · Rosario Mendoza[2] · Roberto Matorras[2] · Naiara Agirregoitia[1]

Received: 22 November 2015 / Accepted: 19 February 2016 / Published online: 7 March 2016

Abstract The endogenous cannabinoid system has been characterized in some female reproductive organs but little is known about the expression and localization pattern of cannabinoid-degrading enzymes in relation to the CB1 cannabinoid receptor in human oocytes. In this study, we focus on the investigation of the presence and differential distribution of fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL) in relation to CB1 during the maturation of human oocytes. We used a total of 290 human oocytes not suitable for in vitro fertilization/intracytoplasmic sperm injection (ICSI): germinal-vesicle (GV) and metaphase-I (MI) stages and metaphase-II (MII) oocytes that had not developed into an embryo after ICSI. Cannabinoid-degrading enzymes and the cannabinoid CB1 receptor were present in human oocytes. Specifically, FAAH was detected in the periphery of the oocyte from the GV to MI stage and co-localized with CB1. Later, by the MII stage, FAAH was spread within the oocyte, whereas MGLL immunostaining was homogeneous across the oocyte at all stages of maturation and only overlapped with CB1 at the GV stage. This coordinated redistribution of cannabinoid system proteins suggests a role for this system in the maturation of the female gamete.

Keywords Meiosis · Resumption · Enzyme · Maturation · Gamete

This work was supported by a grant from the University of the Basque Country (GIU14/26).

Electronic supplementary material The online version of this article (doi:10.1007/s00441-016-2381-2) contains supplementary material, which is available to authorized users.

✉ Ekaitz Agirregoitia
e.agirregoitia@ehu.eus

[1] Department of Physiology, Faculty of Medicine and Dentistry, University of the Basque Country, Barrio Sarriena s/n, Leioa 48940, Bizkaia, Spain

[2] Human Reproduction Unit, Cruces University Hospital, University of the Basque Country, Plaza de Cruces s/n, Barakaldo 48903, Bizkaia, Spain

Introduction

Oocyte meiotic maturation is the process by which immature oocytes become fertilizable eggs. Fully grown oocytes are arrested at the first meiotic prophase, in the germinal vesicle (GV) stage and, once they become meiotically competent, resume meiosis upon receiving specific ovulatory signals. Meiotic maturation continues with the transition from meiosis I to meiosis II until oocytes are then arrested at metaphase of meiosis II (MII); an oocyte does not complete meiosis II until it is fertilized by sperm (Schindler 2011).

The molecular mechanisms responsible for oocyte maturation are not fully understood and several different molecules have been implicated as modulators of the process. Among these molecules, the role of the cannabinoid compounds has been highlighted by some authors (Sun and Dey 2012). Endocannabinoids are fatty acid derivatives that exert their effects by binding to membrane Gi/o protein-coupled receptors CB1 and CB2. Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids. Both are degraded by fatty acid amide hydrolase (FAAH), which is an integral membrane protein with its hydrolytic site facing the lipid bilayer, although 2-AG is also degraded by monoacylglycerol lipase (MGLL), which, in contrast, is a cytosolic enzyme. The distributions of FAAH and MGLL overlap in the central nervous system, but they are not identical. For example, in some cerebral areas, MGLL

is localized presynaptically, whereas FAAH is localized postsynaptically. Nevertheless, one proposal is that, together, endocannabinoid-metabolizing enzymes can complement the distribution of cannabinoid receptors in the brain and that this provides multiple opportunities for local regulation of endocannabinoid tone (Yazulla 2008).

Several papers have been published on the role of cannabinoids in the female reproductive system (de Miguel et al. 1998; Wenger et al. 1999, 2001) and both cannabinoid receptors and degradation enzymes have been found in various parts of the mammalian female reproductive system, namely, the uterus and oviduct (Das et al. 1995; Paria et al. 2001; Wang et al. 2004; Dennedy et al. 2004) preimplantation embryos and the placenta (Park et al. 2003; Helliwell et al. 2004; Habayeb et al. 2008). With regard to oocyte maturation, AEA is known to be present in human follicular fluid (Schuel et al. 2002) and its concentration is higher in follicles with mature oocytes than in those with immature oocytes (El-Talatini et al. 2009). The localization of the cannabinoid receptors and degrading enzymes in rat (Bagavandoss and Grimshaw 2010) and human ovary has led to the hypothesis that AEA plays a role in folliculogenesis, preovulatory follicle maturation oocyte maturity, and ovulation (El-Talatini et al. 2009). Finally, the hypothesis with respect to the role of the cannabinoid system in oocyte maturation has been reinforced by findings concerning the localization of cannabinoid receptors during the nuclear maturation of human oocytes, in which each receptor is re-localized (Peralta et al. 2011).

In view of the presence of AEA in human follicular fluid and the re-localization of cannabinoid receptors observed in the oocyte during its maturation, cannabinoid signaling can be postulated to be involved in the regulation of oocyte meiosis (Peralta et al. 2011). Therefore, to shed further light on this issue, our aim is to characterize, in detail and to compare the expression and distribution of the two AEA-degrading enzymes, FAAH and MGLL and further to explore their relationship with the CB1 receptor in human oocytes during the meiotic resumption of oocytes obtained during in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles.

Materials and methods

Oocyte collection

Human oocytes were obtained from 90 patients (aged 25–40 years; mean 35.02 ± 3.23 years) undergoing IVF/ICSI at the Human Reproduction Unit of Cruces University Hospital. Ethical approval was obtained from the Clinical Research Ethics Committee of the Basque Health System (Osakidetza, CEIC reference number E07/54, 3/2008). Informed consent was obtained from all patients. Overall, 290 oocytes from these patients were analyzed. The main indications for IVF/ICSI were: male factor (62 %), failure of intrauterine insemination (7 %) and idiopathic infertility (10 %). The main associated female conditions were endometriosis (9.5 %) and tubal factor (11.5 %). Patients had no previous history of cannabinoid drug consumption.

All the oocytes used for this study were those discarded from treatment cycles. The majority of oocytes corresponded to immature oocytes (GV and MI) or to MII oocytes from IVF/ICSI procedures in which fertilization failed. Additionally, 8 cases (44 oocytes) were included in which IVF/ICSI could not be attempted because of failure to obtain a sperm sample, since at that time oocyte vitrification was not available in our laboratory. After removal of the corona cumulus cells, the oocytes were stored and transported in minimal media in micro-centrifuge tubes at 4 °C to the laboratory for processing. The zona pellucida of oocytes used for analysis by the reverse transcription plus polymerase chain reaction (RT-PCR) was removed by incubating oocytes in a Tyrode's acidic solution at 37 °C for 30 s in order to detach possible granulose cells.

Our IVF cycle management has been described previously (Matorras et al. 2009). Briefly, it consists in long down-regulation with the gonadotropin-releasing hormone agonist triptorelin (Decapeptyl, Ipsen Pharma, Spain) or the gonadotrophin-releasing hormone antagonist cetrorelix (Cetrotide; Merck Serono, Spain). Then, ovarian stimulation was carried out with recombinant follicle-stimulating hormone (Gonal F; Merck Serono) and highly purified urinary menopausal gonadotropins (Menopur, Ferring, Spain) or recombinant luteinizing hormone (LH), ovulation being triggered with 250 mg recombinant human chorionic gonadotrophin (hCG; Ovitrelle; Merck Serono). Transvaginal ultrasound-guided oocyte retrieval was scheduled 36 h after hCG injection.

All IVF/ICSI procedures and assessments were performed by members of the research group. Oocyte retrieval was performed 35–37 h after hCG administration. Follicles were aspirated with a negative pressure of 115–120 mmHg with a single lumen 18-gauge oocyte pick-up needle (K-OPS-6035-RWH-B-ET; Cook, Spain) under transvaginal ultrasound guidance. Follicular fluids were examined at low magnification (40× to 100×) under a stereomicroscope at 37 °C. The oocyte–cumulus-complexes were incubated for 2 h in IVF Medium (Origio, Denmark) at 37 °C and under 6 % CO_2 in air. Immediately prior to micromanipulation during the ICSI procedure, oocytes were denuded of the cumulus oophorus by brief exposure to 40 IU/L hyaluronidase solution (Hyadase; Origio), followed by mechanical removal of the corona radiate cells with the use of plastic pipettes (denuding pipette; Cook, Spain). Each oocyte was examined under the microscope to assess its maturation stage and integrity. MII oocytes were

defined by the absence of the germinal vesicle and the presence of an extruded polar body. Metaphase II oocytes were microinjected and placed in the culture incubator following the standard IVF culture protocol. Oocyte fertilization assessment involved careful analysis of the two pronuclei and the nucleoli within them, during a single examination 16–18 h after the insemination procedure. Normally fertilized and non-fertilized oocytes were then placed in the incubator for a 24-h culture period until embryo selection for transfer. The immature oocytes (at MI and GV stages) and the non-fertilized MII oocytes were placed in microcentrifuge tubes with a minimal amount of Flushing Medium (Origio) and stored at 4 °C or at −80 °C until used in the present study.

Of the 290 studied oocytes, 59 were at the GV stage, 65 at the MI stage and 166 at the MII stage. MII oocytes were collected on day 1 after IVF/ICSI, when fertilization failed, i.e., they were unfertilized. GV and MI oocytes were collected on the day of oocyte pick up in ICSI cases. During the period of study, the fertilization rate of MII oocytes was 64 % in our ICSI program.

RT-PCR analysis

Total RNA from oocytes (3 samples of ~30 oocytes, from each maturation stage: GV, MI and MII) and cerebral cortex was extracted by using the Dynabeads mRNA-DIRECT Kit (Ambion, Austin, Tex., USA) following the manufacturer's instructions. Immediately after extraction, total RNA was kept frozen at −80 °C until further use. cDNA was obtained with the ImProm-II Reverse Transcription System (Promega, Madison, Wis., USA). Briefly, a mixture of about 150 ng RNA and random primers was heated at 65 °C for 10 min and chilled on ice for 5 min. Then, after addition of the reverse transcription mix, it was annealed at 25 °C for 5 min. First-strand synthesis was carried out at 55 °C for 60 min and the reverse transcriptase was inactivated at 70 °C for 15 min. Primers used for PCR were as follows: human *FAAH*, 5′-AAGCAACATACCCCATGCTC-3′ and 5′-GTTTTGCGGT ACACCTCGAT-3′ (276-bp product; Storr et al. 2009); human *MGLL*, 5′-CAAGGCCCTCATCTTTGTGT-3′ and 5′-ACGTGGAAGTCAGACACTAC-3′ (162-bp product; Ludanyi et al. 2008). Human *ACTB* (β-actin) primers, 5′-TCCCTGGAGAAGAGCTACGA-3′ and 5′-ATCTGCTGGAAGGTGGACAG-3′ (362-bp product; Agirregoitia et al. 2010) were used as internal controls. All primer pairs were located on different exons to avoid amplification of genomic DNA.

PCR was performed under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The mixture was electrophoretically separated on a 2 % agarose gel.

Immunofluorescence

After assessment of the meiotic stage, oocytes were suspended in phosphate-buffered saline plus bovine serum albumin (PBS+BSA; 1 μg/μl) and smeared onto a slide coated with poly-L-lysine, in order to localize the target protein immunocytochemically. They were then fixed with 3 % paraformaldehyde for 10 min followed by incubation in Triton X100 (0.1 %) for 10 min to permeabilize the oocytes. Subsequently, slides were washed three times in PBS and incubated for 20 min in PBS / 10 % (v/v) bovine fetal serum at room temperature. For indirect immunofluorescence staining, slides were incubated with rabbit polyclonal FAAH and MGLL antibodies (Cayman Chemicals, Ann Arbor, Mich., USA) at a dilution of 1:200 overnight at 4 °C. For double-staining, in addition to the anti-FAAH or anti-MGLL antibodies, we used goat polyclonal CB1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at a dilution of 1:50. Slides were then washed in PBS+BSA (1 μg/μl) three times, incubated with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 donkey anti-goat IgG secondary antibodies (Molecular Probes; Eugene, Ore., USA) for 2 h at 37 °C in the dark and washed in PBS+BSA (1 μg/μl) three times with staining of the nuclei with Hoechst 33342 during the second wash to facilitate assessment of the maturational stage of each oocyte. Finally, oocytes were mounted with Fluoromount G (EMS; Hatfield, England) and examined by confocal microscopy. Images were acquired with an Olympus Fluoview FV500 confocal microscope by using a 40× lens (UPLAPO; NA: 0.85). Each fluorochrome was sequentially acquired in order to avoid overlapping of fluorescent emission spectra. Two negative controls were performed by using specific blocking peptides for each antibody (Cayman Chemicals) and by omitting the primary antibody before addition of the secondary antibody to set the background acquisition parameters.

Results

RT-PCR analysis of CB1 and cannabinoid-degrading enzyme mRNA in human oocytes

We detected the presence of *CB1* and *FAAH* but not *MGLL* degrading-enzyme transcripts in human oocytes by using RT-PCR. The expected 276-bp fragment for *FAAH* was detected during all stages of oocyte maturation and in samples of gray matter of human prefrontal cerebral cortex (positive control; Fig. 1a). The 162-bp fragment corresponding to *MGLL* was detected in human cortex (positive control) but not in oocytes at any stage of maturation (Fig. 1b). The housekeeping gene *ACTB* was detected in all tissues (Fig. 1c).

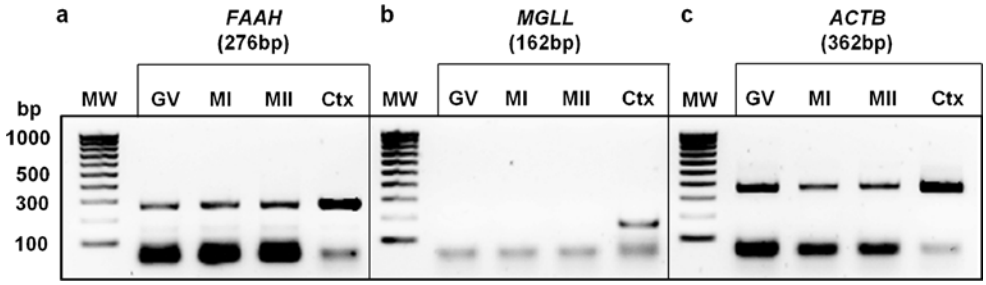


Fig. 1 Ethidium-bromide-stained 2 % agarose electrophoresis gels of the reverse transcription plus polymerase chain reaction (RT-PCR) products for fatty acid amide hydrolase (*FAAH*), monoglyceride lipase (*MGLL*) and β-actin (*ACTB*) in the human oocyte at the germinal vesicle (*GV*), metaphase I (*MI*) and metaphase II (*MII*) stages and in gray matter from the human prefrontal cerebral cortex (*Ctx*). **a** Amplified fragments obtained with primers specific for the human cannabinoid-degrading enzyme FAAH (276-bp band). **b** Amplified fragments obtained with primers specific for the human cannabinoid-degrading enzyme MGLL (162-bp band). **c** ACTB was used as an internal control (362-bp band). Molecular weights (*MW*; *bp*) are indicated *left*. $n = 3$. A representative RT-PCR experiment is shown

Immunocytochemical localization of cannabinoid-degrading enzymes FAAH and MGLL in human oocytes

Immunofluorescence analysis revealed that both FAAH and MGLL were present in the human oocytes. However, the localization of these enzymes showed variations through the stages of the resumption of meiosis in human oocytes. On the one hand, FAAH localization in the oocyte was peripheral at the GV (Fig. 2a) and MI (Fig. 2b) stages, whereas at the MII (Fig. 2c) stage, it was found peripherally but also appeared homogeneously over the entire oocyte (z-stack images in Supplementary Figs. 1, 3). On the other hand, MGLL immunostaining was found homogeneously in oocytes from the GV to MII stages (Fig. 2e-g; see also z-stacks images in Supplementary Figs. 2, 4). When the anti-FAAH and anti-MGLL receptor antibodies were blocked with the specific peptide before addition to the oocyte, no specific fluorescence was observed (Fig. 2d, h).

Immunocytochemical localization of FAAH and MGLL compared with that of CB1 receptor during maturation of human oocytes

In the GV (Fig. 3c) and MI (Fig. 3f) stages, FAAH co-localized with the CB1 receptor at the periphery of the oocyte, although the CB1 receptor was also observed in the cytoplasm at MI (Fig. 3d, f). At the MII stage, the localization of FAAH

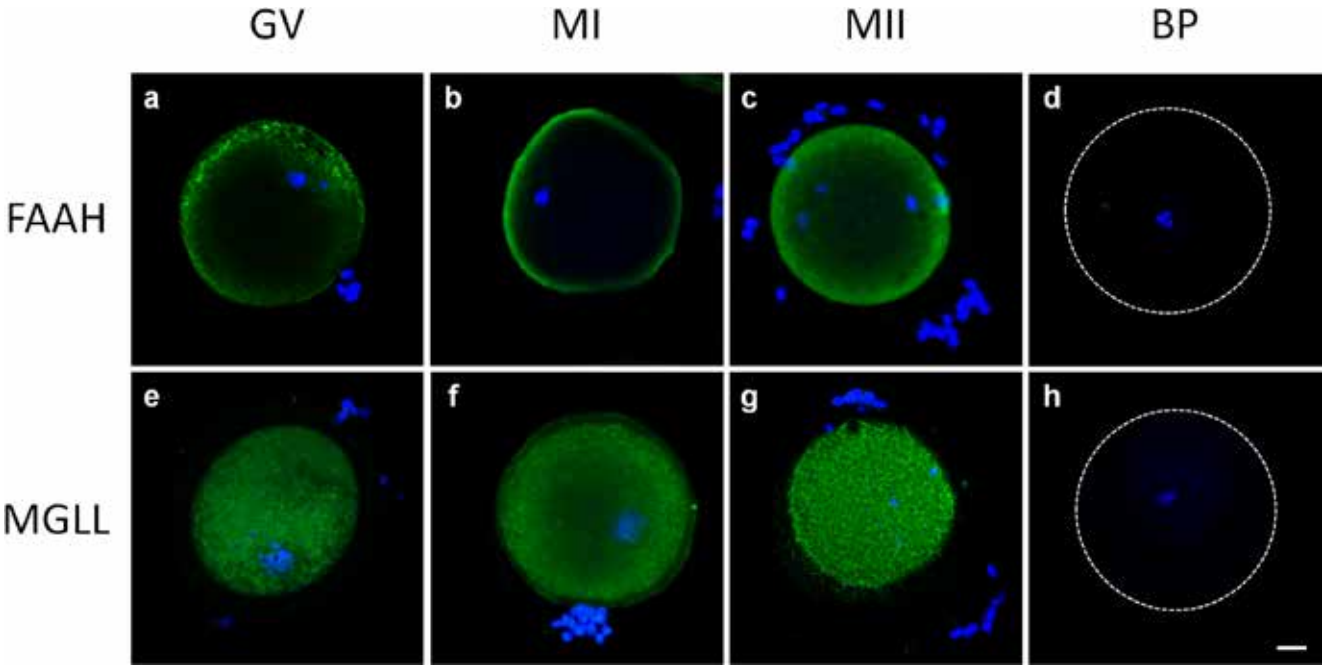


Fig. 2 Immunofluorescence analysis of cannabinoid-degrading enzymes in human oocytes. Distributions of FAAH and MGLL are shown in *green* at (**a**, **e**) the germinal vesicle (GV), (**b**, **f**) metaphase I (MI) and (**c**, **g**) metaphase II (MII) stages. Phase contrast images (**d**, **h**) are shown of the negative control, consisting in pre-adsorption of the primary antiserum with the specific blocking peptide (*BP*). Hoechst-labeled DNA is shown in *blue*. $n = 15$ per stage. Representative photomicrographs are shown. *Bar* 20 μm

had changed (Fig. 3h): the enzyme appeared homogeneously over the entire oocyte, whereas all the CB1 immunostainig appeared at the periphery at MII (Fig. 3g). At MII, FAAH co-localized with CB1 to a lesser extent than at previous stages (Fig. 3i; see also z-stack images in Supplementary Fig. 1). With regard to MGLL, this enzyme showed a weak co-localization with the CB1 receptor at the GV (Fig. 4c) stage, but generally, in other stages, the localization of CB1 and MGLL was different: peripheral for CB1 (Fig. 4d, g) and cytoplasmic for MGLL (Fig. 4e, h; see also z-stack images in Supplementary Fig. 2).

Discussion

The role of the endocannabinoid system has been studied in various mammalian reproductive processes, such as spermatogenesis, sperm motility, fertilization, embryo oviductal transport, preimplantation embryo development, implantation, and post-implantation embryonic growth (Wang et al. 2006a, 2006b; Taylor et al. 2007; Sun and Dey 2012). Even so, the part played by this system in the ovary and in oocyte events has rarely been analyzed (El-Talatini et al. 2009; Bagavandoss and Grimshaw 2010; Peralta et al. 2011). The aim of the present study was to gather further data on the presence and localization of cannabinoid-degrading enzymes in relation to the CB1 cannabinoid receptor during the resumption of meiosis in human oocytes in order to form a basis for explaining a possible role of the cannabinoid system in this process.

Our immunofluorescence analysis of human oocytes has confirmed the presence of cannabinoid-degrading enzymes FAAH and MGLL in these cells. As FAAH is a membrane-bound protein (Deutsch et al. 2002), its observed localization in the oocyte periphery indicates that it is localized to the oolema. On the contrary, MGLL is a cytosolic serine hydrolase (Dinh et al. 2002) and this could explain the homogeneous localization of MGLL over the entire oocyte observed in our experiments.

Several authors have described the presence of cannabinoid system components in the oocyte. In rat ovaries, FAAH and the CB2 receptor have been found in both preantral and antral follicle oocytes, whereas the CB1 receptor is not present (Bagavandoss and Grimshaw 2010). In human ovaries, however, only FAAH has been found localized to the theca cells of secondary and tertiary follicles, whereas CB1 immunostaining appears in oocytes from primordial to secondary follicles but

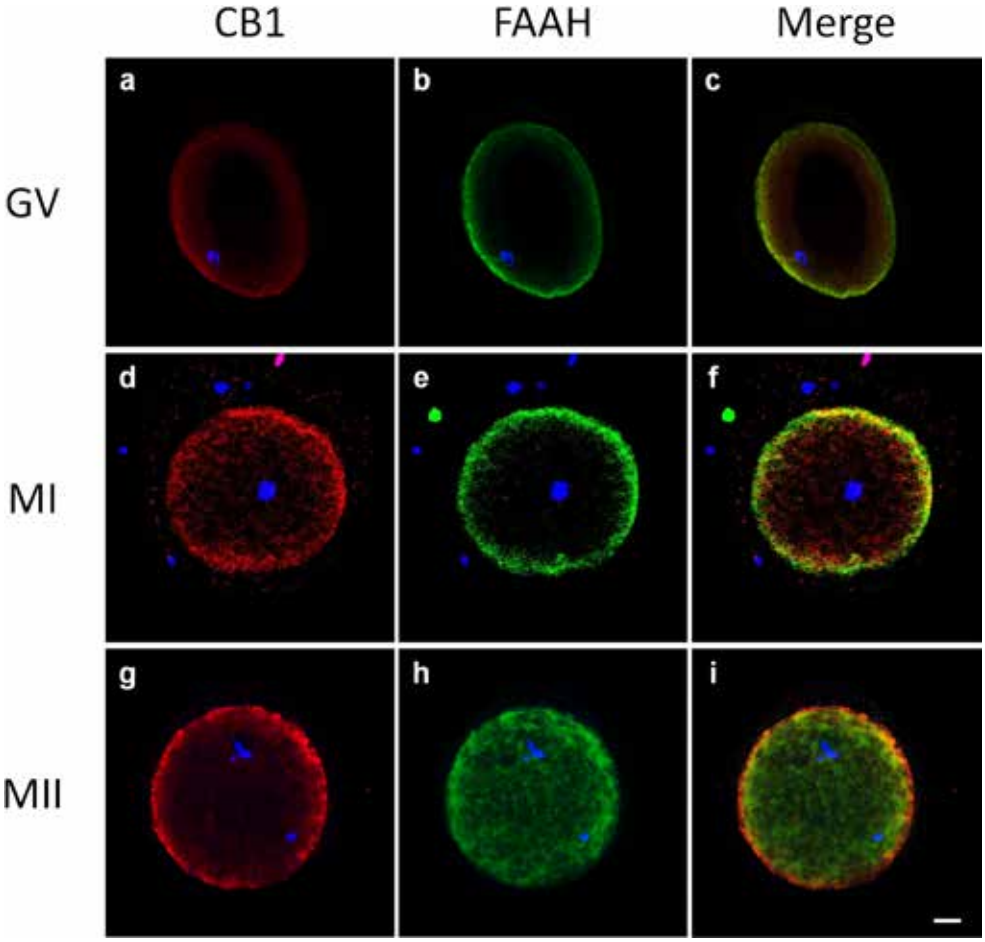


Fig. 3 Immunofluorescence analysis of cannabinoid-degrading enzymes compared with CB1 cannabinoid receptor in human oocytes. Distributions of FAAH (**b**, **e**, **h**), CB1 (**a**, **d**, **g**) and their overlap (**c**, **f**, **i**) are shown in *green*, *red*, *yellow*, respectively, at (**a-c**) the germinal vesicle (GV), (**d-f**) the metaphase I (MI) and (**g-i**) the metaphase II (MII) stages. $n = 15$ per stage. Representative photomicrographs are shown. *Bar* 20 μm

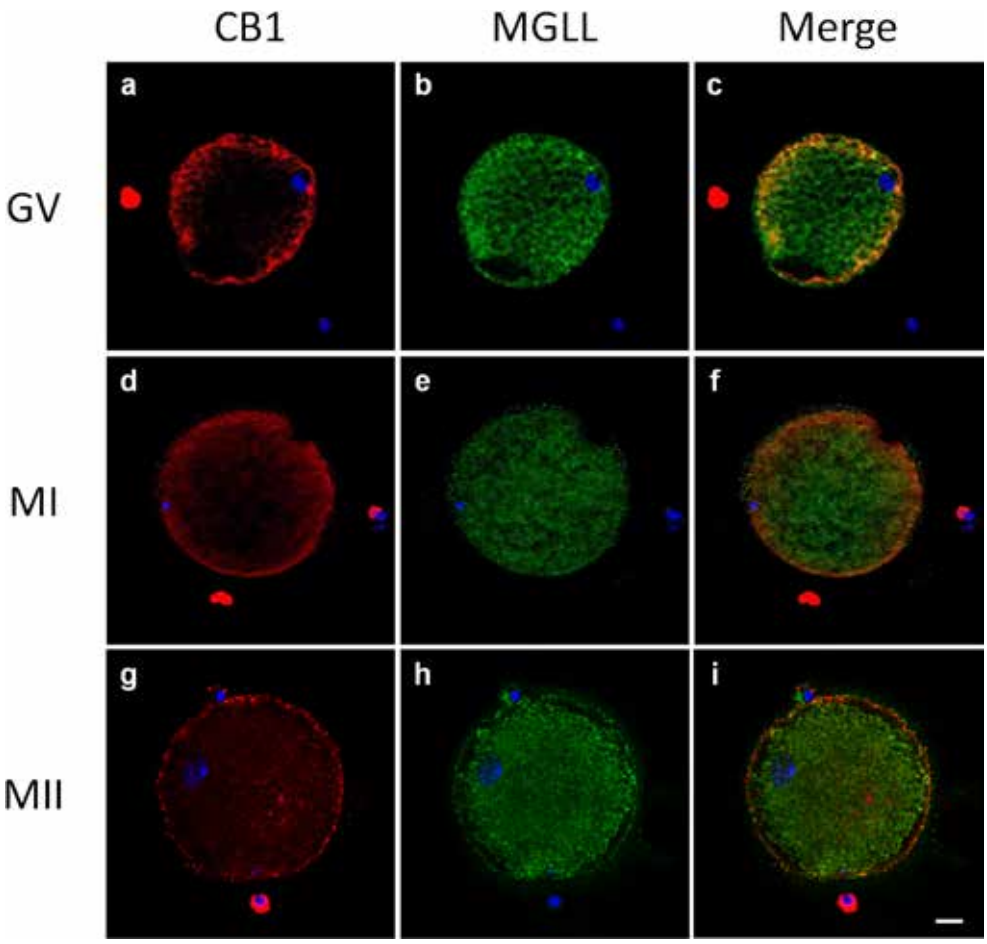


Fig. 4 Immunofluorescence analysis of cannabinoid-degrading enzymes compared with CB1 cannabinoid receptor in human oocytes. Distributions of MGLL (**b**, **e**, **h**), CB1 (**a**, **d**, **g**) and their overlap (**c**, **f**, **i**) are shown in *green*, *red* and *yellow*, respectively, at (**a-c**) the germinal vesicle (GV), (**d-f**) the metaphase I (MI) and (**g-i**) the metaphase II (MII) stages. $n = 15$ per stage. Representative photomicrographs are shown. *Bar* 20 μm

not in tertiary follicles and, in contrast, intense CB2 staining has been observed in oocytes during all stages of follicle maturation (El-Talatini et al. 2009). Finally, in a study focused on human oocytes separated from follicles, the presence of both CB1 and CB2 has been corroborated during the resumption of oocyte meiosis from the GV to MII stages (Peralta et al. 2011). Whether the differences in the localization of CB1, CB2 and FAAH during the follicle and/or oocyte maturation observed in the aforementioned three studies reflect differences between species or are also attributable to differences in methodology (Bagavandoss and Grimshaw 2010; Peralta et al. 2011) is unclear. In any case, taken together, all the evidence to date leads us to believe that the previous non-detection of FAAH in human oocytes (El-Talatini et al. 2009) is attributable to a relatively low level of expression of FAAH rather than its absence. Three statements uphold this view. First, FAAH has previously been detected in rat oocytes (Bagavandoss and Grimshaw 2010) and has again been detected in our present study by using only the human oocyte separated from follicles. Second, the staining observed specifically in the plasma membrane in the present study was achieved by using a specific anti-FAAH antibody and verified with a blocking peptide. Finally, we detected, for the first time, the presence of FAAH mRNA in human oocytes. Further, the observed progression of the localization of the CB1 receptor in the present study is similar to that observed in a previous study of our group with a different antibody (Peralta et al. 2011) and as such, strengthens the evidence for its presence and re-localization pattern during the resumption of oocyte meiosis. In contrast, we were unable to compare our MGLL localization with the results of other studies, since, to the best of our knowledge, this is the first time that MGLL has been localized to the oocyte.

The CB1 and FAAH immunostaining patterns changed during the various stages of meiosis resumption, as occurs with other proteins (Kume et al. 1997; Agirregoitia et al. 2012) and also with the CB2 receptor in mouse spermatogenesis (Grimaldi et al. 2009) and in human oocyte maturation (Peralta et al. 2011). In contrast, MGLL maintained its cytoplasmic location throughout all the observed stages. Moreover, CB1 and FAAH co-localized in the periphery at the GV and MI stages but they overlapped less at MII. An overlapping pattern in the expression of CB1 and FAAH has been reported in other cell types, such as neurons, suggesting that the endocannabinoid system is responsible for an autofeedback control of neurotransmitter release (Bouskila et al.

2012). In meiotic progression, CB1 shows some internalization at MI but is then re-localized to the periphery at MII, whereas FAAH is internalized at the MII stage. This differing re-localization suggests a possible coordinated redistribution of CB1 and FAAH during the resumption of oocyte meiosis in order to regulate local endocannabinoid tone, as has been proposed for other cell types (Yazulla 2008). This is plausible since, in mammals, all of the qualitative and quantitative changes in protein synthesis occurring during oogenesis take place at the time of the resumption of meiosis (Picton et al. 1998). Additionally, Ji et al. (1997) observed modifications in the protein pattern of human oocyte membranes during preovulatory maturation. More interesting still is our study in which we detected CB1 and FAAH in granulosa cells from GV to MII oocytes and their co-localization in some portions of the cell membrane. Moreover, MGLL immunostaining was more homogeneous across the cell and only overlapped with CB1 weakly (Agirregoitia et al. 2015).

The reason for CB1 and FAAH re-localization during meiosis has not been studied at all. Even so, according to the classical theory of G-protein-coupled receptor (GPCR) functionality, these receptors have to reach the cell surface to act and, after agonist binding, GPCRs undergo a rapid "desensitization" and the ligand-receptor complex is internalized prior to being recycled back to the cell surface or being degraded (Cahill et al. 2007). Therefore, taking into account our data for the CB1 receptor and FAAH-degrading enzyme, we can retain our previous hypothesis that CB1 is activated at the GV stage (when CB1 is localized at the plasma membrane) and subsequently internalized when the oocyte reaches the MI stage (when CB1 is also found within the cytoplasm; Peralta et al. 2011). Thus, as FAAH co-localizes with CB1 at the GV and MI stages, it could participate in the degradation of the cannabinoid that is bound to CB1 when the cannabinoid is internalized together with CB1 at MI. Additionally, at least at the GV and MI stages, FAAH might degrade free cannabinoids, which would enter the active site via the membrane (McKinney and Cravatt 2005). Then, according to our results, the receptor could be recycled, being mostly localized at the plasma membrane once again when the oocyte is blocked at the MII stage but FAAH cannot act at the periphery of oocyte because of its internalization. The presence of MGLL in the cytoplasm of oocytes might help FAAH in the degradation of cannabinoids (Yazulla 2008). Nevertheless, the co-localized MGLL and CB1 observed at GV might have a coordinated function, as has also been described in axon terminals of some rat cerebral areas (Gulyas et al. 2004).

The regulation of "AEA tone" by FAAH has been proposed as an important event in the oviduct during early pregnancy (Wang et al. 2006a, 2006b), in blastocyst activation (Wang et al. 2003) and during implantation (Paria et al. 1999), but no hypotheses have been proposed in relation to such a process in oocyte maturation. Even so, the AEA concentration in human follicular fluid has been reported to rise during oocyte maturation (El-Talatini et al. 2009) and this concentration is even higher in the oviduct (Gebeh et al. 2012). In humans, on average, one oocyte completes growth each month and is ovulated in response to a mid-cycle surge of LH and in response to this LH surge, the oocyte resumes meiosis (Hunt and Hassold 2008). Hence, the resumption of meiosis in oocytes occurring in the oviduct, where the AEA concentration is higher than in follicular fluid and the relocation of CB1 and FAAH during nuclear maturation lead to the hypothesis that signaling involving AEA, CB1 and FAAH in the oocyte is involved in the resumption of meiosis. To date, the presence of 2-AG has only been described in the uterus of the female reproductive tract (Wang et al. 2007) but whether it is present in the ovary or the oviduct is unknown; this merits investigation to elucidate the role of MGLL during the resumption of meiosis. In addition, the presence of CB1, FAAH and MGLL in granulosa cells suggests that the endocannabinoids act not only over the oocyte but also over the granulosa cells (Agirregoitia et al. 2015).

In the present study, we found *FAAH* but not *MGLL* mRNA in mature oocytes, although the proteins of both receptors are present during all stages of meiotic resumption. *MGLL* mRNA might be absent because its transcription becomes silent after the resumption of meiosis until the 4- to 8-cell stage (Gosden and Lee 2010) and because the mRNA is selectivity degraded during oocyte maturation (Stitzel and Seydoux 2007). Moreover, the timing and pattern of RNA synthesis in the oocyte is known not necessarily to coincide with translation into an active protein (Picton et al. 1998). Importantly, we do not consider that our observations concerning the behavior of the compounds of the cannabinoid system studied in this work can be attributed to an artifact associated with fertilization failure, since our results from unfertilized oocytes and non-sperm-exposed oocytes are not significantly different (Supplementary Fig. 5).

In conclusion, our study reports, for the first time, the presence of the cannabinoid-degrading enzymes FAAH and MGLL during the meiotic resumption in human oocytes. As CB1 and CB2 are present in oocytes (Peralta et al. 2011) and as AEA is present in follicular and oviductal fluids (Schuel et al. 2002; El-Talatini et al. 2009), our results suggest that oocytes can regulate the "AEA tone" near the microenvironment in which the communication between oocyte and granulosa cells takes place. Further studies are needed to investigate the role of the cannabinoid system during the nuclear maturation of the oocyte, because our results might have an impact on the design of culture media for assisted reproductive technology and treatments, especially for in vitro maturation.

Acknowledgments We thank SGIker (UPV/EHU) for personal and technical support and particularly Ricardo Andrade for his assistance with the confocal microscopy analysis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests

References

Agirregoitia E, Carracedo A, Subiran N, Valdivia A, Agirregoitia N, Peralta L, Velasco G, Irazusta J (2010) The CB(2) cannabinoid receptor regulates human sperm cell motility. Fertil Steril 93:1378–1387

Agirregoitia E, Peralta L, Mendoza R, Exposito A, Ereno ED, Matorras R, Agirregoitia N (2012) Expression and localization of opioid receptors during the maturation of human oocytes. Reprod Biomed Online 24:550–557

Agirregoitia E, Ibarra-Lecue I, Totorikaguena L, Mendoza R, Expósito A, Matorras R, Urigüen L, Agirregoitia N (2015) Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation. Fertil Steril 104:753–760

Bagavandoss P, Grimshaw S (2010) Temporal and spatial distribution of the cannabinoid receptors (CB1, CB2) and fatty acid amide hydroxylase in the rat ovary. Anat Rec (Hoboken) 293:1425–1432

Bouskila J, Burke MW, Zabouri N, Casanova C, Ptito M, Bouchard JF (2012) Expression and localization of the cannabinoid receptor type 1 and the enzyme fatty acid amide hydrolase in the retina of vervet monkeys. Neuroscience 202:117–130

Cahill CM, Holdridge SV, Morinville A (2007) Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. Trends Pharmacol Sci 28:23–31

Das SK, Paria BC, Chakraborty I, Dey SK (1995) Cannabinoid ligand-receptor signaling in the mouse uterus. Proc Natl Acad Sci U S A 92:4332–4336

de Miguel R, Romero J, Munoz RM, Garcia-Gil L, Gonzalez S, Villanua MA, Makriyannis A, Ramos JA, Fernandez-Ruiz JJ (1998) Effects of cannabinoids on prolactin and gonadotrophin secretion: involvement of changes in hypothalamic gamma-aminobutyric acid (GABA) inputs. Biochem Pharmacol 56:1331–1338

Dennedy MC, Friel AM, Houlihan DD, Broderick VM, Smith T, Morrison JJ (2004) Cannabinoids and the human uterus during pregnancy. Am J Obstet Gynecol 190:2–9

Deutsch DG, Ueda N, Yamamoto S (2002) The fatty acid amide hydrolase (FAAH). Prostaglandins Leukot Essent Fatty Acids 66:201–210

Dinh TP, Freund TF, Piomelli D (2002) A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. Chem Phys Lipids 121:149–158

El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC, Konje JC (2009) Localisation and function of the endocannabinoid system in the human ovary. PLoS One 4:e4579

Gebeh AK, Willets JM, Marczylo EL, Taylor AH, Konje JC (2012) Ectopic pregnancy is associated with high anandamide levels and aberrant expression of FAAH and CB1 in fallopian tubes. J Clin Endocrinol Metab 97:2827–2835

Gosden R, Lee B (2010) Portrait of an oocyte: our obscure origin. J Clin Invest 120:973–983

Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, Geremia R, De Petrocellis L, Di Marzo V (2009) The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. Proc Natl Acad Sci U S A 106:11131–11136

Gulyas AI, Cravatt BF, Bracey MH, Dinh TP, Piomelli D, Boscia F, Freund TF (2004) Segregation of two endocannabinoid-hydrolyzing enzymes into pre- and postsynaptic compartments in the rat hippocampus, cerebellum and amygdala. Eur J Neurosci 20:441–458

Habayeb OM, Taylor AH, Bell SC, Taylor DJ, Konje JC (2008) Expression of the endocannabinoid system in human first trimester placenta and its role in trophoblast proliferation. Endocrinology 149:5052–5060

Helliwell RJ, Chamley LW, Blake-Palmer K, Mitchell MD, Wu J, Kearn CS, Glass M (2004) Characterization of the endocannabinoid system in early human pregnancy. J Clin Endocrinol Metab 89:5168–5174

Hunt PA, Hassold TJ (2008) Human female meiosis: what makes a good egg go bad? Trends Genet 24:86–93

Ji YZ, Bomsel M, Jouannet P, Wolf JP (1997) Modifications of the human oocyte plasma membrane protein pattern during preovulatory maturation. Mol Reprod Dev 47:120–126

Kume S, Yamamoto A, Inoue T, Muto A, Okano H, Mikoshiba K (1997) Developmental expression of the inositol 1,4,5-trisphosphate receptor and structural changes in the endoplasmic reticulum during oogenesis and meiotic maturation of *Xenopus laevis*. Dev Biol 182:228–239

Ludanyi A, Eross L, Czirjak S, Vajda J, Halasz P, Watanabe M, Palkovits M, Magloczky Z, Freund TF, Katona I (2008) Downregulation of the CB1 cannabinoid receptor and related molecular elements of the endocannabinoid system in epileptic human hippocampus. J Neurosci 28:2976–2990

Matorras R, Prieto B, Exposito A, Mendoza R, Crisol L, Herranz P, Burgues S (2009) Mid-follicular LH supplementation in women aged 35–39 years undergoing ICSI cycles: a randomized controlled study. Reprod Biomed Online 19:879–887

McKinney MK, Cravatt BF (2005) Structure and function of fatty acid amide hydrolase. Annu Rev Biochem 74:411–432

Paria BC, Zhao X, Wang J, Das SK, Dey SK (1999) Fatty-acid amide hydrolase is expressed in the mouse uterus and embryo during the periimplantation period. Biol Reprod 60:1151–1157

Paria BC, Song H, Wang X, Schmid PC, Krebsbach RJ, Schmid HH, Bonner TI, Zimmer A, Dey SK (2001) Dysregulated cannabinoid signaling disrupts uterine receptivity for embryo implantation. J Biol Chem 276:20523–20528

Park B, Gibbons HM, Mitchell MD, Glass M (2003) Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. Placenta 24:990–995

Peralta L, Agirregoitia E, Mendoza R, Exposito A, Casis L, Matorras R, Agirregoitia N (2011) Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. Reprod Biomed Online 23:372–379

Picton H, Briggs D, Gosden R (1998) The molecular basis of oocyte growth and development. Mol Cell Endocrinol 145:27–37

Schindler K (2011) Protein kinases and protein phosphatases that regulate meiotic maturation in mouse oocytes. Results Probl Cell Differ 53:309–341

Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, Giuffrida A (2002) N-Acylethanolamines in human reproductive fluids. Chem Phys Lipids 121:211–227

Stitzel ML, Seydoux G (2007) Regulation of the oocyte-to-zygote transition. Science 316:407–408

Storr M, Emmerdinger D, Diegelmann J, Yuce B, Pfennig S, Ochsenkuhn T, Goke B, Lohse P, Brand S (2009) The role of fatty acid hydrolase gene variants in inflammatory bowel disease. Aliment Pharmacol Ther 29:542–551

Sun X, Dey SK (2012) Endocannabinoid signaling in female reproduction. ACS Chem Neurosci 3:349–355

Taylor AH, Ang C, Bell SC, Konje JC (2007) The role of the endocannabinoid system in gametogenesis, implantation and early pregnancy. Hum Reprod Update 13:501–513

Wang H, Matsumoto H, Guo Y, Paria BC, Roberts RL, Dey SK (2003) Differential G protein-coupled cannabinoid receptor signaling by anandamide directs blastocyst activation for implantation. Proc Natl Acad Sci U S A 100:14914–14919

Wang H, Guo Y, Wang D, Kingsley PJ, Marnett LJ, Das SK, DuBois RN, Dey SK (2004) Aberrant cannabinoid signaling impairs oviductal transport of embryos. Nat Med 10:1074–1080

Wang H, Dey SK, Maccarrone M (2006a) Jekyll and Hyde: two faces of cannabinoid signaling in male and female fertility. Endocr Rev 27: 427–448

Wang H, Xie H, Dey SK (2006b) Endocannabinoid signaling directs periimplantation events. AAPS J 8:E425–E432

Wang H, Xie H, Sun X, Kingsley PJ, Marnett LJ, Cravatt BF, Dey SK (2007) Differential regulation of endocannabinoid synthesis and degradation in the uterus during embryo implantation. Prostaglandins Other Lipid Mediat 83:62–74

Wenger T, Toth BE, Juaneda C, Leonardelli J, Tramu G (1999) The effects of cannabinoids on the regulation of reproduction. Life Sci 65:695–701

Wenger T, Ledent C, Csernus V, Gerendai I (2001) The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. Biochem Biophys Res Commun 284:363–368

Yazulla S (2008) Endocannabinoids in the retina: from marijuana to neuroprotection. Prog Retin Eye Res 27:501–526

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019
Accepted: 19 August 2019

Original Paper

Tetrahydrocannabinol Modulates *in Vitro* Maturation of Oocytes and Improves the Blastocyst Rates after *in Vitro* Fertilization

Lide Totorikaguena[a] Estibaliz Olabarrieta[a] Angela Patricia López-Cardona[b,c] Naiara Agirregoitia[a] Ekaitz Agirregoitia[a]

[a]Department of Physiology, Faculty of Medicine and Nursing, UPV/EHU, Leioa, Spain, [b]Department of Animal Reproduction, INIA, Madrid, Spain, [c]G.I. Biogénesis, Universidad de Antioquia, Medellín, Colombia

Key Words
Oocyte • Maturation • THC

Abstract
Background/Aims: Among the assisted reproductive techniques, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone to mature exogenously in culture media supplemented with different molecules to promote maturation. In this sense, we are interested in the role that cannabinoids could have as IVM promoters because cannabinoid's molecular pathway is similar to the one by which oocyte's meiosis resumption is activated. With the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC) in the IVM process. ***Methods:*** By immunocytochemistry, we detected the location pattern of cannabinoid receptor type 1 (CB1) and type 2 (CB2) during oocyte maturation in presence or absence of THC, as well as, the staining pattern of p-AKT and p-ERK. We used a genetic/pharmacological approach generating knockout oocytes for CB1 and/or CB2 and they were incubated with THC during the oocyte maturation to visualize the physiological effects of THC, observing the rate of blastocyst achieved by oocyte. ***Results:*** This study confirms that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation. ***Conclusion:*** Our data suggest that THC may be useful IVM supplements in clinic as is more feasible and reliable than any synthetic cannabinoid.

Ekaitz Agirregoitia
Dept. of Physiology, Faculty of Medicine and Nursing UPV/EHU, Leioa, Bizkaia (Spain)
Tel. +34 946 01 3460, E-Mail e.agirregoitia@ehu.eus

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

Introduction

For years now, growing fertility problems and current changes in family patterns have resulted in a wider use of assisted reproduction techniques (ART) among couples trying to get pregnant [1–3]. Even so, the success of ARTs is not yet complete and the research work continues to improve these techniques. Among the ARTs, the *in vitro* maturation of oocytes (IVM) is less developed than other techniques, but its implementation would entail a qualitative advance. This technique consists in the extraction of immature oocytes from antral ovarian follicles with the patient under low hormone stimulation or without hormone. The oocytes are then exogenously matured in culture media supplemented with different molecules to promote maturation [4, 5].

The most important goal of IVM method is that could be the only hope or alternative for a high number of patients unable to tolerate high doses of gonadotropins, such as patients with polycystic ovary syndrome (PCOS) or ovarian hyperstimulation syndrome (OHSS) [6, 7]. In general, clinical pregnancy and implantation rates per embryo transfer have reached 35%–40% and 15%–20%, respectively, in infertile women with PCOS after IVM of immature oocytes [8]. Further candidates for oocyte IVM are patients whose ovarian tissue was frozen due to a pre-existing disease such as cancer, in whom hormone stimulation was not recommended or there was insufficient time to undergo a normal *in vitro* fertilization (IVF) cycle [9]. In addition, by avoiding controlled ovarian stimulation, the IVM procedure eliminates the need for frequent ultrasound monitoring and is thus less costly than conventional IVF [10].

The complexity lies in that the oocyte meiotic maturation is a complex process whereby immature oocytes acquire the characteristics required for successful fertilization and embryogenesis. For that, immature oocytes arrested at the diplotene of prophase I (designed as germinal vesicles -GV-) must resume meiosis until the metaphase II of meiosis (MII) [11].

However, although some mechanisms that lead the reactivation of meiosis are still to be explained, it is known that the oocyte maturation involves the activation of various signal transduction pathways that converge to activate maturation-promoting factor. Among others, there are some evidence that heterotrimeric G-proteins, which inhibit adenylate cyclase, can interact with both PI3K and MAPK to promoting the oocyte maturation [12]. In this sense, we are interested in the role that cannabinoids could have as IVM promoters. On the one hand, the molecular cascade that cannabinoids exerts when they activate the G-protein coupled cannabinoid receptors (inhibition of adenylate cyclase, reducing cAMP, and activation of PI3K/Akt and MAPK patways) is very similar to the one that occurs during oocyte meiotic resumption [13, 14]. On the other hand, some studies described the presence of the cannabinoid system in human [15–19], bovine [20] and murine [21] oocytes, and, at least in the two last species, synthetic cannabinoids were able to modulate the oocyte maturation [20, 21].

Being this the general content, with the intention of advancing in the possible use of cannabinoids as supplements for the media for *in vitro* maturation of oocytes, we intend to deepen the study of the function of the phytocannabinoid Δ-9-tetrahydrocannabinol (THC). The THC is called phytocannabinoid because it is a compound of the plant *Cannabis sativa*, and it has attracted particular attention since its synthetic analogs dronabinol and nabilone are licensed for medicinal use [22]. Even so, the use of phytocannabinoids as THC in clinic is more feasible and reliable than any synthetic cannabinoid [23, 24].

The aim of this study is to characterize the role of the phytocannabinoid THC in the IVM process. To that end, we used a genetic/pharmacological approach generating knockout oocytes for CB1 and/or CB2 receptors. They were modulated pharmacologically during the oocyte maturation to visualize the physiological effects of THC. Our results support the notion that the incubation of oocytes with THC during IVM accelerated some events of that process like the phosphorylation pattern of ERK and AKT and was able to increase the blastocyst rate in response to IVF. Moreover, it seems that both CB1 and CB2 are necessary to maintain a healthy oocyte maturation.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

Materials and Methods

Experimental animals

The adult wild-type, *Cnr1*$^{-/-}$ [25], *Cnr2*$^{-/-}$ [26]and *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$ mice used in this study were kept in an animal house under controlled conditions of temperature (22 ± 1°C) and photoperiod (light/dark cycle 14 h:10 h). Animals were given free access to water and food. All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the *Guide for Care and Use of Laboratory Animals*, endorsed by the Society for the Study of Reproduction and European legislation.

Isolation and in vitro maturation of cumulus–oocyte complexes

Female 8 to 10 week old WT (C57BL/6xCBA) or KO on a C57BL/6N background (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$, and *Cnr1*$^{-/-}$*/Cnr2*$^{-/-}$) mice were superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon, Intervet, Castle Hill, NSW, Australia), and ovaries were collected 46 to 48 h later. The ovaries were cleaned of any connective tissue and placed in handling medium M2 supplemented with 4 mg/ml bovine serum albumin fraction V. Antral follicles were punctured with 30-gauge needles, and immature cumulus–oocyte complexes (COCs) were collected in handling medium. Only COCs with 3 compact cumulus cells were used. COCs were matured for 17 h in TCM-199 supplemented with 10% (v/v) fetal calf serum (FCS) and 10 ng/ml epidermal growth factor at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity.

IVM supplementation with cannabinoid agonist THC

The THC stock solutions were prepared in DMSO. During maturation (17 h), COCs were incubated with different doses of THC (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) to evaluate the effects of activation of cannabinoid receptor by this agonist. Once obtained the most efficient concentration for oocyte maturation, to see the phosphorylation pattern of ERK and AKT proteins and the effect on embryo development, the COCs were incubated with 10^{-7} M (100nM) of THC. COCs containing the same amount of DMSO were used as an incubation control.

Meiotic progression of mice oocytes undergoing IVM with THC

To determinate the impact of THC on germinal vesicle breakdown (GVBD), COCs from experimental group and control with DMSO at 2, 4, 6, 8, 12, 17h of IVM were used as described previously [27]. In sum, COCs were partially denuded in 0, 1% of hyaluronidase (Sigma H3506) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 20 min. Then, were washed twice in PBS and incubated in PBS containing Hoescht 33342 (0.01 mg/ml) for 15 min. Oocytes were then placed in glass slides and squashed with coverslip in order to visualize the nuclear stage under microscopy (Zeiss Axioskop, NY, USA).

Immunofluorescence

For these experiments, 3 females per genotype were used in 3 replicate trials. Once meiotic stages were established, to immunocytochemically localize the CB1 and CB2, 20 WT oocytes per stage were treated as previously described [17]. Briefly, *in vitro* matured oocytes in presence or absence of THC were washed in PBS supplemented with 1% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde (Panreac, Barcelona, Spain) for 10 min at room temperature. The oocytes were then permeabilized by incubation in PBS with 10% (v/v) FCS and 1% Triton X-100 for 45 min at room temperature. After permeabilization, oocytes were incubated overnight at 4°C in PBS containing 1% PVA, 5% normal FCS serum, and 1:100 rabbit polyclonal anti-cannabinoid CB1 (Cayman Chemicals, Ann Arbor, MI, USA) and 1:400 rabbit polyclonal anti-cannabinoid CB2 (Cayman Chemicals, Ann Arbor, MI, USA). After incubation, oocytes were washed twice in PBS containing 1% PVA and incubated in PBS supplemented with 1% PVA, 5% FCS serum, and 1:500 goat polyclonal secondary antibody Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Next, the oocytes were washed 3 times in PBS–1% PVA. In all cases, nuclei were stained with Hoechst 33342 (0.01 mg/ml) during the second wash to facilitate the determination of the maturation stage of each oocyte [28]. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS, Hatfield, United Kingdom) and examined by confocal microscopy (Fluoview FV500; Olympus, Tokyo, Japan). Negative controls were prepared in the same way omitting the primary antibody before addition of the secondary antibody.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

To monitor the activation of pERK1/2 and pAKT signaling during oocyte maturation in the presence of cannabinoids, an average number of 20 oocytes was collected at 0 h, 10 min, 30 min and 1 h to evaluate the initial response, and at 17 h as the optimal time for maturation. Rabbit polyclonal phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) primary antibody was used at a 1:400 dilution and Rabbit phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA) 1:200 primary antibody was used at a 1:400 dilution. Finally, oocytes were mounted in microdrops with Fluoromount G (EMS, Hatfield, United Kingdom) and examined by confocal microscopy (LSM 800, Zeiss, NY, USA). Negative controls were performed in the same way, except for omission of the primary antibody before secondary antibody addition.

Isolation and in vivo maturation of COCs

MII oocytes were collected from 8- to 10-week-old oviducts of female WT (C57BL/6xCBA) or KO (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$) superovulated by intraperitoneal injections of 5 IU equine chorionic gonadotropin (Folligon), followed 48 h later by 7.5 IU of human chorionic gonadotropin (Veterin Corion;Divasa-Farmavic S.A., Spain,). Briefly, at 14 h after human chorionic gonadotropin administration, oviducts were removed from superovulated female mice and placed in a Petri dish containing M2 at 37°C. After washing, collected oviducts were placed in fresh M2 medium, and COCs were released from the ampulla with the aid of Dumont #55 forceps and washed in new M2 medium until fertilization.

In vitro fertilization

Sperm from C57BL/6xCBA male mice aged 6 to 24 wk and of proven fertility were incubated for 1 h in human tubal fluid (HTF) medium under 5% CO_2 at 37°C for capacitation. After *in vitro* or *in vivo* maturation, COCs were transferred to a 500 µl equilibrated HTF drop and overlaid with mineral oil and a 1×10^6 concentration of spermatozoa. All *in vitro* fertilization experiments were repeated 9 times using 3 females per genotype.

Rates of oocyte nuclear maturation and fertilization

To assess whether adding THC affected rates of oocyte nucleus maturation and fertilization, the presence of the first polar body and pronuclear formation were identified respectively. At 24 h after fertilization, all presumptive zygotes that had not divided into 2 cells were fixed in 4% paraformaldehyde for 10 min and then stained with Hoechst 33342 (0.01 mg/ml) for observation with a immunofluorescence microscope (Zeiss Axioskop, NY, USA) under UV light.

In vitro culture of embryos

Five hours after *in vitro* fertilization, 10-25 presumptive zygotes from oocytes of female WT (C57BL/6xCBA) or KO (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$) were washed in HTF medium and cultured in 20-µl drops of equilibrated culture medium KSOMaa overlaid with mineral oil at 37°C under an atmosphere of 5% CO_2 in air with maximum humidity. Embryos were cultured for 5 d, and cleavage rates were assessed on d 1 (24 h after fertilization) and blastocysts on d 4 (96–100 h after fertilization).

TUNEL analysis

Apoptosis was determined by in situ DNA 3 end labelling of histological sections using a non-radioactive labelling method [Terminal deoxynucleotidyl transferasemediated dUTP nick-end labelling assay (TUNEL) from detection Kit, TUNEL POD (ROCHE). The control oocytes, the vehicle (DMSO) group and THC group were processed as per the manufacturer's instructions. The apoptotic cells appear in fluorescent green whereas the nucleus was marked in blue with Hoechst 33342.

Statistical analysis

All statistical tests were performed by using Graphpad software (GraphPad Software, Inc. La Jolla, CA 92037 USA). The mean and standard error of the mean of cleavage rates, blastocyst yields, the percentage change of blastocyst rate between the 4 genotypes were compared by 1-way ANOVA followed by multiple pairwise comparisons by using the Tukey's *post hoc* test in most data. Paired t-test was performed to compare the difference between treatments (without and with THC). Values of $P < 0.05$ were considered significant.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

Statement of Ethics

All experimental procedures using mice were approved by the University of the Basque Country (UPV/EHU, CEEA reference number: M20-2015-016-027-028-173) and were performed according to the Guide for Care and Use of Laboratory Animals, endorsed by the Society for the Study of Reproduction and European legislation.

Results

CB1 receptor location-pattern during the resumption of meiosis and nuclear maturation of oocyte in presence or absence of THC

To analyse if the THC has any effect on the relocation of CB1 during the oocyte maturation, we incubated the immature oocytes with increasing concentrations of THC during 17h and we collected the oocytes at 10 min, 30 min, 1 h and 17 h to observe the CB1 location-pattern.

As it is known, in immature oocytes, CB1 is homogeneously localized over the GV and the receptor is relocated to the periphery of the oocyte after GVBD, when meiosis resume, which happens after more than one hour of maturation (Control of Fig. 1). However, the presence in the incubation media of any tested concentration of THC (from 10^{-9} M to 10^{-6} M) accelerated the relocation of CB1receptor from GV to the periphery of oocytes. Thus, the staining of CB1 in GV disappeared before 1 h of incubation (quicker than in control) and, specifically, the concentration of 10^{-7} M of THC led to accelerate that relocation before 30 min (Fig. 1).

CB2 receptor location-pattern during the resumption of meiosis and nuclear maturation in presence or absence of THC

To continue understanding the role of THC in oocyte maturation and after determining its effect on CB1 receptor relocation, we also examined the effect of THC on location of CB2 receptor. We performed the same experiment explained previously for CB1. CB2 showed a homogeneous distribution-pattern in the whole oocyte with more staining intensity in the periphery of the oocyte at all tested times both in absence or in presence of THC (Fig. 2).

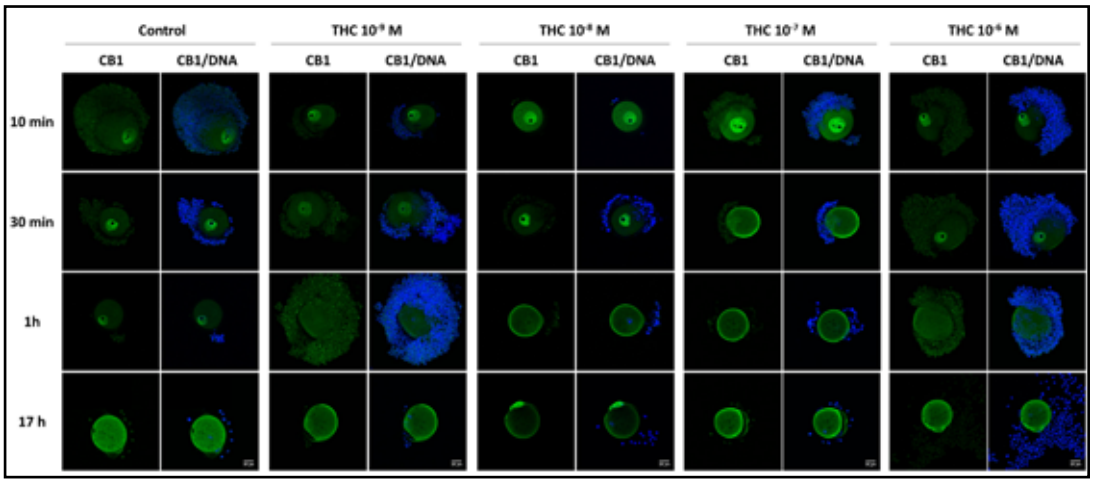


Fig. 1. Immunolocalization of CB1 during the maturation of mouse oocytes. Immature COCs were cultured in vitro in absence of THC and in presence of THC at 1 nM (10-9 M), 10 nM (10-8 M), 100 nM (10-7 M) and 1 μM (10-6 M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had the same staining pattern; representative photomicrographs are shown. Scale bars, 25 μm.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

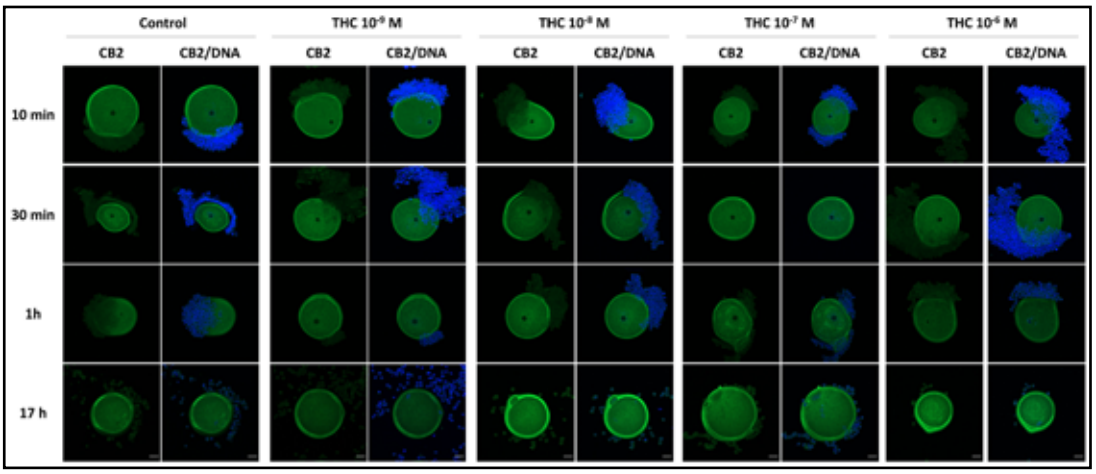


Fig. 2. Immunolocalization of CB2 during the maturation of mouse oocytes. Immature COCs were cultured in vitro in absence of THC and in presence of THC at 1 nM (10-9 M), 10 nM (10-8 M), 100 nM (10-7 M) and 1 µM (10-6 M) during 10 min, 30 min, 1h and 17 h. The distribution of CB1 is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 µm.

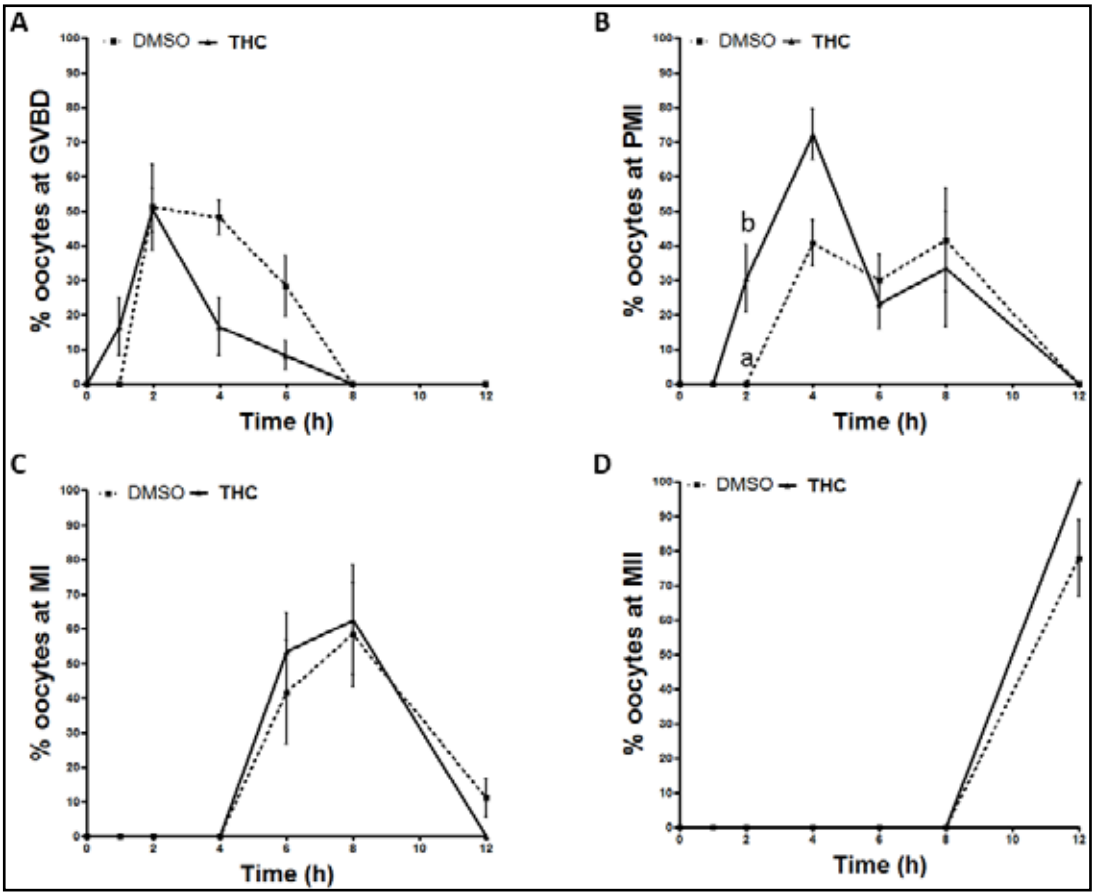


Fig. 3. Changes in nuclear maturation of oocytes. Results are expressed as percentage of oocytes at each stage of maturation at each point: (A) germinal vesicle break down, GVBD; (B) pro-metaphase I stage, PMI; (C) metaphase I stage, MI; (D) metaphase II stage, MII. n = 5 independent experiments of 15 oocytes per treatment. Significant differences between treatments are indicated with different letters; $p<0.05$ in all cases.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

Meiotic progression of mice oocyte's exposed to THC

To determinate if THC has also some effect on oocyte nuclear maturation we fixed oocytes after 0, 2, 4, 6, 8 and 12h of IVM where media was supplemented with 10^{-7} M of THC or vehicle (DMSO). Oocytes nuclear stage was classified in germinal vesicle (GV), germinal vesicle break down (GVBD), pro-metaphase I (PMI), metaphase I (MI) and metaphase II (MII). At 0h all the oocytes were in GV stage (Fig. 3A) and after 2h of IVM a significant higher percentage of PMI was detected when we used THC compared with the oocytes maturated in presence of the vehicle (Fig. 3B). After 4h of IVM the majority of the oocytes were at PMI, but no differences were observed among treatments. Also after 8 h of IVM, all groups reached MI (Fig. 3C) at the same time. After 12 h of maturation a higher percentage of MII stage was found in THC experimental groups compared with control (Fig. 3D) but that difference was not significant. Finally, after 17 hours, all the oocytes of both treatments arrived at MII (data not shown).

Phosphorylation pattern of ERK1/2 during the resumption of meiosis and nuclear maturation in presence or absence of THC

As signaling via MAPKs plays a role in the oocyte maturation and that pathway is commonly modulated by cannabinoids, we used the cannabinoid agonist THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of ERK1/2 compared with oocytes maturated in presence of the vehicle (DMSO).

According to the results obtained in the relocation of cannabinoid receptors during the maturation of oocytes, for these experiments we used the concentration of THC that generated the most changes regarding the relocation of the receptors in comparison to the control (10^{-7} M).

In immature oocytes, ERK1/2 was dephosphorylated but, after 17 h of incubation (the time required for mouse oocyte maturation), ERK1/2 was phosphorylated in oocytes. At 10 min, ERK1/2 appeared phosphorylated only in the granulose cells of oocytes incubated with THC. We did not observe the phosphorylation of granulose cells of control oocytes until 30 min. However, at that time, the COCs incubated with THC had already dephosphorylated its granulose cells but, instead, we could observe the presence of phosphorylated ERK1/2 inside the oocyte. At 1 h of incubation, the oocytes incubated with THC showed more intense phosphorylated ERK1/2 compared with the control and, at that time, the granulose cells of control COCs had already been dephosphorylated (Fig. 4A).

When we used oocytes from knock out mice where the cannabinoids receptors were absent (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$), we did not observe difference in the phosphorylation pattern of ERK1/2 between the oocytes matured in the presence or absence of THC (Fig. 4B, 4C and 4D).

Phosphorylation pattern of AKT during the resumption of meiosis and nuclear maturation in presence or absence of THC

The pathways mediated by AKT are also key in the oocyte maturation and that kinase is also modulated by cannabinoids, so, we used THC to determine whether the activation of the CB1 and CB2 receptors during oocyte maturation could affect the phosphorylation pattern of AKT compared with oocytes maturated in presence of the vehicle (DMSO).

We performed the same experiments as previously explained for pERK1/2. In this case, we observed few differences in the phosphorylation pattern of AKT in response to 10 and 30 min of treatment with THC, where the AKT was phosphorylated in granulose cells while it was undetectable in granulose cells exposed to vehicle. From that moment, the pattern of phosphorylation observed in the COCs treated with THC or vehicle was similar (Fig. 5A).

When we used oocytes from knock out mice where the cannabinoids receptors were absent (*Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$), we did not observe difference in the phosphorylation pattern of AKT between the oocytes matured in the presence or absence of THC (Fig. 5B, 5C and 5D).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

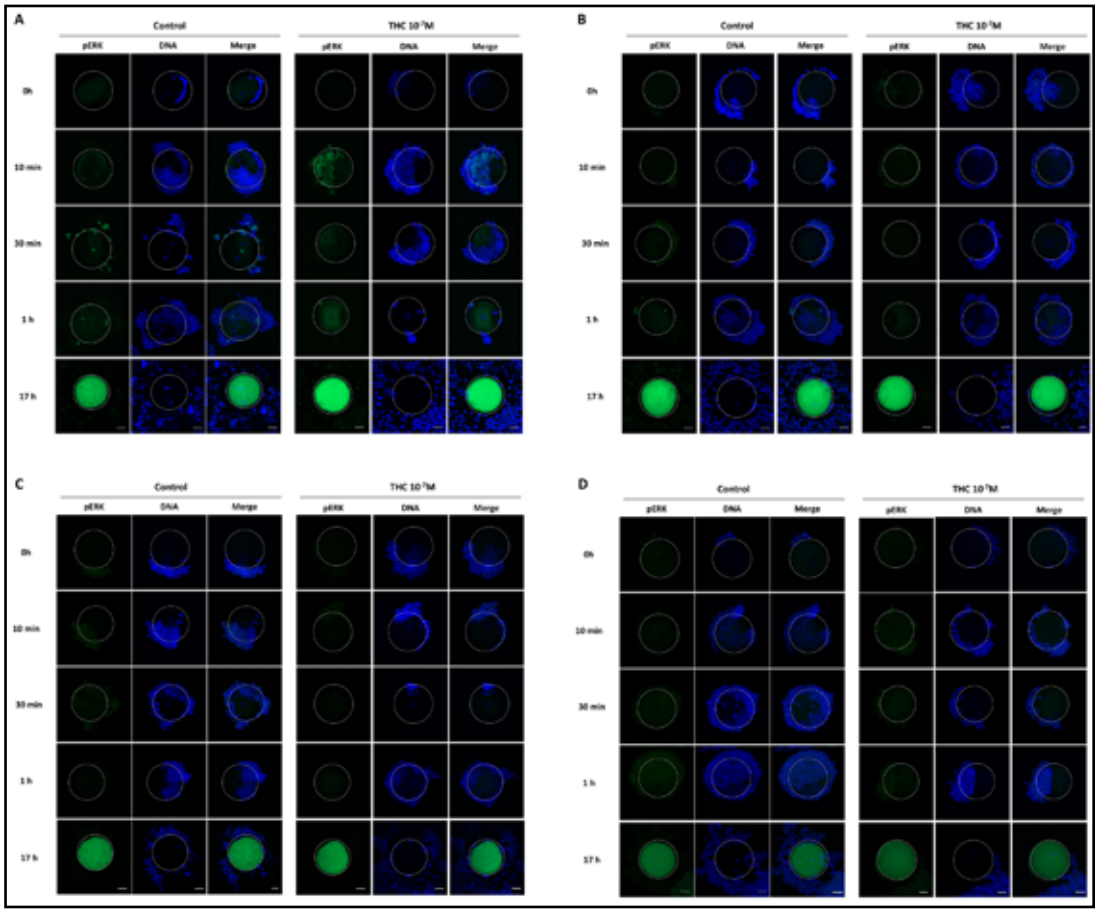


Fig. 4. Phosphorylation of ERK by THC during oocyte maturation. Immature GV oocytes from (A) wild type mice, (B) Cnr1$^{-/-}$ mice, (C) Cnr2$^{-/-}$ mice and (D) Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice were cultured in vitro in presence of 10-7 M of THC or in absence of it (control) for 17 h and the phosphorylation status of ERK (pERK) was observed at 0, 10 min, 30 min, 1 h and 17 h. pERK is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 μm.

Apoptosis analysis in oocytes exposed to THC during maturation

To check if the presence of THC, during the 17 hours it was in contact with the COCs, was harmful, the detection of apoptotic oocytes or granulosa cells was performed using the in situ TUNEL analysis. We did not observe any apoptotic cell in the COCs treated with THC during maturation or in those treated only with the vehicle (DMSO) (Fig. 6).

Blastocyst rate produced from oocytes matured in presence or absence of THC

Our next objective was to test whether exposure of COCs to THC during oocyte maturation would affect the maturation observing the fertilization and/or subsequent embryo development rate. For that purpose, we cultured, *in vitro*, immature COCs during 17 h (the average time to mature mice oocytes) in absence or presence of 10-7 M of THC and, then, we performed the *in vitro* fertilization (IVF).

The incubation with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes. However, THC led to a significant improvement in blastocysts rate since twice as many embryos were produced when we used a 10^{-7} M concentration of THC, compared to vehicle (Fig. 7A).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

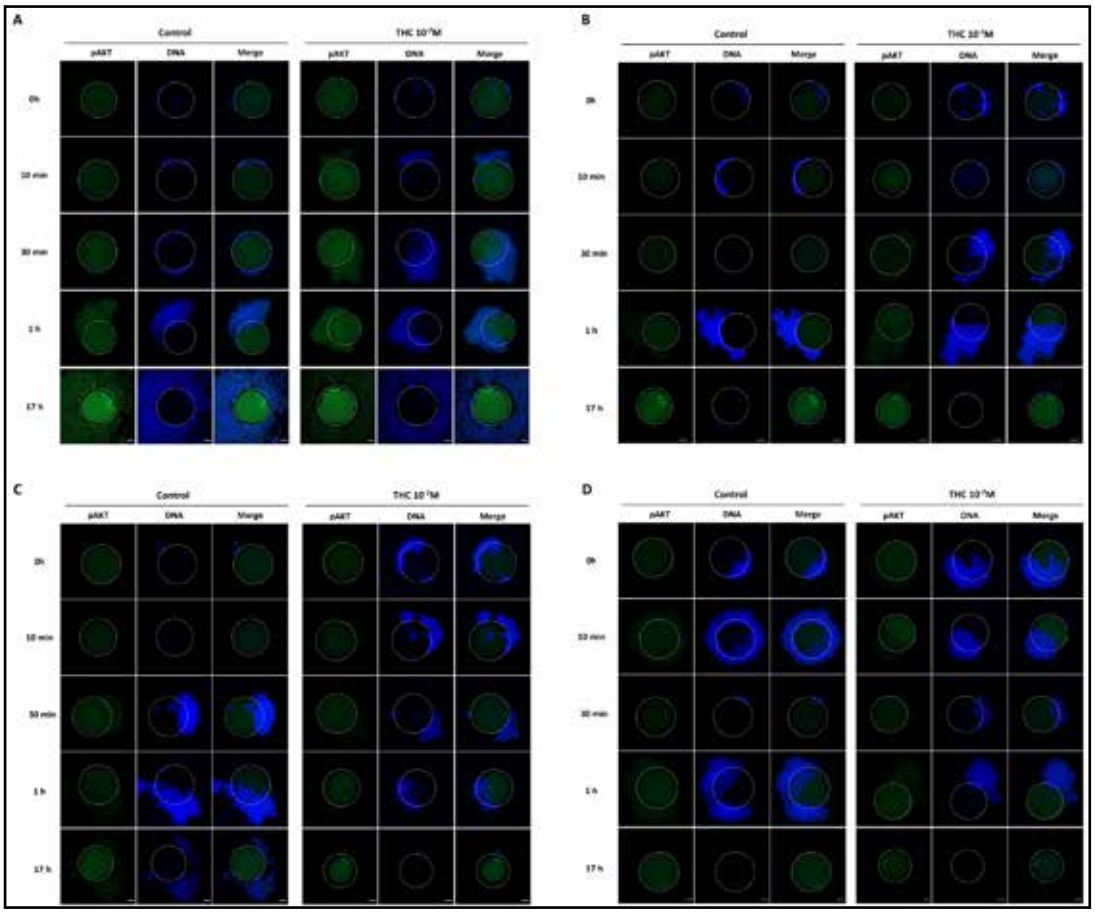
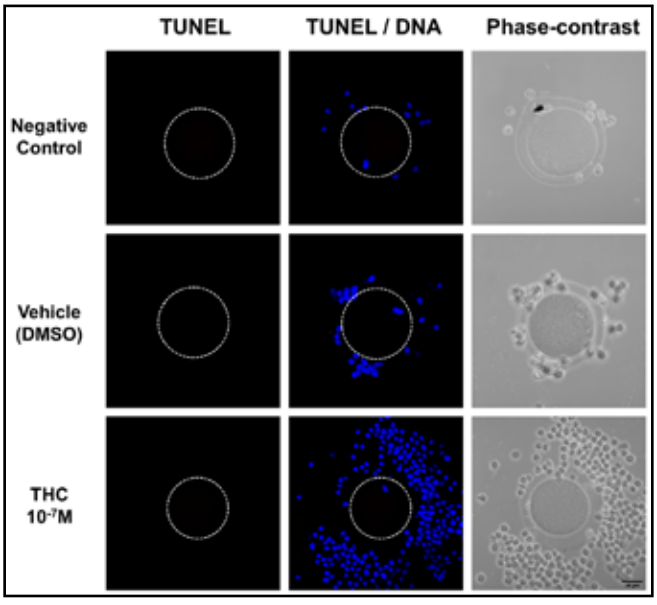


Fig. 5. Phosphorylation of AKT by THC during oocyte maturation. Immature GV oocytes from from (A) wild type mice, (B) Cnr1$^{-/-}$ mice, (C) Cnr2$^{-/-}$ mice and (D) Cnr1$^{-/-}$/Cnr2$^{-/-}$ mice were cultured in vitro in presence of 10-7 M of THC or in absence of it (control) for 17 h and the phosphorylation status of AKT (pAKT) was observed at 0, 10 min, 30 min, 1 h and 17 h. pAKT is shown in green. Hoechst-labelled DNA is shown in blue. n = 5 independent experiments of 15 oocytes per treatment. All of analysed oocytes had same staining pattern; representative photomicrographs are shown. Scale bars, 20 μm.

Fig. 6. Analysis of apoptotic cells, by TUNEL, in COCs treated with vehicle (DMSO) or THC. Apoptotic cells showed in green and DNA, stained by Hoechst 3342, in blue. Representative photomicrographs are shown.



Cellular Physiology and Biochemistry
Cell Physiol Biochem 2019;53:439-452
DOI: 10.33594/000000149
Published online: 23 August 2019

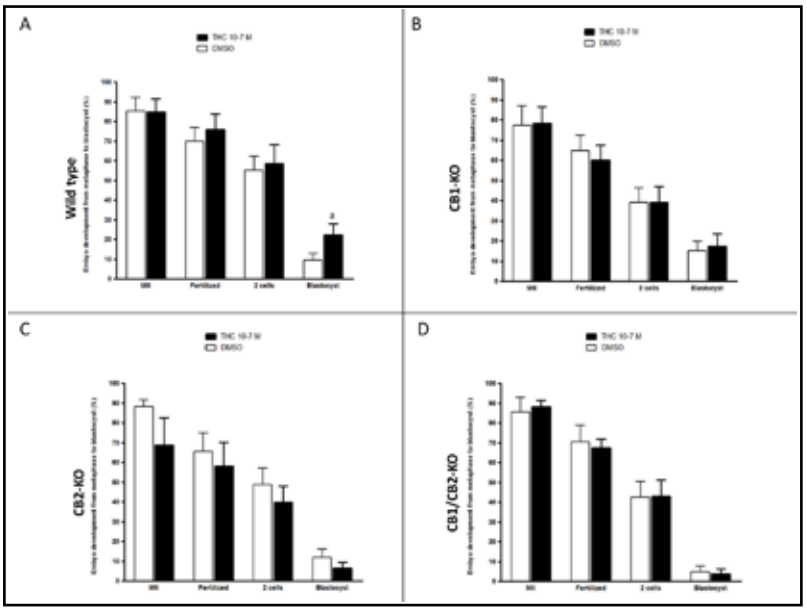


Fig. 7. Response to THC 10^{-7} M during oocyte maturation (17 h) measured as rates of metaphase II oocytes (MII), fertilized oocytes, 2 cell-stage embryos and blastocysts after IVF. The Fig. shown the embryo development observed for oocytes incubated with vehicle (DMSO) (white) and incubated with THC 10^{-7} M (black). The sperm always came from WT mice and the oocytes came from A) wild type (WT) mice, B) $Cnr1^{-/-}$ mice, C) $Cnr2^{-/-}$ mice and D) $Cnr1^{-/-}$/ $Cnr2^{-/-}$ mice. Results are the mean of % ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p<0.005$ in all cases.

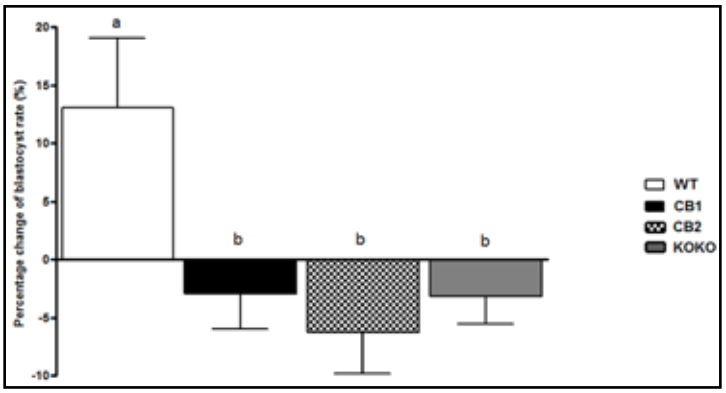


Fig. 8. Percentage change of blastocysts rate between control and treatment. Blastocysts rate changes using oocytes from wild type (WT) mice (white), $Cnr1^{-/-}$ mice (black), $Cnr2^{-/-}$ mice (dotted) and $Cnr1^{-/-}$/$Cnr2^{-/-}$ mice (grey). Results are the mean ± SEM of 10 independent experiments. Significant differences between treatments are indicated with different letters; $p<0.05$ in all cases.

To be able to know if the observed action of THC was carried out by CB1 and/or CB2 receptor, we also performed the IVM with COCs from knock out mice *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$ and *Cnr1–*$^{-/-}$*/Cnr2*$^{-/-}$, followed by the subsequent IVF done using sperm from wild type male mice. That way, we were able to verify that the effect observed on *in vitro* embryo development were effectively attributable to the absence of the cannabinoid receptor during maturation and not to the *in vitro* fertilization (IVF) or embryo culture processes.

We did not observe any improvement on blastocyst rate between the oocytes treated with THC or with vehicle for any genotype (Fig. 7B, C and D). In that sense, taking into account the percentage change of blastocyst rate between control and treatment for each genotype, we observed that the only genotype where incubation of oocytes with THC is beneficial for an increase in the number of blastocysts is the wild type (Fig. 8).

Discussion

Through systematic series of genetic and pharmacologic experiments, we examined whether the use of THC acting via CB1 and/or CB2 modulated oocyte maturation in a mouse

model, and we tried to elucidate the mechanisms of such actions. The choice of using the phytocannabinoid THC has to do with the controversy generated by the use of synthetic cannabinoids [23, 24], which is why it seems that the use of THC in the clinic could be more feasible and reliable. Our observations in wild type, *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$, and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice indicated that, when present during IVM of oocytes, the THC accelerated the relocation of CB1 receptor in oocytes. Moreover, the incubation of oocytes with THC during the IVM produced modulations in ERK1/2 and AKT phosphorylation and increased blastocyst rates achieved. Those effects seem to be achieved through both cannabinoid receptors studied (CB1 and CB2).

The first evidence that linked the endocannabinoid system and the oocyte maturation was that the endocannabinoid AEA (*N*-arachidonoylethanolamide) was present in human follicular fluid [29] and its concentration rose during oocyte maturation [30, 31]. In previous works, it was detected the Cnr1 mRNA transcript and protein of CB1 receptor in oocytes during *in vitro* and *in vivo* maturation [17, 20, 21]. Furthermore, CB1 receptor could be an indicator of oocyte nuclear maturation, because it was clearly localized over GV oocyte and it moves towards the periphery as it changes to the stage MII [17, 20, 21]. In the present work we saw how the presence of THC in the maturation media accelerated that relocation of CB1 receptor from GV to the periphery. That difference in relocation velocity is interesting because, in oocytes matured *in vivo*, CB1 was localized peripherally sooner than in oocytes matured *in vitro* [21] and, as is well established, the *in vitro* maturation process is delayed in comparison with *in vivo* maturation [32]. The presence of THC in the maturation media also accelerated the oocyte nuclear maturation of immature oocytes between 1 and 2 hours of incubation, similar times observed in the aforementioned acceleration of the relocation of CB1 receptor. The meiosis resumption also began earlier (around 2 h) when THC was present in the IVM media of bovine oocytes [20].

Although CB2 did not show any relocation difference during the IVM, we detect a peripheral location of this receptor did not previously found. So, as according to the classic theory of GPCR functionality, to exert its actions, a receptor needs to reach the cell surface [33], both CB1 and CB2 could have any involvement in oocyte maturation. Actually, there are data supporting the notion that GPCR-Gαi (such as CB1 and CB2) is a meiotic maturation inducer [34].

To know if the changes observed during the maturation of oocytes incubated with THC were observed in other processes of IVM, we analysed the phosphorylation-pattern of ERK1/2 and AKT, since those essential kinases regulate the oocyte meiosis progression [35]. Interestingly, the presence of THC during the oocyte maturation accelerated the phosphorylation-pattern of ERK1/2 and, to a lesser extent, the phosphorylation-pattern of AKT. Previously, it was described that the selective CB2 receptor agonist, JWH133, induced the ERK1/2 and AKT phosphorylation cascade in spermatogonia and their progression toward meiosis [36, 37]. In addition, in the previous functional experiments done with cows showed how the presence of THC during the IVM led to accelerate the phosphorylation-pattern of ERK1/2 and AKT kinases [20]. These results are in accordance with the fact that the activation (phosphorylation) of AKT stimulates the meiosis resumption, it is involved in the MI/MII transition and it regulates polar body emission and spindle organization [38–41]. In the same way, the prompt activation of ERK1/2 induces premature chromosome condensation and meiosis resumption as well as pronucleus breakdown [42]. It is interesting to highlight that the activation of CB2 by a treatment with JWH133 induced and accelerated the meiosis progression in fetal oocytes of mice, although they observed how also decreased the pool of primordial and primary follicles [43]. Even so our treatment with THC was *in vitro* and it only lasted 17 hours, and, that could be why we do not observe apoptosis in oocytes or granulosa cells as observed by De Domenico and co-workers [43] after systemic treatment with JWH133.

Finally, our last objective was to test whether all that changes described regarding the exposure of COCs to THC during oocyte maturation would affect the fertilization and/or subsequent embryo development rate in positive or negative manner. Although the incubation

with THC had no significant effect on rates of mature oocytes (MII), fertilized zygotes and 2-cell embryos compared to vehicle-treated oocytes, the THC led to an improvement in blastocysts rate (2 times more number of embryos). That improvement in the amount of blastocysts achieved had not been observed in previous experiments performed with THC in bovine oocytes [20], although it was observed using a synthetic agonist for CB1 in mice oocytes [21]. In addition, the experiments carried out by knockout mice for CB1 and/or CB2 receptors, confirmed that both receptors are involved in the modulation of oocyte maturation by THC since, when one of the two receptors is absent, the THC is not able to generate improvements in the ratio of blastocysts compared to the control.

Acknowledgements

The authors thank Alfonso Gutiérrez-Adán (INIA, Animal Reproduction Department, Madrid, Spain) for critical reading of the manuscript and SGiker (UPV/EHU) for personal and technical support, particularly Ricardo Andrade and Alejandro Díez for his assistance with the confocal microscopy analysis. In addition, the authors wish to thank Guillaume Soonekindt for reviewing the article to improve the grammar and flow of the text.

This work received financial support from the University of the Basque Country (Grants PPGA18/01 and PPGA19/04) L.T. acknowledges the financial support given by University of the Basque Country (PIF15/149). E.O. has carried out all the experimentation without any public or private financial help. A.P.L.C had the financial support given by Universidad de Antioquia, COLCIENCIAS through the Francisco José de Caldas fellowship 512/2010.

Author Contribution: Conceptualization and designed the experiments, L.T., N.A. and E.A.; Methodology and Investigation, L.T., E.O., A.P.L.C. (IVM, IVF, CIV); Methodology and Investigation, (immunocytochemistry) L.T., E.O., A.P.L.C.; L.T. and E.A. wrote the first draft of the manuscript. A.P.L.C., N.A. and E.A. contributed to editing and revising the manuscript. N.A and E.A. supervised the project.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

1 Jose-Miller AB, Boyden JW, Frey KA: Infertility. Am Fam Physician 2007;75:849-856.
2 Gonzalez M, Morgado B: Maternidad a solas por eleccion: primera aproximacion. Anu Psicol 2008;39:119–126.
3 Sociedad Española de Fertilidad (SEF). Registro Nacional de Actividad 2016-Registro SEF. Informe estadístico de Técnicas de Reproducción Asistida 2016. URL: https://www.registrosef.com/
4 Barnes FL, Crombie A, Gardner, DK, Kausche A, Lacham-Kaplan O, Suikkari AM, Tiglias J, Wood C, Trounson AO: Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum Reprod 1995;10:3243–3247.
5 Barnes FL, Kausche A, Tiglias J, Wood C, Wilton L, Trounson A: Production of embryos from in vitro-matured primary human oocytes. Fertil Steril 1996;65:1151–1156.
6 Nader S: Infertility and pregnancy in women with polycystic ovary syndrome. Minerva Endocrinol 2010;35:211–225.
7 González-Ortega C, Piña-Aguilar RE, Cancino-Villarreal P, Pérez-Peña E, Gutiérrez-Gutiérrez AM: Natural-cycle in vitro fertilization (IVF) combined with in vitro maturation in infertile patients with polycystic ovarian syndrome (PCOS) requiring IVF. Taiwan J Obstet Gynecol 2019;58:192–195.
8 Chian RC, Uzelac PS, Nargund G: In vitro maturation of human immature oocytes for fertility preservation. Fertil Steril 2013;99:1173–1181.

9 Nikseresht M, Fallahzadeh AR, Toori MA, Mahmoudi R: Effects of pomegranate seed oil on the fertilization potency of rat's sperm. J Clin Diagnostic Res 2015;9:FF01–FF04.
10 Chang EM, Song HS, Lee DR, Lee WS, Yoon TK: In vitro maturation of human oocytes: Its role in infertility treatment and new possibilities. Clin Exp Reprod Med 2014;41:41–46.
11 Coticchio G, Dal Canto M, Mignini Renzini M, Guglielmo MC, Brambillasca F, Turchi D, Novara PV, Fadini R: Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. Hum Reprod Update 2015;21:427–454.
12 Schmitt A, Nebreda AR: Signalling pathways in oocyte meiotic maturation. J Cell Sci 2002;115:2457–2459.
13 Schindler K: Protein Kinases and Protein Phosphatases that Regulate Meiotic Maturation in Mouse Oocytes, in Kubiak JZ (ed): Cell Cycle in Development. Berlin, Heidelberg, Springer, 2011.
14 Dalton GD, Howlett AC: Cannabinoid CB 1 receptors transactivate multiple receptor tyrosine kinases and regulate serine/ threonine kinases to activate ERK in neuronal cells. Br J Pharmacol 2012;165:2497–2511.
15 Reich R, Laufer N, Lewysohn O, Cordova T, Ayalon D, Tsafriri A: In Vitro Effects of Cannabinoids on Follicular Function in the Rat1. Biol Reprod 1982;27:223–231.
16 Paria BC, Das SK, Dey SK: The preimplantation mouse embryo is a target for cannabinoid ligand-receptor signaling. Proc Natl Acad Sci 1995;92:9460–9464.
17 Peralta L, Agirregoitia E, Mendoza R, Expósito A, Casis L, Matorras R, Agirregoitia N: Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. Reprod Biomed Online 2011;23:372–379.
18 Agirregoitia E, Ibarra-Lecue I, Totorikaguena L, Mendoza R, Expósito A, Matorras R, Urigüen L, Agirregoitia N: Dynamics of expression and localization of the cannabinoid system in granulosa cells during oocyte nuclear maturation. Fertil Steril 2015;104:753-760.
19 Agirregoitia E, Totorikaguena L, Exposito A, Mendoza R, Matorras R, Agirregoitia N: Dynamic of expression and localization of cannabinoid-degrading enzymes FAAH and MGLL in relation to CB1 during meiotic maturation of human oocytes. Cell Tissue Res 2016;365:393-401.
20 López-Cardona AP, Sánchez-Calabuig MJ, Beltran-Breña P, Agirregoitia N, Rizos D, Agirregoitia E, Gutierrez-Adán A: Exocannabinoids effect on in vitro bovine oocyte maturation via activation of AKT and ERK1/2. Reproduction 2016;152:603–612.
21 López-Cardona AP, Pérez-Cerezales S, Fernández-González R, Laguna-Barraza R, Pericuesta E, Agirregoitia N, Gutiérrez-Adán A, Agirregoitia E: CB1cannabinoid receptor drives oocyte maturation and embryo development via PI3K/Akt and MAPK pathways. FASEB J 2017;31:3372–3382.
22 Dinis-Oliveira RJ: Metabolomics of δ9-tetrahydrocannabinol: Implications in toxicity. Drug Metab Rev 2016;48:80–87.
23 Schreiber S, Bader M, Lenchinski T, Meningher I, Rubovitch V, Katz Y, Cohen E, Gabet Y, Rotenberg M, Wolf EU, Pick CG: Functional effects of synthetic cannabinoids versus Δ^9-THC in mice on body temperature, nociceptive threshold, anxiety, cognition, locomotor/exploratory parameters and depression. Addict Biol 2019;24:414-425.
24 Wiley JL, Marusich JA, Huffman JW: Moving around the molecule: Relationship between chemical structure and in vivo activity of synthetic cannabinoids. Life Sci 2014;97:55–63.
25 Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann C, Zieglgänsberger W, Di Marzo V, Lutz B: The endogenous cannabinoid system controls extinction of aversive memories. Nature 2002;418:530–534.
26 Buckley NE, McCoy KL, Mezey É, Bonner T, Zimmer A, Felder CC, Glass M, Zimmer A: Immunomodulation by cannabinoids is absent in mice deficient for the cannabinoid CB2 receptor. Eur J Pharmacol 2000;396:141–149.
27 Khatir H, Lonergan P, Mermillod P: Kinetics of nuclear maturation and protein profiles of oocytes from prepubertal and adult cattle during in vitro maturation. Theriogenology 1998;50:917–929.
28 Gutiérrez-Adán A, Pintado B: Effect of flanking matrix attachment regions on the expression of microinjected transgenes during preimplantation development of mouse embryos. Transgenic Res 2000;9:81–89.
29 Coy P, Grullon L, Canovas S, Romar R, Matas C, Aviles M: Hardening of the zona pellucida of unfertilized eggs can reduce polyspermic fertilization in the pig and cow. Reproduction 2008;135:19–27.
30 El-Talatini MR, Taylor AH, Elson JC, Brown L, Davidson AC, Konje JC: Localisation and Function of the Endocannabinoid System in the Human Ovary. PLoS One 2009;4:e4579.

31 El-Talatini MR, Taylor AH, Konje JC: Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation. Hum Reprod 2009;24:1989–1998.
32 Chian RC, Buckett WM, Tan SL: In-vitro maturation of human oocytes. Reprod Biomed Online 2004;8:148–166.
33 Cahill CM, Holdridge S V., Morinville A: Trafficking of delta-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia. Trends Pharmacol Sci 2007;28:23–31.
34 Mehlmann LM: Stops and starts in mammalian oocytes: Recent advances in understanding the regulation of meiotic arrest and oocyte maturation. Reproduction 2005;130:791–799.
35 Sánchez F, Smitz J: Molecular control of oogenesis. Biochim Biophys Acta 2012;1822:1896–1912.
36 Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, Geremia R, De Petrocellis L, Di Marzo V: The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. Proc Natl Acad Sci 2009;106:11131–11136.
37 Giacomo D Di, De Domenico E, Sette C, Geremia R, Grimaldi P: Type 2 cannabinoid receptor contributes to the physiological regulation of spermatogenesis. FASEB J 2016;30:1453–1463.
38 Tomek W, Smiljakovic T: Activation of Akt (protein kinase B) stimulates metaphase I to metaphase II transition in bovine oocytes. Reproduction 2005;130:423–430.
39 Kalous J, Solc P, Baran V, Kubelka M, Schultz RM, Motlik J: PKB/AKT is involved in resumption of meiosis in mouse oocytes. Biol Cell 2006;98:111–123.
40 Kalous J, Kubelka M, Šolc P, Šušor A, Motlík J: AKT (protein kinase B) is implicated in meiotic maturation of porcine oocytes. Reproduction 2009;138:645–654.
41 Cecconi S, Mauro A, Cellini V, Patacchiola F: The role of Akt signalling in the mammalian ovary. Int J Dev Biol 2012;56:809–817.
42 Fissore RA, He CL, Vande Woude GF: Potential Role of Mitogen-Activated Protein Kinase during Meiosis Resumption in Bovine Oocytes. Biol Reprod 1996;55:1261–1270.
43 De Domenico E, Todaro F, Rossi G, Dolci S, Geremia R, Rossi P, Grimaldi P: Overactive type 2 cannabinoid receptor induces meiosis in fetal gonads and impairs ovarian reserve. Cell Death Dis 2017;8:1–11.