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Controlled ovarian hyperstimulation cycle: alterations in redox status of the follicular fluid

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"La constancia es la virtud por la cual todas las demás virtudes dan fruto"
Arturo Graf

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El trabajo de tesis doctoral que se presenta se ha organizado como a continuación se describe:

- Introducción. Se describen los antecedentes sobre la estimulación ovárica y los tratamientos de fertilización *in vitro* más utilizados. Se dan algunas pinceladas sobre los principales radicales libres y las especies reactivas derivadas del oxígeno y del nitrógeno, así como las vías de eliminación de estas especies o prevención de su formación mediante sistemas enzimáticos antioxidantes. Se establecen las bases a partir de las cuales se definen la hipótesis y los objetivos.

- Bibliografía. Se enumeran las citas bibliográficas consultadas para la introducción.

- Hipótesis y Objetivos. Se propone la hipótesis de partida y se describen los objetivos a desarrollar.

- Resultados y Discusión. Los resultados y la discusión se presentan en 3 capítulos, que se han desarrollado agrupando varios aspectos de la tesis. Constituyen 3 artículos de investigación (uno publicado y el resto enviado o pendiente de publicar).

  1. Capítulo I. En el primer capítulo se analizan marcadores del estado redox del líquido folicular de folículos maduros procedentes de mujeres fértiles que siguen dos ciclos, uno natural o espontáneo y uno de hiperestimulación ovárica controlada (objetivos 1-3 de la tesis). Se analizan la actividad antioxidante total (TAA), la capacidad absortiva de radicales de oxígeno (ORAC), los niveles intrafoliculares de óxido nítrico y de vitamina E (alfa y gamma tocoferol), la composición acílica del líquido folicular y las actividades de los enzimas antioxidantes catalasa, glutatión peroxidasa, superóxido dismutasa (SOD) y de la familia paraoxonasa (PON). Se realiza el análisis estadístico de los resultados para datos apareados, comparándose el ciclo estimulado con el ciclo natural (control).

  2. Capítulo II. En este capítulo se analizan marcadores de las modificaciones oxidativas de proteínas del líquido folicular en ambos ciclos, natural y estimulado (objetivo 4 de la tesis). Se determinan los biomarcadores: semialdehído glutámico (GSA), semialdehído aminoadípico (AASA), N^{ε}-(carboximetil)lisina (CML), y N^{ε}-(carboxietil)lisina (CEL) por cromatografía gas-líquida acoplada a espectrometría de masas. El diseño del estudio es el mismo que en el capítulo 1.

3. Capítulo III. En el último capítulo se caracterizan en células ováricas humanas de la granulosa las proteínas PON1, PON2 y PON3 (objetivo 5 de la tesis). Se analiza su expresión en dichas células, tanto a nivel proteico como del mRNA. Se detectan sus localizaciones intracelulares por microscopia e inmunofluorescencia y se utilizan siRNAs dirigidos contra los genes de la familia *PON* para determinar la especificidad de la metodología utilizada. Estos ensayos se realizan también en células de hepatoma humano HepG2, por ser células en las que se ha descrito que se expresan estos genes, y en las líneas celulares humanas de granulosa COV434, por ser más fácilmente manejables para estudios de expresión.

- Conclusiones. En este apartado se definen las conclusiones finales extraídas de los resultados de la tesis doctoral.

The doctoral thesis work presented has been organized as follows:

- Introduction. The background on ovarian stimulation and the most commonly used *in vitro* fertilization treatments are described. Some brushstrokes are given on the main free radicals and the reactive species derived from oxygen and nitrogen, as well as the pathways of scavenging these species or preventing their formation through antioxidant enzymatic systems. The bases from which the hypothesis and objectives are defined are established.
- References. The bibliographical citations consulted for the introduction are listed.
- Hypothesis and Objectives. The starting hypothesis is proposed and the objectives to be developed are described.
- Results and Discussion. The results and the discussion are presented in three chapters, which have been developed by grouping several aspects of the thesis. They constitute 3 research articles (one published and the rest sent or pending to be published).).

    1. Chapter I. The first chapter analyses markers of the redox state of follicular fluid from mature follicles from fertile women that follow two cycles, one natural or spontaneous, and one of controlled ovarian hyperstimulation (objectives 1-3 of the thesis). Total antioxidant activity (TAA), oxygen radical absorptive capacity (ORAC), intrafollicular levels of nitric oxide and vitamin E (alpha and gamma tocopherol), fatty acyl composition of follicular fluid and activities of the antioxidant enzymes, catalase, glutathione peroxidase, superoxide dismutase (SOD) and the paraoxonase (PON) family are analyzed. The statistical analysis of the results is performed for paired data, comparing the stimulated cycle with the natural cycle (control).

    2. Chapter II. In this chapter, markers of oxidative modifications of follicular fluid proteins in both natural and stimulated cycles are analyzed (objective 4 of the thesis). The biomarkers semialdehyde glutamic (GSA), aminoadipic semialdehyde (AASA), N^{ε}-(carboxymethyl)lysine (CML), and N^{ε}-(carboxethyl)lysine (CEL) are measured by gas-liquid chromatography coupled to mass spectrometry. The design of the study is the same as in chapter I.

3. Chapter III. In the last chapter, the proteins PON1, PON2 and PON3 are characterized in human ovarian granulose cells (objective 5 of the thesis). Their expression in these cells is analyzed at both protein and mRNA levels. Their intracellular locations are detected by confocal microscopy and immunofluorescence, and siRNAs directed against the *PON* family genes are used to determine the specificity of the applied methodology. These assays are also performed in human hepatoma cells HepG2, because they are cells that have been described as expressing these genes, and in human cell lines of granulosa COV434, because they are more easily manageable for expression studies.

- Conclusions. This section defines the final conclusions drawn from the results of the doctoral thesis.

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1. Resumen

Según la Organización Mundial de la Salud (OMS), la infertilidad es “una enfermedad del aparato reproductor definida por la imposibilidad de lograr un embarazo clínico después de 12 meses o más de relaciones sexuales sin protección regular”. Actualmente cada vez son más las parejas que la sufren y por consenso está establecido que el 30% de los casos de infertilidad se deben al factor femenino, el 30% al factor masculino y el 25% restante a causas desconocidas.

Afortunadamente la aparición y el desarrollo de las técnicas de reproducción asistida (TRA) han supuesto un gran avance para hacer frente a esta enfermedad y los datos muestran un aumento considerable del uso de estas técnicas para la consecución de un embarazo a término, siendo España uno de los paises europeos donde más tratamientos se realizan al año. En la mayoría de los casos las TRA implican la realización de ciclos de hiperestimulación ovárica controlada en la mujer. Tras un ciclo ovárico natural o espontáneo se obtiene únicamente un ovocito. La aplicación de estos ciclos de estimulación tiene como objetivo la obtención de un número elevado de ovocitos, con el fin de aumentar las posibilidades de éxito de todo el tratamiento y conseguir embarazos con recién nacidos vivos. Existen varios protocolos de estimulación ovárica que consisten en la administración de agonistas o antagonistas de la GnRH, principal responsable de la regulación del ciclo ovárico, y de gonadotropinas, hormonas capaces de estimular el desarrollo de varios folículos ováricos y de desencadenar la ovulación en el momento idóneo. Estos protocolos se adaptan en función de las caracterisiticas de las pacientes y, aunque son muy efectivos en cuanto a la obtención de ovocitos, también presentan efectos adversos relacionados con la disminución de la calidad de los ovocitos y el riesgo de recién nacidos prematuros y de bajo peso.

El folículo ovárico es una estructura celular del ovario donde se desarrolla el ovocito. Está compuesto por unas finas capas de células ováricas y se encuentra lleno de lo que se conoce como líquido folicular. Este fluido está en contacto con el ovocito, de manera que su composición es de vital importancia para determinar su calidad. Dentro del líquido folicular podemos encontrar proteínas, hormonas, lípidos y aminoácidos, entre otros. Se considera un exudado del plasma, pero también su composición depende de las

secreciones de las células de la granulosa y la teca que conforman el folículo ovárico. El estado antioxidante del líquido folicular puede ser crucial para el correcto desarrollo de los procesos reproductivos, ya que muchos de ellos están regulados en cierta medida por las especies reactivas derivadas del oxígeno (ROS) y los antioxidantes, también presentes en el líquido folicular. Es necesario un equilibrio entre ROS y antioxidantes, ya que el aumento de los primeros daría lugar a lo que se conoce como "estrés oxidativo", muy perjudicial para los sistemas biológicos.

Teniendo en cuenta estos antecendentes se estableció la HIPÓTESIS de esta tesis doctoral: los ciclos de hiperestimulación ovárica controlada mediados por gonadotropinas provocan alteraciones en el estado antioxidante del líquido folicular y, por tanto, del microambiente del ovocito, provocando una menor protección antioxidante.

El principal OBJETIVO de este trabajo fue caracterizar el estado antioxidante del líquido folicular asociado a los ovocitos aspirados de la misma mujer después de un ciclo de ovulación natural (NC) y un ciclo de hiperestimulación ovárica controlada (COH).

Para conseguir este objetivo se reclutó una población de mujeres fértiles (donantes de óvulos). En ambas situaciones (NC y COH) se obtuvo el líquido folicular. Se plantearon los siguientes OBJETIVOS OPERATIVOS:

1. Determinación de glucosa, α- y γ-tocoferol, óxido nítrico, capacidad antioxidante total (TAA y ORAC), actividad de los enzimas antioxidantes: superóxido dismutasa, glutatión peroxidasa, catalasa y del sistema de paraoxonasas: PON1 y PON3.
2. Cuantificación de las proteínas de la familia de paraoxonasa por transferencia western.
3. Determinación de la composición de los ácidos grasos de los lípidos del líquido folicular por cromatografía de gases.
4. Determinación de los biomarcadores de modificaciones oxidativas no enzimáticas de proteínas: semialdehído glutámico, semialdehído aminoadípico, N^{ε}-(carboxietil)lisina y N^{ε}-(carboximetil)lisina.
5. Caracterización de las proteínas del sistema PON en células de la granulosa humana:
    a. Determinación de los productos de transcripción de los genes *PON1*, *PON2* y *PON3* mediante RT-PCR convencional.

b. Cuantificación de las proteínas PON1, PON2 y PON3 mediante transferencia western cuantitativa
c. Análisis de la distribución subcelular de las proteínas PON1, PON2 y PON3 mediante inmunofluorescencia y microscopía confocal.

La población de estudio consistió en 41 mujeres fértiles (donantes de óvulos) previo consentimiento informado. Todas ellas tenían una edad comprendida entre 18-34 años, un IMC de 18-29 kg/m^2, sin endometriosis, abortos recurrentes o enfermedades genéticas o cromosómicas. Cada mujer siguió un NC y un COH con antagonistas de GnRH, según el protocolo establecido por la clínica. Tras la estimulación, en ambos ciclos se indujo la ovulación con agonistas de GnRH y se aspiraron los ovocitos mediante punción ovárica. Tras retirar el ovocito, el líquido folicular se centrifugó para eliminar los restos de células y sangre y se congeló hasta su utilización.

Para la determinación del estado antioxidante del liquido folicular se emplearon técnicas bioquímicas. La actividad antioxidante total (TAA), la capacidad de absorción de radicales de oxígeno (ORAC), la concentración de oxido nítrico, glucosa y las actividades enzimáticas glutatión peroxidasa, catalasa, superóxido dismutasa y PON1 (arilesterasa y paraoxonasa) se determinaron mediante técnicas espectrofotométricas. La concentración de vitamina E y la actividad enzimática simvastatinasa de PON3 se determinaron mediante HPLC. La cuantificación de PON1 y PON3 se realizó mediante inmunotransferencia western, utilizando proteínas recombinantes humanas como patrones. Para determinar la composición acílica se utilizó la técnica de cromatografía de gases con detector de ionización de llama, y para determinar los productos de las modificaciones oxidativas de las proteínas, cromatografía gas-líquido acoplada a espectrometría de masas (GC/MS).

Para realizar el objetivo 5 se utilizaron células primarias de la granulosa humana, obtenidas de muestras biológicas del líquido folicular tras la punción ovárica mediante gradiente de Percoll. También se han utilizado, para la optimización de las técnicas, líneas celulares de granulosa humana COV434 (Sigma-Aldrich, St Louis, USA) y de hepatocarcinoma humano HepG2 (ATCC, Manassas, USA). Con el fin de caracterizar las proteínas del sistema paraoxonasa (PON1, PON2 y PON3) se llevaron a cabo técnicas de biología celular y molecular en los tres tipos celulares. En cuanto a las técnicas de biología celular, se utilizaron técnicas de fraccionamiento por centrifugación diferencial para la

obtención de extractos celulares, sincronización del ciclo celular mediante doble bloqueo con timidina y citometría de flujo para el estudio del ciclo celular. La localización de proteínas se determinó por immunofluoresencia, utilizando microscopía confocal de alta resolución. En relación a técnicas de biología molecular, se analizó mediante RT-PCR el mRNA de PON1, PON2 y PON3. Para comprobar la especificidad de la metodología empleada se realizó el silenciamiento a nivel transcripcional de dichos genes.

El análisis estadístico se llevó a cabo utilizando el paquete estadístico para las ciencias sociales (IBM-SPPS Statistics 20). Todos los datos se expresaron como media ± SEM. La prueba de Kolmogorov-Smirnov se utilizó para determinar la distribución normal de las variables. Las comparaciones estadísticas entre NC y COH se realizaron utilizando la prueba de la t de Student para muestras apareadas y la prueba de Wilcoxon (equivalente no paramétrico). La diferencia entre las medias se consideró estadísticamente significativa cuando la prueba adquiría un valor de $p<0,05$.

Los resultados de este trabajo mostraron que COH afecta al estado antioxidante del líquido folicular, reduciendo su protección antioxidante. Esto se vió reflejado en una reducción de la TAA, la concentración de α-tocoferol y las actividades de PON1 y PON3 en el líquido folicular. La concentración intrafolicular de PON3 también fue menor en COH. Sin embargo, en el caso de PON1, la concentración intrafolicular de PON1 fue significativamente mayor en COH. Esto podría indicar algún tipo de inhibición que conduce a la reducción de la actividad paraoxonasa de PON1. En la bibliografía se ha descrito que la actividad paraoxonasa en el suero disminuye conforme avanza la edad de la mujer, mientras que la concentración de la proteína PON1 se mantiene constante. Si extrapolamos estos resultados a nuestro estudio, se podría sugerir que COH podría conducir a un envejecimiento prematuro del ovario y reforzaría la idea de que el sistema de enzimas PON desempeña un papel en la fertilidad.

En lo referente a la composición acílica de los lípidos del líquido folicular, los resultados mostraron que COH aumenta la proporción de ácido palmítico y disminuye el contenido de ácido linoleico, aumentando significativamente el cociente araquidonato/linoleato, un índice de la vía biosintética del araquidonato a partir de su precursor. Dado que el ácido araquidónico es el principal precursor de prostaglandinas proinflamatorias, los resultados sugieren que COH podría inducir un proceso inflamatorio diferenciado del NC.

COH indujo un aumento de la concentración intrafolicular de ácidos grasos poliinsaturados n-3 (n-3 PUFAs), en particular del ácido docosahexaenoico (DHA). Se ha descrito que los n-3 PUFA se correlacionan positivamente con la edad de la mujer y negativamente con el número de ovocitos maduros. Estos datos y nuestros resultados hacen cuestionarse si COH supone, de alguna manera, la manifestación de características asociadas a un envejecimiento reproductivo.

Se puso a punto la metodología por cromatografía gas-líquida acoplada a espectrometría de masas para la detección y cuantificación de marcadores de las modificaciones oxidativas de proteínas del líquido folicular. Se determinaron los niveles estacionarios de los biomarcadores semialdehído glutámico (GSA), semialdehído aminoadípico (AASA), N^{ε}-(carboximetil)lisina (CML) y N^{ε}-(carboxietil)lisina (CEL). Los resultados indicaron que COH produjo un aumento de la concentración de CEL sin alterar el contenido de CML, GSA y AASA, lo que indica una menor protección frente al daño oxidativo derivado de los procesos de glicoxidación de proteínas.

Debido a las alteraciones observadas en las actividades de los enzimas de la familia de paraoxonasas inducidas por COH, se investigó si las células ováricas de la granulosa expresaban estas proteínas. Los resultados indicaron que, tanto a nivel transcripcional, como proteico las células de la granulosa expresan PON1, PON2 y PON3, lo que refuerza la idea de que estas proteínas tienen un papel clave en la reproducción.

Las CONCLUSIONES obtenidas de esta tesis doctoral son las siguientes:

1. El ciclo de hiperestimulación ovárica controlada (COH) afecta al equilibrio redox del líquido folicular en mujeres fértiles, dando lugar a un fluido menos protegido frente a los radicales libres que el ciclo natural (NC). Estos cambios se reflejan en una menor actividad antioxidante total (TAA), menores concentraciones de α-tocoferol y actividades antioxidantes de la familia de paraoxonasas disminuídas.
2. COH modifica el perfil acílico de los lípidos totales del líquido folicular, aumentando la proporción de ácidos grasos saturados totales y del ácido graso poliinsaturado n-3 docosahexaenoico.
3. Se ha desarrollado un método para la detección y cuantificación de marcadores de diferentes tipos de lesiones oxidativas de proteínas del líquido folicular humano mediante cromatografía de gases acoplada a espectrometría de masas. Se han

determinado por primera vez en este fluido biológico las concentraciones estacionarias *in vivo* de semialdehído glutámico (GSA) y semialdehído aminoadípico (AASA) como marcadores de lesión oxidativa directa catalizada por metal, N^{ε}-(carboxietil)lisina (CEL) como marcador de lesión oxidativa por glicoxidación, y N^{ε}-(carboximetil)lisina (CML) como marcador mixto de lesión oxidativa por glicoxidación y lipoperoxidación. GSA fue el marcador más abundante en el líquido folicular.

4. Las concentraciones de CEL fueron mayores en COH que en NC, lo que sugiere que COH aumenta la susceptibilidad del líquido folicular al daño oxidativo derivado de procesos de glicoxidación de proteínas.

5. Las células de la granulosa humana expresan PON1, PON2 y PON3 a niveles de mRNA y proteico. PON2 se localiza intracelularmente, mientras que PON1 y PON3, además de distribuirse intracelularmente, se secretan activamente al medio por las células de la granulosa. Estos hallazgos refuerzan el papel de estas proteínas en la reproducción.

2. Summary

According to the World Health Organization (WHO), infertility is "a disease of the reproductive system, defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse." Currently, more and more couples have to deal with it, and it is established that 30% of cases of infertility are due to female factor, 30% to male factor and the remaining 25% to unknown causes.

Fortunately, the appearance and development of assisted reproduction techniques (ART) have been a great step forward in dealing with this disease and data show a considerably increase in the use of these techniques to achieve a full-term pregnancy, Spain being one of European countries where more treatments are carried out per year. In most cases, ART involves the use of controlled ovarian hyperstimulation cycles in women. After a natural or spontaneous ovarian cycle, only one oocyte is retrieved. The application of these stimulation cycles aims to obtain a high number of oocytes, in order to increase the success rates of treatments. There are several stimulation protocols that consist of the administration of agonists or antagonists of the GnRH, the main responsible for the regulation of the ovarian cycle, and gonadotropins, hormones that play a key role in the development of ovarian follicles and the triggering of ovulation. These protocols are adapted to the characteristics of the patients and although they are very effective in terms of obtaining a high number of oocytes, they also have adverse effects, related to the decrease in the quality of the oocytes and the risk of premature and low weight births.

The ovarian follicle is a cellular structure in the ovary, in which the oocyte develops. It is formed by thin layers of ovarian cells and is filled with follicular fluid. This fluid is in contact with the oocyte, so that its composition is of vital importance to determine its quality. Follicular fluid contains proteins, hormones, lipids and amino acids, among others. It is considered a plasma exudate, but its composition also depends on the secretions from the granulosa and theca cells that define the ovarian follicle. The antioxidant state of follicular fluid can be crucial for the correct development of the reproductive processes, since many of them are regulated to a certain extent by the reactive oxygen species (ROS) and antioxidants, also available in follicular fluid. A balance between ROS and antioxidants is needed, since an increase in the former would give rise what is known as "oxidative stress", which is detrimental to biological systems.

Taking into account these antecedents, the HYPOTHESIS of this doctoral thesis was: controlled ovarian hyperstimulation cycles mediated by gonadotropins trigger alterations in the redox balance of follicular fluid and, therefore, in the oocyte microenviroment, rendering a reduced antioxidant protection.

The main OBJECTIVE of the present Doctoral Thesis was to characterize the antioxidant status of follicular fluid associated with oocytes aspirated from the same woman after a natural cycle (NC) and a controlled ovarian hyperstimulation cycle (COH).

To achieve this aim, a population of fertile women (oocyte donors) was recruited. In both situations (NC and COH), ovarian mature follicles were aspirated, and, after oocyte separation, the follicular fluid was subjected to different analyses.

The following OPERATIVE OBJECTIVES were raised:

1. Determination of glucose, α- and γ-tocopherol, nitric oxide, total antioxidant capacity (TAA and ORAC), activity of antioxidant enzymes: superoxide dismutase, glutathione peroxidase, catalase, and the PON system (PON1 and PON3).
2. Quantification of PON system proteins by western blot.
3. Determination of fatty acyl composition from the follicular fluid lipids by gas chromatography.
4. Determination of biomarkers of non-enzymatic oxidative modifications of proteins, namely glutamic semialdehyde, aminoadipic semialdehyde, N^{ε}-(carboxyethyl)lysine and N^{ε}-(carboxymethyl)lysine.
5. Characterization of PON system proteins in human granulosa cells:
    a. Determination of the transcription products of the *PON1*, *PON2* and *PON3* genes by RT-PCR
    b. Quantification of PON1, PON2 and PON3 proteins by quantitative western blot.
    c. Analysis of subcellular distribution of PON1, PON2 and PON3 proteins by immunofluorescence and confocal microscopy.

A population of 41 fertile women (egg donors) with prior informed consent was recruited to achieve these goals. They had an age between 18-34 years, a BMI of 18-29 kg/m^2, no endometriosis, recurrent miscarriages or genetic or chromosomal diseases. Each woman

followed a NC and a COH controlled with GnRH antagonists, according to the protocol established by the clinic. After stimulation, ovulation with GnRH agonists was induced in both cycles and the oocytes were aspirated by ovarian puncture. After removal of the oocyte, the follicular fluid was centrifuged to remove remaining follicular cells and blood, and frozen until use.

Biochemical techniques were used to determine the antioxidant state of the follicular fluid. The total antioxidant activity (TAA), oxygen radicals absorbance capacity (ORAC), nitric oxide and glucose concentrations, and the enzymatic activities: glutathione peroxidase, catalase, superoxide dismutase, and PON1 (arylesterase and paraoxonase) were determined by spectrophotometric techniques. The concentration of vitamin E and the simvastatinase activity of PON3 were determined by HPLC. The quantification of PON1 and PON3 was performed by western blot, using human recombinant proteins as standards. Gas-chromatography technique with flame ionization detector was used to determine the fatty acid profile, and the products of the oxidative modifications of proteins (CEL, CML , AASA, GSA) was determined by gas-liquid chromatography coupled to mass-spectrometry (GC/MS).

Primary human granulosa cells, obtained from biological samples of follicular fluid after ovarian puncture using Percoll gradient, were used to carry out objective 5. Human granulosa COV434 (Sigma- Aldrich, St Louis, USA) and hepatocarcinoma HepG2 (ATCC, Manassas, USA) cell lines were also used for the optimization of techniques. In order to characterize the proteins of the paraoxonase system (PON1, PON2 and PON3), cellular and molecular biology techniques were carried out in the three cell types. Regarding cell biology techniques, differential centrifugation fractionation techniques were used to obtain cell extracts, synchronization of the cell cycle by double thymidine block assay, flow cytometry for the study of the cell cycle, and protein localization by immunofluorescence using high resolution confocal microscopy. In relation to molecular biology techniques, the presence of PON1, PON2 and PON3 mRNAs was analyzed by conventional RT-PCR. To verify the specificity of the methodology used, silencing of PON genes at transcriptional level was carried out.

The statistical analysis was carried out using the statistical package for the social sciences (IBM-SPPS Statistics 20). All data were expressed as mean ± SEM. The Kolmogorov-Smirnov test was used to determine the normal distribution of the variables. Statistical comparisons between NC and COH were performed using the Student’s t-test and the Wilcoxon test

(nonparametric equivalent) for paired samples. A value of $p<0.05$ was considered statistically significant.

The results of this work showed that COH affects the antioxidant state of the follicular liquid, decreasing its antioxidant protection. This was reflected in a reduction of the total antioxidant capacity, the concentration of α-tocopherol, and the activities of PON1 and PON3 in the follicular fluid. The intrafollicular concentration of PON3 was also lower in COH. However, in the case of PON1, the intrafollicular concentration of PON1 was significantly higher in COH. This could indicate some type of inhibition that leads to the reduction of PON1 activity. It has been described in the literature that paraoxonase activity in serum decreases as the woman's age progresses, while the concentration of PON protein remains constant. If we extrapolate these results to our study, it could be suggested that COH could lead to a premature ovarian aging and reinforce the idea that the PON enzyme system plays a role in fertility.

With regard to the fatty acids composition, our results showed that COH leads to a higher proportion of palmitic acid and decreases the content of linoleic acid, increasing significantly the ratio arachidonate/linoleate, an index of the biosynthetic pathway of arachidonate from its precursor. Since arachidonic acid is the main precursor of proinflammatory prostaglandins, the results suggest that COH may induce an inflammatory process distinct from NC.

COH induced an increase in the intrafollicular concentration of n-3 polyunsaturated fatty acids (n-3 PUFA), specifically docosahexaenoic acid (DHA). It has been described that n-3 PUFA correlates positively with the woman's age and negatively with the number of mature oocytes. These data and our results raise the question of whether COH implies, in any way, the manifestation of characteristics associated with reproductive aging.

We have developed the methodology by gas-liquid chromatography coupled to mass-spectrometry for the detection and quantification of markers of protein oxidative modifications in follicular fluid, We have determined the steady-state levels of glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), N^{ε}-(carboxymethyl)lysine (CML), and N^{ε}-carboxyethyl)lysine (CEL). The results indicated that COH produced an increase in the concentration of CEL without altering the content of CML, GSA and AASA, which indicates less protection against oxidative damage derived from protein glycoxidation processes.

Due to the alterations we had seen in the activities of the enzymes of the COH-induced paraoxonase family, we investigated whether ovarian granulosa cells expressed these proteins.

The results indicated that, at both transcriptional and protein levels, granulose cells express PON1, PON2 and PON3, reinforcing the idea that these proteins play a key role in reproduction.

The CONCLUSIONS obtained from this doctoral thesis are the following:

1. Controlled ovarian hyperstimulation cycle (COH) affects the redox balance of follicular fluid in fertile women, leading to a less protected fluid against free radicals than from natural cycle (NC). These changes are reflected in a lower total antioxidant activity (TAA), α-tocopherol levels and the antioxidant activities of the paraoxonase family.
2. COH modifies the fatty acid profile of the total lipids from the follicular fluid, increasing the proportion of total saturated fatty acids, and n-3 polyunsaturated docosahexaenoic acid.
3. A method has been developed for the detection and quantification of markers of different types of oxidative lesions of proteins from human follicular fluid by gas chromatography coupled to mass spectrometry. For the first time, the *in vivo* steady-state levels of glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA) as markers of direct metal-catalyzed oxidative lesion, N^{ε}-(carboxyethyl)lysine (CEL) as marker of oxidative lesion by glycoxidation, and N^{ε}-(carboxymethyl)lysine (CML) as mixed marker of oxidative lesion by glycoxidation and lipoperoxidation, have been determined in this biological fluid. GSA was the most abundant marker in the follicular fluid.
4. The concentrations of CEL were higher in COH than in NC, suggesting that COH increases the susceptibility of follicular fluid to oxidative damage derived from protein glycoxidation processes.
5. Human granulosa cells express PON1, PON2 and PON3 both at the mRNA and protein levels. PON2 is intracellularly located, while PON1 and PON3, in addition to being distributed intracellularly, are actively secreted into the medium by granulose cells. These findings reinforce the role of these proteins in reproduction.

INTRODUCTION

INTRODUCTION

1. Infertility and assisted reproduction technology

Infertility is recognised by World Health Organisation (WHO) as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al. 2009). At this time, it can be stablished that infertility has become a global problem as it is estimated that more than 40 million persons all over the world suffer from it (Mascarenhas et al. 2012).

Approximately 9% of the couples have fertility problems (Boivin et al. 2007), whose etiology has a very varied origin (genetic, epigenetic, mitochondrial, immunological, hormonal and environmental) (Tarin et al. 2014). By consensus, 30% of infertility cases are due to female factor, 30% to male factor, 25% to both of them, and 15% are cases of unexplained infertility.

The main causes of male infertility are testicular dysfunction, alterations of the genital tract that affect sperm transport, erection problems, alterations in semen production (azoospermia, oligospermia) and obesity (De Kretser 1997; Reis and Dias 2012; Di Vicenzo et al. 2018).

Regarding women, infertility is mainly due to ovulatory dysfunction, that can be caused by an advanced age (at the age of 35, the reproductive potential decreases and after 40 years, the possibility of pregnancy is less than 10%), endocrine disorders, polycystic ovary syndrome (PCOS) and diminished ovarian reserve. Tubal factor (obstruction of fallopian tubes), uterine or cervical factors (congenital uterine anomaly) and endometriosis are also related to this disease (Practice Committee of the American Society for Reproductive Medicine 2004; Hull et al. 1985).

Reproductive medicine is the medical specialty responsible for diagnosing and treating infertility, preserve fertility, and other reproductive problems. However, over the last decades, the development of Assisted Reproduction Technology (ART) has been a very important breakthrough for the treatment of infertility (Passos 2004; Kamel 2013).

ART consists on all treatments or procedures that include the *in vitro* handling of both human oocytes and sperm, or embryos, for establishing a pregnancy (Zegers-Hochschild et al. 2009) . The main techniques are:

- **Conventional *In Vitro* Fertilization (IVF)**. Through this technique oocyte-sperm fertilization is performed externally, in the laboratory, to subsequently transfer the embryo to maternal uterus (Fig. 1a). The first step in IVF is the ovarian stimulation to obtain a high number of oocytes per cycle. In order to recover oocytes, the woman undergoes a follicular puncture. Once retrieved, several oocytes are selected and a sample of sperm (previously capacitated) is added, so that the fertilization takes place spontaneously. Once fertilized, the development of the embryo is rigourosly contolled and it is implanted by embryo transfer in the maternal uterus.

- **Intracytoplasmic Sperm Injection (ICSI)**. It is a variant of IVF in which a single sperm is selected and injected into an oocyte using specific micropipettes (Fig. 1b). This technique has allowed to successfully achieving pregnancy in couples diagnosed with a severe male factor (Palermo et al. 1992).

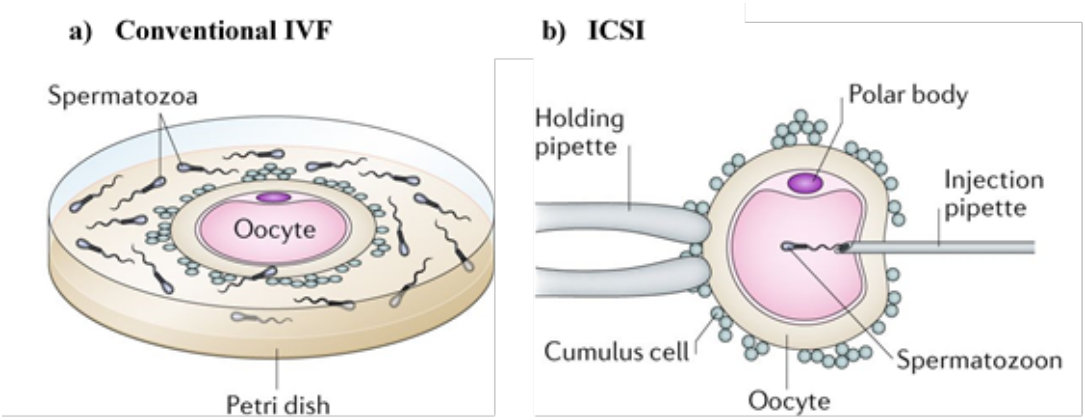

Figure 1. Scheme of a) Conventional IVF and b) ICSI. From Esteves et al. (2018).

In addition to these main techniques, ovodonation, a type of IVF in which oocytes fertilized come from a donor woman, gamete intrafallopian transfer and gamete and embryo cryopreservation are also used.

Even though they are not included in what WHO defines as ART (Zegers-Hochschild et al. 2009), it is important to mention Artificial Insemination (AI), which involves inserting a sperm sample (from anonymous donor or male partner) through the female's

cervix and into the uterus. The most frequent indications of insemination with male partner's sperm are mild or moderate male factor, sterility without apparent cause and serodiscordant couples in which the male is HIV positive (Jindal et al. 2016).

The seminal washing techniques and sperm capacitation (swim-up, MACS…) allow to eliminate the seminal plasma and concentrate small volumes of sperm provided with progressive mobility, and are also of vital importance to offer good results in assisted reproduction programs.

Since the first IVF treatment was successfully carried out in 1978 (Steptoe and Edwards 1978), the use of these assisted reproduction techniques has spread exponentially. The last published study of European Society of Human Reproduction and Embryology (ESHRE) provides us recent data about the situation of assisted reproduction in Europe. In 2014 a total number of 776,556 assisted reproduction treatment cycles were reported (+13.1% than in 2013) and 170,163 children were born. Since the beginning of data collection in 1997, it is estimated that more than 8 million treatment cycles have been carried out and almost 1.5 million children have been born (De Geyter et al. 2018).

Spain is the European country where the most assisted reproduction treatment cycles are conducted (109,275) and 6.4% of national births corresponds to pregnancies achieved through these techniques (De Geyter et al. 2018).

Despite the development of ART and optimization of the treatments, most of the couples achieve success after multiple attempts. Therefore, we must not ignore the psychological and social consequences faced by people who undergo this type of treatments and suffer from infertility, especially women.

For women from developing countries, infertility can be a reason for discrimination, stigma and ostracism (Cui 2010). In most Western countries, there is no such total rejection of infertile women. However, women generally feel guilty when they are not able to achieve pregnancy (Cousineau and Domar 2007) and consider the whole process of assisted reproduction as the most distressed experience of their lives (Freeman et al. 1985). It has also been demonstrated that the prevalence of depression in women suffering from infertility is higher than in fertile women (Domar et al. 1992; Cwikel et al. 2004), and stress generated before, during, and after treatments is one of the main causes of abandonment (Olivius et al. 2002; Brandes et al. 2009). Therefore, it is of great interest to

continue researching in this field, to have a better understanding of infertility and to be able to improve and overcome the difficulties that all of these treatments present.

2. Controlled Ovarian Hyperstimulation (COH)

Controlled ovarian hyperstimulation is a pharmacological treatment in which women are stimulated to induce the development of multiple ovarian follicles to obtain multiple oocytes (Zegers-Hochschild et al. 2009).

2.1 Physiology of natural ovarian cycle

Under normal physiological conditions, that is, in a natural or spontaneous ovarian cycle, the ovary releases only one oocyte. This process is accurately regulated by hypothalamus – hypophysis – ovary neuroendocrine axis with the participation of several hormones.

The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which is the principal regulator of reproductive axis. Its secretion is pulsatile and varies both in frequency and in amplitude throughout the cycle (Marshall et al. 1991).

GnRH binds to its receptor on a population of gonadotropic cells of the adenohypophysis, which promote the synthesis and release of gonadotropins, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), through the bloodstream to the ovary. Rapid GnRH pulse (high frequency, low amplitude) increases LH, and slow GnRH pulse (low frequency, high amplitude) increases FSH gene transcription (Kaiser et al. 1997; Dalkin et al. 1989).

Each cycle can be divided in two phases, follicular phase and luteal phase. During the follicular phase, GnRH pulses are slow. The increase of FSH stimulates the recruitment and development of an ovarian follicle pool. Follicle maturation stimulates the secretion of estradiol (E2) by the ovary, which selectively inhibits FSH release (negative feedback), needed for selection of the dominant follicle, and maintains rapid GnRH pulsatility during the late follicular phase. The persistent rapid GnRH pulses increases LH, which further stimulates E2 secretion, culminating in positive E2 feedback to produce the mid-cycle LH surge. At this point, the mature oocyte releases from the follicle (Fig. 2). After ovulation, luteinization of the ruptured follicle results in progesterone secretion, which reduces the frequency of GnRH pulses. With the demise of corpus luteum, E2, progesterone and

inhibin levels fall, and the GnRH pulse frequency increases, leading to follicular maturation in the next cycle (Fig. 2).

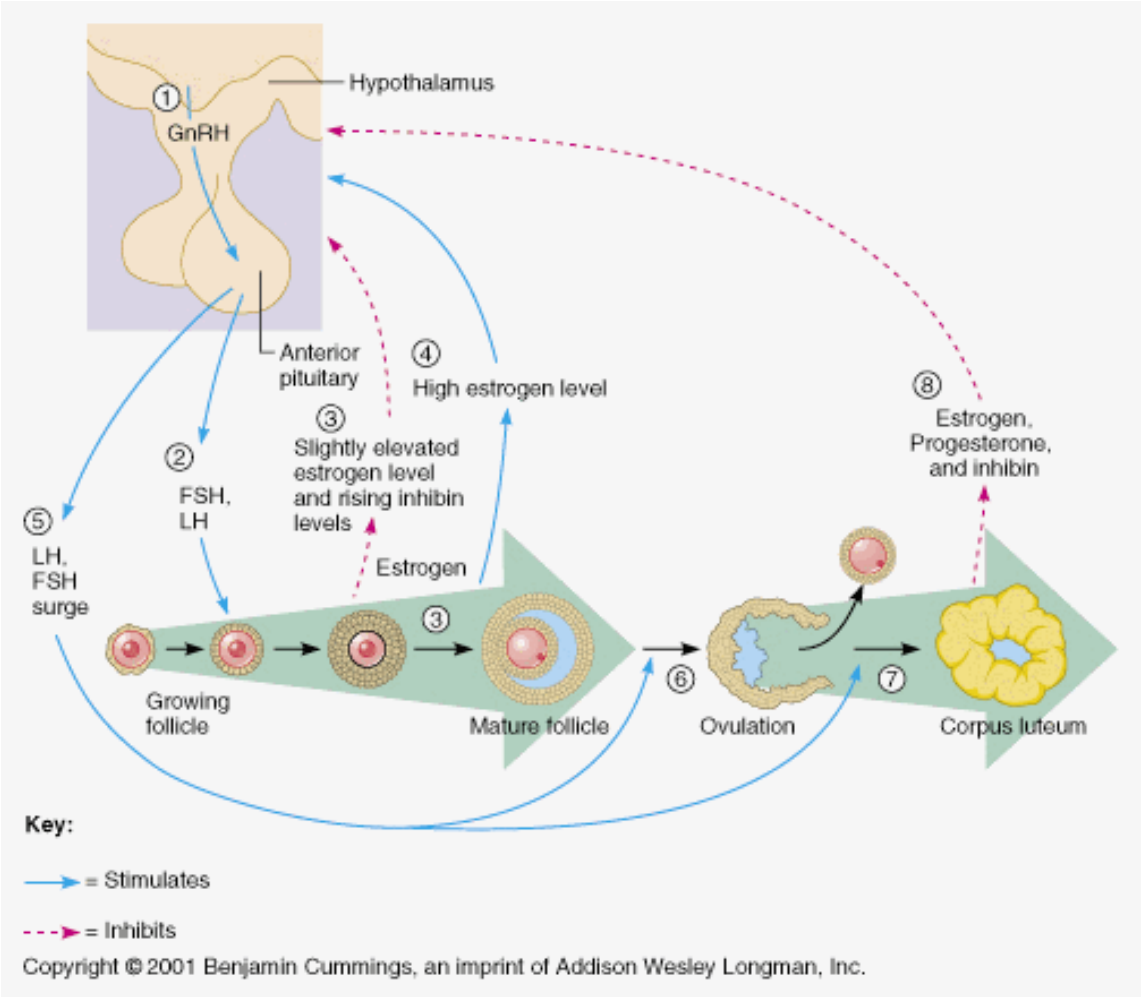

Figure 2. Scheme of a natural ovarian cycle. GnRh is released by hypothalamus and binds to specialized cells in adenohypophysis (1), activating the synthesis and release of FSH and LH (2), which stimulates the recruitment and maturation of follicles. During maturation E2 is synthesized by the ovary to inhibit FSH production (3) and in this way the selection of a single dominant follicle is favored. In the middle of the cycle LH surge (5) is provoked by the high estradiol levels (4) and ovulation takes place. Only one oocyte is released (6) and the follicle that contained it starts its luteneization (7) with a subsequently secretion of progesterone which inhibits GnRH released (8). With the demise of corpus luteum, E2, progesterone and inhibin levels decrease, and the GnRH pulse frequency increases, leading to follicular maturation in the next cycle.

This process is also known as menstrual cycle and lasts 28 days approximately. All these hormonal changes leading by GnRH have a very precise synchronization and their effect not only focus on adenohypophysis and ovaries but also in the uterus. In this organ, the superficial layers of the endometrium are affected by the action of the hormones involved in the cycle and these changes can be grouped in 3 phases: *menstrual phase*, from day 1 to day 4, *proliferative phase*, from day 5 to day 14, and finally *secretory phase*, from day 15 to day 28 (Fig. 3).

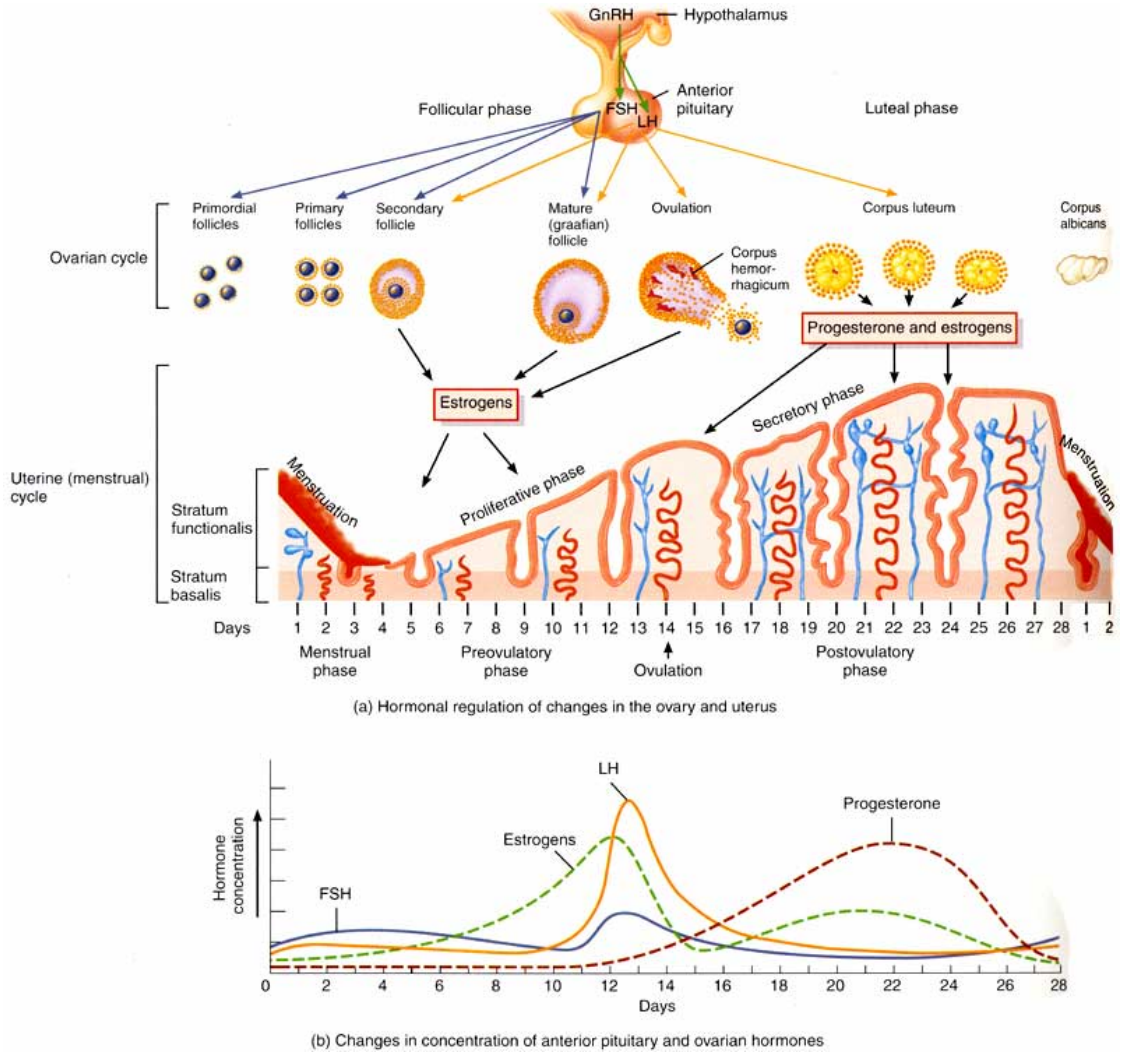

Figure 3. General scheme of the progression of spontaneous ovarian cycle over time. Progression of hormonal different stages and their effects in the ovary and uterus are shown. From Freund (2012)

2.2 Evolution of COH

The origin of ovarian stimulation begins with the discovery of the human chorionic gonadotropin hormone (hCG) in 1927 by Ascheim and Zondek in the urine of pregnant women. In their experiments carried out on immature female mice, they found that the subcutaneous administration of this hormone caused follicular development, luteneization and the haemorrhage into the ovarian stroma. By this time, other studies conducted also by Zondek proposed the existence of two substances, called Prolan A and B (FSH and LH), secreted by the pituitary gland that participated in follicular development and ovulation (Reviewed in Beall and Decherney, 2012 and Lunenfeld 2012).

The discovery of gonadotropins implication in the ovulation cycles prompted great interest among reproductive professionals, who considered these molecules as a possible therapy for infertile women who suffered from anovulation. In 1938, Pregnant Mare Serum Gonadotropin (PMSG) was marketed with the intention of acting as an inducer of ovulation, and several clinical trials were carried out, however the clinical validity of this hormone and of all those originated in animals was questioned (Reviewed in Beall and Decherney, 2012 and Lunenfeld, 2012).

During the decade of 1950's, gonadotropins were extracted from the human pituitary glands (human pituitary gonadotropins - hPG) and from the urine of menopausal women (human menopausal gonadotropins - hMG). hPG was used to induce ovulation with good results (Gemzell et al.1958), but it was discontinued in 1988 due to the limited source of pituitaries and consequently difficulty of satisfying the growing demand for gonadotropins. In addition, this hormone was later related to iatrogenic Creutzfeld-Jakob disease (CJD) (Cochius et al. 1990; Dumble 1992). hMG was firstly used for ovulation induction in 1960 with expected results in vaginal epithelium, endometrium and steroid secretion, although the purity of preparations was very low since they only contained 5% FSH and LH. Lately, hCG was successfully incorporated to the treatment to induce follicular rupture and consequently oocyte release (Lunenfeld 1963). At this point, the interest was focused on increasing the purity of gonadotropins preparations because in the early 1970s, clinicians began to notice that more flexibility was necessary in adjusting the stimulation protocols in an individualized way, using different doses of hormones (Reviewed in Lunenfeld 2004).

By immunochromatography with polyclonal antibodies to hMG it was possible to separate the FSH fraction from the urine, obtaining the product *Metrodine* from Serono Laboratories for commercial use (Lunenfeld and Eshkol 1970) and the development of monoclonal antibodies (more specific) also led to the production of highly purified FSH (*hpFSH - Metrodine HP*). However, the growing worldwide demand for the use of gonadotropins drove research to obtain a different source of these hormones, not based exclusively on the urine of a menopausal woman.

During the development of these purification techniques, the field of genetic engineering advanced quickly and in the 1980s allowed the production of the so-called recombinant gonadotropins rFSH (recombinant FSH) and rLH (recombinant LH). In 1992, the first pregnancy using rFSH was reported (Germond et al. 1992; Devroey et al. 1992).

Parallel to these facts, in 1971 GnRH was described and synthesized by Schally et al. (1971). Its implication in gonadotropins release was the key of actual ovarian hyperstimulation protocols. Since its discovery, hundreds of analogues have been synthesized and its use revolutionized ovarian stimulation for FIV/ICSI. In the 1980s, GnRH agonists became widespread with a radical shift in pregnancy rates (Hughes et al. 1992) and in the 1990s, the incorporation of the GnRH antagonists meant a simplification of the IVF-ICSI protocols (Coccia et al. 2004).

2.3 Analogues of GnRH: mechanisms of action

GnRH is a decapeptide with the following structure: pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2. The replacement or deletion of different amino acids within the original molecule resulted in the discovery of GnRH agonists and antagonists (Fig. 4).

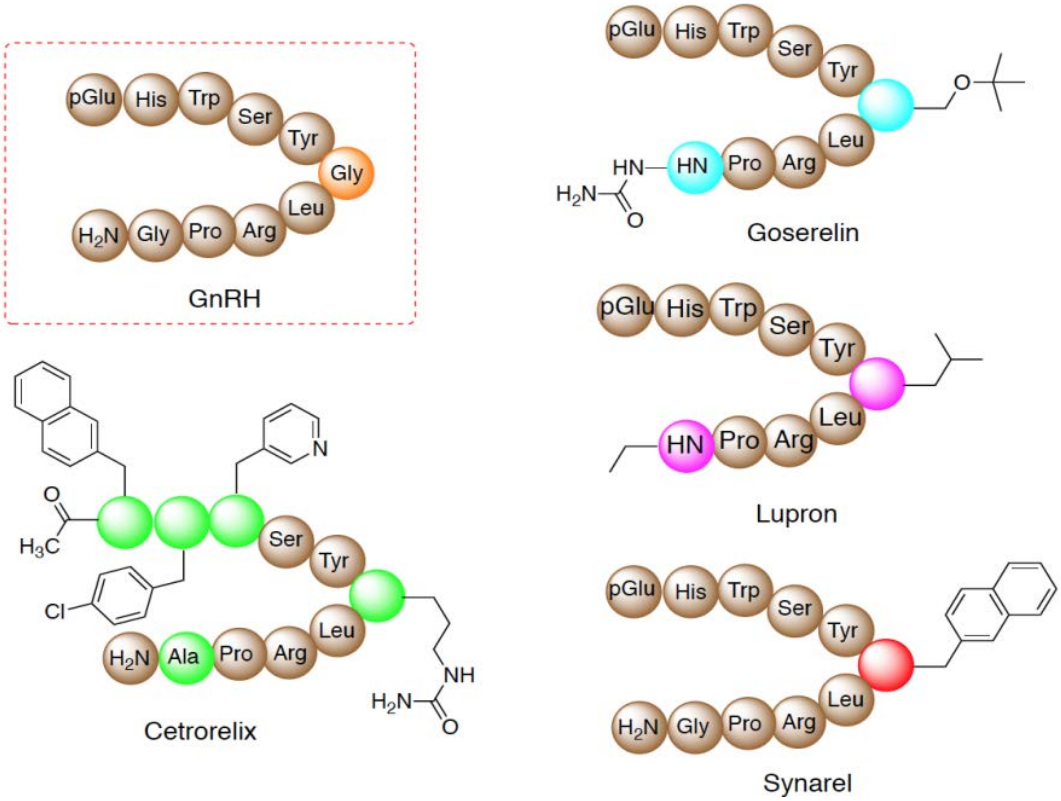

Figure 4. Comparison of native GnRH structure with four analogues. In GnRH agonists (Goserelin, Lupron and Synarel) Gly is substituted by diferente aminoacids and in GnRH anatagonist (Cetrorelix) a higher number of aminoacids are substituted. From Kafarski and Lipok (2015)

Under normal conditions, as explained above, GnRH is released by the hypothalamus, It binds to the membrane receptors of the gonadotropic cells in the adenohypophysis and stimulates the synthesis and release of FSH and LH gonadotropins, which are responsible for follicular maturation process and subsequent ovulation.

GnRH analogues have a longer half-life and more competencies than the native molecule, and their use aim to avoid the spontaneous LH surge that triggers ovulation by suppression of the endogenous synthesis of FSH and LH (Al-Inany et al. 2007). The mechanisms of action of these analogues on the gonadotropic cells of the adenohypophysis differ if they are agonists or antagonists.

GnRH agonists. They have a higher affinity for the GnRH receptor than the native GnRH. Their binding induces the release of large amounts of FSH and LH (flare up) and, at the same time, its effect induces the increase in the number of receptors (upregulation). However, after prolonged use, internalization of the receptor-GnRH agonist complex occurs, which leads to a decrease in the number of GnRH receptors and, therefore, in FSH and LH release (downregulation) (Conn and Crowley 1994) (Fig. 5).

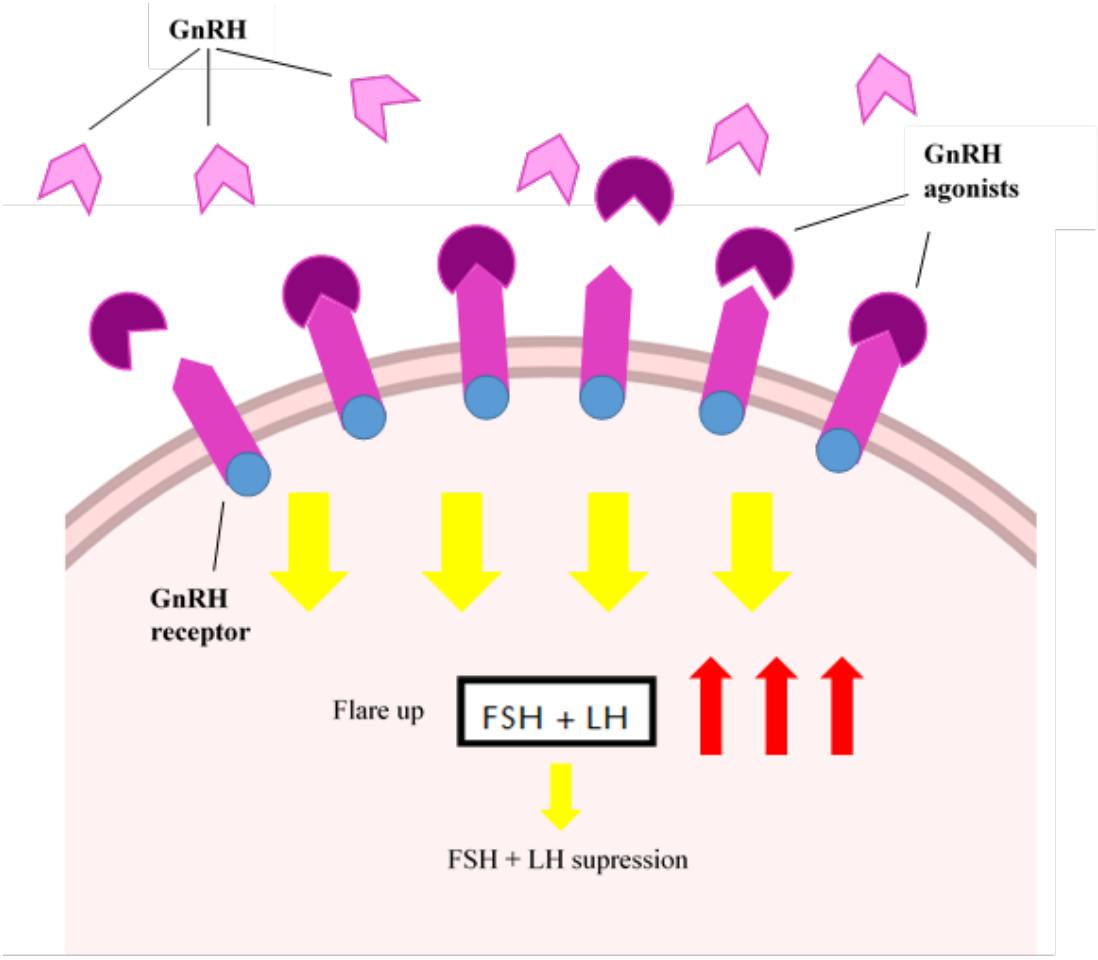

Figure 5. Mechanism of action of GnRH agonists

GnRH antagonists. They bind competitively to GnRH receptor, blocking it and preventing the action of endogenous GnRH pulses on the adenohypophysis. The secretion of gonadotropins decreases within hours of the administration of the antagonists and there is no flare up effect (Conn and Crowley 1994) (Fig. 6).

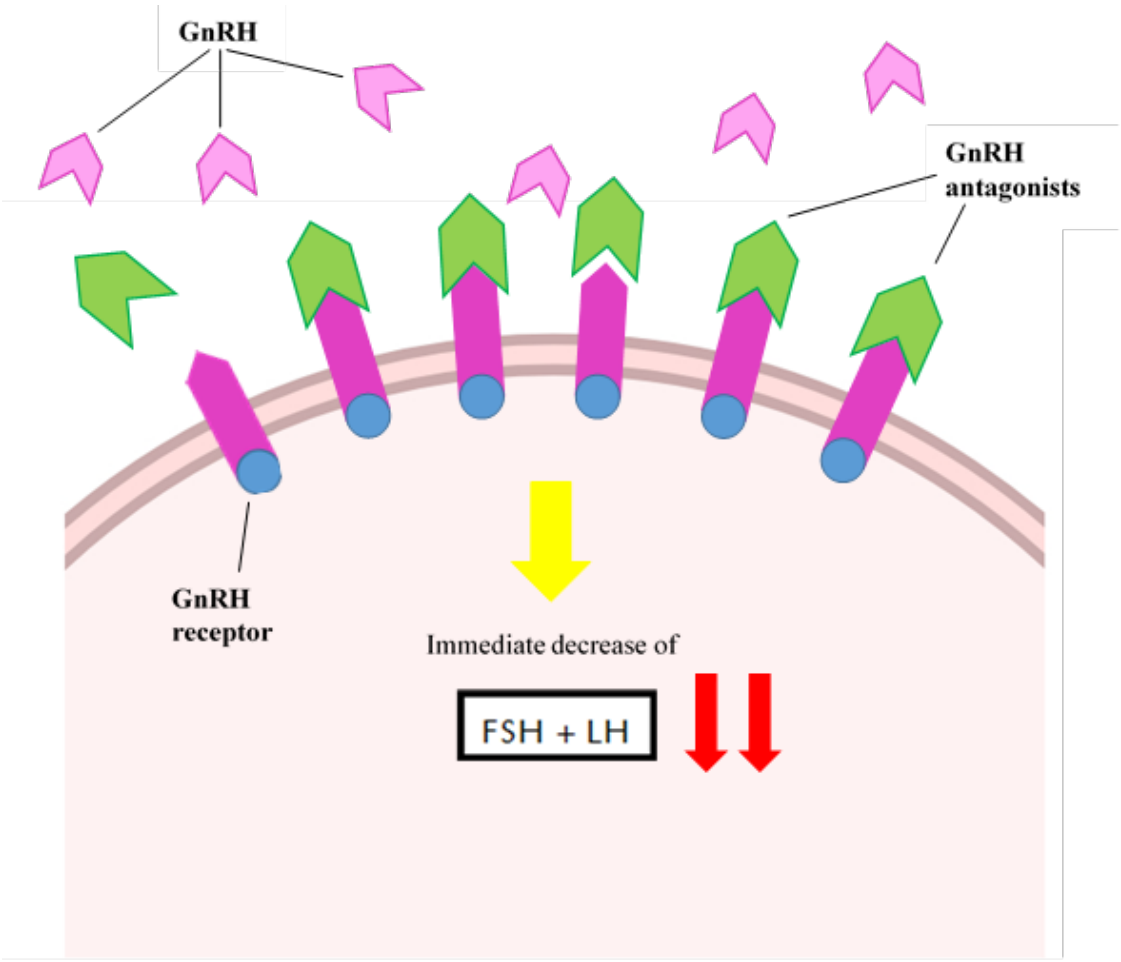

Figure 6. Mechanism of action of GnRH antagonists

2.4 COH protocols

Currently there is a wide variety of COH protocols combining GnRH analogues and gonadotropins due to the individualization of the treatments (Fig. 7). The theoretical basis of all of them is as follows: GnRH agonists or antagonists are administered to the patient to suppress the endogenous release of FSH and LH. Gonadotropins are then administered in a controlled manner to induce the synchronized recruitment and development of the follicles. Finally, hCG or GnRH agonists are administered to induce maturation and ovulation at a specific moment, so as to be able to extract all the oocytes by follicular puncture.

COH most common protocols are:

Agonists protocols

- Long protocol. GnRH agonists are administrated in the follicular or luteal phase of the previous cycle of ovarian stimulation. Once hypophysis suppression is confirmed, the dose of the analogue can be reduced by half and the application of the gonadotropins should begin according to the chosen protocol until the criteria for the hCG administration have been reached. This protocol is usually applied to young women with normal ovarian reserve (Hu et al. 2014).
- Short protocol. GnRH agonist administration begins in early follicular phase of ovarian stimulation cycle. GnRH agonist is administrated on day 2 of menstruation (Hu et al. 2014). The flare up effect provoked by agonist is added to the effect of administering exogenous gonadotropins, increasing follicular recruitment. The dose of agonist is stopped when the follicular maturity is reached and ovulation is induced with hCG. This protocol is usually used for poor responder patients (Kumar and Sharma 2014; Hu et al. 2014).

Antagonists protocols

- Single dose protocol; also called “French protocol”. It was developed by Olivennes et al. (1995). It consists in the administration of a single dose of GnRH antagonist on the 7^{th} or 8^{th} day of ovarian stimulation with gonadotropins, or when the largest follicles reach 14 mm in diameter. If after 72 hours of the application of the antagonist the patient does not get ready for hCG trigger, daily doses of antagonists should be administered until the day of hCG administration.

- Multiple dose protocol: also called "Lübeck protocol" since it was developed by Diedrich et al. (1994) at the Lübeck University Clinic in Germany. It consists of the administration of daily injections with low doses of the GnRH antagonist, starting at the 5th to the 7th day of ovarian stimulation with gonadotrophins, until the day of the injection of hCG.

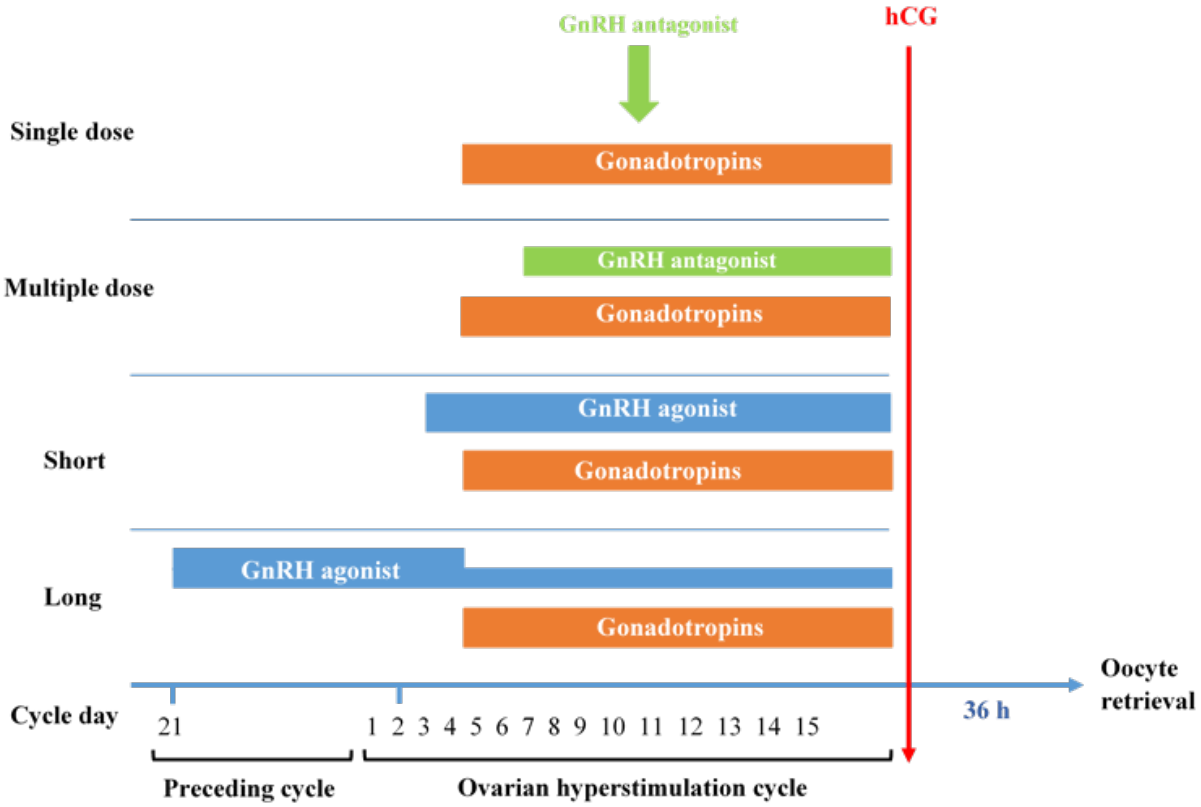

Figure 7. Scheme of GnRH agonist and antagonist protocols.

2.5 Side effects of COH

Despite the fact that COH cycles are routinely used in IVF/ICSI programs, the use of these drugs can have negative effects to patients. Ovarian hyperstimulation syndrome (OHSS) is the most common complication. One of its causes is the increase of vascular endothelial grow factor (VEGF) secreted by granulosa cells induced by hCG. Severe OHSS is associated with higher levels of VEGF (Pellicer et al. 1999).

Obtaining a high number of oocytes after a cycle is something positive from the clinical point of view, but it is also associated with a loss of oocyte quality. It was shown that the retrieval of more than 10 oocytes in women was associated with lower oocyte

quality (Pellicer et al. 1989), and that when more than 13 oocytes were retrieved, pregnancy rates were lower (Van der Gaast et al. 2006). In some cases, there has also been a positive correlation between the use of exogenous gonadotropins and aneuploid embryos (Baart et al. 2009).

ART and COH also influence perinatal outcomes. Multiple pregnancies, low birthweight risk and premature birth are closely related to the use of exogenous gonadotropins (Mak et al. 2016), and a study showed that 4 year old children born following IVF with COH and embryo transfer had higher systolic blood pressure percentils (La Bastide-Van Gemert et al. 2014).

3. Oocyte microenvironment: Ovarian follicles and follicular fluid

3.1 Follicular development

The ovarian follicles are cellular structures in which the oocyte grows and matures until ovulation occurs. They develop in ovarian stroma under a strict regulation and constitute the basic units of female reproductive biology. Until they acquire their competence, they suffer from different morphological, structural and functional changes (Fig. 8).

Primordial follicle formation starts around twentieth week of gestation and continues until just after birth. They are formed by the primary oocyte (stopped at prophase I) surrounded by a cellular monolayer, generally consisting of 4 to 8 cells of fusiform shape (pregranulosa). Subsequently, these cells differentiate into granulosa cells, which proliferate to form a layer of cuboidal cells that completely surround the oocyte, forming the primary follicle. The follicle will increase its size due to high mitotic activity of granulosa cells, which will form a stratified epithelium. Meanwhile, the oocyte initiates the synthesis of the glycoproteins that will form the zona pellucida (extracellular layer surrounding the oocyte). Both cell lines initiate the development of cytoplasmic extensions that cross the zona pellucida to maintain an intercellular communication through gap-type junctions. In addition, a continuous paracrine-type regulation is initiated, which involves products that are secreted by both the oocyte and the granulosa cells into the intercellular space (Albertini et al. 2001).

The secondary follicle is characterised by an increase of size and the differentiation of the theca cells and the basal lamina. At this point granulosa cells begin to express FSH receptors at their membranes and from this stage, follicular development is dependent on both FSH and LH gonadotropins (Lovekamp-Swan and Davis 2003).

The most external layer of granulosa cells starts to form spaces full of liquid. These spaces will coalesce and form the antrum, the main characteristic of antral follicle.

From this moment, the dominant follicle will evolve reaching the maximum level of development, becoming pre-ovulatory follicle or Graafian follicle. At this time, theca and granulosa cells are regionally differentiated and when ovulation occurs due to LH surge, they redifferentiate to luteal cells, giving rise to corpus luteum.

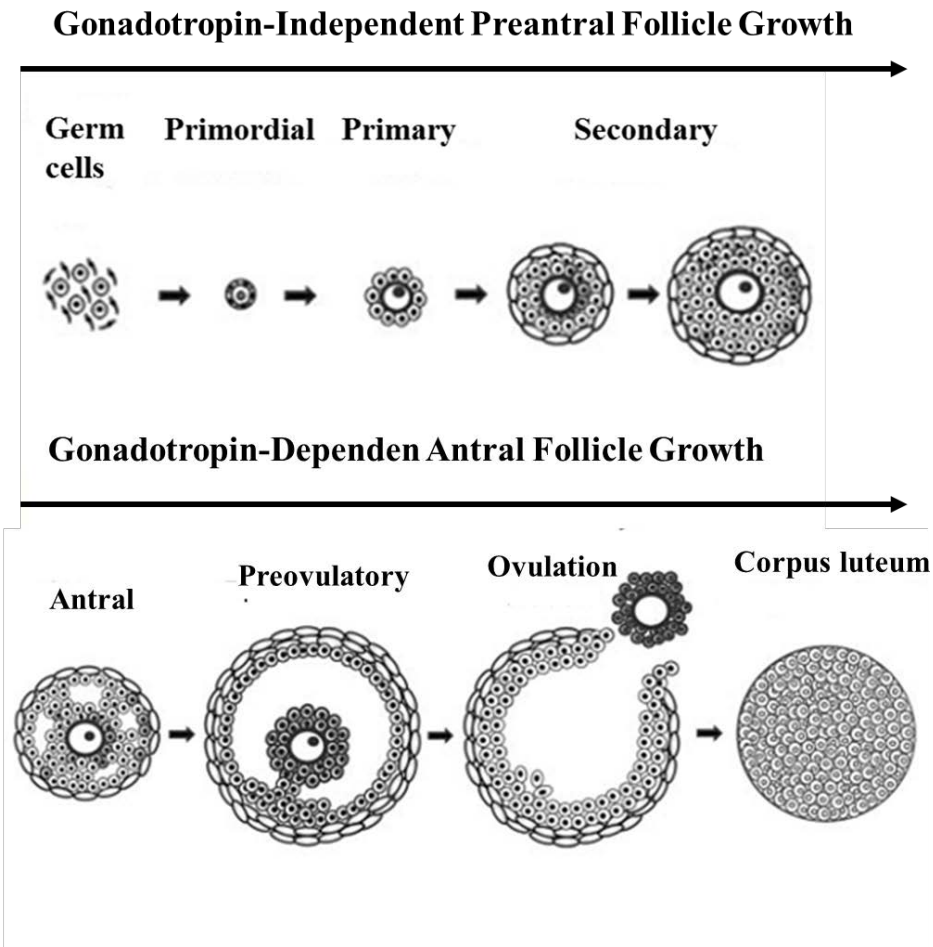

Figure 8. ***Cont.***

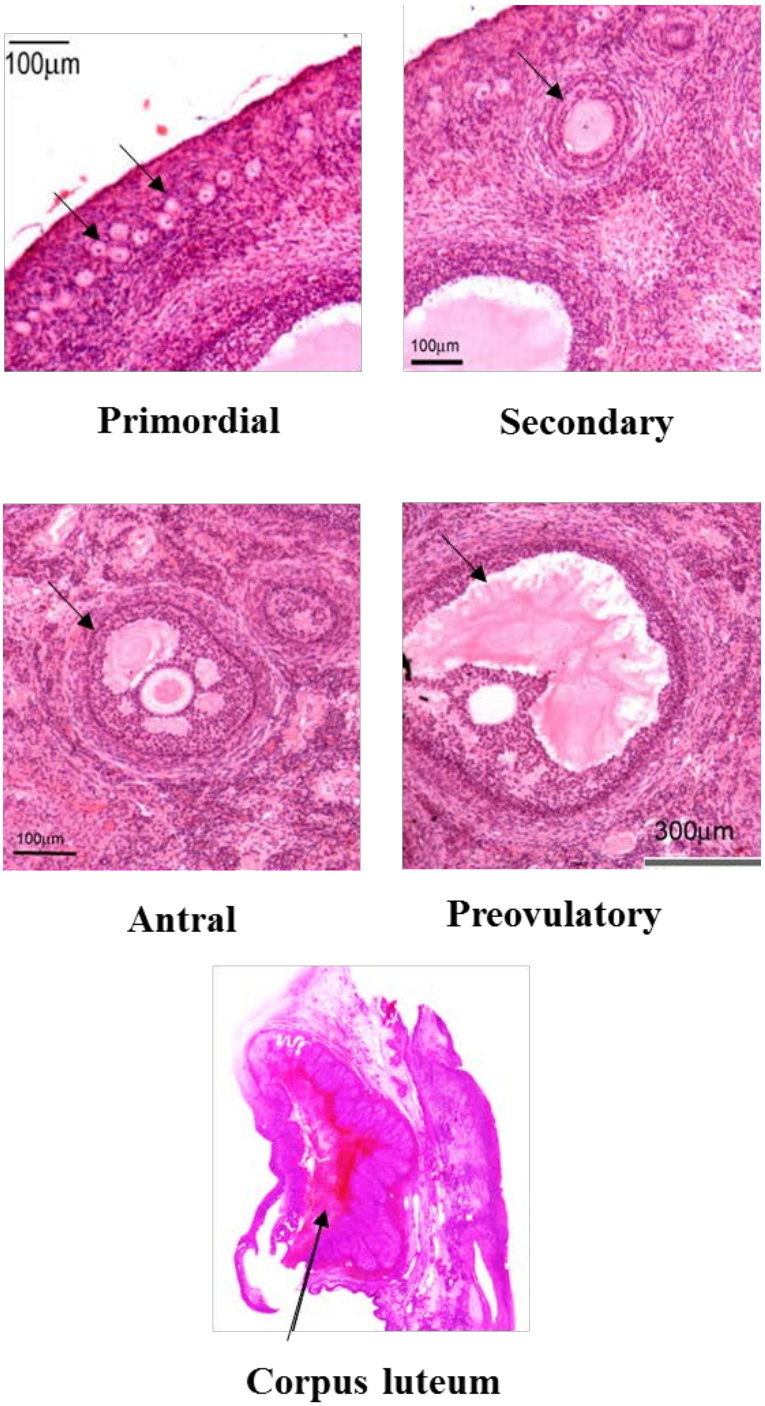

Figure 8. Follicular development. a) Different stages of follicular growth Modified from Hua et al. (2015). b) Histological images of different stages of follicular growth. Available in Histology Guide © Faculty of Biological Sciences, University of Leeds https://www.histology.leeds.ac.uk/

3.2 Graafian follicle

Graafian follicle or pre-ovulatory follicle is the complete developed follicle, which contains the oocyte ready to be released. Its size ranges 15-20 mm diameter and protrudes on the surface of the ovary in the period closest to ovulation. It is formed by multiple layers of granulosa and theca cells that are responsible for controlling follicle development in the preantral stage (Palma et al. 2012). Histologically, several parts are distinguished (Fig. 9).

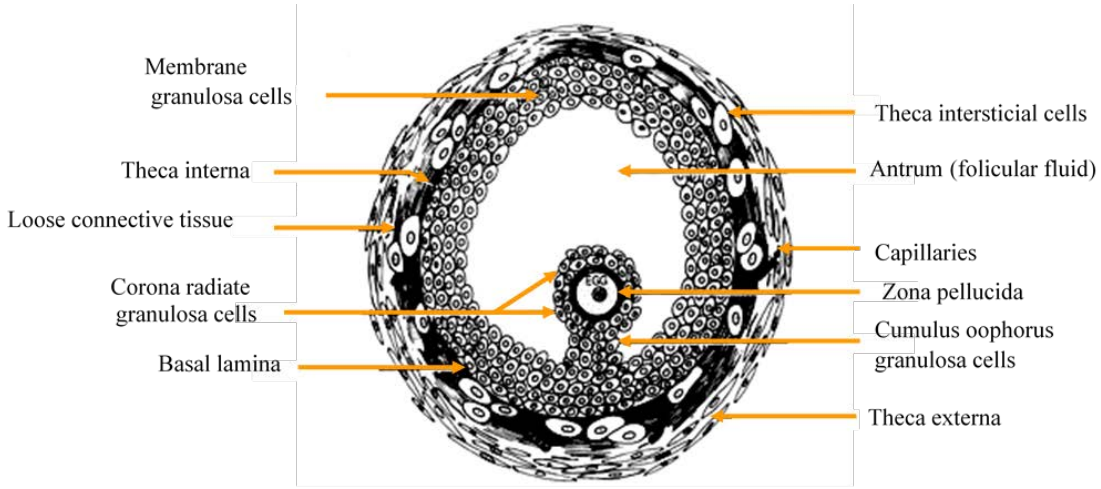

Figure 9. Graafian follicle structure. Modified from Erickson (1983).

Granulosa cells are somatic cells. They form the basic structure of the follicle due to the expression of Stem Cell Factor (SFC), which recognises a specific receptor in oocyte membrane (Abir et al. 2004). They are thought to secrete Oocyte Maturation Inhibitor (OMI), a substance that keeps oocyte arrested in meiosis I (Tsafriri and Pomerantz 1986) as well as numerous growth factors.

Granulosa cells layout leads to several structures within the follicle:

- *Corona radiate*. It consists of two or three layers of granulosa cells that are attached to the zona pellucida, the outermost protective layer of the oocyte.
- *Cumulus oophorus*. It is a cluster of granulosa cells surrounding the oocyte and binding it with the inner granulosa cells layer of the follicle. For fertilization to occur sperm must penetrate this layer.

- *Antrum.* Cavity where the oocyte is located. It is filled with follicular fluid, enriched in lipids and proteins that favors the development of the oocyte.

Theca cells are also important for follicular development. They are believed to origin from fibroblast-like stromal cells in the ovary (Erickson et al. 1985; Honda et al. 2007). Theca cells are capable to produce androgens, which subsequently will be transformed in estrogens by granulosa cells. Their interaction with granulosa cells and the oocyte is mediated by members of the autocrine BMP and TGF-β families and other growth factors.

Theca cells also provide structural integrity to the follicle by the differentiation of two layers:

- *Theca interna*. Cells from this layer are responsible for androgens synthesis. They present small lipid vesicles, an abundant smooth endoplasmic reticulum and mitochondria. This layer is very vascularized.

- *Theca externa*. It is composed mainly of fibrous connective tissue.

3.3 Follicular recruitment

Gonads and germ cells begin to form in the 5th week of intrauterine life. These cells multiply by mitosis and differentiate to oogonias, reaching a number of around 7 million in the fetal ovary at 20 weeks of gestation. During this period oogonias begin meiosis I, becoming primary oocytes and arrest in the diplotene phase (prophase I) due to the formation of primordial follicles. If oocyte-follicle cells interaction do not occur, oocytes are lost via apoptosis (De Pol et al. 1997; Abir et al. 2004). At birth, the population of primordial follicles present in both ovaries is around 2 million and this pool is decreased until the time of menopause at, approximately 50 years, when the pool depletes (Fig. 10). The process that involves follicular development until antral and preovulatory stages is called *follicular recruitment*. Currently, it is considered that there is a) an *initial recruitment* of primordial follicles independent of the action of gonadotropins and dependent of intraovarian growth factors and b) a *cyclic recruitment*, dependent of gonadotropins (especially FSH), which involves the cohort of antral follicles (McGee and Hsueh 2000) (Fig. 11).

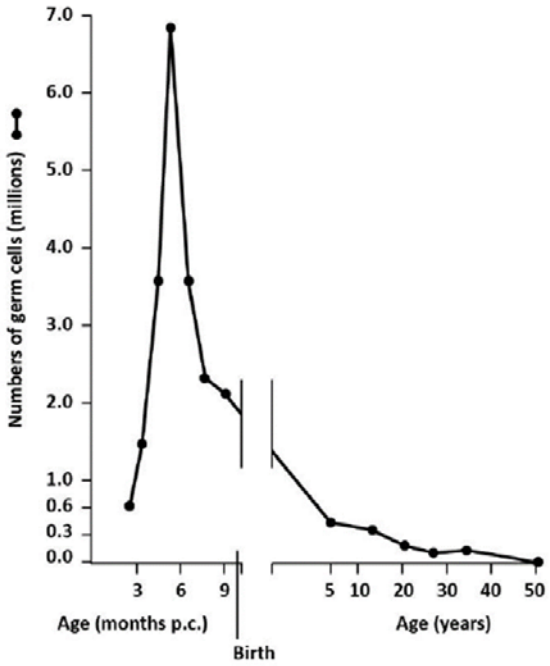

Figure 10. Outline of follicular population over time.

The initial recruitment of primordial follicles begins when the follicles are formed, and is regulated by different paracrine growth factors from granulosa cells (Hsueh et al. 2015). It is believed to be a continuous process. Not all the follicles are stimulated and some of them remain quiescent. Some of the activated follicles will develop until antral stage and others will undergo atresia, decreasing the pool.

After pubertal onset, cyclic recruitment will take place in each menstrual cycle. The presence of high amounts of FSH gonadotropin in the blood is able to rescue a cohort of antral follicles from atresia. These follicles continue growing, and during this stage follicular selection occurs, through which only one of the antral follicles will continue its development until reach the preovulatory stage. Some of the follicles grow faster, follicular cells increase estrogen and inhibin levels and, as it is described before, this leads to a suppression of FSH release by adenohypophysis. This suppression is the key for follicular dominance.

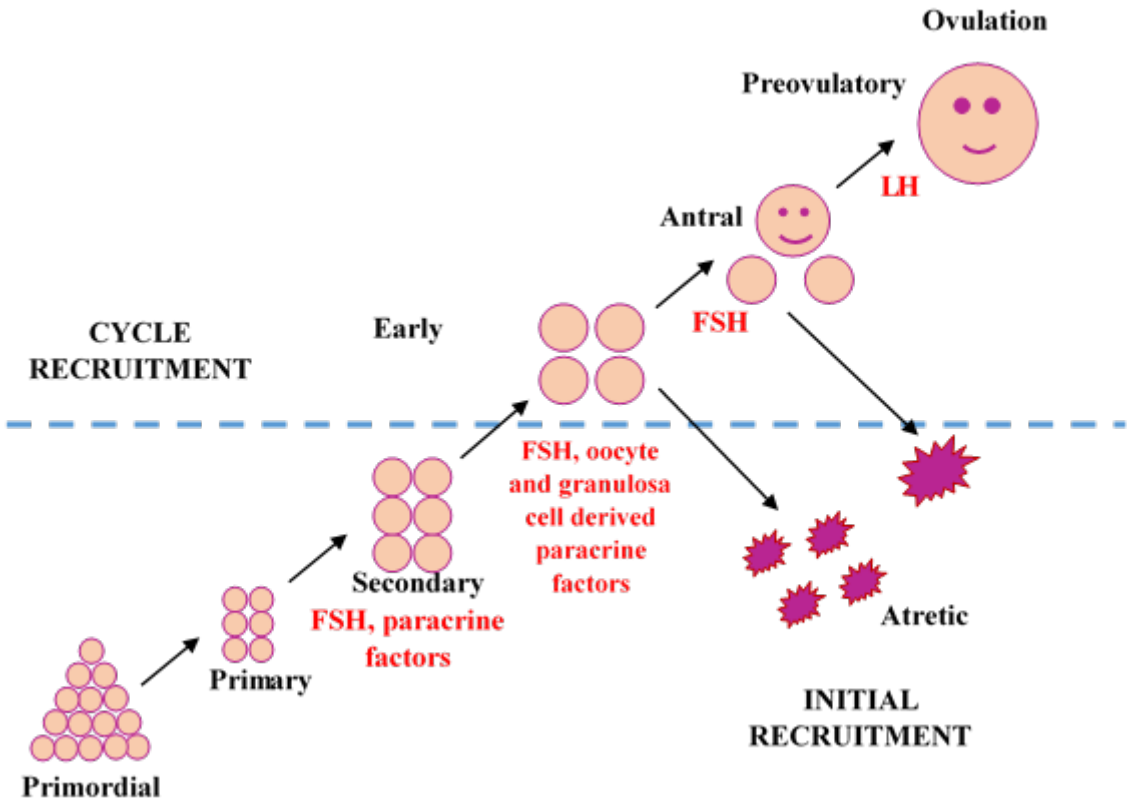

Figure 11. Follicular recruitment and hormonal regulation. Modified from Hsueh et al. (2015).

In this situation of FSH deficit only the dominant follicle will survive due to a higher number of FSH receptors (Fauser and van Heusden 1997), and the rest will undergo atresia due to the absence of FSH.

Ovarian hyperestimulation cycles occur thanks to the administration of exogenous gonadotropins, which are able to avoid this follicular selection process. Administration of higher amounts of exogenous FSH leads all recruited follicles to reach the pre-ovulatory stage.

3.4 Follicular fluid

Follicular fluid is a yellow slightly viscous fluid with a pH higher than 7, formed by a complex mixture of proteins, metabolites and ions (Rodgers and Irving-Rodgers 2010). It represents a plasma exudate whose composition also depends on the secretion and uptake of follicular cells, that is, granulosa and theca cells (Shalgi et al. 1973). The transport of these molecules through the blood-follicle barrier is regulated by their molecular weight and charge (Hess et al. 1998). Therefore, its composition give us an insight of disorders in the secretory processes of granulosa and internal theca cells, as well as alterations in plasma composition due to physiological or pathological processes (Edwards 1974). The oocyte is developed and matured in contact with follicular fluid, so its composition also will play a key role in these processes.

The main components of follicular fluid are polysaccharides, proteins, anticoagulants, lipids, growing factors (Zamah et al. 2015; Hsieh et al. 2009), steroid hormones, and enzymatic and non-enzymatic antioxidants, which participate in the growth and maturation of the oocyte and follicular cells, as well as in the protection from physical damage and oxidative stress (Ambekar et al. 2013).

Regarding proteins, a large number of them have been identified in this fluid by proteomic analysis (Jarkovska et al. 2010; Zamah et al. 2015; Shen et al. 2017). More than 60% of the intrafollicular proteins have extracellular origin, and the rest are located intracellularly or in the plasma membrane (Shen et al. 2017). As for their molecular functions, most proteins have catalytic activity and binding function, followed by receptor and structural functions (Zamah et al. 2015). About 50% of the follicular proteins are involved in immune activity and coagulation (Shen et al. 2017).

HDL constitutes the principal source of lipids in follicular fluid and represent the major lipoproteins. HDL is the main cholesterol supplier for the *de novo* synthesis of steroid hormones, and also have antioxidant capacity thanks to its protein components (Browne et al. 2008). Fatty acids and phospholipids have been also identified (Cordeiro et al. 2014; Ruiz-Sanz et al 2018).

In relation to hormones, progesterone is the most abundant hormonal component of the follicular fluid and is important in the final stages of follicular development and in the ovulation (Gougeon 2010). Gonadotropins, growth hormone (GH), steroids hormones, corticosteroids and prolactin have also a significant presence (Wang 2005).

4. Oxidative stress in physiopathology of female reproduction

4.1 ROS, antioxidants and oxidative stress

Reactive oxygen species are formed by the partial reduction of oxygen. They are very reactive molecules which can be clasified in two groups: radical oxygen species, such as, superoxide anion (O_2^{-}), hydroxyl ($HO^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals, and non-radicals species, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). These last compounds by themselves are not reactive but act as oxidizing agents and in the presence of traces of metal catalysts such as iron or copper can lead to the formation of $HO^{\bullet}$ through the Fenton reaction. Nitrogen-containing oxidants, such as nitric oxide (NO) and peroxinitrite ($ONOO^{-}$), are called reactive nitrogen species (RNS) (Fig. 12).

The mitochondria is the major source of ROS in the cell during ATP production through the oxidative phosphorylation pathway (Goossens et al. 1999), and the enzymatic reactions in which O_2^{-} is produced catalyzed by NADPH oxidases (Van Heerebeek et al. 2002), lipoxygenases (Kuhn and Thiele 1999) and cyclooxygenases (Kuehl and Egan 1980) are also an importante source of ROS.

Both ROS and RNS are essential for biological systems since they modulate numerous cellular functions. However, at high concentrations an imbalance between pro-oxidants and antioxidant defense systems takes place, leading to a situation called “oxidative stress” which is detrimental.

Organisms have developed different defense mechanisms to control the excess of ROS and to maintain the balance. These defense mechanisms include a variety of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Fig. 12) and non-enzymatic antioxidants, such as vitamin C, vitamin E (tocopherols), β-carotene, selenium, zinc and glutathione.

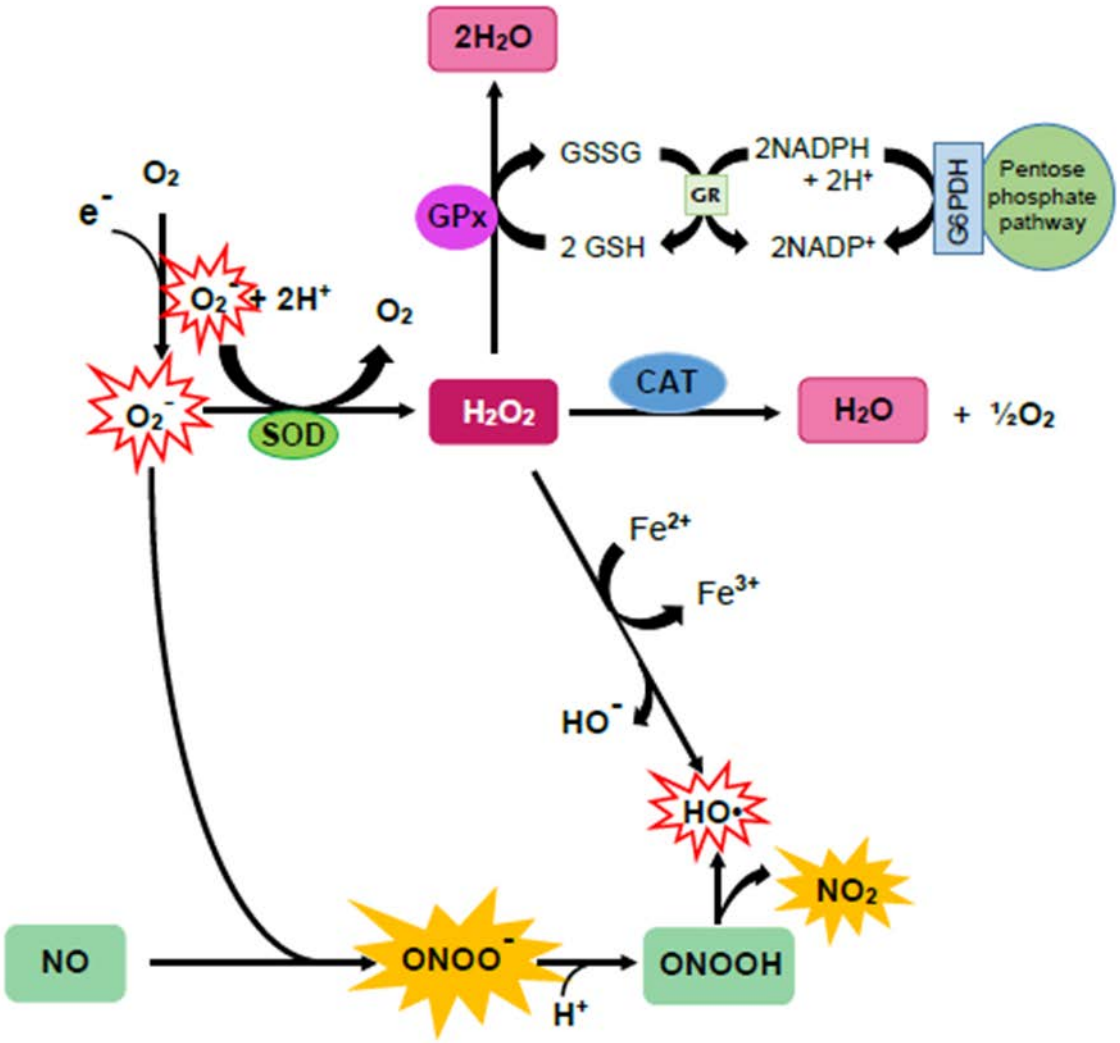

Figure 12. Generation of ROS and RNS and antioxidants system. Scheme shows the reactions by which ROS and RNS are produced, and neutralized by antioxidant enzymes. GPx, gluthation peroxidase; GSH, glutathione; GSSG, glutathione disulfide; GR, GSSG reductase; G6PDH, glucose 6-phosphate dehydrogenase; O_2^-, superoxide anion SOD, superoxide dismutase; NO, nitric oxide; $HO^·$ hydroxyl radical; NO_2, nitrogen dioxide radical; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid.

4.2 Oxidative stress in female reproductive processes

ROS and antioxidants play a key role in regulation of female reproductive processes. From the recruitment of ovarian follicles to pregnancy, the presence of these molecules is crucial (Table 1).

During follicular recruitment, granulosa cells present a high metabolism reflected in an increase in cytochrome P450 activity and steroid biosynthesis (Richards 1994). This turns into an increase in ROS production in preovulatory follicle triggering to blood flow alterations and follicle rupture (Du et al. 2006). ROS are considered to be ovulation inducers. In fact, several studies have confirmed in mice and rabbits the negative effects on ovulation of the absence of ROS (Shkolnik et al 2011; Miyazaki et al. 1991).

ROS are also responsible for apoptosis of non-dominant follicles. As we have shown earlier, during the follicular development, FSH induces the increase of estrogens in the ovary. These estrogens provoke an antioxidant response, upregulating catalase and GSH in the dominant follicle (Behman et al. 2001; Sugino 2006), thus avoiding its degeneration. Angiogenesis is also an important event for follicle growth, and ROS are involved in its regulation (Ushio-Fukai and Alexander 2004).

Oocyte maturation is also determined by ROS, since these species induce the resumption of meiosis I. However, meiosis II is promoted by antioxidants (Behman et al. 2001). Implantation, fertilization of oocytes, formation of blastocyst, luteolysis and luteal maintenance in pregnancy are modulated by ROS. Therefore, any ROS/antioxidant imbalance could lead to damage in all of these processes.

The pathological effects of high ROS concentrations has been studied. ROS-induced oxidative stress can alter embryonic development (Dennery 2007) and lower antioxidant capacity has been related to a decrease in fertilization potential (Oyawoye et al 2003). Pregnancy complications such as recurrent pregnancy loss, preeclampsia, intrauterine growth restriction, fetal death and embryonic resorption have been associated with oxidative stress (Gupta et al 2007). Furthermore, oxidative stress is involved in several reproductive diseases, such as polycystic ovary syndrome (PCOS) and endometriosis. PCOS has been associated with low antioxidant concentrations (Palacio et al 2006) and studies carried out in patients with endometriosis showed increased levels of oxidative stress markers (Jackson et al. 2005; Szczepanska et al. 2003).

Currently, the interest in correlations between systemic and local levels of ROS in IVF therapy and the oxidant–antioxidant balance in the human oocyte's environment, i.e. the follicular fluid is growing considerably. Follicular fluid contains leukocytes, cytokines and macrophages. These cells generate ROS (Attaran et al. 2000), and this, together with the intense metabolism of the granulosa cells during ovulation, represent a focus of active free-radical generation (Brannstrom et al. 1994; Lachapelle et al. 1996).

Table 1. The role of oxidative stress in female reproductive processes. Taken from Lu et al. (2018) Review.

Function	**Reproductive process**
Positive effect	Zn-Cu SOD↑ → Promotion of the development of follicles
	Biosynthesis of ovarian steroids → P450↑ → ROS↑ → Blood flow↑ → Rupture of follicles → Ovulation
	ROS↑ → Promotion apoptosis of non-dominant follicles FSH↑ → E2↑ → CAT and GSH↑ → Protection of cells from apoptosis
	E2 and P ↓ → SOD↓ → OS↑ → Endometrial shedding and implant failure
	ROS↑ → NF-κB↑ → $PGF_{2\alpha}$↑ → Luteum dissolution
	Sperm-ovum binding → ROS↑ → Corpus luteus functional↑ ROS↑ → Antioxidants↑ → Synthesis of progesterone
Negative effect	PCOS: Serum proline activity↑, OS↑ Physiological hyperglycemia → ROS↑ (Monocytes) → TNF-α↑ → NF-κB↑ → Resistance of Insulin↑
	Preeclampsia: Defective placenta → Hypoxia and reperfusion injury → OS↑ → Cytokines↑, Prostaglandins↑ → TAS↓, GPx of placenta↓ V_C↓ → Risk of preeclampsia↑ (MDA↑) ROS↑ → Vasoconstriction↑ → Coagulation activity↓ OS↑ → Vascular endothelial injury↑ → ROS↑ → TNF-α↑, ox-LDL↑ → Endothelial subtypes of activated NAD(P)H oxidase → O_2^-↑ Auto-antibodies of AT1-AA↑ → NAD(P)H oxidase↑ → ROS↑ → O_2^-↑
	Endometriosis: In the peritoneal fluid, MDA↑, IL-6↑,TNF-α↑, IL-8↑, VEGF↑, MCP-1↑, ox-LDL↑. In endometriotic lesions: OS↑ → NF-κB↑ → Inflammation↑ In endometriotic cells: MAPK↑ ROS↑ → IUGR, abortion, fetal malformation

The → indicates that it has an effect on the next step. The ↑ represents and increase and the ↓ represents a decrease**.**

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

Controlled ovarian hyperstimulation cycle (COH) is a widely extended practice used in most of the IVF treatments, due to the increase of the number of oocytes retrieved leading to improve IVF success rates. However, there are many studies reporting the negative effects of COH. In a previous work, we described that controlled ovarian hyperstimulation induced oxidative stress, mainly reflected by changes in the redox status of the serum in terms of total antioxidant activity, susceptibility to *in vitro* oxidation, and the levels of antioxidants such as tocopherol, bilirubin, uric acid, and albumin (Aurrekoetxea et al. *Fertil Steril* **2010**; 94:1279–1286).

It has been reported that oocytes derived from natural cycles show differences from those from stimulated ones in relevant aspects. Ovarian stimulation with exogenous gonadotropins alters the hormonal and biochemical composition of the follicular milieu. Natural cycle (NC) seems to be a good alternative to reduce the risks associated with ovarian stimulation. It does not involve strong medication for women, and the literature reveals a greater potential for embryo implantation. Since the follicular fluid surrounds the oocyte, the study of its chemical composition offers very specific information on the oocyte maturation and consequently its quality and embryonic competence. The antioxidant status of follicular fluid would have significant consequences for oocyte maturation and consequently the quality of embryo.

The HYPOTHESIS of this work is that controlled ovarian hyperstimulation cycles mediated by gonadotropins trigger alterations in the redox balance of follicular fluid and, therefore, in the oocyte microenviroment, rendering a reduced antioxidant protection.

The main OBJECTIVE of the present Doctoral Thesis is to characterize the antioxidant status of the follicular fluid associated with oocytes aspirated from the same woman after a natural cycle (NC) and a controlled ovarian hyperstimulation cycle (COH).

To achieve this aim, a population of fertile women (oocyte donors) was recruited. In both situations (NC and COH), ovarian mature follicles were aspirated, and, after oocyte separation, the follicular fluid was subjected to different analyses.

The following OPERATIVE OBJECTIVES were raised:

1. Determination of glucose, α- and γ-tocopherol, nitric oxide, total antioxidant capacity (TAA and ORAC), activity of antioxidant enzymes: superoxide dismutase, glutathione peroxidase, catalase, and the PON system (PON1 and PON3).
2. Quantification of PON system proteins by western blot.
3. Determination of the fatty acyl composition of the follicular fluid lipids by gas chromatography.
4. Determination of biomarkers of non-enzymatic oxidative modifications of proteins, namely glutamic semialdehyde, aminoadipic semialdehyde, N^{ε}-(carboxyethyl)-lysine and N^{ε}-(carboxymethyl)-lysine.
5. Characterization of the PON system proteins in human granulosa cells:
    a. Determination of the transcription products of the PON1, PON2 and PON3 genes by RT-PCR
    b. Quantification of PON1, PON2 and PON3 proteins by quantitative western blot.
    c. Analysis of subcellular distribution of PON1, PON2 and PON3 proteins by immunofluorescence and confocal microscopy.

RESULTS AND DISCUSSION

CHAPTER I

CHAPTER I: Follicular fluid in gonadotropin-stimulated cycles is less protected against oxidative challenge than in natural cycles.

This chapter has been sent for publication in:

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Abstract

Study question: Are markers of antioxidant status in follicular fluid from an exogenous gonadotropin controlled ovarian hyperstimulation cycle (COH) lower than those from a natural cycle (NC)?

Summary answer: Total antioxidant activity (TAA), alpha-tocopherol levels, and activities of the paraoxonase family PON1 and PON3 are reduced in COH compared with NC. The fatty acid composition of total lipids also changes, increasing the proportion of total saturated fatty acids and n-3 polyunsaturated docosahexaenoic acid (DHA).

What is known already: COH is associated with clinical complications and adverse pregnancy and perinatal outcomes. Studies show that COH triggered systemic oxidative stress in infertile women, which could reflect increased levels of reactive oxygen species in reproductive tissues. However, it remains unknown whether COH alters the physiological antioxidant status of follicular fluid in women with no reproductive dysfunction, i.e. in an ideal oocyte microenvironment.

Study design, size, duration: Longitudinal study in which 41 women (oocyte donors) participated between February 2014 and December 2015.

Participants/materials, setting, methods: Women consecutively underwent a COH with a GnRH antagonist protocol, and a NC. In both cases, GnRH agonist was administered to induce ovulation 36 h before oocyte retrieval. The following antioxidant markers were determined in follicular fluid: TAA, oxygen radical absorbance capacity (ORAC), nitric oxide, α- and γ-tocopherol, and the activities of superoxide dismutase (SOD), catalase, total and Se-dependent glutathione peroxidases (tGPx and Se-GPx), PON1 paraoxonase and arylesterase, and PON3 simvastatinase. The concentrations of PON1 and PON3 proteins were determined using western blot and the fatty acid composition was analyzed by gas chromatography.

Main results and the role of chance: TAA (mean ± SE, 1.70 ± 0.03 mM versus 1.86 ± 0.03 mM, $p<0.05$), α-tocopherol (4.37 ± 0.26 µM versus 5.74 ± 0.30 µM, $p<0.05$), PON1 paraoxonase and arylesterase activities (245 ± 24 nmol/min/ml versus 272 ± 27 nmol/min/ml, $p<0.05$ and 87.2 ± 4.6 µmol/min/ml versus 99.3 ± 4.8 µmol/min/ml, $p< 0.05$, respectively), and PON3 simvastatinase (13.48 ± 0.52 nmol/min/ml versus 16.29 ± 0.72 nmol/min/ml, $p<0.001$) were significantly lower in COH versus NC. Other markers

showed non-statistically significant changes. Fatty acids from COH were more saturated, increasing the palmitic content and decreasing the n-6 and total polyunsaturated fatty acids (PUFAs). The long-chain n-3 PUFA docosahexaenoic acid increased significantly its contribution to the total lipids ($p<0.05$). This study indicates that NC is associated with higher follicle antioxidant protection.

Limitations, reasons for caution: Caution must be taken when extrapolating the results to a physiological natural cycle, since all women received the GnRH agonist to induce ovulation.

Wider implications of the findings: These results provide new insights into the possible prevention of the adverse effects of ovarian hyperstimulation by directing therapeutic applications to the maintenance of the redox balance and antioxidant status, with special attention to the PON system and its function in the ovary.

Study funding/competing interest(s): Work sponsored by the University of the Basque Country UPV/EHU (ref. GIU16/62) and the Basque Government (Department of Economic Development and Competitiveness SPRI, refs. IG-2013 0001214 and IG-2014 0000837). I.P. received a pre-doctoral fee from the Basque Government.

Trial registration number: N/A

Key words: Assisted reproductive techniques; follicular fluid; ovulation induction; oxidative stress; fatty acid composition.

1. Introduction

Since the first IVF treatment was successfully carried out in 1978 (Steptoe and Edwards 1978), the use of assisted reproductive techniques has spread exponentially. Although the first IVF treatment was performed in a natural ovulation cycle (NC), nowadays, a controlled ovarian hyperstimulation cycle (COH) is a widely extended protocol used in most of the IVF treatments. Thus, this treatment greatly increases the number of oocytes retrieved, which improves the success rates of IVF (Pelinck et al. 2002). However, several studies are reporting the negative effects of COH. Its use can lead to clinical complications, such as ovarian hyperstimulation syndrome (Namavar Jahromi et al. 2018) and reduction of embryo viability (Santos et al. 2010). Adverse perinatal outcomes have also been described. A study performed by Seggers et al. (2014) showed that 4 year-old children born following IVF with COH and embryo transfer had higher systolic blood pressure percentiles, suggesting a possible side effect in offspring. Several authors confirmed the higher risk of low birthweight and preterm births in babies conceived after COH-IVF (Mak et al. 2016; Declercq et al. 2015). In contrast, the literature shows that NC reduces the risks associated with ovarian stimulation (Pelinck et al. 2002) and produces oocytes with greater potential for embryo implantation (Gordon et al. 2013; Ferrareti et al. 2012).

The role of reactive oxygen species (ROS)/antioxidants in pathophysiology of reproduction is a major subject of study. In a previous work, we found that COH induced oxidative stress, mainly reflected by changes in the antioxidant/pro-oxidant status of the serum, in terms of total antioxidant activity, susceptibility to *in vitro* oxidation, and the levels of tocopherol, bilirubin, uric acid, and albumin (Aurrekoetxea et al. 2010). One of the main targets of the oxidative attack of free radicals are polyunsaturated fatty acids (PUFAs), including linoleic acid (18:2n-6 *cis*) and α-linolenic acid (18:3n-3). These two fatty acids are the respective precursors of the n-6 and n-3 families of long-chain fatty acids. They have to be supplied by the diet, since humans do not have the Δ^{12}- and Δ^{15}-desaturase enzymes necessary for their synthesis from stearic acid (18:0). In addition to their structural function as components of all biological membranes, PUFAs have important physiological functions, such as increasing membrane fluidity, inducing cellular signaling, and regulating gene expression (Bentsen 2017).

Oocytes derived from NC show differences from those from COH in important aspects, such as epigenetics and ultrastructural integrity (Ventura-Juncá et al. 2015; Lee et al. 2017). Since follicular fluid surrounds the oocyte, the study of the chemical composition and redox balance of this fluid offers very specific information on the oocyte maturation and consequently its quality and embryonic competence. Recently, we have described that N^{ε}-(carboxyethyl)lysine, a marker of the chemical oxidative modifications of proteins, was significantly lower in follicular fluid from NC compared with COH (Pérez-Ruiz et al. 2018). In the present study, we have determined antioxidative markers of follicular fluid from women undergoing both a NC and COH at the time of follicular aspiration with the aim of elucidating whether COH can affect follicular fluid redox balance in comparison with the physiologically ideal milieu in NC.

2. Materials and Methods

2.1. Study design and study population

This is a longitudinal study consisting of 41 women (oocytes donors) that were recruited at the Valencian Institute of Infertility in Bilbao (www.ivi.es, Vizcaya, Spain). Inclusion criteria were: age 18-34 years, BMI (18-29 kg/m^2), no endometriosis, no recurrent abortion, and no genetic or chromosomic diseases. Each woman followed a natural cycle (NC) and a controlled ovarian hyperstimulation cycle (COH). COH was carried out following a clinical protocol with GnRH antagonists (Pérez-Ruiz et al. 2018). Ovarian stimulation was started with the administration of gonadotropins, which usually lasts an average of 9-10 days. The dose administered to each woman was individualized according to the BMI, the response to previous cycles, the age and the number of antral follicles observed by ultrasound before the start of stimulation. The dose fluctuated between 150 and 300 IU, and different types of gonadotropins were used (rFSH or hGM or a combination of both).

During the stimulation period, serial ultrasounds were performed to verify the relevant follicular growth. Ovulation was induced with GnRH agonist (Triptorelin, 0.1 mL, one ampoule/day sc, administered 36 h prior to puncture) when there were at least 3 follicles with an average diameter greater than 17 mm. To conduct a NC an ultrasound on day 8-10 of the cycle was performed to check the size of the follicle and every 2-3 days depending on the follicular development until the follicle reached a size between 17 and

20 mm. At that time, a single dose of a GnRH agonist was administered 36 h before the puncture to induce ovulation.

The Ethics Committee of the University UPV/EHU (Ethics Committee for Research involving Human Subjects, CEISH) approved the human subject protocols (CEISH/96/2011/RUIZLARREA and M30_2015_187_RUIZ LARREA) on 27 February 2012 and 28 October 2015, and the study was carried out according to the UPV/EHU and IVI-Bilbao agreements, references 2012/01 and IVI_02_2015 RUIZ LARREA. The project complies with the Spanish Law of Assisted Reproductive Technologies (14/2006). Written informed consent was obtained from all trial participants.

2.2. Sample collection

To collect follicular fluid samples transvaginal ultrasonography and serum estradiol levels were performed routinely during ovarian stimulation to assess ovarian follicle maturation. Follicular puncture was performed, followed by transvaginal aspiration. After removal of the oocyte, the follicular fluid sample was centrifuged at 3000 g for 10 min to remove debris, blood and cells. Then the supernatant was transferred to sterile polypropylene tubes and stored in liquid nitrogen. Tubes were transported to the University and kept at -80 ºC until assay. In COH two contralateral follicles of the same size were punctured and the corresponding samples of follicular fluid were pooled.

2.3. Total Antioxidant Activity

The total antioxidant activity (TAA) was determined by the $ABTS^{+}$ radical cation decolorization assay adapted to 96-well microplates, as described previously (Aurrekoetxea et al. 2010).

2.4. Oxygen Radicals Absorbance Capacity

The ORAC assay was carried out according to (Huang et al. 2002) using fluorescein as a fluorescence probe and in 96-well microplates, as described previously (Lizcano et al. 2010). All the samples were analyzed at four dilutions and in triplicates. Results were calculated based upon differences in areas under the fluorescence decay curve between blank, samples and standards. Final ORAC values were expressed as mM Trolox equivalents

2.5. Nitric Oxide determination

Nitric oxide (NO) was determined according to the method described by (Miranda et al. 2001) based on Griess reaction and adapted to 96 well plates by (Aurrekoetxea et al. 2010).

2.6. Vitamin E quantitation

α-Tocopherol and γ-tocopherol concentrations were measured by reverse phase HPLC after lipid extraction according to Ruiz-Sanz et al. (2007).

2.7. Antioxidant enzymes activity

All the enzymatic activity assays were carried out by spectrophotometric techniques using Synergy HT^{TM} microplate reader (BioTek, Winooski, VT, USA).

2.7.1. GPx activity

Total and selenium-dependent GPx activities were measured according to the method proposed by Wilson et al. (1989), adapted to 96-well microplates. Briefly, in a final volume of 225 µl, 40 µl of different dilutions of follicular fluid samples were added per well in duplicate. The reaction was started at 30 °C by the addition of 160 µl of reaction mixture containing 34.2 mM potassium phosphate buffer pH 7, 0.94 mM EDTA-Na_2, 1.40 mM sodium azide, 0.45 U glutathione reductase, 0.45 mM GSH, 0.2 mM NADPH, and 25 µl of 0.78 mM H_2O_2 or 0.72 mM cumene hydroperoxide, substrates for selenium-dependent and total activity respectively. The reaction was measured by monitoring the NADPH oxidation at 340 nm every 60 s for 15 min. Reactions rates were derived from the corresponding slopes and the results were expressed as nmol/min/ml by applying the experimental molar extinction coefficient 3.065 $mM^{-1}cm^{-1}$.

2.7.2. SOD activity

SOD activity was determined using a commercial kit (SOD determination Kit, Sigma-Aldrich, Buchs, Switzerland), based on the method proposed by Zhou and Prognon (2006). The assay was adapted to 96-well microplates following the manufactured instructions.

2.7.3. Catalase activity

Catalase activity was measured according to Cohen et al. (1996). Briefly, the activity was measured by the absorbance decrease at 240 nm following the disappearance of H_2O_2. Samples were measured in triplicates at 30 °C and the reaction was started by adding 37.5 mM H_2O_2 in 100 mM potassium phosphate buffer, pH 6.8, to 1 µl of follicular fluid. The reaction was measured by monitoring the change of absorbance at 240 nm every 7 s over 3 min. Reaction rates were derived from the corresponding slopes and the results were expressed as µmol/min/ml by applying the experimental molar extinction coefficient 0.039 $mM^{-1}cm^{-1}$.

2.7.4. PON1 and PON3 activity

Both PON1 paraoxonase (using paraoxon as substrate) and arylesterase (using phenylacetate) were measured spectrophotometrically based on the method described by (Furlong et al. 1989) according to Meijide et al. 2017. All reactions were carried out in triplicate. PON3 activity assay was carried out by reverse-phase HPLC using simvastatin lactone (SVL) as substrate, according to Meijide et al. 2017.

2.8. PON1 and PON3 detection by western blot

To quantify PON1 and PON3 proteins, western blot was carried out according to Meijide et al. 2017. A calibration curve from human recombinant proteins, 2.5-10 ng made with PON1 (ProsPec-Tany TechnoGene Ltd., Ness Ziona, Israel) and 0.625-7.5 ng with PON3 (ThermoFisher Scientific, Waltham, MA, USA), was loaded in each western blot.

2.9. Acyl composition analysis by capillary gas-liquid chromatography/flame ionization detection

Fatty acids from the total lipid fraction of follicular fluid were first transmethylated (Lepage and Roy 1986), separated and quantified by gas chromatography with a flame ionization detector, as described previously (Ruiz-Sanz et al. 2018). Results were expressed as the percentage of the total fatty acids (in moles). The following parameters were calculated: average chain length (ACL) = [Σ ($total_{14}$%×14) + ... + (Σ% $total_n$ × n)]/100 (n = number of carbon atoms), double bond index (DBI) = (Σ % of unsaturated fatty acids × number of double bounds of each unsaturated fatty acid)/100, and

peroxidizability index (PI) = [(% monoenoics × 0.025) + (% dienoics × 1) + (% trienoics × 2) + (% tetraenoics × 4) + (% pentaenoics × 6) + (% hexaenoics × 8)]/100.

2.10. Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (IBM-SPPS Statistics 20). All data were expressed as mean ± SEM. The Kolmogorov-Smirnov test was used to determine the normal distribution of the variables. Statistical comparisons between NC and COH were performed using the Student's *t* test for paired samples and the Wilcoxon test (non-parametric equivalent). A value of $p<0.05$ was considered statistically significant.

3. Results

The women enrolled in the study were 29 ± 0.7 years old (mean ± SEM) and had a mean body mass index of 23 ± 0.6 Kg/m^2 (mean ± SE). The NO concentration, ORAC, and the antioxidant activities of SOD, total and Se-dependent GPx and catalase showed no significant differences between NC and COH (Table 1). However, follicular fluid from NC showed a markedly higher TAA compared with that from COH ($p<0.001$, Fig. 1).

Table 1. Markers of follicular fluid antioxidant status in NC and COH.

	NC (mean ± SEM)	COH (mean ± SEM)
NO (µM)	60.0 ± 4.5	53.2 ± 3.3
ORAC (mM)	12.5 ± 0.4	12.6 ± 0.4
SOD (U/ml)	20.4 ± 2.1	18.7 ± 1.7
tGPx (nmol/min/ml)	41.5 ± 2.6	44.8 ± 2.7
Se-GPx (nmol/min/ml)	35.2 ± 2.2	36.4 ± 1.8
Catalase (µmol/min/ml)	35.0 ± 3.6	35.5 ± 2.4

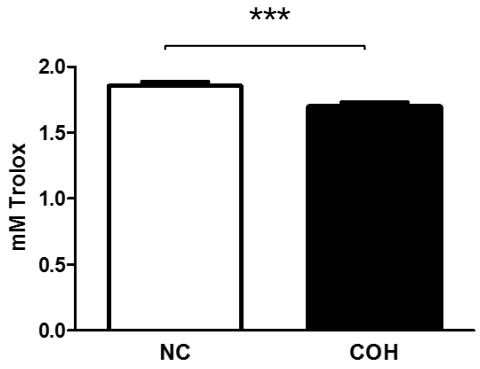

Figure 1. Total antioxidant activity in follicular fluid from women undergoing both NC and COH. Bars represent the mean + SEM. ***p<0.001.

α-Tocopherol levels were also significantly higher in NC (p<0.001), whereas γ-tocopherol was similar in both groups (Fig. 2).

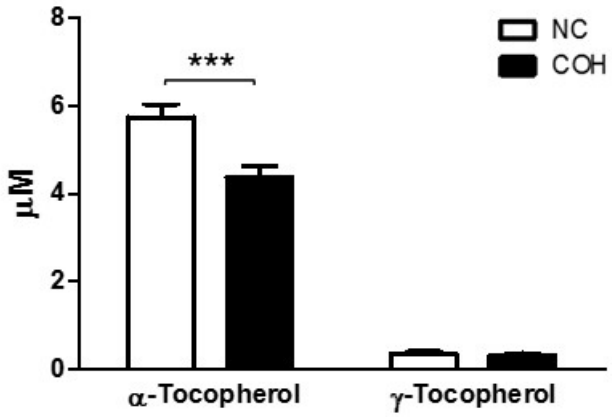

Figure 2. Vitamin E levels in follicular fluid from women undergoing both NC and COH. Bars represent the mean + SEM. ***p<0.001.

Arylesterase and paraoxonase activities of PON1 (p<0.05), and PON3 simvastatinase (p<0.001) were higher in follicular fluid from NC (Fig. 3).

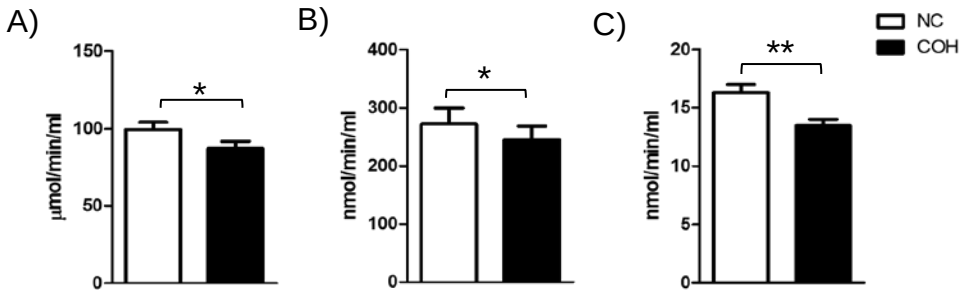

Figure 3. A) PON1 arylesterase, B) PON1 paraoxonase, and C) PON3 simvastatinase activities of follicular fluid from NC and COH. Bars represent the mean + SEM. *p<0.05, **p<0.01.

PON proteins were also quantified in follicular fluid by western blot. As it is shown in Fig. 4, a standard curve of human recombinant proteins was loaded in each assay to quantify the protein in the samples. In contrast to the results from PON1 activities, data showed that the protein levels of PON1 were significantly higher in follicular fluid in COH (14.6 ± 0.8 vs 9.6 ± 0.5 mg/ml). In the case of PON3, the protein expression levels were increased in NC (1.7 ± 0.1 vs 1.4 ± 0.1 mg/l), as could be expected from the results of its catalytic activity.

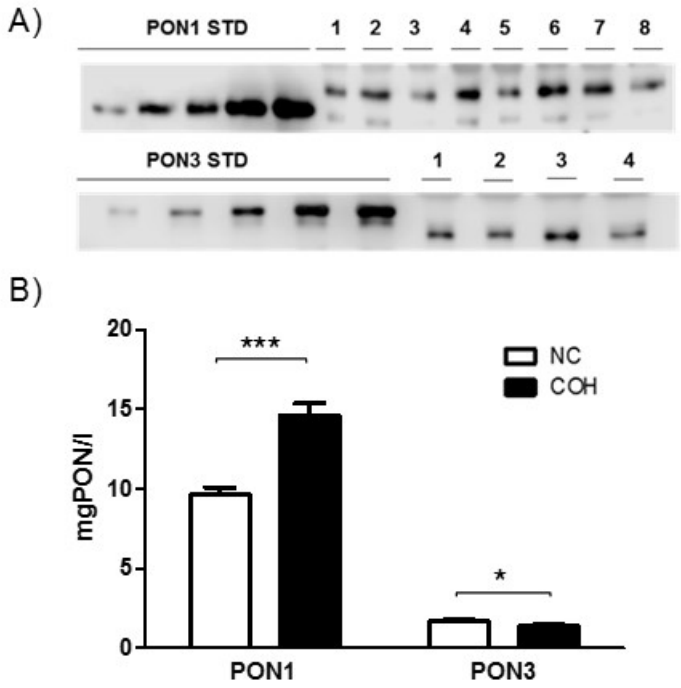

Figure 4. PON1 and PON3 expression in follicular fluid. A) PON1 and PON3 expression levels in different samples of follicular fluid analyzed by western blot. Each lane corresponds to increasing quantities (2.5 - 10 ng PON1, 0.625 - 7.5 ng PON3) of the human recombinant proteins (STD) and follicular fluid samples from NC (lanes 1, 3, 5, 8 for PON1, and 1, 3 for PON3) and COH (lanes 2, 4, 6, 7 for PON1, and 2, 4 for PON3). B) Mean values + SEM of PON1 and PON3 (mg/l) of the quantified samples. *p<0.05, ***p<0.001.

A total of 33 fatty acids were quantified in follicular fluid (Table 2). As it was previously reported (Ruiz-Sanz et al. 2018), PUFAs represented the main fraction of fatty acids (43%), and linoleic acid was the most abundant fatty acid (29%). Total fatty acids in the follicular fluid from COH were less unsaturated than those from NC due to a lower proportion of PUFAs and a higher content of saturated fatty acids. Although the content of linoleic acid (18:2n-6 *cis*) was higher in NC, long-chain n-6 PUFAs (eicosadienoic, docosapentaenoic, and docosahexaenoic acids) were increased in COH. In this sense, the 20:4n-6/18:2n-6 ratio, a general parameter referring to the arachidonic acid biosynthetic pathway from its essential precursor, was significantly elevated in the COH group. A higher percentage of n-3 docosahexaenoic acid (22:6n-3, DHA) was observed in COH,

contributing to the general reduced n-6/n-3 ratio in this group. We did not find significant differences in the derived average chain length, and double bond and peroxidizability indices between groups.

Table 2. Fatty acid composition of follicular fluid from NC and COH.

	NC	COH	P value
SAT	35.69 ± 0.46	36.82 ± 0.38	0.009
Lauric (12:0)	0.21 ± 0.03	0.20 ± 0.02	0.400
Myristic (14:0)	0.76 ± 0.07	0.75 ± 0.06	0.866
Pentadecanoic (15:0)	0.22 ± 0.02	0.19 ± 0.01	0.247
Palmitic (16:0)	22.20 ± 0.39	23.57 ± 0.37	0.000
Heptadecanoic (17:0)	0.33 ± 0.03	0.37 ± 0.04	0.622
Stearic (18:0)	9.20 ± 0.24	9.03 ± 0.19	0.526
Nonadecanoic (19:0)	0.09 ± 0.01	0.08 ± 0.01	0.484
Arachidic (20:0)	0.33 ± 0.02	0.33 ± 0.01	0.943
Heneicosanoic (21:0)	0.28 ± 0.02	0.26 ± 0.01	0.386
Behenic (22:0)	0.93 ± 0.02	0.95 ± 0.04	0.574
Tricosanoic (23:0)	0.33 ± 0.01	0.35 ± 0.02	0.298
Lignoceric (24:0)	0.72 ± 0.03	0.70 ± 0.03	0.616
Pentacosanoic (25:0)	0.07 ± 0.02	0.04 ± 0.00	0.069
MUFAs	20.69 ± 0.47	20.98 ± 0.39	0.550
Myristoleic (14:1n-5)	0.04 ± 0.00	0.04 ± 0.00	0.862
Trans-palmitoleic (16:1n-7 *trans*)	0.05 ± 0.01	0.04 ± 0.01	0.414
Palmitoleic (16:1n-7 *cis*)	1.09 ± 0.06	1.10 ± 0.09	0.886
Elaidic (18:1n-9 *trans*)	0.11 ± 0.02	0.09 ± 0.01	0.342
Oleic (18:1n-9 *cis*)	16.16 ± 0.41	16.21 ± 0.33	0.908
Vaccenic (18:1n-7 *cis*)	1.46 ± 0.04	1.56 ± 0.04	0.037
Eicosenoic (20:1n-9)	0.28 ± 0.02	0.31 ± 0.02	0.293
Nervonic (24:1n-9)	1.49 ± 0.05	1.64 ± 0.04	0.033
PUFAs	43.61 ± 0.67	42.20 ± 0.49	0.013
n-6 PUFAs	40.90 ± 0.76	39.33 ± 0.47	0.010
Linoelaidic (18:2n-6 *trans*)	0.07 ± 0.01	0.07 ± 0.00	0.520
Linoleic (18:2n-6 *cis*)	29.46 ± 0.71	27.22 ± 0.55	0.001
γ-Linolenic (18:3n-6)	0.28 ± 0.02	0.23 ± 0.02	0.077
Eicosadienoic (20:2n-6)	0.36 ± 0.01	0.41 ± 0.02	0.026
Dihomo-γ-linolenic (20:3n-6)	1.96 ± 0.09	2.07 ± 0.11	0.335
Arachidonic (20:4n-6)	8.21 ± 0.33	8.70 ± 0.38	0.085
Docosatetraenoic (22:4n-6)	0.35 ± 0.04	0.38 ± 0.02	0.464
Docosapentaenoic (22:5n-6)	0.20 ± 0.01	0.24 ± 0.01	0.005
n-3 PUFAs	2.72 ± 0.18	2.87 ± 0.17	0.346
α-Linolenic (18:3n-3)	0.16 ± 0.01	0.17 ± 0.01	0.443

Eicosapentaenoic (20:5n-3)	0.38 ± 0.07	0.35 ± 0.06	0.777
Docosapentaenoic (22:5n-3)	0.33 ± 0.01	0.33 ± 0.02	0.857
DHA (22:6n-3)	1.85 ± 0.12	2.03 ± 0.12	0.036
n-6/n-3 ratio	16.61 ± 1.19	14.68 ± 0.85	0.022
UNSAT	64.30 ± 0.46	63.18 ± 0.38	0.009
DBI	137.58 ± 1.52	136.91 ± 1.55	0.572
ACL	18.02 ± 0.02	18.02 ± 0.02	0.822
PI	0.90 ± 0.02	0.91 ± 0.02	0.340
20:4n-6/18:2n-6	0.28 ± 0.01	0.32 ± 0.02	0.010

Results are expressed as the percentage of total moles of measured fatty acids and are the mean ± standard error of the mean. ACL, average chain length; DBI, double bond index; DHA, docosahexaenoic acid; MUFAs, monounsaturated; PI, peroxidizability index; PUFAs, polyunsaturated; SAT, saturated; UNSAT, unsaturated.

4. Discussion

The present results show that COH modified the antioxidant status of follicular fluid. In particular, several antioxidative markers were lower, among them α-tocopherol levels, the TAA and paraoxonase activities. That is, the follicular fluid from COH had a weaker antioxidant protection than from NC. In a recent proteomic study, Wu et al (2015) analyzed follicular fluid from both NC and COH of infertile women and found differentially expressed proteins. The upregulated proteins were related to immune and inflammatory responses in the ovary, physiological processes associated with free radical management. According to the authors, the presence of increased levels of these proteins suggested that women undergoing COH could develop aberrant immune and inflammatory responses during the stimulation cycle leading to negative effects. Several proteomic studies have documented the presence of SOD, catalase and GPx enzymes in human follicular fluid (Angelucci et al. 2006; Twigt et al. 2012). Papler et al. (2014) carried out a study comparing cumulus gene expression between modified natural cycle and COH and observed that there were differences in the expression of many genes involved in oxidation-reduction processes such as GPx3 and SOD. These genes were significantly upregulated in cells from NC. However, we did not find any differences in their enzymatic activities, which could be due to post-translational regulatory processes. Most of these type of modifications by nitration, which usually take place during inflammation, give rise to reduced SOD activity (Yamakura and Kawasaki 2010).

Several studies have demonstrated the presence of a NO generator system in the ovary (granulosa cells, teca cells, and oocyte) of different species and in follicular fluid (Tamanini et al. 2003), suggesting the function of this molecule in the control of ovarian physiology. Anteby et al. (1996) determined that the NO concentration in follicular fluid of women subjected to an IVF program increased as the volume of the follicle increased, with NO playing a role in follicular maturation and ovulation (Basini and Grasselli 2015). It has also been observed that the expression of mRNAs of different NOS isoforms could be subjected to regulation by gonadotropins (Van Voorhis et al. 1995; Matsumi et al. 2000). In the current study, we did not find significant differences in NO levels in follicular fluid from NC and COH, suggesting that COH did not affect the physiological levels of NO in the ovary. It may not be a surprising finding if the main role of NO is focused on folliculogenesis and we take into account that follicular fluid was retrieved from mature follicles in both cycles.

Vitamin E exerts free radical scavenging activity. Previous reports have suggested that α-tocopherol has a role in the development of the oocyte, since the presence of the vitamin in follicular fluid from women undergoing IVF negatively correlated with embryo fragmentation score (Browne et al. 2009). Palini et al. (2014) analyzed the relationship between ovarian stimulation and IVF outcomes, and found a positive association between α-tocopherol levels and the pregnancy rate. In this work, we have observed that the concentration of α-tocopherol was significantly higher in follicular fluid from NC, thus also suggesting oxidative aggression exerted by the exogenous gonadotropin treatment reflected in the follicular fluid. The condition of oxidative stress may be triggered not only by increased ROS production, but also as consequence of local decrement of antioxidant levels.

In the present work, follicular fluid from COH showed significantly lower PON1 paraoxonase and arylesterase activities, as well as PON3 simvastatinase than from NC. Quantitative analysis by western blot and recombinant human PON proteins as standards indicated that the intrafollicular PON3 concentration was, as expected, lower in COH. However, in the case of PON1, its concentration was even higher compared with NC, indicating some type of inhibition leading to reduction of PON1 activity. Remarkably, it has been reported that serum PON1 paraoxonase activity decreased with age, with the protein concentrations remaining constant (Seres et al. 2004). These data and our results

suggest that COH could lead to a premature aging and give more strength to the idea that the PON enzyme system plays a role in fertility.

Saturated fatty acids such as palmitic and stearic acids were detrimental for reproduction in animals, inhibiting granulosa and theca cell proliferation and inducing apoptosis (McKeegan and Sturmey 2011). On the other hand, unsaturated fatty acids exerted positive effects on animal reproduction. Although not similar in animal models, the levels of linoleic acid in human follicular fluid directly correlated, and arachidonic acid negatively correlated with fertility rates (Shaaker et al. 2012). Linoleic acid was also associated with proper embryo development (Haggarty et al. 2006). In our study, COH induced a higher proportion of palmitic acid, and a lower linoleic acid content, significantly increasing the arachidonate/linoleate ratio, an index of the arachidonate biosynthetic pathway from its precursor. Since arachidonic acid is the parent compound of pro-inflammatory prostaglandins, the results suggest that COH could induce an inflammatory process distinct from NC. Moreover, elevated concentrations of arachidonic and linoleic acid derivatives in the follicular fluid at the time of oocyte retrieval significantly decreased the ability of oocytes to form pronuclei after ICSI (Ciepiela et al. 2015).

COH also led to higher intrafollicular concentrations of long-chain n-3 PUFAs, among them DHA. n-3 PUFAs contain more than one double bond and are therefore susceptible to oxidation. In this sense, an increase has been described in malondialdehyde (a lipid peroxidation index) following the administration of n-3 PUFA supplements (Kelley et al. 2014). The oxidation of n-3 PUFAs entails the production of reactive compounds capable of reacting with cysteine, histidine, and lysine residues of cellular proteins and forming covalent adducts which cause damage to proteins and thus compromise their functions. In a recent study, we analyzed by gas liquid chromatography/mass spectrometry markers of oxidative protein modifications in follicular fluid from NC and COH. We reported that there were no significant differences of the N^{ε}-(carboxymethyl)lysine biomarker (indicative of oxidative modification by lipoperoxidation and glycoxidation processes) between both cycles (Pérez-Ruiz et al. 2018). Therefore, an increased lipid peroxidation does not seem to be the main cause of the actions induced by COH. On the other hand, n-3 PUFAs alter the organization of lipid rafts of the cell membranes, affecting their physicochemical properties and the functions of proteins associated with them (Kelley et

al. 2014; Kwiecien et al. 2014). Lipid rafts are involved in oocyte maturation and fertilization (Chattopadhayay et al. 2010; Rashidi et al. 2014; Singh et al. 2013), and they inversely correlated with the n-3 PUFAs content of the membranes from different model systems (Shaikh et al. 2012). In a previous work, we analyzed the fatty acid composition of follicular fluid from fertile oocyte donors and infertile women undergoing ovarian stimulation. n-3 PUFA correlated positively with the woman's age and negatively with the number of total and metaphase II oocytes (Ruiz-Sanz et al. 2018). The question arises as to whether COH could suppose in some way the manifestation of characteristics associated with reproductive aging. Some features obtained in our lipid analysis data revealed an enrichment of the 23:0 (not statistically significant) and 24:1n-9 fatty acids in the COH groups; theses acyl moieties are particularly present in sphingomyelins. Cordeiro et al. (2018) have recently reported a higher abundance of sphingomyelins in the follicular fluid from the aging group compared to that from younger women. Based on the literature and our results, the general recommendation to take supplements with n-3 PUFAs in IVF should be reviewed.

In conclusion, this study indicates that COH affects the redox balance of follicles. Follicular fluid from COH is less protected against free radicals than from NC, which could have detrimental consequences in the oocyte development and maturation, its quality and embryonic competence in pregnancy rates and IVF success. Therefore, further research is needed on the changes that COH triggers in the follicular microenvironment, and, in particular, on the role that PON proteins play in the ovary.

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CHAPTER II

CHAPTER II: Analysis of protein oxidative modifications in follicular fluid from fertile women: natural *versus* stimulated cycles.

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Abstract

Oxidative stress is associated with obstetric complications during ovarian hyperstimulation in women undergoing *in vitro* fertilization. The follicular fluid contains high levels of proteins, which are the main targets of free radicals. The aim of this work was to determine specific biomarkers of non-enzymatic oxidative modifications of proteins from follicular fluid *in vivo*, and the effect of ovarian stimulation with gonadotropins on these biomarkers. For this purpose, 27 fertile women underwent both a natural and a stimulated cycle. The biomarkers, glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), N^{ε}-(carboxymethyl)lysine (CML), and N^{ε}-(carboxyethyl)lysine (CEL), were measured by gas-liquid chromatography coupled to mass spectrometry. Results showed that follicular fluid contained products of protein modifications by direct metal-catalyzed oxidation (GSA and AASA), glycoxidation (CML and CEL), and lipoxidation (CML). GSA was the most abundant biomarker (91.5%). The levels of CML amounted to 6% of the total lesions and were higher than AASA (1.3%) and CEL (1.2%). In the natural cycle, CEL was significantly lower ($p<0.05$) than in the stimulated cycle, suggesting that natural cycles are more protected against protein glycoxidation. These findings are the basis for further research to elucidate the possible relevance of this follicular biomarker of advanced glycation end product in fertility programs.

Keywords: assisted reproduction; follicular fluid; oxidative stress; aminoadipic semialdehyde;glutamic semialdehyde; gas chromatography-mass spectrometry; female infertility.

1. Introduction

Free radicals are highly reactive molecular species that are inherent to aerobic life. Proteins are one of the major targets of free radical attack, not only because of their high concentration in all compartments, but also because some amino acids (tyrosine, methionine, arginine, proline and lysine) are prone to non-enzymatic modifications (Amici et al. 1989). Proteins are involved in practically all physiological processes, and their oxidative modifications may have detrimental effects in the organism (Stadtman and Levine 2003; Chiu et al. 2011). A hallmark of oxidative protein damage is the introduction of carbonyl groups into amino acid residues. Carbonyl groups in proteins may directly arise by metal-catalyzed oxidation of specific amino acids. Alternatively, during the protein glycation and the peroxidation of polyunsaturated fatty acids, reactive aldehydes (methylglyoxal, glyoxal, and 4-hydroxynonenal) are formed. These compounds react with residues of lysine in proteins generating covalent adducts, thus contributing to total carbonylation. Therefore, quantification of total carbonyl groups in proteins constitutes a global marker of protein oxidation independent of the radical initiator (Griffiths et al. 2002; Augustyniak et al. 2015).

Multiple residues in proteins are susceptible to non-enzymatic modifications, which presents a challenge in undertaking the analysis, but also the possibility of getting a fingerprint. Thus, the metal-catalyzed oxidation renders glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA) as main carbonyl products (Requena et al. 2001). Lysine residues in proteins are targets for specific covalent addition of reactive compounds derived from glucose metabolism (Ames 2008) and lipid peroxidation (Fu et al. 1996). As a consequence, the N^{ε}-(carboxymethyl)lysine (CML) and N^{ε}-(carboxyethyl)lysine (CEL) advanced glycation end products (AGE) are formed, the last one arising specifically from non-enzymatic decomposition of glycolytic intermediates (Ahmed et al. 1997). All these compounds represent specific indices of in vivo oxidative modifications of amino acids in proteins.

Controlled ovarian stimulation with exogenous gonadotropins is generally used in assisted reproduction in order to recover a synchronous cohort of mature oocytes and thus improve the results of in vitro fertilization. However, the treatment involves clinical problems, such as pregnancy and perinatal complications (Mak et al. 2016; Zhu et al. 2016), multiple pregnancies (Fauser et al. 2005), and ovarian hyperstimulation syndrome

(Rik and Smitz 1992). Ovarian hyperstimulation also causes deterioration of the quality and maturity of oocytes in some patients, including patients with polycystic ovary syndrome (PCOS) and unexplained poor responders (Aboulghar et al. 1997). The follicular fluid is the microenvironment for the developing oocyte and is enriched in proteins. A large number of proteins have been identified in this fluid by proteomic analysis (Jarkovska et al 2010; Zamah et al. 2015; Shen et al. 2017). More than 60% of the intrafollicular proteins have extracellular origin, and the rest are located intracellularly or in the plasma membrane (Shen et al. 2017). As for their molecular functions, most proteins have catalytic activity and binding function, followed by receptor and structural functions (Zamah et al. 2015). About 50% of the follicular proteins are involved in immune activity and coagulation (Shen et al. 2017). As indicated above, proteins are the main targets of the attack of reactive oxygen species (ROS), and oxidative stress could compromise their function. In a previous work, we described that controlled ovarian stimulation led to oxidative stress, mainly reflected by changes in the redox status of the serum in terms of total antioxidant activity, susceptibility to *in vitro* oxidation, and the levels of antioxidants such as tocopherol, bilirubin, uric acid and albumin (Aurrekoetxea et al. 2010). It has been reported that oocytes derived from natural cycles show differences from those from stimulated cycles in relevant aspects, such as epigenetics and ultrastructural integrity (Ventura-Juncá et al. 2015; Lee at al. 2017). Ovarian stimulation with exogenous gonadotropins alters the composition of the follicular milieu (von Woff et al. 2014; Kollman et al. 2017). However, the effect of the hormonal intervention on the protein integrity is unknown. The aim of this work was to analyze biomarkers of in vivo non-enzymatic oxidative modifications of proteins from human follicular fluid arising from specific pathways, and the impact of ovarian stimulation on these biomarkers. The measured protein oxidation indices were GSA and AASA, which are specific protein carbonyls from metal-catalyzed oxidations, CEL for glycoxidation, and CML for mixed glyco- and lipoxidation.

2. Materials and Methods

2.1 Study design and study population

Twenty-seven fertile women (with at least one living child) were recruited from 28 February 2014 to 16 December 2015 in the "Oocyte donor program" of the Valencian Institute of Infertility in Bilbao (IVIRMA, www.ivi.es, Vizcaya, Spain). Women were under the age of 35, had a normal physical examination, body mass index between 18 kg/m^2 and 25 kg/m^2, and normal karyotype. Criteria for participation in the study were no history of chromosomal or genetic diseases, negative infectious disease screening, regular menstrual cycles (26–35 days), and no history of endometriosis or recurrent miscarriages.

Women underwent both a cycle of controlled ovarian hyperstimulation and a natural cycle. For the stimulated cycles, pituitary desensitization was achieved by administration of GnRH antagonists, as described previously (Soares et al. 2005). Briefly, women were given pretreatment with a contraceptive pill (3 mg of drospirenone plus 0.03 mg of ethinyl estradiol (Yasmin; Schering, Madrid, Spain) during the cycle before the scheduled oocytes retrieval. Ovarian stimulation was initiated 5 days after pill discontinuation. A daily dose of 0.25 mg of GnRH antagonist (Cetrotide, Serono Laboratories, Madrid, Spain) was administered on day 6 of stimulation, or when the largest follicle was at least 14 mm in diameter. GnRH antagonist administration was maintained until the induction of ovulation. Ovarian stimulation with gonadotropins (recombinant follicle-stimulating hormone, highly purified human menopausal gonadotropin, or a combination of both) was established for an average of nine or ten days. Ovarian response was assessed by vaginal ultrasound examination every 2 days. Ovulation was induced by single subcutaneous administration of a GnRH agonist (2 mg triptorelin acetate, Gonapeptyl, Ferring S.A., Madrid, Spain) when at least three leading follicles were >17 mm in diameter. Transvaginal oocyte retrieval was scheduled 36 h later.

For the natural cycle, follicle size was assessed by vaginal ultrasound on day 8–10 of the cycle. Thereafter, follicular development was followed every 2 or 3 days until the follicle was between 17 and 20 mm in diameter. Then, ovulation was induced by single subcutaneous administration of triptorelin acetate, and oocytes were retrieved 36 h later.

2.2 Ethical Approval

The Ethics Committee of the University UPV/EHU (Ethics Committee for Research involving Human Subjects, CEISH) approved the human subject protocol (CEISH/276/2014/RUIZLARREA), and the study was performed according to the UPV/EHU and IVI-Bilbao agreement. The project complies with the Spanish Law of Assisted Reproductive Technologies (14/2006). Written informed consent was obtained from all trial subjects for participation in the study.

2.3 Sample Collection

For each patient, follicular fluid from the sole follicle (NC) or from two contralateral follicles (COH) was obtained. Samples visually contaminated with blood were discarded. In the stimulated cycle, samples of follicular fluid from the same woman were pooled. After centrifuging the samples (3000 g for 10 min), the supernatant was collected and stored in liquid nitrogen. The samples were taken to the University and kept at −80 °C until analysis.

2.4 Measurement of GSA, AASA, CML, and CEL

The oxidative modifications products of proteins were determined by gas liquid chromatography coupled to mass spectrometry (GC/MS) with selective ion monitoring, following the method described by Pamplona et al (2005) adapted to follicular fluid. Two milliliters of chloroform:methanol (2:1 v/v, 3x) in 0.01% butylated hydroxytoluene were added to 20 µl of follicular fluid. Trichloroacetic acid (10% final concentration) was added to precipitate proteins by centrifugation. The proteins were reduced overnight with 1 ml of 500 mM NaBH4 (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foaming agent. The proteins were precipitated by adding 1 ml of 20% trichloroacetic acid and the solution centrifuged. The following isotopically labelled internal standards were then added: [$^2H_8$]lysine (*d*8-Lys; CDN Isotopes), [$^2H_4$]-CML, [$^2H_4$]-CEL, [$^2H_5$]-5-hydroxy-2-aminovaleric acid (HAVA) for GSA quantification, and [$^2H_4$]-6-hydroxy-2-aminocaproic acid (HACA) for AASA quantification. The samples were hydrolyzed at 155 ºC for 30 min in 1 ml of 6 N HCl, and then vacuum dried. The *N*,*O*-trifluoroacetyl methyl ester derivatives of the hydrolyzed proteins were prepared by sequential treatment with methanolic HCl and trifluoroacetic anhydride (Von Wolff et al. 2014). Briefly, 1 ml of 6.5% acetyl chloride in methanol was added to the sample, and the reaction was maintained at 65 ºC for 30 min. After vacuum-drying, one milliliter of

trifluoroacetic anhydride was added and the reaction proceeded for 1 h at room temperature. The resultant *N,O*-trifluoroacetyl methyl ester derivatives were dried and resuspended in dichloromethane for gas-chromatographic studies. The GC/MS analyses were carried out on an Agilent 6890N gas chromatograph equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a 5973 mass selective detector (Agilent Technologies, Barcelona, Spain). The injection port was maintained at 275 °C; the temperature program was set at 110 °C for 5 min, with the temperature then rising by 2 °C/min to 150 °C, then by 5 °C/min to 240 °C, then by 25 °C/min to 300 °C, and finally held at 300 °C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. The compounds were detected by selected ion-monitoring (SIM) GC/MS. The ions used were: lysine and *d*8-lysine, m/z 180 and 187, respectively; HAVA and *d*5-HAVA (stable derivatives of GSA), m/z 280 and 285, respectively; HACA and *d*4-HACA (stable derivatives of AASA), m/z 294 and 298, respectively; CML and *d*4-CML, m/z 392 and 396, respectively; CEL and d4-CEL, m/z 379 and 383, respectively. The amounts of products were expressed as the ratio of μmol GSA, AASA, CEL, or CML per mol of lysine.

2.5 Glucose Measurement

Intrafollicular glucose was determined by glucose oxidase assay kit (Falcorgent GLU from A. Menarini Diagnostics, Barcelona, Spain).

2.6 Statistical Analysis

The statistical package SPSS 24.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. The population size analysis was performed with G*Power 3.1 software (Düsseldorf, Germany) (Faul et al. 2007). Sample size was calculated as a function of the power level (0.8), the pre-specifiedsignificance level a (0.05), and the population effect size to be detected with a probability of 0.95 (in this case, medium effect size). Taking these parameters into account, a total sample size of 27 was computed. Data were expressed as mean ± standard error of the mean (SE). Statistical significance for the differences of the means was estimated by parametric Student's t-test and the Wilcoxon test (non-parametric equivalent) for paired data. The Pearson's correlation test was used to analyze associations between quantitative variables. The threshold for statistical significance was set to $p<0.05$.

3. Results

Calibration curves for GSA, AASA, CML, CEL, and lysine were derived in order to have quantitative data by analyzing increasing amounts of the non-deuterated compound in the presence of a constant amount of the corresponding deuterated compound. In the case of direct oxidations by free radicals, HAVA (for GSA quantification), and HACA (for AASA quantification) were used as standards, since during the reduction and acid hydrolysis processing of the samples the parent compounds are converted into HACA (GSA) and HAVA (AASA). Correlation coefficients higher than 0.998 were obtained for all the analyzed protein oxidation indices (Fig. 1).

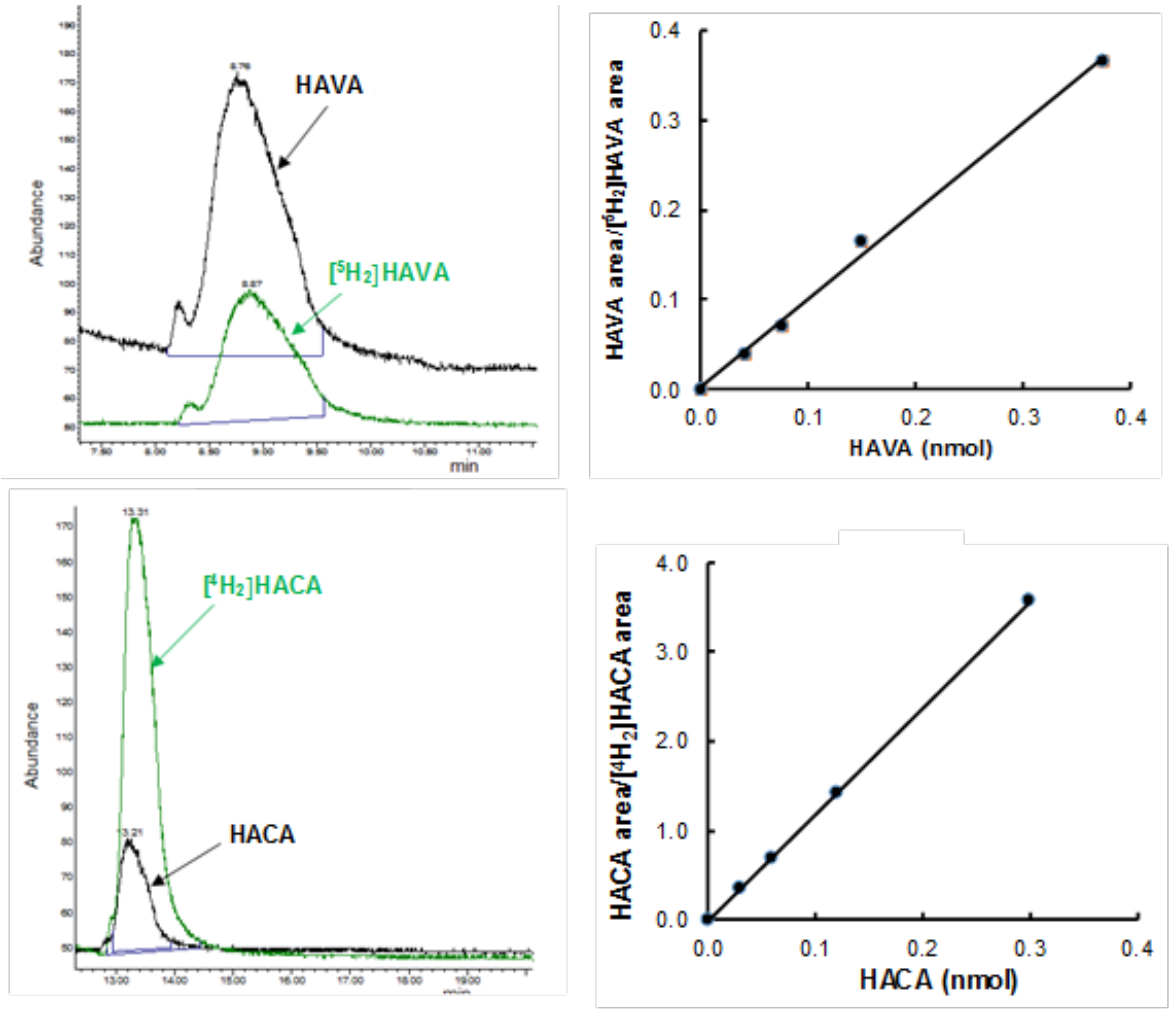

Figure 1. *Cont.*

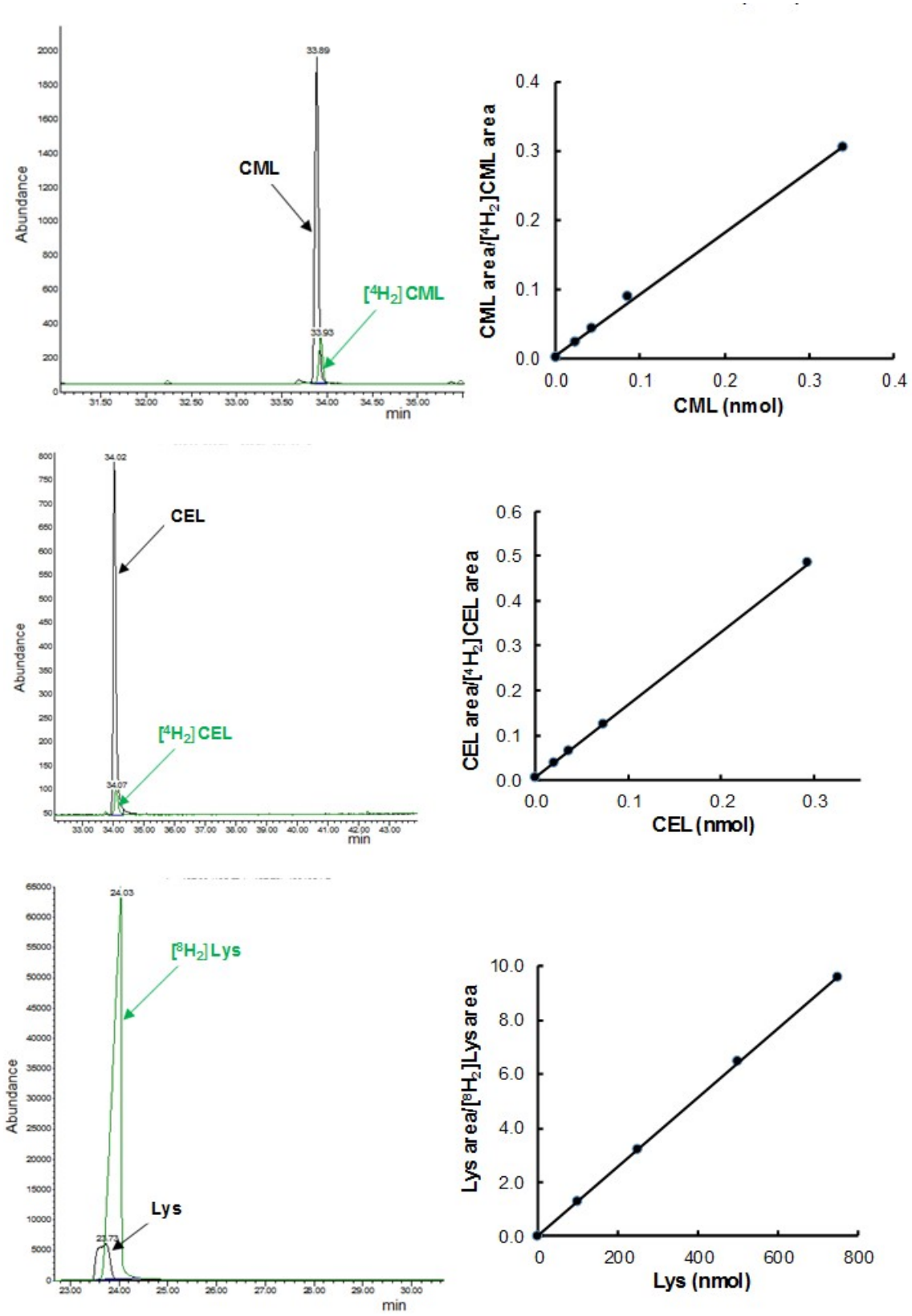

Figure 1. Chromatograms and correlation curves of biomarkers of protein oxidative modifications. Deuterated (green) and non-deuterated (black) compounds were detected in SIM mode. Standard curves with linear correlation coefficients higher than 0.998 were derived from the analyses. HAVA ([$^2H_5$]-5-hydroxy-2-aminovaleric acid) and HACA ([$^2H_4$]-6-hydroxy-2-aminocaproic acid) are the compounds

directly detected by GC/MS after sample processing according to Mat and Meth, and correspond to GSA (glutamic semialdehyde) and AASA (aminoadipic semialdehyde), respectively. CML: N^{ε}-(carboxymethyl)lysine; CEL: N^{ε}-(carboxyethyl)lysine.

Proteins of follicular fluid contained oxidation products resulting from metal-catalyzed oxidation, glycoxidation, and lipoxidation, since the four mentioned biomarkers were detected in all the analyzed samples (Fig. 2). An analysis of their distribution revealed that the most abundant products were those derived from metal-catalyzed oxidation, GSA and AASA (92.8% of the total measured markers). GSA was the most abundant protein lesion (91.5%), corresponding to a mean of 5.1 altered residues per 1,000 lysines. The levels of the other analyzed biomarkers were an order of magnitude lower than GSA, reaching between 0.064 and 0.34 damaged residues per 1,000 lysines. The levels of CML, a protein oxidation biomarker originated from glycoxidative and lipoxidative processes, amounted to 6% of the total lesions and were higher than the levels of AASA (1.3%) and CEL (1.2%).

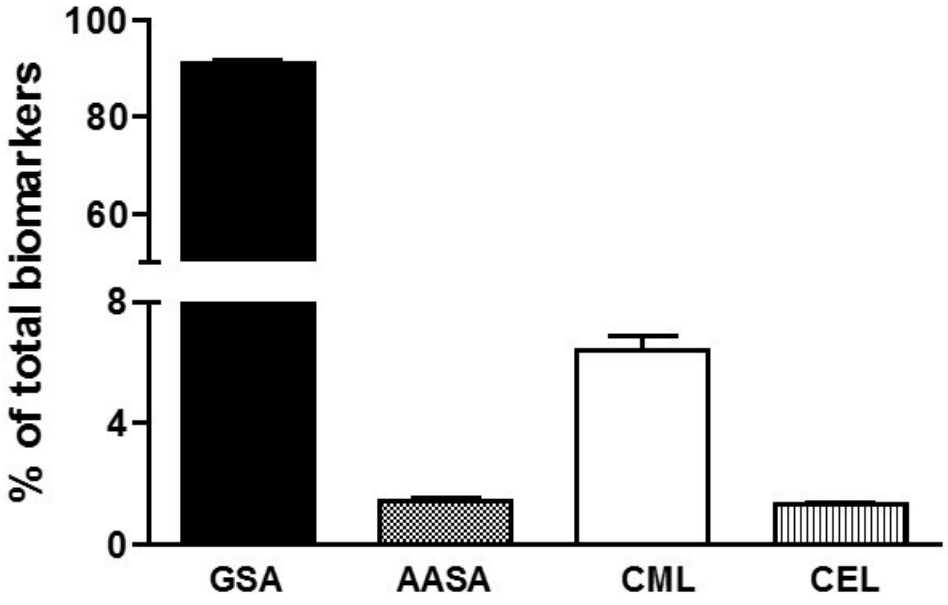

Figure 2. Biomarkers of protein oxidative modifications in follicular fluid. Values were measured in samples from a natural cycle. Bars represent the mean + SE of the percentage of total protein oxidative modifications.

Considering the all measured oxidation indices in natural and stimulated cycles, several correlations between biomarkers were observed (Figure 3). Thus, AASA was significantly correlated with the CEL index of glycoxidative modifications of proteins (p<0.0001). The CML biomarker also correlated with CEL (p<0.05).

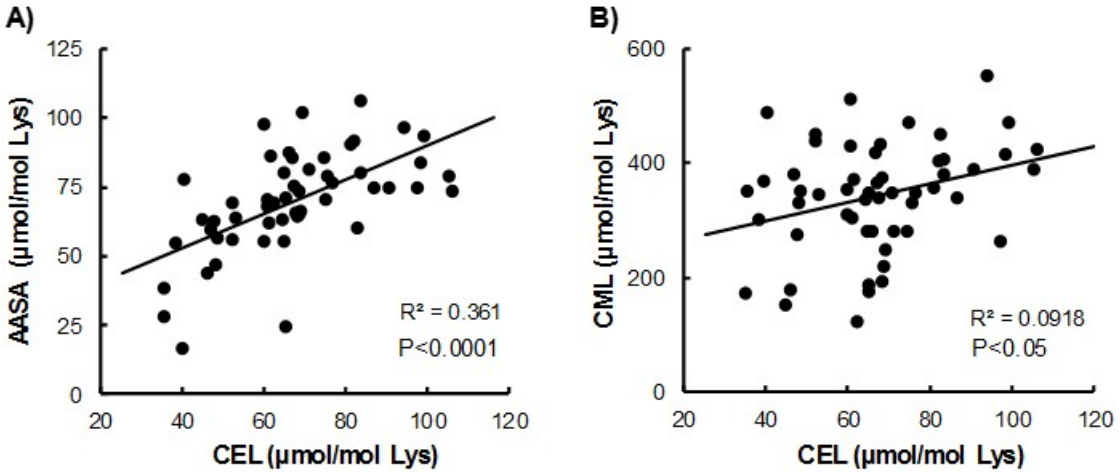

Figure 3. Correlations of (A) AASA and (B) CML with CEL in follicular fluid. Samples were obtained from natural and stimulated cycles.

We used this methodology to determine the possible effect of controlled ovarian hyperstimulation on the protein redox status in the follicular milieu. Results revealed no differences for the metal-dependent protein modifications (Figure. 4A,B), nor were the mean values for CML adducts different between the natural cycle (337 ± 18 µmol/mol Lys) and the stimulated cycle (350 ± 19 µmol/mol Lys) (Figure. 4C). However, the CEL content in follicular fluid from stimulated cycles was significantly higher than that from natural cycles (8%, p<0.05, Figure 4D).

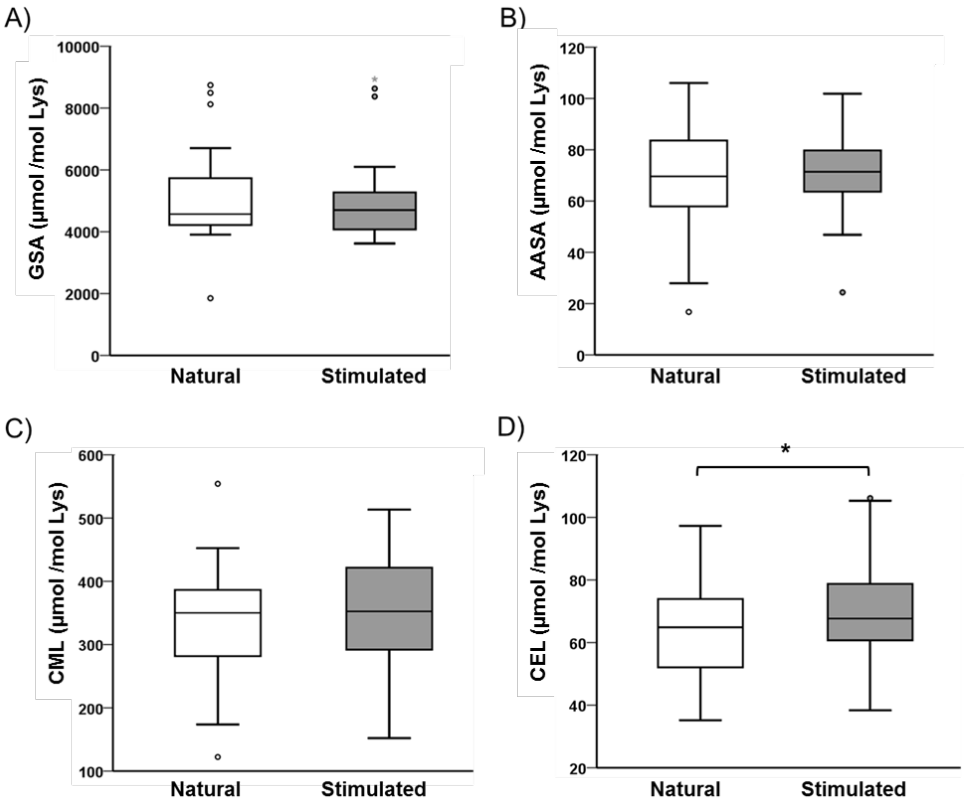

Figure 4. Biomarkers of protein oxidative modifications in follicular fluid from women undergoing both a natural cycle and a stimulated cycle. The box and whiskers graphs represent values for **A)** GSA; **B)** AASA; **C)** CML; and **D)** CEL. The box extends from the 25th to 75th percentiles. The line in the middle of the box corresponds to the median, and the whiskers are drawn down to the 5th percentile and up to the 95th percentile. Open circles represent the outliers of the distribution. Values are expressed as µmol biomarker/mol Lys residue. **p*<0.05.

In view of the differences found between natural and stimulated cycles for the index of the modifications of proteins by glycoxidation, we measured glucose in follicular fluid in both cycles (Figure 5.). Results showed that the levels of glucose were significantly higher ($p<0.05$) in the natural cycle (69 ± 4 mg/dl) than in the stimulated cycle (59 ± 3 mg/dl).

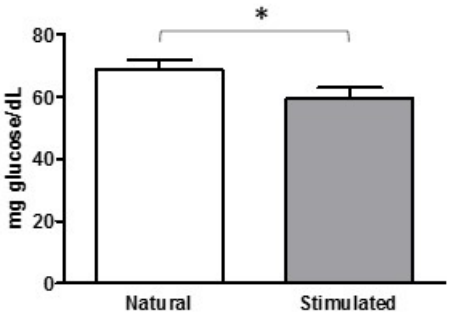

Figure 5. Glucose concentration in follicular fluid from women undergoing both a natural cycle and a stimulated cycle. Bars represent the mean + SE. **p*<0.05.

4. Discussion

In this study, we have described the detection and quantification of specific biomarkers of non-enzymatic oxidative modifications of follicular fluid proteins that arise by different pathways. ROS are products of the cellular metabolic activity that at physiological concentrations play important roles at local and systemic levels. However, non-neutralized ROS cause oxidative damage to lipids, proteins, and nucleic acids, leading to aberrant molecular activities (Roy et al. 2017).

Oxidative damage to proteins by ROS can result in cleavage of the polypeptide backbone, cross-linking, and modifications of the side chains of amino acids. Various types of protein oxidative modifications are induced directly by ROS or indirectly by reactions with secondary products of oxidative stress. Variations in the steady-state levels of oxidatively modified proteins in vivo can be due to differences in the rates of oxidant generation, antioxidative defenses, protein repair and degradative capacity, or susceptibility of proteins to oxidative damage (Stadtman and Levine 2003; Levine et al. 1990).

Our data support the presence of markers derived from protein oxidation in follicular fluid samples from both natural and stimulated cycles determined by gas-liquid chromatography-mass spectrometric. Other authors have reported the presence AGE-modified proteins in follicular fluid, but the analyses were based on immunological techniques (Jinno et al. 2011; Yao et al. 2018). The follicular fluid composition reflects metabolic processes and the hormonal microenvironment in which the oocyte develops (Wiener-Megnazi et al. 2004). Besides granulosa cells, leukocytes and macrophages can also be found in the follicular microenvironment. All of these cell types are capable of generating ROS (Attaran et al. 2000). The expression of antioxidant enzymes in cells that synthesize steroids, such as granulosa, thecal, and luteal cells, has also been reported (Carbone et al. 2003; Agarwal et al. 2008). As a consequence, the oocyte is exposed in vivo to oxidative stress and ROS scavenging activities present in the follicular fluid.

In the present study, it was clearly shown that the steady-state content of protein carbonyls originated from metal-catalyzed oxidation (GSA and AASA) was higher than the levels of the CEL and CML glycoxidation products. Moreover, the intrafollicular amount of GSA, a product of the oxidation of arginine and proline (Requena et al. 2001), was higher than that of AASA (which originates from lysine). Taken together, these

results indicate that the protein residue oxidation is highly selective and suggest that the degree of oxidation reflected by these biomarkers depends on the amino acid composition, their susceptibility to oxidation (directly affected by the structural characteristics and folding of the protein), and/or the pro-oxidant stimulus. In line with our results, Temple et al. (2006) reported that iron-induced in vitro oxidation of human albumin led to the conversion of only two of the 59 lysine residues to AASA, and when hypochlorous acid was used as oxidizing agent five different lysine modification sites were identified.

The increase in protein carbonylation may result not only from direct oxidation of the macromolecule by free radicals but also by the involvement of reactive dicarbonyl compounds such as glyoxal and methylglyoxal. These compounds originate during the triose phosphate metabolism, by lipid peroxidation and by myeloperoxidase-dependent reactions (Anderson et al. 1999; Naudí et al. 2013). Electrophilic aldehydes are well-known by-products of the non-enzymatic peroxidation of polyunsaturated fatty acids, usually arachidonic and linoleic acids, and are also formed from glycolytic intermediates. These aldehydes are highly reactive with protein residues of histidine, cysteine, and lysine, forming Michael's adducts, which may result in impairment of enzyme activities and interference with signaling pathways (Uchida and Stadtman 1992; Monroy et al.2013; Lord et al. 2015). Amino acid residues, such as lysine, can be modified by adduct formation with aldehydes derived from the peroxidation of polyunsaturated fatty acids, particularly aldehydes with α, β-unsaturated functional groups (Esterbauer et al. 1991). Among them, glyoxal, a precursor to CML, yields chemically stable adducts with lysine residues. CML was initially described as a product of carbohydrate autoxidation, but it is now well documented that it is also a compound derived from lipid peroxidation in the presence of proteins, and its amount depends on the degree of unsaturation and oxidizability of fatty acids (Kalea, Schmidt and Hudson 2009) We have detected CML in the follicular fluid of women at a concentration about 10-fold lower than the metal-dependent modifications.

Neither the CML nor the GSA and AASA levels in the follicular fluid were modified as a consequence of controlled ovarian stimulation. By contrast, we observed a higher content of CEL in follicular fluid from stimulated cycles. We do not know the physiological relevance of this 8% increase, although a similar increase of CEL has been reported in brain cortex samples from patients with Alzheimer's disease (Pamplona et al. 2005). It is still not known whether the damaged proteins associated with this marker have

specific functions in the reproductive process. AGEs in the follicular fluid not only represent a loss of function of those AGE-modified proteins but are also an additional source of ROS. AGEs circulate in the medium (i.e., follicular fluid) and bind to plasma membrane receptors, such as the receptor for AGEs (RAGE), which is a member of the immunoglobulin superfamily of receptors (Bierhaus et al. 2005). The AGE/RAGE interaction activates the nuclear factor kappa-B and other downstream pathways, resulting in production of ROS and pro-diabetic, pro-inflammatory, pro-thrombotic, and pro-atherogenic responses (Fu et al. 1996).

In a recent study, a proteome analysis of follicular fluid from natural and stimulated cycles revealed that eight proteins were differentially expressed in stimulated cycles (Wu et al. 2015). The upregulated proteins were related to immune and inflammatory responses, suggesting that these processes might play a role in the adverse effects of ovarian hyperstimulation. The treatment with exogenous gonadotropins leading to multiple oocyte retrieval would contribute to ROS increase, and consequently their detrimental effects on proteins.

The CEL increase in the stimulated cycle was accompanied by a substantial reduction in follicular glucose levels. This decrease could be due to the fact that the sugar is being metabolized to intermediates that generate methylglyoxal, which reacts with intrafollicular proteins, thus forming stable adducts. The ovarian stimulation protocol has been shown to alter the intrafollicular levels of hormones associated with fertility parameters, in particular estradiol, luteinizing hormone, and anti-Müllerian hormone. The treatment with exogenous gonadotropins reduced these hormones, reflecting poorer outcomes of oocyte maturation and quality, and embryo implantation (von Wolff et al. 2014). It is unknown whether the hormonal alterations are related to the changes observed in CEL levels or if they are independent factors that may contribute to the in vitro fertilization (IVF) outcomes. The present work is a pilot study. If the results are validated using a larger sample size, the measurement of CEL could have implications in assisted reproduction. The identification of the specific glycoxidatively modified proteins and the knowledge of the associated functional changes in fertility parameters are a subject of research to develop. The information derived from those data could result in direct implications for the techniques and outcomes of assisted reproduction. It also remains to be established whether similar changes are reflected in other biological fluids, such as blood.

Conclusions

The GC/MS method described in this article provides a means for the specific quantification of CEL and CML adducts and oxidative modifications of amino acids (GSA and AASA) occurring in follicular fluid proteins. Differences between the natural and stimulated cycles were found for the CEL glycoxidation-modified protein biomarker, with the lowest index observed for the natural cycle. These findings are the basis for future research and open new perspectives for establishing the possible relevance that this intrafollicular biomarker of in vivo protein modifications has in determining success of IVF in assisted reproduction programs.

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CHAPTER III

CHAPTER III: PON expression in human granulosa cells: unexpected nuclear localization of PON1 and PON3.

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Abstract

Paraoxonases (PON1, PON2 and PON3) are hydrolytic enzymes with antioxidant activities. PON1 and PON3 are predominantly synthesized in the liver, secreted to the bloodstream and transported by lipoproteins, mainly associated to high-density lipoproteins, while PON2 is an intracellular protein. Most of the references regarding the clinical implications of PON deal with cardiovascular diseases. However, the role of these paraoxonase proteins in reproduction remains unknown. Recently, PON1, PON2, and PON3 have been localized in ovarian follicular fluid. This fluid is formed in part by low molecular weight components of the blood and products from local synthesis of ovarian cells, such as granulosa cells. The aim of this work was to characterize for the first time PON1, PON2 and PON3 proteins in human granulosa cells. We used conventional RT-PCR to analyze mRNA levels and western blotting using commercial human recombinant PON proteins as standards to quantify the proteins. Immunocytochemistry and confocal microscopy were applied to analyze the intracellular distribution of the PON proteins. Due to the difficulties of working with primary granulosa cells, HepG2, a human hepatoma cell line that highly expresses paraoxonases, and the human immortalized granulosa COV434 cell line were used to optimize methodologies. The results showed that granulosa cells express PON1, PON2, and PON3, and that PON secreted proteins are also localized in the nucleus.

1. Introduction

The use of assisted reproduction techniques has increased exponentially during the last years in developed societies due to the problem of infertility. Although free radicals and reactive oxygen species (ROS) are necessary in different steps during female reproduction, high concentrations of these species are associated with reproductive diseases and infertility (Al-Gubory et al.2010). To maintain these species at non-harmful stationary concentrations, both enzymatic and non-enzymatic antioxidants act coordinately in our body. Among the antioxidant enzymes are paraoxonases (PONs). The human paraoxonase gene family comprises three members, which are located adjacently in a cluster on the long arm of chromosome 7 (La Du 1996; Primo-Parmo et al. 1996; She et al. 2012). The genes encode PON1, PON2, and PON3 proteins, which show antioxidant activities. PON1 and PON3 are predominantly synthesized in the liver, secreted to the bloodstream, and transported by lipoproteins, mainly associated to HDL. In contrast, PON2 is intracellularly located in a wide variety of tissues and cells (Primo-Parmo et al. 1996; Ng et al. 2003).

Most of the references regarding the clinical implications of the PON proteins deal with cardiovascular diseases. In the blood, PON1 and 3 exert antioxidant actions against the oxidative modifications of LDL, thus protecting against atherosclerosis. However, the role of PONs in fertility and their possible location in reproductive tissues are unknown. In a previous work, we reported that follicular fluid of developing follicles in the ovary from patients undergoing ovarian stimulation presented catalytic activities of PON1 and PON3, These activities were higher as the follicle grew. We also found differences in PON3 activity between unfertile patients and fertile oocyte donors, and the activity was higher in donors (Meijide et al. 2017). Follicular fluid is formed in part by elements from the blood, small enough to pass the hematofollicular barrier, but also contains other components that are locally secreted by the ovary cells. The quantification of extracellular PON1 and PON3 proteins in serum and follicular fluid from the same woman at the time of oocyte retrieval suggested that their activities in follicular fluid were not simply due to a higher HDL infiltration rate from the serum (Meijide et al. 2017). So far, there are no data concerning the expression of these proteins in human ovary cells. In this work, we

propose to analyze the expression of PON1, PON2, and PON3 in ovarian granulosa cells. To this end, the cells were purified from follicle samples of women undergoing ovarian stimulation at oocyte retrieval. We have used RT-PCR techniques to detect mRNAs of the *PON* genes, quantitative western blot to quantify the proteins, and immunofluorescence and confocal microscopy techniques to analyze their subcellular location. In order to optimize the methodologies employed, and due to the reduced sample volume of granulosa cells, we used the stable cell line HepG2, with high *PON* expression, and the human ovary COV434 cell line. To study the genes expression specificity of the methodology used, silencing target mRNA by transfection of cells with small interfering RNA (siRNA) was performed.

2. Material and methods

2.1 Human samples

Samples were collected from 20 IVF female patients of the IVI-RMA assisted reproduction clinic in Bilbao. Women underwent controlled ovarian hyperstimulation by the protocols of the clinic (Pérez-Ruiz et al. 2018). At the time of oocyte retrieval, samples were obtained by follicular puncture. The oocyte was separated from the follicular fluid and the rest of ovary cells. This biological material, that constitutes a medical waste, was the starting biological material to isolate granulosa cells.

The Ethics Committee for Research involving Human Subjects of the University (CEISH) approved the protocol (M30_2015_187_RUIZ LARREA, 28th October 2015). Written informed consent was obtained from all subjects participating in the study, which was conducted in accordance with the guidelines of the Helsinki Declaration.

2.2 Isolation and culture of granulosa cells

Granulosa cells were isolated from blood cells using Percoll solutions (40% and 60%) prepared in McCoy's medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS). The biological samples were centrifuged at 600 g for 10 min at room temperature. The cellular pellet was resuspended in McCoy's medium and 1 ml of the mixture was carefully layered on polystyrene tubes containing Percoll gradient (2:2:1, v/v/v). The tubes were centrifuged at 600 g for 20 min at room temperature. After centrifugation, the cells were collected from the interface. This process

was repeated once. The isolated cells were washed from Percoll by centrifugation. Finally, granulosa cells were used immediately.

The purified cells were cultured in Petri dishes in McCoy medium with 10% FBS, to which apotransferrin (5 mg/ml), Δ^4-androstendione (0.1 μM), $HNaCO_3$ (2.2 g/l) and HEPES (20 mM) were added. All these materials were purchased from Sigma-Aldrich (St. Louis, USA). The culture was maintained in the incubator (5% CO_2) at 37 °C for 48 h and subsequently, cells were processed for RNA extraction, western blot and immunocytochemical analysis.

2.3 COV434 and HepG2 culture

The human ovary COV434 granulosa cells (Sigma-Aldrich, St. Louis, USA) and human HePG2 hepatoma cells (ATCC, Manassas, USA) were cultured in Dulbecco's Modified Eagle Medium and Eagle's Minimum Essential Medium (Sigma-Aldrich, St. Louis, USA), respectively. Both media were supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 U/ml penicilin, and cells lines were cultured (5% CO_2) at 37 °C in six well plates.

2.4 siRNA and transfection

The siRNA sequences targeting *PON1*, *PON2*, and *PON3* were designed using the siDESIGN Center bioinformatics tool, http://dharmacon.horizondiscovery.com/design-center/ (Wang and Mu 2004) (Table 1), and were purchased from Sigma-Aldrich, St. Louis, (USA) together with scrambled siRNA. Transfection of PON and scrambled siRNAs were performed using the RNAiMAX lipofectamine transfection reagent (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Briefly, filtered Opti-MEM I medium was added to a 6-well plate with a final siRNA concentration of 0.9 μM (siRNA PON1), 0.3 μM (siRNA PON2), and 0.6 μM (siRNA PON3). RNAiMAX was then added to each well and the plate was incubated for 20 min at room temperature. After incubation, 400,000 cells/well were seeded in complete culture medium without antibiotics. The cells were maintained for 48 h at 37 °C (5% CO_2).

Table 1. Sequences of siRNA targeting *PON1*, *PON2*, and *PON3*.

	Sense 5´→3´	***Antisense 5´→3***
PON1		
siRNA 1	GGAUUAAAGUAUCCUGGAA	UUCCAGGAUACUUUAAUCC
siRNA 2	GGAAGUAAAUUUGAUGUAU	AUACAUCAAAUUUACUUCC
siRNA 3	GCAUGCUAAUUGGACUUUA	UAAAGUCCAAUUAGCAUGC
PON2		
siRNA 1	GGGCAAAUGUUGUUUACUA	UAGUAAACAACAUUUGCCC
siRNA 2	CCAAGGGCACGGGAAUUAA	UUAAUUCCCGUGCCCUUGG
PON3		
siRNA 1	GGAUUAAAGUAUCCUGGAA	UUCCAGGAUACUUUAAUCC
siRNA 2	CCUUAUUGAGGAACUUGAA	UUCAAGUUCCUCAAUAAGG
Scrambled		
siRNA	UUCUCCGAACGUGUCACGU	ACGUGACACGUUCGGAGAA

2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from cells with TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's protocol, which is a modification of the method described by (Chomczynski and Sacchi 1987). RNA concentration was assessed using NanoDrop (Thermo Fisher Scientific, Waltham, USA). RNA was treated with extension grade DNase I (Thermo Fisher Scientific, Waltham, USA) to eliminate possible DNA contamination of the sample according to manufacturer's instructions.

The cDNA was obtained from 1.5 µg of RNA by the retrotranscription reaction using the Superscript III first strand synthesis system for RT-PCR kit according to the manufacturer's instructions.

2.6 PCR analysis

The PCR was performed on a Biometra thermocycler using primers designed with the aid of the bioinformatics tool Primer-Blast, https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Ye et al. 2012) (Table 2). To detect PON1 and PON2 conventional PCR was used and in the case of PON3 nested PCR was carried out using two set of primers.

Table 2. Sequences of the primers designed for PCR.

Gene	*Forward (5' → 3')*	*Reverse (5' → 3')*
PON1	CCAGTCTTCTTACCAAACACGA	GGGTTGAAGCTCTTTATTCCA
PON2		
variant 1	TTAGTGTGGGTCTAAAATTCCC	TGGGTGGTTTACAACAAAGAG
variant 2	TACTAATGATGGATCTAAAAGAAGAAAAAC	GTATTCTTGAATTCGTTGTCTATG
PON3		
Set 1	AGATGTTCCTGGCGTTTAG	ACCTCCCTTGGGCTGTAGAA
Set 2	GAGAAGTGGAGCCAGTAGA	ATTTCTAGCGCTTGTGCCCT

PON1 and PON2 PCRs were carried out in a final volume of 40 µl containing 1x Immobuffer, 2.5 mM Cl_2Mg, 200 µM/nt dNTP mix, 5% DMSO, 0.5 µM of each primer, 1.25 U of IMMOLASE^{TM} DNA Polymerase (Bioline, Luckenwalde, Germany) and 5 µl of cDNA previously diluted. PON3 nested PCR was carried out in the same conditions, but without DMSO.

Gene target		Denaturing	Hybridization	Extension	Cycles
PON1	Temperature	94 °C	58.6 °C	72 °C	35
	Time	1 min	1 min	1 min	
PON2 *variant 1*	Temperature	94 °C	52.5 °C	72 °C	30
	Time	1 min	1 min	1 min	
PON2 *variant 2*	Temperature	94 °C	54.5 °C	72 °C	30
	Time	1 min	1 min	1 min	
PON3	Temperature	94 °C	54 °C	72 °C	30
PCR 1	Time	1 min	1 min	1 min	
PCR 2	Temperature	94 °C	55 °C	72 °C	30

Time	1 min	1 min	1 min

Table 3. Final conditions of the amplification of *PON1*, *PON2* variants and *PON3* transcription products by PCR.

The products resulting from amplifications were separated by horizontal electrophoresis on 2% agarose gels and SYBR safe staining. The bands were visualized by optical densitometry using the Bio-Rad Molecular Imager FX system.

2.7 Protein quantification by western blot

At the indicated time, the culture medium was removed (reserved for further analysis) and the cells were washed twice with cold phosphate buffered saline (PBS). Cells were lysed in ice for 30 min with lysis buffer, consisting on 20 mM Hepes pH 7.5, 1 mM NaF, 10 mM EGTA, 40 mM β-glycerophosphate, 1% NP-40, 2.5 mM $MgCl_2$, 2 mM orthovanadate, and 1 mM dithiothreitol, to which 10 µl/ml of a protease inhibitor cocktail (Sigma-Aldrich, St Louis, USA) was added just before using. Cellular fragments were removed by centrifugation at 10000 x g for 5 min at 4 °C, and the supernatant was used for analysis. Total protein was quantified by staining with Coomasie's bright blue (Bradford 1976) and detection of PON1, PON2 and PON3 was carried out according to Meijide et al. (2017). Cellular protein (10 µg, 40 µg and 80 µg) was loaded to detect PON2, PON1, and PON3, respectively.

To detect PON1 and PON3 in the medium, the medium was enriched with minority proteins using Bio-Rad's ProteoMiner kit by affinity column chromatography as described in Meijide et al. (2017) and 30 µl of the resulting sample was loaded.

2.8 Cellular synchronization by double thymidine block

Cellular synchronization at G1/S boundary was performed using thymidine (Sigma-Aldrich, St Louis, USA) follow the method proposed by (Chen and Deng 2018). After cell cycle resuming, cells were collected at 0, 2, 4, 8, 16, 24 and 48 h for further analyses.

2.9 Cell cycle analysis

Cells seeded for the synchronization assay were collected fixed overnight at 4 °C in 70% ice-cold ethanol. After washing with ice-cold PBS, cells were stained with a 25 µg/ml

propidium iodide solution in the presence of 200 µg/ml RNase A for 45 min at 37 °C in the dark. The cell cycle distribution of the cells was determined by flow cytometry in a Beckman Coulter Gallios Flow cytometer in the General Research Services SGIker of the UPV/EHU, with a total acquisition of 10,000 events. The percentage of cells in different phases of the cell cycle was analyzed by Summit 4.3 software (Dako, Hovedstaden, Denmark).

2.10 Immunocytochemical assays

Cells were cultured on round covers under the conditions previously described. After 48 h of culture cells were fixed with 3.7% formaldehyde for 15 min at room temperature. The cells were were washed thrice and permeabilized with 0.5% Triton X-100 for 5 min at room temperature and blocked with 10% SBF for 1 h. Samples were incubated overnight at 4 °C with anti-PON1 (1:100), anti-PON2 (1:100) (R & D systems, Minneapolis, USA) and anti-PON3 (1:100) (Abcam, Cambridge, UK) primary monoclonal antibodies. In addition, the samples were incubated with monoclonal antibodies for the detection of mitochondria with anti-ATP5a (Abcam, Cambridge, UK) at a dilution of 1:1000. For synchronization studies samples anti-PON1 was used (Thermo Fisher Scientific, Waltham, USA). After several washes with PBS, the secondary antibodies were added in a 1:200 dilution. All of them were purchased from Thermo Fisher Scientific (Waltham, USA). Anti-rat anti-IgG conjugated with Alexa Fluor488 was added for the detection of PON1 and PON2. Anti-rabbit anti-IgG conjugated with Alexa Fluor488 for the detection of PON3. Anti-mouse anti-IgG conjugated to Alexa Fluor 633 for the detection of mitochondria and anti-mouse anti-IgG antibody conjugated to Alexa Fluor 488 was used to detect PON1 in synchronization assays. (Thermo Fisher Scientific, Waltham, USA) The nuclei of the cells were stained with 5 µg/ml DAPI (Sigma-Aldrich, St Louis, USA). Finally, the samples were placed on a slide with glycergel mounting medium (Dako, CA, USA). The images were acquired by ZEISS LSM 880 confocal microscopy with Airyscan high resolution mode at the microscopy service of the UPV/EHU. These images were processed with the ImageJ-win32 software (https://imagej.nih.gov/ij/).

2.11 Statistical analysis

Quantitative data were expressed as the mean ± standard deviation of at least three experiments using the Microsoft Office 2010 Excel spreadsheet. The significance of the differences between the means was determined by the Student's t-test. Differences were considered statistically significant when $p<0.05$.

3. Results

3.1 Detection of PON1, PON2 and PON3 mRNAs by RT-PCR

Tests were performed with different concentrations of the PCR mixture, number of cycles and hybridization temperatures (data not shown). The final conditions of the PCR are described in the *Materials and methods* section. The amplification of the PON transcripts with specific primers for each of the family members resulted in representative fragments of each transcript (PON1, PON2 and PON3). The primers used for the amplification of the PON1 mRNA were specifically attached to the sequence between exon 1 and exon 4 of the gene, amplifying a 527 bp fragment. This fragment was detected in the three cell types studied (Fig. 1). The amplification was specific.

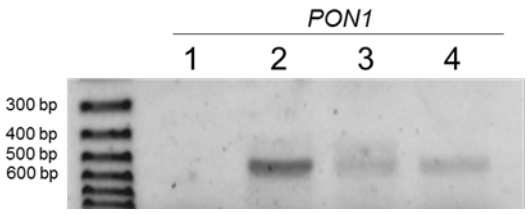

Figure 1. Detection of PON1 mRNA in HepG2, COV434 and granulosa cells. Lanes 1, 2, 3 and 4 conrrespond to blank, HepG2, COV434 and granulosa cells, respectively. Amplification fragment has a size of 527 bp.

Two alternatively spliced transcript variants encoding different isoforms have been described for *PON2* (Mochizuki et al. 1998). Variant 1 (v1) represents the longer transcript and encodes the longer isoform 1. Variant 2 (v2) uses an alternate, in-frame splice site in the coding region, compared to v1. It encodes isoform 2, which is shorter than isoform 1. We have designed a PCR protocol capable to differentiate both variants. The cDNA amplification obtained from mRNAs resulting from PON2 transcription was also positive for the three cell types, achieving fragments of 209 bp (v1) and 127 bp (v2) (Fig. 2).

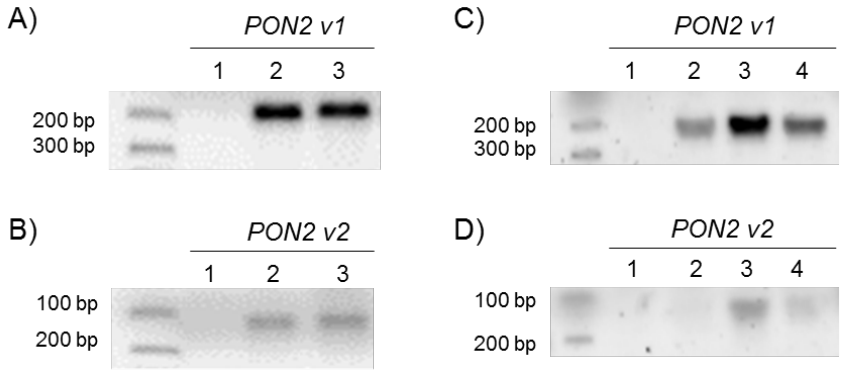

Figure 2. Detection of PON2 mRNA in HepG2, COV434 and granulosa cells. A) and B) Amplification of PON2 variant 1 (209 bp) and variant 2 (127 bp) in HepG2 (line 2) and granulosa cells (line 3). C) and D) Amplification of PON2 variant 1 (209 bp) and variant 2 (127 bp) in COV434. Lanes 2, 3 and 4 are from different COV434 samples. Blank (lane 1).

Finally, the PCR used for the detection of PON3 resulted in a product of 476 bp (Fig. 3). This sequence for which primers are specific is found between exon 2 and exon 6 of the *PON3* gene.

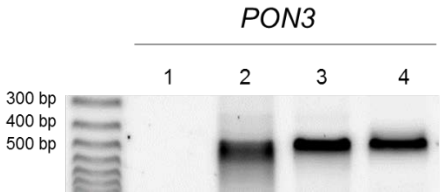

Figure 3. Detection of PON3 mRNA in HepG2, COV434 and granulosa cells. Lanes 1, 2, 3 and 4 correspond to blank, HepG2, COV434 and granulosa cells, respectively. Amplification fragment has a size of 476 bp.

3.2 Quantification of PON1, PON2 and PON3 by western blot

The protein expression of the components of the *PON* family was analyzed by quantitative western blot, using standard curves with human recombinant commercially available proteins (Fig. 4). PON1 and PON2 were detected in HepG2, COV434, and

granulosa cells. However, PON3 was detected only in HepG2 (Fig. 4). In the case of PON1, a single band corresponding to a 43 kDa molecular weight protein was observed. For this member of the PON family, the concentrations in HepG2 and granulosa cells were similar (Table 1).

For PON2, there were two bands correponding to proteins of a molecular weight close to 40 kD, due to the alternative splicing process mentioned above. This process results in two isoforms of the same protein, which differ by less than 3 kDa according to their amino acid sequences (Horke *et al.*, 2007). Both variants of the same protein are distinguished in the two cell types studied, indicating the expression of both variants (Fig. 4B). Since PON2 is an intracellular protein, its concentrations were higher than those of PON1 (Table 1).The expression detected in HepG2 and COV434 appeared to be greater than that detected in granulosa cells, although the differences were not statistically significant.

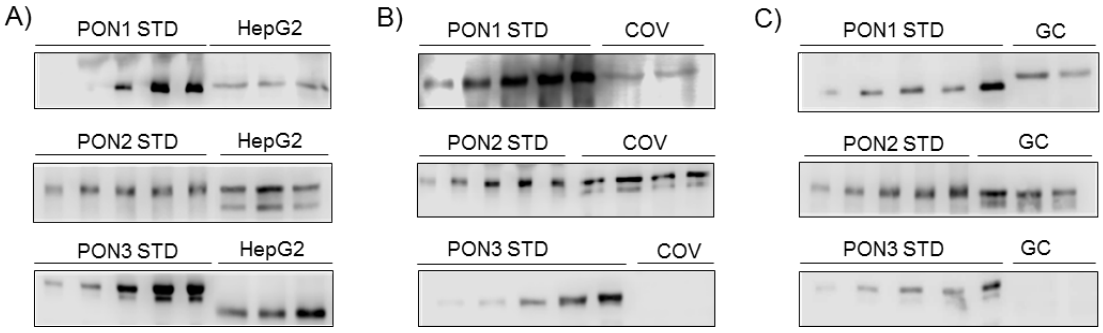

Figure 4. Immunoblots showing the expression of PON1, PON2, and PON3 proteins in A) HepG2, B) COV434, and C) granulosa cells (GC). Lanes STD indicate increased concentrations of the corresponding commercially available standard human recombinant proteins from which the standard curves were derived. Each of the HepG2, COV434 and GC lanes were from different samples.

With respect to PON3, the protein band in HepG2 was clearly visible. However, PON3 could not be detected by immunoblotting in COV434 and granulosa cells (Fig. 4B and C). This could be due to the low intracellular concentration of this secretion protein, which renders its detection by western blot unsatisfactory results.

	ng/mg protein		
Cell type	PON1	PON2	PON3
HepG2	0.10 ± 0.02	2.33 ± 0.70	0.06 ± 0.03
COV434	0.078 ± 0.003	2.29 ± 0.20	—

Granulosa cells	0.18 ± 0.04	1.49 ± 0.31	—

Table 2. Concentrations of PON1, PON2 and PON3 in HepG2, COV434, and granulosa cells.

Taking into account that PON3 is a secretion protein, we analyzed it in the conditioned medium after 48 h of incubation. In order to detect minority proteins in the conditioned environment, it was necessary first to enrich them, partially reducing the concentration of more abundant proteins by affinity column chromatography. When analyzing the conditioned medium of the granulosa cells, a band corresponding to PON3 could be detected by western blot (Fig. 5). The concentration of the protein was 18.4 ± 7.3 ng/ml after 48 h of incubation.

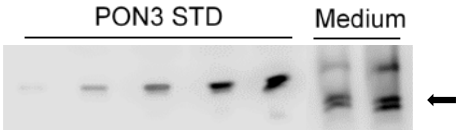

Figure 5. Immunoblot showing the expression of PON3 in the conditioned medium of granulosa cells for 48 h. The PON3 STD lanes correspond to increasing concentrations of human recombinant PON3, from which the standard curve was derived. The last two lanes correspond to two different samples.

We also analyzed PON1 in the conditioned medium of the granulosa cells after 48 h of incubation. Results revealed a band of 43 kDa representative of PON1 (Fig. 6). Its concentration was 158 ± 32 ng/ml, which indicates a secretion 9-fold higher than that of PON3.

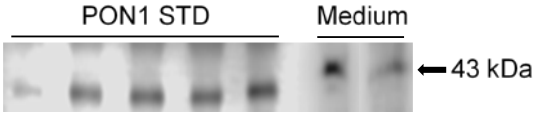

Figure 6. Immunoblot showing the expression of PON1 in the conditioned medium of granulosa cells for 48 h. The PON1 STD lines correspond to increasing concentrations of human recombinant PON1, from which the standard curve was derived. The last two lines correspond to two different samples.

3.3 Silencing PON1, PON2, and PON3 using siRNAs

Silencing studies were carried out using siRNAs designed for this purpose. Given the difficulty of handling fresh granulosa cells for silencing studies, we used HepG2 cells, which had responded similarly to granulosa cells in terms of paraoxonase expression.

Specific siRNAs were designed for PON1, PON2 and PON3 mRNAs. In addition, a random siRNA was added as control. In all cases, it was possible to verify that the silencing did not occur due to nonspecific interferences from the siRNA molecule itself. The silencing percentage for each of the siRNAs was calculated from the optical densities of the corresponding bands obtained in the RT-PCR assays (Fig. 7, Table 5). RT-PCR results obtained in HepG2 cells 48 h post-transfection with siRNAs targeted specifically to the PON gene member showed that there was reduction in mRNA expression of PON1, PON2 and PON3, compared to cells treated with non-silencing siRNA (20% reduction for PON1, 64-70% for PON2 and 100% for PON3).

	% silencing mRNA			
	PON1	**PON2**		**PON3**
Cell type		**siRNA 1**	**siRNA 2**	
HepG2	19.6	69.6	64.4	100

Table 5. PON1, PON2 and PON3 mRNA siRNA silencing in HepG2. Data correspond to % of silenced mRNA.

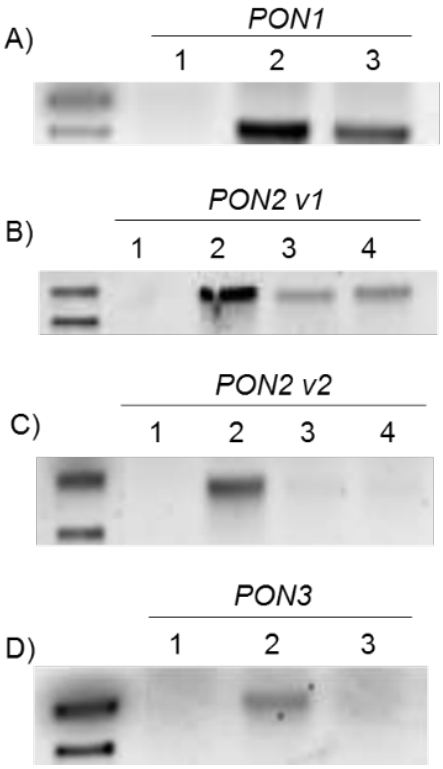

Figure 7. Amplification by RT-PCR of mRNA in HepG2 showing effects of *PON* silencing. Results correspond to cellular 48 h-post-transfection with *PON* siRNAs. A) *PON1*, B) *PON2* variant 1, C) *PON2* variant 2, and D) *PON3*. Lane 1, blank; lane 2, random siRNA; lanes 3 and 4, specific siRNA targeting *PON1*, *PON2* and *PON3*.

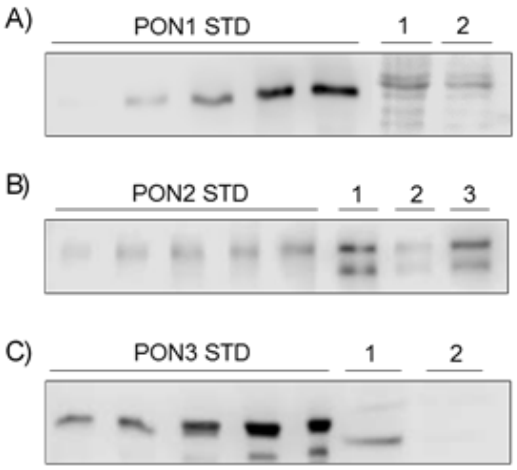

Figure 8. PON1, PON2 and PON3 proteins siRNA silencing in HepG2. Results correspond to cellular 48 h-post-transfection with *PON* siRNAs. A) *PON1*, B) *PON2* variants 1 and 2, C) *PON3*. Lane1, random siRNA; lanes 2 and 3, mixture of siRNA specific against A) PON1, B) PON2 and C) PON3.

The reduction of the expression of the PON genes at the protein level after silencing treatment with siRNA was confirmed by quantitative western blot analysis (Fig. 8). The

protein concentrations decreased by 33% (PON1), 82% and 17% (PON2), and 100% (PON3), compared with cells treated with non-silencing siRNAs.

3.4 Immunocytochemical detection of PON1, PON2 and PON3

Protein intracellular distribution studies showed different locations for PON1, PON2 and PON3. In HepG2, COV434, and granulosa cells, PON1 was diffusely localized in the cytoplasm, apparently excluded from mitochondria. It is very remarkable the nuclear staining, avoiding nucleolus and nuclear membrane spaces (Fig. 9). PON3 showed a similar pattern of intracellular localization, but the presence in mitochondria could not be discarded in COV434 (Fig. 11). PON2 was distributed in the cytoplasm with a punctated pattern, largely associated with mitochondria. It was also appreciated a perinuclear enrichment, probably with a partial nuclear membrane localization, in HepG2 and COV434 (Fig. 10).

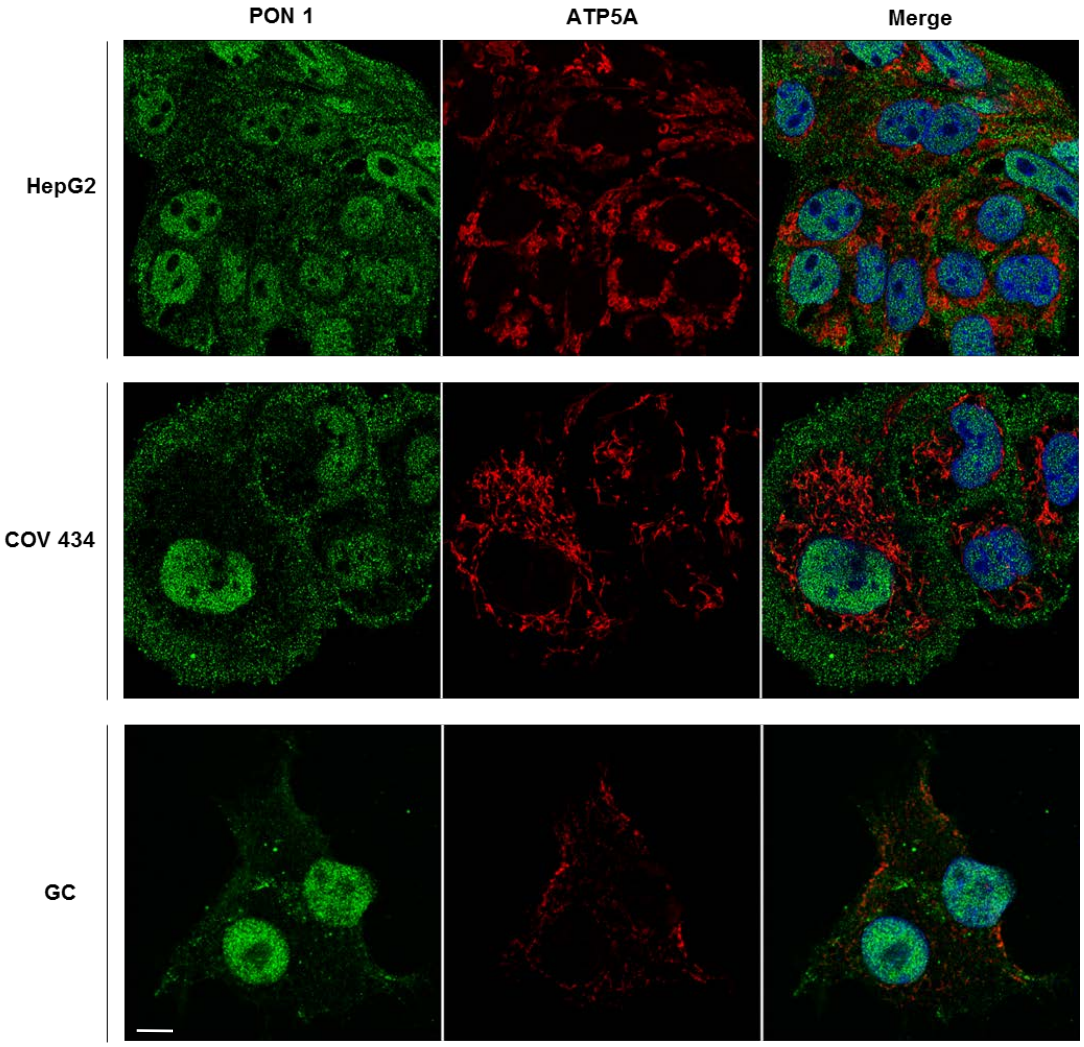

Figure 9. Intracellular distribution of PON1 in HepG2, COV434 and granulosa cells (GC). PON1 protein appears in green, while the mitochondria are red. The nuclei were counterstained with DAPI (blue). White scale bar corresponds to 5 µm.

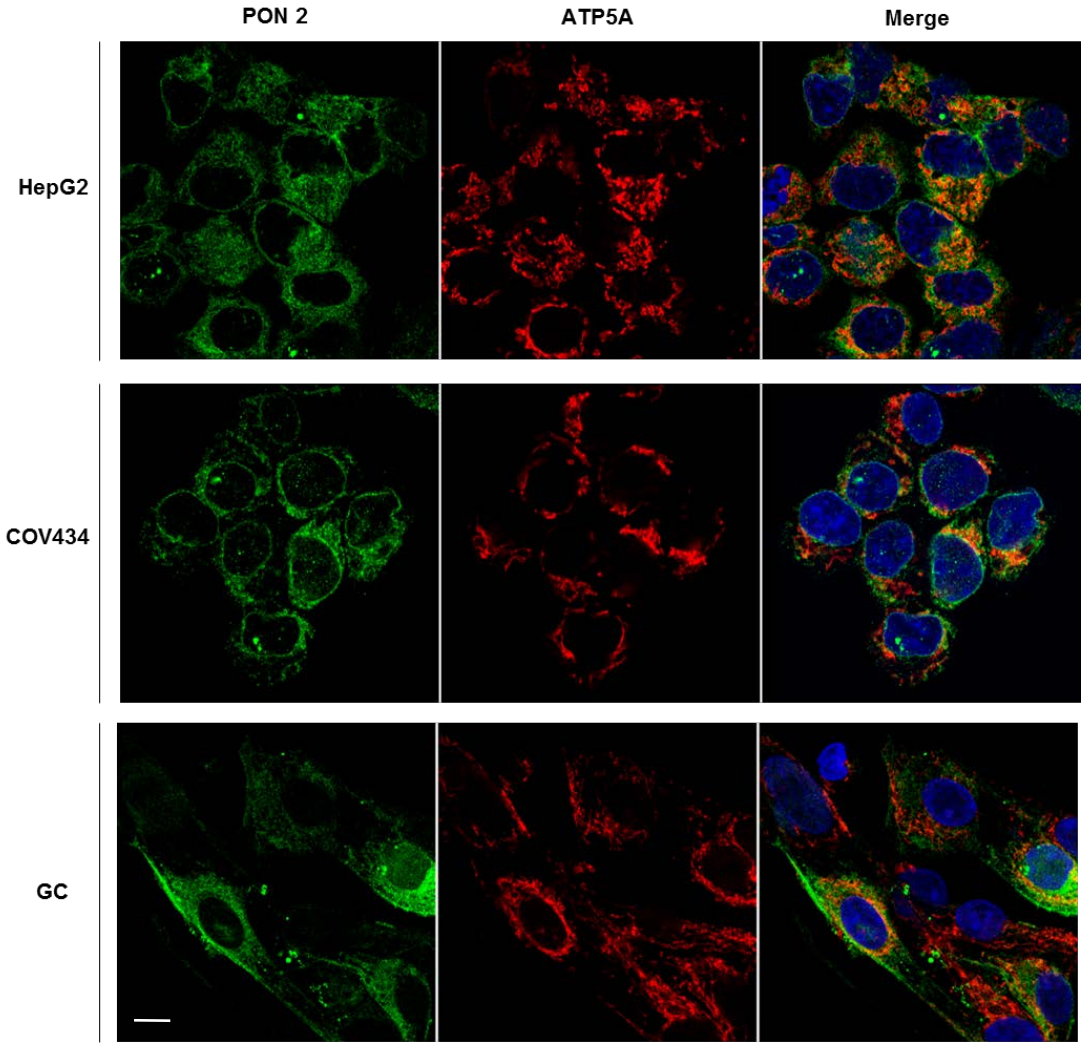

Figure 10. Intracellular distribution of PON2 in HepG2, COV434 and granulosa cells (GC). PON2 protein appears in green, while the mitochondria are red. The nuclei were counterstained with DAPI (blue). White scale bar corresponds to 5 µm.

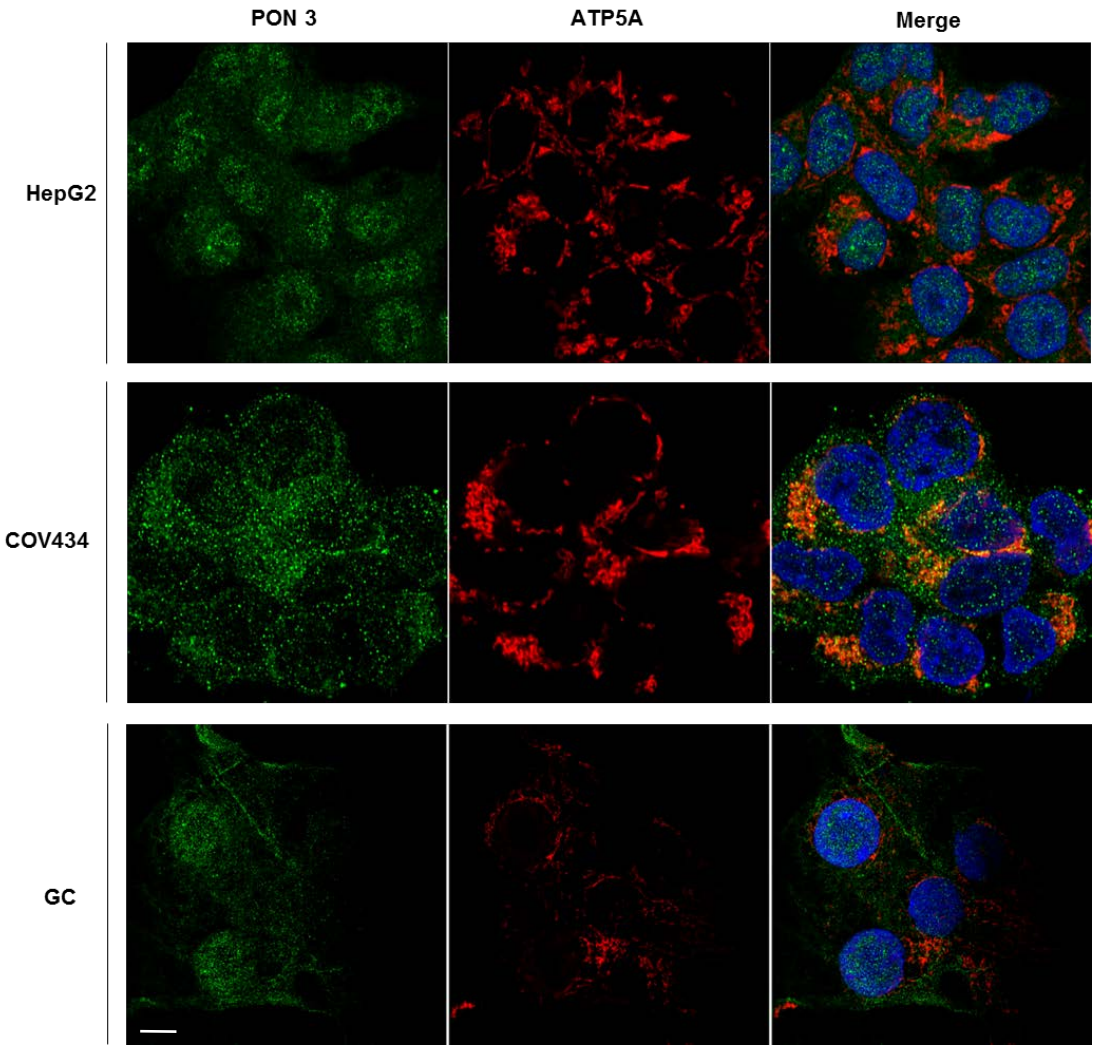

Figure 11. Intracellular distribution of PON3 in HepG2, COV434 and granulosa cells (GC). PON3 protein appears in green, while the mitochondria are red. The nuclei were counterstained with DAPI (blue). White scale bar corresponds to 5 µm.

3.5 Synchronization of the cell cycle and localization of PON1

Cell cycle blocking with thymidine synchronizes cells at G1/S boundary, as can be shown in the graphs at time 0 (Figs. 12 and 13). At this time, the distribution of PON1 was established in discrete granules in the nucleus in both HepG2 and COV434 cells. Along time, after cell cycle resuming, some more extranuclear staining could be appreciated. There was a heavy staining of the whole dividing-cell, excluding the condensed chromosomes (Fig. 12 and 13).

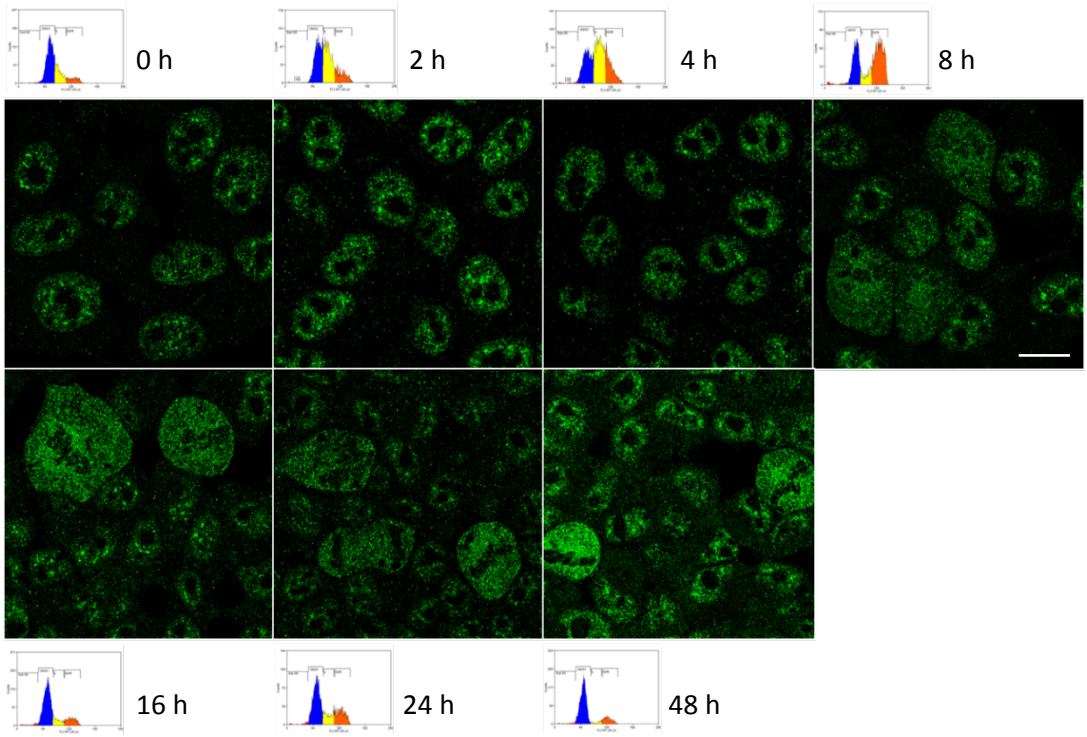

Figure 12. Intracellular distribution of PON1 in HepG2 after cell cycle synchronyzation. Confocal images were taken at the indicated times after cell cycle resuming. Small graphs inserted represents cell distribution along the different cell cycle steps. The white scale bar corresponds to 5 µm.

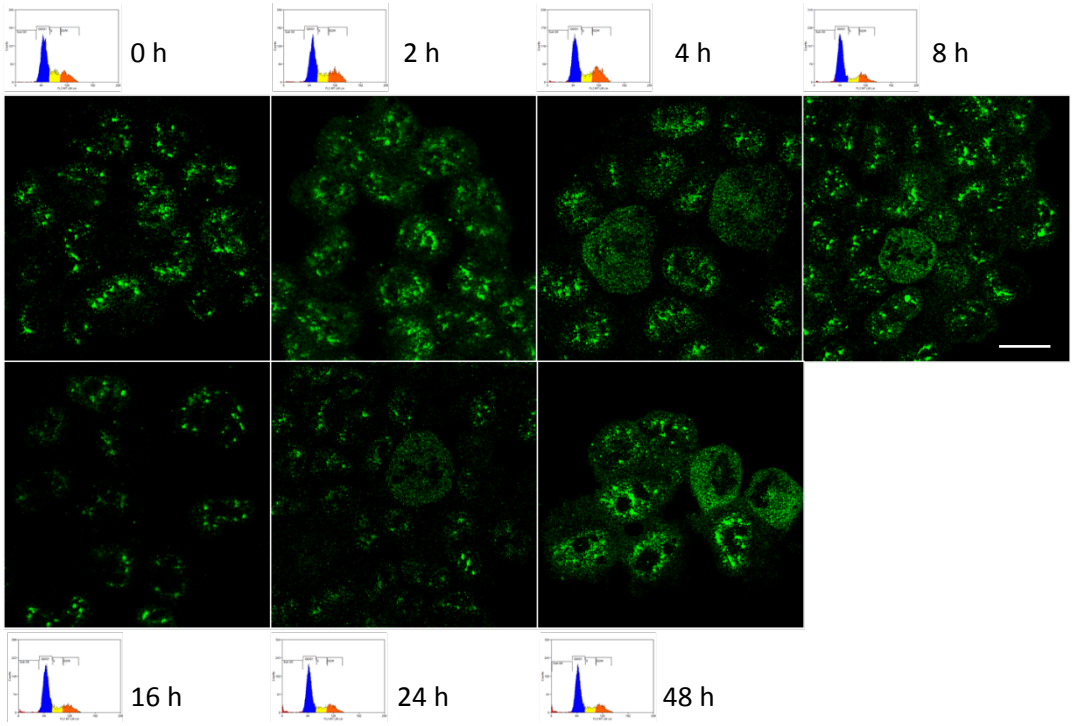

Figure 13. Intracellular distribution of PON1 in COV434 after cell cycle synchronyzation. Confocal images were taken at the indicated times after cell cycle resuming. Small graphs inserted represents cell distribution along the different cell cycle steps. The white scale bar corresponds to 5 µm.

4. Discussion

In this study, we have analyzed the possible expression of the *PON* family gene in human ovarian granulosa cells. Our results indicate that these cells express the genes at the transcriptional and protein levels. Granulosa cells contained the mRNAs of *PON1*, the two variants of *PON2*, and *PON3*, as was demonstrated by the RT-PCR methodological approach. In addition, we have found by western blot and immunocytochemistry that the proteins PON1 and PON2, and to a lesser extent PON3, were expressed in ovarian cells. To our knowledge, to date, no such studies have been documented in humans. Marsillach et al. reported in mouse the presence of PON1, PON2 and PON3 proteins in the ovary (Marsillach et al. 2008). They found a stronger staining of PON1 and PON3 than PON2 in the follicular fluid, and a rather weak staining of PON1, PON2, and PON3 in granulosa cells. It was not an study on isolated cells, but an histological analysis of ovarian tissue sections. In dairy cows, PON2 and PON3 mRNAs were expressed in granulosa cells, but PON1 mRNA could not be detected (Schneider et al. 2013). The authors proposed that the activity of PON1 measured in follicular fluid could be due to the PON1 protein associated with plasma HDL that are transferred from blood to follicular fluid. In our study, PON1 and PON3 were detected in the conditioned medium that was collected after incubating the granulosa cells for 48 h. These results reinforce the idea that they are secretion proteins that are synthesized by ovarian cells and transported to the medium independently of HDL from serum origin.

PON2 was the most abundant member of the family in the cells, while PON1 was present to a lesser extent, and PON3 could not be detected by western blot. The immortalized human granulosa COV434 cell line behaved like the primary cells. Thus, PON1 and PON2 were quantified by western blot inside the cells, but PON3 was not detected. This lack of detection was not the result of unsuccessful methodology, as the protein was firmly quantitated in HepG2. The silencing performed in HepG2 reinforces the specificity of the methodology used. Taking into account that granulosa cells follow the same expression pattern as HepG2 for these enzymes, the silencing results obtained in HepG2 could be extrapolated to granulosa cells. It has been reported that COV434 cell line retains many of the characteristics of primary granulosa cells. It contains FSH receptors, lacks the LH receptor, and exhibits physiological responses of natural granulosa cells, making this line the cell of choice for investigating many of the properties and functions of human granulosa cells (Zhang et al. 2000).

Regarding the function of these proteins in the follicle, antioxidants are necessary to maintain harmless levels of ROS in the oocytes, which is essential for the production of a healthy embryo (Guerin et al. 2001). Paraoxonases may have a role in this protection, as the enzyme activity of PON1 was positively related to the quality of the embryo and the number of blastomers in women undergoing *in vitro* fertilization (Browne et al. 2008). Similarly, the exogenous addition of recombinant PON1 during the *in vitro* maturation of bovine oocytes enhanced embryonic development (Rincon et al. 2016). In our study, the concentration of PON1 in granulose cells was higher than in hepatoma HepG2 or ovarian COV434 cell lines. The protein not only appeared in the conditioned medium, but was also localized by immunocytochemistry in the cytosol and, surprisingly, in the nucleus in the three cell types studied. The blockage of the cell cycle induced redistribution of the protein.

PON2 is the family member that is expressed intracellularly in most tissues. The protein levels of PON2 are higher in females than in males, according to studies in mice (Furlong et al. 2016). Estrogens seem to positively regulate PON2, since in ovariectomized mice the levels of PON2, both at transcriptional and protein levels, decrease in organs such as striated muscle, cerebral cortex and liver, reaching the same levels as in males. This means that males are more sensitive to oxidative stress (Furlong et al. 2016). In the present work, we have detected PON2 in granulosa cells, both at transcriptional and protein levels, in concentrations similar to those found in HepG2. PON3 is poorly investigated and the least characterized member. However, the significant PON3 activity found in follicular fluid (Browne et al. 2008; Rashidi et al. 2014) and the fact that its simvastatinase activity was higher in fertile than in infertile women (Meijide et al. 2017) suggest the involvement of the enzyme in fertility.

In addition of being associated with HDL (Christoffersen et al. 2006), VLDL and chylomicrons (Furhman et al. 2005), PON1 has been described in blood microparticles (Bastos-Amador et al. 2012), exosomes (Principe et al. 2013), and extracellular space (Sorenson et al. 1995). Similar to PON1, PON3 was localized on the HDL surface (Reddy et al. 2001) but it has also been localized in the inner mitochondrial membrane (Schweikert et al. 2012). Subcellular PON2 distributions includes the plasma membrane (Hagmann et al. 2014), the endoplasmic reticulum (Shakhparonov et al. 2018), the nuclear envelope (Horke et al. 2007), lysosomes (Levy et al. 2007) and the inner mitochondrial

membrane (Devarajan et al. 2011). In this study we have detected PON1 and PON3 not only in the conditioned medium, but also, localized by immunocytochemistry, in the cytosol and, surprisingly, in the nucleus in the three cell types studied. To date, this is the first evidence of a subcellular location of PON1 and PON3 in the nucleus. These results are consistent with a work in which the intracellular activity of PON1 was mainly detected in nuclear and microsomal fractions, although the enzyme activity was also detected in mitochondria and cytosol (Gonzalvo et al. 1998). However, it should be considered that the subcellular location data provided in the aforementioned study were obtained from the analysis of cell fractions isolated by differential centrifugation, that is, by indirect methods.

COMPARTMENTS (https://compartments.jensenlab.org) is a weekly updated web resource that integrates evidence on protein subcellular localization from manually curated literature, high-throughput screens, automatic text mining, and sequence-based prediction methods. According to this resource, applying text mining, all PON proteins achieved a score of 2 (0-5 scale) in the confidence for localization in the nucleus. We have tried cNLS Mapper software (http://nls-mapper.iab.keio.ac.jp) for nuclear localization signal (NLS) prediction within PON1 and PON3 proteins (Kosugi et al. 2009). Results show one predicted bipartite NLS within the PON1 sequence (score 4) and another one within the PON3 sequence (score 3.1). The software utility states that a score of 3, 4, or 5 corresponds to a protein that is predicted to be localized in both the nucleus and the cytoplasm.

In another order of arguments, analysis of the reported interactions for PON1 (7 total unique interactions) and PON3 (2 total unique interactions) in the Biological General Repository for Interaction Datasets (BioGRID 3.5, https://thebiogrid.org) showed nuclear transcription factor Y subunit gamma (NFYC) as the common partner for both proteins (Stark et al. 2006). This obtained result corresponds to an inferred interaction by affinity capture from cell extracts by either polyclonal antibody or epitope tag and the associated interaction partner is identified by mass spectrometric methods. NFYC is a protein localized in the nucleus (Romier et al. 2003).

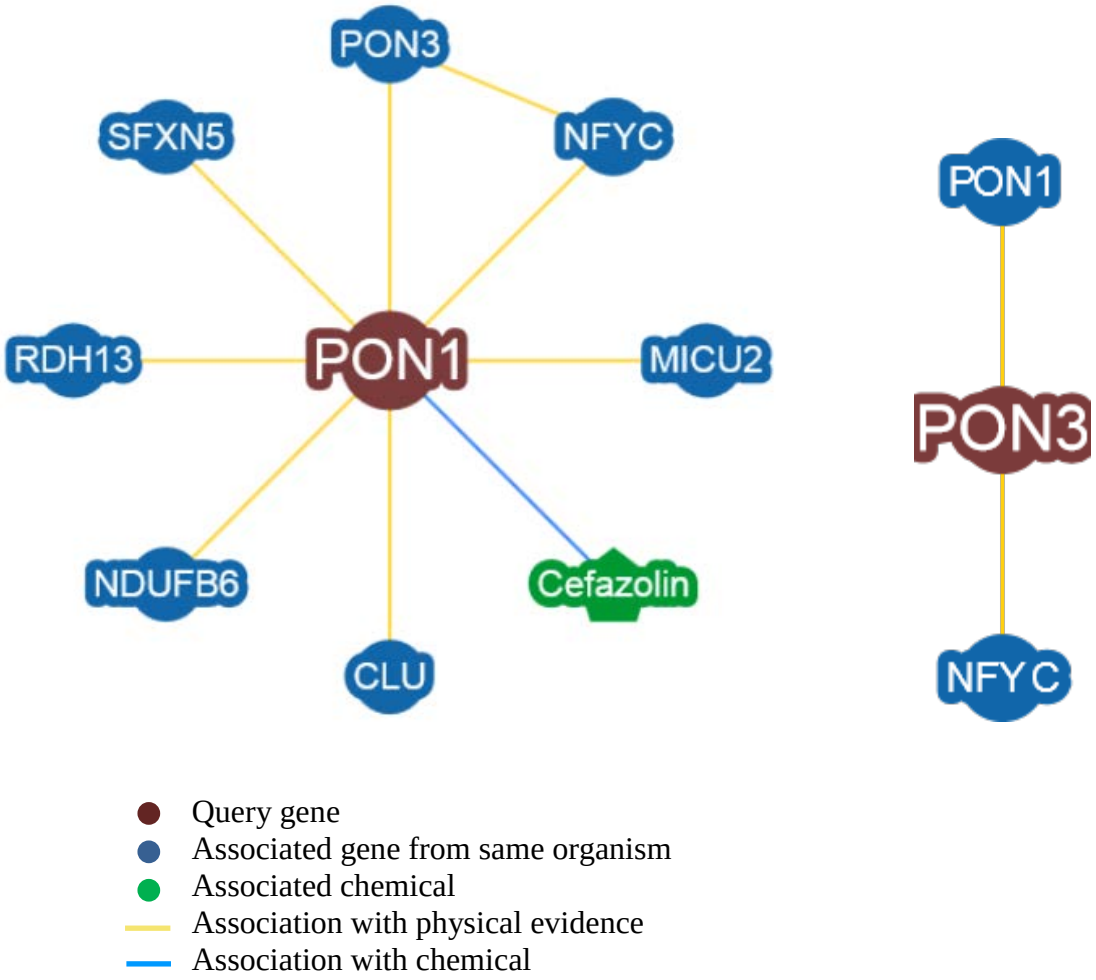

Figure 14. Network of interactions for PON1 and PON3 according to BioGRID 3.5.

Several studies focused on the regulation of *PON1* gen expression showed that nuclear transcription factors play a key role (Ponce-Ruiz et al. 2017). In addition, interaction studies carried out by Huttlin et al. (2017) demonstrated a strong interaction between nuclear factor Y (NF-Y) and PON1, and also a potential binding site at position -162 for nuclear factor 1 (NF-1) has been reported (Kim et al. 2009). All these data are consistent with the presence of PON1 in the nucleus.

The changes observed for PON1 in the nucleo-cytoplasmic distribution after blocking HepG2 and COV434 cells at G1/S boundary suggests a role for PON1 in the intracellular mellieu. At G1/S phase shynchronization, PON1 was located mainly in the nucleus in small clusters. After 48 h, when the cell cycle was recovered, the protein location seemed to be also the cytoplasm. This occurred both in HepG2 and COV434 cells. The role of the expression and/or intracellular distribution of PON1 along the cell cycle, and vice versa,

is an area of research still to be addressed. In this focus, PON1 overexpression supported metastatic progression of lung cancer by decreasing G1/S ratio and cell senescence (Aldonza et al. 2017). In contrast, PON1 knocking down caused cells to be arrested.

Confocal microspy results for PON3 in COV434 cells also suggest a mitochondrial localization in our study. In this sense, overexpressed PON3 diminished the intramitochondrial formation of superoxide anion by directly interacting with Q10 coenzyme (Schweikert et al. 2012), reinforcing the possible association with this organelle.

By immunocytochemistry, we have detected that PON2 has a perinuclear distribution and its location may be associated with the mitochondria. Considering that mitochondria is the major source of free radicals, the fact that PON2 is located in this organelle supports the idea that this enzyme protects cells against oxidative stress (Furlong et al. 2016). A study carried out by Horke et al. (2007) demonstrated the presence of PON2 in nuclear enveloped and ER in vascular cells, which is consistent with the perinuclear distribution we have found. The proteins in the endoplasmic reticulum are also targets of oxidative stress, so the action of PON2 in this organelle would participate in reducing ROS levels. It has been suggested that each of the two isoforms of PON2 may have a different location inside the cell (Horke et al. 2007), which would be compatible with their distribution in both the mitochondria and endoplasmic reticulum.

Conclusions

For the first time, we have demonstrated that human ovarian granulosa cells express *PON1*, *PON2* and *PON3* genes, both at the transcriptional and protein levels. PON3 was mainly secreted into the medium. Therefore, the PON enzyme activities and the proteins previously detected in follicular fluid seem to have their origin in part in the granulosa cells, thus contributing to maintaining the redox balance in the endocrine medium, necessary for the ovary to develop its functions properly and ultimately for the reproductive process.

5. References

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CONCLUSIONS

CONCLUSIONS

1. Controlled ovarian hyperstimulation cycle (COH) affects the redox balance of follicular fluid in fertile women, leading to a less protected fluid against free radicals than from natural cycle (NC). These changes are reflected in a lower total antioxidant activity (TAA), α-tocopherol levels and the antioxidant activities of the PON1 and PON3 paraoxonase family.

2. COH modifies the fatty acid profile of the total lipids from the follicular fluid, increasing the proportion of total saturated fatty acids, and the n-3 polyunsaturated docosahexaenoic acid.

3. A method has been developed for the detection and quantification of markers of different types of oxidative lesions of proteins from human follicular liquid by gas chromatography coupled to mass spectrometry. For the first time, the *in vivo* steady-state levels of glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA) as markers of direct metal-catalyzed oxidative lesion, N^{ε}-(carboxyethyl)lysine (CEL) as marker of oxidative lesion by glycoxidation, and N^{ε}-(carboxymethyl)lysine (CML) as mixed marker of oxidative lesion by glycoxidation and lipoperoxidation have been determined in this biological fluid. GSA was the most abundant marker in the follicular fluid.

4. The concentrations of CEL were higher in COH than in NC, suggesting that COH increases the susceptibility of follicular fluid to oxidative damage derived from protein glycoxidation processes.

5. Human granulosa cells express PON1, PON2 and PON3 both at the mRNA and protein levels. PON2 is intracellularly located, while PON1 and PON3, in addition to being distributed intracellularly, are actively secreted into the medium by granulose cells. These findings reinforce the role of these proteins in reproduction.