TESIS DOCTORAL

TERAPIAS AUTÓLOGAS EN DERMATOLOGÍA REGENERATIVA:

LA TECNOLOGÍA DEL PLASMA RICO EN FACTORES DE CRECIMIENTO

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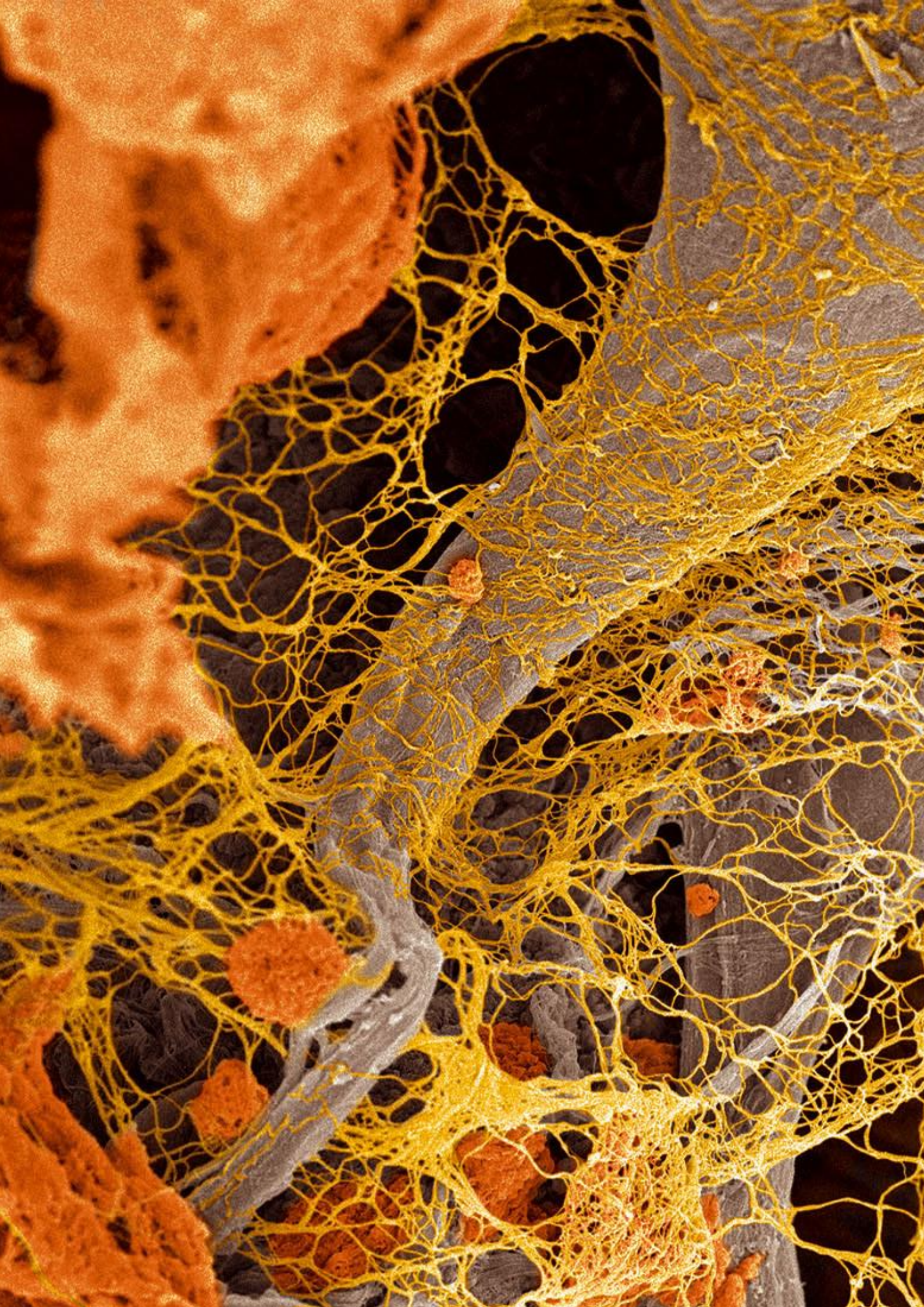

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A mi padre y a mi madre

“The good thing about science is that it is true whether or not you believe in it”

Neil deGrasse Tyson

ABREVIATURAS

6-4PP: pyrimidine photoproduct

8-OHdG: oxo-deoxyguanosine

ADAM: A disintegrin and metalloprotease

ADN: ácido desoxiribonucléico

ADP: adenosin difosfato

ADP: adenosin-difosfato

AEMPS: Agencia Española del Medicamentos y Productos Sanitarios

Akt: proteína kinasa B

AP-1: proteína activadora-1

ARP2/3: actin related protein 2/3

ASC: adipose derived stem cells

ATP: adenosin trifosfato

AURKB: aurora kinase B

BAD: Bcl2 associated death promoter

BAX: Bcl2 associated X protein

Bcl-2: B cell lymphoma-2

BPL: buenas practicas de laboratorio

CCL y CXCL: chemokine ligand

CCNB1: ciclina B1

CDC20: cell división cycle protein-20

CDK: kinasa dependiente de ciclina

CPD: cyclobutane pyrimidine dimers

CTAP-III: connective tissue activating peptide-III

CTGF: factor de crecimiento de tejido conectivo

ECM: matriz extracelular

EGF: factor de crecimiento epidérmico

ERK: extracelular signal-regulated kinase

FAK: focal adhesión kinasa

FBS: suero bovino fetal

FDA: Food and Drug Administration

FDH: fibroblastos dérmicos humanos

FGF: factor de crecimiento fibroblástico

Fn: Fibronectina

FvW: Factor von Willebrand

G*: complex modulus

G´: storage modulus

G´´: loss modulus

GM-CSF: granulocyte-macrophage colony-stimulating factor

HA: ácido hialurónico

HGF: factor de crecimiento hepatocítico

IFN-γ: Interferon gamma

IGF: factor de crecimiento parecido a insulina

IL: interleuquina

JNK: c-Jun N-terminal kinase

MAPK: mitogen activated protein kinase

MeSH: medical subjects headings

MLCK: myosin light chain kinase

MMP: metaloproteasa

$NADH^{-}$/NADPH: nicotinamida adenina dinucleótico

NF-κB: nuclear factor kappa B

PAR-1: protease activated receptor-1

PDGF: factor de crecimiento derivado de plaquetas

PF-4: factor plaquetario-4

PF-4: platelet factor-4

PI3K: phosphatidylinositol 3 kinase

PLC: phospholipase C

PLK1: polo like kinase 1

PRGF: Plasma rico en factores de crecimiento

PRGF-Gel: PG

PRGF-Serum: PS

PRP: Plasma rico en plaquetas

RANTES: regulated on activation normal T cell expressed and secreted

ROS: especies reactivas del oxígeno

RT-PCR: reacción en cadena de la polimerasa con transcriptasa inversa

Shh: sonic hedgehog

SMA: smooth muscle actin

SMC2/4: structural maintenance chromosome

SOD-1: superoxido dismutasa-1

tan δ: elasticidad

TEM: microscopía electrónica de transmisión

TGF-β: factor de crecimiento transformante beta

TIMP: inhibidor de metaloproteasa

TNF-alpha: tumor necrosis factor alpha

TSP-1: tromboespondina-1

TSP-1: trombospondina-1

UV: ultravioleta

V1: versican 1

VEGF: factor de crecimiento vascular endotelial

Vn: Vitronectina

Wnt: wingless integrated

β-TG: beta tromboglobulina

η: viscosity complex

PUBLICACIONES CIENTÍFICAS

La presente tesis doctoral se basa en las siguientes publicaciones originales, a las que se hace referencia mediante números romanos (I-VII). Estos trabajos se incluyen en el apartado de "Diseño experimental y Resultados".

I. Anitua E, Pino A, Orive G. **Plasma rich in growth factors promotes dermal fibroblast proliferation, migration and biosynthetic activity**. *Journal of Wound Care*. 2016; 25 (11):680-687.

II. Anitua E, Pino A, Orive G. **Plasma rich in growth factors inhibits ultraviolet B induced photoageing of the skin in human dermal fibroblast culture**. *Current Pharmaceutical Biotechnology*. 2016; 17 (12):1068-1078.

III. Anitua E, Pino A, Jaén P, Orive G. **Plasma rich in growth factors enhances wound healing and protects from photo-oxidative stress in HDF and 3D skin models**. *Current Pharmaceutical Biotechnology*. 2016; 17 (6):556-570

IV. Anitua E, Pino A, Troya M, Jaén P, Orive G. **A novel personalized 3D injectable protein scaffold for regenerative medicine**. *Journal Materials Science. Materials in Medicine*. 2017; 29 (1):7

V. Anitua E, Troya M, Pino A. **A novel protein based autologous topical serum for skin regeneration**. *Journal of Cosmetic Dermatology*. 2019. Ahead of print

VI. Anitua E, Pino A, Troya M. **Biological stability of Plasma rich in growth factors derived autologous topical serum after three months storage**. *Journal of Drugs in Dermatology*. 2018; 17 (10):1115-1121

VII. Anitua E, Pino A, Orive G. **Opening new horizons in regenerative dermatology using platelet based autologous therapies**. *International Journal of Dermatology*. 2017; 56 (3):247-251

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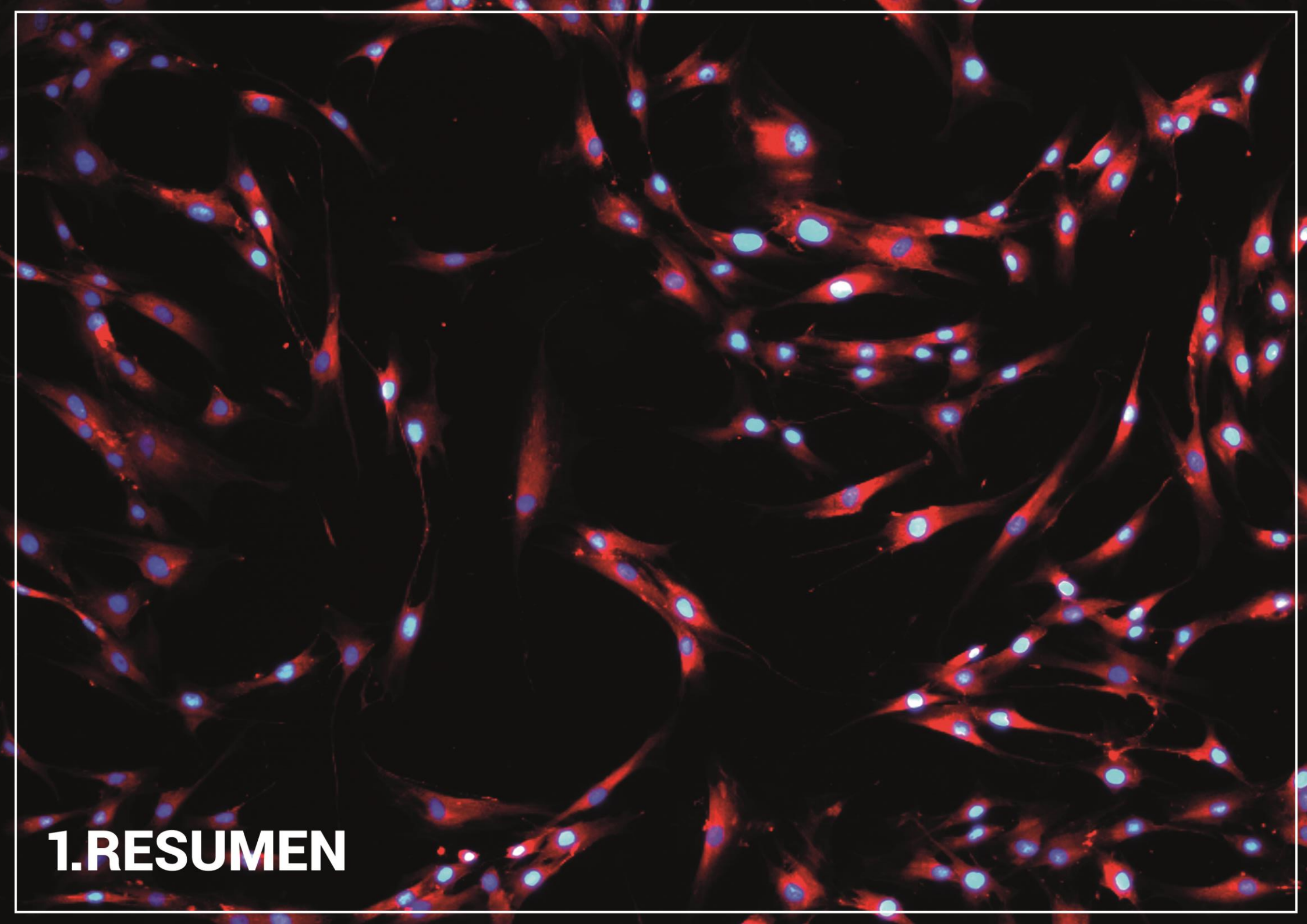


1.RESUMEN

Resumen

Resumen

La medicina regenerativa definida como “un área interdisciplinar de la medicina dirigida a la reparación, reposición o regeneración de células, tejidos u órganos dañados estructural o funcionalmente”, se postula como un campo emergente con la capacidad de aportar soluciones eficaces que mejoren la calidad de vida de los pacientes. Este hecho es especialmente relevante teniendo en cuenta el constante incremento de la expectativa de vida de la población. A lo largo de los últimos años han surgido un gran número de herramientas biotecnológicas tales como la ingeniería tisular, las células progenitoras (células madre), la terapia génica, la nanotecnología y las terapias biológicas. Este conjunto de terapias pretende regenerar tejidos y órganos y con ello mejorar el bienestar de los pacientes. Otra de las líneas de investigación más prometedoras, es la basada en el potencial regenerativo del plasma rico en plaquetas. Esta tecnología deriva de la extracción de un pequeño volumen de sangre del paciente que es centrifugada y procesada *in situ* para obtener un concentrado plaquetario capaz de dar lugar a diferentes formulaciones farmacéuticas enriquecidas en factores de crecimiento y proteínas bioactivas. El plasma rico en plaquetas es un tratamiento personalizado dirigido a estimular la regeneración tisular, ya que la acción de este conjunto de mediadores sobre la zona dañada regula directamente los mecanismos y fases que gobiernan la regeneración de tejidos. Por lo tanto, las terapias autólogas derivadas de concentrados plaquetarios representan un cambio de concepto hacia una estrategia terapéutica individualizada y centrada en orientar favorablemente las primeras y fundamentales fases del proceso regenerativo.

El potencial biológico de este tipo de tratamientos ha sido estudiado en múltiples áreas de la medicina tales como la cirugía oral y maxilofacial, la traumatología y medicina del deporte e incluso la oftalmología. Otra de las aplicaciones emergentes del plasma rico en plaquetas en los últimos años es su potencial regenerativo en el tejido cutáneo. Siguiendo la directriz actual de la biomedicina, la dermatología regenerativa presenta una tendencia hacia la medicina personalizada. Sin embargo, existe aún un largo camino por recorrer dado que muchos de los tratamientos existentes en la actualidad son paliativos y no contemplan la restitución celular completa del tejido dañado. Además, el coste de los tratamientos contra afecciones de alta prevalencia es muy elevado, por lo que se requieren alternativas de mayor relación coste-eficacia. En este sentido, la tecnología del plasma rico en plaquetas puede suponer un punto de partida que ofrezca un nuevo enfoque biológico dirigido a tratar diversas afecciones cutáneas desde una perspectiva autóloga. No obstante, también existe cierta controversia en relación a los concentrados plaquetarios en cuanto a la inconsistencia de los protocolos de obtención y a la ausencia de caracterización de alguno de los

preparados descritos en la literatura. Esta falta de consenso en términos de estandarización del tratamiento conduce a la persistencia de interrogantes sobre su potencial biológico, a la indefinición de los mecanismos de acción de esta herramienta terapéutica y en conjunto a cierta controversia sobre su uso y potencial terapéutico.

La tecnología del plasma rico en factores de crecimiento (PRGF) surge como un plasma rico en plaquetas que está respaldado por un sólido bagaje de evidencias científicas recogidas durante más de veinticinco. El análisis detallado de las propiedades intrínsecas de las plaquetas, junto con un protocolo optimizado para su concentración, activación y liberación proteica, ha permitido el desarrollo de una tecnología de gran versatilidad y potencial terapéutico. Asumiendo estos hechos, el uso del PRGF en el área de la dermatología regenerativa puede representar un hito a la hora de afrontar los diferentes retos anteriormente descritos.

En el presente trabajo de investigación se pretende aislar y caracterizar diferentes líneas de fibroblastos dérmicos a modo de sistema experimental sobre los que testar y poner a punto el PRGF como suplemento de cultivo celular. Además, se realizará una caracterización hematológica y proteica del PRGF obtenido de una población heterogénea de donantes y se analizarán sus mecanismos biológicos a nivel celular. A modo de condición patológica cutánea recurrente, también se estudiará el potencial terapéutico de los factores de crecimiento autólogos en situaciones de estrés oxidativo tanto *in vitro* como en modelos de piel 3D humana. Finalmente, se profundizará en la versatilidad de la tecnología PRGF con el fin de desarrollar nuevas formulaciones farmacéuticas derivadas de la sangre del paciente. Dichos biomateriales deberán complementar el arsenal dermatológico disponible en la actualidad, actuando por un lado a modo de matriz 3D biomimética que simule un entorno tisular sano y por otro lado permitiendo una aplicación tópica basada en factores de crecimiento autólogos.

En resumen, se pretende estudiar y evaluar el PRGF como una nueva herramienta terapéutica en dermatología regenerativa.

2. INTRODUCCIÓN

A lo largo de las últimas décadas la esperanza de vida media de la población ha aumentado de forma considerable hasta alcanzar aproximadamente los 70-80 años de edad [1]. De hecho, el informe anual del sistema nacional español de salud de 2018 indica que en la actualidad los hombres y mujeres alcanzarán los 82,8 y 85,6 años respectivamente, de los cuales aproximadamente 63 se vivirán de forma saludable. Esto convierte a España en uno de los países más longevos del mundo superado solo por Japón. Un aumento en la longevidad acarrea un mayor desgaste de los tejidos del organismo como los pertenecientes al sistema musculo-esquelético, sistema nervioso, sistema vascular, etc. Uno de los órganos más afectados y en el que más esfuerzos se están desarrollando para contrarrestar este envejecimiento progresivo, es la piel. La comunidad científica se afana en diseñar, desarrollar y optimizar terapias y tecnologías que favorezcan y promuevan la regeneración y restauración tisular [2].

A lo largo de los últimos años, el campo de la dermatología regenerativa ha emergido como una disciplina clave en el desarrollo de tecnologías innovadoras en materia biomédica. Este campo interdisciplinar, está dirigido tanto a la investigación *in vitro* como a aplicaciones clínicas centradas en la reparación, reposición y regeneración de fenotipos celulares cutáneos y sus tejidos asociados. La dermatología regenerativa traslacional supone en definitiva el punto de convergencia entre la investigación clínica y la experimentación básica avanzada, utilizadas ambas para mejorar el abordaje de patologías cutáneas y optimizar la planificación terapéutica que repercute directamente en la calidad de vida del paciente [3].

2.1. LA PIEL

2.1.1. Generalidades

La piel es un órgano de gran complejidad formado por multitud de tipos celulares que interaccionan entre sí de forma coordinada para mantener su integridad. Se trata de un tejido dinámico y altamente sofisticado que cubre una superficie aproximada de $2m^2$ y comprende alrededor del 17% del peso corporal [4]. Aunque su función principal es la de barrera biológica frente a agresiones del medio externo como los rayos ultravioleta (UV), lesiones, infecciones cutáneas o alteraciones homeostáticas, participa de forma activa en un amplio abanico de procesos fisiológicos. Estas funciones incluyen la termorregulación del organismo, la prevención frente a la pérdida de fluidos, electrolitos o proteínas, la percepción sensorial, la síntesis de moléculas bioactivas y la defensa inmunitaria.

Desde un punto de vista biomecánico, la piel se comporta como un material viscoelástico no lineal, heterogéneo y anisótropo que está sometido a pre-cargas

derivadas de la tensión ejercida por el andamiaje entre la matriz extracelular y el sistema musculo-esquelético [5]. Por lo tanto, es vital que los procesos de reparación cutánea aborden no solo la regeneración del defecto tisular, sino que promuevan la correcta organización tridimensional del tejido conectivo subyacente a fin de garantizar su viabilidad y funcionalidad.

Durante el desarrollo embrionario, el ectodermo da lugar a estructuras epiteliales de la piel como la epidermis, la unidad pilosebácea-apocrina y las glándulas sudoríparas-ecrinas. Los melanocitos, nervios y receptores sensoriales especializados se originan en el neuroectodermo. El resto de elementos como los fibroblastos, las células de Langerhans, los vasos sanguíneos y linfáticos y el tejido musculo/adiposo derivan del mesodermo [6].

2.1.2. Anatomía funcional

La piel posee funciones cruciales para la supervivencia del organismo. Tanto es así que la pérdida de un 20% del tejido cutáneo puede provocar la muerte del individuo. Además, está en constante remodelación y sus diferentes capas están integradas en tres densas redes que la conectan con el resto de los sistemas del organismo: el sistema linfático, los vasos sanguíneos y las terminaciones nerviosas. Estas redes aportan nutrientes, oxígeno y eliminación de sustancias toxicas de la piel y son vitales para el balance de fluidos, la respuesta inmune y la mecanotransducción. A su vez conectan la piel con una compleja red neuro-endocrina que es clave en diferentes procesos fisiológicos [7].

El tejido cutáneo interactúa con el medio ambiente mientras que protege al organismo de amenazas externas y mantiene su integridad estructural. Estas amenazas incluyen la radiación UV tras una exposición solar, la desecación, el sobrecalentamiento, traumas mecánicos, agentes infecciosos, toxinas e irritantes químicos entre otros. Al mismo tiempo, la piel debe ser lo suficientemente flexible como para permitir el crecimiento y el movimiento del organismo. Esta barrera, aparte de prevenir la pérdida de calor y la sobreacumulación de fluidos, constituye también el mayor reservorio de agua y energía del organismo gracias a la grasa subcutánea. Además, la piel opera como un órgano excretorio que elimina sustancias de desecho a través del sudor y el sebo [8].

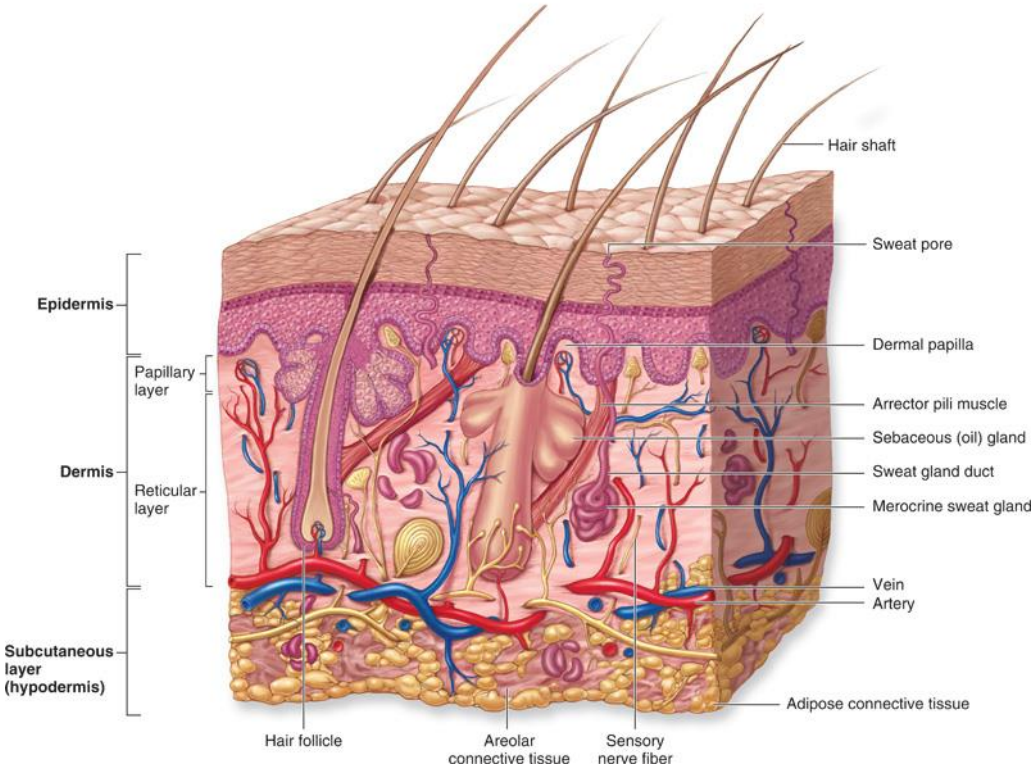


Figura 1: Esquema ilustrativo de las diferentes capas de la piel y las numerosas estructuras biológicas que la constituyen. Adaptado de [9].

La dermis presenta una matriz extracelular rica en colágeno que da soporte y nutre la epidermis. Está densamente inervada por amplias redes vasculares interconectadas a lo largo del plexo papilar y plexo reticular. La dermis también está complementada por un extenso sistema de vasos linfáticos que controla el balance homeostático. Este amplio sistema de perfusión cutáneo garantiza el acceso de eritrocitos, nutrientes y células del sistema inmunitario a la piel jugando a la vez un papel crítico junto con las glándulas sudoríparas en la termorregulación y balance de fluidos asociados a procesos de vasoconstricción/vasodilatación [10].

Las refinadas propiedades sensoriales de la piel derivan de una densa red de fibras nerviosas interconectadas que abundan en la epidermis y que se encuentran en constante remodelación. Este sistema neuronal, junto con orgánulos altamente especializados y receptores sensitivos, envía señales electroquímicas al córtex sensorial aportando información acerca de la presión, temperatura, dolor o vibraciones en el medio ambiente [11].

2.1.3. Estructura y fisiología

La piel posee una estructura heterogénea, altamente organizada y estratificada en tres principales capas llamadas epidermis (epitelio de cobertura), dermis (vascularizada, rica en anejos cutáneos y estructuras nerviosas) e hipodermis (tejido subcutáneo). Algunas propiedades específicas de la piel como el color o la distribución superficial difieren según la edad del individuo, el sexo o la región corporal analizada. Se pueden diferenciar dos tipos principales de piel: piel glabra (sin pelo) y piel pilosa (con pelo). La piel glabra posee una epidermis gruesa y una dermis delgada y se encuentra en los dedos, la palma de la mano, la planta de los pies y los labios. Este tipo de piel tiene una gran sensibilidad a causa de una alta densidad de inervación derivada de la presencia de mecano-receptores como corpúsculos de Meissner/Paccini/Ruffini y discos de Merkel. Por otro lado, la piel pilosa cubre la mayor parte de la superficie corporal y posee una epidermis delgada frente a una dermis más gruesa. Este tipo de piel tiene una mayor densidad de anejos cutáneos como las glándulas sudoríparas, glándulas sebáceas y folículos pilosos [12].

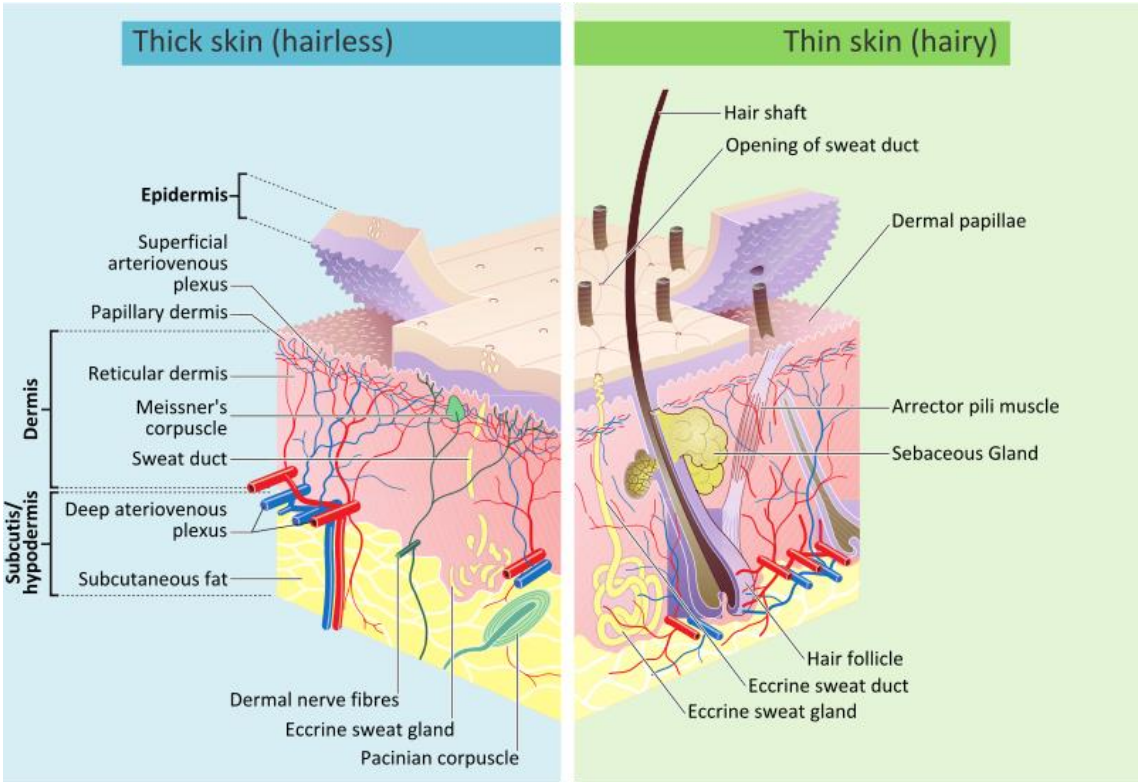


Figura 2: Principales tipos de piel en función de su localización anatómica. Piel glabra (hairless) y piel pilosa (hairly). Adaptado de [9].

2.1.3.1. Epidermis

La capa externa de la piel, la epidermis, contiene principalmente queratinocitos y poblaciones menores de melanocitos y células del sistema inmune como las células de Langerhans. Es el epitelio de cobertura cutáneo y tiene una fisiología pavimentosa, estratificada y queratinizada en constante regeneración. La epidermis constituye un sistema dinámico cuya estructura y metabolismo tiene dos principales funciones: proteger la piel de daños externos y mantener la hidratación de los tejidos internos [11]. De la profundidad a la superficie se distinguen los siguientes estratos:

- Capa basal: constituida por una hilera de células cilíndricas que se asienta sobre la membrana dermo-epidérmica, y cuya división o mitosis da origen a las células germinativas.
- Capa espinosa: compuesta por varias hileras de células poliédricas unidas mediante desmosomas, que a medida que ascienden se van aplanando. El espacio intercelular está formado por una sustancia de sostén estructural llamada glucocálix.
- Capa granulosa: formada por 2-3 hileras de células aplanadas, que contienen en su citoplasma gránulos basófilos de queratohialina.
- Capa córnea: constituida por capas apiladas de células muertas aplanadas, anucleadas, sin orgánulos citoplasmáticos, íntimamente unidas entre sí. Éstas se desplazan hasta desprenderse en la superficie, proceso que comúnmente se conoce como descamación de la piel. La capa cornea es la capa protectora por excelencia, ofrece a la vez rigidez y cierta flexibilidad para no fisurarse con los movimientos; es impermeable a los fluidos internos y externos y se opone a la penetración de moléculas exógenas.

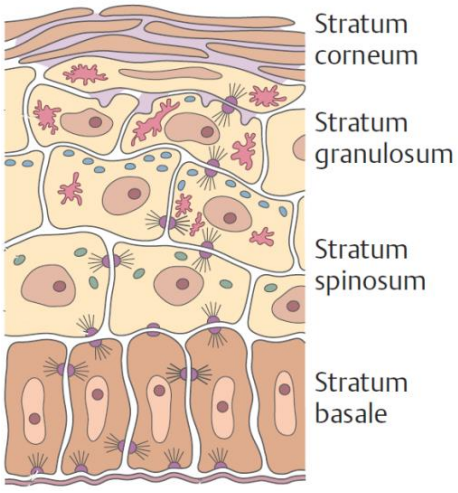


Figura 3: Capas de la epidermis. Adaptado de [13].

Estas capas las conforman distintos fenotipos de una misma célula, el queratinocito, que en su proceso madurativo ascendente evoluciona hacia una muerte celular programada. El queratinocito se va diferenciando y adquiere morfologías y funciones particulares hasta llegar a formar la capa córnea (el corneocito, biológicamente es una célula muerta con gran contenido de queratina). El tiempo de tránsito desde la célula basal al corneocito y su desprendimiento final es de aproximadamente 30 días. Los queratinocitos epidérmicos originan una capa de células en la membrana basal a partir de la cual migran hacia la superficie cutánea y mientras ascienden producen la proteína queratina junto con una amplia variedad de lípidos [14].

2.1.3.2. Membrana basal (unión dermo-epidérmica)

La membrana basal, también conocida como la unión dermo-epidérmica, es una zona de conexión altamente intrincada con una arquitectura de enlaces articulados entre epidermis y dermis. Estas uniones generadas por queratinocitos epidérmicos y fibroblastos dérmicos están constituidas por proteínas estructurales de la matriz extracelular (colágeno tipo IV y VII, laminina, glicosaminoglicanos) componentes de los hemidesmosomas (integrinas y plectinas) y diversas moléculas de adhesión. Esta complejidad radica en que la membrana basal sirve simultáneamente como un sistema eficiente de unión de la epidermis al sustrato mientras que permite el acceso de nutrientes, oxígeno, anticuerpos, complemento y células inmunes a través de la dermis. Por lo tanto, la membrana basal representa un sistema de anclaje dermo-epidérmico que aporta elasticidad y flexibilidad cutánea y que interviene en la comunicación molecular y el tráfico celular. De hecho, alteraciones puntuales en la continua remodelación de esta interfaz dinámica, pueden derivar en diferentes patologías cutáneas [15].

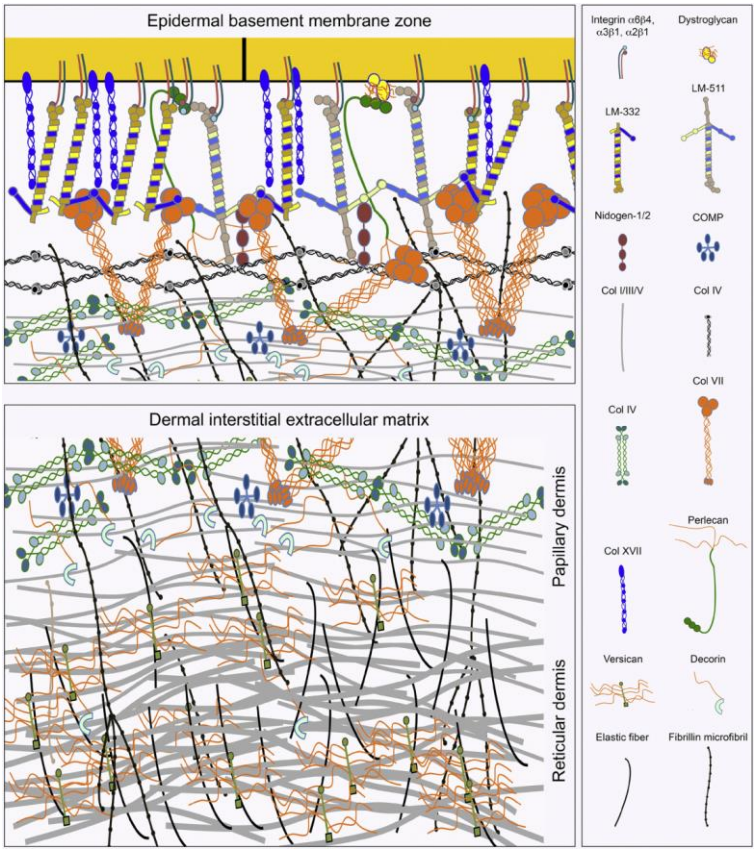


Figura 4: Esquema ilustrativo del entramado estructural de la membrana basal y dermis. Adaptado de [15].

2.1.3.3. Dermis

La dermis es una capa densa e irregular de tejido conectivo de 2-3mm de grosor por debajo de la epidermis. Se trata de un tejido diferenciado que está constituido principalmente por células, fibras estructurales y matriz extracelular. La matriz extracelular está compuesta principalmente por elastina y colágeno. Ambas macroproteinas aportan elasticidad, resistencia a la tracción, flexibilidad y soporte mecánico a la piel. Otras proteínas poliméricas como los glicosaminoglicanos y el ácido hialurónico mantienen el turgor cutáneo gracias a sus propiedades higroscópicas [16].

Desde un punto de vista morfológico y funcional, la dermis se divide en la dermis papilar, encargada del intercambio nutritivo y metabólico con la epidermis, y la dermis reticular o profunda, cuya función es aportar resistencia mecánica. La dermis papilar, subyacente a la epidermis, es la sección más fina del tejido conectivo y comprende aproximadamente el 10% del grosor dérmico. Contiene fibrillas de colágeno y elastina distribuidas de forma laxa y con una mayor cantidad de sustancia fundamental amorfa. La dermis reticular en cambio, es un tejido de alta densidad y de gruesas fibras asociadas a una superposición de poblaciones celulares y vasos sanguíneos. La principal fuente de rigidez mecánica la aporta la dermis reticular mientras que flexibilidad de la

dermis papilar funciona a modo de adhesivo deformable entre la membrana basal (o unión dermo-epidérmica) y la dermis [17].

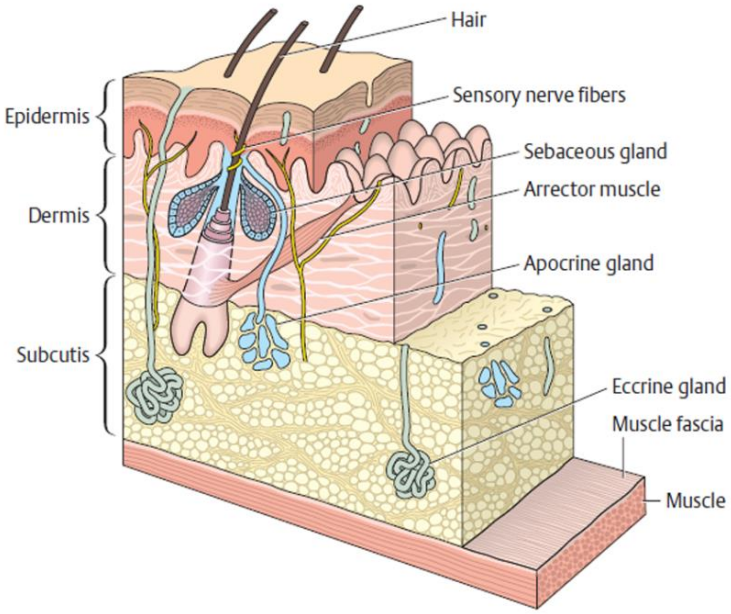


Figura 5: representación tridimensional de la piel. Adaptada de [13].

Los fenotipos celulares predominantes de la dermis son:

- Fibroblastos: representan la célula principal de la dermis y se encargan de sintetizar fibras estructurales y mantener la homeostasis del tejido conectivo circundante.
- Miofibroblastos: son las células responsables de la contracción y retracción del tejido de cicatrización.
- Mastocitos: células que forman parte del sistema defensivo que al ser estimulados liberan mediadores de la inflamación como la histamina, heparina o serotonina.
- Macrófagos: derivados de los monocitos sanguíneos, son responsables de la fagocitosis de agentes extraños y de la presentación de antígeno en las reacciones inmunes.
- Linfocitos: células encargadas de la inmunovigilancia.

Las principales estructuras de sostén presentes en la dermis son:

- Fibras de colágeno: es la macroproteína más importante de la dermis y aporta firmeza mecánica. La síntesis de colágeno se realiza a nivel intracelular en los fibroblastos y su organización en fibrillas y fibras tiene lugar en la matriz extracelular.
- Fibras elásticas: se componen de proteínas microfibrilares con una matriz de elastina y forman una red que aporta elasticidad.
- Matriz extracelular: la matriz extracelular llena los espacios entre los componentes fibrosos y celulares de la dermis y es rica en agua, electrolitos, proteínas plasmáticas y polisacáridos. Su alto contenido en glicosaminoglicanos como el ácido hialurónico le confiere un papel clave en el balance homeostático del agua ya que actúa como humectante y lubricante entre las redes estructurales durante los movimientos de la piel.

La dermis es un tejido altamente vascularizado y posee una gran densidad de terminaciones nerviosas y fibras sensoriales que forman una red especializada alrededor de receptores específicos. La mayoría de los anejos cutáneos como folículos pilosos, músculos piloerectores, glándulas sebáceas y glándulas sudoríparas se encuentran en esta capa de la piel.

2.1.3.4. Hipodermis

La hipodermis (o grasa subcutánea), es una capa de tejido laxo por debajo de la dermis. Deriva embriológicamente de la mesénquima y el fenotipo celular predominante es el adipocito. Estas células están altamente especializadas y sirven a modo de reserva de grasa. También regulan la ingesta de nutrientes, el metabolismo energético y la sensibilidad a la insulina a través de la secreción de leptina y adipoquinas. El tejido subcutáneo aporta insulación térmica y sirve como reservorio de energía. Tiene también una función estructural al absorber impactos mecánicos que protegen los órganos y estructuras internas. Es un tejido altamente vascularizado y capaz de metabolizar o sintetizar hormonas esteroideas y neuropéptidos. Además, permite el desplazamiento y movilidad de la piel sobre los planos profundos [7].

2.1.4. Localización y función de las células madre cutáneas

Los procesos de re-epitelización y reparación de la piel requieren poblaciones celulares que migren y regeneren los diferentes defectos tisulares. Es sabido que la epidermis se renueva aproximadamente cada cuatro semanas, gracias a lo cual es posible mantener la homeostasis y la correcta función del tejido cutáneo. Estos procesos derivan principalmente de la mitogénesis y diferenciación de células madre epidérmicas, las cuales contribuyen al reabastecimiento de queratinocitos superficiales en procesos de reparación. Las células madre se caracterizan por su capacidad de auto-renovación prolongada y su potencial para diferenciarse en células maduras funcionales. Es conocido que las células madre pueden dividirse mayoritariamente entre pluripotentes (capaces de dar lugar a cualquier tipo celular del organismo, incluidas las germinales) o multipotentes (con una capacidad de diferenciación menor que las pluripotentes) [18].

La piel posee diferentes nichos de células madre multipotentes localizadas principalmente en la región del promontorio capilar y las áreas interfoliculares de la superficie epidérmica. La región del promontorio capilar constituye un nicho de células madre capaces de dar lugar a todos los linajes celulares del folículo piloso. El folículo piloso se forma durante la embriogénesis como un apéndice de la epidermis. La condensación de las células de la papila dérmica (células mesenquimales especializadas), estimula la formación de una yema o promontorio por parte de células progenitoras de la membrana basal que alternativamente proliferan y migran hacia el bulbo piloso. En función del ciclo en el que se encuentra el folículo piloso, este nicho genera células germinales de la matriz bulbar que se diferencian para dar lugar a las diferentes estructuras del vello como la vaina radicular. Cuando el crecimiento capilar degenera (fase telógena), las células madre del promontorio entran en estado quiescente y detienen su actividad mitogénica hasta que vuelve a activarse el ciclo capilar (fase anágena) [19].

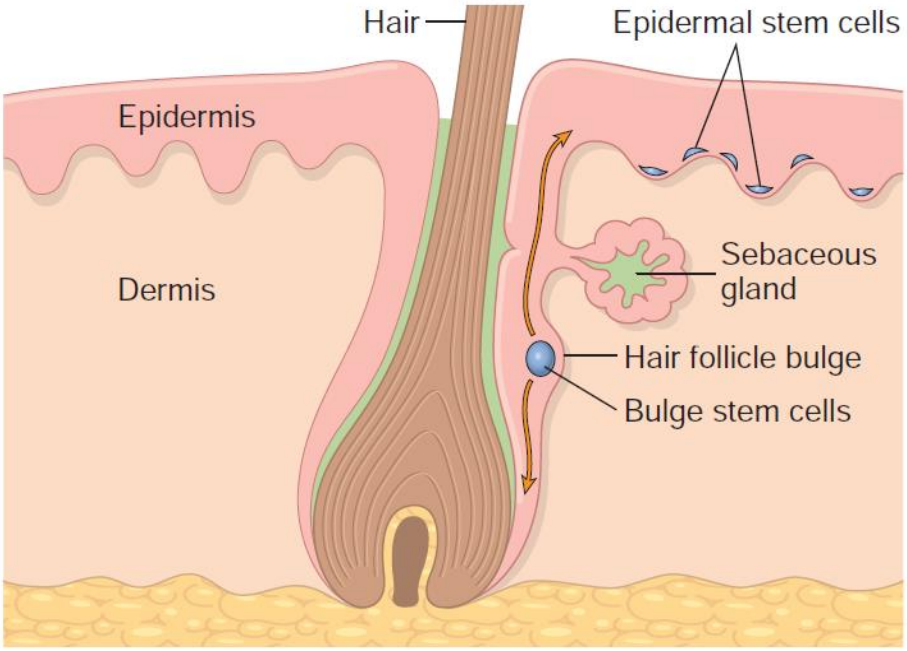


Figura 6: Localización de células madre cutáneas. Adaptada de [20].

Las células madre interfoliculares sin embargo, están distribuidas de forma individual a lo largo de la epidermis regulando la amplificación y el transito celular que dará lugar a la diferenciación de las capas de la piel. Estas células madre residentes, se adhieren a la membrana basal en un microambiente idóneo que permite controlar el destino celular induciendo su quiescencia, proliferación y auto-renovación o diferenciación/migración a capas superiores de la epidermis. Por lo tanto, las células madre interfoliculares mantienen la homeostasis cutánea reabasteciendo continuamente las células supra-basales terminales, mientras que las células muertas del estrato corneo se desprenden de la superficie de la piel [21].

2.2. MECANISMOS DE REPARACIÓN Y REGENERACIÓN CUTÁNEA

2.2.1. Deterioro de la piel

Para que el tejido cutáneo pueda ejercer sus múltiples funciones con eficacia, la piel necesita unas condiciones fisiológicas y estructurales óptimas entre las que se encuentra un correcto balance de temperatura, pH, hidratación, elasticidad, sebo y rugosidad. La mayoría de estos parámetros dependen del funcionamiento y coordinación de células residentes como los fibroblastos dérmicos, queratinocitos epidérmicos y adipocitos [22]. Los procesos de regeneración cutánea y cierre de heridas engloban una compleja cascada de eventos celulares entre los que se encuentran la proliferación, migración y diferenciación celular, así como la remodelación de la matriz

extracelular [23]. Sin embargo, el paso de los años junto con un amplio abanico de afecciones cutáneas (heridas crónicas, úlceras, quemaduras, enfermedades inflamatorias, trastornos autoinmunes) provocan el deterioro paulatino de la piel. Estos factores finalmente se traducen en la incapacidad del tejido para progresar de forma óptima a través de las diferentes fases de la regeneración cutánea como la hemostasia, inflamación transitoria, formación de tejido de granulación, epitelización y neovascularización [24].

2.2.1.1. Cambios estructurales

Los principales signos estructurales del deterioro cutáneo están asociados a cambios en el grosor y composición de las diferentes capas que forman la piel. Hasta la edad de 20 años, el grosor de la epidermis y dermis aumenta debido a la síntesis ininterrumpida de fibras de colágeno y elásticas y a la remodelación/deposición de matriz extracelular. Sin embargo, el paso del tiempo y la aparición de diferentes afecciones dermatológicas afecta decisivamente a la renovación de proteínas estructurales, e induce la disminución de la celularidad y vascularización del tejido [25]. Además, el ratio de recambio de queratinocitos decrece, provocando una morfología celular alterada asociada a una disminución en la actividad enzimática de los melanocitos. Las células de Langerhans presentan menos terminaciones dendríticas lo que supone una pérdida en su capacidad de captación de antígenos. A nivel de estrato córneo, la producción de sebo decrece y la pérdida de hidratación alcanza niveles que inhabilitan la descamación superficial efectiva traduciéndose en una pérdida de agua transepidérmica [26]. La función barrera de la epidermis también se ve alterada por la disminución de lípidos como la ceramida, lo que la hace más susceptible a rupturas post-traumáticas. La membrana basal, sufre el aplastamiento de la unión dermo-epidérmica que deriva en un menor número de las crestas papilares. Este reducción de interdigitaciones entre la epidermis y la dermis la hace menos resistente a fuerzas de cizalla y además el intercambio de nutrientes y oxígeno entre las dos capas se ve alterado de forma negativa [27].

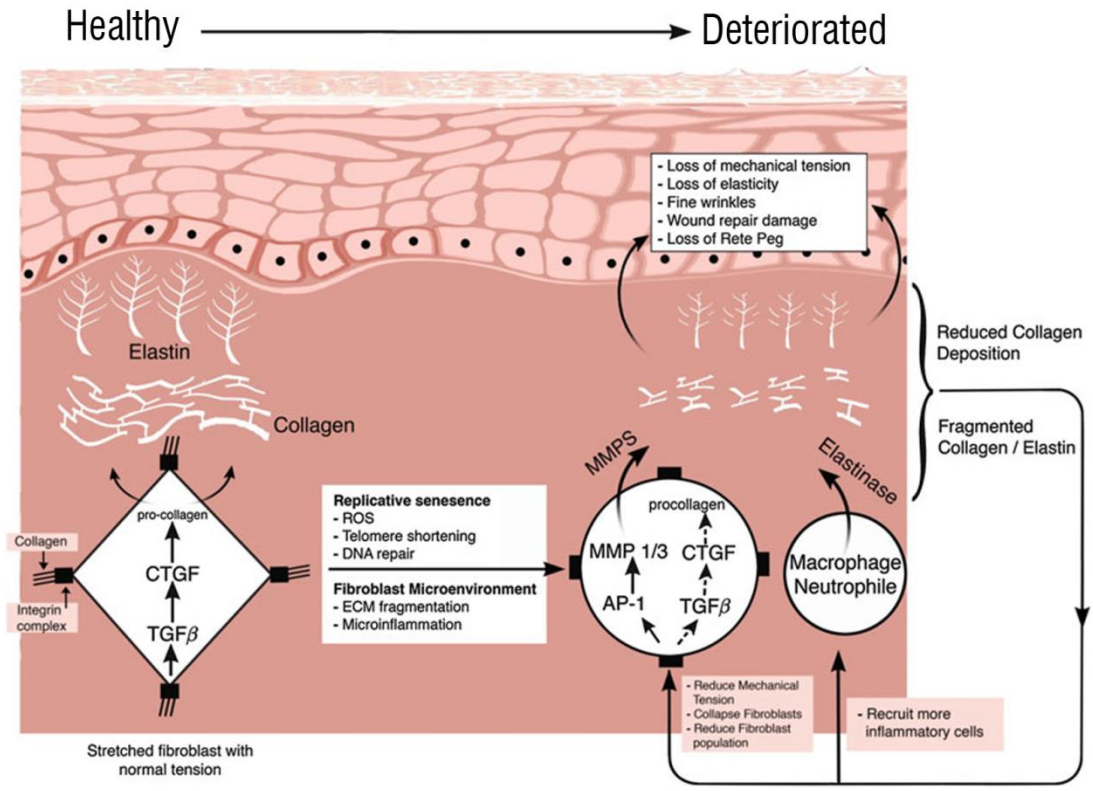


Figura 7: Alteraciones estructurales de la piel deteriorada. Adaptada de [28].

La cantidad de colágeno, elastina y ácido hialurónico disminuye, por lo que la integridad estructural de la piel decae. Esto se debe tanto a la degradación enzimática de macroproteínas como a una reducción en la síntesis de fibras tridimensionales por parte de los fibroblastos dérmicos. Además, las fibras de colágeno se vuelven gruesas, densas y peor organizadas, lo que repercute en las propiedades mecánicas de la piel. El recambio de fibras elásticas también se ve afectado, lo que supone no solo una pérdida de rigidez, sino también alteraciones en receptores sensoriales residentes como los corpúsculos de Paccini y Meissner. De forma paralela, la cantidad y distribución de la grasa subcutánea de la hipodermis también se ve alterada, dando lugar a la pérdida de protección de estructuras internas y consecuentemente la termorregulación del organismo sufre cambios reseñables [29].

2.2.1.2. Cambios fisiológicos

Los cambios fisiológicos de la piel deteriorada engloban alteraciones bioquímicas, modificaciones en la permeabilidad, vascularización, respuesta a lesiones, respuesta inmune e inflamatoria, capacidad auto-reparadora y alteraciones epigenéticas.

- Alteraciones bioquímicas: a medida que la piel se deteriora se reduce la síntesis de vitamina D y el pH cutáneo pasa de ser ligeramente ácido a básico. Estos procesos aumentan la vulnerabilidad frente a la colonización bacteriana y afectan a la barrera húmeda de la piel ya que la capa ácida formada por aminoácidos, sales y otras sustancias se ve alterada [25].
- Permeabilidad: la permeabilidad de la piel depende tanto de la hidratación del estrato córneo como del nivel y composición de los lípidos intracelulares. Estos factores junto con una adecuada microcirculación que permita la eliminación de deshechos regulan los procesos de absorción percutánea. La sequedad cutánea derivada del deterioro paulatino de la piel, provoca una reducción del contenido lipídico tisular interfiriendo en los procesos de absorción de sustancias hidrofóbicas [30].
- Vascularización y termorregulación: en la piel deteriorada se produce la regresión de los capilares, así como una desorganización de pequeños vasos, afectando significativamente a la circulación sanguínea. A su vez, la pérdida de crestas epiteliales genera una disminución en la densidad de la red vascular que aporta oxígeno, intercambio de nutrientes y sustancias de desecho. La degeneración del plexo vascular, que también está asociada a una reducción de la sudoración ecrina, provoca en última instancia una disfunción de la termorregulación del organismo que se traduce en predisposición a hipotermias y a la incapacidad de respuesta vasoconstrictora o vasodilatadora por parte de las arteriolas dérmicas [31].
- Respuesta inmune e inflamatoria: ambos mecanismos de defensa cutánea se ven afectados con el deterioro progresivo de la piel. La respuesta inflamatoria es más lenta y menos intensa, por lo que algunos signos de daño tisular permanecen camuflados dificultando el diagnóstico eficaz de ciertos problemas dermatológicos. Por otro lado, la reducción del número de células de Langerhans en la epidermis, junto con la caída de niveles circulantes de linfocitos, células-T y células-B reducen la respuesta inmune de la piel y alteran las reacciones de hipersensibilidad retardada. Este fenómeno también está asociado al aumento de moléculas autoinmunes y a la disminución de anticuerpos funcionales en el torrente sanguíneo [27,32].

- **Alteraciones epigenéticas**: estas alteraciones consisten en modificaciones que afectan al empaquetamiento del ácido desoxirribonucléico (ADN) y a la traducción de proteínas ya sea por modificaciones covalentes de las histonas nucleares o la metilación de residuos de cisteína. Estos factores causan cambios fenotípicos característicos que intervienen negativamente en el funcionamiento del tejido llegando a provocar, en algunos casos, procesos cancerosos [33].
- **Regeneración y respuesta a lesiones**: a diferencia de la piel sana donde se produce un recambio córneo total cada dos semanas, la piel deteriorada tarda aproximadamente el doble. Este hecho refleja la dificultad del tejido cutáneo deteriorado a la hora de responder a lesiones y de auto-repararse. Los procesos de proliferación, migración y metabolismo celular son menos eficaces y la disminución de la síntesis de fibras estructurales junto con la reducida capacidad contráctil de los fibroblastos dérmicos, generan un tejido de granulación de baja integridad biomecánica. Por lo tanto, las úlceras no solo corren el riesgo de cronificarse sino que se transforman en heridas recurrentes debido a la mala calidad del tejido cicatrizado [7].

Función	Alteración fisiológica
Función barrera	Incremento en el tiempo de renovación del estrato córneo
Percepción sensorial	Pérdida de sensibilidad y aumento del picor
Termo-regulación	Reducción de la producción de sudor
Respuesta a lesiones	Menor respuesta inflamatoria, reducción de la capacidad de cierre de heridas, menor re-epitelización, aumento de la vulnerabilidad a traumas mecánicos
Permeabilidad	Reducción de la absorción percutánea, reducción de la producción de sebo, menor vascularización, menor limpieza química
Función inmune	Menor número de linfocitos circulantes, mayor riesgo de reacciones de hipersensibilidad
Otros	Menor producción de vitamina D, menor elasticidad

Tabla 1: Alteraciones fisiológicas de la piel deteriorada. Adaptada de [28].

En resumidas cuentas, la dermatología regenerativa tiene como objetivo contrarrestar el amplio abanico de alteraciones estructurales y fisiológicas que impiden la correcta

regeneración cutánea. Para tal fin, actúa sobre las diferentes fases que se suceden e intercalan desde la aparición de una lesión hasta la regeneración y reposición de un tejido sano y funcional.

2.2.2. Mecanismos de reparación y regeneración cutánea

Existen múltiples causas de lesión, daño o trauma que dan lugar a una pérdida de la integridad tanto anatómica como funcional de la piel. En respuesta a este daño o agresión se ponen en marcha una serie de mecanismos dirigidos a restaurar el área dañada, entre los que se distingue la reparación tisular frente a la regeneración. La reparación tisular consiste en la restauración de un tejido sin que éste conserve su arquitectura, morfología ni función original. Debido a que el tejido lesionado no recupera su estado inicial, sus propiedades tanto físicas como biomecánicas son claramente inferiores a las del tejido sano. Generalmente este proceso tiene como resultado una cicatrización. En cambio, la regeneración tisular promueve la sustitución de las células dañadas por otras nuevas que son estructural y funcionalmente idénticas a las originales, por lo que las propiedades del tejido neoformado son idénticas a las del tejido original [34].

Asumiendo estos preceptos, el principal objetivo terapéutico no debe ser reparar sino regenerar; lo que implica reconstruir la forma y restaurar la función. Una de las razones por las que un tejido cicatriza en lugar de regenerarse puede residir en que aunque exista la población celular necesaria para la regeneración, se carece de las señales estimuladoras que intervienen en la proliferación y diferenciación de esas células. Por tanto, puede que un tejido sea capaz de regenerarse autónomamente si se activan las señales estimuladoras necesarias o se inhiben las que impiden que se pongan en marcha los mecanismos de regeneración. La cicatrización de heridas y la reparación tisular son mecanismos fisiológicos que consisten en una compleja cascada secuencial de procesos biológicos solapados que se producen de manera dinámica y regulada. Se corresponden con la aparición de diferentes tipos celulares en la herida o lesión y son controlados por numerosas citoquinas y factores de crecimiento. Estas moléculas son las que proporcionan las señales locales necesarias responsables de regular los mecanismos y vías que coordinan la cicatrización de heridas y la regeneración tisular [35].

Cuando el tejido cutáneo sufre una agresión se inicia una hemorragia, seguido de la formación de un coágulo de fibrina, una respuesta inflamatoria, una fase de proliferación celular, angiogénesis, epitelización y finalmente la síntesis de nueva matriz extracelular. Así, se puede hablar de cuatro fases principales:

- Hemostasia: se produce inmediatamente tras el daño y generalmente se completa en horas, dando lugar a la formación de un coágulo.
- Fase inflamatoria: comienza tras la hemostasia y normalmente finaliza en las primeras 24 a 72 horas tras la lesión, aunque se puede prolongar de 5 a 7 días.
- Fase proliferativa y reparativa: se produce de 1 a 3 semanas tras el daño.
- Fase de remodelación: tiene lugar aproximadamente 3 semanas después y se puede prolongar en el tiempo desde meses hasta varios años para lograr una integridad estructural y funcional óptimas.

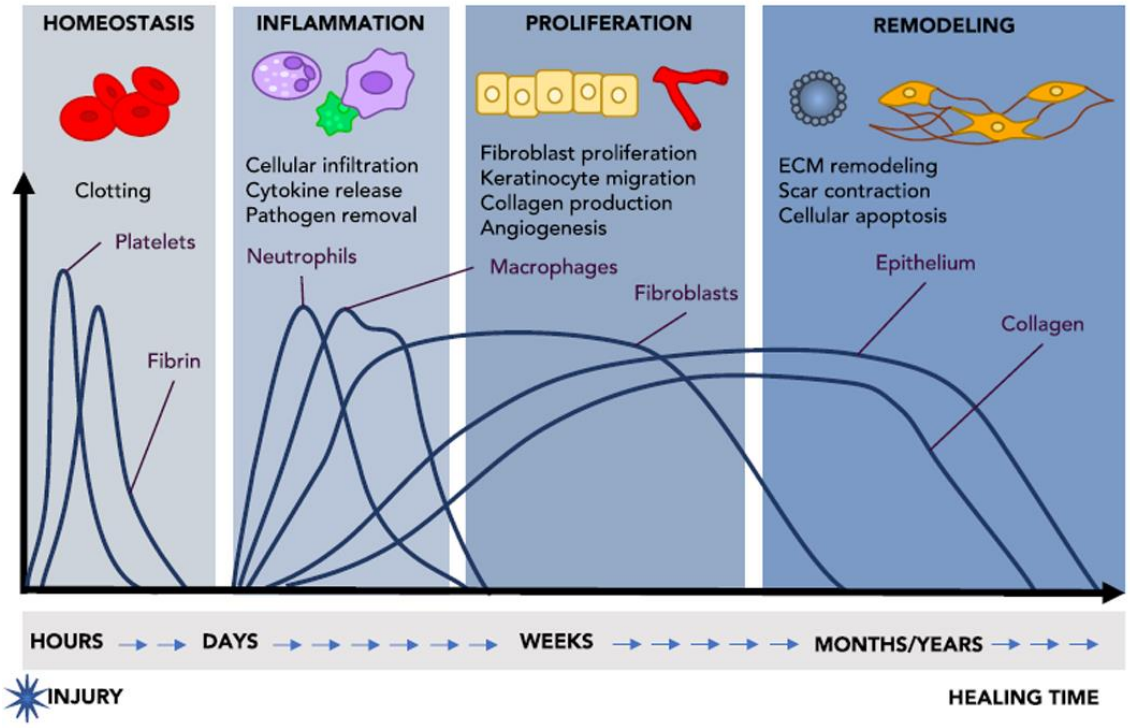


Figura 8: Diagrama cronológico del proceso de reparación y regeneración cutáneo. Adaptado de [36].

2.2.2.1. Respuesta vascular y hemostasia

La hemostasia se define como el conjunto de mecanismos mediante los cuales se controla la pérdida de sangre por parte del organismo. Es el resultado de un equilibrio entre el mantenimiento de la sangre en estado fluido y una reacción inmediata ante un daño vascular que hace que se detenga la pérdida sanguínea y se repare la pared vascular.

Inmediatamente tras la lesión se produce un sangrado como consecuencia de la ruptura de vasos sanguíneos y por tanto el primer paso tras el daño es controlar la hemorragia. Las plaquetas son las primeras células en llegar y juegan un papel primordial en el desarrollo de una hemostasia óptima. Como respuesta a este daño se

activan y se adhieren a moléculas expuestas a los vasos sanguíneos, principalmente colágeno, membranas basales de los capilares y microfibrillas sub-endoteliales. La adhesión plaquetaria requiere de la presencia de una proteína plasmática, el Factor de von Willebrand (FvW), que actúa como puente entre las plaquetas y la pared del vaso [23]. Tras la activación, las plaquetas cambian de una morfología redondeada a una forma alargada con pseudópodos y sus gránulos alfa se fusionan con la membrana plasmática plaquetaria liberando mediadores como serotonina, adenosin-difosfato (ADP) y proteínas adhesivas como el fibrinógeno y la fibronectina. Estos mediadores junto con la trombina generada localmente inducen una mayor agregación y secreción plaquetaria formándose el denominado tapón plaquetario. Si el defecto vascular es pequeño, este tapón plaquetario resulta suficiente para detener la pérdida de sangre aunque si por el contrario, la lesión es extensa, se necesita un coágulo para frenar la hemorragia [35].

Tanto el tejido dañado como los monocitos activados y las células endoteliales expresan factores tisulares superficiales que participan en el proceso de coagulación. Además, las plaquetas activadas secretan Factor V desde los gránulos alfa, que se une al Factor X para producir el activador de protrombina, el cual, en presencia de calcio (Ca^{2+}), provoca la transformación de protrombina en trombina. Ésta cataliza la producción de monómeros de fibrina a partir del fibrinógeno que en presencia del Factor XIII (factor estabilizante de la fibrina) y de Ca^{2+} como cofactor, forma hebras de fibrina. La trombina también se une a los receptores en la superficie de las plaquetas y activa al Factor VIII sérico contenido en los gránulos alfa, el cual forma un complejo con el Factor IX en la superficie de las plaquetas. Como resultado de la agrupación de las hebras de fibrina se forma un coágulo sanguíneo que consiste en una malla de fibrina, que contiene el agregado plaquetario, así como eritrocitos y leucocitos atrapados en su interior. La retracción del coágulo se produce por la contracción de las fibras de actina y miosina plaquetarias, proceso que ocurre entre 20 minutos y 1 hora tras el cierre del vaso sanguíneo afectado. Durante la retracción del coágulo se liberan las moléculas contenidas en los gránulos alfa de las plaquetas, entre ellas, tromboxano y serotonina, que provocan la vasoconstricción y favorecen la hemostasia [37].

La polimerización de la fibrina se produce por medio de la trombina, formándose un coágulo que previene posteriores hemorragias y que actúa como una estructura para las células que acuden al lugar de la lesión, entre ellas neutrófilos, monocitos, fibroblastos y células endoteliales, además de servir como reservorio de factores de crecimiento y citoquinas. Finalmente, y como parte del proceso de reparación tisular, la fibrina es digerida de forma enzimática, fenómeno conocido como fibrinolisis [38].

2.2.2.2. Fase inflamatoria

A medida que la sangre fluye a través de los vasos dañados, se forma un hematoma que rellena el espacio tisular con plaquetas. Las plaquetas activadas liberan varios factores de crecimiento y citoquinas que favorecen la migración, proliferación y diferenciación celular, así como la síntesis de matriz extracelular. Inmediatamente tras la formación del coágulo se generan las señales celulares necesarias que desencadenan una respuesta por parte de los neutrófilos que acuden al lugar de la lesión. Siendo las primeras células en llegar, rápidamente ofrecen protección frente a infecciones, fagocitando los posibles microorganismos y ayudando a la eliminación de los desechos y restos del tejido desvitalizado [39]. A continuación, llegan los linfocitos T y monocitos, diferenciándose estos últimos en macrófagos, que se convierten en el tipo celular predominante y que participan en diferentes aspectos de la remodelación tisular: producción de factores de crecimiento (influyen en el crecimiento y en la respuesta de los fibroblastos), producción de factores angiogénicos (ayudan en la revascularización de la herida) y producción de factores que intervienen en la reestructuración y remodelación de la matriz extracelular.

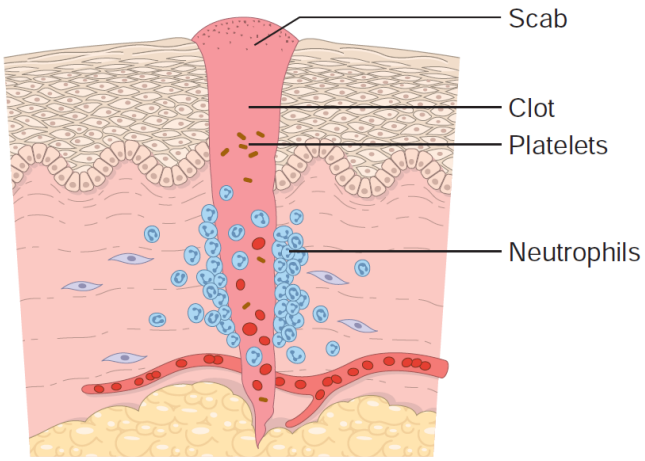


Figura 9: Fase inflamatoria del proceso de reparación y regeneración cutáneo. Adaptado de [20].

Los macrófagos activados clásicamente (M1) inducidos por interferón-γ (IFN-γ) y/o tumor necrosis factor-alfa (TNF-alfa) son efectores proinflamatorios y tienen funciones bactericidas, mientras que otros macrófagos (M2), están involucrados en la resolución de la inflamación y la curación del tejido. Los macrófagos activados son importantes para que se dé el paso a la fase proliferativa y ayudan a los neutrófilos en su labor a la vez que secretan factores implicados en sucesivos estadios. Cuando la labor de los neutrófilos ha finalizado entran en apoptosis y son fagocitados por los propios macrófagos. Los macrófagos liberan factores quimiotácticos para los fibroblastos que

acuden al lugar de la lesión y comienzan a proliferar generando una matriz extracelular que forma una cicatriz para reemplazar el tejido dañado. El colágeno es el principal componente de la matriz extracelular y un elemento clave en la reparación del defecto causado por la lesión, ya que restaura la estructura y función tisular [40].

2.2.2.3. Fase proliferativa y reparativa

Los queratinocitos epidérmicos que se localizan en las inmediaciones de la lesión comienzan a proliferar gracias a la estimulación mediante EGF (factor de crecimiento epidérmico) y TGF-β (factor de crecimiento transformante-β) liberados por las plaquetas activadas y macrófagos, dando lugar a una barrera protectora frente a la pérdida de fluidos y a la invasión por parte de más bacterias. Las células endoteliales de los vasos sanguíneos próximos a la región lesionada también proliferan en respuesta al VEGF (factor de crecimiento vascular endotelial) formando nuevos capilares que se extienden por el lugar de la lesión, y con ello inician el proceso angiogénico que contrarresta la hipoxia local [41].

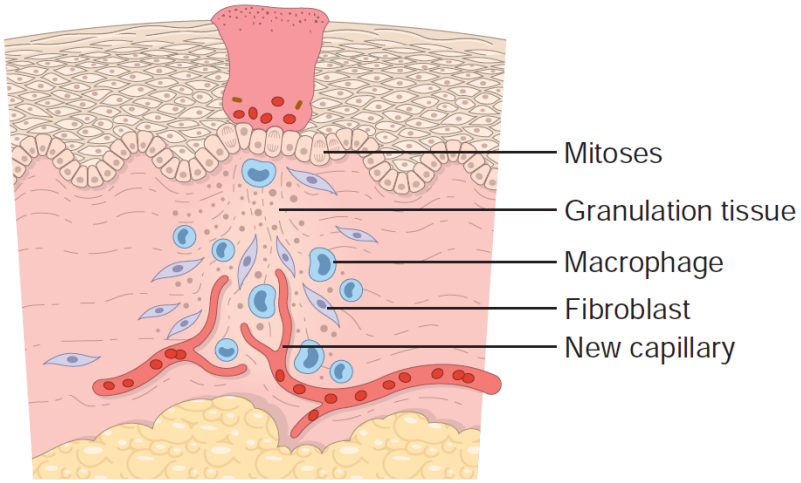


Figura 10: Fase proliferativa del proceso de reparación y regeneración cutáneo. Adaptado de [20].

Los fibroblastos dérmicos migran al lugar dañado y comienzan a sintetizar colágeno y a proliferar gracias al efecto del PDGF (factor de crecimiento derivado de plaquetas) y del EGF entre otros. Estas células se anclan a la matriz mediante integrinas y sintetizan una matriz provisional compuesta por colágeno, glicosaminoglicanos y fibronectina. El TGF-β1 estimula la síntesis de colágeno tipo I por parte de los fibroblastos y reduce la producción de metaloproteasas (MMPs). Este conjunto de hecho favorece la eliminación del tejido dañado y su sustitución por tejido específico de la zona donde se ha producido la lesión [42].

2.2.2.4. Fase de remodelación

Esta fase consiste en la deposición de matriz, principalmente colágeno y elastina, de una manera organizada y controlada. Si existiera una carencia de estas macroproteinas estructurales, la fuerza tensil de la herida se vería afectada y si por el contrario, se produjera una deposición excesiva, se generaría una cicatriz hipertrófica o queloide dando lugar en ambos casos a una estructura alterada y una pérdida de función. En términos prácticos, este proceso de remodelación cuenta con los fibroblastos que por un lado los construyen la nueva matriz extracelular que servirá como soporte para que acudan nuevas células y con los vasos sanguíneos que transportarán el oxígeno y nutrientes necesarios para mantener el metabolismo celular [43].

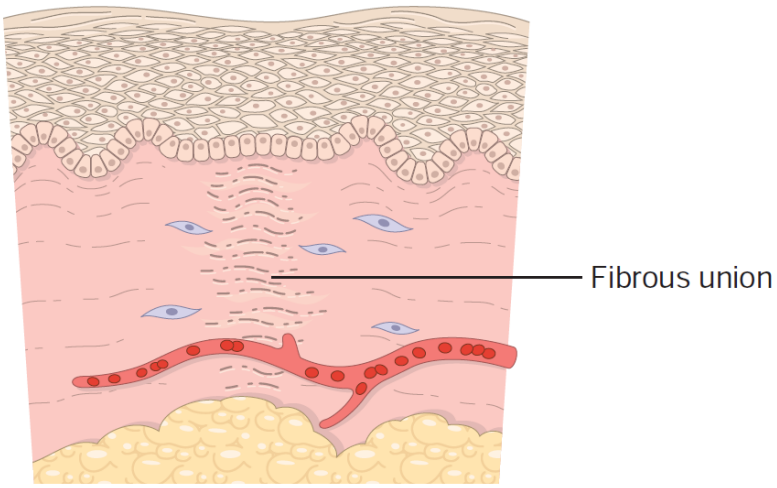


Figura 11: Fase de remodelación del proceso de reparación y regeneración cutáneo. Adaptado de [20].

El coágulo de fibrina formado en la fase inflamatoria es sustituido por un tejido de granulación que consiste en un tejido conjuntivo muy vascularizado, carente de nervios pero rico en fibroblastos, capilares y células inflamatorias residuales, que proporciona un ambiente metabólicamente activo y que ayuda a la reparación tisular. Los macrófagos y plaquetas liberan factores de crecimiento como PDGF, TGF-β1 y FGF (factor de crecimiento fibroblástico) que estimulan a las células residentes a proliferar, migrar al lugar de la lesión y sintetizar glicosaminoglicanos, proteoglicanos y colágeno. El tejido de granulación está formado principalmente por fibras de colágeno tipo III que progresivamente y gracias a la acción de las MMPs va siendo sustituido por colágeno tipo I. Éste es un proceso controlado de degradación del antiguo colágeno y síntesis del nuevo donde es también importante la participación de los TIMPs para asegurar un correcto equilibrio a fin de lograr una remodelación y reparación tisular satisfactoria [23]. Durante esta etapa de remodelación, el tejido neoformado se reestructura y reorganiza para asemejarse lo máximo posible al tejido original, disminuyendo la

densidad celular, la vascularización y eliminándose el exceso de matriz. Las fibras de colágeno de la nueva matriz de reparación se orientan de modo que se maximice la fuerza tensil y se recupere al máximo posible la disposición original. Pueden necesitarse varios años para que se complete totalmente el proceso regenerativo [44].

En resumen, los mecanismos de reparación y regeneración cutánea se inician inmediatamente después de que se produzca una lesión gracias a la secreción en la zona dañada de una gran cantidad de factores de crecimiento, citoquinas y proteínas principalmente desde las plaquetas activadas. La formación de un coágulo sanguíneo formado por una red de fibrina y diversas proteínas tales como vitronectina, fibronectina y trombospondina, previene una posible hemorragia, proporciona una barrera frente a los patógenos y sirve como matriz para las células que acuden al lugar de la lesión. Este mismo coágulo actúa como reservorio de los factores de crecimiento que serán necesarios en posteriores fases del proceso de reparación [45].

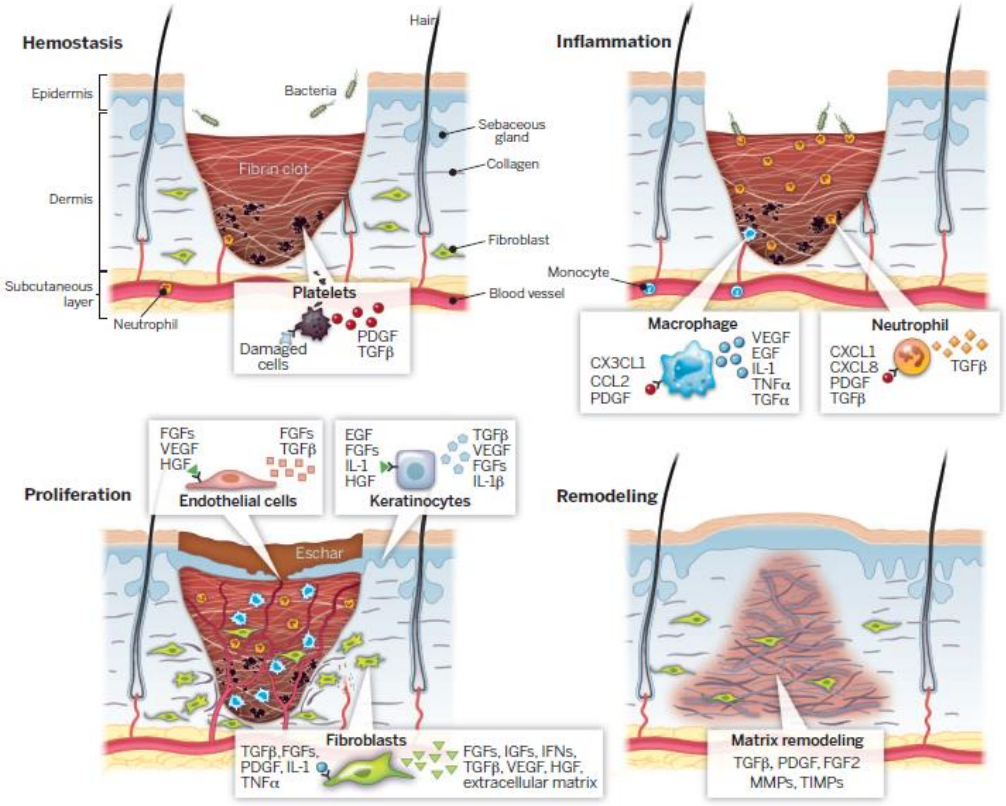


Figura 12: Diagrama representativo de las cuatro fases de la reparación y regeneración de heridas. Adaptado de [46].

Sin embargo, en todo proceso regenerativo suceden alteraciones en el medio local de la lesión con repercusiones en las diferentes fases de la reparación [47,48]. Estas alteraciones pueden clasificarse según:

- Variables específicas de la lesión: lugar anatómico, presencia/ausencia de infección, nivel de vascularización/oxigenación, estrés mecánico, edema.
- Variables sistémicas: estado nutricional, edad, sexo, estrés psicológico, movilidad.
- Medicación o exposiciones tóxicas: sustancias anticancerígenas (quimioterapia), sustancias anti-inflamatorias no esteroideas, glucocorticoides, radioterapia, fumador/no fumador, alcohol/drogas recreativas.
- Enfermedades y patologías: diabetes, enfermedades autoinmunes, estasis venosas, predisposición a queloides, enfermedades genéticas cutáneas, estado inmunocomprometido, obesidad, vasculitis, neuropatía, enfermedades infecciosas.

El deterioro progresivo o repentino de la piel puede por tanto alterar significativamente la funcionalidad tisular provocando entre otros efectos, el retraso en la llegada de neutrófilos y macrófagos al lugar dañado y un aumento de la actividad proteolítica. La actividad fibroblástica también se ve alterada derivando en una disminución de la mitogénesis, retrasando la neovascularización, desregulando la apoptosis celular y empeorando la integridad biomecánica de la lesión.

2.3. TERAPIAS AUTÓLOGAS EN DERMATOLOGÍA REGENERATIVA: TECNOLOGÍA DEL PLASMA RICO EN FACTORES DE CRECIMIENTO

Como medida de prevención y/o tratamiento activo frente a multitud de patologías, la ingeniería de tejidos, las terapias biológicas, la terapia celular o incluso la terapia génica son algunas de las innovaciones que la medicina regenerativa pone al servicio de los pacientes con el fin de promover la reparación y sustitución de células, tejidos u órganos dañados. Muchos de estos avances han demostrado su potencial en áreas como la dermatología regenerativa, la cual tiende crecientemente hacia una medicina personalizada capaz de abordar de forma segura y eficaz una gran variedad afecciones cutáneas. En este sentido, el desarrollo de terapias autólogas basadas en productos derivados de la sangre del paciente ha irrumpido con fuerza en el ámbito científico debido en gran medida a su potencial regenerativo y a su perfil de bioseguridad.

2.3.1. Generalidades

La tecnología del plasma rico en factores de crecimiento (PRGF) es una de las terapias basadas en plasma y plaquetas de más amplio recorrido a lo largo de los últimos años. La utilización del PRGF se inició en la década de los 90 en el ámbito de la medicina oral y maxilofacial con el fin de regenerar hueso en los alveolos o defectos post-extracción

dentarios [49] . Esta tecnología está basada en la extracción de un reducido volumen de sangre del paciente que es centrifugado en unas condiciones específicas para obtener diferentes fracciones enriquecidas en plaquetas y proteínas plasmáticas. Dichas plaquetas contienen en su interior un gran número y diversidad de factores de crecimiento y mediadores moleculares que se aplican en el propio paciente y estimulan la regeneración tisular. Tras observar clínicamente propiedades relevantes tales como su potencial regenerativo y anti-inflamatorio, se dedujo que su potencial terapéutico podría ser extensivo y aplicable a otras áreas de la medicina [50]. De hecho, desde el año 2013, el sistema PRGF está registrado como "medicamento de uso humano" por la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) para múltiples aplicaciones tales como la cirugía maxilofacial, lesiones musculo-esqueléticas, afecciones oftalmológicas y tratamiento de úlceras cutáneas. Indudablemente los beneficios clínicos unidos a su biocompatibilidad y versatilidad, han propiciado la autorización de su uso a nivel terapéutico [51].

Las plaquetas constituyen una fuente potencial de múltiples factores de crecimiento y proteínas autólogas que se han empleado como moléculas terapéuticas para la regeneración de un amplio rango de tejidos. Debido a la complejidad de estos procesos, es necesaria la presencia de un reservorio de proteínas y citoquinas, así como de un control en su liberación para favorecer al máximo posible su potencial terapéutico. El PRGF conforma un scaffold de fibrina que actúa como un vehículo para la liberación de múltiples factores de crecimiento en el lugar de la lesión.

La elección de las plaquetas como reservorio protéico y por tanto, el desarrollo de preparaciones ricas en plaquetas surgió de manera fortuita debido al interés inicial por sus propiedades adhesivas y hemostáticas [52]. Inicialmente, los productos ricos en plaquetas estaban dirigidos a sustituir los coágulos sanguíneos por unas preparaciones enriquecidas en plaquetas que, una vez activadas, podían secretar una gran cantidad de factores de crecimiento y proteínas en el medio circundante, desencadenando los mecanismos de regeneración tisular. Eliminando los eritrocitos y leucocitos, esta preparación aprovecharía al máximo las propiedades de las plaquetas y los morfógenos almacenados, permitiendo una cicatrización y regeneración acelerada [53]. Las proteínas plasmáticas y las plaquetas (junto con los factores de crecimiento que albergan en su interior) son por tanto la base del efecto terapéutico del PRGF. En consecuencia, es necesario entender el papel que juegan las principales moléculas de secreción plaquetaria y las rutas de señalización celular que se activan en respuesta a su aplicación cutánea para hacerse una idea de la base biológica de esta tecnología y sus múltiples mecanismos de acción.

2.3.2. Las plaquetas

Las plaquetas, también denominadas trombocitos, son los componentes sanguíneos más pequeños y están especializados en la función hemostática ya que circulan en el torrente circulatorio evitando el sangrado y previniendo potenciales hemorragias derivadas de la rotura de un vaso sanguíneo [54]. Son producidas en grandes cantidades a partir de los megacariocitos que son células poliploides de la serie blanca que se diferencian y maduran en la médula ósea. En la circulación sanguínea las plaquetas están en reposo, presentando una forma de disco biconvexo, con un diámetro de 2-5 µm y 0,5 µm de grosor. Entre las funciones plaquetarias fundamentales destacan la hemostasia primaria y la trombosis que se lleva a cabo de dos maneras diferentes: primero, a través de sus propiedades adhesivas y cohesivas que conducen a la formación de un tapón hemostático y segundo, a través de la activación de mecanismos de coagulación. Además de la participación de las plaquetas en la hemostasia, cada vez hay más evidencias de su intervención en procesos de gran relevancia tales como la reparación tisular, la cicatrización de heridas, la remodelación vascular y en las respuestas tanto inflamatoria como inmune [55].

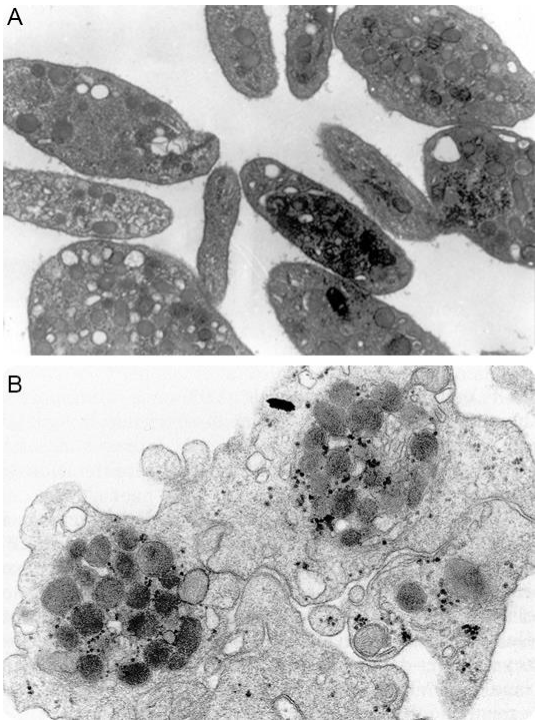


Figura 13: A) Imagen a microscopía electrónica de transmisión (TEM) de plaquetas en estado de reposo. B) Imagen TEM de plaquetas activadas liberando su contenido en factores de crecimiento. Adaptado de [50]

Las plaquetas poseen importantes funciones secretoras por lo que su contribución en la regeneración tisular se produce a través de la liberación de diferentes proteínas y otras moléculas que se encuentran almacenadas en las distintas poblaciones de

gránulos localizados en su citoplasma. Estas proteínas y metabolitos activos se liberan bien por difusión a través de la membrana o bien se secretan por exocitosis generando vesículas secretoras que se fusionan con la membrana plasmática de la plaqueta. Así, tales sustancias o bien son secretadas o se presentan asociadas a la superficie de la plaqueta activada [54].

Hay tres tipos de gránulos secretorios que están presentes en las plaquetas en número variable, abundando los gránulos tipo alfa que son los más importantes y característicos. Estos gránulos se mantienen intactos en reposo y durante la activación plaquetaria se centralizan previamente a la liberación de su contenido. A continuación se detallan los tipos de gránulos y su contenido [56-58]:

1. Lisosomas o gránulos lisosomales (gránulos lambda λ), bastante escasos y de 150 a 200 nm de diámetro, contienen hidrolasas ácidas pudiendo actuar como compartimentos para la digestión endosomal.
2. Gránulos delta o cuerpos densos: en número de 3 a 8 por plaqueta, de 250 a 300 nm de diámetro y con gran variabilidad morfológica. Las sustancias almacenadas y liberadas desde estos gránulos no sólo son importantes cofactores en la agregación plaquetaria, sino que también están implicadas en otros procesos biológicos. Contienen ADP (adenosin difosfato), ATP (adenosin trifosfato), Ca_{2+}, pirofosfato, serotonina, histamina y catecolaminas.
3. Gránulos alfa: existen aproximadamente de 50 a 80 por plaqueta y son los más grandes en tamaño ya que tienen un diámetro aproximado de 200 a 500 nm. Contienen multitud de proteínas bioactivas, muchas de las cuales tienen un papel fundamental en la hemostasia y/o cicatrización de heridas. Se pueden diferenciar varios grupos moleculares atendiendo a sus propiedades funcionales.
    - Proteínas adhesivas como el factor de von Willebrand (FvW), el fibrinógeno (Fg), la fibronectina (Fn) y la vitronectina (Vn) y glicoproteínas como la trombospondina-1 (TSP-1) que durante la hemostasia se unen a los receptores de plaquetas y participan directamente en la formación del trombo, además de formar parte de la matriz extracelular.
    - Factores de coagulación como los Factores V y VIII, antitrombina III y el inhibidor de la vía del factor tisular entre otros.
    - Proteínas fibrinolíticas como el plasminógeno y el inhibidor del activador del plasminógeno tipo I que además de regular la fibrinolisis se unen a la Vn aumentando las interacciones célula-matriz. También interactúan con integrinas favoreciendo los mecanismos de migración celular.

- Proteasas como las metaloproteasas (MMPs) cuya expresión puede ser regulada por factores de crecimiento, citoquinas y quimiocinas plaquetarias. En las plaquetas hay, entre otras, MMP-2, -9, ADAM-10 y -17 (A Disintegrin and Metalloprotease) y sus inhibidores como los inhibidores tisulares de metaloproteasas 1-4 que participan en la angiogénesis, remodelación vascular, regulación de la coagulación y equilibrio anabólico-catabólico.
- Quimiocinas como el factor plaquetario-4 (PF-4) y la β-tromboglobulina (β-TG) que intervienen en la regulación de la angiogénesis, y otras como la interleuquina 8 (IL-8) y RANTES (regulated on activation normal T cell expressed and secreted) que atraen leucocitos y activan a otras plaquetas. Las quimiocinas o citoquinas quimiotácticas son una gran familia de pequeñas proteínas que inducen la quimiotaxis de las células, además de cumplir otras funciones importantes en la proliferación y apoptosis de diferentes poblaciones celulares, en la morfogénesis tisular, la hematopoyesis, la angiogénesis y en el desarrollo de respuestas inmunes específicas. Las plaquetas constituyen la fuente más abundante de PF-4 y β-TG y más importante aún, las pueden liberar rápidamente cuando sean necesarias ya que se almacenan en los gránulos alfa como precursores inactivos y se vuelven disponibles con la activación plaquetaria. También hay citoquinas que conducen a la inflamación y a la producción de integrinas, así como a una síntesis de interleuquinas y otras quimiocinas.
- Proteínas antimicrobianas denominadas trombocidinas, que son productos derivados de quimiocinas CXC y que tienen un importante papel en la prevención de infecciones locales.
- Glicoproteínas de membrana como el CD40L y la P-selectina. Esta última se localiza exclusivamente en la membrana de los gránulos alfa de las plaquetas en reposo y es tras la secreción plaquetaria, cuando queda expuesta y distribuida por toda la superficie de la plaqueta. La p-selectina y el CD40L, ambas glicoproteínas dependientes de la activación plaquetaria, participan en la agregación y adhesión plaquetaria y en las interacciones entre las plaquetas, los monocitos y los neutrófilos.
- Factores de crecimiento como el factor de crecimiento derivado de las plaquetas (PDGF), factor de crecimiento transformante β (TGF-β), factor de crecimiento epidérmico (EGF), factor de crecimiento fibroblástico (FGF), factor de crecimiento vascular endotelial (VEGF), factor de crecimiento parecido a insulina (IGF), factor de crecimiento hepatocítico (HGF) y factor de crecimiento de tejido conectivo (CTGF), entre otros. Estas proteínas están implicadas en

multitud de mecanismos celulares como la quimiotaxis, proliferación, diferenciación y angiogénesis.

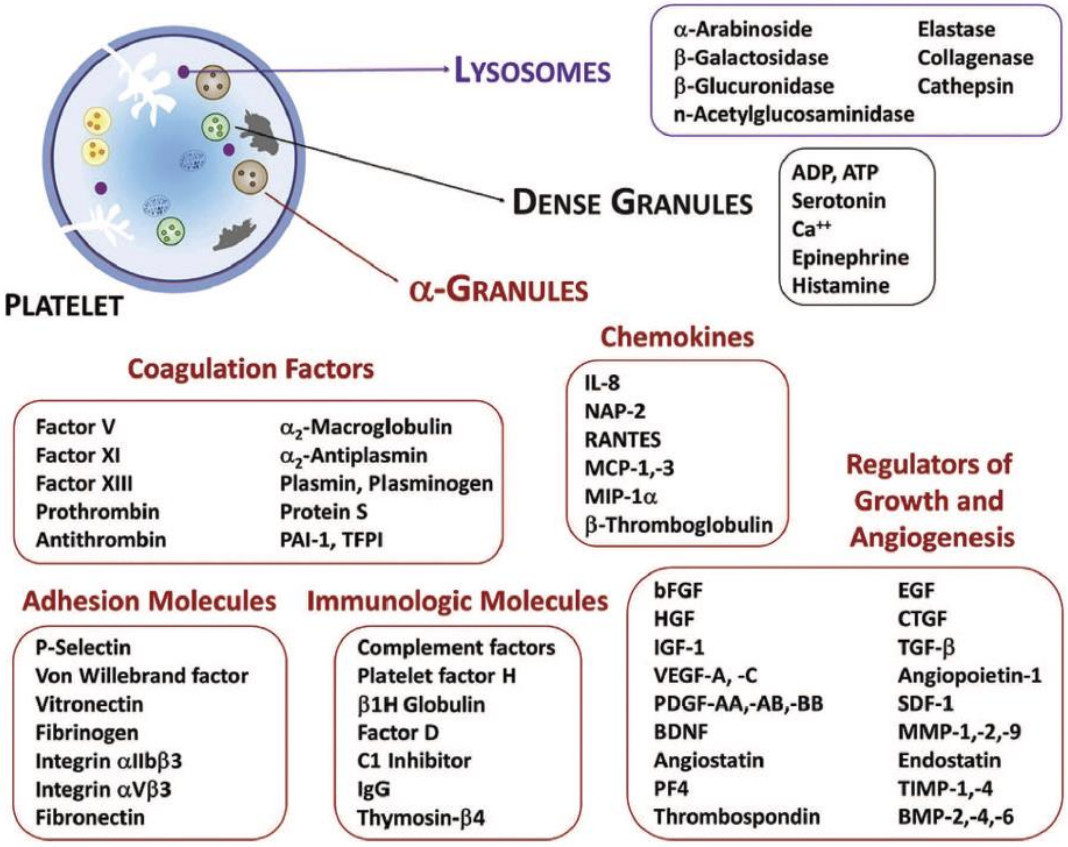


Figura 14: Carga molecular y proteica de las plaquetas. Adaptado de [59].

2.3.3. Factores de crecimiento

Los factores de crecimiento forman parte de un gran número de polipéptidos que transmiten señales que afectan a la actividad celular, actuando generalmente como moléculas de señalización y favoreciendo la comunicación entre células. Son proteínas hidrosolubles de bajo peso molecular, lo que facilita su difusión y su función como agentes señalizadores para las células. Forman parte de un complejo entramado y de una vasta red de comunicaciones inter e intracelular que influye en funciones tan críticas como son la mitosis, la síntesis de matriz y la diferenciación tisular. Los factores de crecimiento interaccionan con receptores específicos situados en la membrana citoplasmática de las células diana, desencadenándose diversos procesos biológicos tras su unión [60]. Así, estos morfógenos pueden estimular la mitosis de células quiescentes haciendo que abandonen la fase G0 del ciclo celular y entren en fase G1,

además de estimular la proliferación, mantener la supervivencia celular, estimular la migración e inducir la diferenciación [61].

Una vez que un factor de crecimiento se ha unido a su receptor en la célula diana, se desencadena un sistema de transducción de señales intracelulares que en última instancia alcanza el núcleo y produce una respuesta biológica. Cuando se establece la unión receptor-ligando, el receptor se activa mediante un cambio conformacional. El ligando se une al dominio extracelular del receptor y el dominio intracelular del mismo activa el sistema de transducción de señales. Una proteína intracelular denominada factor de transcripción, se activa como parte de este proceso y migra hasta el núcleo donde se une al ADN nuclear e induce la expresión de uno o varios genes. La expresión de estos nuevos genes es lo que en última instancia provoca un cambio en las características de la célula. El tipo de activación, así como el factor de transcripción específico varía dependiendo de la célula diana, de la combinación con su receptor y de la competencia biológica de la célula.

Los principales factores de crecimiento que intervienen en los procesos de reparación y regeneración de la piel son:

Factor de Crecimiento Derivado de las Plaquetas (PDGF)

El PDGF es una glicoproteína catiónica termoestable de unos 30-32 kDa compuesta por cadenas peptídicas unidas por puentes disulfuro. No sólo se almacena en los gránulos alfa de las plaquetas, abundando en éstas las isoformas PDGF-AB y –C, sino que también lo sintetizan monocitos, macrófagos, fibroblastos, células musculares lisas y células endoteliales. El PDGF juega un importante papel durante la embriogénesis así como en multitud de mecanismos tanto fisiológicos como patológicos en la edad adulta [45].

Los niveles de PDGF se ven reducidos en heridas cutáneas crónicas. Se ha demostrado que este factor de crecimiento es susceptible al microambiente proteolítico característico de úlceras crónicas, debido en gran medida a la acción catalítica de las metaloprotesas [62]. Esto supone un obstáculo que retrasa la curación de heridas ya que el PDGF tiene un rol significativo en las diversas fases de la regeneración de la piel. Tras la lesión, el PDGF es liberado por degranulación plaquetaria y estimula la mitogenicidad y quimiotaxis de los neutrófilos, macrófagos, fibroblastos y células del músculo liso al lugar afectado [63].

El PDGF fue el primer factor que mostró ser quimioatrayente para las células que migran hacia el lugar de una lesión. Es mitogénico para los fibroblastos en parte debido

a su capacidad para inducir la síntesis de IGF-I. El PDGF también hace que aumente la producción de fibronectina y ácido hialurónico en la piel por parte de los fibroblastos. No induce directamente la síntesis de procolágeno, sino que estimula a las células diana para que produzcan TGF-β1, lo que hace que se inicie la síntesis de colágeno. La liberación de PDGF es imprescindible para la cicatrización de heridas y durante este proceso, está altamente regulado por el TGF-β1 [64].

Factor de Crecimiento Transformante Beta (TGF-β)

La superfamilia del TGF-β engloba varias proteínas multifuncionales que regulan muchos y variados aspectos de la función celular, incluyendo la proliferación, diferenciación y reconocimiento celulares, la adhesión y motilidad, la síntesis de matriz extracelular, la angiogénesis, la respuesta inmune y la apoptosis celular. La unión del ligando a su receptor desencadena la cascada de información intracelular mediante la fosforilación de proteínas Smad que una vez activadas, se translocan al núcleo y conjuntamente con otros cofactores, regulan la transcripción de los genes diana.

El TGF-β es un factor pleiotrópico que interviene en la cicatrización de heridas y la síntesis de colágeno y matriz extracelular. De hecho, los niveles de este factor de crecimiento están significativamente reducidos en úlceras crónicas de larga duración, debido en gran parte al microambiente proteolítico característico de estas lesiones [62]. Durante la curación de heridas cutáneas, el TGF-β interviene de forma importante en procesos de inflamación, angiogénesis, reepitelización y desarrollo de tejido conectivo. Además está involucrado en la protección del tejido sano adyacente a la lesión y prepara la herida para la formación del nuevo tejido de granulación [65]. Esta proteína también promueve la regeneración de la piel mediante la inducción de la contracción perilesional y la síntesis de colágeno, mientras que reduce los niveles de metaloproteasas catalíticas. Aunque el TGF-β es imprescindible para la reparación tisular, su exceso conduce a un aumento en la deposición de matriz extracelular y a una fibrosis por lo que sus niveles deben estar óptimamente controlados en el lugar de la lesión [66].

Factor de Crecimiento de Tejido Conectivo (CTGF)

El Factor de Crecimiento de Tejido Conectivo (CTGF), posee propiedades mitogénicas y quimiotácticas y se ha visto que está implicado en múltiples funciones celulares tales como adhesión, migración, proliferación y diferenciación, así como en la angiogénesis y en la síntesis de matriz en el tejido conectivo. Tras una lesión cutánea, la expresión de CTGF aumenta para promover la formación de tejido de granulación, la reepitelización

y la reorganización de la matriz extracelular. Este morfógeno es un potente inductor de proteínas estructurales de la dermis como el colágeno tipo I, fibronectina y de sus receptores, las integrinas, además de activar la señalización del TGF-β1 mediante la unión a dicho factor y de mediar en su expresión participando así en los procesos fibróticos [67].

Factor de Crecimiento Epidérmico (EGF)

El EGF es posiblemente uno de los factores de crecimiento mejor caracterizados en los procesos de regeneración cutánea. Los principales miembros de la familia proteica involucrados en la curación de heridas son el EGF, TGF-alfa y EGF ligado a heparina. Estos ligandos se unen a su receptor principal, un receptor tirosina kinasa que se encuentra localizado en la epidermis, siendo su presencia más abundante a lo largo de la membrana basal [68].

El EGF es liberado por las plaquetas, macrófagos y fibroblastos y actúa de forma paracrina sobre los queratinocitos. Las plaquetas contienen cantidades importantes de EGF y cuando son liberadas, actúan localmente en las fases iniciales de la cicatrización de heridas. Uno de los mecanismos de acción del EGF es incrementar la expresión de keratina 6 y keratina 16, involucradas en rutas de señalización para la estimulación temprana de la epitelización y mantenimiento de la integridad tisular de heridas. El EGF induce la la migración y proliferación de células epiteliales y fibroblastos y aumenta la síntesis de proteínas como la fibronectina, además de ser mitogénico para los queratinocitos [45].

En situaciones de defectos cutáneos como heridas agudas o úlceras crónicas, se produce una sobreexpresión de EGF que acelera la reepitelización tisular e incrementa la fuerza tensil que ayuda a la contracción de las heridas. A pesar de que esta proteína es uno de los morfógenos constitutivos de fluidos ulcerosos, se ha demostrado que es susceptible al microambiente proteolítico que se genera en dichas lesiones. De hecho, diversos estudios han demostrado que la aplicación tópica de EGF recombinante acorta los plazos de curación de ulceras venosas o de tipo diabético [69].

Factor de Crecimiento del Endotelio Vascular (VEGF)

El VEGF es el más potente y específico regulador del desarrollo vascular durante la embriogénesis y de la formación de vasos sanguíneos en el adulto. Aumenta la permeabilidad vascular y es un mitógeno específico de células endoteliales, además de un factor de supervivencia para ellas. El VEGF se une a receptores tirosina kinasa de la superficie de los vasos sanguíneos y juega un papel clave en la curación de heridas ya

que promueve eventos tempranos de la angiogénesis como la migración y proliferación endotelial [70].

La hipoxia, característica del microambiente de numerosas úlceras crónicas, es el principal mecanismo de regulación del VEGF a lo que se suman otros morfógenos como el TGF-β, EGF, FGF, IGF, PDGF y algunas interleuquinas, estimulando su producción. Esto sugiere que la liberación paracrina o autocrina de estos factores colabora con la hipoxia local en la regulación del VEGF [71]. De hecho, lesiones cutáneas crónicas como las úlceras diabéticas, estasis venosas o úlceras por presión presentan isquemias locales que han llegado a ser tratadas ocasionalmente con VEGF recombinante como modalidad terapéutica [72]. El resultado del efecto angiogénico del VEGF a nivel de la dermis es la restauración de la perfusión tisular, el restablecimiento de la microcirculación y el incremento de oxígeno en la herida.

Factor de Crecimiento Fibroblástico (FGF)

La familia del FGF comprende un amplio grupo de factores de crecimiento polipeptídicos estructuralmente relacionados y que consta de unos 23 miembros. Los FGFs más abundantes en el tejido adulto son el factor de crecimiento fibroblástico ácido (FGF-1 o alfa-FGF) y el básico (FGF-2 o β-FGF). Algunos de los FGFs están entre los más importantes reguladores de la angiogénesis, del desarrollo embrionario, migración, proliferación y diferenciación celular [73].

Aunque los niveles de FGF-2 aumentan tras una herida cutánea aguda, su presencia disminuye en lesiones ulcerosas crónicas. Este morfógeno tiene un rol fundamental en la reepitelización de la piel y remodelación de la dermis a través de la síntesis de proteínas estructurales. El FGF regula la migración y diferenciación de sus células diana y controla la deposición de matriz extracelular. Además, desempeña un papel fundamental en la cicatrización de heridas ya que estimula la motilidad de queratinocitos y proliferación de los fibroblastos que dan lugar al tejido de granulación [74]. El FGF incrementa la transcripción de factores de transcripción involucrados en la detoxificación de especies reactivas del oxígeno (ROS). Este mecanismo ayuda a reducir los niveles de radicales libre que promueven la apotosis de queratinocitos en el lecho ulceroso de heridas crónicas, preservando su función en la cicatrización de heridas [75].

Factor de Crecimiento Insulínico (IGF)

Los IGFs son polipéptidos con una gran homología en su secuencia con la proinsulina. Se expresan ampliamente en prácticamente todos los tejidos del cuerpo. El IGF es endocitado y almacenado en los gránulos alfa de las plaquetas, habiendo así un IGF de

origen plaquetario que se libera junto con el resto de los factores de crecimiento durante la activación plaquetaria. Los IGFs juegan un papel muy importante en la regulación del metabolismo y crecimiento celular, además de intervenir en la motilidad, diferenciación y en prácticamente todas las funciones celulares. Tienen un fuerte efecto anabólico y mitogénico, pero también se han demostrado sus potentes efectos inhibitorios en la apoptosis. El IGF también se une a la fibronectina y vitronectina de la matriz extracelular con una afinidad comparable a la que tiene por su receptor, inmovilizándose el factor para que actúe estimulando localmente la migración celular [76].

Factor de Crecimiento de Hepatocítico (HGF)

El HGF se aisló inicialmente a partir de fibroblastos siendo un factor que estimulaba la motilidad de células epiteliales. Los componentes de matriz extracelular fibronectina y vitronectina, participan en la inmovilización del HGF en una localización determinada creando un gradiente de concentración lo que conlleva un aumento de la migración endotelial. El HGF actúa de manera autocrina, paracrina y endocrina y estimula la migración y proliferación celular, además de ser un factor angiogénico por sí mismo o actuando sinérgicamente con el VEGF [77]. Otra de las funciones del HGF es su papel en procesos anti-inflamatorios mediados por la reducción de la translocación al núcleo de las células del factor nuclear NF-kB.

2.3.4. Propiedades y obtención del PRGF

En 2007, se introduce por primera vez el término plasma rico en plaquetas en la base de datos del Medical Subjects Headings (MeSH) de los institutos de salud de Estados Unidos. Esta base de datos contiene más de treinta mil términos médicos que son revisados con una periodicidad anual y que reflejan los cambios tecnológicos y nuevos procedimientos en la práctica médica. La definición que da la MeSH del plasma rico en plaquetas es la de: "una preparación consistente en plaquetas concentradas en un volumen limitado de plasma. Es utilizado en varios procedimientos quirúrgicos de regeneración de tejidos, en los que los factores de crecimiento de las plaquetas mejoran la curación de heridas y la regeneración tisular". Sin embargo, el uso de los derivados hemáticos se remonta a 1972 cuando Matras demostró el potencial de la fibrina como sellante y agente hemostático [78]. Una década después, Staindl utilizó la trombina como activador de un coágulo rico en fibrinógeno el cual presentaba una firme adherencia al lugar de la lesión, control del sangrado y mejora en la regeneración post-operatoria [79]. Ya en 1990 Gibble et al. introdujo el concepto autólogo de gel de fibrina y años mas tarde, Marx et al. aplicaron la técnica de centrifugación y separación

por gradiente de densidad para generar concentrados plaquetarios que incrementaban la funcionalidad de injertos óseos [80]. Sin embargo, existían ciertas limitaciones inherentes a la tecnología como el elevado volumen de sangre necesaria, la necesidad de trombina bovina inmunogénica para promover la activación plaquetaria y la presencia de leucocitos en el preparado final. Para superar estas limitaciones, el doctor Eduardo Anitua desarrolló a partir de 1999, un procedimiento de centrifugación única en el que partiendo de pequeños volúmenes de sangre fue posible elaborar de forma reproducible un plasma rico en plaquetas libre de leucocitos y activado mediante cloruro de calcio. Dicho preparado biológico se denominó Endoret-PRGF [49].

Hoy en día existe un importante número de preparaciones ricas en plaquetas en función del laboratorio que las haya desarrollado, optimizado y patentado. Aunque el principio de separación y concentración plaquetaria es similar, existe una clasificación que hace distinciones entre los denominados gold standard, es decir, los más utilizados y de mayor prestigio/recorrido científico. Esta clasificación llamada PAW Classification System hace referencia a cuánto se concentran las plaquetas (**P**latelet concentration), al tipo de activador plaquetario utilizado (**A**ctivator type) y a la presencia o ausencia de leucocitos en la solución final (**W**hite blood cell content) [81].

Nombre	Laboratorio	Centrifugación	Volumen de sangre	Volumen de PRP	Concentración plaquetaria	Leucocitos
Plasma based PRP systems	Arthrex	5min	16ml	4-7ml	2X-3X	No
ACP	Cascade	6min	9ml	4,5ml	1,3X-1,7X	No
PRGF-Endoret	BTI	8min	9ml	3-4ml	2X-3X	No
GPS II/III	Biomet	12-15min	30-60ml	3-6ml	2X-8X	Superior a sangre
Smart PReP	Harvest	12-15min	20-60ml	3-10ml	3X-7X	Superior a sangre
Magellan	Arteriocyte Medtronic	14-20min	30-60ml	3-10ml	3X-7X	Superior a sangre
Accelerate	Exactech	12min	30-60ml	3-10ml	7X-10X	Superior a sangre

Tabla 2: Características de los principales sistemas de obtención de preparados ricos en plaquetas.

En la presente tesis se ha utilizado la tecnología PRGF para obtener un preparado autólogo rico en plaquetas y evaluar su potencial como posible terapia en dermatología regenerativa. La tecnología del PRGF consiste en la obtención de un volumen limitado de plasma autólogo enriquecido en plaquetas, es decir, con una concentración de plaquetas por encima de los niveles basales y que también mantiene intactos los factores de coagulación en sus valores fisiológicos.

Tras la extracción de sangre en tubos con citrato sódico al 3,8% como anticoagulante, se centrifuga a una velocidad de 580g durante 8 minutos en la centrífuga BTI System V (BTI Biotechnology Institute, Vitoria, España) para garantizar un gradiente de densidad óptimo mientras que se evita la fragmentación de las membranas celulares. Esta centrifugación origina la formación de tres capas principales: la serie roja (glóbulos rojos) en la parte inferior del tubo, una capa intermedia blanquecina (leucocitos o "buffy coat"), y una capa superior amarillenta que contiene el plasma y las plaquetas. Las formulaciones derivadas del sistema PRGF evitan la inclusión de la serie blanca ya que numerosos estudios sugieren que la inclusión de un concentrado leucocitario que libera citoquinas pro-inflamatorias, lejos de ayudar a la regeneración puede derivar en reacciones adversas, sobre todo en situaciones de estrés tisular [82]. A su vez, a nivel experimental y clínico se puede subdividir la columna plasmática en dos fracciones: la fracción 2 o F2 (los 2mL por encima del buffy coat) que es rica en plaquetas llegando a concentraciones de entre 2 y 3 veces por encima del nivel fisiológico; y la fracción 1 o F1 (el volumen por encima de la F2) que mantiene la concentración plaquetaria de la sangre periférica.

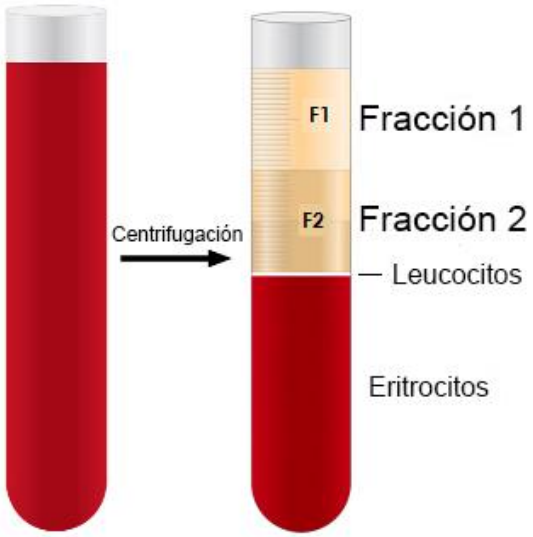


Figura 15: La centrifugación de la sangre provoca la separación de sus fases por gradiente de densidad.

Una vez aisladas las fracciones plasmáticas y para que las plaquetas liberen el contenido de los gránulos alfa es necesario añadir cloruro cálcico ($CaCl_2$). La tecnología PRGF, permite la elaboración de diferentes formulaciones terapéuticas en función del protocolo de activación y la fracción o fracciones plasmáticas utilizadas. De esta forma, en la actualidad existen diversas formulaciones autólogas que derivan del PRGF cuyas características concretas se detallan a continuación:

Plasma rico en factores de crecimiento líquido

Se trata de una formulación que posee una naturaleza líquida transitoria y que polimeriza tras activar la F2 del PRGF con cloruro cálcico. Esta formulación se puede infiltrar en superficie cutánea, tejidos del aparato locomotor, articulaciones, etc. Se utiliza también para bioactivar superficies de implantes dentales y prótesis de todo tipo con el fin de acelerar su oseointegración. Una vez dentro del tejido, las plaquetas activadas liberarán su contenido rico en factores de crecimiento generando a su vez una malla tridimensional que permitirá la migración de las células adyacentes hacia la lesión.

Figura 16: Plasma rico en factores de crecimiento líquido para infiltración tisular o bioactivación de superficies.

Coágulo semisólido

Esta formulación funciona a modo de esponja bioenriquecida rica en factores de crecimiento. Este coágulo puede ser colonizado por células del propio paciente y es ideal para rellenar defectos y promover la regeneración tisular en tratamientos de úlceras cutáneas, alveolos dentales, ingeniería de tejidos, etc. El coágulo semisólido constituye un soporte de fibrina tridimensional, biocompatible y con un alto contenido en factores de crecimiento. La formación de esta matriz mimetiza la última etapa de la coagulación en la que el fibrinógeno se convierte en fibrina por acción de la trombina. Esta última, en presencia de calcio rompe el fibrinógeno originándose los monómeros de fibrina y también activa al Factor XIII que favorece el entrecruzamiento de las hebras de fibrina y la formación del coágulo. Así, esta red tridimensional de fibrina asociada con fibronectina y vitronectina proporciona una matriz provisional que actúa como

soporte de las células que acuden al lugar de la lesión (neutrófilos, monocitos, fibroblastos, células progenitoras y células endoteliales).

Figura 17: Coágulo semisólido para relleno de defectos.

Membrana de fibrina

Una vez finalizado el proceso natural de retracción de la fibrina de forma *ex vivo*, el coágulo se convierte en una membrana de fibrina natural con unas propiedades biomecánicas y hemostáticas idóneas para sellar defectos y realizar suturas quirúrgicas estimulando al mismo tiempo la epitelización de la lesión.

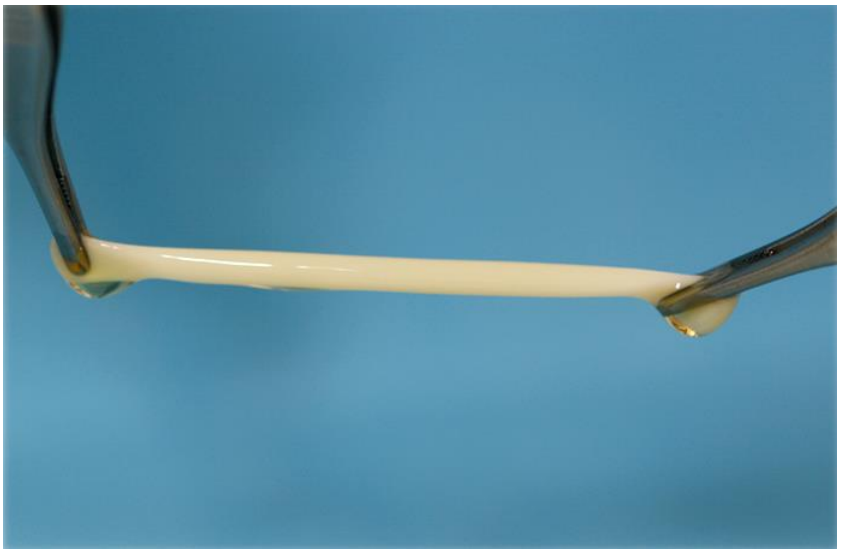


Figura 18: Membrana de fibrina para sellar y realizar suturas.

Sobrenadante rico en factores de crecimiento

La retracción del coágulo semisólido provoca la liberación total de su contenido, el cual está altamente enriquecido en factores de crecimiento. Una vez filtrado, este sobrenadante líquido es libre de elementos celulares y contiene el extracto puro del interior de las plaquetas. Actualmente se emplea para el cultivo *in vitro* de células primarias y como colirio oftalmológico para tratar un gran número de patologías de la superficie ocular.

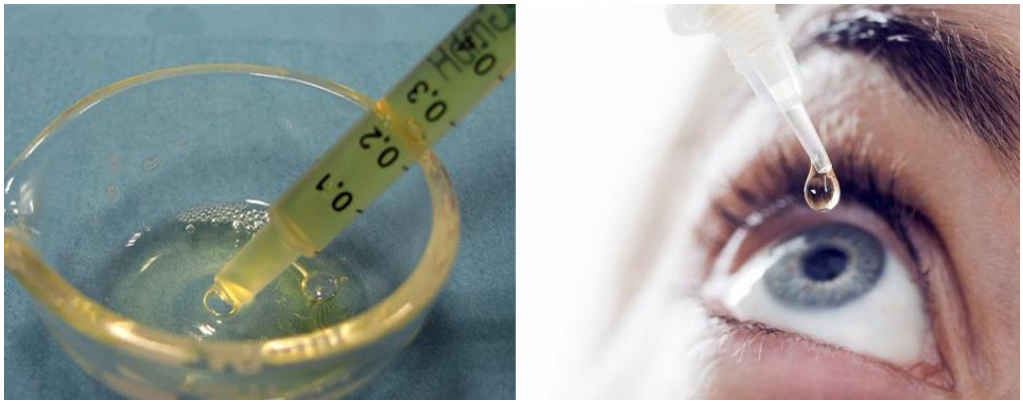


Figura 19: Sobrenadante rico en factores de crecimiento para cultivos celulares y colirio ocular.

2.3.5. Aspectos clave en la elaboración del PRGF

La tecnología del PRGF presenta ciertos requisitos y propiedades idóneas para su uso con fines regenerativos. El coágulo y la membrana de fibrina derivadas del PRGF, presentan fuerza tensil y elasticidad a la vez que consistencia y capacidad adhesiva. Esto permite su manipulación y su colocación en el lugar de la lesión para que los morfógenos actúen de forma local mientras que la fibrina se reabsorbe de manera natural [83]. Por otro lado, el uso de $CaCl_2$ en vez de trombina de origen bovino, garantiza la correcta activación plaquetaria ya que la trombina no solo desencadena una excesiva agregación plaquetaria que reduce los niveles de factores de crecimiento finales sino que su uso está asociado al desarrollo de anticuerpos y coagulopatías [84].

La presencia o ausencia de la denominada serie blanca en los preparados ricos en plaquetas es uno de los aspectos que más controversias suscita. Sin embargo, evidencias cada vez más numerosas señalan que los concentrados leucocitarios pueden repercutir negativamente en el proceso de regeneración tisular. Estas células contienen numerosas citoquinas pro-inflamatorias como IL-1, IL-6 TNF-alfa e IFN-γ, entre otras, que ejercen un papel quimio-atrayente para neutrófilos, macrófagos y demás células del sistema inmunitario que a su vez alimentan la situación inflamatoria mediante la liberación de más interleuquinas [85]. La presencia de un número elevado de

glóbulos blancos en el plasma rico en plaquetas puede tener efectos negativos, ya que liberan metaloproteasas que degradan la matriz y radicales libre altamente reactivos que dañan el tejido sano que rodea el área lesionada provocando la aparición de tejido fibrótico [86]. Además, la ausencia de la serie blanca favorece la estabilidad del coágulo de fibrina lo que se traduce en un mejor comportamiento biomecánico. La presencia de leucocitos o eritrocitos por el contrario, desestabiliza la unión altamente ordenada de monómeros de fibrina que conforma esta red tridimensional [50].

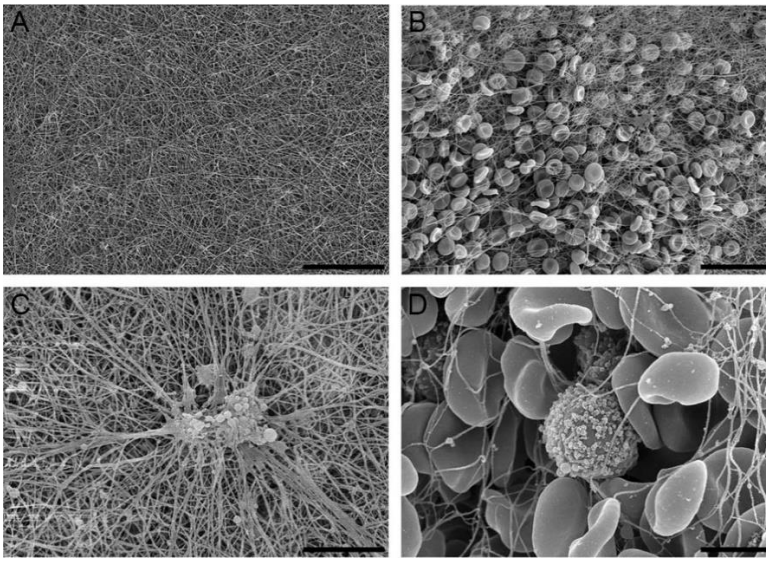
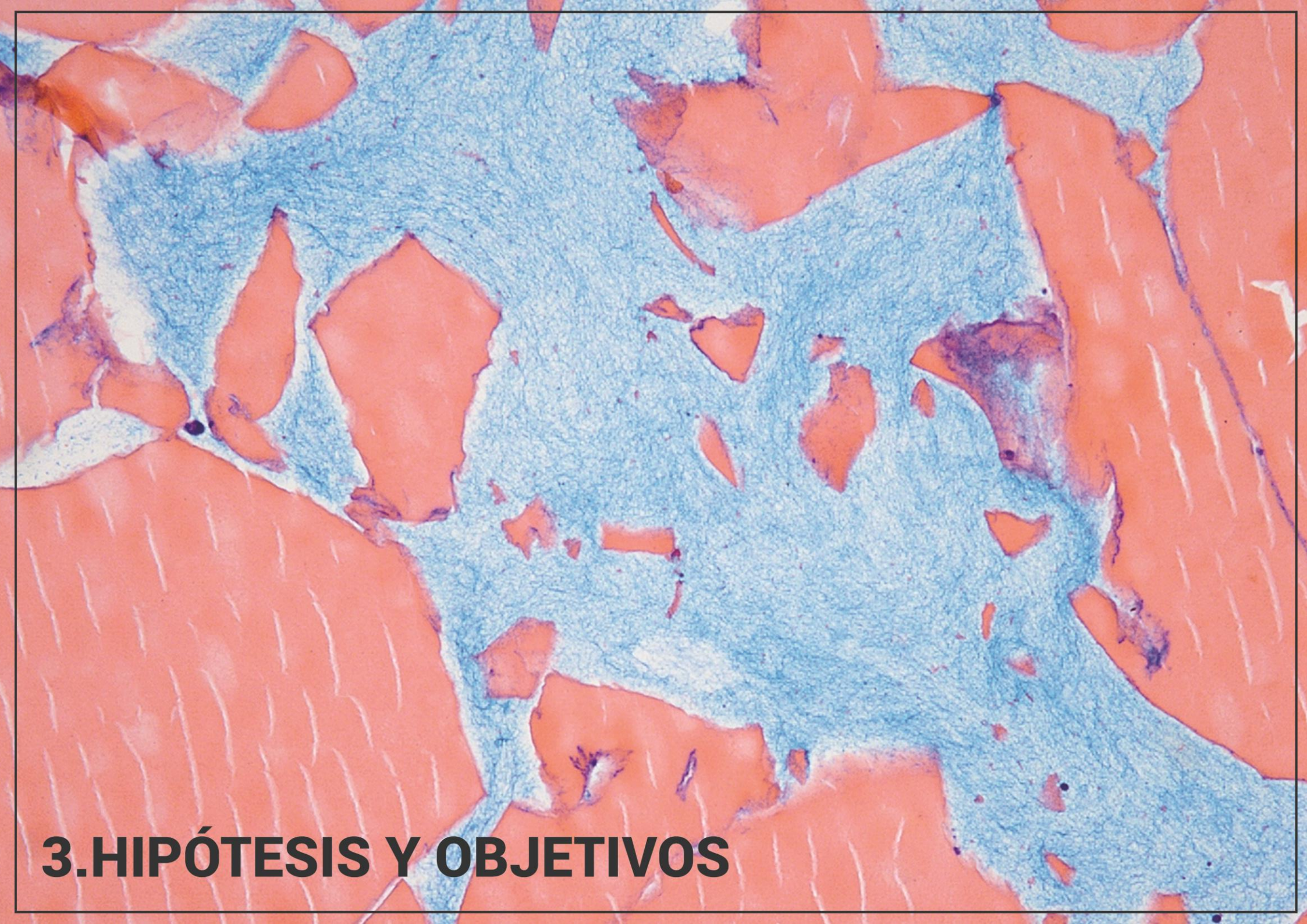


Figura 20: Caracterización mediante microscopía electrónica de barrido (SEM) de la matriz de fibrina en ausencia (A, C) o presencia (B, D) de leucocitos. Barras de escala para A-B: 25µm. Barras de escala para C-D: 5µm. Adaptada de [82].

A su vez, el objetivo de la tecnología de los plasmas ricos en plaquetas es concentrar el número de plaquetas por encima de los niveles fisiológicos. Por esta razón algunos sistemas de elaboración han aumentado el tiempo y la velocidad de centrifugación de la sangre para obtener concentraciones elevadas entre 4 y 6 veces superiores a la sangre. Incluso existen protocolos que promueven la doble centrifugación (centrifugación primero de la sangre y centrifugación del plasma después) para alcanzar niveles aún más altos. Sin embargo estudios de caracterización de concentrados plaquetarios, indican que mientras que niveles bajos de concentración no provocan una respuesta celular suficiente [87], aumentar el número de plaquetas en exceso puede reducir la síntesis de moléculas fundamentales en el proceso de reparación tisular, lo que equivale paradójicamente a una disminución en su eficacia [88].

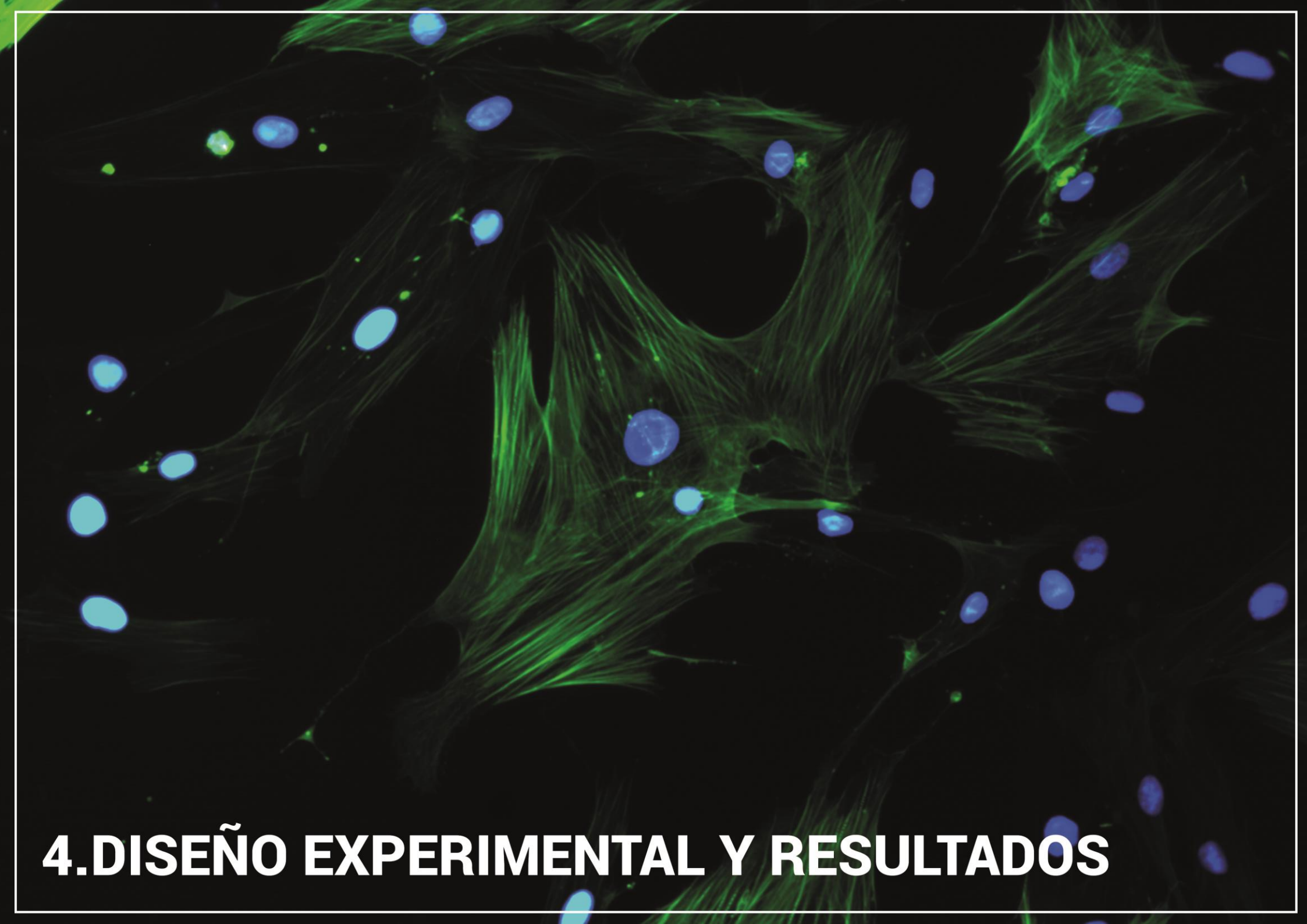


3.HIPÓTESIS Y OBJETIVOS

El plasma rico en factores de crecimiento (PRGF) permite la liberación de factores de crecimiento que resultan claves en la remodelación y regeneración cutánea. En base al potencial regenerativo observado en otras áreas de la medicina, se establece la hipótesis de que las proteínas bioactivas liberadas tras la activación del PRGF, así como la matriz de fibrina derivada de la cascada de coagulación plasmática, puedan interactuar con los diferentes fenotipos celulares y elementos estructurales de la piel y promover así procesos de regeneración tisular. Esta terapia biológica es una nueva herramienta de medicina regenerativa personalizada cuyo fin es la regeneración de la zona cutánea dañada y su posterior restablecimiento.

En la presente tesis doctoral se establecen los siguientes objetivos:

- Obtener, aislar y caracterizar diferentes líneas celulares de fibroblastos dérmicos a modo de sistema experimental sobre los que testar y poner a punto el PRGF como suplemento de cultivo.
- Caracterizar de forma hematológica, proteica y comparativa el PRGF y analizar su potencial biológico a nivel celular.
- Estudiar el efecto protector y regenerador de los factores de crecimiento autólogos en condiciones de estrés celular *in vitro*.
- Analizar *ex vivo* el potencial regenerativo del PRGF en modelos organotípicos de piel 3D humana sometidos a condiciones de estrés oxidativo.
- Desarrollar y caracterizar una formulación biomimética de tipo gel tridimensional estable a partir del PRGF con propiedades biomecánicas óptimas para la reposición de defectos tisulares y el crecimiento celular.
- Desarrollar y caracterizar una formulación tópica almacenable a partir del PRGF con propiedades reológicas óptimas para su extensión superficial y absorción percutánea. Determinar el periodo de bioestabilidad de la formulación autóloga.



4.DISEÑO EXPERIMENTAL Y RESULTADOS

Artículo I

Plasma rich in growth factors promotes dermal fibroblast proliferation, migration and biosynthetic activity.

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Title: Plasma rich in growth factors promotes dermal fibroblast proliferation, migration and biosynthetic activity

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Conflict of interest:

Eduardo Anitua, Ander Pino and Gorka Orive are scientists at BTI Biotechnology Institute. This biotechnology company has developed the technology of plasma rich in growth factors.

Abstract

Objective: The use of plasma rich in growth factors (PRGF) has gained importance in many medical fields due to its regenerative potential. Here, we evaluated the effects of PRGF on primary skin fibroblasts regarding cell proliferation, migration and secretion of growth factors. Additionally, we decided to study whether the age of the patients from whom PRGF-Endoret was prepared may influence the final biological outcomes.

Method: Human dermal fibroblasts were isolated from three healthy volunteers, and PRGF was prepared from blood of 3 young and 3 elderly donors. The effects of PRGF on cell proliferation and migration was evaluated using different PRGF concentrations (5%, 10% and 20%) and PRGF prepared from young (<35 years old) and medium-advanced age donors (>50 years old). Biosynthetic behavior of cells was also analyzed measuring VEGF, TGFβ1 and procollagen type I secreted levels after PRGF treatment.

Results: Mean platelet enrichment reached 2.4X and 2X in young and elderly PRGFs respectively. A dose dependent response was observed in proliferation assays achieving the highest outcome with 20% PRGF. Migration was also promoted in cells but not in a dose dependent manner. Cell proliferation and migration outcomes obtained with both types of PRGF-Endoret (prepared from young and elderly patients) were significantly higher compared to non-stimulated group ($p<0.05$). No statistical significances were observed between PRGF-Endoret groups. Production of VEGF, TGFβ and procollagen type I was significantly increased by cells treated with PRGF. With the exception of VEGF, no statistical significances were observed between PRGF-Endoret groups.

Conclusion: Results from this study conclude that PRGF is safe and effective in stimulating skin regeneration by enhancing proliferation, migration and expression of pivotal bioactive molecules involved in wound healing and hemostasis.

Keywords: regeneration, wound healing, skin

Introduction

The skin is the largest organ in the human body, covering an area of approximately 2 m². The main function of skin is to act as a protective barrier isolating the organism from the environment and thus protecting it from infections and dehydration. To achieve an optimal condition, the skin needs a balance between different factors including temperature, pH, hydration, elasticity, sebum production, scaling level and roughness, most of which depend on the function and fate of resident cells such as dermal fibroblasts, keratocytes and adipocytes.1,2

Wound healing and skin regeneration processes involve a complex cascade of cellular events including proliferation, migration, differentiation and extracellular matrix synthesis.3 The correct function of these cellular mechanisms, allows the normal transition between the different phases of the skin regeneration: hemostasis, inflammation, granulation tissue formation, epithelization, neovascularization and extracellular matrix components deposition.4,5 It is well known that a wide range of pathologies (chronic wounds, ulcers, burns, autoimmune diseases) and ageing are involved in the deterioration of the skin.

In the last few years, several regenerative medicine approaches have been tested with the aim of promoting wound healing and dermal/epidermal regeneration. Some examples include tissue engineered skin 6,7, artificial collagen scaffolds 8-10, hyaluronic acid coated matrices 11,12, stem cell therapy 13 and the use of recombinant growth factors. 14,15

Another interesting alternative lies on the application of autologous proteins and biomaterials for regenerative purposes. The use of plasma and platelet-derived proteins and growth factors has shown to be useful for promoting tissue repair and healing. 16,17 Plasma rich in growth factors (PRGF-Endoret) technology is based on preparing a platelet enriched plasma volume from patient's own blood that can be used therapeutically. 18-20 Recent results demonstrate the potential of PRGF-Endoret in the treatment of several disorders in a wide range of medical fields such as dermatology 21-25, aesthetic surgery 26,27, orthopedic surgery 28, sports medicine 29,30, ophthalmology 31,32, neurobiology 33 and oral/maxillofacial surgery. 34,35

In the present study, we have evaluated the biological effects of PRGF-Endoret on human primary skin fibroblasts. Plasma rich in growth factors prepared from different donors, was assessed over the main biological events of the wound healing process including cell proliferation, migration and secretion of growth factors. Furthermore, we decided to study whether the age of the patients from whom PRGF-Endoret was prepared may influence the final biological outcomes. To address this, all the

experiments on the cells were carried out in parallel using both PRGF-Endoret prepared from young donors (<35 years old) and PRGF-Endoret prepared from medium-advanced age donors (>50 years old).

Materials and methods

Cell isolation

Dermal fibroblasts were isolated from skin biopsies of three healthy patients during joint surgery. Written informed consent was obtained from all patients before the biopsy was performed. Cells were isolated following our standard protocol. Briefly, skin biopsies collected in phosphate buffered saline supplemented with antibiotics were minced and treated with 0.3% collagenase II (Gibco Life Technologies, Gaithersburg, MD, USA) at 37°C for 90 min with gentle stirring. The resulting cell suspension was filtered and centrifuged at 460 g for 10min. Cells were seeded into culture flasks and maintained with Dulbecco`s modified Eagle`s medium (DMEM)/F12 (1:1 volume) (Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 2mM Glutamine, 50µg/ml Gentamicin and 2.5µg/ml Amphotericin B (Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere at 37°C with 5%CO2. After reaching confluence cells were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco Life Technologies, Gaithersburg, MD, USA). Cell viability was assessed by trypan blue dye exclusion. All experiments were performed using cells from these three donors between the third and fifth passages.

Characterization of human dermal fibroblasts

The fibroblast-like morphology of cells was checked by phase-contrast microscopy. Cells were characterized by immunofluorescence using three antibodies against typical dermal fibroblast markers: Collagen Type I (Chemicon-Millipore, Billerica, MA, USA), Fibronectin and Vimentin (Sigma-Aldrich, St. Louis, MO). The cells were also tested against typical epithelial cell marker P-Cytoketarin and against alfa-Smooth muscle actin (alfa-SMA) (Sigma-Aldrich, St. Louis, MO) in order to check the spontaneous differentiation to myofibroblasts in culture.

Cells were plated on 24-well plates at 10000 cell/cm2 density. After 72h of culture with Dulbecco`s modified Eagle`s medium (DMEM)/F12 (1:1 volume) (Gibco Life Technologies, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 2mM Glutamine and 50µg/ml Gentamicin (Sigma-Aldrich, St. Louis, MO) (referred to below as the culture medium), cells were fixed in for

10 minutes in 4% formaldehyde for Collagen type I antigen, in methanol pre-cooled at -20°C for Fibronectin and alfa-SMA antigens and in methanol:acetic acid (3:1) for Vimentin an P-Cytokeratin antigens. Cells for type I collagen staining were permeabilized with 1% Triton X-100, in PBS for 10 minutes. Cells were then blocked with FBS (10% in PBS) for 30 minutes, and incubated for 1 hour with the primary antibodies in the following dilutions: 1:20 for Collagen Type I, 1:50 for Vimentin, 1:500 for P-Cytokeratin and 1:800 for Fibronectin and alfa-SMA. Next, cells were incubated with their appropriate secondary antibodies, goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen, Grand Island, NY, USA). Finally, cells nuclei were stained with Hoechst 33342 (Molecular Probes-Invitrogen, Grand Island, NY, USA), mounted, and visualized under a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany). Control isotype was performed by substituting the primary antibodies by 10% of FBS diluted in PBS. All markers and images taken for each well were performed in triplicate in order to calculate de percentage of positive cells for each antibody.

Plasma Rich in Growth Factors (PRGF-Endoret) preparation and characterization

The study was performed following the principles of the Declaration of Helsinky. Blood from 3 young healthy donors (under 35 years old) and 3 healthy donors over 50 years old was collected after obtaining informed consent and was put into 9 mL tubes containing 3.8% (wt/vol) sodium citrate. For PRGF-Endoret preparation, the blood was centrifuged (BTI System IV, Vitoria, Spain) at 580g for 8 minutes and then plasma column was fractioned into fraction 1(F1) and fraction 2 (F2). F2 is defined as the 2 cm3 platelet rich plasma just above the leukocyte buffy coat, and F1 is defined as the remaining plasma volume above the F2. Platelet poor F1 fraction was discarded and only the platelet rich F2 fraction was used in our assays. Platelet concentration in peripheral blood and in F2 fraction was measured with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France). The collected F2 fraction was incubated with PRGF activator (BTI Biotechnology Institute, SL, Miñano, Spain) at 37°C in glass tubes for 1 hour. When PRGF is activated, fibrinogen is transformed into fibrin by the action of thrombin and the PRGF activator. This leads to fibrin clot formation which is followed by a clot contraction phase thus releasing a fibrin-free liquid supernatant. The fibrin-free supernatants released were collected after centrifugation at 1000g for 10 minutes. Finally, the supernatant volume was aliquoted under laminar air flow conditions and stored at -80°C until use. Several growth factors involved in skin regeneration were measured by commercially available Enzyme-linked immunosorbent

assay kits (ELISA) (R&D Systems, Minneapolis, MN) : Platelet derived growth factor (PDGF), Epidermal growth factor (EGF), Insulin like growth factor (IGF), Fibroblast growth factor-b (FGFb) and Hepatocyte growth factor (HGF). Platelet number can vary slightly between donors so results are expressed as "concentration of growth factor released per platelet", in order to accurately analyze the differences between the two groups. All experiments were performed in triplicate.

Dermal fibroblast biosynthesis

Human dermal fibroblasts at passage 4 were seeded after subculture in 24 well plates at 5000 cell/cm2. Cells were maintained in culture medium for 96 hours until confluence. After this period of time, culture medium was removed, cells were washed with PBS and incubated with serum-free culture medium supplemented with 0.1% (v/v) FBS as a control of non-stimulation, 20% (v/v) Young PRGF or 20% (v/v) Elderly PRGF for 72 hours. After the stimulation period, culture medium was collected and centrifuged at 460g for 10min, and stored at -80°C until assayed. The amount of Vascular Endothelial Growth Factor (VEGF), Transforming growth factor β1 (TGFβ1) (R&D Systems, Minneapolis, MN) and extracellular matrix proteins Pro-collagen type I (Takara, Shiga, Japan) and Hyaluronic acid (Corgenix, Broomfield, CO) were measured by Enzyme-linked immunosorbent assay kits. All samples were measured in triplicates.

Cell Proliferation assay

Subconfluent cultures of cells were detached and cell viability was assessed by trypan blue dye exclusion. Passage 3 cells were plated at a density of 10000 cells/cm2 in 96 well optical bottom black culture plates and cultured for 72 hours with serum-free culture medium supplemented with 0.1% (v/v) FBS as a control of non-stimulation, 5% (v/v) Young PRGF or 5% Elderly PRGF, 10% Young PRGF or 10% Elderly PRGF and 20% (v/v) Young PRGF or 20% (v/v) Elderly PRGF. Density of cells in culture was estimated using the CyQuant Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA). Briefly, medium was removed and wells were washed carefully with phosphate buffered saline (PBS) and the microplates were frozen at -80°C overnight for efficient cell lysis in the CyQuant assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Ku/ml) diluted in cell lysis buffer during 1 hour at room temperature. Then, 2x CyQuant GR dye/cell lysis buffer was added to each sample well, mixed gently and incubated for 5 minutes at room temperature protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Germany). A DNA standard curve ranging from 7.8 to 1000

ng/mL was included in all fluorescence quantifications. All treatments were assayed in triplicates.

Cell Migration assay

Human dermal fibroblasts at passage 4 were seeded after subculture in culture inserts (Ibidi, Martinsried, Germany) previously placed on a 24 well plate at high density (30000 cell/cm2). These inserts consisted of two wells as cell growth areas (0.22cm2/well) and a cell-free gap (500±50 μm) between them. Cell cultures were maintained with culture medium for 48 hours until confluence. After this period, culture inserts were removed carefully and two separated cell monolayers leaving a cell-free gap were created. Cells were washed with PBS and incubated with serum-free culture medium supplemented with 0.1% (v/v) FBS as a control of non-stimulation, 5% (v/v) Young PRGF or 5% Elderly PRGF, 10% Young PRGF or 10% Elderly PRGF and 20% (v/v) Young PRGF or 20% (v/v) Elderly PRGF for 24 hours. After this period, treatments were removed and cells nuclei were stained with 1/500 Hoechst 33342 (Molecular Probes-Invitrogen, Grand Island, NY, USA) in PBS for 10 minutes. Phase contrast images of the gap were captured at time 0 hours and phase contrast and fluorescence images of the gap were captured at time 24 hours of incubation using a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The migratory cells found in this gap were measured using the Image J Software (NIH, Bethesda, MD). The results were expressed as the number of cells migrated per square centimeter of area. All treatments were assayed in triplicates.

Statistical analysis

After checking the normal distribution (Shapiro Wilks test) and homoscedasticity (Levene test) from groups, differences between groups were analyzed using the T-Student or variance analysis (ANOVA) test. In those cases in which the conditions for the application of parametric tests were not achieved (no normal distrubution and/or no homocedasticity), significant differences were analyzed using Mann Whitney and Krustal Wallis tests. Statistical differences between groups were accepted for *P values of 0.05 and **P values of 0.01.

Results

Cell isolation and characterization

Isolated and cultured cells showed fibroblast-like elongated and spindle-shaped appearance. Immunofluorescence microscopy confirmed that fibroblasts were uniformly positive for Collagen type I, Vimentin and Fibronectin, whereas slightly positive for alfa-SMA and negative for the endothelial cell marker P-Cytokeratin. These results confirm the dermal fibroblast nature of the isolated cells (Fig 1 and 2).

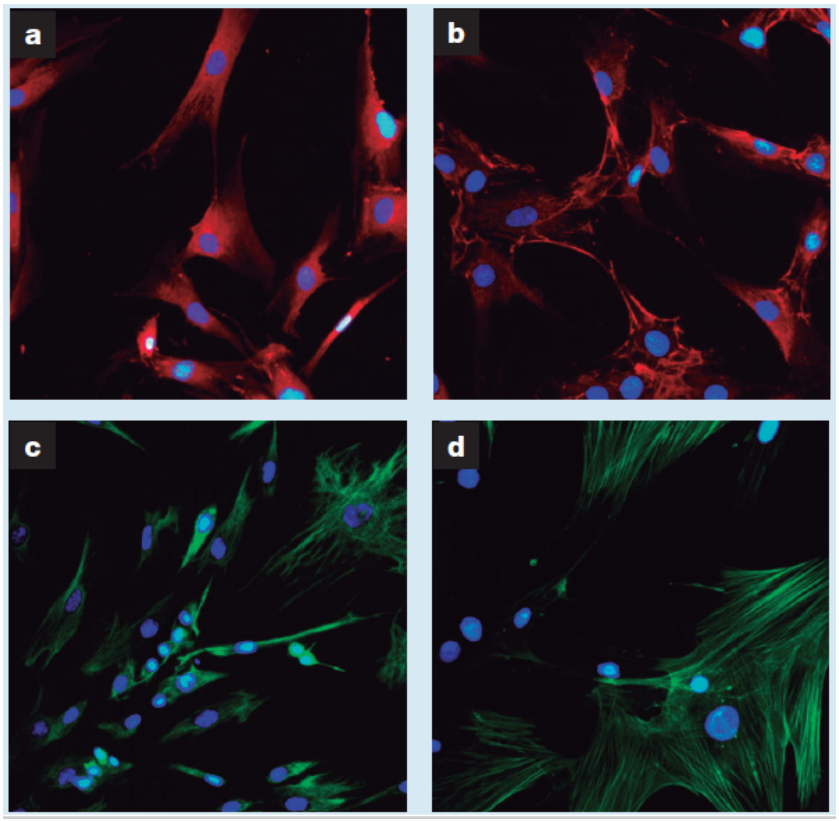

Figure 1: Immunofluorescence characterization of human dermal fibroblasts. Cells were tested for collagen type I (a), fibronectin (b), vimentin (c) and alfa-SMA (d).

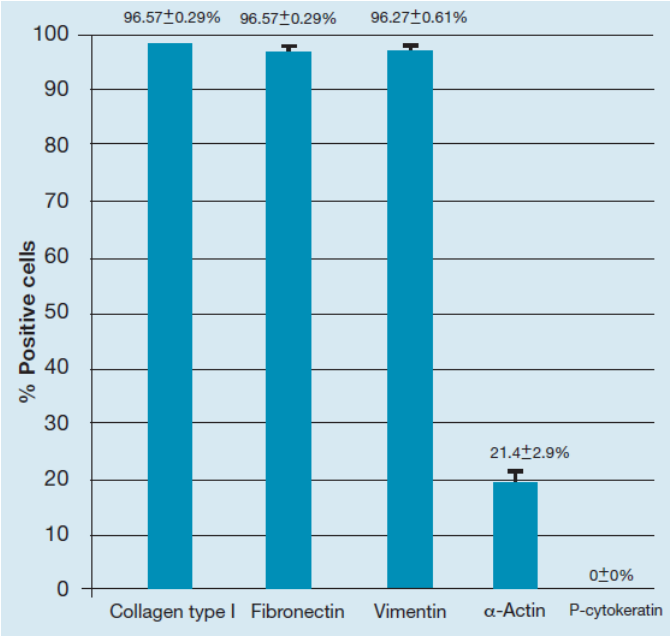

Figure 2: Results showed the dermal fibroblast nature of isolated cells.

PRGF-Endoret cell enrichment and characterization

Blood from three young donors and three elderly donors was obtained and F2 of PRGF was prepared. Blood and PRGF samples were analyzed in a hematology analyzer and results of mean platelet concentration were obtained. Young donor´s PRGF reached a 2.4X mean platelet enrichment and elderly donor´s PRGF reached a 2.0X mean platelet enrichment (Table 1). There were no significant differences between both PRGF-Endoret groups. Additionally, concentrations of several key growth factors for skin regeneration were measured, including PDGF, EGF, IGF, FGFb and HGF (Table 1). No significant differences in growth factor concentration levels were observed when young and elderly PRGFs were compared. None of the PRGF formulations contained leukocytes in a substantial amount.

Donor	Age (years)	Platelets in blood (platelet/µl)	Platelets in PRGF (platelets/µl)	Platelet enrichment	Mean enrichment	[PDGF-ab]/ platelet x10^3	[EGF]/ platelet x10^3	[IGF-I]/ platelet x10^4	[FGFβ]/ platelet x10^5	[HGF]/ platelet x10^3
1	25	147,000	308,000	2.1X		27.7	2.1	4.2	6.0	1.5
2	35	221,000	638,000	2.9X	2.4X	46.4	1.6	1.4	4.6	1.0
3	34	185,000	391,000	2.1X		27.4	1.6	2.9	2.7	1.3
4	52	154,000	293,000	1.9X		29.3	1.6	3.3	5.7	1.5
5	57	105,000	154,000	1.5X	2.0X	43.8	2.7	5.3	10.1	3.1
6	56	143,000	385,000	2.7X		25.8	1.4	2.2	2.1	2.2

Table 1: Plasma rich in growth factors (PRGF) platelet count and enrichment from 18-35 and 50+ age groups.

Dermal fibroblast biosynthesis

The expression of several growth factors and extracellular matrix proteins from cells stimulated by PRGF-Endoret obtained from young or elderly patients was assayed (Fig 3). After 72 hours of incubation, VEGF, TGFβ1 and Pro-collagen type I levels were measured. In all cases, PRGF-Endoret treated cells expressed significantly higher levels of these bioactive molecules compared to non-stimulated cell populations ($p<0.01$). With the exception of VEGF levels, no significant differences in TGFβ1 and Pro-collagen type I levels were observed after stimulation with the different PRGF-Endoret formulations.

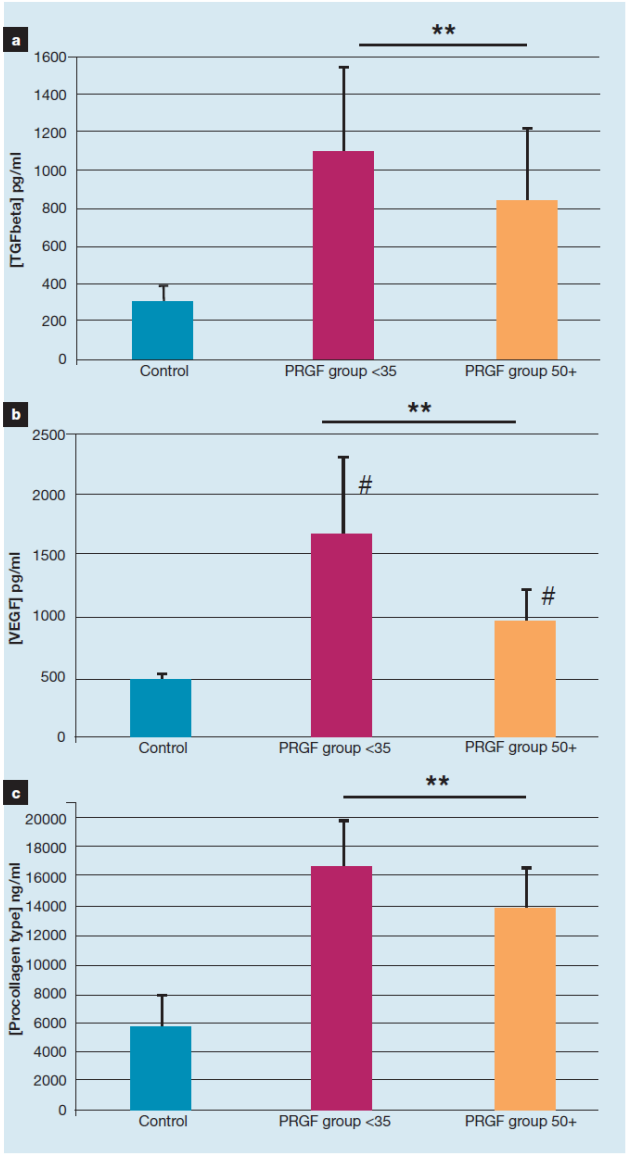

Figure 3: Effect of plasma rich in growth factors (PRGF) on dermal fibroblast biosynthesis and extracellular matrix protein expression. Confluent cells were cultured with 20% PRGF from groups <35 and 50+ for 72 hours. Results showed that both growth factors TGF-β1 (a) and VEGF (b) were significantly overexpressed

($p<0.01$) in PRGF cultured cells compared to control treated ones. Similarly, the ECM protein procollagen type I (c) was significantly overexpressed ($p<0.01$) in PRGF cultured groups. Additionally, no statistical differences were observed between both groups in TGF-β1 and procollagen type I levels. However, VEGF levels were significantly lower ($p<0.01$) in PRGF from group +50 treated cells. **$p<0.01$ versus control grooup; #$p<0.01$ between PRGF from groups <35 and 50+.

Cell proliferation

After 72 hours of treatment, a dose dependent response was observed in cell proliferation levels. In fact, cell proliferation was significantly enhanced with each PRGF-Endoret dose (5%, 10% and 20%) compared to the non-stimulated control ($p<0.01$). Increasing concentrations of PRGF-Endoret lead to a significantly higher proliferation level of dermal fibroblasts ($p<0.01$), with the best results observed at 20% PRGF treatment in which a 2.3-fold increase was reached (Fig 4 and 5). In addition, cell proliferation outcomes obtained with both types of PRGF-Endoret (prepared from young and elderly patients) were significantly higher compared to non-stimulated group ($p<0.05$) at 72 hours. No statistical significances were observed between PRGF-Endoret groups (Fig 6).

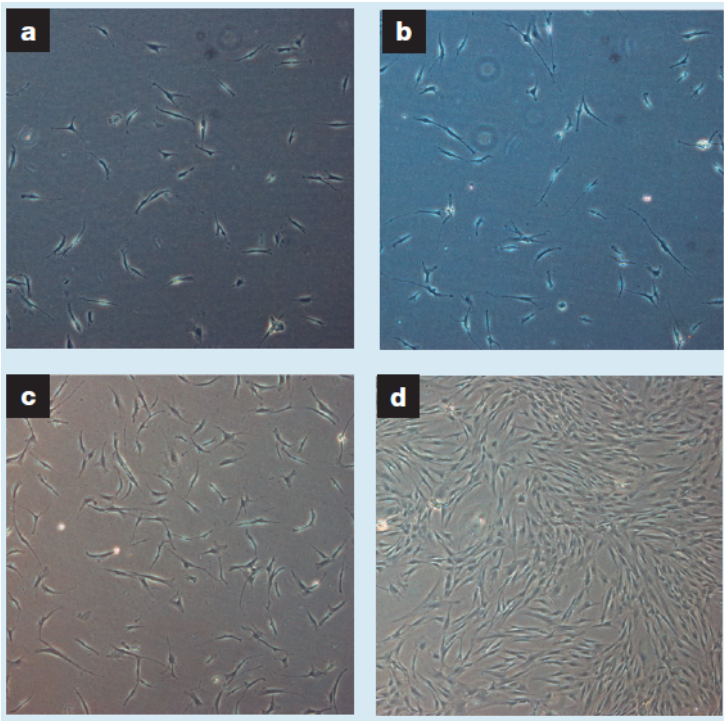

Figure 4: Effect of plasma rich in growth factors (PRGF) on dermal fibroblast proliferation after 72 hours. Phase contrast photomicrogtaphs illustrating the proliferation rate of cells cutured with control medium (a), 5% PRGF (b), 10% PRGF (c) and 20% PRGF (d) are shown.

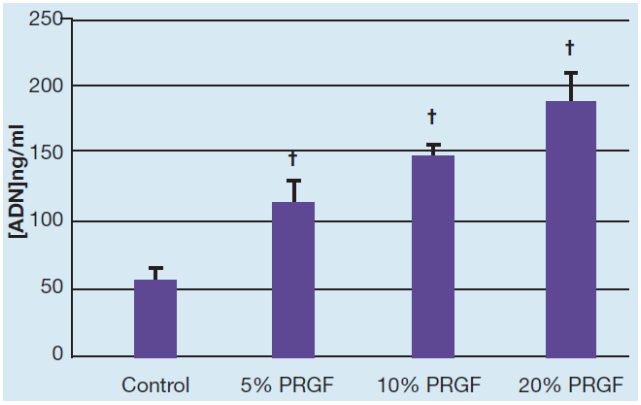

Figure 5: Each treatment showed significantly higher response than the control group (†p<0.01). A dose-dependent response was observed reaching the highest proliferation at 20% plasma rich in growth factors (PRGF) treatment.

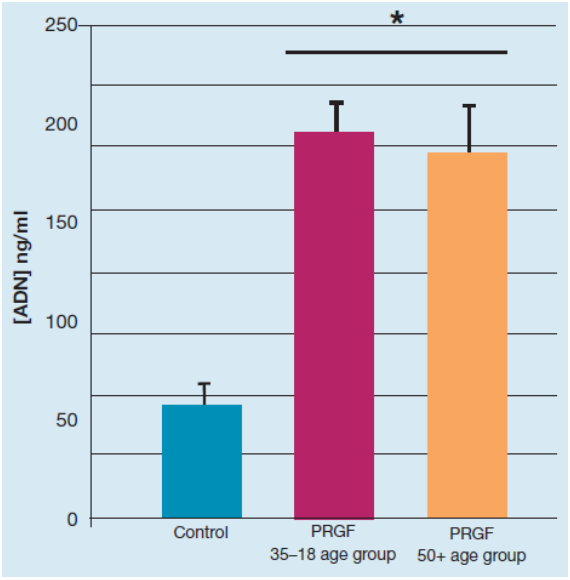

Figure 6: Effect of plasma rich in growth factors (PRGF) from 18-35 and 50+ age groups on the proliferation rate of dermal fibroblasts. Cells were cultured for 72 hours with 20% PRGF. Both treatments showed significantly higher outcomes than the control group (*p<0.05), but no statistical significance was observed between the age groups.

Cell migration

Cell migration was significantly enhanced with each PRGF-Endoret dose (5%, 10% and 20%) compared to the non-stimulated control (p<0.01). Treatment with PRGF-Endoret resulted in a mean 8-fold increase in cell migration outcomes (Fig 7 and 8). Furthermore, cell proliferation results obtained with both types of PRGF-Endoret (prepared from young and elderly patients) were significantly higher compared to non-stimulated group (p<0.05) at 24 hours. No statistical significances were observed between both PRGF-Endoret groups (Fig 9).

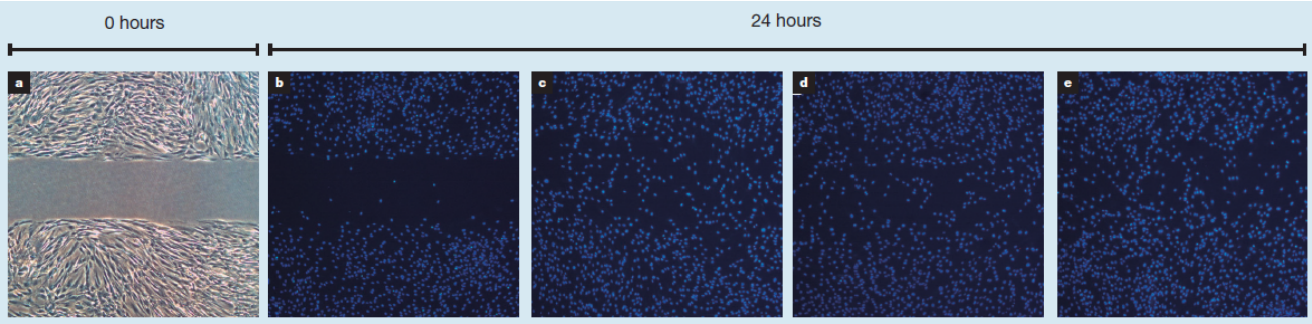

Figure 7: Effect of plasma rich in growth factors (PRGF) on dermal fibroblast migration after 24 hours. Hoechst stained fluorescence microphotographs are shown comparing time 0 hours untreated cells and time 24 hours cells at different PRGF dose levels. Brightfield image (a), control (b), 5% F2 (c), 10% F2 (d), 20% F2 (e).

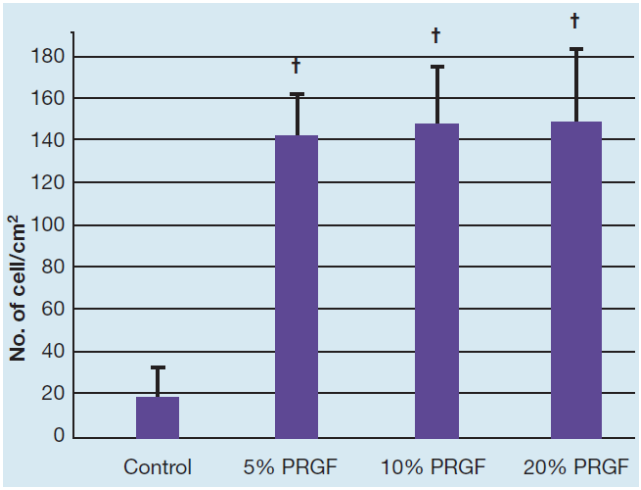

Figure 8: Results showed strong differences between plasma rich in growth factors (PRGF) treated cells and control group (†p<0.05). However, no dose-dependent response was observed between different PRGF concentrations tested.

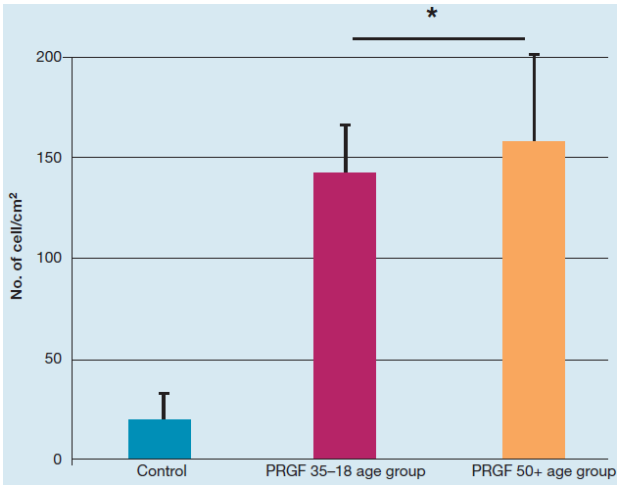

Figure 9: Effect of plasma rich in growth factors (PRGF) from two age groups on the migration rate of dermal fibroblasts. Cells were cultured for 24 hours with 20% PRGF. Both treatments showed significantly

higher outcomes than the control group (*p<0.05), but no statistical significance was observed between the age groups.

Discussion

Growth factors secreted by platelets and those present in human plasma play a major role in many processes involved in the wound healing cascade promoting cell mitosis, migration and extracellular matrix remodeling.18 Upon activation, platelets release a wide variety of proteins and cytokines which have key effects in dermal/epidermal regeneration.21 Some of these proteins include platelet derived growth factor (PDGF), transforming growth factor β1 (TGFβ1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF).36, 37 This pool of morphogens promote damaged tissue regeneration by inducing proliferation, migration, angiogenesis, biosynthesis and extracellular matrix remodeling.38,39

Results from the present study show that PRGF-Endoret prepared from young patients (<35 years old) or from moderate to advance aged patients (>50 years old) significantly enhances the biological behavior of human dermal fibroblasts regarding cell proliferation, migration and the biosynthetis of auto/paracrine proteins and extracellular molecules. Interestingly, no significant differences were observed between age-dependent PRGF-Endoret treatments, reinforcing the idea that this biological approach might be either used in young or elderly patients. However, the present study is based on preliminary *in vitro* data involving dermal fibroblast culture. Additional clinical studies with a greater sample size must be carried out before assuming that plasma rich in growth factors technology is a clinically effective treatment for patient of all ages. Patients of different age ranges treated with PRGF should be compared regarding wound healing efficacy in pathologies such as ulcers or burns. The degree of reepithelization surface, post-surgical infection events and recovery time could be acceptable variables in order to detect clinically relevant differences. Although based on preliminary *in vitro* studies with a relatively low sample size, our findings suggest that growth factor levels and pivotal biologic processes for tissue regeneration might not be influenced by the age of the patient.

In the last decade, major efforts have been devoted to understand, characterize and optimize the formulation of PRGF-Endoret. This approach consists in a 100% autologous formulation composed of donor's plasma and platelets with some distinguishing properties including the absence of pro-inflammatory leukocytes and a

moderate concentration of platelets. The latter has been related to optimal biologic benefit (2X mean platelet enrichment).18,40,41 Additionally it has been already tested the effect of platelet poor plasma and other combinations between plasma fractions in other primary cell types such as gingival fibroblasts, synovium cells or tenocytes. 19 These studies reveal that decreased biological outcomes are achieved when platelet poor plasma or lower concentrated plasma fractions are used. Therefore this preliminary work tries to answer the question whether the platelet rich fraction of PRGF (F2) has a relevant biologic effect.

In our study, we demonstrate that PRGF technology stimulates the essential processes of wound healing and dermal/epidermal regeneration. On the one hand, it enhances the proliferation rate in a dose dependent manner, achieving the highest regenerative potential at a concentration of 20% (2.3 fold increase). Dermal fibroblast migration is also stimulated, but in contrast to proliferation results, not in a dose dependent manner. These findings are consistent with other studies using platelet released supernatants.42-44 Furthermore, attending to our results, we can confirm that the age of the donor does not interfere with the trophic potential of dermal fibroblasts cultured with PRGF-Endoret. In both cases, cell proliferation and migration were enhanced in between 3.5-fold and 8.3-fold compared to the control group. Clinical outcomes have shown that age is not a major limitation when using platelet and plasma-based products for the treatment of different dermatological pathologies.20,45

Another interesting observation was that cell treatment with both PRGF-Endoret formulations increased the secretion of proangiogenic and mitogenic factors (VEGF and TGFβ1), and structural extracellular matrix proteins (Procollagen type I). These results are similar to others that reported high levels of VEGF and HGF 19,46 and Procollagen type I carboxyterminal peptide and pro-alfa collagen chains Collagen type I alfa1/alfa2 47 production from dermal fibroblasts after platelet rich plasma incubation. The angiogenic and mitogenic nature of VEGF and TGFβ1, have been widely demonstrated in several studies 4,38, and probably these growth factor stimulating action, could be the reason of the angiogenic and wound closure effects reported in numerous *in vivo* studies and clinical trials. 22,23,26 Furthermore, procollagen type I is the mayor structural protein found in ECM of the skin, and it plays a pivotal role in wound healing and hemostasis 48, so the increase seen in this macromolecule after PRGF treatment is consistent with studies reporting an improvement in wound healing and tissue regeneration under the same conditions tested. 11,24 Moreover, although we have not seen a decrease in TGFβ1 and procollagen I secretion when using elderly

PRGF, a decrease in VEGF production has been reported. This could be a natural consequence of the aging effect in PRGF, but regardless the results obtained in elderly treatments, PRGF has shown an improving effect in all cases compared to the control ones. So we can conclude that, even though the secretion of some bioactive molecules is lower when using elderly PRGF, the results are in any case better than the control treatment. This affirmation can be sustained by a number of studies in which PRGF has been used in either young or elderly patients with excellent results. 49

Other studies have tried to shed some light over the biomolecular mechanisms behind the regenerative activity of platelet rich plasma preparations. Some findings suggest that growth factors present in PRGF such as FGFb , enhance the dermal fibroblast phenotype by promoting the apoptosis of persistent myofibroblasts thus reducing the fibrotic tissue development. This mechanism is related with the down regulation of PI3K/Akt pathways as well as with the up regulation of Rho/Rho kinase signaling. 50 HGF has also proved to promote scarless tissue regeneration by means of Smad 7 protein overexpression along with a decrease of Smad 2 and Smad 3 phosphorylation . 51 Other studies have concluded that PRP might be involved in the reduction of the inflammatory phase of wound healing due to the attenuation of the NFκB pathway. 52 Wound contraction has additionally been related with the activity of PDGF and other cytokines, where the activation of PAR-1 and PLC induce the extracellular calcium influx via L-type calcium channel and the calcium/CaM-MLCK and Rho kinase activation pathways. 53 Moreover, platelet rich plasma has been shown to activate several anti-apoptotic regulators such as Bcl-2 protein, thus exerting an important cell death preventive effect. 54 In line with these findings, some studies suggest that plasma rich in growth factors enhances the antioxidant activity within the wound area by the overexpression of the antioxidant response element (ARE) via Nrf2 nuclear factor upregulation, ending up in a reduction of local reactive oxygen species levels. 55 The increased bioactivity reported in the present study, could be finally related with the ERK/Akt pathway activation and CDK4/Cyclin D1 overexpression, as it has been demonstrated by studies where the use of autologous growth factors led to an increased cell growth. 56

Conclusions

Although further studies are needed to elucidate the underlying mechanism by which PRGF works, taken together, these findings suggest that this technology is safe and capable of stimulating skin regeneration by enhancing proliferation, migration and expression of pivotal bioactive molecules involved in wound healing and hemostasis. A full proteomic characterization of the PRGF would help to clarify some of its cellular targets and metabolic pathways. In fact, it would be interesting to selectively suppress some of the growth factors within this pool of morphogens in order to accurately detect the exact biologic role of each plasmatic protein. These *in vitro* findings could lead to a next step in plasma rich in growth factor technology allowing to selectively increase or delete some of its molecules thus improving the efficiency of PRGF depending on the pathology required. Additionally *in vivo* and clinical research is needed to clearly state PRGF technology as an effective treatment for dermatological disorders where cutaneous tissue regeneration is pivotal. Randomized and controlled clinical trials with appropriate sample size are encouraged with the aim of testing the safety and efficacy of plasma rich in growth factors for the treatment of poor response dermatologic pathologies such as chronic ulcers and burns.

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Artículo II

Plasma rich in growth factors inhibits ultraviolet B induced photoageing of the skin in human dermal fibroblast culture.

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Title: Plasma rich in growth factors inhibits ultraviolet B induced photoageing of the skin in human dermal fibroblast culture

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Conflict of Interest:
E Anitua is the scientific director of, G.O. and A.P. are scientists at BTI-Biotechnology Institute, the company that has developed the PRGF-Endoret technology.

Abstract

Background: Ultraviolet irradiation is able to deeply penetrate into the dermis and alter fibroblast structure and function, leading to a degradation of the dermal extracellular matrix.

Objectives: The regenerative effect of plasma rich in growth factors (PRGF) on skin ageing was investigated using UVB photo-stressed human dermal fibroblasts as an *in vitro* culture model.

Method: PRGF was assessed over the main indicative features of ultraviolet B irradiation, including ROS formation, cell viability and death detection, apoptosis/necrosis analysis and biosynthetic activity measurement. Four different UV irradiation protocols were tested in order to analyze the beneficial effects of PRGF.

Results: Ultraviolet irradiation exhibited a dose dependent cytotoxicity and dose of 400mJ/cm^2 was selected for subsequent experiments. PRGF increased the cell viability and decreased the cell death comparing to the non-treated group. The apoptosis and necrosis were significantly lower in PRGF treated fibroblasts. ROS production after UV irradiation was significantly reduced in the presence of PRGF. Procollagen type I, hyaluronic acid and TIMP-1 levels were higher in the when treated with PRGF.

Conclusion: This preliminary *in vitro* study suggests that PRGF is able to prevent UVB derived photo-oxidative stress and to diminish the cell damage caused by ultraviolet irradiation.

Keywords: plasma rich in growth factors, dermal fibroblasts, photo-oxidative stress, skin regeneration

Introduction

Healthy and good looking skin plays a key role in the psychological welfare of modern societies due to its incidence in self-image and self-steem. However, as life expectancy increases, skin ageing is becoming a main concern in developed countries. Skin ageing can be attributed to intrinsic and extrinsic factors that taken together lead to cumulative alterations on cutaneous structure which are commonly manifested by increased wrinkling, laxity, loss of tensile strength and sagging [1]. Intrinsic (chronological) factors include genetics, hormonal changes and metabolic processes whereas the main extrinsic factor remains on sun exposure derived ultraviolet irradiation.

UV light can be classified into UV-A, UV-B and UV-C. Although the UVC is the most dangerous one, it is mainly absorbed by the ozone layer. However, UVA and most importantly UVB are essential components of sunlight that generate severe oxidative stress in dermal fibroblasts and epidermal keratocytes. This photodamage comes via interaction with intracellular chromophores that cause the activation of downstream cascades resulting in genetic damage, connective tissue degradation and replicative senescence [2]. Some histological studies have revealed that photodamaged skin is associated with the accumulation of amorphous elastin fibers beneath the epidermal-dermal junction (actinic elastosis), reduced collagen type I and III levels and increased breakdown of extracellular matrix (ECM) proteins such as hyaluronic acid [3]. Furthermore, UVB exposure has been shown to stimulate collagenase production by human dermal fibroblasts and to down regulate matrix metalloprotease inhibitors such as TIMP-1 [4]. However, the major pathogenic agents triggered after UV damage are the Reactive Oxygen Species (ROS) which can alter gene activity and protein function after photo-oxidative stress [5].

In the last few years, several biological compounds have been tested with the aim of preventing cutaneous damage from ultraviolet radiation. Some examples include chromenes from seawedds [6], plant extracts [7-11], mushrooms preparations [12], chitooligosaccharides [13], retinoic acid [14], polyphenols [15], and vegetable derived flavonoids [16]. Moreover, some regenerative medicine approaches have been studied with the same purpose using stem cell therapy [17] and recombinant growth factors [18]. In line with these innovative techniques another interesting alternative lies on the application of autologous platelet rich plasma. Plasma rich in growth factors (PRGF-Endoret) technology is based on the recovery of a small volume of the patient´s own blood which is afterwards centrifuged and activated in order to obtain autologous

formulations enriched in proteins and growth factors [19-21]. Recent results demonstrate the potential of PRGF-Endoret in the treatment of several disorders in a wide range of medical fields such as dermatology [22-26], aesthetic surgery [27-28], orthopedic surgery [29], sports medicine [30-31], ophthalmology [32-34], neurobiology [35] and oral/maxillofacial surgery [36-37]. The pool of active proteins present in PRGF promote tissue regeneration and activate a wide range of biological processes such as cell recruitment, proliferation, migration, differentiation, neovascularization and ECM deposition [38-39].

In this preliminary *in vitro* study we have evaluated the effect of PRGF prepared form different donors on photo stressed human dermal fibroblasts. Several ultraviolet and PRGF treatments were assessed over the main biological outcomes in response to photo-oxidative stress.

Materials and methods

Cell isolation

Dermal fibroblasts were isolated from skin biopsies of three healthy patients during joint surgery (29, 32 and 31 years old respectively). Written informed consent was obtained from all patients before the biopsy was performed. Cells were isolated and viability was assessed following our standard protocol [20].

Human dermal fibroblast characterization

The fibroblast-like morphology of cells was checked by phase-contrast microscopy and characterized by immunofluorescence following our standard protocol [20].

Plasma Rich in Growth Factors (PRGF) preparation and characterization

The study was performed following the principles of the Declaration of Helsinki. Blood from 8 healthy donors (between 25 and 56 years old being the mean age 44±14 years) was collected after obtaining informed consent. Following a single spin protocol, the platelet rich fraction of blood plasma (F2) was collected and after platelet activation the released supernatants were stored at -80°C as previously described [34]. Once the PRGF was obtained, several growth factors involved in skin regeneration were measured by commercially available Enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems, Minneapolis, MN): Hepatocyte growth factor (HGF), Platelet derived growth factor (PDGF), Fibroblast growth factor-b (FGFb), Transforming growth factor

β1 (TGFβ1), Insulin like growth factor (IGF), Vascular endothelial growth factor (VEGF) and Epidermal growth factor (EGF). All experiments were performed in triplicate using PRGF from these 8 donors.

Cell culture and UVB irradiation

Four different irradiation protocols were followed in order to test the beneficial effects of PRGF against UVB radiation (Fig. 1): A) Protection, B) Regeneration, C) Protection+Regeneration and D) Prevention+Protection. All the protocols included a synchronization period in which human dermal fibroblasts were seeded at high density with serum-free culture medium for 24 hours. For "A" protocol, cells were irradiated with UVB in the presence of serum-free culture medium supplemented with 20% PRGF (referred to below as PRGF treatment) and maintained 24 hours with serum-free culture medium (referred to below as basal medium). For "B" protocol, cells were irradiated with UVB in the presence of PBS and cultured afterwards with PRGF treatment for 24 hours. For "C" protocol, dermal fibroblasts were exposed to UVB radiation in the presence of PRGF and cultured over 24 hours with the same treatment. For "D" protocol cells were incubated with PRGF 24 hours following the synchronization period and afterwards were irradiated with UVB in the presence of the same treatment. Finally, the cells were maintained in basal medium for 24 hours. Negative control cells were maintained with complete medium and did not receive UVB radiation. Positive control cells were cultured with basal medium instead of PRGF.

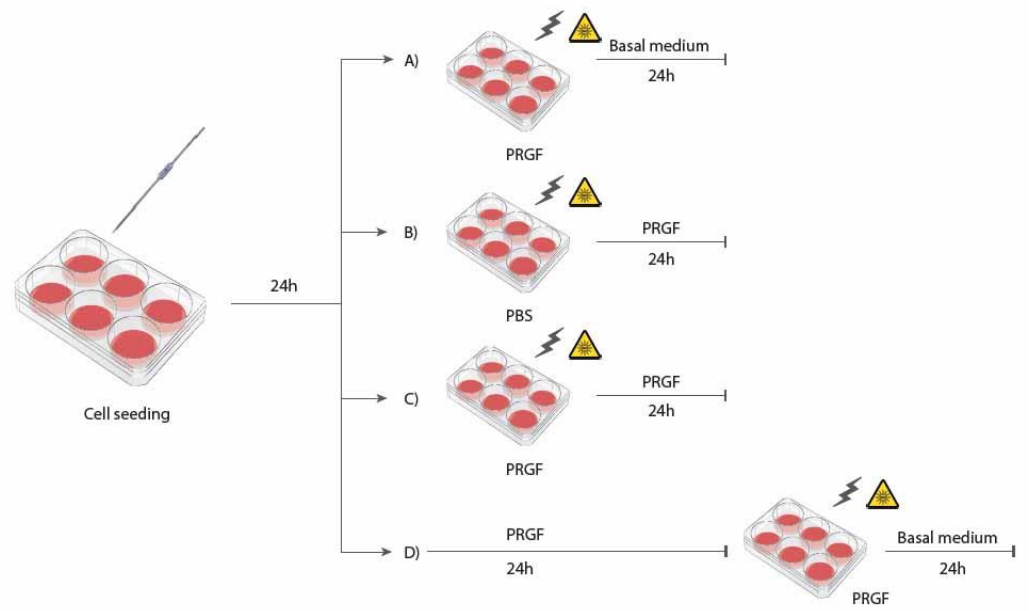

Fig.1. Four different irradiation protocols were followed in order to test the beneficial effect of PRGF against UVB: A) Protection, B) Regeneration, C) Protection+Regeneration and D) Prevention+Protection.

Phototoxicity assay

Passage 5 cells were plated at a density of 10000 cells/cm^2 in 24 well culture plates and maintained for 24 hours with basal medium. Cells were exposed to different doses of UVB in the presence of PBS: 600mJ/cm^2, 400mJ/cm^2, 200mJ/cm^2, 100mJ/cm^2 and 10mJ/cm^2. Afterwards, the cells were maintained in basal medium for 24 hours. The UVB source was a UV lamp (Vilber Lourmat, Marne la Vellee, France) which was used to deliver an energy spectrum with high intensity in the UVB region (312nm). Cell survival percentage was measured by the spectrophotometric Cell viability reagent WST-1 assay (Roche, Mannheim, Germany). Apart from analyzing cell survival percentages, the optimal UVB dose was selected for the following experiments.

Determination of morphological and structural changes

Morphological and structural changes in dermal fibroblasts culture were investigated to determine the cell damage caused by UVB irradiation. Cells were seeded and maintained in basal medium for 24 hours. Afterwards, cells were exposed to UVB radiation in the presence of PRGF and cultured over 24 hours with the same treatment. Finally, cells were immunolabeled for collagen type I, vimentin and fibronectin and visualized with fluorescence microscope as described above.

Cell survival assay

24 hours after seeding, cells were irradiated with an ultraviolet-B dose of 400 mJ/cm^2 following the irradiation protocols described above. Finally cell survival percentage was measured by the spectrophotometric Cell viability reagent WST-1 assay (Roche, Mannheim, Germany).

Live/dead cell analysis

After 24 hours of high density seeding, cells were irradiated with UVB (400 mJ/cm^2) in the presence of PRGF treatment and maintained 24 hours with basal medium. Finally live and dead cells were marked with a sensitive two-color fluorescence assay Live/Dead Cell Imaging Kit (Molecular Probes-Invitrogen, Grand Island, NY, USA). Cells were visualized under x10 objective with a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany) and photographs were taken over three random microscopic fields per well. The digitalized images were analyzed using the Image J Software (NIH, Bethesda, MD). Green stained dermal fibroblasts were counted as live cells and red stained ones were counted as dead cells.

Cell apotosis/necrosis assay

With the aim of distinguishing apoptotic from necrotic fibroblasts among dead cells, the immunophotometric Cell Death Detection ELISA plus kit (Roche, Mannheim, Germany) was used. After 24 hours of high density seeding, cells were irradiated with an ultraviolet-B dose of 400 mJ/cm^2 following the irradiation protocols described above. When each protocol concluded, culture medium was collected and identified as necrotic fraction while the adherent cells were lysed and centrifuged at 200g for 10 minutes. The supernatant was identified as apoptotic fraction as manufacturer´s indications.

Detection of Reactive Oxygen Species (ROS) production

The production of ROS by human dermal fibroblasts after UVB radiation was measured using a fluorimetric quantitative Reactive oxygen species assay kit (Abcam, Cambridge, UK). Cells were seeded at high density in 96 well optical bottom black culture plates and after 24h, cells were irradiated with as described above. After each irradiation protocol, production of ROS was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies) every 20 minutes during two hours.

Dermal fibroblast biosynthesis

The biosynthetic potential of proteins such as Pro-collagen Type I, Hyaluronic acid and TIMP-1 ere measured after UVB radiation. Cells were seeded with complete medium until confluence. Afterwards, cells were irradiated as described above. After each irradiation protocol, culture medium was collected and centrifuged at 460g for 10min, and supernatant was stored at -80°C until assayed. The amount Pro-collagen type I (Takara, Shiga, Japan), Hyaluronic acid (Corgenix, Broomfield, CO) and TIMP-1 (R&D Systems, Minneapolis, MN) was measured by Enzyme-linked immunosorbent assay (ELISA) kits.

Statistical analysis

The normal distribution and the homoscedasticity from groups was checked (Shapiro Wilks and Levene test respectively). Afterwards, T-Student or variance analysis (ANOVA) test was used to analyze differences between treatment groups. When statistical parametric test where not feasible, Mann Whitney and Krustal Wallis tests were performed to analyze significant differences. Statistical differences between groups were accepted for P values of 0.05 and 0.01. All data shown are represented as mean values ±SD.

Results

Cell isolation and characterization

Isolated and cultured cells showed fibroblast-like elongated and spindle-shaped appearance. Immunofluorescence microscopy confirmed that fibroblasts were uniformly positive for Collagen type I, Vimentin and Fibronectin, whereas slightly positive for alfa-SMA and negative for the endothelial cell marker P-Cytokeratin. These results confirm the dermal fibroblast nature of the isolated cells (Data not shown).

PRGF-Endoret cell enrichment and characterization

Blood from 8 healthy donors was withdrawn and F2 of PRGF was prepared. Blood and PRGF samples were analyzed in a hematology analyzer. Mean platelet enrichment reached 2.4X over peripheral blood. Additionally, concentrations of several key growth factors for skin regeneration were measured, including TGFβ1, PDGF, EGF, VEGF, IGF, FGFb and HGF (Table 1).

	Platelet enrichment			Growth factor content						
Donor age	Platelets in blood plate-lets/µlx10³	Platelets in PRGF plate-lets/µlx10³	Platelet enrichment	TGFβ-1 (ng/ml)	PDGF-AB (ng/ml)	EGF (pg/ml)	VEGF (pg/ml)	IGF (ng/ml)	FGFb (pg/ml)	HGF (pg/ml)
26	147	308	2.1X	33	9	634	-	128	18	451
25	221	638	2.9X	93	30	991	344	90	30	579
38	185	391	2.1X	31	11	609	234	115	11	526
34	190	463	2.4X	43	14	604	262	92	9	502
53	154	293	1.9X	26	9	462	-	97	17	458
58	105	154	1.5X	28	7	426	69	82	16	478
57	143	385	2.7X	35	10	543	95	86	8	849
59	112	408	3.6X	47	10	725	72	87	12	651
44 ±14	**157 ±40**	**380 ±141**	**2.4X ±0.7**	**42 ±22**	**13 ±7**	**624 ±176**	**179 ±116**	**97 ±16**	**15 ±7**	**562 ±134**

Table 1: Platelet enrichment and concentration of growth factors in PRGF

Phototoxicity assay and UVB dose selection

After UVB irradiation a dose dependent response was observed in cell survival outcomes (Fig. 2). The highest survival percentages were observed when dermal fibroblasts were exposed to low levels of UVB irradiation (77.3%±2.4 survival at 10mJ/cm^2) while the lowest survival percentages were achieved at high dosages (18.6%±0.5 survival at 600mJ/cm^2). As expected, increasing dosage of UVB radiation, lead to a decrease in cell survival percentages. Additionally, UVB dose of 400mJ/cm^2 was selected for subsequent experiments.

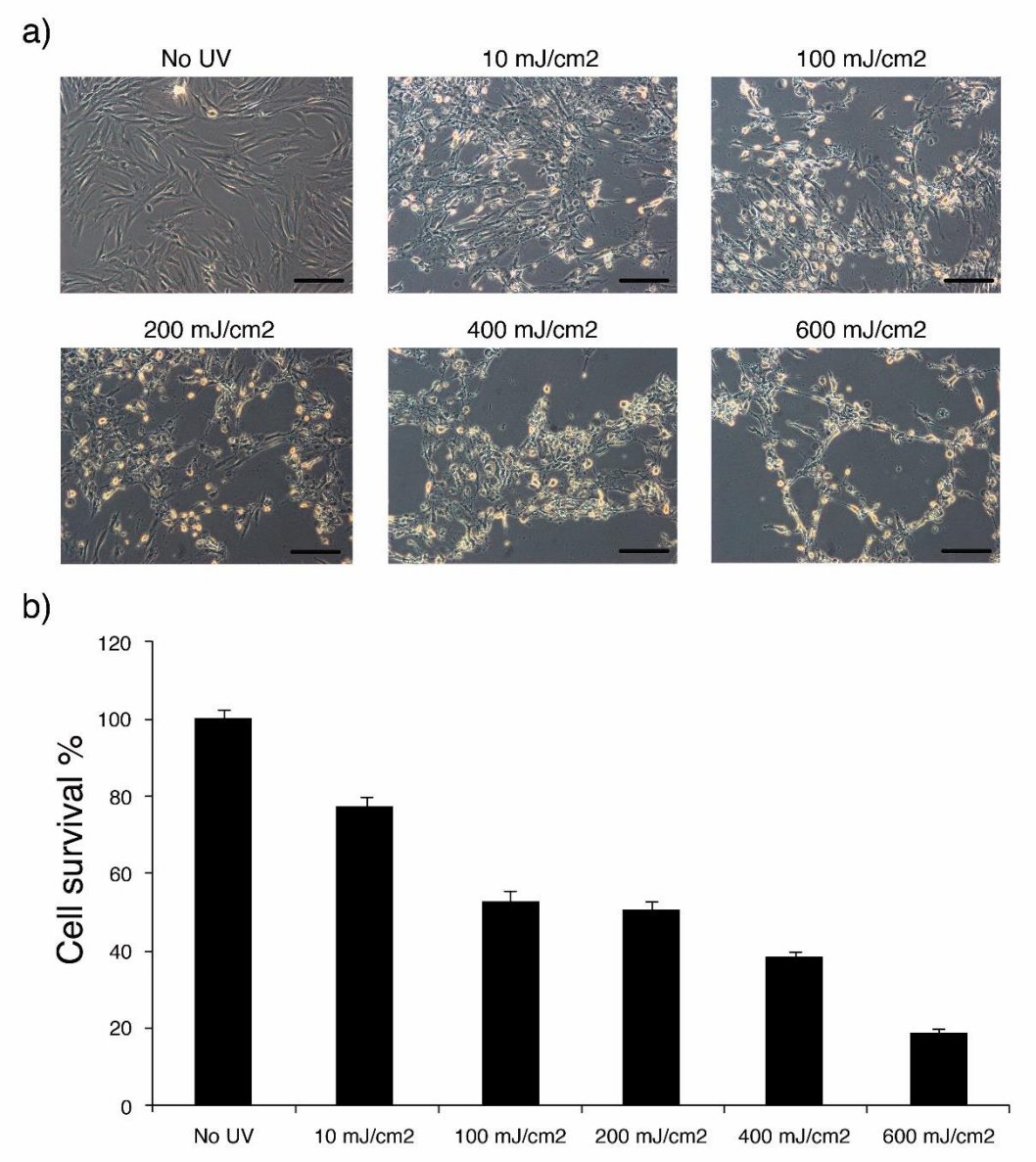

Fig.2. (a) Representative microphotographs taken under phase contrast microscopy illustrating the induction of phototoxicity on dermal fibroblasts after different doses of UVB. Scale bar= 200µm. (b) The survival percentages decrease when UVB doses applied increase, denoting a dose dependent response.

Morphological and structural changes

The subjective observation of morphological and structural changes in dermal fibroblasts can point out the cellular damage developed after UVB irradiation. Non-irradiated cells displayed typical fibroblast morphology with elongated shape and exhibited normal monolayer appearance. However, the tridimensional arrangement of extracellular matrix proteins such as Collagen type I, Fibronectin and Vimentin, involved in structural stability and homeostasis, suffered severe damage after UVB irradiation (Fig. 3). In contrast, when dermal fibroblasts were treated with PRGF, even under the same irradiation dose, their morphological features kept similar to non-

irradiated ones. UV+PRGF treated cell nuclei, showed more euchromatic appearance hence more active and functional than non-treated ones. Additionally, irradiated cells presented dense cytoplasms with heterochromatic nuclei, indicating apoptotic processes that were confirmed in subsequent analyses. Unlike in the absence of treatment, PRGF treated fibroblasts established cell to cell contact via Collagen type I and Vimentin, while the surrounding Fibronectin mesh maintained intact its tridimensional structure. In fact, fiber breakage level was higher among UVB irradiated cells, showing weaker cell-substrate adhesion when compared to UV+PRGF groups. Similar results were obtained when testing the remaining UVB protocols (data not shown).

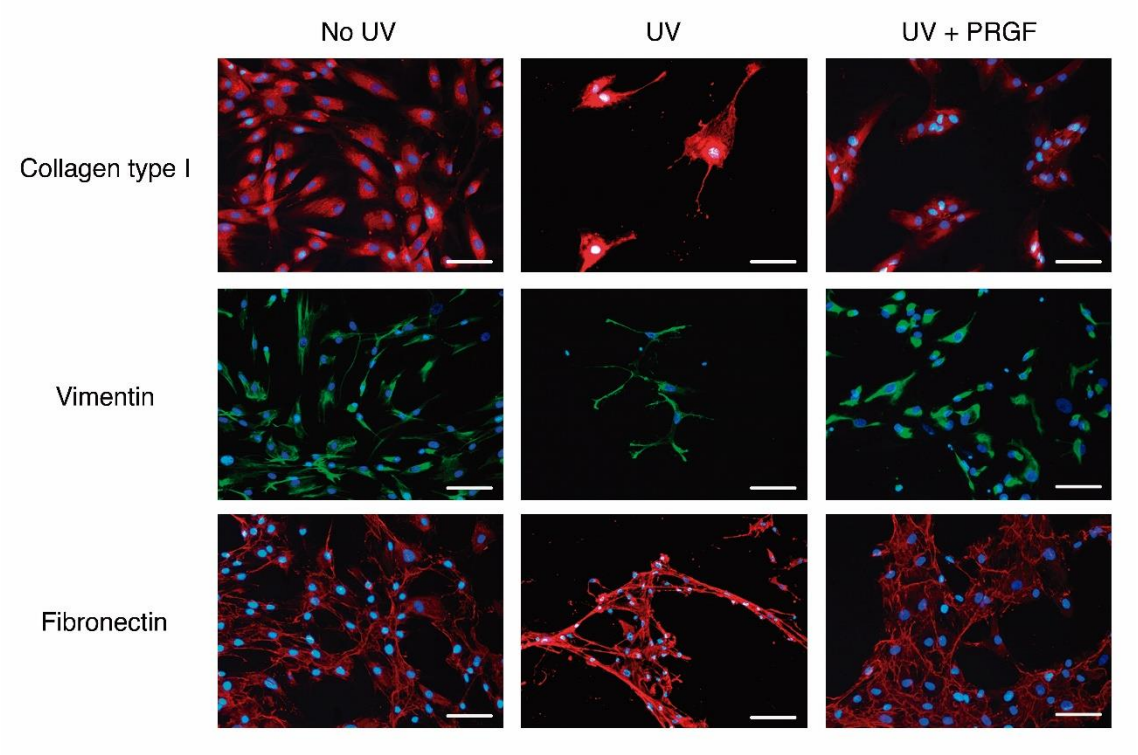

Fig.3. Representative microphotographs taken under fluorescence microscopy of three typical extracellular markers for dermal fibroblasts: Collagen Type I, Vimentin and Fibronectin. Cells were cultured in standard conditions (left), under UVB irradiation (middle) and under UVB irradiation plus PRGF (right). Scale bar= 50μm.

Cell survival

Survival of dermal fibroblasts after UVB irradiation was measured by the cell viability WST-1 assay (Fig. 4). With PRGF, significant improvement was observed for each Protection, Regeneration, Protection+Regeneration and Prevention+Protection protocol achieving survival rates of 51%±7, 49%±13, 58%±18 and 61%±16 respectively

(p<0.01). However, without PRGF treatment, viability decreased significantly by means of 24%±8, 19%±7, 17%±2 and 26%±4 respectively.

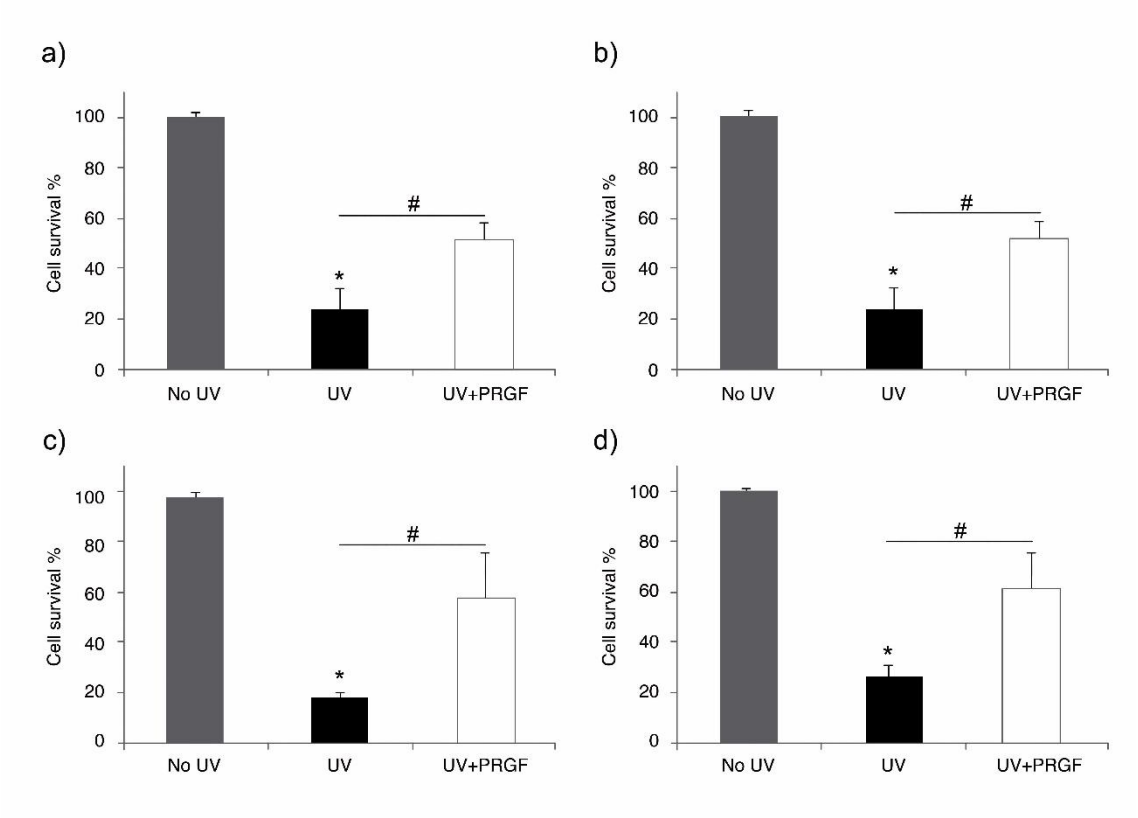

Fig.4. The viability of dermal fibroblast decreased significantly after UVB irradiation for each treatment protocol (p<0.01) (a) Protection, (b) Regeneration, (c) Protection+Regeneration and (d) Prevention+Protection. However, PRGF treated cells achieved significantly higher survival percentages compared to the no treated ones under the same irradiation conditions for every protocol tested (p<0.01). * p<0.01, significant differences between cell survival percentages achieved for non-irradiated and UVB irradiated cells. # p<0.01, significant differences between cell survival percentages achieved for PRGF and non-PRGF groups.

Live/dead cell analysis

After ultraviolet irradiation, fibroblasts were stained with a Live/Dead cell Imaging Kit and images taken under fluorescence microscope were analyzed with the Image J Software (Fig. 5). As expected, in the absence of UVB irradiation, the percentage of live cells (97%±2) was higher compared to the dead ones (3%±2). However, under identical ultraviolet dose, PRGF treated fibroblasts kept a positive live/dead ratio (79%±4/21%±4) while a reciprocal ratio was observed among the non-treated ones (32%±4/68%±4). In fact, life percentage among PRGF treated cells was statistically higher than for non-treated ones (p<0.01).

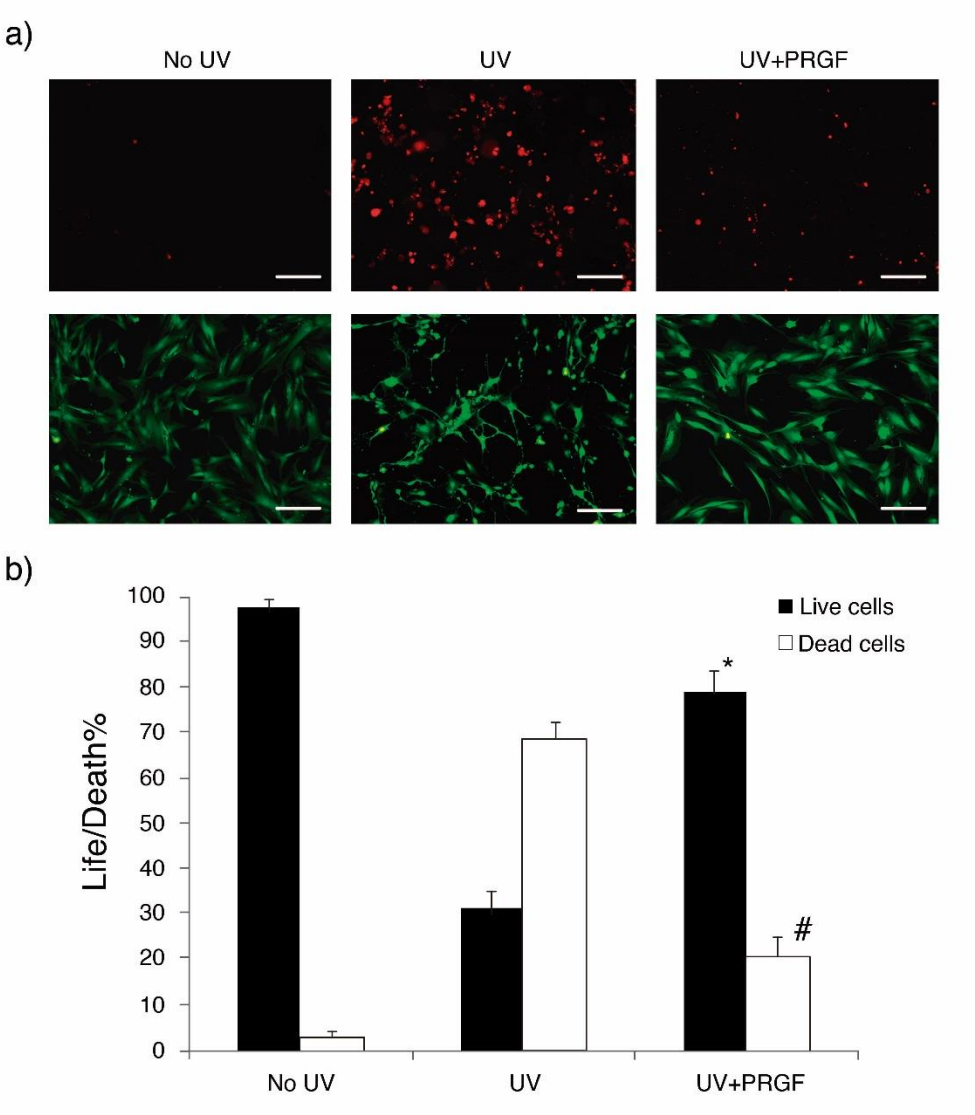

Fig.5. (a) Representative microphotographs taken under fluorescence microscopy showing dead cells (red stain, above) and live cells (green stain, below) 24 hours after UVB irradiation. Scale bar= 100µm. (b) Percentage of live cells is higher than the dead ones in absence of UVB, but in the presence of irradiation, death percentage increases. However, under UVB irradiation, PRGF treated cells keep a positive live/dead ratio while among non-treated ones, a negative live/dead ratio is observed. Interestingly, under the same irradiation dose, life percentage is significantly higher ($p<0.01$) and death percentage is significantly lower ($p<0.01$) if PRGF treatment takes place. * $p<0.01$, significant differences between life percentages achieved for PRGF and non-PRGF groups. # $p<0.01$, significant differences between death percentages achieved for PRGF and non-PRGF groups.

Apoptosis and necrosis determination

After ultraviolet irradiation, apoptotic and necrotic cells were analyzed using the Cell Death Detection ELISA plus kit (Fig. 6). In one hand, under no irradiation, baseline levels of apoptosis and necrosis were significantly lower comparing to irradiated cells ($p<0.01$) while in the other hand, after 24 hours of ultraviolet dose, both death

processes increased significantly. Additionally, the same cell death pattern was observed for each irradiation protocol, in which apoptotic cells overcome necrotic ones. However, PRGF managed to reduce cell death because apoptosis and necrosis levels among PRGF treated cells were significantly lower comparing to non-treated ones ($p<0.01$).

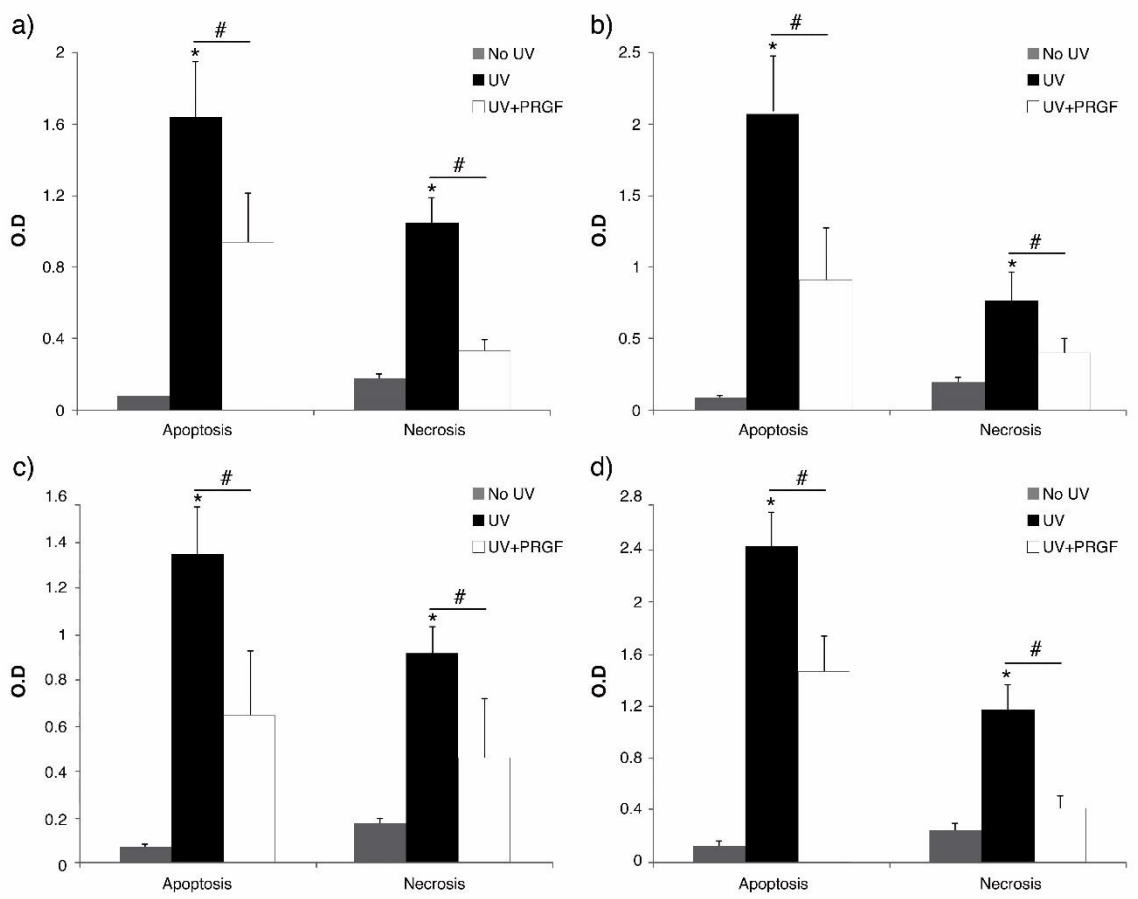

Fig.6. Apoptotic and necrotic levels reached by UV irradiated cells were significantly higher than those without irradiation ($p<0.01$) for each ultraviolet protocol (a) Protection, (b) Regeneration, (c) Protection+Regeneration and (d) Prevention+Protection. However, under the same UV dose, PRGF treated cells exhibited significantly lower levels of apoptosis and necrosis comparing to non-treated ones ($p<0.01$). * $p<0.01$, significant differences between apoptotic and necrotic outcomes achieved for irradiated and non-irradiated cells. # $p<0.01$, significant differences between apoptotic and necrotic outcomes achieved for PRGF and non-PRGF groups.

Detection of Reactive Oxygen Species (ROS) production

Intracellular production of ROS was analyzed after four UV protocols as described above. After ultraviolet dosage, fluorescence units (F.U) were measured every 20 minutes during 2 hours. At every time point of each protocol, ROS production was significantly lower when dermal fibroblasts were cultured with PRGF ($p<0.01$) (Fig. 7). It has to be considered that for Protection and Prevention+Protection, ROS

measurements started immediately after UVB irradiation, whereas for Regeneration and Protection+Regeneration, measurements took place after 24 hours in order to allow the PRGF to accomplish its regenerative effect. Anyhow, for each treatment, statistical differences between PRGF and non-treated cells were found ready by minute 0 and were maintained over 2 hours ($p<0.01$). Finally, non-irradiated cells, maintained a basal ROS production significantly lower than both groups of irradiated cells ($p<0.01$).

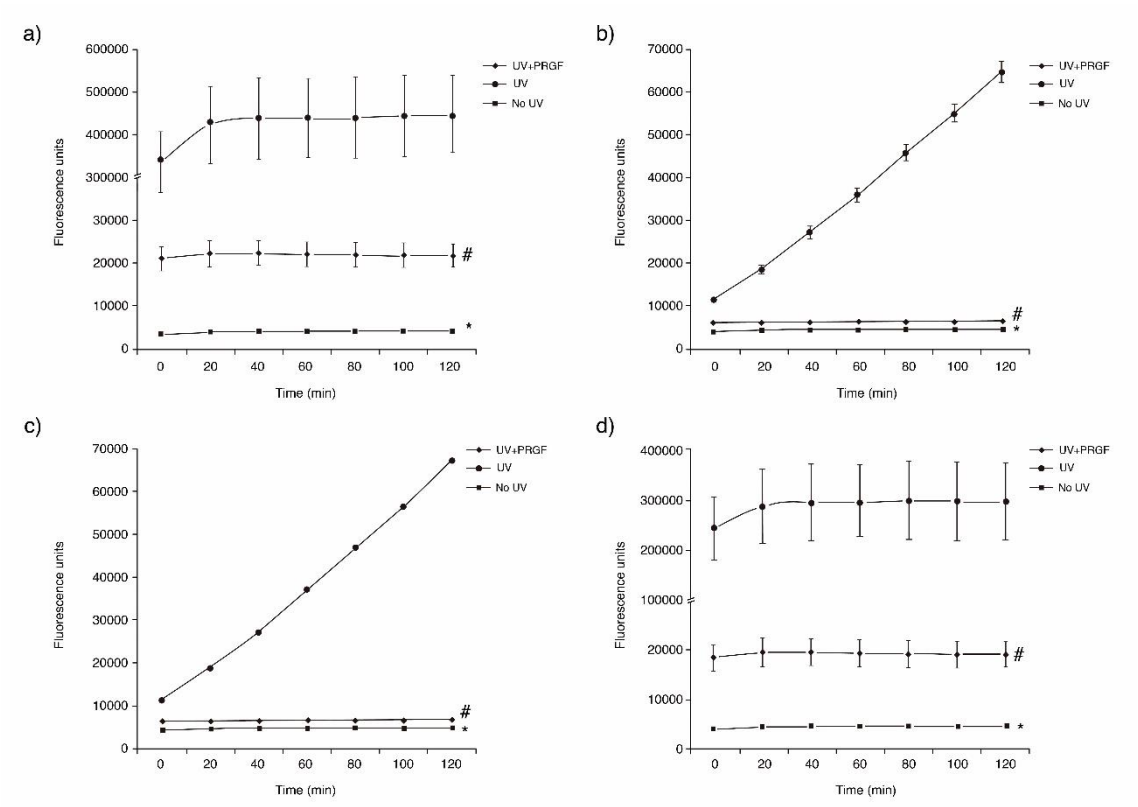

Fig.7. Reactive oxygen species production after ultraviolet irradiation was significantly enhanced comparing to non-irradiated cells by ($p<0.01$). However, after identical UVB doses ROS generation was significantly reduced by PRGF treatment comparing with non-treated ones for each treatment protocol ($p<0.01$) (a) Protection, (b) Regeneration, (c) Protection+Regeneration and (d) Prevention+Protection. At every time point, PRGF decreased ROS production of photo-stressed dermal fibroblasts. * $p<0.01$, significant differences between ROS outcomes achieved for non-irradiated and UVB irradiated cells. # $p<0.01$, significant differences between ROS outcomes achieved for PRGF and non-PRGF groups.

Dermal fibroblast biosynthesis

The biosynthetic activity of dermal fibroblasts after UV exposure was analyzed. Once every irradiation protocol concluded, expression Procollagen type I, Hyaluronic acid and TIMP-1 was measured by commercially available enzyme linked immunosorbent assays (Fig. 8). After the ultraviolet irradiation, the biosynthetic activity of dermal fibroblasts decreased significantly for every bioactive macromolecule ($p<0.01$) .

However, the presence of PRGF maintained a significantly higher expression of these proteins compared to the non-treated cells ($p<0.01$). The outcomes of PRGF group kept similar for each irradiation protocol and for each bioactive molecule with the exception of Hyaluronic acid, in which statistical differences were found only in Protection+Regeneration and Prevention+Protection protocols.

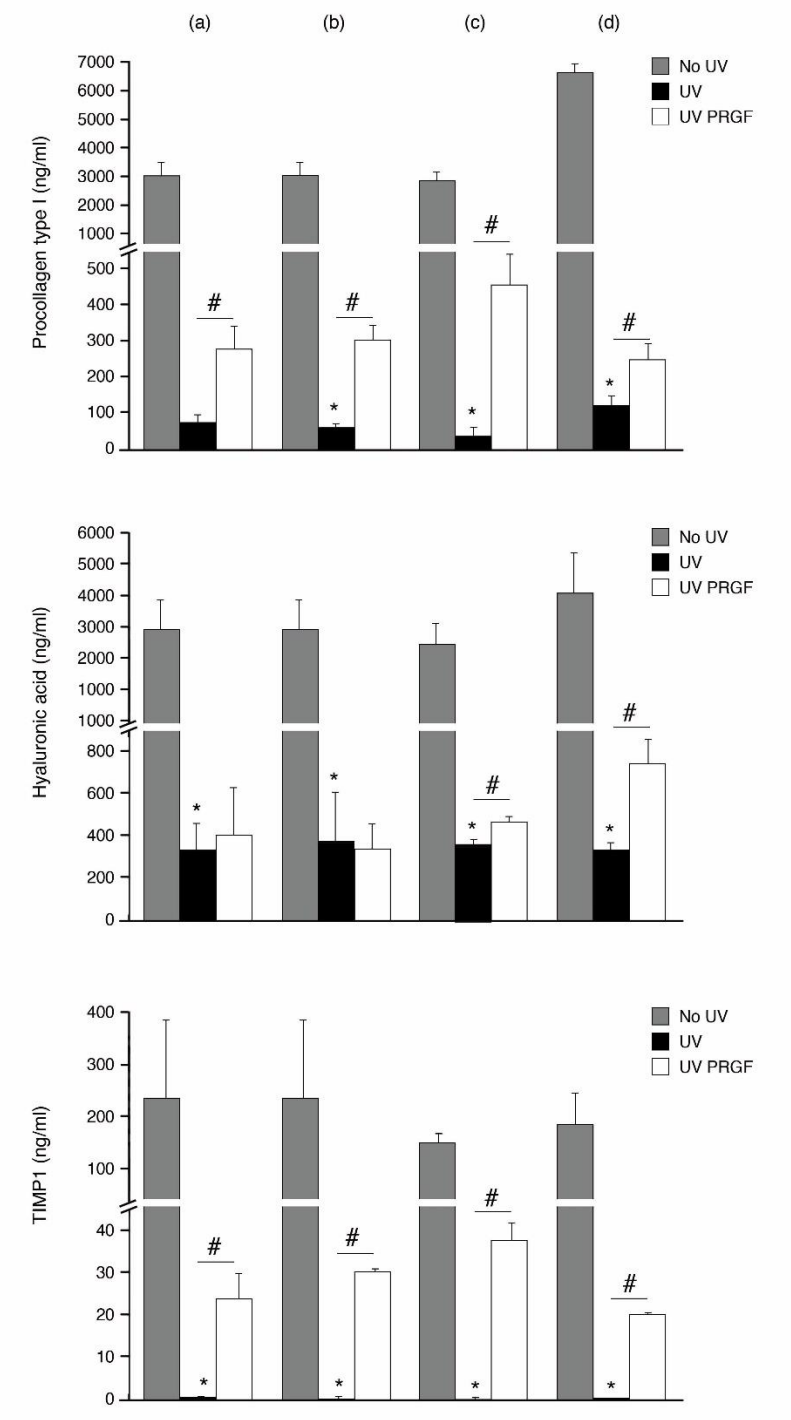

Fig.8. Effect of ultraviolet irradiation and PRGF treatment on dermal fibroblast biosynthesis and extracellular matrix protein expression. Cells were irradiated and cultured with PRGF following four different protocols (a) Protection, (b) Regeneration, (c) Protection+Regeneration and (d)

Prevention+Protection. UVB irradiation significantly reduced the biosynthesis of every macromolecule tested (* $p<0.01$). However, results showed that for every protocol tested, PRGF maintained the expression of Procollagen type I (*above*) and TIMP-1(*below*) above the non-treated cells ($p<0.01$). Hyaluronic acid expression (*center*) among PRGF treated cells was significantly higher for (c) and (d) protocols while no differences were found for (a) and (b) protocols (# $p<0.01$).

Discussion

Growth factors secreted by platelets and those present in human plasma play a major role in tissue regeneration [19]. Upon activation, platelet content of PRGF, release a wide variety of proteins and cytokines which have key effects in dermal/epidermal regeneration [22]. Our results show that some of these proteins include TGFβ1, PDGF, EGF, VEGF, IGF, FGFb and HGF. This pool of morphogens is able to promote damaged tissue regeneration by inducing proliferation, migration, angiogenesis, biosynthesis and extracellular matrix remodeling [38-39]. In the present study, three primary cell lines of human dermal fibroblasts were isolated, and following the manufacturer´s instructions, *in vitro* culture conditions such as initial seeding densities were optimized before each assay.

Fibroblast survival decreased in an ultraviolet dose dependent manner, being 400mJ/cm^2 and 600mJ/cm^2 the most harmful doses. These results are consistent with other UV dose screenings [40]. Adult Europeans get 10 to 20kJ/m^2 of ultraviolet irradiation per year [41], and our 400mJ/cm^2 dose approximately corresponds to an exposure of 10 to 20 weeks. Several irradiation protocols have been used to analyze the protective effect of different compounds against ultraviolet damage [7, 42]. In this study, we performed a preliminary screening of UVB irradiation protocols. Our results showed similar behaviors between some combinations of PRGF and UV, so we finally decided to include four protocols as the most representative ones. Protocol A (protection due to PRGF treatment during UV irradiation) would mimic a sunlight overexposed person just after taking PRGF treatment. Protocol B (regeneration due to PRGF application after photodamage) would mimic a person who decides to take PRGF treatment few days after have being under sunlight. Protocol C (protection and regeneration due to the presence of PRGF during and after the irradiation dose) would mimic the situation of a person who takes PRGF treatment just before sun exposure and few days after being under irradiation. Protocol D (prevention and regeneration due to the application of PRGF before and during UV dose) would mimic the situation of a person taking PRGF treatment some days before and at the moment of sun overexposure. It must be taken into account that for each UV protocol the negative

control group was identified as “No UV” while the positive control group was identified as “UV”. Although the incorporation of a test group showing the effect of PRGF without UV irradiation would be interesting, previous studies have already supported the beneficial effects of plasma rich in growth factors among non-stressed human dermal fibroblasts [20]. It is important to state that the present study is based on preliminary *in vitro* data trying to simulate a physiological condition of cutaneous photo-oxidative damage. Although some studies have suggested the clinical benefits of plasma rich in growth factors for the treatment of overexposed skin, additional randomized and placebo controlled clinical trials are needed to state PRGF as an effective technology for photoaged tissues [43].

In line with other studies, our results showed that after UV irradiation, morphological changes and tridimensional arrangement of dermal fibroblasts worsened [13] and lost their proliferative potential [40]. However, when PRGF treatment took place, fibroblast culture recovered a normal monolayer appearance. In fact, cell survival was 2.6 fold higher among PRGF treated cells while cell death was 3.2 fold higher among non-treated ones.

Additionally, cell death was analyzed to compare apoptotic and necrotic pathways, and PRGF showed to significantly decrease both processes. Apoptotic and necrotic outcomes were 1.9 fold and 2.5 fold lower when PRGF treatment took place. These results are consistent with some studies which have demonstrated that dermal fibroblasts undergo apoptotic fate after ultraviolet irradiation by means of overexpression of Caspases 3, 8 and 9 and induction of premature senescence [15, 44]. Moreover, decrease in cell viability could be related to, the UV derived breakdown of DNA strands due to the shortening of telomeres [45]. Furthermore, it has been demonstrated that UV activates p53, increasing the chance of pyrimidine dimerization, promoting single strand breaks of nuclear DNA [15].

Most of these apoptotic and necrotic events are in first instance triggered by the overproduction of Reactive oxygen species (ROS) that include: superoxide anions ($O_2^{\cdot-}$), peroxide hydrogen (H_2O_2) and hydroxyl radicals ($HO^{\cdot}$) [46]. Although these free radicals are constantly generated in keratinocytes and fibroblasts, they are rapidly removed by antioxidants and different enzymes. However, when UV irradiation takes place, endogenous UV absorbing chromophores including $NADH^{-}$/NADPH, tryptophan, riboflavin and trans-urocanic acid, produce overflow of ROS by energy transfer cascades, overwhelming the organism´s endogenous antioxidant defense mechanism [41]. Interestingly, plasma rich in growth factors has shown to reduce significantly ROS

production levels decreasing an average of 13 fold when fibroblasts were cultured with PRGF.

This finding could be related with our results, demonstrating that PRGF is able to maintain high levels of key ECM proteins such as Procollagen type I and Hyaluronic acid while keeping functional levels of TIMP-1. The HA production was 1.4 fold higher being only the C and D protocols statistically significant, while a mean Procollagen type I I and TIMP-1 increase of 4.8 fold and 53 fold was obtained respectively for every PRGF treatment. In fact many studies have showed that after UV irradiation and ROS generation, a variety of signal transduction pathways are activated, ending up in disequilibrium between MMP overexpression and TIMP downregulation [6, 13, 47]. Finally, this unbalance among ECM remodeling enzymes provoke the degradation of Procollagen type I and Hyaluronic acid. Interestingly, not only TIMPs are able to prevent UV derived ECM degradation, but also n-acetylglucosamines like Hyaluronic acid itself have proven to be able to suppress collagenases activation [48]. So, PRGF treatment could be an effective treatment by means of preventing the degradation of Procollagen type I and maintaining the expression of Hyaluronic acid and TIMP-1 after photo-oxidative stress. However, our findings could be related to an increase in cell viability, hence allowing more cells the chance to synthetize ECM proteins. Therefore proteomic and genetic patterns in response to PRGF treatment must be elucidated in order to accurately define its biological influence. It may probably act as an inhibitor of AP-1 transcription factor preventing the phosphorylation of ERK1/2 and JNK, two of the most important upstream modulators of the MAP Kinase pathways [4]. This signaling cascade could be a target for future PRGF related research.

Conclusion

PRGF has shown to prevent some of the photoageing related events when simulated in *in vitro* models. However, further studies are needed to understand the biomolecular mechanisms by which PRGF prevents some of the most indicative features of ultraviolet irradiation. Our results confirm that PRGF is able to reverse the photo-oxidative stress produced by ultraviolet B irradiation, keeping fibroblast morphology and viability, while maintaining ECM protein production and more importantly, reducing ROS formation.

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Artículo III

Plasma rich in growth factors enhances wound healing and protects from photo-oxidative stress in FDH and 3D skin models.

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Title: Plasma rich in growth factors enhances wound healing and protects from photo-oxidative stress in dermal fibroblasts and 3D skin models.

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Conflict of Interest:

E Anitua is the scientific director of, G.O. and A.P. are scientists at BTI-Biotechnology Institute, the company that has developed the PRGF-Endoret technology.

Abstract

Background: Optimal skin repair has been a desired goal for many researchers. Recently, plasma rich in growth factors (PRGF) has gained importance in dermatology proving it´s beneficial effects in wound healing and cutaneous regeneration.

Objective: The anti-fibrotic, pro-contractile and photo-protective effect of PRGF on dermal fibroblasts and 3D skin models has been evaluated.

Method: The effect against TGFβ1 induced myofibroblast differentiation was tested. Cell contractile activity over collagen gel matrices was analyzed and the effect against UV derived photo-oxidative stress was assessed. The effectiveness of PRGF obtained from young aged and middle aged donors was compared. Furthermore, 3D organotypic skin explants were used as human skin models with the aim of analyzing *ex vivo* cutaneous preventive and regenerative photo-protection after UV exposure.

Results: TGFβ1 induced myofibroblast levels decreased significantly after treatment with PRGF while the contractile activity increased compared to the control group. After UV irradiation, cell survival was promoted while apoptotic and ROS levels were noticeably reduced. Photo-exposed 3D explants showed higher levels of metabolic activity and lower levels of necrosis, cell damage, irritation and ROS formation when treated with PRGF. The histological integrity and connective tissue fibers showed lower signals of photodamage among PRGF injected skin models. No significant differences for the assessed biological outcomes were observed when PRGF obtained from young aged and middle aged donors were compared.

Conclusion: These findings suggest that this autologous approach might be useful for antifibrotic wound healing and provide an effective protection against sun derived photo-oxidative stress regardless the age of the patient.

Keywords: plasma rich in growth factors, wound healing, skin regeneration, photo-oxidative stress.

Introduction

Cutaneous tissue repair and regeneration are pivotal events for the maintenance of skin integrity. Physical injury or harmful agent exposure such as sun derived UV light, trigger a cascade of processes that begin to work simultaneously in order to reestablish the barrier function. Cutaneous regeneration involves the coordination of dermal fibroblasts and epidermal keratinocytes in order to restore the multilayered tissue that constitutes the skin. Their organized interaction during tissue remodeling is essential to recover the damaged area [1]. During injury repair some of these fibroblasts differentiate into myofibroblasts, and are responsible for wound contraction, extracellular matrix (ECM) deposition and fiber organization [2]. However, the presence of these myofibroblastic cells in final stages of wound healing triggers the scarring tissue development resulting in adverse medical consequences including loss of function, restriction of movement, growth decrease, poor aesthetics and adverse psychological effects [3]. Additionally, overexposure to sunlight has shown to generate severe oxidative stress among the aforementioned cell types. The ultraviolet light interacts with intracellular chromophores which ultimately form and release reactive oxygen species (ROS) to the cytoplasmic and extracellular environment. These pathogenic agents including hydrogen peroxide and free radicals, interfere in signal transduction pathways leading to genetic damage. Furthermore, these agents also reduce cell proliferation, increase apoptotic/necrotic processes and promote connective tissue degradation of the skin [4]. In fact, UV irradiation has proved to induce the cleavage of collagen and elastic fibers of the papillary and reticular dermis through the overstimulation of collagenases and downregulation of matrix metalloprotease inhibitors [5].

Several approaches are being evaluated with the aim of promoting wound healing and tissue regeneration after cutaneous damage. Some of these therapies include treatments for burns [6], chronic non healing wounds [7], diabetic ulcers [8] and photo-damaged skins [9]. However, inhibition of scar formation is a pending subject in dermal/epidermal regeneration treatments and so far the existing results are poor [10].

The application of intradermal infiltrations of plasma rich in growth factors (PRGF) introduces a new endogenous therapy based on the formation of a three dimensional fibrin scaffold beneath the patient´s skin. This *in situ* polymerized autologous mesh, has proved to bind several key growth factors released by the alfa granules of the patient´s own platelets [11]. The fibrin clot acts as a transient delivery system that offers a sustained supply of plasmatic proteins, cytokines and morphogens that

ultimately promote local tissue regeneration [12]. The potential benefits of this therapy have also been evaluated in dermatology [13] and other medical fields including, orthopedic surgery [14], sports medicine [15], ophthalmology [16], neurobiology [17] and oral and maxillofacial surgery [18]. This technology recovers a small volume of the patient´s own blood which is afterwards centrifuged and activated in order to obtain an autologous formulation enriched in proteins and growth factors free from pro-inflammatory leukocytes [19]. Additionally, it has been recently demonstrated that PRGF has a significant antimicrobial effect and its application might be potentially useful for infected ulcers, sunburns and postoperative complications [20].

In the present study, the biological effects of PRGF on human dermal fibroblasts and organotypic 3D skin models have been evaluated. Firstly, plasma rich in growth factors was characterized and assessed over the contractile activity of dermal fibroblasts paying special attention to the effect against TGβ1-induced myofibroblast differentiation. Additionally, the protection against UV derived photo-oxidative stress was studied. Due to the advanced age of patients with dermatological disorders [21], it was decided to study whether the age of the patients from whom PRGF was prepared may influence the final biological outcomes. To address this, fibroblast culture experiments were carried out in parallel using both PRGF prepared from young or medium-advanced age donors. Finally, multilayered and full thickness 3D skin explants were intradermally injected with PRGF and the biological protection of the growth factor releasing scaffold against solar damage was assessed.

Materials and Methods

Plasma Rich in Growth Factors (PRGF-Endoret) preparation and characterization

The study was performed following the ethical principles for medical research contained in the Declaration of Helsinki amended in 2008. Blood from 4 healthy young aged donors (31±6 years old) and from 4 healthy middle aged donors (57±3 years old) was collected after obtaining informed consent and was put into 9 mL tubes containing 3.8% (wt/vol) sodium citrate. Smokers and donors with infectious processes, platelet derived blood disorders, previous cancerous diseases or current treatment with corticoids or immunomodulators were considered non-suitable for plasma rich in growth factor donation. For PRGF preparation, the blood was centrifuged (BTI System IV, Vitoria, Spain) at 580g for 8 minutes and the plasma column was fractioned into fraction 1(F1) and fraction 2 (F2). F2 is defined as the 2 cm^3 platelet rich plasma just above the leukocyte buffy coat, and F1 is defined as the remaining plasma volume

above the F2. Platelet poor F1 fraction was discarded and only the platelet rich F2 fraction was used in our studies. Platelet concentration in peripheral blood and in F2 fraction was measured with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France).

Briefly, PRGF activator (BTI Biotechnology Institute, SL, Miñano, Spain) was added to the collected F2 fraction and was incubated at 37°C in glass tubes for 1 hour. The released supernatant was collected after centrifugation at 1000g for 10 minutes. Several growth factor levels between young aged and middle aged PRGF were compared by commercially available Enzyme-linked immunosorbent assay kits (ELISA) (R&D Systems, Minneapolis, MN): Transforming growth factor β1 (TGFβ1), Hepatocyte growth factor (HGF), Platelet derived growth factor-AB (PDGF-AB), Epidermal growth factor (EGF), Insulin like growth factor-I (IGF-I) and Fibroblast growth factor-b (FGFb). Platelet number can vary slightly between donors so results are expressed as "concentration of growth factor released per platelet", in order to accurately analyze the differences between the two groups. After PRGF-Endoret characterization, the following 2D assays were developed using human dermal fibroblast cultures: antifibrotic effect, procontractile activity and photoprotection assays (Fig. 1A).

On the other hand, for 3D assays, just activated liquid F2 fraction of a healthy donor (27 years old) was injected into human skin models for *in situ* scaffold formation. These organotypic skin explants, were exposed to UV light to test the photopreventive and photoprotective effect of PRGF-Endoret (Fig. 1B). Five organotypic skin models were used for each treatment group.

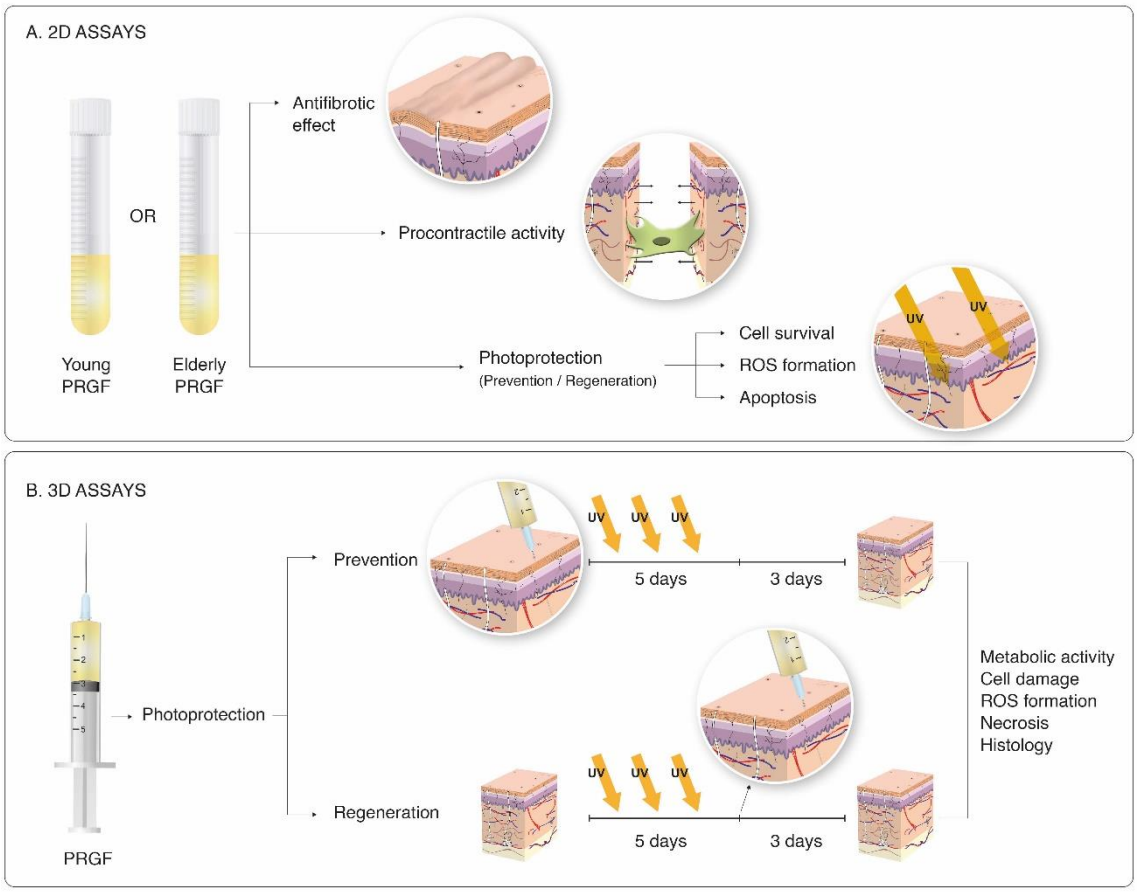

Fig.1. Experimental design of the study. (A) 2D and (B) 3D assays are shown. For 2D assays, human dermal fibroblasts were used to evaluate the antifibrotic effect, procontractile activity and photoprotective effect of PRGF obtained from either young aged or middle aged donors. For 3D assays, organotypic skin explants were intradermically injected with PRGF before or after UV irradiation to evaluate its photopreventive and photoregenerative effect respectively.

2D Assays

Human dermal fibroblast isolation and characterization

Dermal fibroblasts were isolated from skin biopsies of three healthy patients during joint surgery. Written informed consent was obtained from all patients before the biopsy was performed. Cells were isolated following our standard protocol. Briefly, skin biopsies collected in phosphate buffered saline supplemented with antibiotics were minced and treated with 0.3% collagenase II (Gibco Life Technologies, Gaithersburg, MD, USA) at 37°C for 90 min with gentle stirring. The resulting cell suspension was filtered and centrifuged at 460 g for 10min. Cells were seeded into culture flasks and maintained with Dulbecco`s modified Eagle`s medium (DMEM)/F12 (1:1 volume) (Gibco) supplemented with 15% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 2mM Glutamine, 50µg/ml Gentamicin and 2.5µg/ml Amphotericin B (Sigma-Aldrich, St. Louis, MO) in a humidified atmosphere at 37°C with 5%CO2. After reaching confluence,

cells were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco). Cell viability was assessed by trypan blue dye exclusion.

Their fibroblast-like morphology was checked by phase-contrast microscopy. Cells were also characterized by immunofluorescence using three antibodies against typical dermal fibroblast markers: Collagen Type I (Millipore, Billerica, MA, USA), Fibronectin,Vimentin and alfa-SMA (Sigma-Aldrich). Cells were plated on 24-well plates at 10000 cell/cm2 density. After 72h of culture with Dulbecco`s modified Eagle`s medium (DMEM)/F12 (1:1 volume) (Gibco) supplemented with 15% fetal bovine serum (FBS) (Biochrom), 2mM Glutamine and 50µg/ml Gentamicin (Sigma-Aldrich) (referred to below as "culture medium"), cells were fixed for 10 minutes in 4% formaldehyde for Collagen type I antigen, in pre-cooled methanol at -20°C for Fibronectin and alfa-SMA antigens and in methanol:acetic acid (3:1) for Vimentin antigen. Cells for Collagen type I staining were permeabilized with 1% Triton X-100, in PBS for 10 minutes. Cells were then blocked with FBS (10% in PBS) for 30 minutes, and incubated for 1 hour with the primary antibodies in the following dilutions: 1:20 for Collagen Type I, 1:50 for Vimentin, and 1:800 for Fibronectin and alfa-SMA. Next, cells were incubated with their appropriate secondary antibodies, goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen, Grand Island, NY, USA). Finally, cells nuclei were stained with Hoechst 33342 (Molecular Probes-Invitrogen, Grand Island, NY, USA), mounted, and visualized under x20 lens with a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany). A negative control of the secondary antibody was performed by eliminating the previous incubation with the primary antibody in order to ensure the correct fluorescence detection.

Protective and reverse effect against myofibroblast differentiation

To test the protective effect against myofibroblast differentiation, subcultured human dermal fibroblasts at passage 5 were seeded at 5000 cell/cm^2 in 24 well plates and incubated with the following treatments for 72 hours: 1) Basal medium (serum-free culture medium with 0.1% (v/v) FBS) and TGFβ1 (2.5ng/mL) (Millipore) as a myofibroblast differentiation stimulation treatment, 2) Basal medium with TGFβ1 (2.5ng/mL) and 20% Young aged PRGF, and 3) Basal medium with TGFβ1 (2.5ng/mL) and 20% Middle aged PRGF. After 72 hours of treatment period, cells were fixed in methanol pre-cooled at -20°C. Afterwards, cells were blocked with FBS (10% in PBS) for 30 minutes, and incubated for 1 hour with the primary antibody mouse anti-alfa-SMA (Sigma-Aldrich) at 1:800, followed by the incubation with goat anti-mouse IgG-Alexa

Fluor 488 (Molecular Probes-Invitrogen). Finally, cells nuclei were stained with Hoechst 33342 (Molecular Probes-Invitrogen), mounted, and visualized under a fluorescence microscope (Leica Microsystems). Myofibroblast cell counting was determined by taking photographs over three random x10 microscopic fields per well. The digitalized images were analyzed using the Image J Software (NIH, Bethesda, MD). Blue dye positive cells were counted to obtain total cell number and blue dye positive plus green dye alfa-SMA positive cells were counted as myofibroblasts. Cells showing any kind of greenish stain were considered alfa-SMA positive cells. All treatments and images taken for each well were performed in triplicate.

To test the reverse effect against myofibroblast differentiation, subcultured human dermal fibroblasts at passage 5 were seeded at 5000 cell/cm^2 in 24 well plates and incubated for 72 hours with myofibroblast differentiation stimulation treatment: basal medium and TGFβ1 (2.5ng/mL) (Millipore). After the pretreatment period, wells were washed with PBS and culture medium was replaced by the following treatments for 72 hours: 1) Basal medium and TGFβ1 (2.5ng/mL) (Millipore) as a myofibroblast differentiation stimulation treatment, 2) Basal medium supplemented with TGFβ1 (2.5ng/mL) and 20% Young aged PRGF, and 3) Basal medium supplemented with TGFβ1 (2.5ng/mL) and 20% Middle aged PRGF. After 72 hours of treatment period, cells were immunolabeled and images were analyzed as described above. All treatments and images taken for each well were performed in triplicate.

The myofibroblast count detected in TGFβ1 treatment group was set to 100% and the percentage of myofibroblasts in TGFβ1+Young/Middle aged PRGF groups was analyzed.

Collagen matrix contraction assay

24 well culture plates were coated with 0.2%BSA in water for 30 minutes and after two washes with sterile water, plates were left drying overnight. Collagen solution was prepared mixing 8 parts of collagen from bovine skin (Sigma-Aldrich) with 1 part of 10xPBS as manufacturer´s instructions. The pH was adjusted to 7.5-7.2 with sterile NaOH 0.1M. Human dermal fibroblasts at passage 4 were resuspended in the collagen solution and plated in pre-coated plates at high density (50000 cell/cm^2). Cells were incubated at 37°C for 1 hour until collagen matrix gelation. Afterwards, fibroblast embedded collagen gels were incubated with basal medium as a control of non-stimulation, 20% (v/v) Young aged PRGF or 20% (v/v) Middle aged PRGF. In order to observe the contraction of collagen gels, images were taken with x4 magnification at time 0, 2, 4, 6, 8 and 24 hours with a digital camera (Lumix, Panasonic DCM-FZ28,

Osaka, Japan). The contractile activity (contraction %) of the fibroblasts was measured with the Image J Software analysis (Bethesda). The results were expressed as the gel area percentage compared to time 0. All treatments were assayed in triplicates.

Cell survival after photo-oxidative stress

A UV lamp (Vilber Lourmat, Marne la Vellee, France) delivering a high intensity energy spectrum (680 μW/cm^2) in the UV-B region (312nm) was used for photo-oxidative stress induction.

To test the preventive effect of PRGF against ultraviolet radiation (PRGF+UV), passage 4 cells were plated at a density of 10000 cells/cm^2 in 24 well culture plates and maintained for 24 hours with culture medium. Afterwards cells were incubated with 20% Young/Midlle aged PRGF for 24 hours and briefly irradiated during 10 minutes with UVB (400 mJ/cm^2). Finally, cells were maintained in basal medium for 24 hours and cell survival percentage was measured by the spectrophotometric Cell Viability WST-1 assay (Roche, Mannheim, Germany). WST-1 reagent was added following a 1:10 dilution as manufacturer´s indications and cells were incubated for 1 hour. The collected medium was added to 96 well culture plates, where absorbance was measured using a microplate reader with a filter for wavelength between 420 and 480nm.

To test the regenerative effect of PRGF against UVB radiation (UV+PRGF), passage 4 cells were plated at a density of 10000 cells/cm^2 in 24 well culture plates and maintained for 24 hours with culture medium. Afterwards, cells were irradiated with an UVB dose of 400 mJ/cm^2 in the presence of PBS and subsequently incubated with 20% Young/Middle aged PRGF for 24 hours. Finally, cell viability was measured as described above.

Negative control cells were maintained with complete medium and did not receive UVB radiation. Positive control cells were cultured with basal medium instead of PRGF.

Reactive Oxygen Species (ROS) production

The production of ROS by human dermal fibroblasts after UVB radiation was measured using the Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK). This kit uses the cell permeant reagent 2,7-dicholofluorescein diacetate (DCFDA) as a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell.

Passage 4 cells were plated at high density (40000 cells/cm^2) in 96 well optical bottom black culture plates and were maintained with culture medium for 24 hours. Afterwards, cells were irradiated with an ultraviolet-B dose of 400 mJ/cm^2 following the irradiation protocols described above (preventive and regenerative protocols). Finally, after each irradiation protocol, production of ROS was measured by adding the DCFDA reagent as manufacturer´s indications and fluorescence was determined using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies) after two hours.

Apoptosis after photo-oxidative stress

With the aim of determining the apoptosis level, the immunophotometric Cell Death Detection ELISA plus kit (Roche) was used. The assay is based on a quantitative enzyme linked immunoassay using monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono and oligonucleosomes present in the apoptotic fraction of cell lysates. Passage 5 cells were plated at a density of 20000 cells/cm^2 in 24 well culture plates and maintained for 24 hours with culture medium. Afterwards, cells were irradiated with an ultraviolet-B dose of 400 mJ/cm^2 following the irradiation protocols described above (preventive and regenerative protocols). When each protocol concluded, culture medium was discarded and the adherent cells were lysed and centrifuged at 200g for 10 minutes. The supernatant was identified as apoptotic fraction as manufacturer´s indications. Briefly, the ELISA assay was developed for the detection of apoptotic cells.

3D Assays

Organotypic skin explant culture and ultraviolet irradiation

Organotypic skin explant cultures (HOSEC) were used as human skin models to the *ex vivo* analysis of cutaneous photodamage. These 10mm diameter explants were cultured with basal medium leaving the epidermis in direct contact with the air. The aforementioned preventive and regenerative effect of PRGF against UV irradiation was tested using a solar simulator lamp SOL 500 (Dr Honle UV Technology, Germany), with a spectral range corresponding to natural sunlight (295-3000 nm). The UV light output was measured by an UV-meter (Dr Honle UV Technology) and adjusted to 3.2-3.6 mW/cm^2. The explants were irradiated with a daily dose of 10J/cm^2 for five days (50 minutes of lamp exposure per day). The *in situ* scaffold formation was achieved by the intradermal injection of just activated PRGF (70µl per explant) using 32G needles.

For “prevention” studies (PRGF+UV), plasma rich in growth factors was injected before UV exposure while for “regeneration” studies (UV+PRGF), it was injected after the last UV dose. Both protocols were followed by a culture period of three days until the analysis of the explants (Fig. 1B). Negative control explants were maintained with basal medium and did not receive UV radiation. Positive control explants did receive UV radiation but were not treated with PRGF. Five organotypic skin models were used for each treatment group.

Metabolic activity

Skin explants metabolic activity was measured by the fluorimetric resazurin reagent (Sigma-Aldrich). After each irradiation protocol (prevention and regeneration), explants were cultured for 1 hour with 6μM of resazurin reagent as manufacturer´s instructions. Metabolically active cells are able to reduce resazurin and release the fluorescent agent resorufin by intracellular oxidoreductases. The culture medium was collected and added to 96 well culture plates, where fluorescence was measured using a fluorescence microplate reader (excitation 530-560nm and emission 590nm).

Cell damage

Cell damage induced by UV light exposure was determined by the Colorimetric Cytotox 96 Assay (Promega, Madison, USA). When cell membranes are compromised by pho-oxidative stress, the cytosolic enzyme lactate dehydrogenase (LDH) is released to the surrounding medium. In the presence of the working reagent, surrounding LDH induces formazan production which can be measured by a microplate reader. After each irradiation protocol, the level of LDH was determined in a microplate reader with a filter for wavelength between 490 and 500nm.

Skin irritation

Following the CIELab chromatic colour model, the skin redness after each irradiation protocol was measured by a colorimeter (Konica Minolta CS-200, Madrid, Spain). An increase in the value of explant redness suggests a potential irritation effect due to UV derived photo-damage. Irritation percentage decrease due to PRGF treatment was assessed after three days of the last UV dose.

Necrosis

At the end of the UV dose and PRGF treatments, epithelial cells from the organotypic explants were isolated by enzymatic digestion using 3.5mg/ml of collagenase type I (Molecular Probes-Invitrogen) and 5% trypsin-EDTA (Sigma-Aldrich) in basal medium

for 4 hours at 37ºC. Afterwards, the cellular suspension was filtered through 70µm pore filters in order to clean the cell isolate from tissue debris and elastic fibers. Basal medium containing 10^6 cells/mL, was incubated with 5µg/mL of propidium iodide (PI) (Sigma-Aldrich) for 5 minutes as a necrosis marker. Finally, the percentage of positive cells for PI was analyzed by flow cytometry (Cytomics FC500-MCL, Beckman Coulter, IN, USA).

Reactive oxygen species (ROS) production

To accurately determine de preventive effect of PRGF against ROS formation, the explants were analyzed just after the last UV dose, whereas for the regenerative effect, the analysis was done after three days of PRGF treatment. Epithelial cells from the skin models were isolated as described above. Briefly, basal medium containing 10^6 cells/mL was incubated with 10µM of H_2DFFDA (Molecular Probes-Invitrogen) in Hank´s solution (Sigma-Aldrich) for 30min. The H_2DFFDA is oxidized to the fluorescent molecule DFF by the action of ROS molecules like hydrogen peroxide and hydroxyl radicals, formed after UV exposure. The percentage of ROS positive cells was analyzed by flow cytometry (Beckman Coulter) in order to compare the ROS production between PRGF treated and non-treated skin explants.

Histological and histomorphometric analysis

The histological analysis was made by an objective and blind expert. Prior to the UV exposure, a skin explant was used for the *in situ* PRGF scaffold visualization. A single indian ink drop was used to stain the PRGF just activated. Thirty minutes after intradermal injection, the explant was fixed in 4% formaldehyde for 24 hours, paraffin-embedded and sectioned at 5µm thickness followed by a counterstain with Hematoxylin-Eosin (H-E) (Sigma-Aldrich).

At the end of the UV and PRGF treatments, the organotypic culture explants were fixed as described above. Interstitial edema and epidermal thickness were analyzed by H-E staining (Sigma-Aldrich). The overall increase in the white-empty area between connective tissue fibers was measured as UV provoked interstitial edema. Additionally, several microphotographs covering the entire length of the explants were used to analyze the mean epidermal thickness differences between treatments. Proliferating reticular dermal cells were detected by Ki67 immunolabeling (Abcam) and expressed as Ki67+ cell%/mm^2. The amount, tridimensional organization and integrity of collagen fibers were also qualitatively analyzed using masson´s trichrome stain (Labolan, Navarra, Spain) and the immunolabeling of collagen type I (Abcam). Finally, the

decrease of elastic fibers due to UV radiation was also measured by the orcein stain (Labolan) and expressed as orcein+ fiber area percentage.

For histological evaluation, images at low and high power magnifications were obtained with a digital camera Leica DFC 300 FX (Leica Microsystems) coupled to a light microscope Leica DMLB. Images, were analyzed by the Image J Software analysis (Bethesda).

Statistical analysis

After checking the normal distribution (Shapiro Wilks test) and homoscedasticity (Levene test) from groups, differences between groups were analyzed using the T-Student or variance analysis (ANOVA) test. In those cases in which conditions for applying of parametric tests were not achieved, significant differences were analyzed using Mann Whitney and Krustal Wallis tests. Statistical differences between groups were accepted for P values of 0.05. Results were expressed as mean ± standard error.

Results

PRGF-Endoret characterization

Blood from young and elderly donors was withdrawn and F2 of PRGF was prepared. Samples were analyzed in a hematology analyzer and both young and middle aged donors reached 2.4X mean platelet enrichment over peripheral blood. None of the PRGF formulations contained leukocytes.

Several key growth factors for skin and other tissue regeneration were measured, including TGFβ1, HGF, PDGF, EGF, IGF and FGFb. No significant differences in growth factor concentration levels were observed when young and middle aged PRGFs were compared (Fig. 2).

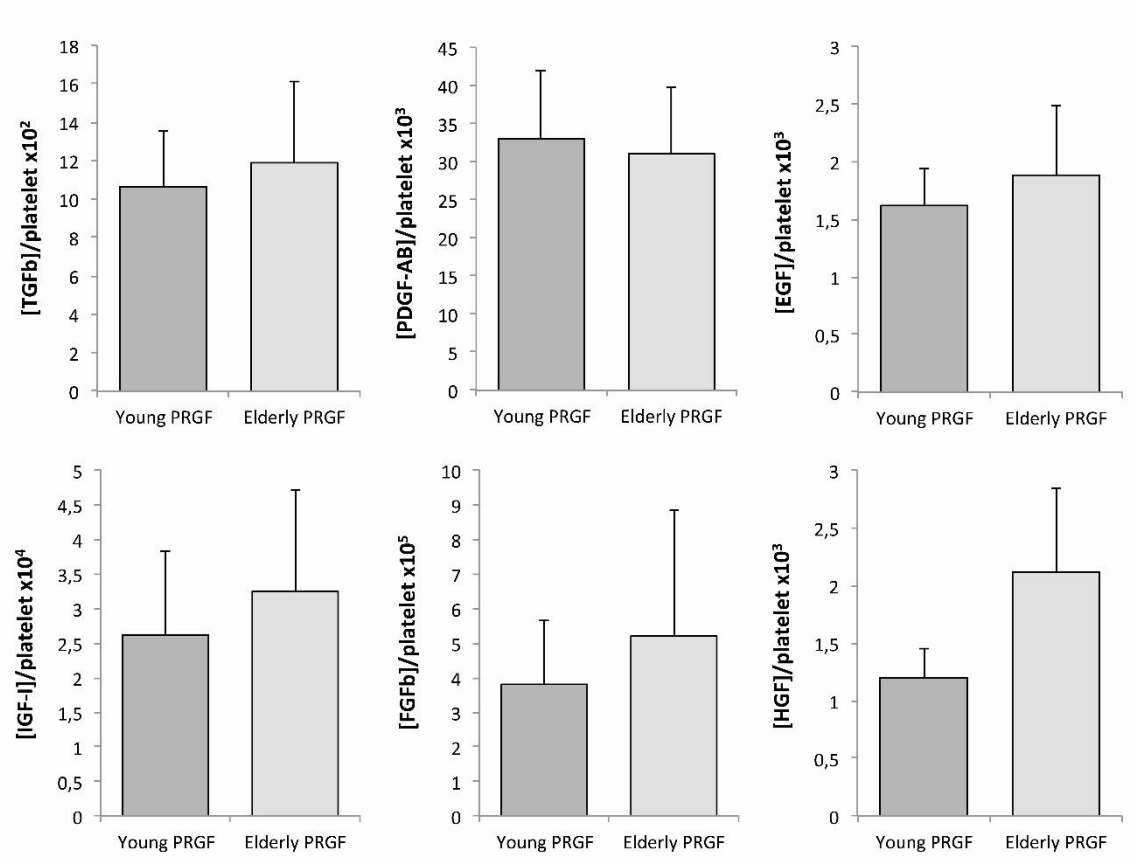

Fig.2. Comparison of growth factor content between PRGF obtained from four young aged and four middle aged donors. No statistical differences were found between groups in any growth factor content: TGFβ1, PDGF-AB, EGF, HGF, IGF-I and FGFb. For normal distribution following data, statistical differences were analized using t-student test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used.

Cell isolation and characterization

Isolated and cultured cells showed elongated fibroblast-likeand a spindle-shaped appearance. Immunofluorescence microscopy confirmed that fibroblasts were uniformly positive for Collagen type I, Vimentin and Fibronectin. These results confirm the dermal fibroblast nature of the isolated cells (Data not shown).

PRGF protects and reverts the TGFβ1 induced myofibroblast differentiation while induces the contractile activity of dermal fibroblasts

The effect of PRGF on the protection and reversion of TGFβ1 induced myofibroblastic differentiation was evaluated. To evaluate the protective effect, immunofluorescence images were analyzed 72 hours after the treatment period and percentages of myofibroblast differentiation were obtained. Both young and middle aged PRGF treatments even in the presence of TGFβ1 reduced the myofibroblast differentiation rates to 34±9% and 32±7% respectively ($p<0.05$) (Fig. 3A and 3B). To evaluate the

reverse effect, immunofluorescence images were analyzed after the aforementioned pretreatment and 72 hours after having been cultured with PRGF plus TGFβ1 or TGFβ1 alone. Both young and middle aged PRGF treatments triggered a decrease in myofibroblast count up to 23±4% and 20±1% respectively (Fig. 3C and 3D).

These results demonstrate that both young and middle aged PRGF counteracted the effect of TGFβ1 induced myofibroblast differentiation, suggesting an anti-fibrotic effect. Significant differences were found between PRGF and TGFβ1 treated groups for both protection and reversion protocols ($p<0.05$).

Collagen gels containing embedded fibroblasts were prepared to test their contractile activity when cultured with PRGF. Contractile activity was analyzed by taking photographs at 2, 4, 6, 8 and 24 hours as previously described. Results showed that those cells cultured with young or middle aged PRGF increased their contractile activity compared to non-treated ones (Fig. 3E and 3F). Statistically significant differences started to appear at 4 hours and were maintained until the 24 hours of the experimental design ($p<0.05$). Gel area percentage obtained at 24h were 11±3% and 14±2% for young and middle aged PRGF and 59±11% for control group (2.2 fold increase).

No statistical differences were found between outcomes of young and middle aged PRGF groups.

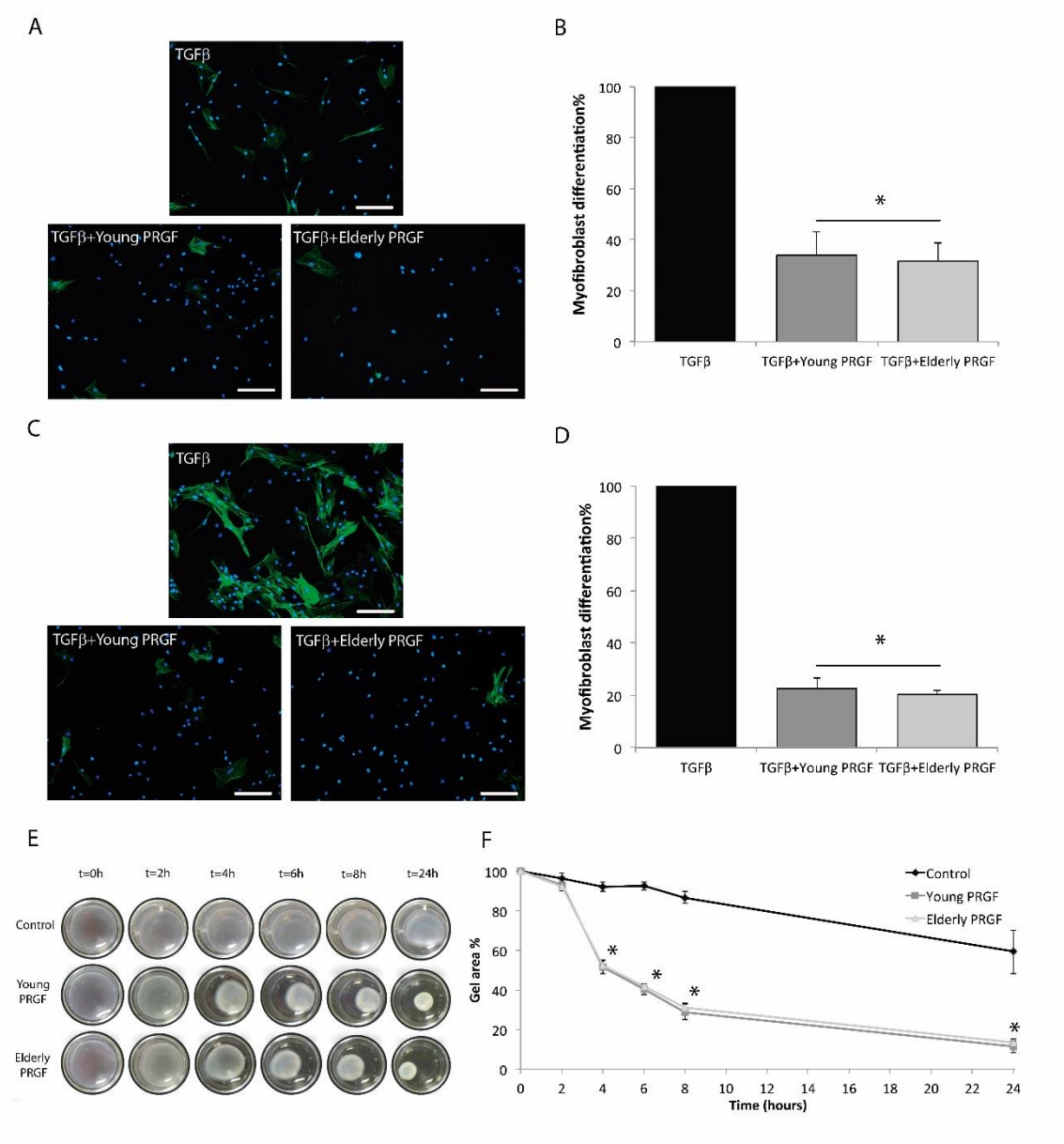

Fig.3. Biological mechanisms of PRGF in 2D assays. (A) Representative images of the protective effect of PRGF against TGFβ1 induced myofibroblast differentiation. Immunofluorescence of alfa-SMA shows greenish positive cells. (B) Both young aged and middle aged PRGF showed to decrease the myofibroblast differentiation rate (*p<0.05) when co-treated with TGFβ1 for 72 hours (protection). (C) Representative images of the reversal effect of PRGF against TGFβ1 induced myofibroblast differentiation. (D) Both young aged and middle aged PRGF showed to decrease the myofibroblast differentiation rate (*p<0.05) when pretreated with TGFβ1 alone for 72 hours and cultured for 3 more days with TGFβ1+PRGF (reversion). (E) Representative photographs showing the collagen gel contraction process achieved by PRGF treated dermal fibroblasts. (F) Cells embedded in collagen gels were cultured with either young aged or middle aged PRGF. Results in indicated that PRGF enhances significantly the contractile activity of dermal fibroblasts, reducing the diameter of collagen gels (*p<0.05). Statistical differences began at 4 hours and were maintained during 24 hours. PRGF treatments include 4 different PRGF preparations obtained from 4 young aged donors (under 35 years old) and 4 additional PRGF preparations obtained from 4 middle aged donors over 50 years old. No statistical differences were found between young aged

and middle aged groups for any of the aforementioned analyzed parameters. For normal distribution following data, statistical differences were analized using t-student test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used. Cells were seeded at 5000 cells/cm^2 for myofibroblast differentiation assays and at 50000 cells/cm^2 for contraction assays. Immunofluorescence images are taken at 10x magnification. Scale bars: 100µm

PRGF increases cell survival rate while inhibits ROS formation and apoptosis after photo-oxidative stress

Firstly, cell viability after UVB irradiation was analyzed for prevention and regeneration protocols. When cells were treated with young/middle aged PRGF previous to the ultraviolet exposure, 59±17% and 62±16% of fibroblasts survived respectively compared to the 26±4% of the non-treated ones ($p<0.05$) (Fig. 4A and 4B). Moreover, when young/middle aged PRGF was added after UVB irradiation, dermal fibroblasts achieved survival rates of 44±12% and 53±13% respectively compared to the 19±7% of the non-treated ones ($p<0.05$) (Fig. 4C and 4D). In both cases, PRGF treatment demonstrated to be effective against photo-oxidative stress.

The intracellular production of reactive oxygen species was also analyzed. Both young and middle aged PRGF decreased significantly the production of ROS no matter if the treatments were applied before or after the UVB dose ($p<0.05$) (Fig. 4E and 4F). It has to be considered that for prevention protocol, ROS formation was analyzed at the end of UVB irradiation, whereas for the regeneration one, measurements took place after 24 hours thus to allowing the PRGF to accomplish its regenerative effect. For both protocols, ROS levels were statistically lower than their respective UV groups.

Finally the apoptotic level after UVB doses was compared for the different treatment groups. The results showed that young/middle aged PRGF was able to significantly decrease the number of apoptotic cells after photo-oxidative stress ($p<0.05$). As in the aforementioned experiments, both preventive and regenerative PRGF protocols demonstrated to provide cell culture with a biological protection against UVB irradiation (Fig. 4G and 4H).

No differences were found between young and middle aged PRGF outcomes for any of the biological mechanisms tested.

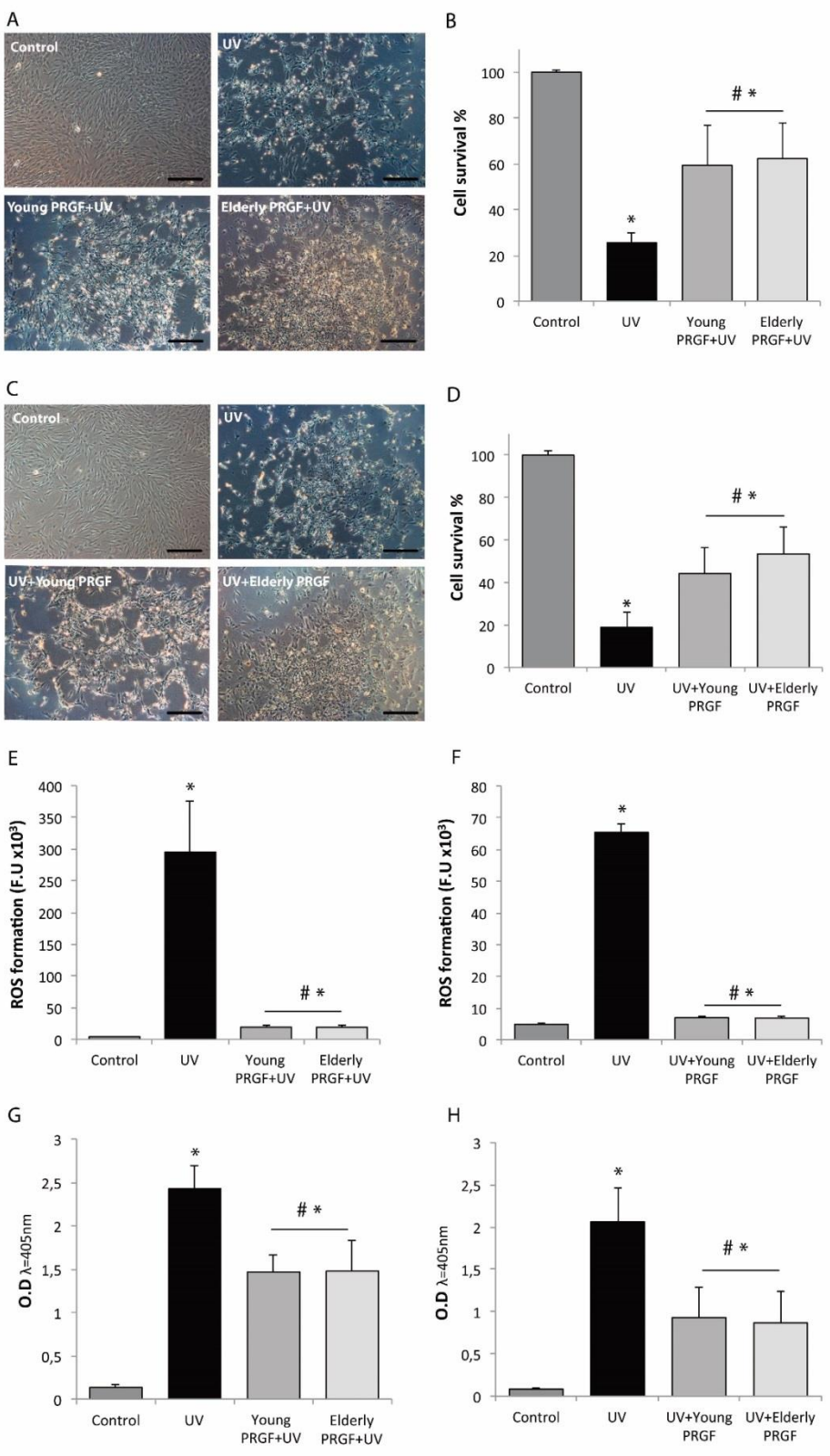

Fig.4. Biological response of human dermal fibroblasts to photo-oxidative stress. (A) Prevention: representative images showing the preventive effect of PRGF against UVB irradiation. (B) Prevention: although the UVB showed to decrease the cell survival significantly (*p<0.05), PRGF was able to maintain higher levels of viable cells when added before the photo-oxidative stress (#p<0.05). (C) Regeneration: representative images showing the regenerative effect of PRGF against UVB irradiation. (D) Regeneration:

although the UVB decreasedcell survival significantly (*p<0.05), PRGF treatment promoted higher amount of viable cells when added after the photo-oxidative stress (#p<0.05). (E) Prevention: while ROS production was increased after UVB irradiation (*p<0.05), PRGF was able to prevent significantly reactive oxygen species formation when added 24h before the UVB dose (#p<0.05). (F) Regeneration: the UVB light increased ROS production (*p<0.05), however, PRGF was able to decrease those levels when added during 24 hours after UVB irradiation(#p<0.05). (G) Prevention: although several apoptotic mechanisms were triggered after UVB irradiation (*p<0.05), PRGF prevented cell death when added before the photo-oxidative stress. (H) Regeneration: similar behavior was detected among apoptotic cells when PRGF was added after the UVB dose (#p<0.05). (*p<0.05) indicates significant differences compared to non-irradiated Control group. (#p<0.05) indicates significant differences compared to UV irradiated group. PRGF treatments include 4 different PRGF preparations obtained from 4 young aged donors (under 35 years old) and 4 additional PRGF preparations obtained from 4 middle aged donors over 50 years old. No statistical differences were found between young aged and middle aged groups for any of the aforementioned analyzed parameters. For normal distribution following data, statistical differences were analized using ANOVA test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used. Cells were seeded at 10000 cells/cm^2 for cell survival assays, at 40000 cells/cm^2 for ROS detection assays and at 20000 cells/cm^2 for apoptosis assays. Phase contrast images are taken at 5x magnification. Scale bars: 200µm

PRGF prevents and regenerates solar damage in 3D organotypic skin explants

To test the preventive and regenerative potential of PRGF against solar damage, 3D orgatotypic skin explants were intradermically injected before and after UV radiation. Once the activated PRGF was injected, an *in situ* generated fibrin matrix was formed, releasing multiple key growth factors for cutaneous regeneration including TGFβ, HGF, FGFb, PDGF, IGF and EGF (Fig. 5A). The fibrin scaffold showed a multilayered distribution that covered mainly the papillary and reticular dermis although some areas of the hypodermis were also reached (Fig. 5B).

As shown in Fig. 5C, the metabolic activity of cells within the explant was significantly reduced by solar damage and only the regenerative treatment of PRGF was able to restore optimal levels (79±4%) compared to the non-treated ones (50±7%) (p<0.05). Cell membrane integrity was also tested by the surrounding LDH measurement and although sun exposure showed to increase the cell damage for every treatment group, both preventive and regenerative PRGF protocols were able to reduce significantly LDH levels from 209±11% of the non-treated group to 155±25% and 167±12% respectively (p<0.05) (Fig. 5D). Additionally, skin irritation status of photo-stressed explants was measured by a skin colorimeter, and results indicated that while non-treated explants reached 122±3% of redness index, PRGF regenerated and prevented skin models maintained baseline levels similar to the non-exposed ones (93±8% and 107±5% respectively) (p<0.05) (Fig. 5E). Furthermore, the percentage of necrotic cells

was analyzed by flow cytometry after UV exposure, and only the regenerative PRGF protocol demonstrated to maintain similar necrosis levels to non-irradiated explants. These outcomes were significantly lower than those reached by non-treated and preventive PRGF treated skin models ($p<0.05$) (Fig. 5F). Finally, reactive oxygen species producing cells within photo-damaged skin models were detected by flow cytometric analysis. As Fig. 5G indicates, both preventive and regenerative PRGF protocols maintained baseline ROS levels, similar to non-irradiated explants (15±5% and 19±1% respectively), while significantly higher outcomes were detected for non-treated ones (41±8% and 40±1% respectively) ($p<0.05$).

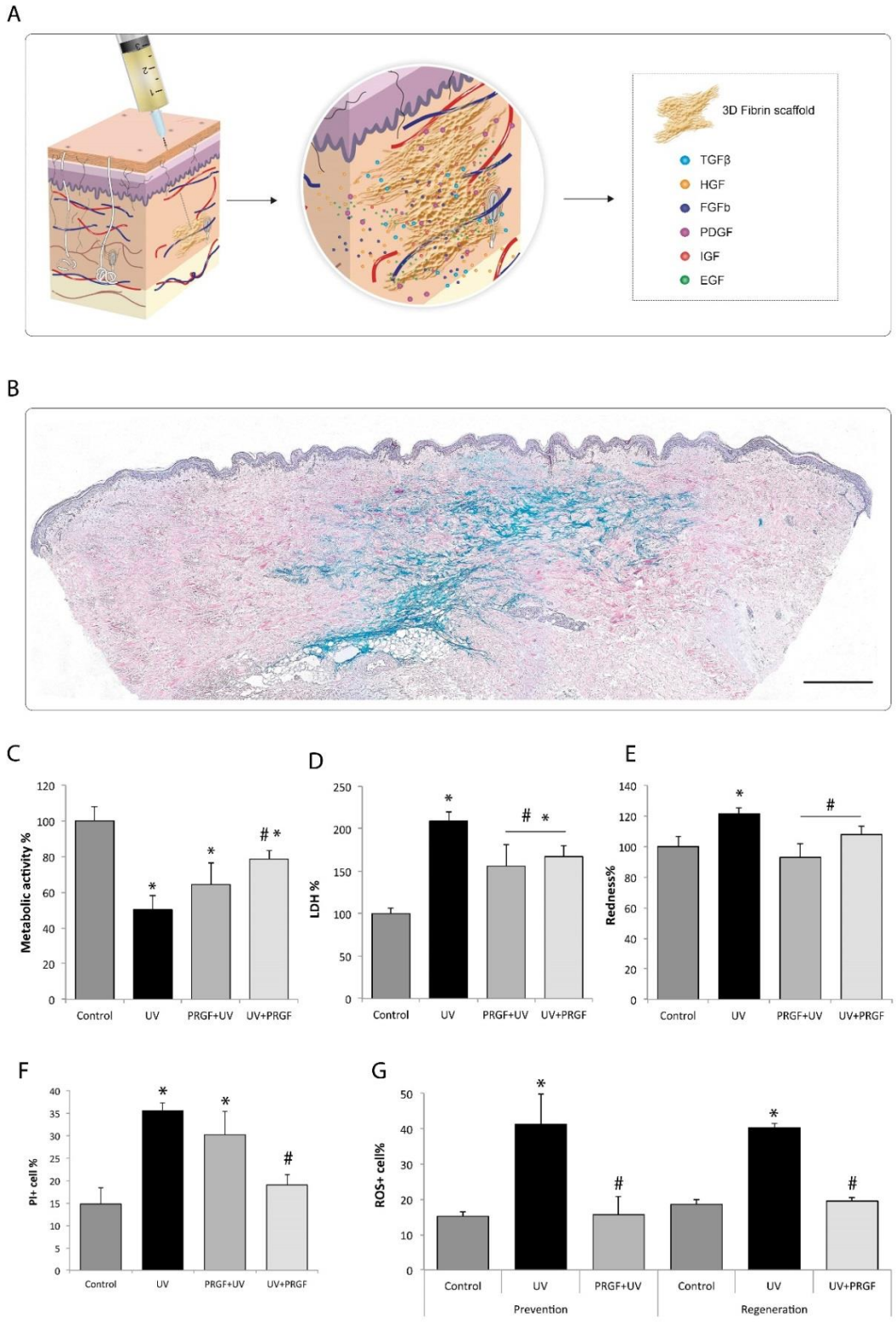

Fig.5. Biological response of organotypic skin explants to photo-oxidative stress. (A) Schematic diagram of the *in situ* scaffold formation and growth factor release after PRGF intradermal injection. (B) Indian ink stained PRGF scaffold distribution across the papillary and reticular dermis. (C) Sun exposure decreased the metabolic activity of skin explants (*p<0.05) while only the regenerative PRGF treatment applied after UV dose was able to significantly restore metabolic activity levels (#p<0.05). (D) Although cell membranes were significantly compromised after the UV dose (*p<0.05), both preventive and regenerative PRGF

protocols showed to decrease the cell damage caused by photo-oxidative stress due to a decrease in the surrounding LDH levels (#p<0.05). (E) Both preventive and regenerative PRGF treatments, reduced the skin irritation level compared to the UV treated group (#p<0.05), whose redness index was significantly higher than non-irradiated explants (*p<0.05). (F) Sun exposure increased the necrosis of skin explants (*p<0.05), however, the regenerative PRGF treatment was able to reduce significantly the necrotic cell percentage (#p<0.05). (G) Although reactive oxygen species formation was induced by photo-oxidative stress (*p<0.05), both preventive and regenerative protocols showed to decrease ROS development after UV dose, compared with their respective non-treated groups (#p<0.05). Five organotypic skin models were used for each treatment group. PRGF+UV represent the preventive effect of plasma rich in growth factors when added before the UV dose. UV+PRGF represent the regenerative effect of plasma rich in growth factors when added after the UV dose. (*p<0.05) indicates significant differences compared to non-irradiated Control group. (#p<0.05) indicates significant differences compared to UV irradiated group. For normal distribution following data, statistical differences were analized using ANOVA test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used.Histological image is taken at 2.5x magnification. Scale bar: 800µm.

PRGF maintains the histological integrity of sun exposed 3D organotypic explants

Photo-damaged and PRGF treated skin models were histologically stained with H-E in order to determine the interstitial edema percentage and the mean epidermal thickness. As shown in Fig. 6A and 6B, edema levels increased significantly up to 39±1% after sun exposure, while baseline levels were maintained for both preventive and regenerative protocols (28±2% and 26±3% respectively) ($p<0.05$) when PRGF was injected. Regarding the epidermal width, a significant decrease was observed from 100±16µm of their baseline thickness to 54±8µm after UV exposure (Fig. 6C and 6D). When PRGF was injected, both protocols showed to increase the epidermal integrity compared to the non-PRGF treated group (82±9µm and 96±16µm respectively) ($p<0.05$). Moreover, proliferating reticular dermal cells were quantified by Ki67 immunolabeling. Results showed that although solar damage reduced significantly the percentage of bioactive cells per mm^2 from 4.48±0.6% to 1.94±1.2% ($p<0.05$), the regenerative PRGF treatment was able to increase the proliferating levels up to 8.73±2.6% ($p<0.05$) (Fig. 6E and 6F).

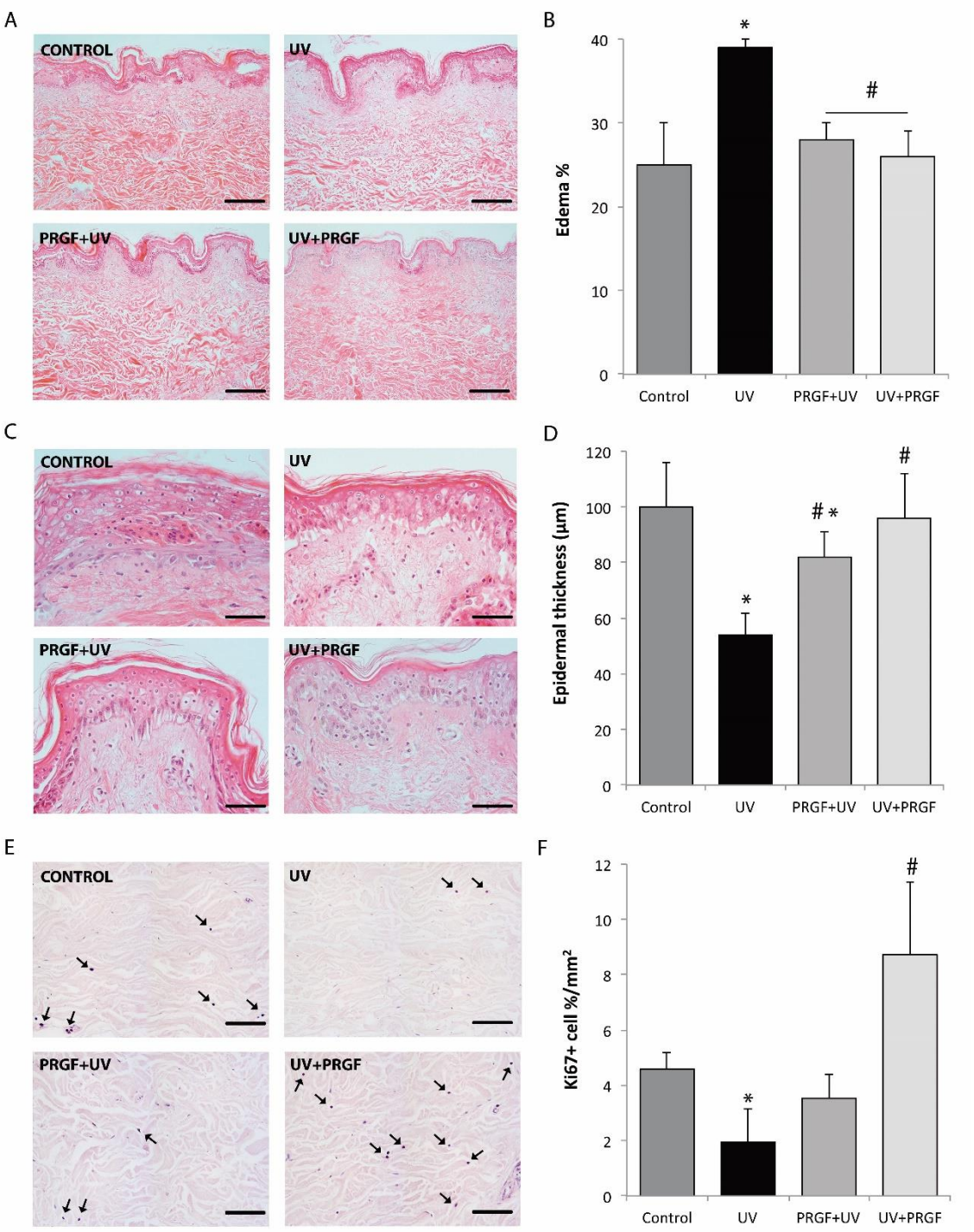

Fig.6. Histomorphometric analisys of organotypic skin explants after solar exposure. (A) Representative micrographs of H-E stained models after UV and PRGF treatments (10x magnification). (B) The interstitial edema increased after UV irradiation (*p<0.05) while both preventive and regenerative PRGF treatments showed to significantly reduce the edema surface percentage (#p<0.05). (C) Representative micrographs of H-E stained epidermal layers after UV and PRGF treatments (40x magnification). (D) Although the epidermal thickness decreased significantly after sun exposure (*p<0.05), both PRGF protocols increased the mean width of the epidermis compared to the non-PRGF treated group (#p<0.05). (E) Representative micrographs of Ki67 immunolabeled explants with arrow indicated Ki67+ cells (20x magnification). (F) Proliferating dermal cells decreased after UV dosage (*p<0.05), however, regenerative PRGF treatment

was able to increase significantly Ki67+ cell percentage (#p<0.05). Five organotypic skin models were used for each treatment group. PRGF+UV represent the preventive effect of plasma rich in growth factors when added before the UV dose. UV+PRGF represent the regenerative effect of plasma rich in growth factors when added after the UV dose. (*p<0.05) indicates significant differences compared to non-irradiated Control group. (#p<0.05) indicates significant differences compared to UV irradiated group. For normal distribution following data, statistical differences were analized using ANOVA test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used.Scale bars: 200μm for (A) images, 50μm for (C) images and 100μm for (E) images.

Furthermore, the maintenance of the connective tissue organization after photo-oxidative stress was qualitatively analyzed by masson trichrome stain and collagen type I immunolabeling. As it is observed in Fig. 7A, collagen type IV fibers beneath the basement membrane of the epidermis suffered a noticeable breakdown after UV irradiation. However, both preventive and regenerative PRGF treatments were able to restore the sun derived photo-damage by enhancing the volume of collagen fibers hence showing a better organized papillary and reticular dermis. Additionally, collagen type I images of Fig. 7A presented a severe cleavage process of this type of ubiquitous collagen along dermal layers of photo-exposed explants. Nevertheless, PRGF treatment protocols seemed to prevent the deterioration of collagen I, showing an optimal tridimensional organization and similar amount of fibers compared to non-irradiated skin models. Finally, the volume decrease of elastic fibers was also analyzed using the orcein stain (Fig. 7B and 7C). After sun exposure, orcein+ fiber area percentage decreased significantly from 11.6±0.4% to 8.8±1.3% ($p<0.05$). However both preventive and regenerative PRGF treatments showed to increase elastic fiber surface by 12.9±0.8% and 13±1.1% respectively ($p<0.05$). It can be additionally observed how these elastic fibers emanate from the dermal/epidermal junction (DEJ) and coalesce within reticular dermis in non-irradiated and in both PRGF protected explants. In the contrary, with no PRGF treatment, the UV irradiation splits those fibers and disrupts the bundle connection between layers.

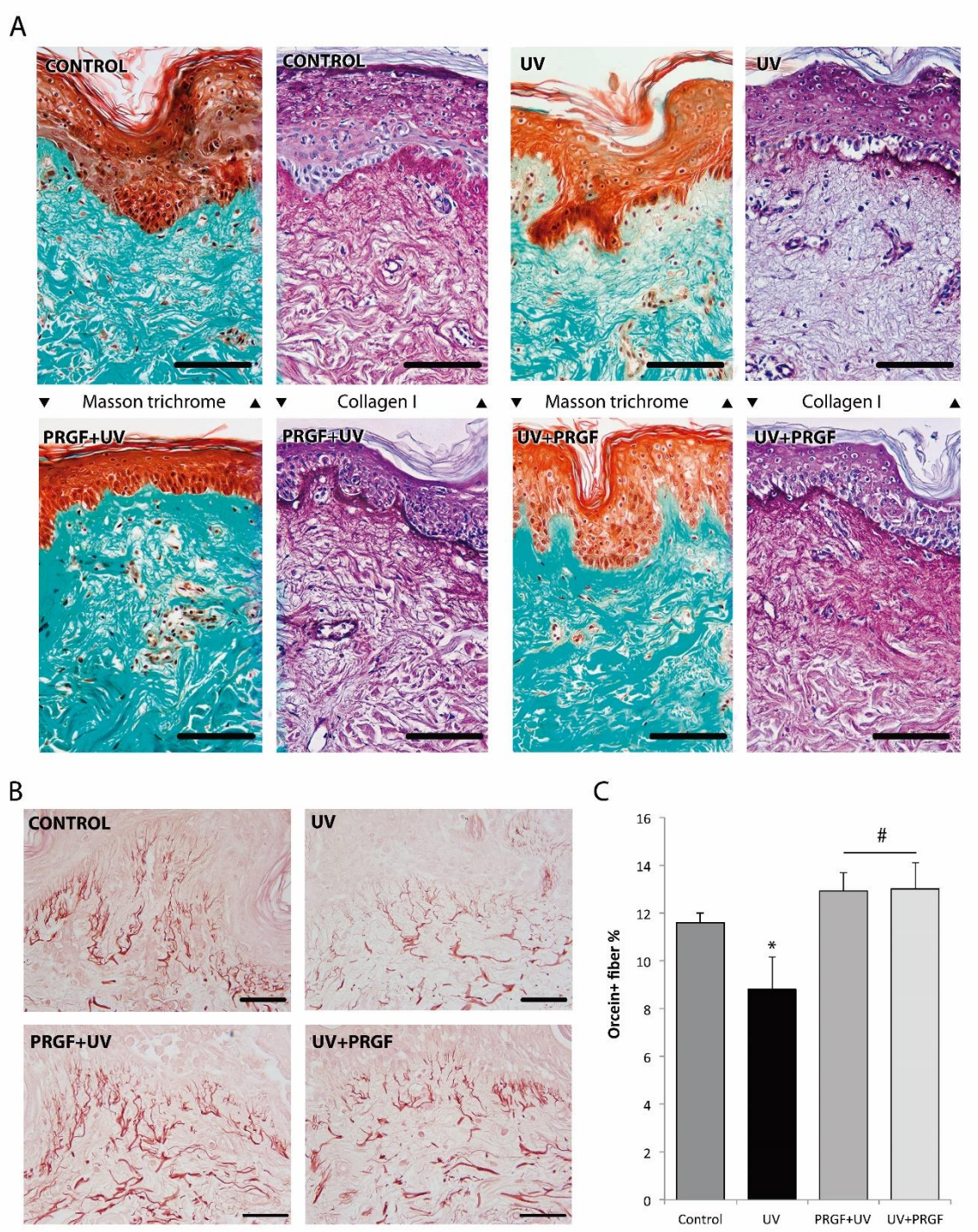

Fig.7. Structural maintenance of collagen and elastic fibers after photo-oxidative stress. (A) Representative micrographs of masson´s trichrome stained (left) and collagen type I immunolabeled (right) skin explants (40x magnification). Results of UV irradiated explants, showed a severe breakdown of collagen IV fibers beneath the basement membrane along with an advanced cleavage process of collagen I bundles of the dermis. However, both preventive and regenerative PRGF treatments were able to protect the tridimensional integrity of the ECM matrix by maintaining the volume and organization of papillary and reticular fibers. (B) Representative micrographs of orcein stained elastic fibers after UV and PRGF treatments (40x magnification). (C) Although the elastic fiber surface percentage decreased significantly after sun exposure (*p<0.05), both PRGF treatments maintained a high orcein+ connective tissue

(#p<0.05). Five organotypic skin models were used for each treatment group. PRGF+UV represent the preventive effect of plasma rich in growth factors when added before the UV dose. UV+PRGF represent the regenerative effect of plasma rich in growth factors when added after the UV dose. (*p<0.05) indicates significant differences compared to non-irradiated Control group. (#p<0.05) indicates significant differences compared to UV irradiated group. For normal distribution following data, statistical differences were analized using ANOVA test. For non-parametric data, Mann Whitney and Krustal Wallis tests were used.Scale bars: 50μm.

Discussion

Skin regeneration and wound healing process involve a wide range of coordinated events that ultimately end up in cutaneous tissue repair [22]. Here, the technology of plasma rich in growth factors as an innovative therapy for skin regeneration has been evaluated. Upon activation, platelets release a balanced pool of key growth factors including TGFβ, PDGF, EGF, HGF, IGF and FGFb that interestingly reached similar levels when comparing PRGF from young and middle aged donors. Moreover, no differences were found between donors for any of the biomechanisms assessed, so the suitability of the therapeutic application of PRGF regardless the age of the patient is reinforced. Several findings have demonstrated that it´s regenerative potential is based on the transient fibrin scaffold formed after PRGF injection were the heparan sulfate domains sequester the aforementioned growth factors thus being able to sustain a gradual release of bioactive molecules [11].

One important concern in cutaneous wound healing is scar formation due to fibrotic tissue development by persistent myofibroblasts [3]. TGFβ1 has been identified as one of the most potent inductors of fibroblast differentiation into alfa-SMA-expressing cells. When these myofibroblasts do not undergo their apoptotic fate, they can severely impair tissue function by excessive ECM protein secretion, pro-inflammatory cytokine release and profibrotic factor expression [23]. Our results showed that even in the presence of TGFβ1, PRGF was able to reduce and revert the myofibroblast phenotype. These findings are consistent with others in which PRGF has shown its ability to enhance wound healing preventing it from fibrotic tissue development being useful for the scarless regeneration of gingival [24], ocular [25], tendon [26] and muscle tissue [27]. Interestingly, it has been observed that FGFb enhances the fibroblast phenotype by promoting the apoptosis of persistent myofibroblasts [10]. These studies suggest that this mechanism is related with the down regulation of PI3K/Akt pathways as well as with the up regulation of Rho/Rho kinase signaling. Furthermore, several studies have reported the release of latent TGFβ1 from self-

generated stores in ECM when the skin is exposed to mechanical forces and tensile activities, similar to those suffered when a cutaneous injury takes place [28]. If the TGFβ1 feedback gets unbalanced, fibrotic tissue might develop and a hypertrophic or a keloid scar could replace the healthy area. However, HGF has been reported to be an effective inhibitor of the myofibroblast differentiation. HGF induces the Smad 7 protein overexpression which leads to an inhibition of TGFβ1 induced myofibroblast transformation by means of decreasing Smad 2 and Smad 3 phosphorylation [29].

After cutaneous injury, stress fibers are expressed and together with mechanical tensions over the damaged ECM, the orientation of fibroblasts along the axis of the wound is triggered, ending up in optimal tissue contraction [30]. Therefore, the contractile activity of dermal fibroblasts was tested in the presence of PRGF. Results showed significant differences between PRGF treated and non-treated groups achieving 2.2 fold higher contraction rates after 24 hours of cell culture. These findings are consistent with other studies in which platelet rich plasma or recombinant growth factors enhanced tridimensional gel contraction [10]. In fact PDGF has proven to be an important inductor of ECM contraction [31]. Moreover, according to a recent report, "thrombin (platelet)-rich plasma" is able to promote gel shrinkage by PAR-1 and PLC activation, extracellular calcium influx via L-type calcium channel and the calcium/CaM-MLCK and Rho kinase activation pathways [32].

Interestingly, this *in situ* polymerized autologous mesh has also proved to protect against UV derived photo-oxidative stress. An UVB light source to irradiate 2D cultured human dermal fibroblasts and a solar simulator lamp to overexpose 3D organotypic human skin models (HOSEC) have been used. For both assays, the preventive and regenerative effect of PRGF was assessed with the aim of resembling the situation of pre and post photo-damage treatments respectively. Our results demonstrated that PRGF increased cell survival and reduced apoptotic levels when comparing to non-treated fibroblasts. Additionally, the metabolic activity and irritation level of skin explants improved after PRGF treatment, while necrotic tissue and cell membrane damage was significantly reduced. These results may be attributable to the strong mitogenic response shown by several cell types treated with PRGF due to the effect of key growth factors such as VEGF, HGF, PDGF, FGFb and IGF [33]. More studies are needed to elucidate the underlying mechanisms behind PRGF, including the regulatory effect on the overexpression of apoptotic caspases and the prevention of the DNA strand cleavage and pyrimidine dimerization after UV exposure [34]. Nevertheless, there is some evidence regarding the anti-apoptotic effect of PRGF in different *in vitro* hostile microenvironments [35]. Interestingly the hallmark of photo-oxidative stress,

ROS formation, which is responsible for the activation of ERK1/2 and JNK upstream modulators of MAP kinase pathways [5], has shown to be significantly decreased after PRGF treatment in both 2D and 3D models. These results suggest that plasma rich in growth factors could enhance the organism´s own antioxidant activity by reducing intracellular levels of free radicals such as peroxide hydrogen and superoxide anions. In fact, other studies have recently demonstrated that PRGF is able to activate detoxifying and antioxidative enzymes by the overexpresion of the antioxidant response element (ARE) via Nrf2 nuclear factor upregulation in human osteoblasts [36]. Finally, the histomophometric analysis of irradiated 3D explants, showed a protective effect of PRGF over the structural integrity in terms of reduction of interstitial edema and maintenance of the epidermal thickness. Moreover, better organized and less deteriorated collagen and elastic fibers were found in PRGF protected skin models. These results are consistent with other pilot studies in which photo-aged patients treated with PRGF developed a thickening of the demo/epidermal layer along with a reduction in the photo-elastotic area and an augmentation of CD34+ deep dermal dendrocyte number [37]. The persistence of a three dimensional fibrin scaffold beneath the irradiated epidermal layer, serves as a provisional matrix for cell growth [38] as our Ki67+ cell outcomes have shown. Therefore, even under solar irradiation, reticular dermal cells might counteract the unbalance between collagenase overexpression and tissue inhibitor matrix metalloprotease (TIMP) down-regulation [39]. In fact, other studies have shown that PRGF is able to promote collagen type I and hyaluronic acid biosynthesis by human dermal fibroblasts [40], so the intradermal application of this autologous therapy could be an effective treatment in the maintenance of the damaged ECM after photo-oxidative stress. However, both the biomolecular mechanism and clinical treatment protocol of PRGF need to be further investigated as different outcomes have been achieved regarding the preventive and regenerative groups. While both PRGF protocols showed to maintain cell membrane integrity and decrease ROS formation, only the regenerative protocol reported a significant improvement in the metabolic activity and necrotic cells among irradiated explants. Randomized and placebo controlled clinical studies are encouraged as they would probably shed light on these and other key features of plasma rich in growth factor therapy.

Conclusion

Although additional studies are needed to elucidate all the underlying mechanism by which this autologous technology regulates skin regeneration, our findings suggest that plasma rich in growth factors (PRGF-Endoret) offers a promising therapy that enhances dermal fibroblast mediated wound contraction preventing it from fibrotic tissue development. Additionally, we have demonstrated that PRGF might enhance the photo-protective mechanisms triggered after UV exposure both in 2D and 3D skin models. Finally, as no differences were found between PRGF donors, it can be assumed that PRGF-Endoret technology is efficient regardless the age of the patient.

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Artículo IV

A novel personalized 3D injectable protein scaffold for regenerative medicine.

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Title: A novel personalized 3D injectable protein scaffold for regenerative medicine

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Conflict of interest:

Eduardo Anitua, Ander Pino, Gorka Orive and María Troya are scientists at BTI Biotechnology Institute. This biotechnology company has developed the technology of plasma rich in growth factors.

Abstract

Biomaterials should be designed to closely resemble the characteristics and functions of the native extracellular matrix to provide mechanical support and signals to direct biological events. Here we have developed a novel injectable plasma rich in growth factors (PRGF-Endoret)-based formulation that combines a thermal-denaturation step of plasma with an autologous fibrin crosslinking. Rheological and mechanical properties were evaluated. Additionally, the microstructure and biological capacity of the biomaterial was also characterized. This novel formulation exhibited ideal mechanical properties and a gel-like behavior with the ability to progressively release its growth factor load over time. The results also suggested that the novel injectable formulation is non-cytotoxic, biocompatible and suitable for cell ingrowth as it is deduced from the fibroblast proliferation within the scaffold. Finally, stimulation of both cell proliferation and matrix proteins synthesis demonstrated the regenerative potential of this autologous protein based injectable scaffold.

Keywords : Platelet rich in growth factors, autologous, mechanical properties, injectable, 3-D culture.

Introduction

Tissue engineering has promoted the use of 3D scaffolds as cell and drug delivery vehicles like a possible solution to the current shortage of transplantable organs. Ideally, biomaterials should be designed to closely resemble the characteristics and functions of the native extracellular matrix (ECM) which not only provides mechanical support for cells, but also supplies signals that direct several biological events including cell attachment, proliferation, differentiation and survival [1, 2].

The specific characteristics and functions of each tissue require versatile and highly tunable biomaterials to be applied in different applications [3]. However, raw materials should fulfill the requirements for an ideal tissue engineered scaffold including: non-toxicity, biocompatibility, controlled biodegradability and appropriate mechanical properties that mimic those of the replaced tissue [2]. Naturally occurring biomaterials perform most of the above stated criteria with the added advantage that minimize the immune and inflammatory response. Cell-matrix adhesions are crucial for cell survival and during cell adhesion and spreading, mechanical and contractile forces are generated and transmitted to the matrix. Cells are also subjected to exogenous forces including chronic loading and fluid shear stress [4, 5]. Mechanical and rheological properties of biomaterials are of special interest as they must resemble the native mechanical environment to withstand and favor the physiological forces derived from cell activity. In order to improve their clinical relevance, biomaterials must also provide a sustained and localized delivery system of biologically active molecules such as growth factors, critical signaling molecules that play a central role in successful tissue regeneration [6].

Scaffolds may also be used as either injectable or transplantable implants. Injectable biomaterials are becoming increasingly popular as they provide a minimally invasive approach with the ability of filling complicated shape defect of any size [7-9]. For these injectable materials, rheological characterization is of great importance as viscoelasticity is a prerequisite. They have to be sufficiently deformable prior to or during injection but elastic enough following injection to resist shear deformation forces once implanted [10, 11]. Currently, injectable biomaterials are being applied in diverse areas of tissue engineering [12, 13].

Autologous plasma rich in growth factors (PRGF-Endoret) is a versatile technology that provides a biological system of patient's own mediators that optimize tissue regeneration. The different PRGF-Endoret-derived endogenous formulations [14] enable the clinical use of this approach in several medical fields ranging from dentistry

and orthopedics to ophthalmology among others [14-16]. Interestingly, PRGF-Endoret provides a three-dimensional, biocompatible and biodegradable fibrin scaffold that has been widely used as a hemostatic and sealant agent [17]. Additionally, it works as a growth factor delivery system and it plays a role as cell carrier for tissue engineering applications [18, 19]. Some of the fibrin scaffold characteristics include a relative rapid degradation and retraction which could be an advantage for surgical applications and cell and growth factor delivery.

However, some other medical applications require shape-specific scaffolds that maintain structural stability and mechanical integrity for long periods of time [20]. To overcome the limitations currently associated with the fibrin retraction process, we have developed an autologous protein based injectable scaffold. This is a novel injectable PRGF-Endoret-based formulation that provides long-term shape and volume stability for specific applications. This new biomaterial provides an autologous biodegradable platform system with the ability to deliver bioactive molecules and support cells thus enhancing tissue regeneration.

In this study a new injectable PRGF-Endoret-based hydrogel has been developed to overcome the fibrin retraction process. The development of this new biomaterial involves the combination of a thermal-denaturation step of plasma with an autologous fibrin crosslinking. Additionally, the mechanical properties and biological capacity of this new scaffold have been also characterized.

Material and methods

The study was performed following the principles established in the Declaration of Helsinki amended in 2013.

Autologous protein based injectable scaffold preparation

The autologous protein based injectable scaffold was obtained as follows. After informed consent was given, blood from 3 healthy volunteers was harvested into 9-ml collection tubes containing 0.4 ml of 3.8% (wt/v) trisodium citrate as anticoagulant. Samples were centrifuged at 580 g for 8 min (BTI System IV, BTI Biotechnology Institute, S.L., Álava, Spain) at room temperature. Part of the tubes were used to draw off the whole plasma column avoiding the buffy coat and incubated at 76ºC for 12 minutes, to obtain the precursor gel of the new scaffold. Meanwhile, 2 ml of plasma (fraction F2), just also above the buffy coat, was collected from the remaining tubes. Thereafter, F2 samples were activated with PRGF-Endoret Activator (Endoret

Dentistry) at a relative rate of 20 µl per 1 ml of gel that is to be prepared. Immediately after that, the novel scaffold was created by mixing the precursor gel and the activated F2 in a ratio of 5:1.

Characterization

SEM and histology analysis

Scanning electron microscopy (SEM) was employed to evaluate the 3D structure of the novel injectable scaffold. Samples were fixed with 2.5% glutaraldehyde and postfixed with osmium tetroxide (1% OsO4 in 0.1 M cacodylate) and finally dehydrated through ascending alcohol concentrations. Thereafter, gels were subjected to critical point drying (Autosamdri 814. Tousimis, Rockville), gold sputter coated and imaged using an electron microscope (S-4800; Hitachi, Japan).

The novel injectable PRGF-Endoret formulation was also histologically stained for hematoxylin and eosin (H&E). Briefly, scaffolds were fixed in 10% formalin for 24h. They were then embedded in paraffin and the obtained blocks were cut into 6 µm slices. Samples were then stained for H&E (Sigma-Aldrich, St. Louis, MO) and observed with a Leica DM IRB optical microscope (Leica Microsystems, Wetzlar, Germany).

Swelling ratio

Scaffolds were incubated for 90 min at 37ºC to allow sufficient crosslinking to occur. After that, scaffolds were weighed to obtain the initial weight (W_0) and then incubated in phosphate buffered saline (PBS) at 37ºC to allow any swelling to occur. At different time points (24h, 48h and 72h), samples were removed from PBS and wet weight was measured (W_x). The swelling ratio was calculated as follows:

$$\text{Swelling ratio (\%)} = ((W_x - W_0)/W_0)*100$$

In vitro degradation study

Degradation upon exposure to tissue plasminogen activator (tPA) was studied over a time period of 12 days by mass loss. Plasminogen is activated into plasmin through the action of tPA which finally induces the degradation of plasmatic fibrin. Briefly, the autologous protein based injectable scaffolds were prepared as described in section 2.1 and incubated for 90 min at 37ºC to allow sufficient crosslinking. Samples were then immersed in PRGF-Endoret for 24h at 37ºC. These were then weighed to obtain the initial weight (W_0) and transferred into PRGF-Endoret containing tPA (0.25 µg/ml [21], AbCam Cambridge, UK) at 37ºC. Weight loss was recorded at timed intervals (1, 2,

5, 7 and 12 days) and media were renewed at 7th day. Samples immersed in PRGF-Endoret without tPA were used as non-degradation control.

Rheological properties

A Kinexus ultra+ rheometer (Malvern, Malvern, UK) was used to measure the viscoelastic properties of the autologous protein based injectable scaffold. The novel injectable PRGF-Endoret formulation was obtained as described above. Strain sweeps from 0.01 to 100% at 1Hz were performed to establish the linear viscoelastic range (LVR). Rheological properties were recorded as a function of oscillatory frequency between 0.1 and 10 Hz and 0.5% shear strain which was found to be in the LVR. The temperature was held at 25ºC.

Mechanical testing

Mechanical testing of samples were conducted on a MicroTester 5548 (Instron, Barcelona, Spain) equipped with a 50 N load cell. Vertical compressive stress force analysis of the autologous protein based injectable scaffolds was performed at 2-minute linear descent (2.5mm to 0.9mm) at room temperature. The Young's modulus and the maximum tension force were obtained after analysis of the stress-tension curve. The injectability was examined by an extrusion force test. Force was applied to push the material through a 25-gauge needle at a rate of 1 mm/s and at room temperature.

Growth factor measurements

Growth factor content

The autologous protein based injectable scaffolds were incubated for 60 min at 37ºC to allow complete activation of platelets. The hydrogels were then centrifuged at 14000g for 15 minutes to obtain the liquid extract. The supernatant obtained was stored at -80ºC until use. Enzyme-linked immunosorbent assay (ELISA) kits were used to evaluate the concentrations of epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-I), platelet-derived growth factor AB (PDGF-AB) and transforming growth factor beta-1 (TGF-β1) (R&D Systems, Minneapolis, MN) on the scaffold extracts.

Immunohistochemical analysis

The main growth factors present in the autologous protein based injectable scaffolds were histologically analyzed. Briefly, samples were fixed in 10% formalin for 24h followed by paraffin embedding and sectioning (4 μm). Immunohistochemical

examination was performed using the following primary antibodies: EGF (Abcam), IGF-I (Santa Cruz Biotechnology, Heidelberg, Germany), PDGF-AB (Santa Cruz Biotechnology) and TGF-β1 (Abcam). After overnight incubation at 4ºC (IGF-I and TGF β1) or 1 h at room temperature (EGF and PDGF-AB), primary antibody staining was revealed using Peroxidase Substrate Kit (DAB) (Vector laboratories, Peterborough, UK).

Growth factor release kinetics

The release kinetics of several growth factors from the novel injectable PRGF-Endoret formulation was determined by ELISA assays. Briefly, autologous protein based injectable scaffolds were added to a 6-well plate and incubated for 90 min at 37ºC to allow sufficient crosslinking to occur. After that, Dulbecco's modified Eagle's medium (DMEM)/F-12(Gibco-Invitrogen, Grand Island, NY, USA) was added and samples were maintained at 37 °C in a humidified 5 % CO_2 atmosphere. After 3, 5 and 24 h and 7 days of incubation, medium was centrifuged at 500 g for 10 minutes and the obtained supernatant was distributed in aliquots and stored at -80ºC until use. Quantification of EGF, IGF-I, PDGF-AB and TGF-β1 (R&D System) was performed according to the manufacturer's instructions.

In vitro cell studies

Cell culture

Two primary human dermal fibroblasts cultures were isolated as previously described [22]. Fibroblasts were cultured in complete medium: DMEM/F-12 supplemented with 15 % fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany), 2 mM glutamine and 50 µg/mL gentamicin (Sigma-Aldrich). Cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere. Cells between the fifth and the sixth passages were used in the experiments.

Cytotoxicity evaluation

Autologous protein based injectable scaffolds were incubated for 90 min at 37ºC to allow sufficient crosslinking to occur. After that, the biomaterials were immersed in complete medium for 24h at 37ºC to obtain their leachates. After this period, cells seeded at 20000 cells/cm^2 were incubated with the scaffold leachates for 24h at 37 °C in a humidified 5 % CO_2 atmosphere. Cell viability was measured using the WST (tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay (Sigma-Aldrich). The cytotoxicity was defined as the

relative viability (%), without hydrogel leachate in culture media considered as 100% viability.

Cell encapsulation

Primary human dermal fibroblasts were encapsulated in the new scaffold. Briefly, cells pellets were resuspended in the F2 fractions just before activating and mixing with their corresponding precursor gels. 1 ml of encapsulated cells (170000 cells/ml) was added to the wells of 12-well plates. Plates were maintained at 37ºC allowing for crosslinking. After 90 minutes, 2 ml of complete medium were pipetted into the wells and incubated at 37 °C in a humidified 5 % CO_2 atmosphere. The culture medium was renewed the next day and every 72h for 7 consecutive days.

Three-Dimensional cell culture

Cell proliferation inside the cell-gel constructs after 1, 4 and 7 days was evaluated using the WST colorimetric assay (Sigma-Aldrich). At each time point, each cell-gel construct was incubated with WST reagent at 37ºC for 4h. Absorbance at 450/620 nm was directly proportional to the number of living cells. Autologous protein based injectable scaffolds without cells were used as controls.

SEM and histology analysis was also performed on the cell-gel constructs at day 7 as already described above.

Live/dead assay

Viability of encapsulated cells at day 1, 4 and 7 after culture was assessed using a live/dead cell imaging kit (Molecular Probes-Life technologies, Grand Island, NY, USA), according to the manufacturer's instructions. After 30 minutes incubation at room temperature, live and dead cells were imaged with a fluorescent microscope (Leica DM IRB) with excitation filters of 488 nm (green, live cells) and 570 nm (red, dead cells).

Proliferation assay

The bioactivity of gel extracts was determined by assessment of primary human dermal fibroblasts proliferation. Cells were seeded at a density of 9000 cells/cm^2 on 96-well optical-bottom black plates and maintained in culture medium containing 0.2% FBS for 24 h. The medium was then replaced with serum-free medium supplemented with either 0.2% FBS, as a control of non-stimulation, or 20% gel extract for 72 h. Cell proliferation was assessed by Cyquant cell proliferation assay (Molecular Probes-Life technologies) according to the manufacturer's protocol.

Hyaluronic acid and type I collagen synthesis

The biological effect of the new injectable PRGF-Endoret extract was also evaluated on the synthesis of extracellular matrix proteins. Dermal fibroblasts were seeded on 6-well plates until reaching confluence. Cells were then incubated for 7 days with the corresponding treatments: culture medium with 0.2%FBS or culture medium with 20% scaffold extract. Treatments were renewed on the third day. Conditioned culture media were collected at day 3 and 7, centrifuged for 10 min at 500 g and stored at -80ºC until use. Additional wells were kept without cells to determine the initial amount of hyaluronic acid (HA) added with each treatment. ELISA kits (Corgenix, Broomfield, CO) were used to determine the HA concentration in conditioned culture media.

The quantification of type I collagen was performed by Western blot. At day 7, total protein was extracted and western blot analysis was performed as previously described [23].The blots were incubated with primary antibody against type I collagen (Santa Cruz Biotechnology) overnight at 4ºC and with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse) (Santa Cruz Biotechnology) for 1h at room temperature. Blots were then developed by chemiluminescence with Supersignal West Pico substrate (Thermo Scientific-Pierce Biotechnology, Rockford, USA) using an image analyzer (Chemidoc image analyzer, Bio-Rad Laboratories). The Stain-Free technology was used as loading control method [24].

Statistical analysis

Statistical analysis is based on two-tail unpaired t-test for the cytotoxicity evaluation, proliferation assay and hyaluronic acid synthesis. Type I collagen synthesis and the degradation study data were analyzed via Mann-Whitney test. Repeated measures analysis of variances was used to evaluate the swelling ratio and growth factor release kinetics and Kruskal-Wallis for data derived from 3-D cell culture. Results are reported as mean ± standard deviation. The significant level was set as $p < 0.05$. All data were analyzed using GraphPad InStat 3.10 software.

Results

Morphology and microstructure analysis

Starting from a translucent yellow plasmatic fraction, the autologous protein based scaffold was prepared and extruded through a 25 gauge needle. The newly formed biomaterial showed a yellowish gel opaque appearance with the capacity to maintain a three-dimensional shape (Fig 1A). To observe the inner structure, H-E stained cross sections were analyzed under a light microscope. As it is shown in Fig 1B, high molecular weight plasmatic proteins formed solid thermal aggregates enclosed by a stable fibrin network. The surface of this enzymatic-free crosslinked scaffold was further analyzed using scanning electron microscopy (SEM). High magnification images evidence a fibrin mesh that totally fits the three-dimensional gel deposits of polymerized proteins, thus providing irregular shaped spaces with interconnected fibers suitable for cell ingrowth (Fig 1C-D).

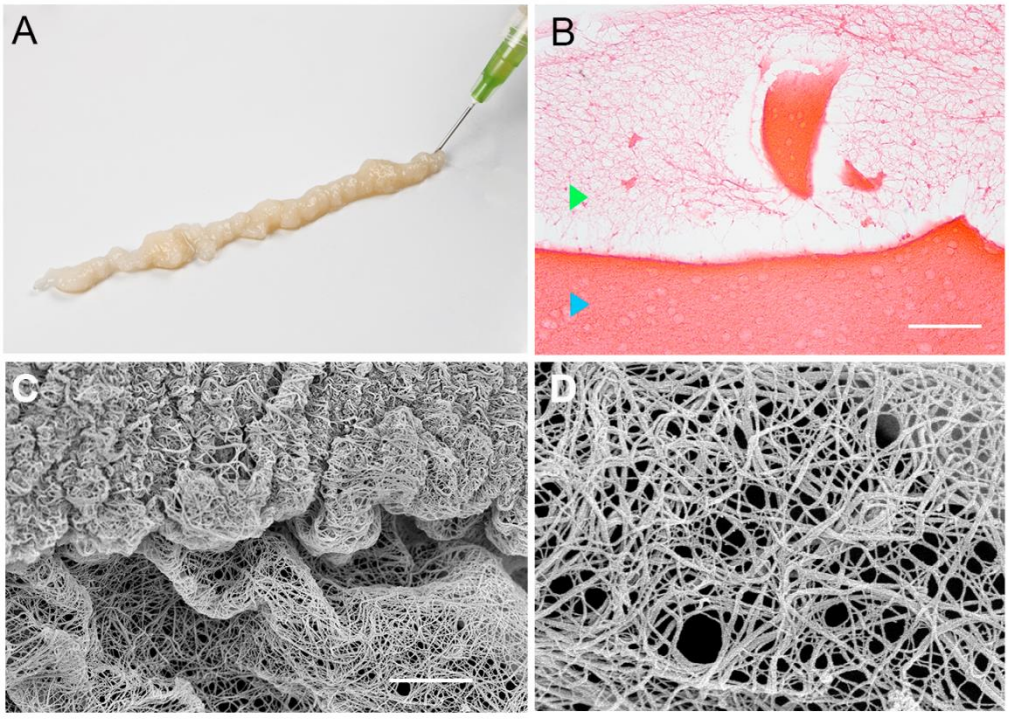

Fig. 1. Macroscopic view of the autologous protein based injectable scaffold (A). H-E stained cross sections (B) and SEM microstructure analysis (C-D) show how thermal shock derived gel deposits are closely integrated with the fibrin network rich in growth factors, resulting in a three-dimensional scaffold with bioactive potential. Magnified image of the fibrin rich area of the autologous protein scaffold (AP-scaff) observed in image C (D). Scale bars: 50 μm for B and 4 μm for C images. Fibrin network ( ) and gel deposits ( ).

Absorption ability and enzymatic degradation rate

The new injectable PRGF-Endoret formulation showed an hydration level of 91±1%. The swelling ratio test was used to evaluate the saline absorption ability of the new scaffold. Results demonstrated that the scaffold was able to sustain a saline binding behavior up to 72h and a total of 15%±2 swelling ratio was achieved (Fig 2A). This saline binding ability could be attributed to both the hydrophilicity and the three-dimensional maintenance of the biomaterial structure [25].

The new hydrogels were also incubated with tPA, the enzyme that catalyzes the conversion of plasminogen to plasmin, to evaluate the potential of the new formulation to be degraded upon exposure to enzymes found in vivo. We compared the weight loss of samples incubated in PRGF-Endoret with those incubated in PRGF-Endoret supplemented with tPA enzyme (Fig 2B). The biostability of the fibrin crosslinked protein gel was noticeably high as 63% of the initial weight was still measurable at day 12. The control group without fibrinolytic enzyme showed only a slight biodegradation response maintaining a stable weight percentage of 87 after 12 days.

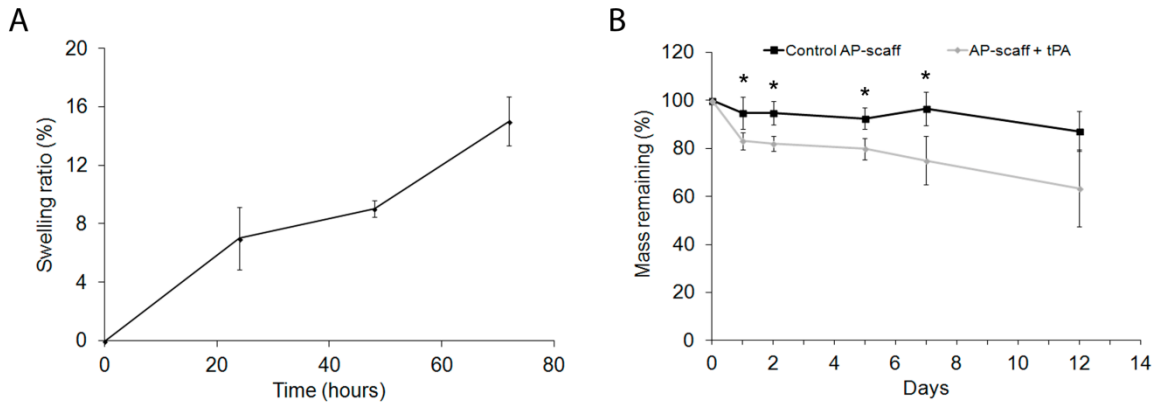

Fig. 2. Swelling ratio of the autologous protein scaffold (AP-scaff) maintained in PBS during 72h (A). Biodegradation rate of AP-scaff after incubation in PRGF-Endoret with or without tissue plasminogen activator during 12 days (B). *Statistically significant differences compared to AP-scaff incubated for 1, 2 and 5 days in PRGF-Endoret supplemented with tPA ($p < 0.05$).

Rheological and mechanical properties

With the aim of determining the biomechanical suitability of the new hydrogel to behave as a three-dimensional scaffold, the novel biomaterial was subjected to shear stress and compression forces after being extruded through a high gauge needle (25G). Once the linear viscoelastic range of the protein gel was settled (Fig 3A), key rheological parameters such as “storage modulus” (G´), “loss modulus” (G´´), and “complex modulus” (G*) were determined at 0.5% shear strain within an oscillatory

frequency of 0.1-10Hz (Fig 3B-C). The elasticity (Tan δ) and the "viscosity complex" (η) of the biomaterial were also evaluated (Fig 3D-E). The storage and loss modulus ranged from 1061±400 Pa to 1735±629 Pa and from 184±64 Pa to 298±111 Pa respectively. These results evidence the viscoelastic nature of the gel and its ability to partially recover its original shape after deformation. The total energy needed to deform the biomaterial (complex modulus), reached values from 1077±404 Pa to 1761±638 Pa showing a high stiffness once extruded. The new scaffold showed a gel-like behavior as tan (δ) values remained between 0.176±0.03 and 0.172±0.01. The viscosity complex (η) ranged from 1715±643 Pa.s to 28±10 Pa.s suggesting that it resembles a pseudoplastic hydrogel with the ability to remain in the injection site after extrusion, rather than spreading out [26]. Table 1 highlights the rheological properties at standard values at 1Hz and 0.5% shear strain.

Rheological properties	Elastic modulus (G') (Pa)	1373 ± 498
	Viscous modulus (G'') (Pa)	225 ± 84
	Complex modulus (G*) (Pa)	1391 ± 505
	Tan δ	0.164 ± 0.013
	Complex viscosity η* (Pa.s)	221 ± 80
Mechanical properties	Maximum tension force (KPa)	0.9 ± 0.2
	Young's modulus (KPa)	1.4 ± 0.4
	Mean extrusion force (N)	12.1 ± 2.7
	Maximum load	38.2 ± 15.8

Table 1. Rheological and mechanical properties of the autologous protein based injectable sacaffold. Maximum tension force is given from a 2-minute linear descent (2.5 mm to 0.9 mm) at 38% strain and at room temperature. Rheological properties were determined at 1Hz, 0.5% shear strain and 25ºC.

Increasing vertical compression forces were applied over the new biomaterial with the aim of determining its mechanical strength. The tension force generated during progressive non-confined pressing resulted in an increase from 4±7 Pa to 803±261 Pa (Fig 3F-G). Table 1 summarizes some of the mechanical characteristics of the hydrogel. Results showed that the new hydrogel is a highly cohesive scaffold suggesting that it might behave as a long standing gel deposit once implanted in the living tissue. The injectability was also measured using a 25 gauge needle, resulting in a median extrusion force and maximum load that enables the hydrogel to be easily applied with precise dosage (Table 1).

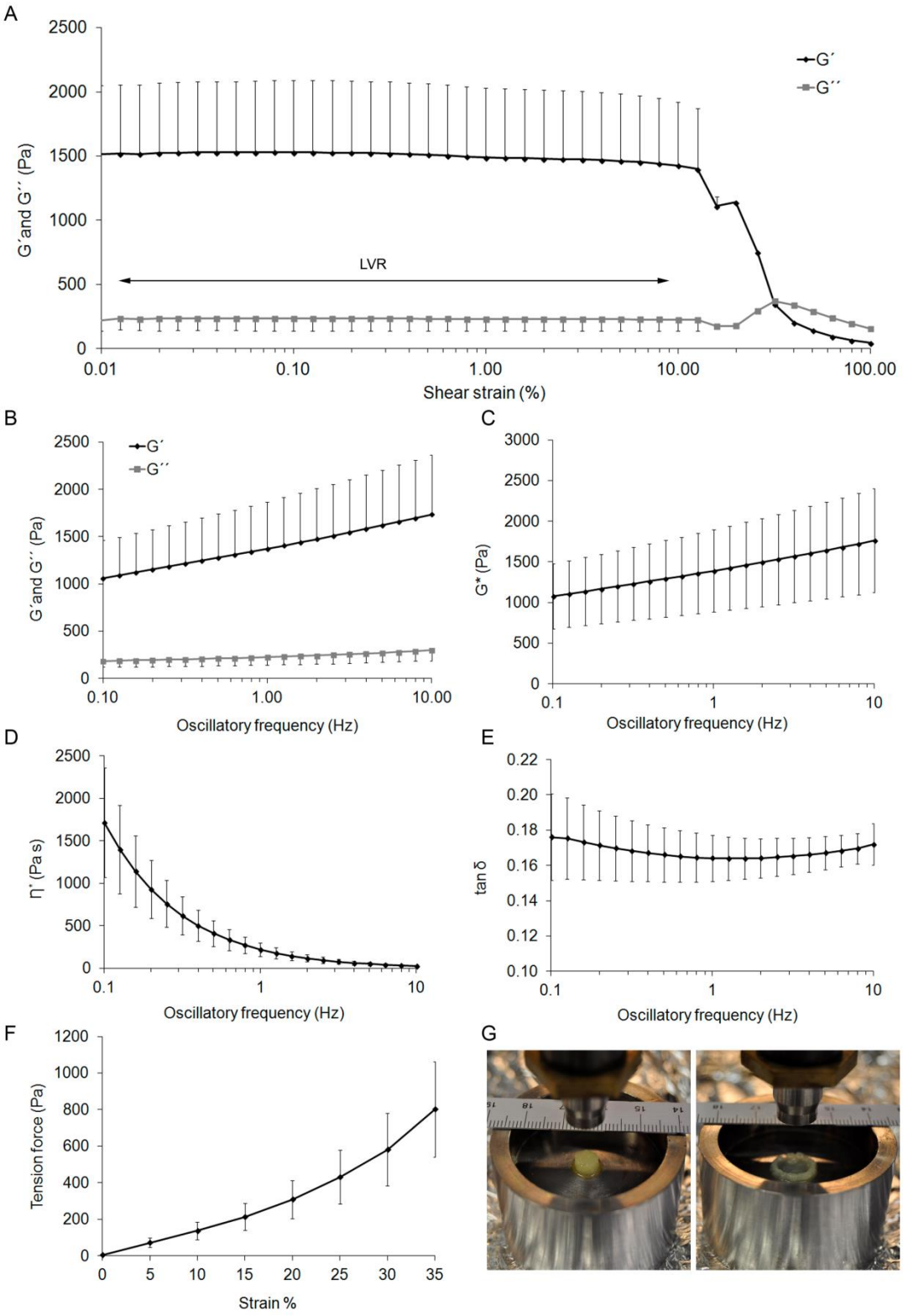

Fig. 3. Rheological and mechanical characterization of the autologous protein scaffold (AP-scaff). Definition of the viscoelastic linear range (LVR) of AP-scaff prior to the rheological and mechanical

parameter measurements (A). Amplitude sweep data showing the “storage modulus” (G´) and the “loss modulus” (G´´) as a function of oscillatory frequency (Hz) at 0.5% shear strain (B). A plot of the magnitude of “complex modulus” (G*) versus frequency (Hz) at 0.5% shear strain (C). Flow curve showing the “viscosity complex” (η) and the elasticity spectra (Tan δ) of the biomaterial as a function of oscillatory frequency (Hz) at 0.5% shear rate (D-E). Mechanical tension force slope of AP-scaff during non-confined pressure test (Pa) (F-G).

Growth factor content and release kinetics

The growth factor content of the novel hydrogel was quantified in order to determine its biologic potential. An overview of the microscopic location of some of the key morphogens for tissue regeneration was firstly examined. As it is shown in Fig 4A, EGF, IGF-I, PDGF-AB and TGF-β1 are mainly distributed over the crosslinked fibrin network. The immunohistochemical results reveal that these growth factors are located around platelet aggregates and along the numerous protein binding domains of the autologous fibrin mesh. Colorimetric assays corroborated these findings as high concentrations of growth factors were quantified in the extracts of gels obtained from different donors (Fig 4B). Additionally, the release kinetics of growth factors from the autologous protein based injectable scaffold was studied (Fig 4C). EGF and TGF-β showed a sustained release percentage ranging from 30±1% after 3 hours to 55±1% at day 7 and 28±6% after 3 hours to 58+4% at day 7 respectively ($p<0.05$). Alternatively, PDGF-AB and IGF-I seemed to reach a plateau starting from 34±1% and 31±4% at 3 hours to 49±2% and 48±2% at 24 hours respectively ($p<0.05$).

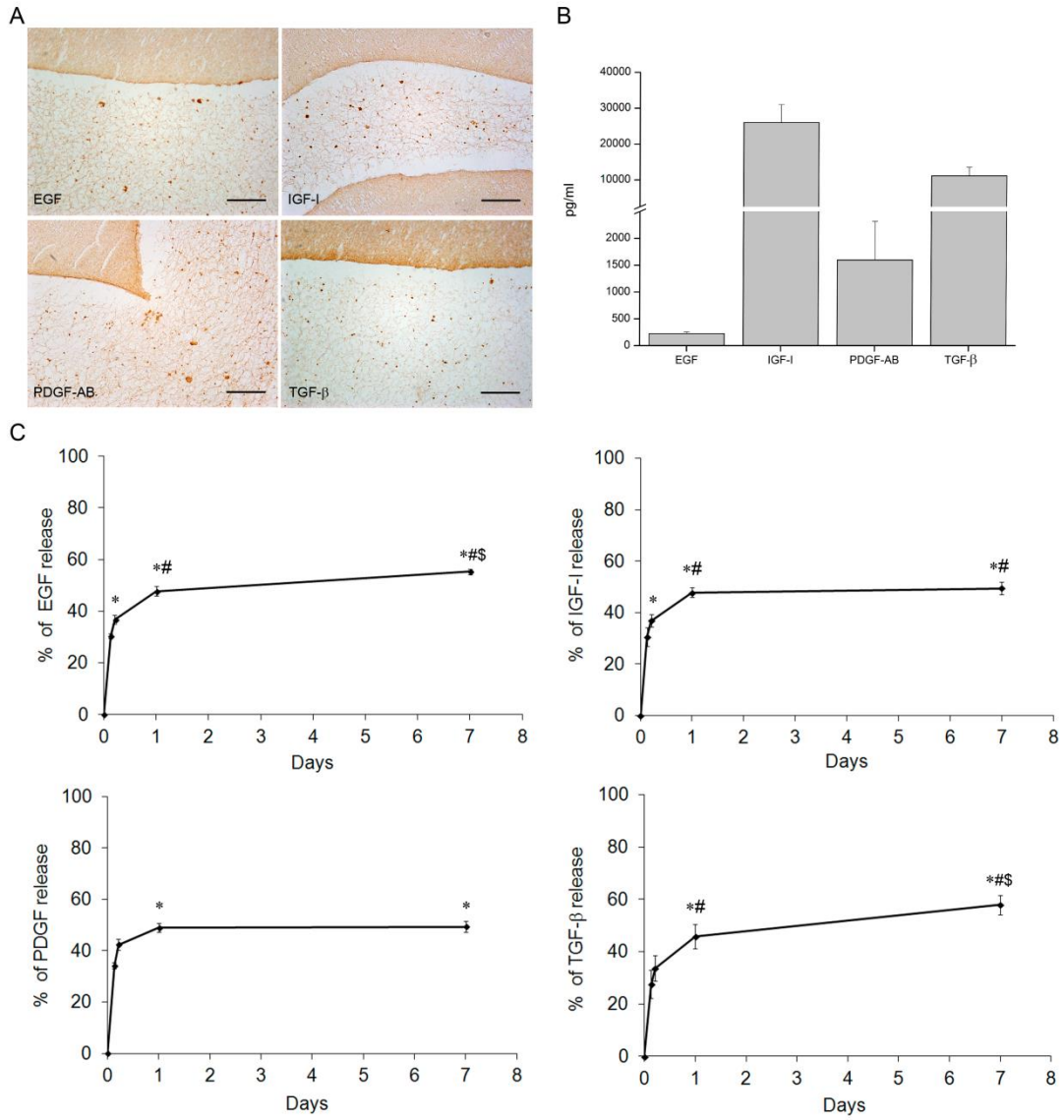

Fig. 4. Histological and quantitative analysis of growth factor content and release kinetics from the new hydrogel. Representative micrographs of EGF, IGF-I, PDGF-AB and TGFβ1 immunolabeled autologous protein scaffold samples at 40x magnification (A). Total growth factor content analysis obtained from extracts of the new biomaterial (B). Release kinetics of different growth factors from the injectable hydrogel incubated at 37ºC during 3, 5 and 24h and 7 days. *Statistically significant differences compared to 3h. # Statistically significant differences compared to 5h. $ Statistically significant differences compared to 24h. ($p<0.05$). Scale bars: 50µm.

Biocompatibility assays and three-dimensional cell culture

The biocompatibility of the novel PRGF-Endoret based scaffold was evaluated. Human dermal fibroblasts were cultured with scaffold leachates and after *in vitro* cytotoxicity measurements, no reduction in the viability percentage was observed when comparing with the control group (Fig 5A). Additionally, cells were encapsulated within the protein gel and cultured in three-dimensional arrangement during consecutive days.

Cell growth was analyzed at day 1, 4 and 7 inside the biomaterial and as it is shown in Fig 5B, a significant increase in cell number was observed at day 7 when compared with the first day of culture ($p<0.05$). The viability of cells co-cultured within the novel biomaterial was also analyzed using live/dead dye staining and visualized under fluorescence microscope (Fig 5C). Living cells fluoresced in green while dead cells were visualized in red fluorescence. Merged images showed that fibroblasts dispersed uniformly throughout the surface and cavities of the novel biomaterial and exhibited good morphology. A noticeable increase in cell growth and colonization area was observed between days 1 and 7 of co-culture, with good biocompatibility and hardly any detectable cell death. Histological and SEM microphotographs were taken after 7 days of cell-gel co-culture (Fig 5D). H-E stained cross sections revealed fibroblastic shape cells attached to fibrin rich surfaces of the scaffold where the crosslinked network fits the numerous spaces between gel deposits. From SEM images of the scaffold surface it could be observed heterogeneous and multi-layered structures of polymerized proteins coated with fibrin fibers where cells were capable to adhere and grow. These cells showed to proliferate inside the macro-porous spaces that were suitable for *in vitro* colonization after fibrin network crosslinking occurred. These results suggest that the new hydrogel is non-cytotoxic, biocompatible and suitable for cell ingrowth.

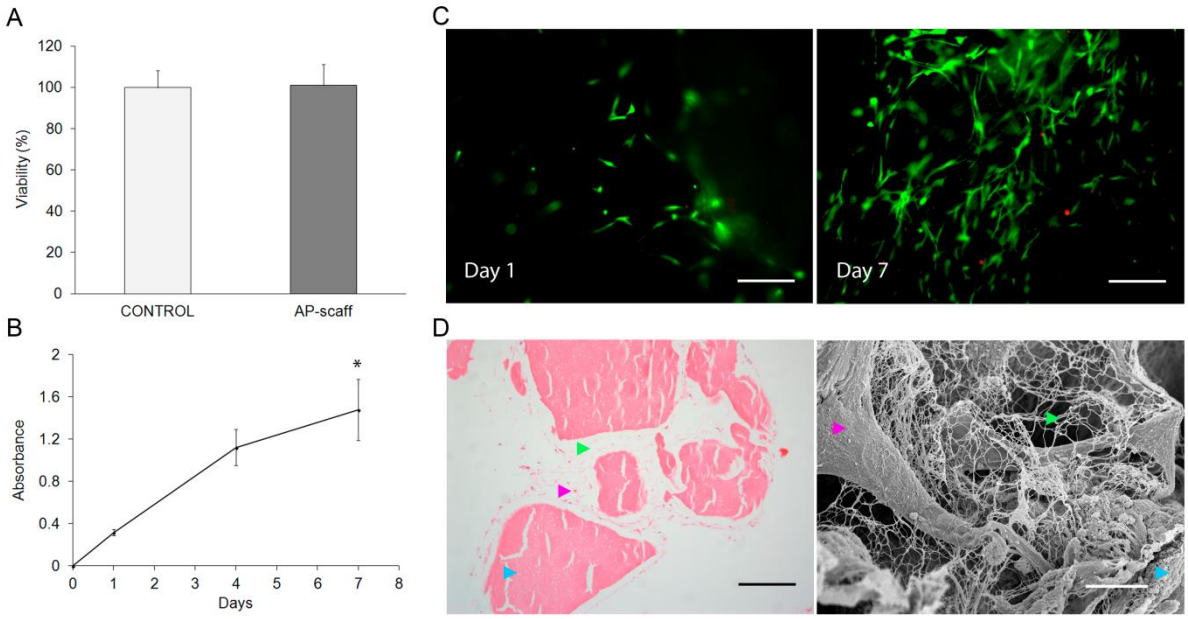

Fig. 5. Biocompatibility assays demonstrated that the new biomaterial has no cytotoxic effect as no reduction in the viability percentage was observed when comparing with the control group (A). Encapsulated cells within the protein gel were able to proliferate during consecutive days inside the autologous protein scaffold (AP-scaff) (B). Representative fluorescence microphotographs of

encapsulated fibroblasts proliferating in a three-dimensional arrangement during 7 days. Live cells in green and dead cells in red. (C). Histological H-E stained cross section of cell colonies proliferating inside AP-scaff (D left) and representative SEM microphotograph showing cell adhesion to the protein and fibrin surface of the hydrogel (D right), both after 7 days of culture. *Statistically significant differences compared to 1 day ($p<0.05$). Scale bars: 100µm for (C) images, 200µm for H-E stained (D) image and 10µm for SEM (D) image. Fibrin network ( ), gel deposits ( ) and proliferating fibroblasts ( ).

Bioactivity evaluation

The regenerative potential of scaffold extracts was determined by cell proliferation and biosynthetic activity assessment. As it is shown in Fig 6 A-B, cell proliferation was significantly enhanced after 72 hours of culture as 1.3 fold increase in total DNA was observed when compared to control group ($p<0.05$). Moreover, the expression of two key extracellular matrix proteins such as HA and type I collagen was analyzed by ELISA and Western blot, respectively. After 7 days, treated cells showed significantly higher levels of HA when compared to non-treated cells ($p<0.05$) (Fig 6C). Similarly, the biosynthesis of procollagen type I after 7 days of culture, showed to be significantly increased in gel-extract treated fibroblasts ($p<0.05$) (Fig 6D-E).

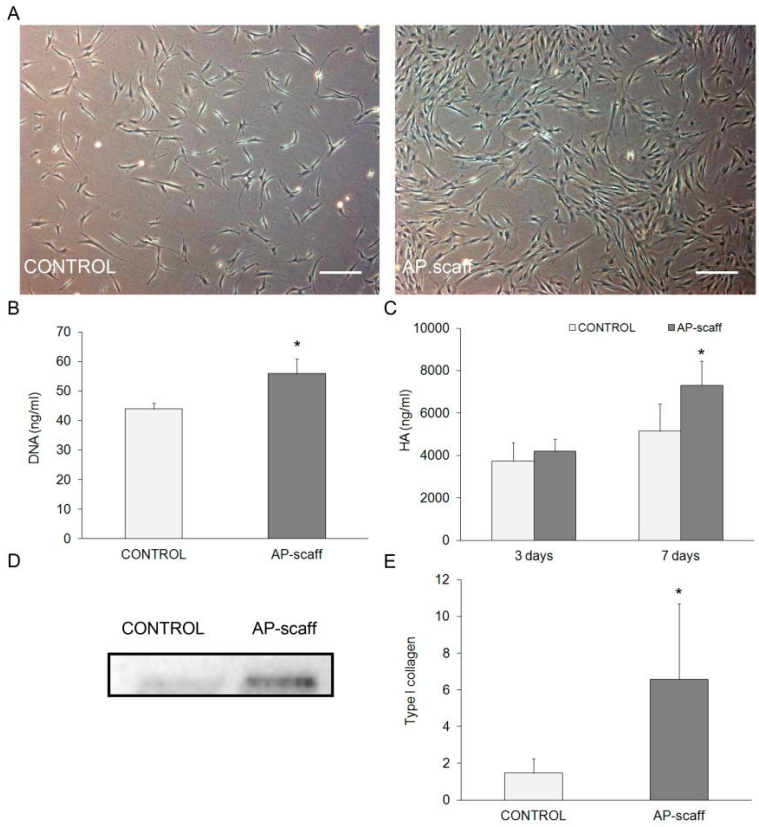

Fig. 6. Representative phase contrast microphotographs of fibroblast proliferation after 72h of cell culture with the new biomaterial extract (A). Fluorimetric analyses revealed that fibroblasts cultured with the

autologous scaffold (AP-scaff) extract reached a higher proliferation rate compared to the control group (B). Immunoenzymatic and western blot results showed that cells cultured with the extract of the new hydrogel, synthetized higher amounts of structural molecules like hyaluronic acid (C) and type I collagen after 7 days respectively (D-E). *Statistically significant differences compared to control group ($p<0.05$). Scale bars: 200µm.

Discussion

Plasma rich in growth factors technology (PRGF-Endoret) is based on the withdrawal of a small volume of the patient´s own blood which is afterwards centrifuged and activated in order to obtain an autologous clot enriched in proteins and growth factors [27]. However, from the biomechanical point of view, this technology is limited by the natural fibrin retraction process resulting in a transient membrane once implanted on the receptor tissue. In order to open up new application areas, the use of longer lasting three-dimensional biomaterials has gained an increasing clinical importance with several approaches that combine platelet derived proteins and structural scaffolds to prolong the fibrin´s short-temporary nature, thus enhancing its overall mechanical properties. Such constructs include platelet rich plasma loaded poly-lactide-ethylene-glycol-lactide gels [28], platelet rich plasma loaded chitosan-gelatin sponges [29], fibrin hydrogels functionalized with cartilage extracellular matrix (ECM) [30] or platelet rich plasma-hyaluronic acid based blends [31]. Nevertheless, these approaches require exogenous agents and chemical cross-linkers that may hinder the regenerative potential of autologous growth factors and increase the risk of immune reactions.

The present study evaluates a protein based injectable hydrogel that combined with the platelet enriched plasmatic fraction leads to a tunable platform that serves as a long-lasting scaffold for tissue regeneration. This gel-type matrix is based on the thermal polymerization of plasmatic proteins and the biologic cross-linkage of fibrin fibers. Similar proteinaceous biomaterials have been already used in various therapeutic approaches including tissue engineering, drug delivery, adhesion barriers and wound dressings [32, 33]. Heat-aggregated protein chains have proved to stand as solid gels with strong mechanical properties due to the formation of hydrogen bonds between newly formed beta-sheets, transformation of alfa helices to random coils and the presence of disulfide bonds among exposed cysteine groups [34-36]. To our knowledge this is the first 100% autologous injectable scaffold based on an endogenous protein gel enriched in growth factors that may be used with regenerative purposes.

After activation, platelets aggregate and plasmatic fibrinogen progressively evolves into an organized fibrin network. The microstructure analysis of the autologous scaffold evidenced the presence of three-dimensional protein-gel deposits interconnected with numerous fibrin threads. As it has been reported in other studies, irregular sized microporous spaces were also observed after protein matrix formation, providing a suitable adhesion surface and colonization niches for cell ingrowth [37, 38]. Fibroblastic attachment, proliferation and long term adaptation demonstrated that the new hydrogel can support cell growth with high metabolic activity and viability due to both the sustained delivery of growth factors and its structural support. In fact, other reports have outlined the ability of these type of injectable biomaterials to act as a localized delivery systems of previously loaded cell populations throughout an *in situ* solidification process into the desired tissue bed [39]. Our results suggest that the porous lamellar matrix spaces within the scaffold increased the nutrient absorption capacity thus allowing an optimal environment for tissue regeneration [40]. It is hard to reproduce the conditions that scaffolds encounter when implanted into living tissues, however, *in vitro* degradation studies can serve to accurately evaluate the potential of this type of biomaterials. In this context, tPA is an enzyme found *in vivo* that has been previously used in clot lysis assays [21]. After subsequent days of enzymatic biodegradation, results revealed that the hydrogel may act as a long-term scaffold once implanted in the living tissue. This type of biomaterials may be suitable for medical applications that involve a sustained released of growth factors and bioactive proteins. Additionally, these scaffolds meet the appropriate requirements to provide shape and volume stability for long periods of time. The absence of cytotoxicity also offers a biologic advantage over other exogenous biomaterials due to the lack of waste molecules that are usually leached out [41].

Injectable three-dimensional scaffolds that are minimally invasively injected into the living tissue are subjected to the interplay and sum of shear stress and vertical compression/stretching forces. From a rheological point of view, these biomaterials should be viscous enough at high shear rates to be accurately delivered through a therapeutic needle while elastic enough to maintain a suitable structure for longstanding tissue regeneration [42]. The prevalence of the storage over the loss modulus observed might be a direct consequence of the crosslinking at the molecular level that macroscopically is translated into a viscoelastic behavior of the material that tends to maintain its original shape rather than creeping and spreading like a viscous fluid [43]. An optimal rheological profile with suitable viscosupplementation has been related to the present PRGF-Endoret based hydrogel as a thinning phenomenon was

observed due to progressive polymeric chain disentanglement and stress axis alignment after shear rate application [31]. Additionally, the independency of the angular viscosity toward frequency sweeps confirmed that the elastic component of the biomaterial keeps it as a stable gel deposit once extruded with no expected undesired flow beyond the application space [44]. These rheological results further prove the injectability of the new scaffold at room temperature, and in situ gel forming potential for *in vivo* applications [28]. From a mechanical point of view, the progressive cohesiveness displayed by the autologous gel, not only evidenced the natural stiffness of the biomaterial but also the strong inner adhesion forces that avoid particles to separate from the original deposit during the tissue regeneration period [11].

The transient fibrin mesh derived from PRGF-Endoret technology has proved to bind several key growth factors released by the alfa granules of the patient´s own platelets [45]. Likewise this fibrin membrane, the new scaffold has showed to act as a delivery system that offers a sustained supply of plasmatic and platelet derived proteins, cytokines and morphogens that ultimately promote local tissue regeneration. The fibrin network spaces between gel deposits of the biomaterial sequester growth factors such as EGF, IGF-I, PDGF-AB and TGFβ1 via heparin sulfate domains thus being able to provide a gradual release of bioactive molecules [46]. These proteins are reported to be involved in reepithelization processes, stimulate cell migration, and present a potent mitogenic role [47]. Results of the present study have shown that the extracts, rich in growth factors, of the protein based injectable scaffold promote cell proliferation and ECM deposition via type I collagen and HA biosynthesis. Collagen is one of the major constituents of extracellular matrix and provides structural support to resident cells in both soft and hard tissues. These findings are consistent with other studies that demonstrate the high proliferative activity of different cell phenotypes cultured with platelet derived autologous products [48, 49, 22, 50]. Other reports have also showed the structural remodeling potential of plasma rich in growth factors by means of procollagen carboxiterminal peptide neoformation, pro-alfa1/2 collagen chain expression, glucosaminoglican production, elastin fiber reorganization and co-regulation of matrix metalloproteases and tissue inhibitor metalloproteases [51-53].

Our findings demonstrated that the new hydrogel not only might be a suitable scaffold for cell ingrowth and mechanical support, but also behave as a biologically active structure able to release a wide range of autologous growth factors involved in tissue regeneration. Numerous studies have tried to shed some light on the biomolecular mechanisms of platelet derived products in the main stages of the wound healing process. Anti-fibrotic tissue regeneration and reduction of the inflammatory phase for

example, have been associated with reduced Smad2/3 phosphorylation and attenuation of NFκB pathway respectively after growth factor therapy [54, 55]. Moreover, platelet rich plasma has shown to activate several anti-apoptotic regulators such as Bcl-2 protein, thus exerting an important cell death preventive effect [56]. Other studies suggest that PRGF-Endoret enhances an antioxidant activity by the overexpression of the antioxidant response element (ARE) via Nrf2 nuclear factor upregulation, ending up in a reduction of local reactive oxygen species (ROS) [57]. Increased bioactivity of platelet rich plasma treated cells could also be related with the ERK/Akt pathway activation and CDK4/Cyclin D1 overexpression as well as CDK1/2, PLK1 and E2F1 transcription factor gene upregulation [58, 59]. Recent reports have additionally outlined the pivotal role of some of the molecules released after platelet activation, such as HGF and VEGF, in the contribution of angiogenesis and the activation of surrounding endothelial cells to establish new blood vessels from pre-existing vasculature [60]. In contrast to the safety profiles of other three-dimensional scaffolds, the new hydrogel might offer a preventive effect against common bacterial contamination, as cationic antimicrobial peptides, collectively known as platelet microbicidal proteins, are released after platelet degranulation [61, 62]. Several studies have underlined the role of platelet derived products as antibacterial agents due to the presence of molecules such as platelet factor 4, connective tissue-activating peptide III, platelet basic protein, thymosin beta 4, fibrinopeptide A/B and RANTES that play a role inhibiting the synthesis of bacterial survival proteins [63, 64].

Conclusion

Here, we report a protein based scaffold derived from plasma rich in growth factors technology. This biomaterial is clearly aimed at clinical practice as it provides an autologous, safe and easy to prepare *in situ* formulation. The nature of this hydrogel is based on the thermal polymerization of plasmatic proteins and the natural crosslinkage of the fibrin network that occurs after platelet activation. Additional *in vivo* reports will be needed to fully characterize the functionality of this new biomaterial. Clinical studies will assess the efficacy and safety of this tunable platform that may improve the regenerative potential of current cell therapy and growth factor delivery approaches. However, present results conclude that this new biomaterial has desirable mechanical properties for cell ingrowth in three-dimensional arrangement while provides a sustained release of bioactive molecules that promotes surrounding tissue regeneration.

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Artículo V

A novel protein based autologous topical serum for skin regeneration.

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Title: A novel protein based autologous topical serum for skin regeneration

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Conflict of interest:

Eduardo Anitua is the scientific director of, and Ander Pino and Maria Troya are researchers at BTI Biotechnology Institute, the company that has developed the PRGF-Endoret technology

Abstract

As skin ages a functional decrement occurs. To avoid future vulnerability to dermatologic diseases an optimal cutaneous regeneration is mandatory. Biological therapies based on blood derived autologous proteins are gaining attention of scientists and dermatologists. A novel 100% autologous topical serum has been developed using plasma rich in growth factors technology. The physicochemical characterization and the biologic potential of the novel formulation have been studied. Rheological and mechanical properties and the biological capacity of the formulation were characterized. Human dermal fibroblast culture and 3D organotypic skin explants were used as *in vitro* and *ex vivo* cutaneous models respectively. The autologous topical serum presented an optimal spreadability index and appropriate shear thinning behavior that allowed an easy handling and rapid integration within the cutaneous tissue. The formulation has a high growth factor load with the ability to progressively penetrate into the dermo/epidermal layers of the skin. It is biocompatible and promotes cell proliferation and chemotactic activity. The autologous topical serum promotes the biosynthetic activity of cells by the stimulation of collagen and hyaluronic acid expression. These findings present an *in situ* and easy to prepare autologous topical serum based on the patient´s own blood with physicochemical and bioactive properties useful for skin regeneration purposes.

Keywords: skin regeneration, platelet rich plasma, growth factors, topical application.

Introduction

The skin covers an area of approximately 2 m², being the largest organ in the human body. Its main function is to act as a protective barrier isolating the organism from the environment and thus protecting it from infections and dehydration. However, the mechanistic, protective and restorative properties of the cutaneous tissue become impaired with age due to a multifactorial process derived from both intrinsic and external factors [1]. Functional decrements as a result of chronological ageing include reduced wound heling potential, decreased cutaneous immune responsiveness and delayed recovery of barrier function after damage. The natural ageing process is triggered by a programmed tendency of keratinocytes and fibroblasts to stop dividing, a lower production of structural proteins and a reduced biomechanical strength [2]. Alternatively, external environmental factors include: exposure to sun derived ultraviolet radiation, pollutants (ozone, smog or particulate matter) and lifestyle factors such as smoking or alcohol consumption. Some of the main consequences of the extrinsic ageing converge in biochemical and molecular pathways that increase free radical and metalloprotease imbalance within the cutaneous tissue. Hence there is a progressive reduction in water binding capacity that changes the permeability of the skin due to an increased production of reactive oxygen species that ultimately provoke xerosis, loss of elasticity, slower turnover of epidermal cells and atrophy [3]. Additionally, as skin ages the vasculature progressively atrophies and supporting dermis is deteriorated while collagen and elastin fibers become sparse and disorganized. These changes leave vulnerable skin areas increasingly susceptible to future dermatologic diseases with a steadily decreasing ability to cutaneous renewal [4,5].

Patient number in need of optimal cutaneous regeneration is increasing due to the rise of life expectancy in the last decades, currently averaging 77.6 years [6]. In this line, several regenerative medicine approaches have been tested with the aim of promoting wound healing and dermal/epidermal regeneration. Some examples include tissue engineered skin [7,8], artificial collagen scaffolds [9-11], hyaluronic acid coated matrices [12,13], stem cell therapy [14] and the use of recombinant growth factors [15,16]. In fact, recent findings suggest that physiologically balanced growth factors derived from heterologous dermal fibroblast culture techniques may be integrated as active pharmaceutical ingredients within a topically applied vehicle with regenerative purposes [17]. In this regard, the use of the patient´s own blood and platelet derived proteins have shown to be useful for promoting tissue repair and healing [18,19]. These therapies are based on the withdrawal of a small blood volume in order to obtain the platelet rich plasma fraction (PRP) after a single or double centrifugation step. Upon

activation, platelets release a balanced pool of key growth factors including transforming growth factor-β (TGFβ), platelet derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin like growth factor (IGF), fibroblast growth factor-b (FGFb) and angiopoietin-I (Ang-I) [20].

Recent studies highlight the therapeutic effect of plasma rich in growth factors technology (PRGF) not only in regenerative dermatology [21], but also in the treatment of several disorders in a wide range of medical fields such as orthopedic surgery [22], sports medicine [23,24], ophthalmology [25,26], neurobiology [27] and oral/maxillofacial surgery [28,29]. There is growing evidence about the different therapeutic products that can be obtained from the this autologous technology such as semi-solid fibrin membrane, injectable formulation, gel or liquid supernatant [19,30]. However, currently there is no availability of a 100% autologous topical ointment with adequate rheological properties and an optimal protein balance for superficial cutaneous applications.

In the present study an autologous topical serum (ATS) derived from PRGF technology is described. The physicochemical characterization as well as the biologic potential of the novel formulation is evaluated over human dermal fibroblast culture and 3D organotypic human skin explants.

Material and methods

The study was performed following the principles established in the Declaration of Helsinki amended in 2013.

Preparation of ATS

After informed consent was obtained, blood from 3 healthy volunteers was harvested into 9-ml collection tubes containing 0.4 ml of 3.8% (wt/v) trisodium citrate as anticoagulant. Samples were centrifuged at 580 g for 8 min (BTI System IV, BTI Biotechnology Institute, S.L., Vitoria, Spain) at room temperature, and the whole plasma column, avoiding the leucocyte rich buffy coat, was collected. Part of the plasma was subjected to 76ºC for 12 minutes to provoke plasmatic protein gelation. The remaining plasma was incubated with PRGF-activator (BTI) at 37ºC for 1h in order to trigger the fibrin clot retraction process. The PRGF supernatant rich in growth factors was obtained. The ATS was then prepared by vigorously mixing both products in a 2:1 ratio.

pH and viscosity evaluation

A commercial topical cream and ATS samples were used to determine and compare their pH and viscosity. pH analysis was performed with a pH-meter GLP 21+ (Crison, Barcelona, Spain). Viscosity was measured with a rheometer (AR 1000 TA Instruments, New Castle, USA). One mL of sample was applied to the sample holder and viscosity was determined over a large range of shear stress (from 0.10 to 100 s^{-1}).

Spreadability

The spreadability of the ATS was determined by measuring the spreading behavior of 1g of formulation placed between two horizontal plastic plates [31,32]. A weight of 128g was put on the upper plate for 1 min. The diameter of the circle before and after the serum spreading was determined. The spreadability of a commercial topical cream was also evaluated. Spreadability was calculated as follows:

A_f/A_i

where A_f is the area of the serum after spreading and A_i is the area of the serum before spreading.

Growth factors

Determination of total growth factor content

ATS extracts were obtained after sample centrifugation at 14000g for 15 minutes. Enzyme-linked immunosorbent assay (ELISA) kits were used to evaluate the concentrations of epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-I), platelet-derived growth factor AB (PDGF-AB) and transforming growth factor beta-1 (TGF-β1)(R&D Systems, Minneapolis, MN) on the ATS extracts.

Growth factor release kinetics

Topical formulations were added to a 6-well plate. Dulbecco's modified Eagle's medium (DMEM)/F-12(Gibco-Invitrogen, Grand Island, NY, USA) was added after a 90 minute incubation at 37ºC to prevent serum from soaking up the medium. Samples were then maintained at 37 °C in a humidified 5 % CO_2 atmosphere. Media were collected after 3, 5 and 24 h and 7 days of incubation and centrifuged at 500 g for 10 minutes to eliminate the debris. Samples were then stored at -80ºC until use. ELISA assays were used to quantified EGF, IGF-I, PDGF-AB and TGF-β1 (R&D System) according to the manufacturer's instructions.

Ex vivo skin penetration

Three-dimensional human organotypic skin explants (hOSEC) were used to conduct this *ex vivo* assay. The explants were immersed in phosphate-buffered saline (PBS) at 51ºC for 1 minute to separate the epidermis from the dermis. The epidermis was then placed in a Franz diffusion cell. Before performing the percutaneous absorption study, both transepithelial resistance and capacitance were verified in order to be higher than 1000 Ω and less than 50 nF (respectively). One thousand microliters of the formulation was applied into the donor compartment. Twenty four hours after application, the whole volume of the receptor chamber (5 ml) was collected, the epidermis was washed and lysed and both stored at -80ºC until use. EGF and PDGF-AB were measured by ELISA, according to manufacturer protocol (R&D System).

In vitro evaluation

Cell culture

Primary human dermal fibroblasts cultures were isolated from skin biopsies as previously described [33]. Fibroblasts were cultured in complete medium: DMEM/F-12 supplemented with 15 % fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany), 2 mM glutamine and 50 µg/mL gentamicin (Sigma-Aldrich, Madrid, Spain). Cells were incubated at 37 °C in a humidified incubator with 5% CO_2. Cell viability was assessed by trypan blue dye (Sigma-Aldrich, Madrid, Spain) in hemoctytometer. Two cell lines between the fifth and the sixth passages were used.

Cell Proliferation

Cell proliferation was determined by a commercially avalaible fluorescence-based kit (Cyquant cell proliferation assay, Molecular Probes-Life technologies), according to the manufacturer's protocol.

Briefly, cells were cultured in 96-well optical-bottom black plates at a density of 10000 cells/cm^2 with medium containing 0.2% FBS. After 24h, media was removed and treatments were added: serum-free medium supplemented with either 20% ATS extracts or 0.2% FBS, as a control of non-stimulation. Cells were maintained for 72h. After incubation, cells were washed with PBS and frozen. Cell proliferation was assessed according to the manufacturer´s instructions. Fluorescence intensity was quantified using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany) with excitation at 485 nm and emission at 535 nm.

Chemotaxis

The chemotactic potential of ATS was also determined by a commercial fluorimetric kit (CytoSelect™ 96-Well Cell Migration Assay, 8 µm, Cell Biolabs, San Diego, USA).

A polycarbonate membrane chamber (8 µm pore size) serves to separate wells into two compartments and as a barrier to discriminate migratory cells from non-migratory cells. Dermal fibroblasts were seeded at a density of 10000 cells/well in serum-free medium into the top compartment. Both treatments, serum-free medium supplemented with either 20% ATS extracts or 0.2% FBS, were placed in the bottom compartment. After 24h, quantification of migration induced by the chemoattractants was performed by detecting cells in the bottom compartment using a fluorescence microplate reader (Twinkle LB 970) with excitation at 485 nm and emission at 535 nm.

Extracellular matrix synthesis

ATS extracts were used to evaluate its effect on dermal fibroblasts synthesis of extracellular matrix proteins. Confluent cells were incubated with the corresponding treatments: serum-free medium supplemented with either 20% ATS extracts or 0.2% FBS for 7 days. Treatments were renewed on the third day. Conditioned culture media collected at day 3 and 7 and centrifuged for 10 min at 500 g were used for hyaluronic acid (HA) quantification. Cells were also lysed on day 7 of treatment to obtain the protein extract to determine the amount of type I collagen synthesized.

ELISA for hyaluronic acid

HA levels on conditioned culture media were measured by an Enzyme-Linked Immunosorbent Assay (ELISA), according to manufacturer protocol (Corgenix, Broomfield, CO). Wells with treatments but without cells were maintained as control for the Elisa kit.

Western Blotting for type I collagen

Mammalian protein extraction reagent supplemented with protease and phosphatase inhibitors (Pierce Biotechnology, Bonn, Germany) and the BCA assay (Pierce Biotechnology) was used to determine total protein concentration. Western blot analysis was performed as previously described [34]. Blots were incubated with primary antibody against type I collagen (Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4ºC and with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse) (Santa Cruz Biotechnology) for 1h at room temperature. Blots were developed by chemiluminescence with Supersignal West Pico substrate (Thermo Scientific-Pierce Biotechnology, Rockford, USA) and bands were detected with an

image analyzer (Chemidoc image analyzer, Bio-Rad Laboratories). The Stain-Free technology was used as loading control method [35].

Statistical analysis

Data were reported as mean ± standard deviation. Statistical analysis of the results was performed with the software SPSS v15.0 (SPSS, Chicago, IL). Spreadability, proliferation and chemotaxis data were analyzed via two-tail unpaired t-test. Kruskal-Wallis and Mann-Whitney test were used for the release kinetics. Mann-Whitney test was used for data derived from type I collagen synthesis assay. Repeated measures analysis of variances was used to evaluate hyaluronic acid synthesis.

Results

Preparation of ATS

Blood from three healthy volunteers was used to prepare their respective autologous topical serums. Prior to the preparation, PRGF samples were analyzed in a hematology analyzer. Subjects reached 2.1x ± 0.3 mean platelet enrichment over the peripheral blood (Table 1).

Donor	Gender	Age	Platelets in blood (plt/µLx10^3)	Platelets in plasma (plt/µLx10^3)	Platelet enrichment
1	Male	40	250	466	1.9x
2	Female	32	246	600	2.4x
3	Male	45	178	382	2.1x

Table 1: platelet enrichment in PRGF before autologous topical serum preparation

Macroscopic and physicochemical evaluation

The autologous protein based ointment was assessed for its macroscopic characteristics and organoleptic qualities such as aspect, color and odor. The topical serum had a smooth texture and homogeneous oil-in-water emulsion nature. This semisolid hydro-lotion presented an opaque yellowish appearance. After soft superficial application, no smell was detected and the topical serum rapidly got integrated within the cutaneous tissue (Fig 1A).

The pH values of ATS were found to be in the range of the neutral 7.4-7.8 compared to the slightly acidic commercial cream of pH 5.7 (Fig 1B). The spreadability of ATS was

twofold of the commercial cream as 7.4±2 index versus 3.8±0.6 index was observed respectively ($p<0.05$) (Fig 1C-D). Both formulations showed a shear thinning behavior that is common in pseudoplastic topical gels, ranging from higher viscosity values at low shear rate (250 and 300 Pa.s respectively), to lower viscosity values at high shear rate (4 and 22 Pa.s respectively). The formulations showed and appropriate viscosity that allowed an easy handling and optimal application onto cutaneous surface (Fig 1E).

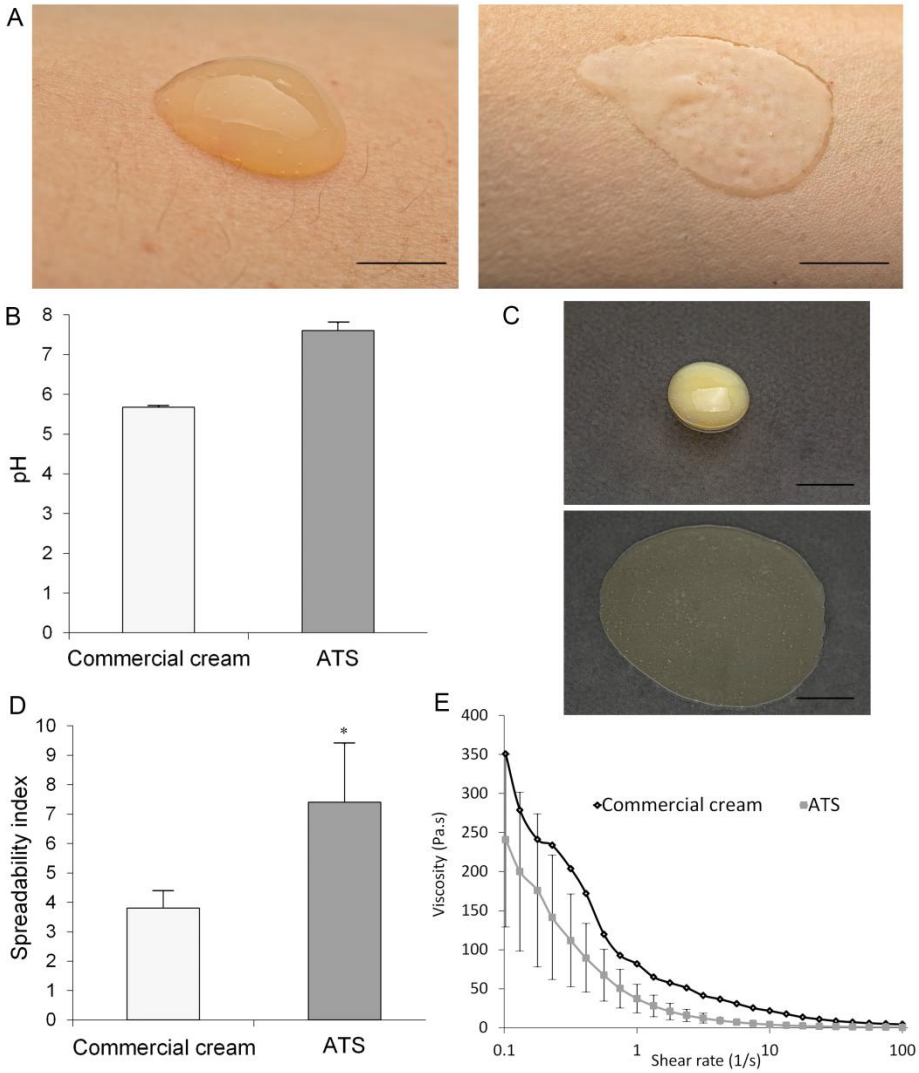

Figure 1. Macroscopic view of the autologous topical serum applied on cutaneous tissue (A). Neutral pH values of ATS compared to a slightly acidic commercial cream (B). Representative images of ATS before and after the spreadability test (C). Spreadability test performed over ATS and a commercial cream (D). Viscosity curves showing the thinning behavior of both topical formulations at increasing shear rate (E). Scale bars: 0.5 cm for A and 1cm for C. *Statistically significant differences compared to commercial cream ($p<0.05$).

Growth factor content and release kinetics

The growth factor content within the novel topical formulation was quantified in order to determine its biologic potential. Several key proteins involved in cutaneous regeneration were measured by enzyme linked immunosorbent assay. As it is shown in Fig 2A, high levels of EGF, IGF-I, PDGF-AB and TGF-β1 were detected in ATS extracts. Additionally, the release kinetics of different morphogens from ATS was studied (Fig 2B-C). PDGF-AB and TGF-β1 showed a sustained release percentage ranging from 57±13% after 3 hours to 79±5% at day 7 and 41±18% to 72±9% respectively ($p<0.05$). Alternatively, EGF and IGF-I seemed to reach a plateau of 53±16% and 76±24% at 1 hour and 24 hour respectively.

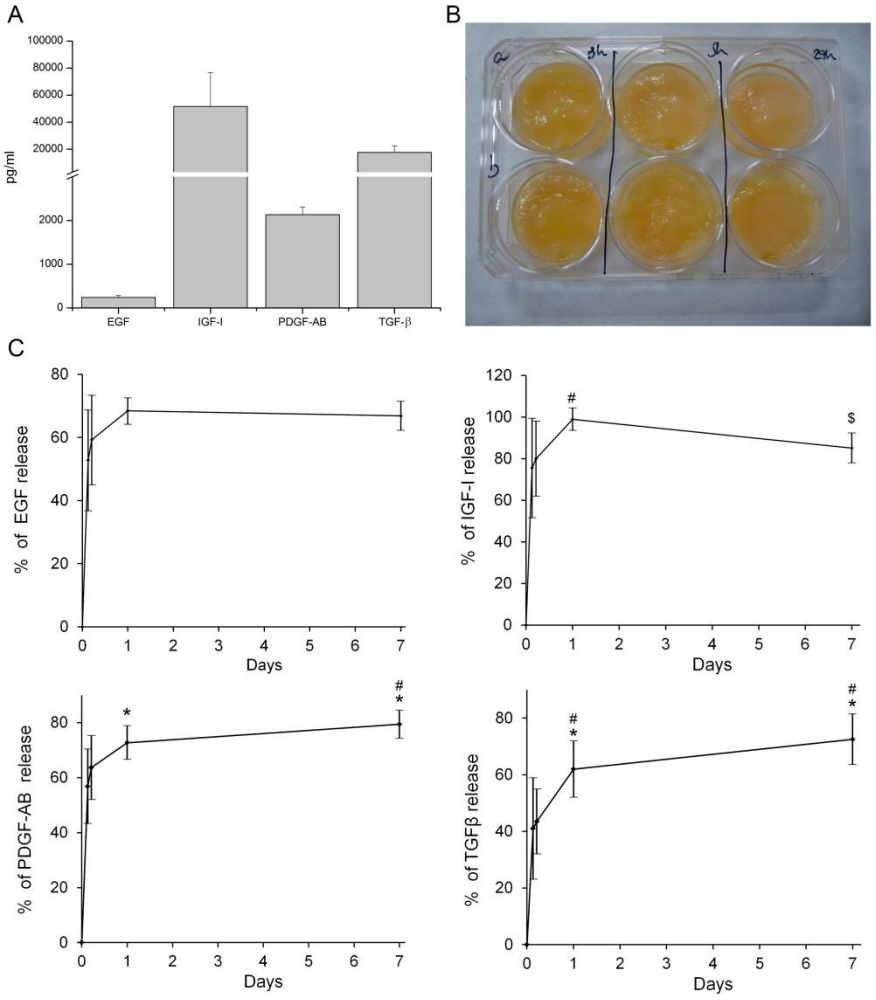

Figure 2. Quantitative analysis of growth factor content and release kinetics from the novel topical formulation. Total growth factor content analysis from extracts of ATS (A). Representative photograph of wells with the ATS tested in the release kinetics assay before the addition of the basal medium (B). Release kinetics of different growth factors from the topical serum incubated at 37ºC during 3, 5 and 24h and 7 days (C). * Statistically significant differences compared to 3h. # Statistically significant differences compared to 5h. $ Statistically significant differences compared to 24h ($p<0.05$).

Skin absorption

To test the cutaneous penetration potential of ATS, 3D organotypic human skin explants were used as a bioequivalent *ex vivo* model. Samples were applied on the epidermis of the Franz diffusion cell system and growth factor levels were measured after 24 hours (Fig 3A-B). Results indicated a cutaneous penetration rate of 29±11% and 12±10% for EGF and PDGF-AB respectively. Growth factors were detected in both the epidermal and dermal layers of the skin (Fig 3C).

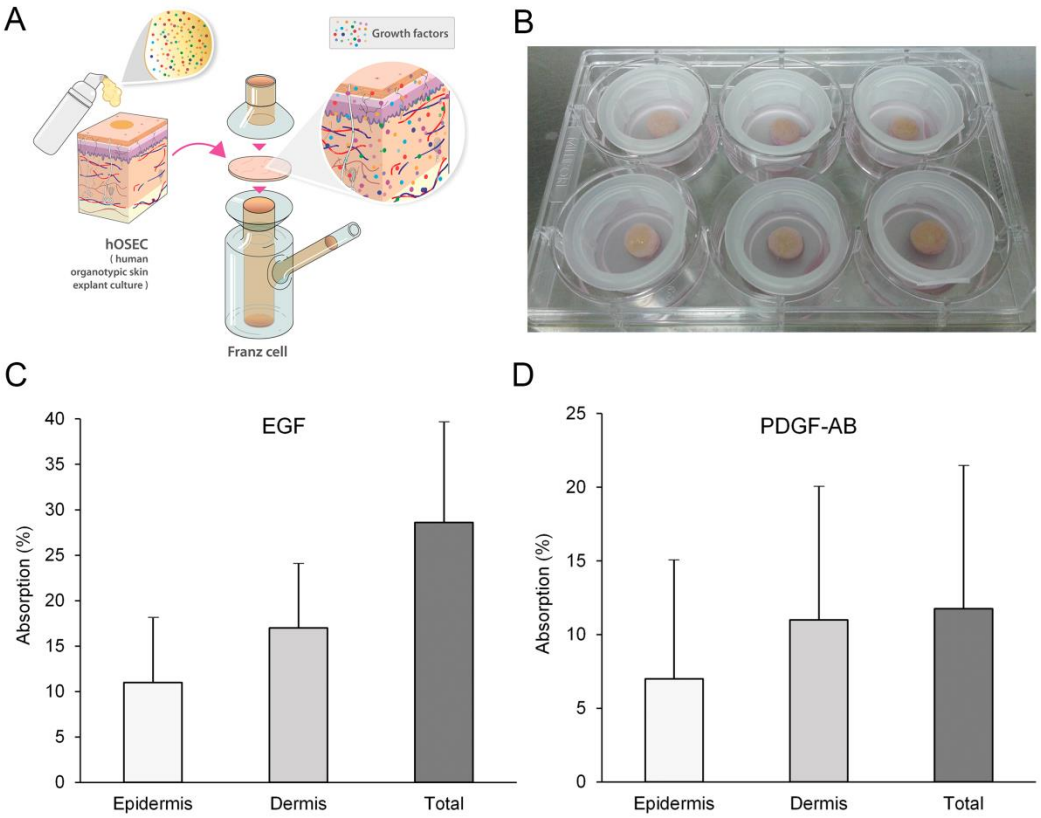

Figure 3. Representative illustration showing the skin absorption assay using Franz cell system and 3D organotypic human skin explants (A). Macrophotographs of the cutaneous constructs used to test the skin penetration test of ATS (B). Epidermal, dermal and global penetration of epidermal growth factor (EGF) and platelet derived growth factor-AB (PDGF-AB) after 24 hours of cutaneous topical application (C-D).

Bioactivity evaluation

The regenerative potential of ATS was determined by proliferative, chemotactic and biosynthetic activity assessment over human dermal fibroblasts. As it is shown in Fig 4A-B, cell proliferation was significantly enhanced after 72 hours of culture as 1.5 fold increase in total DNA was observed when compared to control group ($p<0.05$). The chemotactic assay also showed a 1.5 fold higher DNA signal in the bottom compartment of the polycarbonate membrane chamber after 24 hours of treatment,

suggesting an increased migration of cells towards the ATS containing wells (Fig 4C) ($p<0.05$).

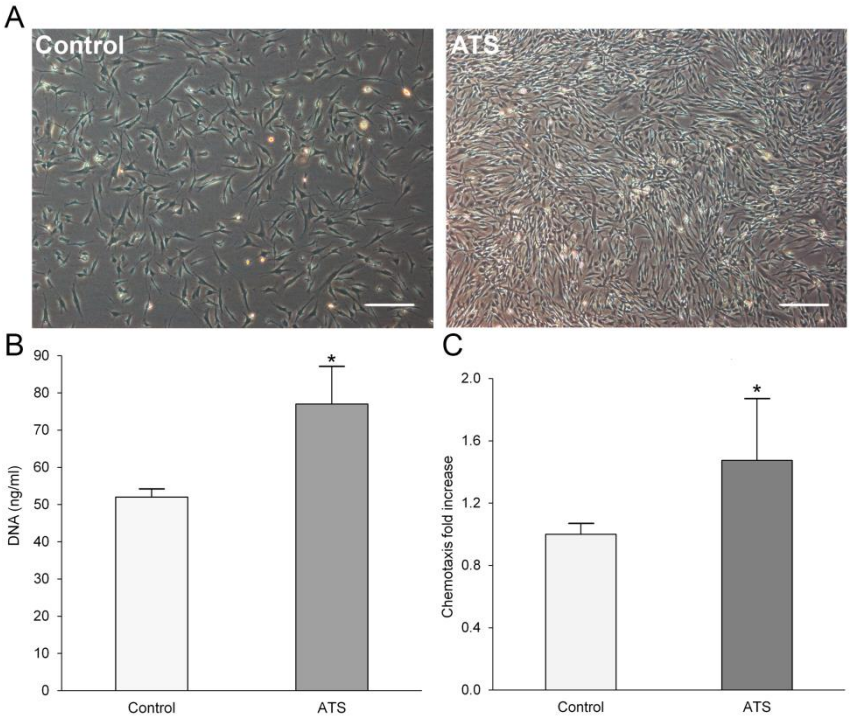

Figure 4. Representative phase contrast microphotographs of fibroblast proliferation after 72h of cell culture with the new formulation extract (A). Fluorimetric analyses revealed that dermal fibroblasts cultured with ATS reached a higher proliferation rate compared to the control group (B). The cell motility assay showed a significantly higher chemotaxis effect of ATS over dermal fibroblasts compared to the control group (C). Scale bars: 200µm.

Moreover, the expression of two key extracellular matrix proteins such as HA and type I collagen was analyzed by ELISA and Western blot respectively. After 3 and 7 days, treated cells showed significantly higher levels of HA when compared to non-treated cells ($p<0.05$) (Fig 5A). Similarly, the biosynthesis of procollagen type I after one week of culture, showed to be significantly increased in ATS treated fibroblasts ($p<0.05$) (Fig 5B).

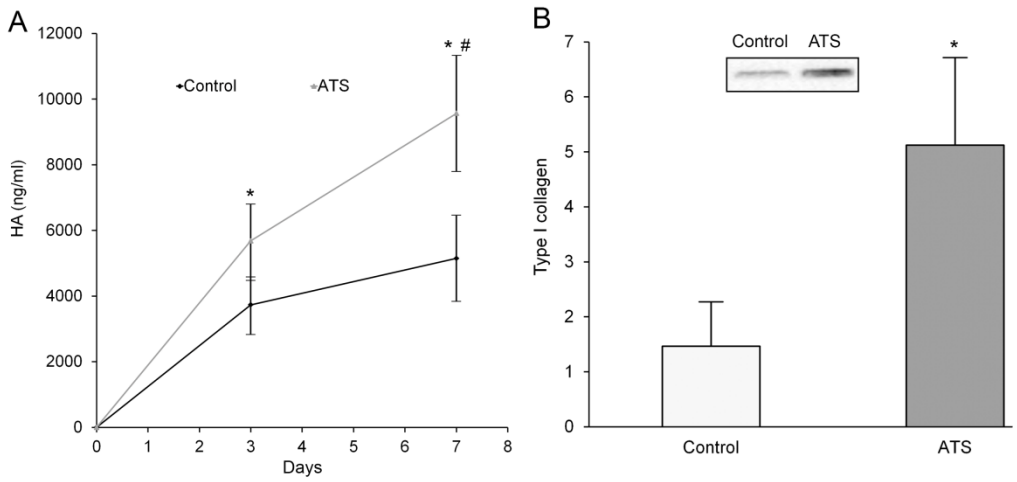

Figure 5. Immunoenzymatic and western blot results showed that cells cultured with the extract of the new topical serum, synthetized higher amounts of cutaneous structural molecules like hyaluronic acid and type I collagen after 3 and 7 days (A-B). *Statistically significant differences compared to control group ($p<0.05$). # Statistically significant differences compared to day 3 ($p<0.05$).

Discussion

Ageing population is increasing at an accelerating rate. These demographic changes are leading to relevant dermatological concerns in the medical field. Aged skin become thin, transparent, wrinkled and dry and displays a loss of structural integrity and physiological function [36]. The main cellular change in skin ageing is the reduction of collagen levels and the development of elastosis that results in the atrophy of the dermal extracellular matrix [36,37]. These alterations impair the skin's mechanical, protective and restorative properties [37]. Both intrinsic and extrinsic factors are involved in skin ageing [38-40]. Intrinsic changes are genetically determined. However, the extrinsic ageing is mainly due to environmental factors that include, pollution, smoking, ultraviolet radiation, diet and repetitive muscle movements [37,41,42]. The synergistic effects of these intrinsic and extrinsic factors lead to multiple clinical conditions with significant-associated morbidity including eczema, itching, dermatitis, autoimmune diseases, vascular disorders and cutaneous neoplasm [41,43,44]. Therefore, there is a striking need to develop safe and effective treatments to overcome the negative effect of skin ageing. A wide variety of formulations have been proposed. In recent years, the applications of topical growth factors have emerged as an attractive therapeutic alternative designed to accelerate wound healing [37,42,45].

The present study evaluates a novel autologous topical serum (ATS) derived from PRGF technology. This novel formulation presents an opaque yellowish appearance with a

smooth and homogeneous texture enriched in autologous growth factors. This semisolid formulation exhibited a pH of 7.6 ± 0.2 which is in the close range of neutral pH, avoiding any skin irritation and compatible with pH of the skin, that is slightly acidic. Pharmaceutical semisolid preparations usually serve as vehicle for topically applied drugs. The rheological properties of these formulations play the most important role in their final behavior [46,47]. In fact, spreadability directly affects to the delivery of the correct dose of the drug and thus to the efficacy of the topical therapy [48]. In this study, spreadability of the ATS was significantly higher than that of the commercial formulation and thereby the autologous serum showed an increased surface available for drug permeation. These results are in line with the rheological results as increasing the viscosity of the delivery vehicle decreases its spreadability [46], hence the ATS presented a lower viscosity. Both formulations exhibited a type of non-newtonian behavior known as pseudoplastic or shear thinning which means that viscosity decreases with the increase of shear rate. Shear thinning products allow easy application thus enhancing availability for drug penetration [48-50].

Growth factors have the potential to control many cellular processes involved in wound healing through an intricate network of intracellular signaling pathways [29,51,52]. Administering topical growth factors attempt to reverse the manifestations of skin aging upregulating the activity of cells responsible for dermal remodeling [37]. The novel autologous topical serum, as derived from PRGF technology, contains a mixture of autologous proteins, growth factors and cytokines. In this study four of the main growth factors were quantified. EGF, IGF-I, PDGF-AB and TGF-β1 are reported to stimulate cell proliferation, cell migration, reepithelization, differentiation, granulation tissue formation, and ECM formation [19,37,53,54]. In fact, EGF has been of particular interest for the treatment of surgical wounds, burns and diabetic ulcers [53,55,56]. Furthermore, PDGF and TGF-β1 from the ATS showed sustained release kinetics for at least one week. One of the main concerns with growth factors is their ability to penetrate the epidermis [42]. The major barrier layer in skin is the stratum corneum that provides a physical barrier and maintains homeostasis by controlling the loss of water and protecting against exogenous microorganisms, substances and radiation [57,58]. Drugs must first penetrate this layer to interact with specific receptors on keratinocytes in order to initiate a signaling cascade that affects fibroblasts among others [37,42]. Growth factors and cytokines are hydrophilic molecules with a high molecular weight that limits their ability to penetrate the stratum corneum [37,59]. However, other permeation routes exist such as skin appendices including hair follicles and sweat glands, or even compromised skin [59,60]. The assessment of percutaneous

permeation provides better insight for the successful development of new formulations [57]. Franz diffusion cells method was applied in this study to determine the EGF and PDGF-AB absorption in human organotypic skin explant culture. These growth factors from the ATS were able to penetrate into the stratum corneum and subsequent lower layers of the skin. The penetration rate of EGF (29±11%) was more than twice than that of PDGF (12±10%). Despite percentages were not very high, it must be taken into account that these data are derived from a single application. However, the treatment with this autologous serum involves repeated administrations thus leading to an increase growth factors penetration increasing their regenerative potential. To overcome the difficulty associated with permeation, delivery systems as drug carriers have been developed providing more effective treatment options. Albumin is the main human plasma protein and an ideal candidate for therapeutically drug delivery. It is a natural transport protein with a remarkable ligand binding capacity. These properties together with the high solubility, stability, biocompatibility and availability provide rationale for the therapeutically use of albumin as a drug delivery approach [61-63]. Therefore, in this study, the ability of growth factors to penetrate the stratum corneum may be partly due to the fact that ATS derives from plasma and holds a high albumin content.

The growth factor content of this new formulation confers it biological properties. Here we have reported that this autologous topical serum significantly stimulated cell proliferation, chemotaxis and matrix synthesis of dermal fibroblasts. Fibroblasts are the major cellular constituent of the skin dermal layer, that produce collagen and other extracellular matrix proteins thus maintaining skin structure and integrity. In addition, dermal fibroblasts have the ability to promote skin regeneration in ageing skin [64]. Communication between cells and ECM is now considered to be essential. Dermal ECM consists mostly of type I collagen and provides tensile strength and resiliency to skin [65,66]. With ageing, fibroblast function and collagen content decreases, while matrix metalloproteinases levels increase thus affecting cell-matrix interactions. All this, results in a loss of mechanical properties and tissue integrity which in turn contributes to fragility and impaired wound healing in aged skin [65-67]. We have demonstrated that the autologous topical serum significantly stimulated the synthesis of type I collagen and hyaluronic acid. TGF-β plays a central role in ECM biosynthesis by regulation of type I collagen synthesis [59,66,68], and as mentioned above, the new autologous formulation contains TGF-β that could be the one behind this collagen synthesis stimulation.

In summary, skin is the largest organ of the human body and provides an essential protective barrier. However, both intrinsic and extrinsic factors are involved in skin

ageing and lead to multiple clinical conditions. Therefore, there is an urgent need to develop safe and effective treatments to overcome those negative effects. The therapeutic effect of the autologous PRGF technology has been proved in several medical fields. The present study evaluated a novel autologous topical serum derived from PRGF technology. This smooth and homogeneous formulation exhibited a neutral pH and good spreadability with a type of non-newtonian behavior known as shear thinning. Its growth factor content is able to penetrate into the stratum corneum and to stimulate several biological processes. Therefore, this study showed that the autologous topical serum could potentially be employed as a novel delivery system to increase bioavailability and therapeutic effect of growth factors for skin regeneration. Nevertheless, further research is still needed in order to determine the product stability and clinical applications.

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Artículo VI

Biological stability of Plasma rich in growth factors derived autologous topical serum after three months storage.

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Title: Biological stability of plasma rich in growth factors derived autologous topical serum after 3 month storage

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Declaration of interest statement:

Eduardo Anitua is the scientific director of, and Ander Pino and Maria Troya are researchers at BTI Biotechnology Institute, the company that has developed the PRGF-Endoret technology

Abstract

In this study, the stability of a novel autologous topical serum (ATS) derived from plasma rich in growth factors technology (PRGF) has been evaluated. As skin ages, mechanical, protective and restorative properties decrease leading to multiple clinical conditions. In recent years, topical administration of growth factors has emerged as a promising therapeutic alternative to promote wound healing and skin regeneration. Determination of stability is a crucial step in the formulation process in order to develop an effective product. Blood from 8 healthy donors was harvested and the autologous topical serum was obtained. Resulting ATS samples were either kept fresh or stored for 1, 2 and 3 months at 4ºC. Physical properties and growth factor content were determined in ATS samples at each time of storage. The effect on human dermal fibroblast proliferation and the sterility of the samples was also studied. All the analyzed parameters remained stable along the storage time while pH values increased slightly with respect to fresh samples. No microbial contamination was detected in any of the samples. Preservation of the autologous topical serum up to 3 months under refrigeration does not affect either its physical or mechanical properties or neither alters the growth factors´ composition, thus preserving its biological potential. This achievement enables patients with chronic disorders to maintain their treatment with a lower frequency of blood extractions without affecting the efficacy of PRGF therapy.

Keywords: platelet rich plasma, skin regeneration, topical formulation, growth factor, stability.

Introduction

A new tendency in dermatologic formulations is the use of bioactive proteins for wound healing and cutaneous restoration purposes. These bio-products are aimed at counterbalancing the skin aging exposome defined as "external and internal factors and their interactions, affecting a human individual from conception to death as well as the response of the human body to these factors that lead to biological and clinical signs of skin aging".[1] Skin is a barrier organ that is subjected to a lifelong exposure of a large variety of genetic and environmental factors including sun radiation, pollutants and nutrition. As skin ages, mechanical, protective and restorative properties decrease leading to multiple clinical conditions. In recent years growth factors and cytokines have emerged as intriguing therapeutic modalities with burgeoning interest in their potential to serve as actives for cutaneous regeneration.[2] In line with these novel approaches, morphogens from human platelet origin are gaining the attention of scientists due to their safe and autologous profile.[3]

Platelet derived autologous products are based on the withdrawal of a small volume of patients' own blood in order to obtain a platelet rich plasma fraction. The latter has been shown to promote wound healing and tissue regeneration in response to a sustained release of bioactive proteins that act at the site of injury after platelet degranulation.[4] The pharmaceutical products that are obtained using plasma rich in growth factors technology (PRGF) provide 100% autologous biological tools including a semi-solid fibrin clot, an injectable formulation, a suturable membrane, a liquid supernatant, eyedrops and a long-lasting 3D scaffold.[5-7] These formulations are *in situ* prepared and are applied over the affected area. They have proven to be clinically relevant in many medical fields such as dermatology, orthopedic surgery and sports medicine, oral and maxillofacial surgery, neurobiology and ophthalmology.[8-13]

Cutaneous surface disorders usually represent chronic diseases often establishing their treatment on a long term basis. As a consequence, is pivotal that therapies used for the management of these pathologies preserve their functional and biological stability for several weeks in order to be used on a daily dosage. Long term storage-PRGF formulations such as autologous eye drops have been already tested for their stability. These formulations allow a sustained biologic therapy for ocular disorders such as dry eye without the inconvenience of repeated blood extractions.[14] Recently, an autologous topical serum (ATS) based on the patient´s own blood has also been developed overcoming the need of daily regimen dermatological treatments. The latter has proved to contain a high load of key proteins in dermo/epidermal

regeneration such as platelet derived growth factor-AB (PDGF-AB), transforming growth factor-β1 (TGF-β1), insulin-like growth factor-I (IGF-I) and edpidermal growth factor (EGF). This pool of morphogens penetrates into the skin and promotes damaged tissue regeneration by inducing cell proliferation, migration, angiogenesis, biosynthesis and extracellular matrix (ECM) remodeling.[15,16]

The objective of the present study was to investigate the stability of the novel autologous topical serum during prolonged and refrigerated storage periods of 1 month, 2 months and 3 months. No preservatives or exogenous chemicals are used with the aim of safeguarding the 100% autologous nature of this ATS, therefore several key properties were analyzed in order to guarantee the suitability of the formulation once stored.[17-20] A physicochemical evaluation was performed including a perceptive assessment of organoleptic attributes such as color, odor and texture. The sensory profile of the product was accompanied by the determination of its pH and rheological characteristics once stored. The biologic activity of the ATS was also determined in terms of growth factor content and human dermal fibroblast mitogenic potential. The sterility of the formulation was additionally examined by microbiological contamination detection. The physicochemical and biological stability of the ATS might facilitate the daily use of topical PRGF technology for cutaneous regeneration and improve the safety, cost-effectiveness and comfort of this biological therapy.

Material and methods

The study was performed following the principles established in the Declaration of Helsinki amended in 2013.

Preparation of the autologous topical serum

The autologous topical serum (ATS) was obtained as follows. Briefly, blood from 8 healthy volunteers was harvested after informed consent was obtained. Samples were centrifuged at 580 g for 8 min (BTI System IV, BTI Biotechnology Institute, S.L., Vitoria, Spain) at room temperature, and the whole plasma column was collected, avoiding the leukocyte rich buffy coat. Part of the plasma was incubated with PRGF-activator (BTI) at 37ºC for 1h until complete retraction of the fibrin clot occurred in order to obtain the enriched growth factor supernatant. The remaining plasma was heated to 76ºC for 12 minutes. Subsequently, the ATS was obtained by vigorously mixing both products in a 2:1 ratio. Finally, aliquots from this autologous formulation were used as fresh samples (t_0) or stored at 5ºC±4ºC for 1 (t_1), 2 (t_2) or 3 months (t_3).

Physicochemichal evaluation of the autologous topical serum

Organoleptic properties

The organoleptic features (physical appearance, texture, color, odor, phase separation and adherence) of the ATS samples stored at different time points were examined. These characteristics were evaluated by visual observation.

pH values

pH analysis was performed in the ATS samples stored at each time with a pH-meter GLP 21+ (Crison, Barcelona, Spain). pH measurement was determined at room temperature.

Viscosity measurement

A rheometer (AR 1000 TA Instruments, New Castle, USA) was used to determine the viscosity of the samples during the stability study. One ml of each sample was applied to the sample holder and viscosity was determined at a shear rate of 1 s^{-1}. The tests were carried out at 20ºC.

Spreadability

The spreadability of the ATS samples stored at different time points was examined. Spreadability was determined by measuring the spreading diameter of 1g of sample placed between two horizontal plastic plates for one minute. A weight of 128 g was carefully applied on the upper plate. Spreadability was calculated by using the following formula:

$$A_f/A_i$$

where, A_f is the area of the serum after spreading and A_i is the area of the serum before spreading.

Growth factor content

Several growth factors involved in cutaneous regeneration were quantified in the extracts of the ATS samples stored at different time points. Briefly, samples were centrifuged at 14000 g for 15 minutes in order to obtain the liquid extracts. The concentration of epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-I), platelet-derived growth factor AB (PDGF-AB) and transforming growth factor beta-1 (TGF-β1) was measured using commercially available enzyme-linked immunosorbent

assay (ELISA) kits (R&D Systems, Minneapolis, MN),according to the manufacturer's instructions.

Sterility analysis

Microbiological examination was made in accordance with requirements of European Pharmacopoeia. Two hundred and fifty microliters from each serum sample stored at different time points were collected to check the sterility. Bact-ALERT iAST and Bact-ALERT iNST culture media were used for qualitative determination of aerobic and anaerobic microorganisms respectively. After samples inoculation, flasks were incubated at 32.5 ºC ± 2.5 ºC for 7 days and monitored for determination of the growth of microorganisms.

Cell proliferation

The biological effect in cell proliferation was also determined in order to evaluate the ATS stability over time. Two primary human dermal fibroblast cell cultures, isolated as previously described,[21] were used in passage 5. Fibroblasts were seeded at a density of 8000 cells/cm^2 in 96-well optical-bottom black plates with Dulbecco's modified Eagle's medium ((DMEM)/F-12) (Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 15 % fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany), 2 mM glutamine and 50 µg/mL gentamicin (Sigma-Aldrich, St Louis,MO, USA) for 24h. The medium was then replaced with serum-free medium supplemented with 20% ATS extracts from each donor and time of storage, including fresh samples extracts (t_0). Cells were cultured for 72 hours. After incubation, cells were carefully washed with phosphate-buffered saline (PBS) and frozen at -80°C for efficient cell lysis. Cell proliferation was assessed by Cyquant cell proliferation assay (Molecular Probes-Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± standard deviation. After the analysis of the data distribution and homoscedasticity, analysis of variance (ANOVA) was used to assess the differences in the spreadability, growth factor content and proliferation among the four different time points (t_0, t_1, t_2 and t_3). Kruskal-Wallis and Mann-Whitney tests were used to evaluate the differences in the pH values. Differences in viscosity were also analyzed via Kruskal-Wallis test. The significant level was set as $p < 0.05$. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc, Chicago, IL)

Results

Preparation of the ATS

Blood from eight healthy volunteers was used to prepare the autologous topical serum. After hematologic analysis, plasma rich in growth factors samples reached 2x ± 0.3 mean platelet enrichment over the peripheral blood.

Physicochemical evaluation

The ATS was assessed for its organoleptic properties at the different storage points tested: fresh formulation, 1 month, 2 months and 3 months. As it is summarized in Table 1, the autologous serum remained unaltered showing an opaque yellowish and homogeneous hydro-lotion appearance with smooth and viscous texture. The solid/liquid emulsion suffered no disruption over time and kept its adherent properties with no distinctive odor.

Storage time	Phisical appearance	Texture	Colour	Odour	Phase separation	Adherence
0	Homogeneous hydro-lotion	Smooth and viscous emulsion	Opaque yellowish apperance	Odorless	No	Adherent to vertical surface
1 month	Homogeneous hydro-lotion	Smooth and viscous emulsion	Opaque yellowish apperance	Odorless	No	Adherent to vertical surface
2 months	Homogeneous hydro-lotion	Smooth and viscous emulsion	Opaque yellowish apperance	Odorless	No	Adherent to vertical surface
3 months	Homogeneous hydro-lotion	Smooth and viscous emulsion	Opaque yellowish apperance	Odorless	No	Adherent to vertical surface

Table 1. Physicochemical evaluation of PRGF-derived autologous topical serum stored at different time points.

The pH value increased from 7.7 ± 0.13 at the beginning to 8.2 ± 0.03 after refrigeration ($p<0.05$) showing the neutral to slightly basic nature of the ATS (Fig 1A). After rheological and spreadability test analysis, no statistical differences were found among the different storage times, showing an average of 24.8 ± 6.4 Pa.s and 6.3 ± 0.6 index respectively (Fig 1B-C).

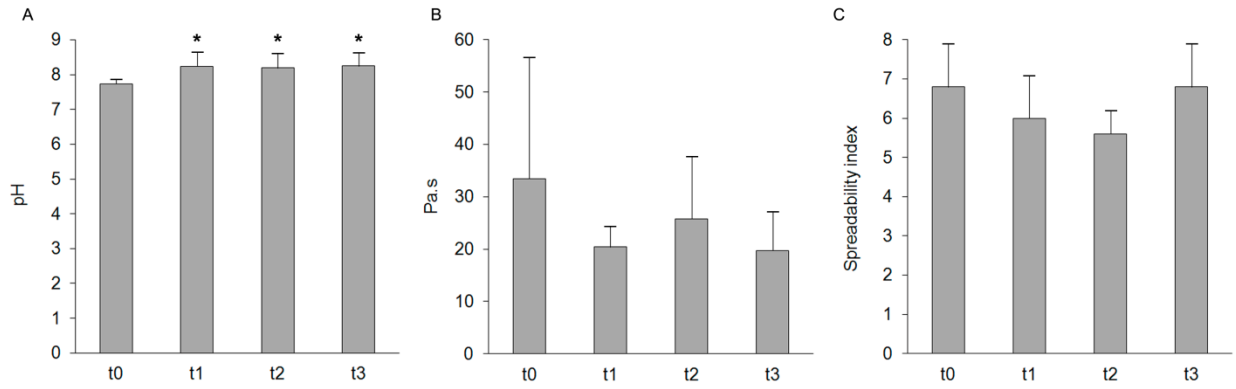

Figure 1. pH (A), viscosity (B) and spreadability (C) of PRGF-derived autologous topical serum samples stored at different time points. *Statistically significant differences compared to fresh samples ($p<0.05$).

Biological evaluation

The growth factor content within the autologous topical serum was determined including several key proteins involved in skin regeneration. As it is described in Table 2, no statistical differences were found in any of the growth factors measured among the different storage times. After enzymatic immunosorbent assays, EGF, IGF-I, PDGF-AB and TGF-β1 reached mean values of 138 ± 14 pg/ml, 43 ± 7 ng/ml, 1541 ± 126 pg/ml and 18 ± 1 pg/ml respectively.

Temperature	Storage time	EGF (pg/ml)	IGF-I (ng/ml)	PDGF-AB (pg/ml)	TGF-β1 (ng/ml)
N/A	t_0	140 ± 28	51 ± 13	1661 ± 408	19 ± 5
4ºC	t_1	142 ± 45	45 ± 10	1550 ± 503	17 ± 4
	t_2	152 ± 36	43 ± 16	1588 ± 424	18 ± 7
	t_3	117 ± 32	34 ± 10	1365 ± 393	18 ± 5

Table 2. Concentration of several growth factors in PRGF-derived autologous topical serum samples stored at different time points. No significant differences were observed among the distinct storage times.

The regenerative potential of the ATS was determined by the assessment of its ability to stimulate human dermal fibroblasts proliferation. As it is shown in Fig 2A-B, no statistical differences were detected in cell proliferation after treatment with the ATS extracts stored at different storage times. A mean DNA concentration of 42 ± 1.4 ng/ml was detected after 72 hours of culture.

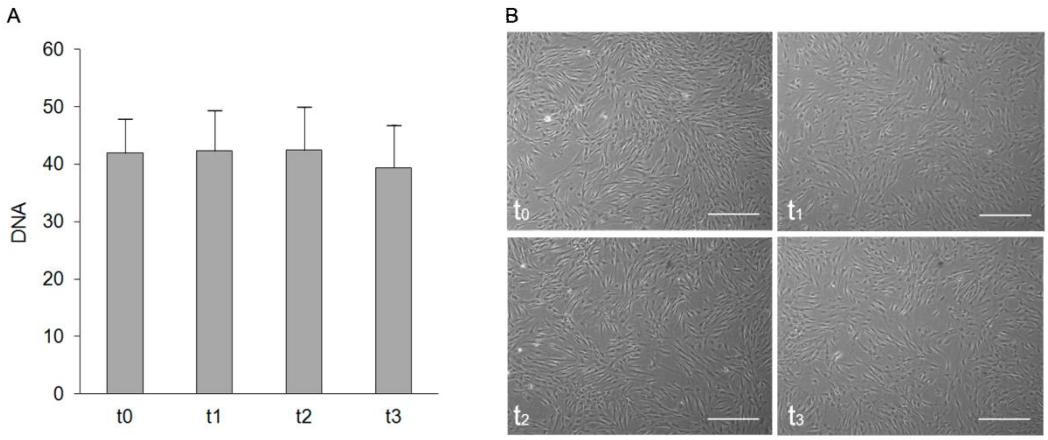

Figure 2. (A) Quantification of the proliferation of human primary dermal fibroblasts cultured with fresh PRGF-derived autologous topical serum (t_0) and with PRGF-derived autologous topical serum stored for 1 (t_1), 2 (t_2) and 3 months (t_3) at 4ºC. (B) Representative phase contrast photomicrographs of the proliferation of human primary dermal fibroblasts cultured with the different PRGF-derived autologous topical serum samples. Scale bar: 500 µm.

Microbiological examination of samples stored at different time points was finally performed to check the sterility of the ATS under the aforementioned conditions. As it is shown in Table 3, no contamination was detected in any of the samples tested, indicating the sterility of the formulation.

Type	Microorganism	ATCC code	Control	0 months	1 month	2 months	3 months
Aerobic	*Aspergillus brasiliensis*	16404	Yes	No	No	No	No
Aerobic	*Bacillus subtilis*	6633	Yes	No	No	No	No
Aerobic	*Candida albicans*	10231	Yes	No	No	No	No
Aerobic	*Pseudomonas aeruginosa*	9027	Yes	No	No	No	No
Aerobic	*Staphylococcus aureus*	6538	Yes	No	No	No	No
Aerobic	*Streptococcus pyogenes*	19615	Yes	No	No	No	No
Aerobic	*Micrococcus luteus*	4698	Yes	No	No	No	No
Anaerobic	*Clostridium sporogenes*	19404	Yes	No	No	No	No
Anaerobic	*Propionibacterium acnes*	11827	Yes	No	No	No	No

Table 3. Microbial contamination assay. No contamination was detected in any of the tested samples.

Discussion

Skin is the largest organ of the human body and provides physical, metabolic and immunological protection thus maintaining homeostasis.[22] As skin ages, those

mechanical, protective and restorative properties may be depleted leading to multiple clinical conditions.[23-25] Developing safe and effective strategies aimed at overcoming the deleterious effects of skin ageing is a clinical challenge. In recent years, topical administration of growth factors has emerged as a promising therapeutic alternative to promote wound healing and skin regeneration.[23,26,27] The use of the autologous technology PRGF has been widely used in several medical fields [28] including in dermatology.[29-32] To determine the shelf-life of a new formulation is a significant step in the manufacturing process in order to develop an effective product with the required therapeutic effects.[22,33] In this study, the stability of the novel autologous topical serum derived from PRGF technology has been evaluated. Previously, the stability of other PRGF formulations, including autologous eye drops, were already demonstrated for up to 6 months at -20ºC.[34]

Results from our study showed that this novel formulation remained physically stable among the different storage times. Similar appearance with no apparent change in its texture, colour and odour was observed in the storage conditions evaluated. In addition, no phase separation was detected. Conversely, pH values of the stored samples did significantly increase with respect to the values of the fresh samples. However, all values remained in the close range of neutral pH thus ensuring its compatibility with the slightly acidic skin pH and avoiding irritation. The rheological properties of pharmaceutical products are crucial to determine and optimize their final behavior.[35] These properties are usually related to the stability, drug delivery and applicability of formulations what ultimately affects the therapeutic efficacy.[36,37] In this study, fundamental concepts of rheology such as spreadability and viscosity remained stable throughout the different storage periods.

Skin wound healing is a well-orchestrated process that requires complex interactions among a variety of cells, growth factors and cytokines.[23,26,38] Growth factors are endogenous polypeptides that regulate cellular function by binding to their receptors. This specific binding activates relevant signaling pathways that in turn, induce the transcription and synthesis of several molecules involved in tissue regeneration.[16,26] Due to their role in wound healing, many studies have focused on the topical administration of growth factors as a promising strategy to promote skin regeneration. In fact, PDGF-BB has been extensively used for treating diabetic ulcers [39-41] and it was the first FDA-approved product in the growth factors family. EGF has also been of particular interest for the treatment of skin wound healing.[42-45] In this study, no statistically significant differences were found in the growth factors concentration (EGF, IGF-I, PDFG-AB and TGF-β) between fresh samples and those stored for 1, 2 and 3

months at 5ºC±4ºC. These growth factors are reported to be involved in many cell processes such as cell proliferation, cell migration, reepithelization, differentiation, granulation tissue formation, and ECM formation.[26,38,46,47] The growth factor stability observed in this study preserves the biological activity of the autologous topical serum. In fact, there were no statistically differences in human primary dermal fibroblast proliferation rate after treatment with both fresh and refrigerated samples.

Finally, despite this autologous formulation does not use preservatives in order to avoid the risk of chemical toxicity, no microbial contamination was found in any of the ATS samples, neither in fresh nor in refrigeration stored samples. In fact, the antibacterial effect of the plasma rich in growth factor technology has been already reported.[48,49]

In summary, this study revealed that the storage up to 3 months does not affect either the physical or mechanical properties and neither alters the growth factors´ composition of the autologous topical serum, thus preserving its biological potential. Therefore, the findings of the present work strongly suggest that the use of the autologous topical serum derived from PRGF technology could extend at least 3 months post-preparation if maintained under refrigeration. This new formulation should be stored in multiples single-day bottles to optimize the product stability and minimize the risk of contamination. This achievement reduces the frequency of blood extractions and enables patients with chronic disorders to maintain a daily use of clinical dosage without affecting the efficacy of the therapy. Nevertheless, further research should be conducted in order to extend the interval of storage.

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Artículo VII

Opening new horizons in regenerative dermatology using platelet based autologous therapies.

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Title: Opening new horizons in regenerative dermatology using platelet-based autologous therapies

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Conflict of Interest:

E Anitua is the scientific director of, G.O. and A.P. are scientists at BTI-Biotechnology Institute, the company that has developed the PRGF-Endoret technology.

Abstract

Biological therapeutic therapies are gaining the attention of scientists and medical doctors. Accumulating evidence suggest that blood-derived autologous therapies are safe and effective treatments for skin repair and wound healing. The fibrin network formed after plasmatic activation and the autologous growth factors released when platelets degranulate, constitute a real biological medicine that has been shown to promote cell recruitment, stimulate new blood vessel formation, reduce inflammation as well as protect from local infections. This perspective highlights recent basic and clinical results published on blood-derived autologous therapies in the field of regenerative dermatology and discusses potential challenges and future prospects.

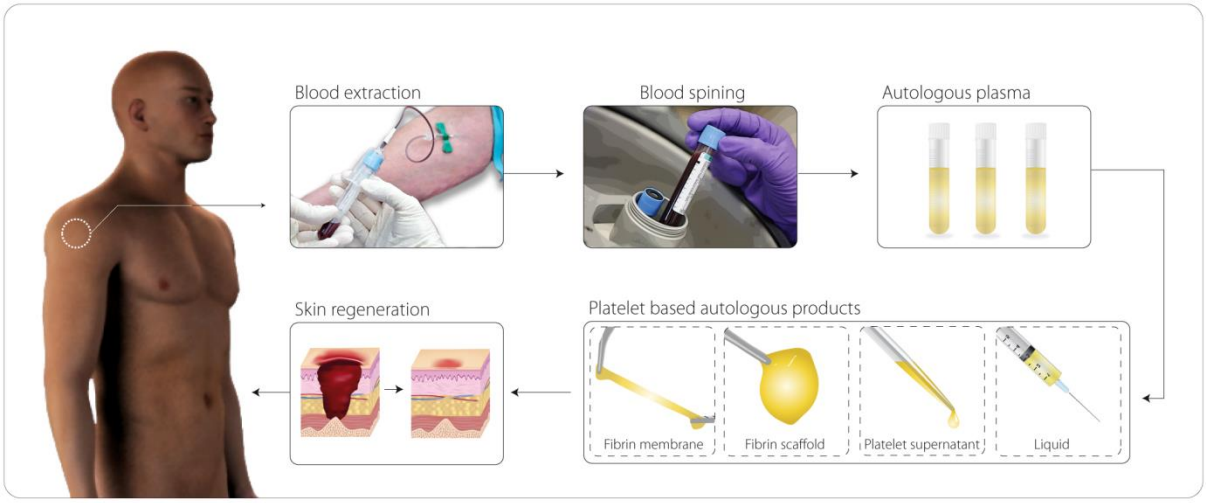

Figure 1: Schematic protocol followed for the preparation of platelet-based autologous products in regenerative dermatology.

Introduction

Biological therapies, tridimensional scaffolds and tissue-engineered composites are some of the current novel innovations that regenerative medicine offers for the repair, regeneration or replacement of injured cells, tissues and organs. Some of these exciting new approaches are focused on dermatology, providing new avenues in the personalized regenerative medicine. One example is the progress been made in blood-derived therapies such as platelet derived autologous products, which are based on the withdrawal of a small blood volume in order to obtain a platelet rich plasma fraction Fig. 1). The latter has been shown to promote wound healing and tissue regeneration [1]. The process for the *in situ* preparation of autologous platelet based bio-products isn´t yet a totally standardized method. 20 to 40 mL of peripheral blood is usually obtained in tubes with sodium citrate as an anticoagulant. Blood undergoes a centrifugation step varying from 200g to 600g that separates three layers: an upper layer that contains the platelet rich plasma fraction (2-3X platelet enrichment), an intermediate layer of buffy coat rich in leucocytes and the bottom layer that consists mostly of red blood cells. Some protocols include a second centrifugation step in order to increase the platelet concentration, however some studies suggest that a moderate platelet enrichment has higher biologic potential [2]. Although the leucocyte layer is sometimes included in the final therapeutic dose, recent reports have outlined that they could be related with pro-inflamatory processes [3-5]. Finally platelets are usually activated with the addition of calcium chloride or thrombin, triggering the sustained release of autologous growth factors an morphogens from their alfa granules. Herein, we summarize some of the newest advances related to the use of blood-derived autologous therapies for the treatment of skin related disorders. Additionally, potential challenges, limitations and future perspectives are also discussed.

Recent Progress in the Field

The reestablishment and improvement of blood flow when tissue function is compromised is a key feature that depends on the development, correct branching and stabilization of newly formed blood vessels. A recent report has outlined the pivotal role of some of the growth factors, cytokines and chemokines released after platelet activation in the contribution of angiogenesis and the activation of endothelial cells to stablish new vessels from pre-existing vasculature [6]. Some of these growth factors include vascular endothelial growth factor (VEGF), fibroblast growth factor-b (FGFb), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF),

epidermal growth factor (EGF) and angiopoietin-I (Ang-I) among others. These proteins may be involved in coordinated processes that require the migration of leading cells that guide the direction of primary capillaries and the differentiation of trailing cells who stablish the lumen of blood vessels. In fact, it has been observed that the angiopoietin-I/Tie-2 signaling pathway may induce the pro-angiogenic response of blood-derived autologous therapies *in vivo*.

Recent reports involving the use of these autologous therapies could have significant implications for future therapies of ulcers and burns [7; 8]. When comparing the percentage of wound closure over time, both studies concluded that autologous platelet rich therapies were able to accelerate cutaneous regeneration in comparison with control groups. Further histomorphometric analyses revealed that at short follow up periods, acute wounds treated with platelet rich therapies showed development of stratum spinosum/granulosum with abundant keratohyaline granules and differentiated keratinocytes. At later stages, they showed decreased hypertrophic epidermal thickness and flattened epithelial cells with anuclear keratinized keratinocytes overlying the epidermis. Granulation tissue index of treated wounds was also significantly higher, along with an increased amount of early blood vessels and more abundant collagen fibers indicating a rapid differentiation to a normal cutaneous structure [7]. Histologic evidence of accelerated re-epithelization and dermal regeneration was also observed in second degree burns injuries. A significant decrease of early healing stage indicators such as neutrophils, resident/migrating macrophages and TGFβ+cells was detected 21 days after treatment with autologous platelet-based therapies. Additionally, the natural reduction of blood vessels along with an increase of matrix metalloproteinase-2 signal indicated that the last healing phase (remodeling), was sooner reached in this experimental group compared with control group. Finally, the evidence of newly formed epidermal thickness and the amount of dermal collagen deposition showed that second degree burns healed significantly faster after applying autologous platelet-based therapy [8]. These results could be explained taking into account the high biological activity of some of the proteins supplied by autologous platelet-based therapies including PDGF, a potent chemoattractant and mitogen for fibroblasts; VEGF and FGF, which stimulate collagen synthesis and angiogenesis; TGFβ, which stimulates mesenchymal, epithelial and endothelial cell growth and proliferation; EGF, which enhances the re-epithelization and angiogenesis; fibronectin and vitronectin which act as cell-adhesion molecules for the migration of epithelial and vascular cells and fibrin, which promotes hemostasis, aids in the stabilization of grafted materials and inhibits bacterial growth [7; 8].

Research on aesthetic dermatology

The use of platelet-based autologous therapies in different dermo-aesthetic fields like skin rejuvenation, acne scars, contour defects and striae distensae is not new [9-12]. However, the current knowledge on their safety profile and potential therapeutic effects is expanding due to the methodologically optimized recent clinical trials [13-17]. The synergy between these autologous therapies and recombinant growth factors such as FGF-b has been recently evaluated for example, over more than two thousand patients for the treatment of wrinkles and depressed areas of the skin [13]. Based on global aesthetic improvement and wrinkle severity rating standardized scales (GAIS and WSRS respectively), the results of this retrospective clinical study showed after six months of follow up that a single combined treatment increased the volume of not only the skin, but also adipose and subcutaneous tissues. The therapy´s effectiveness became evident after an average of 65 days and even wrinkles of high WSRS grade revealed an acceptable improvement, being 97% and 98% the patient and physician satisfaction score respectively at the end of the study.

The use of platelet-based autologous therapies has recently been object of a significant breakthrough over the treatment of hair related disorders such as pattern hair loss or alopecia areata. During the last months several clinical studies including a meta-analysis involving the management of androgenetic alopecia (AGA) have been published [14-17]. Concise PubMed and Google Scholar database search was performed on October 2015 and March 2016 resulting in an overall standardized mean difference of 0.51 [95% confidence interval (CI):0.14, 0.88; I^2=0%] in favor of the autologous regenerative therapies [14]. Some of the latest reports include randomized, double blind, half head and placebo-controlled clinical trials in more than forty patients. After standardized phototrichogram data gathering, the authors concluded that repeated treatments with autologous platelet-based therapies led to a higher increase in mean hair density (hairs/cm^2) compared to the control areas after follow up periods of 3 and 6 months [15; 16]. Following a demographic analysis, it seemed that male patient under his forties with hair loss of 25 years and a positive AGA family history was the best responder to the treatment with autologous therapies [15].

Additional immunohistochemical evaluations of scalp biopsies showed a significant increase of epidermal thickness after treatment with platelet-based autologous therapies. An increase of proliferating Ki67+ basal keratinocytes and follicular bulge cells was also detected along with a higher number of newly formed dermal blood

vessels [16]. Some of the potential biomolecular pathways modulated by autologous growth factors on hair regrowth have been addressed recently [17]. When growth factors such as EGF or PDGF bind to its respective transmembrane tyrosine kinase receptor of dermal papilla cells, the MAPK/ERK pathway has been shown to be significantly upregulated. Afterwards a complex intracellular signaling cascade is triggered, promoting transcription of genes involved in cellular proliferation, differentiation and survival. After growth factor release, phosphoinositide 3-kinases (PI3ks) phosphorylate PIP2 into PIP3 who acts as a secondary messenger activating an anti-apoptotic signaling molecule known as AKT. Apart from promoting cellular growth, metabolism and angiogenesis, AKT is able to inhibit programmed cell death by means of downregulation of pro-apoptotic mediators such as BAD and BAX and overexpression of anti-apoptotic proteins like Bcl-2. Therefore, the final increase of cell survival in response to platelet-based autologous therapies may be related to a prolonged anagen follicle growth phase within the natural hair cycle progress.

Designing autologous composites for dermal regeneration

In line with the rapid development of biocompatible scaffolds and tridimensional matrices for tissue regeneration, researchers have recently found that platelet-based autologous therapies can be used as an aid to biologically improve biomaterials and composites for dermal regeneration and wound healing [18-20].

Some studies have demonstrated the beneficial effect of autologous platelet-based therapies mixed with either collagen or gelatin hydrogels for the treatment of ischemic skin disorders with decreased blood flow such as chronic ulcers or surgical flaps [18; 19]. Both *in vitro* and *in vivo* data showed that these bio-scaffolds were able to promote wound healing with complete re-epithelization within 3 weeks along with hair growth, sweat gland formation and increased blood vessel surface. The controlled growth factor release promoted the recruitment of dermal keratinocytes and mesenchymal stem cells from adjacent healthy tissue thus inducing angiogenesis and new tissue formation [18]. In fact, immunohistochemical analyses revealed an increase in newly formed tube length and capillary area with more abundant branching points and a wider microvascular network within the treated region that could be related to the upregulation of MEK-ERK and AKT pathways [19]. Additionally, a chitosan-gelatin sponge loaded with tannins and autologous platelet-based therapies has been recently tested as an optimal surgical wound dressing that enhances rapid healing and contraction.

Fully replaced epidermal and dermal layers with abundant fibroblasts and newly formed capillaries and collagen fibers were detected at short follow up periods [20].

Future prospects and challenges

The field of autologous platelet-based therapies is expanding rapidly, however several challenges persists. For example, additional studies involving full proteomic characterization of platelet releasates and concise transcriptional gene response after their application are needed to fully understand the biomolecular mechanism behind the regenerative effect of these therapies. Moreover, due to number of commercially available kits and homemade protocols, a more standardized method for preparation is mandatory to properly correlate clinical results with the composition of the platelet-based therapy. The optimal therapeutic dosage of these biologic therapies is also a current field of study. Some screenings have tested 2 to 6 repeated doses with intervals of 2 to 4 weeks between treatments with encouraging results [17]. The volume of patient´s own blood required depends on the chosen preparation method and the size of the treated area, but generally varies from 20 to 80mL per dose. However further clinical trials are needed to clearly state the optimal therapeutic dose and treatment protocol for each dermatological disease [21]. Regarding the safety profile of platelet-based autologous therapies, numerous dermatologic reviews have reported no side effects apart from transient edema and complications related to the blood extraction [1; 12; 14]. Some adverse reactions have been nevertheless reported when the protocol used involves exogenous agents such as commercial fibrin sealants, bovine thrombin or recombinant growth factors such as becaplermin [22]. Although the use of commercial or allogenic platelet concentrates undergo strict regulation and standardized criteria such as nucleic acid testing (NAT) and viral screening, a 100% autologous platelet based product guaranties the safety profile of the therapy without risks of viral infection, cross reactions, or zoonotic disease transmission [22; 23].

New advances in the field are also expected including the potential combination of these autologous therapies with mesenchymal stem cells. Adipose derived stem cells (ASC) have been described as multilineage potential cells with regenerative effects attributed to soluble factors released after clinical implantation. However, the stemness of ASC has shown to be significantly impaired by several factors such as systemic diseases like diabetes or harsh environments found in chronic wounds where local hypoxia, oxidative stress, inflammation and avascularity dramatically reduce their therapeutic effect. In these microenviroments, ACS have shown to exhibit poor survival

rates with higher susceptibility to apoptosis and decreased release kinetics of growth factors such as VEGF, HGF or IGF-I [24].

A recent study have nevertheless demonstrated that the co-administration of autologous platelet-based therapies and ASC prevents from stem cell death and promotes cutaneous tissue regeneration compared to ASC therapy alone [25]. At short follow up periods, wounds treated with this synergic treatment showed higher healed areas along with an enhanced paracrine effect of growth factors such as VEGF, HGF and FGF-2. The increased cell survival of the administered ASC induced a prolonged proliferation and growth factor release leading to a higher migration of local mesenchymal and epithelial cells which produced extracellular structures for wound healing. Additionally, the increased pericyte recruiting and the persistence of ACS near the capillaries triggered the formation of new blood vessels and the stabilization of an extended microvascular network.

The reports outlined herein should be interpreted in light of certain limitations. Some clinical studies could have been performed over a higher number of patients in order to reinforce the hypothetical benefits of platelet-based autologous therapy. In addition, the prolonged effect of autologous growth factors have not extensively been studied as 6 months follow up periods and 7 to 12 days post-treatment data have sometimes been reported for clinical and *in vivo* results respectively. Long term observations as well as comparative studies between different platelet derived treatments are encouraged. Healthy animal models suffering from experimental wounds were used in several studies, highlighting another limitation when trying to compare the final results with patients with delayed healing situations such as diabetes or ischemia.

Given the regenerative potential of platelet-based autologous treatments, future clinical therapeutic applications have already been launched in different directions. Growth factors released after platelet activation may help to regenerate damaged tissue after skin radiation therapy [26; 27]. Minimal invasive new formulations are also being evaluated in order to topically apply autologous proteins with the aim of accelerated wound healing [28]. Additionally, an increasing interest is being focused in the structural properties and bioactive characteristics of plasmatic fibrin scaffolds as biocompatible inks for 3D skin bio-printing purposes [29].

The biologic activity of autologous growth factors along with the biomechanical stiffness of plasmatic proteins after fibrin mesh formation, have showed to offer a unique microenvironment for autologous mesenchymal stem cells. The latter provides

a great deal of hope for patients suffering from ulcers and burns. Although, the skin and hair-related injuries shows no signs of abating, recent exciting results with platelet-based autologous therapies may evoke a glimmer of hope for the patients suffering from these conditions.

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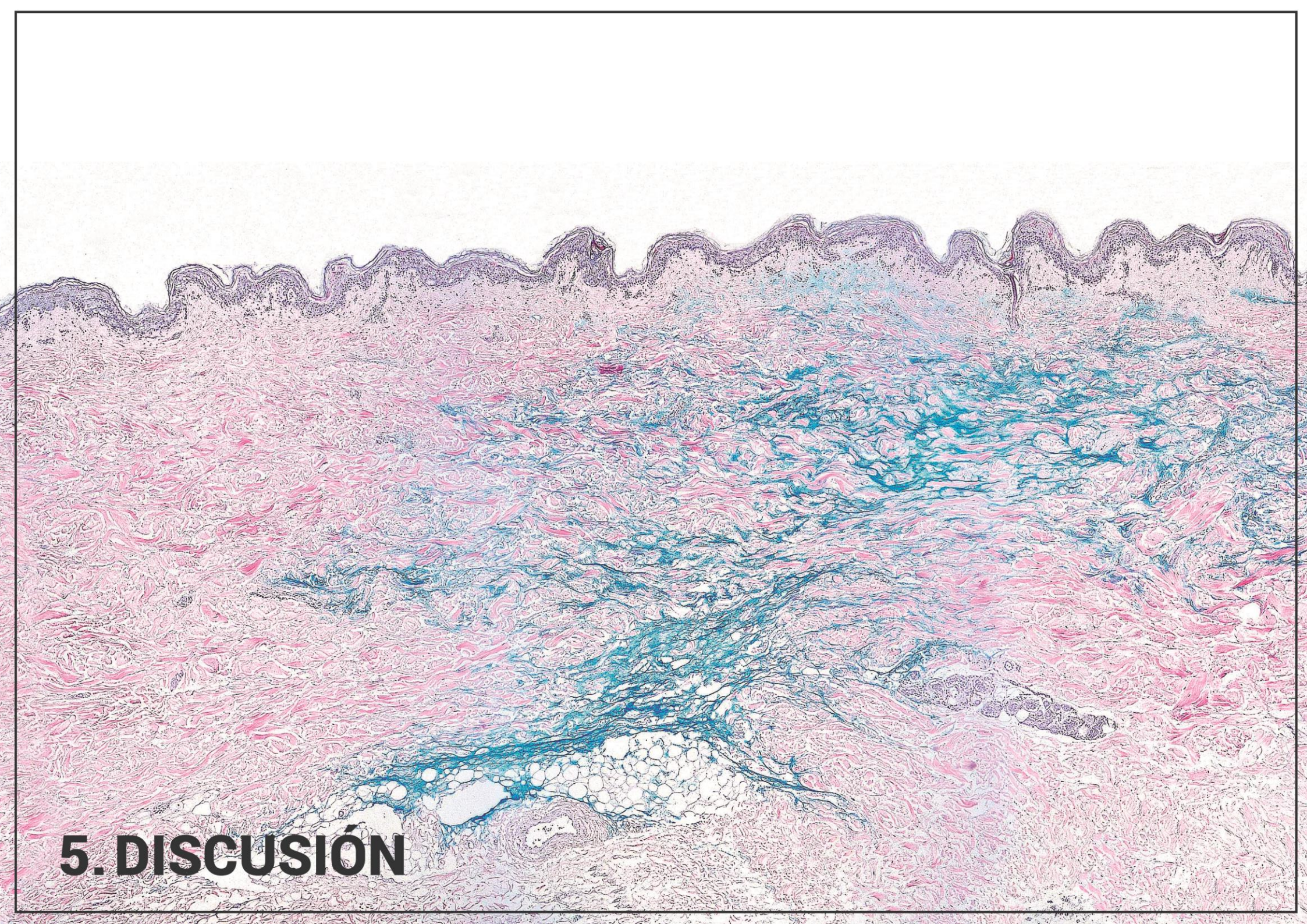


5. DISCUSIÓN

En las últimas décadas, el aumento progresivo de la edad media de la población ha traído como consecuencia una alta prevalencia de afecciones cutáneas crónicas y degenerativas. Este problema supone un gasto aproximado del 2% del presupuesto sanitario en países de la Unión Europea [89]. La regeneración de la piel es un proceso necesario para la restitución y reparación del tejido una vez sufrida una lesión cutánea [45]. Una gran variedad de proteínas, incluidas las citoquinas y factores de crecimiento están involucradas en este proceso, aunque la restauración exitosa de la función tisular original se basa en una secuencia orquestada de fases más que en la acción aislada de estos elementos. La interacción de los factores bioactivos en el tejido dañado resulta crucial para lograr una terapia efectiva [57]. Además, la eficacia de los tratamientos dermatológicos actuales ronda el 50% siendo necesarios tratamientos continuados que disparan el coste final [90]. En definitiva, es evidente que existe una necesidad real de avances biomédicos que sean seguros y mínimamente invasivos dirigidos a abordar la gran variedad de patologías cutáneas existente. Dichas terapias deberían reducir la morbilidad y promover la correcta recuperación funcional del tejido afectado con el fin de mejorar la calidad de vida del paciente. En este sentido el plasma y las plaquetas humanas han demostrado ser un reservorio clave de productos terapéuticos esenciales que engloban componentes fibrilares, celulares y proteicos en una proporción óptima difícilmente disponible por otras vías.

5.1. Caracterización del PRGF

La tecnología del plasma rico en factores de crecimiento ha sido desarrollada para extraer el máximo rendimiento de los procesos naturales de regeneración tisular presentes en el organismo. La eficacia y seguridad de este tratamiento autólogo ha sido ampliamente testada en el ámbito dermatológico [37,91-95], aunque numerosos estudios también avalan su uso en otras áreas de la medicina como la cirugía oral y maxilofacial, oftalmología, neurobiología, traumatología y medicina del deporte [49,96-103]. En el presente trabajo de investigación se ha podido caracterizar y cuantificar el contenido en factores de crecimiento del PRGF obtenido a partir de diversos donantes para su puesta a punto como suplemento celular en cultivos de líneas primarias de fibroblastos dérmicos. Nuestros resultados evidencian la ausencia de leucocitos pro-inflamatorios en los preparados finales, así como una concentración plaquetaria de 2,4±0,7 respecto a la sangre periférica. Estos datos respaldan con análisis recientes que demuestran que el plasma rico en plaquetas obtenido a partir de del protocolo PRGF alcanza niveles de óptimos de recuperación plaquetaria de hasta el 66±7%,

manteniendo intactas las propiedades fisiológicas de las plaquetas como la respuesta al estrés hipotónico, activación, agregación, carga proteica y ratio de coagulación/retracción [104]. De hecho, estudios adicionales demuestran que una centrifugación sanguínea controlada a bajas revoluciones como la que se lleva a cabo para la obtención del PRGF garantiza el correcto fraccionamiento de las fases plasmáticas e incrementa el potencial biológico del producto final [105].

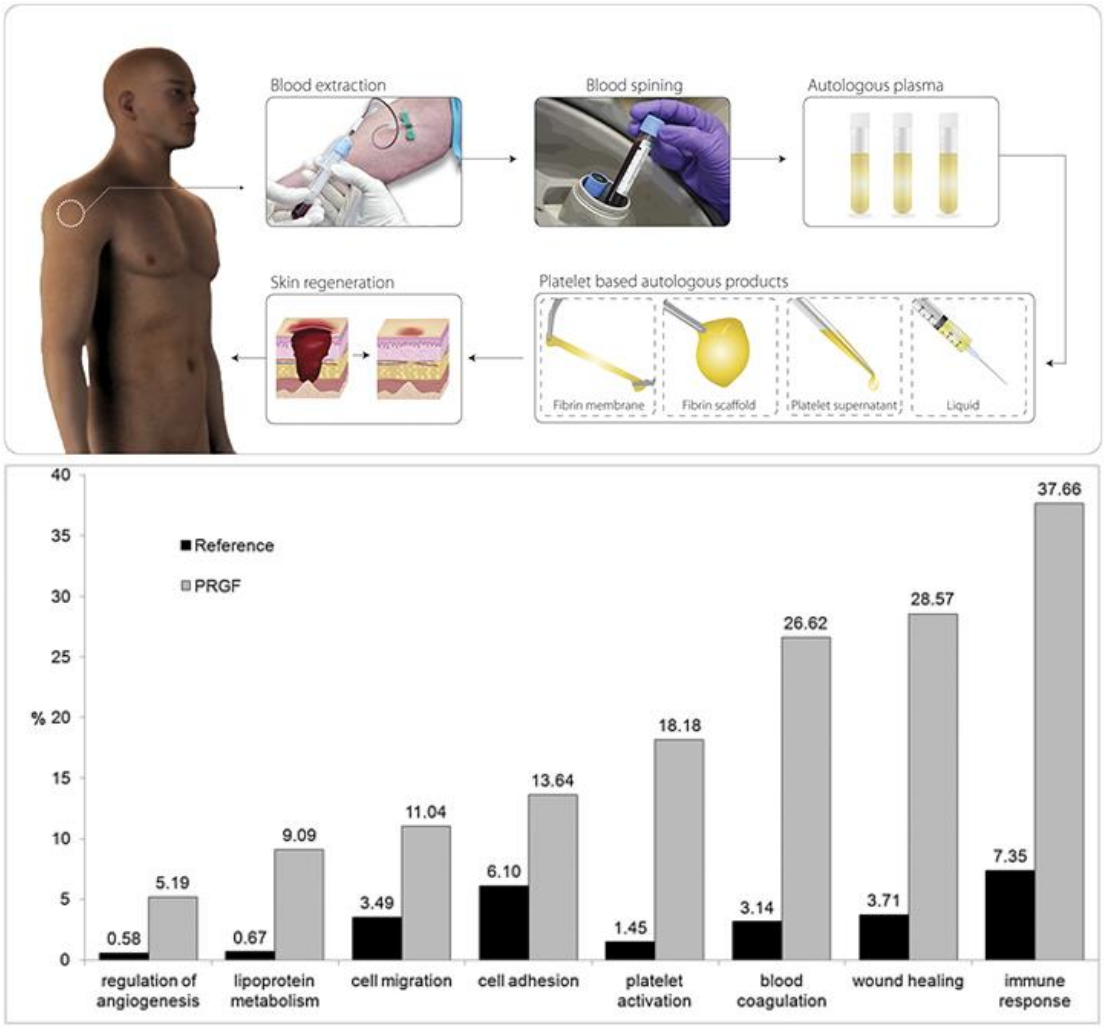


Figura 21: Diagrama ilustrativo de la tecnología PRGF en dermatología regenerativa y análisis proteómico de los principales componentes biológicos estimulados tras su aplicación terapéutica. Adaptado de [106,107]

Para la presente tesis y previo al comienzo con los ensayos *in vitro*, se analizó y comparó paralelamente el contenido en factores de crecimiento del PRGF obtenido a partir de voluntarios jóvenes y de edad avanzada (≤35 y ≥50 respectivamente). Los resultados indicaron que ambos grupos presentaban niveles similares de factores de crecimiento claves en la regeneración cutánea como el TGFβ-1, PDGF-AB, IGF-I, EGF, HGF y FGFb [108]. Esto podría relacionarse con el hecho de que numerosos estudios clínicos avalen la eficacia de los derivados plaquetarios en aplicaciones cutáneas que

afectan a poblaciones ampliamente heterogéneas [109-111]. Se podría decir que el coágulo de fibrina actúa a modo de "reservorio biológico" que no solo es capaz de liberar factores de crecimiento, sino que tal y como se deduce de análisis proteómicos recientes, presenta un amplio catálogo de moléculas íntimamente conectadas al andamiaje fibrilar [106]. Algunas de estas moléculas como la alfa1-antitrisina y la alfa1-antiquimotripsina actúan a modo de defensa frente a enzimas proteolíticas y tienen un papel importante en la curación de heridas crónicas [112,113]. Otra glicoproteína multifuncional como la tromboespondina-1 (TSP-1), juega un papel clave en la modulación de la neovascularización de tejidos dañados y retrasa la fibrinolisis prolongando la vida media de los morfógenos derivados del PRGF [114,115]. Íntimamente asociado al coágulo plasmático se encuentran también la vitronectina y fibronectina, involucradas tanto en la quimotaxis y supervivencia celular como en la captación proteica, prolongando la biodisponibilidad de señales reparadoras [74,116]. Diversos análisis proteómicos indican también que el coágulo derivado del PRGF posee proteínas activas de la fase aguda de la reparación y regeneración tisular, migración y adhesión celular. Además, otros agentes presentes en este interactoma son los factores de activación del metabolismo lipídico, cuyo papel en los mecanismos de curación de heridas y retraso de la fibrinólisis se han demostrado relevantes [117,118].

5.2. Potencial biológico del PRGF en modelos de piel *in vitro*

Para el presente trabajo de investigación se aislaron y caracterizaron diferentes líneas celulares de fibroblastos dérmicos humanos primarios (FDH) como modelo *in vitro* del tejido cutáneo. Los fibroblastos dérmicos son una población celular dinámica y diversa, esenciales para la integridad anatómica y funcional de la piel. No solo producen y organizan la matriz extracelular de la dermis, sino que también se comunican entre sí y con otros tipos celulares jugando un papel crucial en la regeneración cutánea. Este grupo heterogéneo celular reside principalmente en la dermis papilar y dermis reticular, aunque también se encuentran asociados a la papila dérmica de folículos pilosos. Sintetizan una gran variedad de proteínas de anclaje y estructurales de la piel como la decorina, biglicanos, tenascina, laminina, versicano, entactina, colágeno tipo I, III, IV, VI, XII, XIV, XVI, etc [119]. Estas moléculas se organizan de forma ordenada para conformar la ECM de la epidermis, membrana basal y dermis. Los FDH establecen entre ellos interacciones paracrinas y autocrinas mediante la liberación de señales que regulan su actividad biológica y activan rutas de transcripción como la proteína activadora-1 (AP-1) y cascadas de señalización Wnt (wingless integrated) que modulan los procesos de reparación tisular [120]. Las citoquinas quimiotáticas liberadas en zonas dañadas atraen a estas células en una primera oleada para poner en marcha los

mecanismos de regeneración tisular y formación de nuevos vasos sanguíneos. Además, los fibroblastos interactúan paralelamente de forma activa con los queratinocitos epidérmicos transformando gradualmente el microambiente inflamatorio post-traumático en un tejido de granulación sano. De hecho, estas conexiones intercelulares dirigen en gran medida la restauración del tejido conectivo cutáneo, coordinando diversos mecanismos de proliferación, migración y diferenciación celular [22].

Es de sobra conocido que existen diferentes factores que afectan al deterioro cutáneo. Algunos como el envejecimiento, la nutrición, el tabaco o la luz ultravioleta pueden derivar en disfunciones fibroblásticas que fomentan situaciones patológicas en la piel. En estas condiciones, los FDH presentan fenotipos anormales y muestran tasas de proliferación reducida asociados a quiescencia, así como senescencia temprana, patrones de liberación proteica alterada y desregulación de la actividad enzimática [121]. En este sentido, análisis proteómicos sobre FDH bajo senescencia replicativa indican que algunas proteínas del complejo de condensación cromosómico como SMC2/4 (structural maintenance chromosome) se ven significativamente afectadas, lo que se traduce en una disminución de la longitud de los telómeros [122]. Además, la degradación de la matriz extracelular (ECM) afecta de forma directa a la morfología de los fibroblastos dérmicos dando lugar a células colapsadas con poco citoplasma y puntos de anclaje mecánicos prácticamente ausentes. Tanto es así, que en zonas afectadas la conectividad a redes fibrilares y la superficie celular se ven reducidas en un 80% y un 75% respectivamente [123]. Por lo tanto, la degradación gradual y acumulativa de la ECM que deriva de actividades catalíticas de las metaloproteasas y del estrés oxidativo, afecta de manera sustancial al correcto funcionamiento de los fibroblastos y a la integridad cutánea.

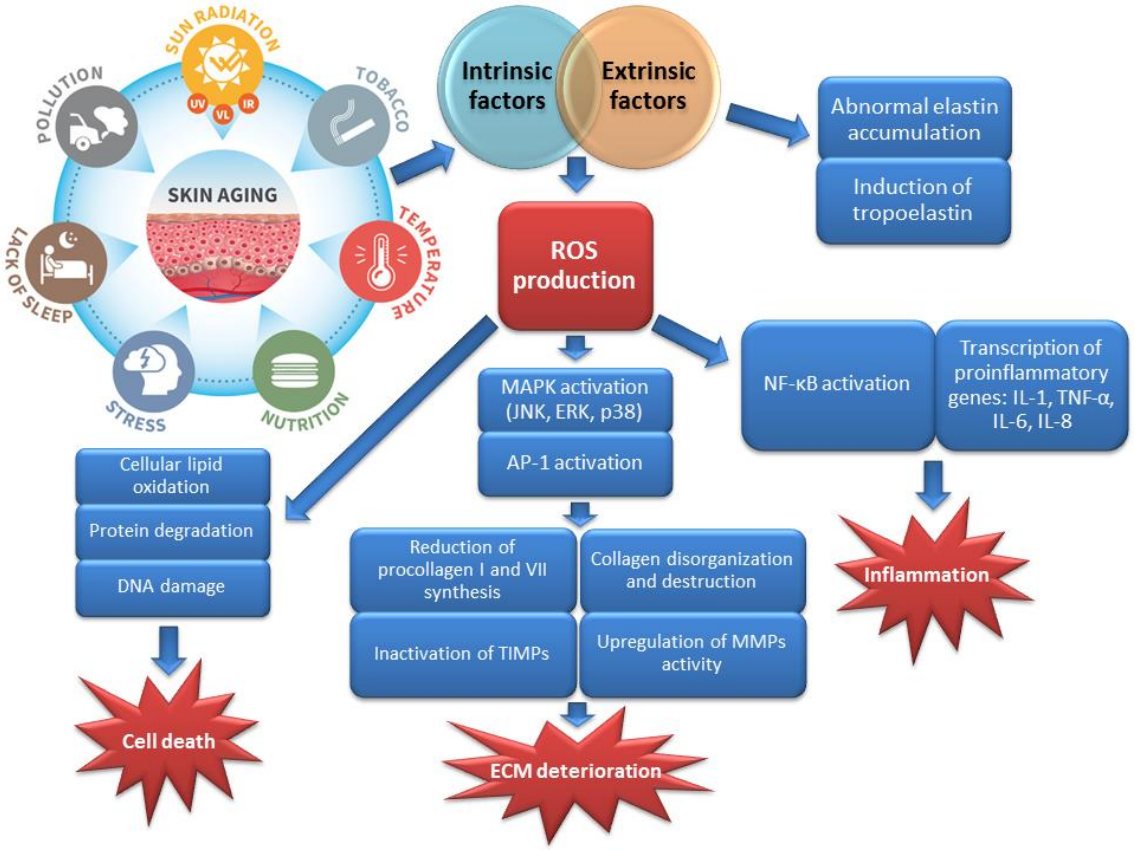


Figura 22: Diagrama esquemático de los principales cambios bioquímicos y rutas moleculares de señalización de la piel que dirigen el deterioro cutáneo en respuesta a factores intrínsecos y extrínsecos. Adaptado de [124,125]

Los ensayos llevados a cabo en el presente trabajo sobre modelos *in vitro* de FDH, demuestran que la tecnología PRGF estimula algunos de los procesos esenciales de la actividad celular. La tasa de proliferación aumenta de una manera dosis-dependiente, alcanzando su máximo potencial regenerativo a una concentración del 20%. El nivel de migración celular también se ve significativamente incrementado tras 24 horas de cultivo, siendo hasta ocho veces superior al control. Previamente se ha concluido que el incremento de la motilidad celular en respuesta al plasma rico en plaquetas puede estar relacionado con un aumento de la actividad nuclear tras la activación de la ruta ERK/Akt (extracelular signal-regulated kinase/protein kinase-B) y la sobreexpresión de reguladores del ciclo celular como la CDK4 (ciclin dependent kinase-4) y Cyclina D1 [126,127]. La familia de las MAPK kinasas (mitogen activated protein kinase), entre las que se encuentran las JNK (c-Jun N-terminal kinase) y las ERK han sido identificadas como factores clave en diversos procesos tróficos. En este sentido, algunos estudios han demostrado que el plasma rico en plaquetas promueve la mitogénesis modulando la fosforilación/desfosforilación cruzada de ERK1/2-JNK [128,129] y controlando la morfogénesis de células madre cutáneas a través de las vías Wnt/β-catenin y Shh (sonic

hedgehog) [130]. Por otro lado, existe un incremento en la expresión de proteínas reguladoras de la fase G1 del ciclo celular como la ciclina A/E y CDK2 en respuesta a los factores de crecimiento liberados por el plasma rico en plaquetas. Estas moléculas señalizadoras inducen la progresión de fibroblastos quiescentes a través de la división celular, lo que se traduce en una aceleración de los procesos de reparación del tejido dañado [126]. De hecho, análisis genéticos recientes han identificado más de 360 genes relacionados con la mitosis, migración y diferenciación celular que están sobre-regulados en respuesta al tratamiento con plasma rico en plaquetas, entre los que se encuentran CDK1, PLK1 (polo like kinase-1), CDC20 (cell división cycle protein-20), CCNB1 (ciclina B1), AURKB (aurora kinase B) y CDK2 [131]. En el presente trabajo, también hemos observado como el PRGF induce un efecto quimiotáctico sobre los FDH que puede estar relacionado en gran medida con el concepto "cell-homing" observado en otros estudios [132]. Este concepto defiende la idea de que una vez infiltrado en el tejido, el PRGF ayuda a reclutar células circundantes del propio paciente hacia la zona afectada [133].

Nuestros resultados indican que el PRGF aumenta la síntesis paracrina de moléculas pro-angiogénicas y mitogénicas como el VEGF y TGF-β, así como la deposición de proteínas de la ECM de la piel como el procolágeno tipo I y ácido hialurónico. Estos resultados son consistentes con otros estudios en los que se ha observado un aumento del metabolismo del VEGF y HGF así como un efecto potenciador del procolágeno tipo I asociado a sus cadenas alfa1/2 tras el tratamiento con plasma rico en plaquetas [134-136]. Análisis por RT-PCR (reacción en cadena de la polimerasa con transcriptasa inversa) también han corroborado un aumento de la expresión genética de colágeno tipo III y fibronectina en fibroblastos dérmicos cultivados con plasma rico en plaquetas [137]. De hecho, se ha demostrado que numerosos factores de crecimiento y citoquinas presentes en el PRGF como el PDGF, EGF, TGF-β, IL-1alfa/β, IL-15, CCL2/5 (chemokine ligand 2/5) y CXCL10 (chemokine ligand 10) inducen la formación de polímeros estructurales de la piel por parte de los FDHs [138]. Otros proteoglicanos relacionados con procesos de adhesión y diferenciación celular como el V1 (versican-1) también son sintetizados en mayor cantidad en respuesta al cultivo con derivados plaquetarios [139].

Un obstáculo importante en la regeneración cutánea es la formación de tejido fibroso. La fibrosis es una situación patológica que está asociada a una descoordinación de la fase de remodelación tisular comprometiendo la funcionalidad del tejido y alterando la arquitectura tridimensional de la piel. Durante las fases tempranas del cierre de heridas, los FDH se diferencian en un tipo celular especializado y efímero denominado miofibroblasto. Estas células se encargan de producir una matriz provisional rica en

glicosaminoglicanos y generan la fuerza contráctil necesaria para el cierre de la lesión [140]. La presencia de niveles elevados de TGF-β1 ha sido identificada como un potente inductor de la diferenciación celular hacia el fenotipo miofibroblástico rico en filamentos de alfa-SMA (smooth muscle actin). A medida que progresa la regeneración del defecto, los miofibroblastos desaparecen por mecanismos de muerte programada y son sustituidos por una segunda oleada de FDHs que inician la formación de un tejido de granulación rico en colágeno. Sin embargo, si los miofibroblastos no experimentan dichos procesos apoptóticos, pueden perjudicar severamente la función tisular debido a una secreción excesiva de ECM, liberación de citoquinas pro-inflamatorias y expresión de factores pro-fibróticos [141].

Nuestros resultados indican que incluso en presencia de exceso de factores pro-fibróticos, el PRGF es capaz de reducir y revertir el fenotipo miofibroblástico. Estos resultados son consistentes con otros estudios en los que se ha evidenciado la capacidad del PRGF de promover la regeneración anti-fibrótica de tejido musculo-esquelético, gingival y ocular [142-145]. Además, se ha observado que moléculas como el FGFb, inducen el fenotipo fibroblástico y activan la apoptosis de miofibroblastos tardíos. Estos estudios sugieren que dicho mecanismo está relacionado con la desregulación de la ruta PI3K (phosphatidylinositol 3 kinase)/Akt y la sobrerregulación de la cascada Rho/Rho kinasa [146]. Por otro lado, cuando la piel se expone a un estrés mecánico como el generado tras una herida, se produce una la liberación de TGF-β1 latente desde reservorios de la ECM. Si se alcanza un desequilibrio en el ciclo metabólico del TGF-β1, puede depositarse un exceso de tejido fibroso y el tejido sano acaba siendo sustituido por cicatrices queloides o hipertróficas [147]. En este sentido, estudios realizados sobre fibroblastos hipertróficos señalan que el plasma rico en plaquetas actúa a través de mecanismos de retroalimentación negativa del TGF-β1, reduciendo la transcripción de genes CTGF que están asociados con desordenes fibro-proliferativos de la piel [148]. Algunos factores de crecimiento como el HGF y VEGF también han demostrado disminuir la transición miofibroblástica mediante la sobreexpresión de la proteína Smad 7 y el antagonismo de la señal TGF-β/Smad 3 respectivamente [149,150]. De hecho, análisis recientes realizados por espectrometría de masas sugieren que el PRGF disminuye la expresión de proteínas como alfa-SMA, desmina, vinculina, cortactina y ARP2/3 (actin related protein 2/3), características del fenotipo de miofibroblastos [151].

Tras una lesión cutánea se expresan fibras de estrés actínicas y se generan tensiones mecánicas a lo largo de la ECM dañada. Esto provoca la orientación de los fibroblastos a lo largo del eje del defecto con el objetivo de ejercer una contracción de la herida que

ayude a la re-epitelización [152]. Nuestros datos derivados del co-cultivo en geles de colágeno, indican que el PRGF duplica la capacidad contráctil de los fibroblastos dérmicos tras 24 horas de tratamiento. Este mecanismo puede estar relacionado con la inducción de la contracción de la ECM promovida por el PDGF y con su interacción sobre receptores PAR-1/PLC (protease activated receptor-1/phospholipase C) [153]. Otros estudios también hablan del efecto pro-contráctil del plasma rico en plaquetas a la hora de promover el flujo de calcio extracelular y activar las cascadas calcio/CaM-MLCK (myosin light chain kinase) y Rho kinasas sobre fibroblastos dérmicos [154].

Categoría funcional	Proteínas	Función biológica
Proteínas adhesivas	VWF, Fg, Fn, Vn, TSP-1, TSP-2, laminina-8	Interacción en contacto celular, hemostasis y coagulación, constituyentes de la matriz extracelular
Factores coagulantes y sus inhibidores	Factor V/Va, factor XI, multimerina, proteína S, kininógeno, proteasa nexin 1 y 2, TFPI, inhibidor de proteína C, gas6	Producción de trombina, procoagulantes y proliferación celular
Factores fibrinolíticos y sus inhibidores	Plasminógeno, PAI-I, u-PA, alfa2-antiplasmina, HRG, TAFI, alfa2-macroblobulina	Producción de plasmina y modelaje vascular
Proteasas y antiproteasas	MMP-1, -2, -4, -9, ADAMTS13, ADAMS10, ADAMS17, TIMPs 1-4, inhibidor plaquetario de FIX, inhibidor C1, antitripsina alfa-1	Angiogénesis, modelaje vascular, regulación de la coagulación, regulación del comportamiento celular
Factores de crecimiento y mitogénicos	PDGF (A, B y C), EGF, IGF-1, VEGF (A y C), bFGF, HGF, BMP-2, -4, -6, CTGF, SCUBE1, IGFBP3	Quimiotaxis, proliferación celular, diferenciación y angiogénesis
Quimiocinas, citoquinas y otros	TGF-β1 y β2, IL-1, RANTES, IL-8, MIP-1alfa, MIP-2, LIX, GRO-alfa, ENA78, SDF-1alfa, MCP-1, MCP-3, PF4, PBP, β-tG, NAP-2, CXCL7, TARC, angiopoyetina-1, HMGB1, IL-6sR, endostatina, osteonectina, sialoproteina ósea, osteoprotegerina	Regulación de la angiogénesis, quimiotaxis, modelaje vascular, interacciones celulares formación de hueso
Proteínas anti-microbianas	Trombocidinas y quinocidinas	Propiedades bactericidas y fungicidas
Glicoproteinas de membrana	alfaIIbβ3, alfavβ3, GPIb, PECAM-1, la mayoría de los constituyentes de la membrana plasmática, receptores de los agonistas primarios, CD40L, factor tisular, P-selectina, TLT-1	Agregación y adhesión de plaquetas, endocitosis de proteínas, inflamación, generación de trombina, interacciones entre plaquetas y leucocitos
Otros	Sulfato de condroitina-4, albúmina, inmunoglobulinas, semaforina-3A, BSDL	Varios

Tabla 3: Contenido y función de proteínas señalizadoras y moléculas bioactivas presentes en los gránulos alfa plaquetarios

Existe una amplia evidencia del efecto angiogénico de los factores de crecimiento autólogos en el tratamiento de heridas isquémicas incluyendo tejidos anóxicos con pobre aporte vascular [145,155,156]. Estos efectores son capaces de modular el desarrollo de brotes endoteliales, promover su transformación en vasos sanguíneos y guiar su maduración final en una red capilar funcional. Este proceso complejo está regulado a través de la ruta angiopoietina-I/Tie-2 que promueve la migración de capilares primarios y la diferenciación de células adyacentes. Además, factores como el PDGF,

HGF y VEGF participan de forma activa a la hora de establecer un lumen de nuevos vasos sanguíneos que nutra el tejido de granulación [157]. Análisis recientes también han concluido que el PRGF posee un marcado efecto anti-inflamatorio que puede estar relacionado con la representación significativa de proteínas de fase aguda asociadas a la atenuación de la ruta NF-κB (nuclear factor kappa B) y el metabolismo de endocanabinoides periféricos [106,158]. Estos elementos cooperan en la resolución de la inflamación aportando a su vez un efecto anti-nociceptivo que se traduce en una reducción del dolor [159,160]. Otro mecanismo crucial que contribuye a la regeneración de defectos cutáneos es el efecto antibacteriano de los derivados plaquetarios. Diversos trabajos han resaltado la presencia de proteínas microbiocidas en las formulaciones plasmáticas como RANTES, beta-defensina-2, CTAP-III (connective tissue activating peptide-III), PF-4 (platelet factor-4) y timosina b-4 [161-163]. Dichas moléculas juegan un rol defensivo frente a diversos tipos de cepas bacterianas y ayudan a promover la correcta regeneración de heridas cutáneas contaminadas [164-166].

Nuestros resultados por lo tanto, se suman a la creciente evidencia científica que avala el uso de derivados plaquetarios como suplementos autólogos para el cultivo y expansión de fibroblastos dérmicos en modelos de piel *in vitro* [167,168].

5.3. Uso del PRGF en condiciones de estrés oxidativo y modelos *ex vivo* de piel 3D

Al igual que cualquier otro órgano, la piel envejece intrínsecamente con el paso del tiempo. Pero a diferencia de otras estructuras, el tejido cutáneo está expuesto a multitud de condiciones medioambientales que fomentan el estrés oxidativo tales como la radiación solar, la contaminación del aire, el tabaco y los componentes foto-sensibilizantes [169]. La piel que se encuentra protegida de la exposición solar (nalgas, antebrazos, etc) sufre procesos de envejecimiento cronológicos caracterizados por cambios relativos en la pérdida de funcionalidad y apariencia visual. Aquellas zonas habitualmente expuestas al estrés medioambiental en cambio, se deterioran prematuramente y son susceptibles de desarrollar diversas patologías cutáneas. La exposición prolongada a la luz ultravioleta derivada de la radiación solar, genera diferentes niveles de estrés oxidativo en la piel que desencadenan multitud de procesos moleculares involucrados en la muerte celular y degradación del tejido. Estos elementos forman parte del denominado "proceso de foto-envejecimiento" y tienen un impacto significativo en la capacidad auto-reparadora de la piel, en su función termorreguladora y sobre todo en la integridad de la matriz extracelular. Las fibras de colágeno y elastina que confieren estabilidad y resiliencia a la piel, experimentan una fragmentación gradual que deteriora las propiedades mecánicas del tejido y altera la

función celular. El foto-envejecimiento también afecta a la curación de heridas, pigmentación, inervación, inmunidad, vascularización y homeostasis del tejido subcutáneo. En conjunto, las alteraciones de la piel relacionadas con el estrés oxidativo desembocan en un aumento de la fragilidad tisular y el desarrollo de patologías cutáneas [169].

La luz ultravioleta se clasifica en UV-A, UV-B y UV-C en función de su longitud de onda e intensidad [170]. Aunque la UVC es la más peligrosa, es absorbida mayoritariamente por la capa de ozono. Sin embargo, la UVA y más significativamente la UVB son componentes esenciales de la radiación solar que generan un severo estrés oxidativo en fibroblastos dérmicos y queratinocitos epidérmicos. El fotodaño se produce a través de interacciones con cromóforos intracelulares que causan la activación de cascadas moleculares derivando en alteraciones genéticas, degradación del tejido conectivo y senescencia replicativa [171]. Estudios histomorfométricos revelan que la piel fotodañada presenta acúmulos de fibras elásticas amorfas por debajo de la unión dermo-epidérmica (elastosis actínica), reducción de colágeno tipo I-III y un incremento de la fractura de proteínas estructurales de la ECM como el ácido hialurónico [172]. Además, la exposición a UVB estimula la producción de colagenasas e inhibe la expresión inhibidores de MMP como TIMP-1 [173]. Sin embargo, los principales elementos citotóxicos que se desencadenan por el estrés oxidativo tras una exposición a rayos UV son las especies reactivas del oxígeno (ROS). Estos radicales libre se liberan al microambiente citoplasmático y extracelular y alteran la actividad genética y función proteica de la piel mediante su interacción con diversas vías de transducción [174].

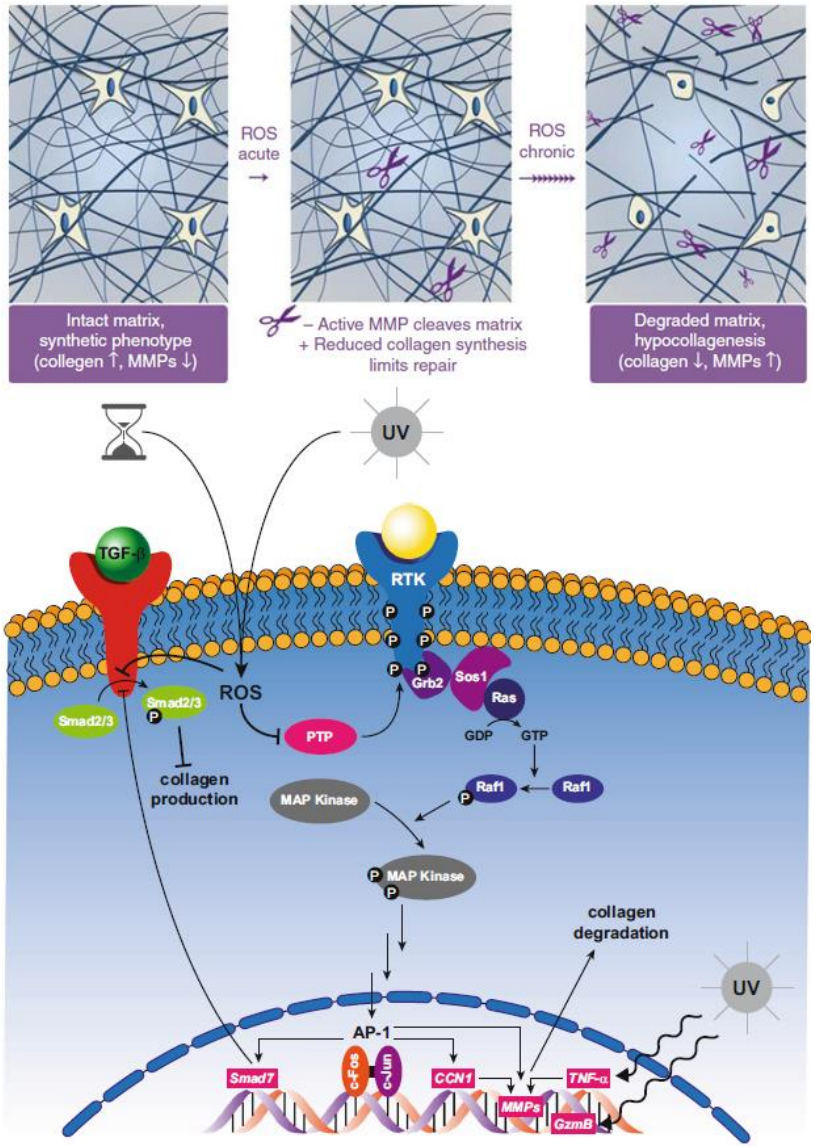


Figura 23: Ilustración esquemática de las rutas intracelulares activadas y estructuras tisulares afectadas por los radicales libre (ROS) generados tras una sobreexposición a luz ultravioleta. Adaptado de [169,175]

Nuestros resultados indican que la supervivencia celular disminuye de una forma dosis-dependiente, siendo las radiaciones de 400mJ/cm^2 y 600mJ/cm^2 las más dañinas. Un europeo medio, recibe una dosis de entre 10 y 20kJ/m^2 de radiación UV cada año. Por lo tanto, la dosis de 400mJ/cm^2 de UVB, a la que se someten los FDH en nuestros ensayos corresponde a una exposición continuada de entre 10 y 20 semanas. Este protocolo, está basado en otros cribados *in vitro* diseñados para testar diferentes elementos como agentes fotoprotectores [176-179]. En la línea de otros estudios, nuestros resultados demuestran que tras una radiación UV se produce una disminución del potencial proliferativo de los FDHs asociado a un deterioro morfológico del citoesqueleto [177,180]. Sin embargo, el tratamiento con PRGF revierte esta situación y la población en monocapa recupera su fenotipo estándar. De hecho, la supervivencia en

cultivo es 2,6 veces superior en aquellas células tratadas simultáneamente con PRGF+UV mientras que el índice de muerte celular es 3,2 veces superior en ausencia de plasma. Los FDH sufren procesos de apoptosis y necrosis celular en respuesta a la radiación UV por medio de la sobreexpresión de las caspasas 3, 8 y 9, lo que deriva en una senescencia prematura [181,182]. Esta reducción en la viabilidad celular está a su vez relacionada con la fragmentación que sufre el ADN nuclear tras una exposición UV y el consiguiente acortamiento de los telómeros [183]. De hecho, se ha demostrado que la radiación solar incrementa las probabilidades de generar dimeros de pirimidina que crean rupturas en las hebras del ácido desoxirribonucléico [181]. Sin embargo, nuestros resultados indican que los factores de crecimiento autólogos reducen los marcadores de apoptosis y necrosis tras una exposición UV, alcanzando niveles de 1,9 y 2,5 veces por debajo del control positivo respectivamente.

La mayoría de los eventos necróticos asociados a la luz UV son desencadenados en primera instancia por la sobreproducción de especies reactivas del oxígeno que incluyen aniones superóxidos (O_2^-), peróxido de hidrógeno (H_2O_2) y radicales hidroxilo (HO^-) [184]. Aunque estos radicales libres son constitutivamente generados por queratinocitos y fibroblastos, son rápidamente eliminados mediante moléculas antioxidantes y enzimas especializadas. No obstante, cuando la piel es sometida a una sobreexposición de radiación solar, los cromóforos encargados de absorber la luz UV (triptófano, riboflavina, ácido trans-urocánico y $NADH^-$/NADPH) generan una sobreproducción de ROS a través de cascadas de transferencia energética que desborda las defensas antioxidantes endógenas del organismo [178]. Tal y como se ha podido observar, el PRGF ha demostrado reducir significativamente los niveles de ROS en presencia de luz UV hasta 13 veces por debajo del control positivo.

La correcta deposición de la ECM de la piel se regula mayoritariamente por un equilibrio entre síntesis/degradación de fibras colágenas por la acción combinada de MMPs y TIMPs. Diversos estudios han demostrado sin embargo, que la radiación UV, provoca en un desequilibrio entre MMP/TIMP que se traduce en una deposición anormal de ECM [185]. Nuestros resultados indican que incluso en condiciones de estrés oxidativo, el PRGF estimula la síntesis de pro-colágeno tipo I y HA (ácido hialurónico) entre 1,4 y 4,8 veces por encima del control positivo mientras que se mantienen niveles funcionales de TIMP-1. Estos resultados son consistentes con otros estudios que señalan la capacidad del plasma rico en plaquetas a la hora de promover la expresión de MMP-1 y MMP-3 a través de rutas ERK-dependientes e inducir la liberación de proteínas ancladas a la ECM como el IGF y GM-CSF (granulocyte-macrophage colony-stimulating factor) [126]. Estas enzimas se encargan fundamentalmente de eliminar

fragmentos de fibras estructurales dañadas y por lo tanto participan en la correcta deposición de nuevo tejido de granulación. El plasma rico en plaquetas también incrementa la expresión de receptores integrina-β1, lo cual afecta positivamente a la regulación de kinasas como FAK (focal adhesión kinasa) y MAPK, traduciéndose en un aumento de la activad de las prolidasas que actúan en el metabolismo del colágeno de la piel [186]. De hecho, estos mecanismos podrían estar relacionados con el efecto inhibidor de los factores de crecimiento autólogos sobre el factor de transcripción AP-1, previniendo la fosforilación de ERK1/2 y JNK, dos de los moduladores más importantes de la ruta de las MAP kinasas [173].

En la presente tesis doctoral y con el objetivo de testar el potencial regenerativo del PRGF sobre el tejido cutáneo, se han realizado una serie de ensayos *ex vivo* utilizando modelos de estrés oxidativo asociado a radiación solar sobre explantes de piel 3D. El desarrollo de cultivos organotípicos tridimensionales proporciona un recurso de gran importancia para estudios básicos de la biología de la piel, el testeo de productos de aplicación tópica y a modo de sustitutos cutáneos en ingeniería de tejidos [187]. Estos modelos de piel humana han sustituido progresivamente la necesidad del uso de animales de experimentación *in vivo* y son ampliamente utilizados en la industria farmacéutica y dermo-cosmética ya que aportan valiosa información acerca de los mecanismos de acción de numerosos principios activos aplicados de forma sistémica o local [188]. Los modelos cutáneos de grosor completo albergan la totalidad de los tipos celulares y estructuras de la piel y reflejan una instantánea de las interdependencias fisiológicas y funcionales en el momento de la generación del explante. Estos constructos representan una herramienta clave a la hora de estudiar los procesos biológicos más relevantes de la homeostasis cutánea, así como evaluar las consecuencias moleculares resultantes de desequilibrios patológicos como la radiación UV y su respuesta a moduladores farmacológicos [189].

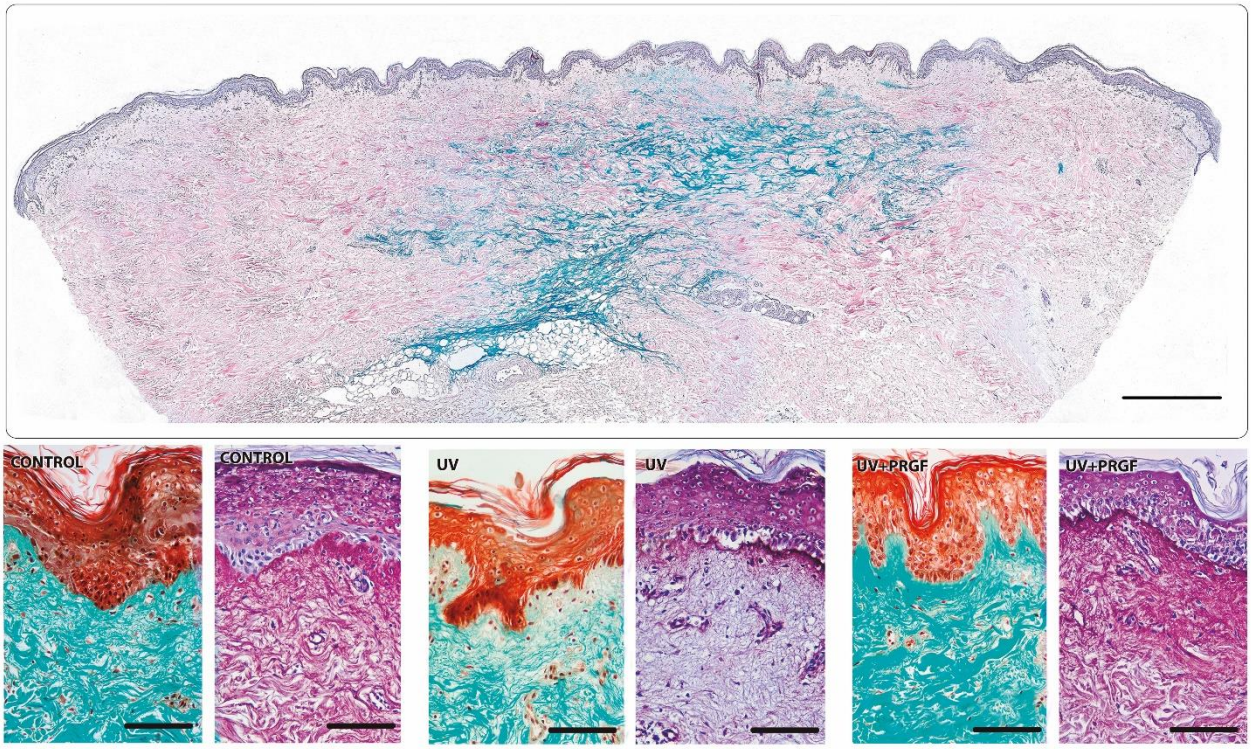


Figura 24: Difusión del PRGF infiltrado a nivel intradérmico en explante de piel 3D (imagen superior). Efecto regenerador del PRGF sobre las fibras de colágeno después de una sobreexposición a radiación UV (imágenes inferiores). Adaptado de [190].

El diseño experimental empleado se ha centrado en evaluar la eficacia preventiva y regeneradora del PRGF infiltrado a nivel intradérmico antes o después de someter al explante de piel a una radiación UV. Los resultados indican que la actividad metabólica y la inflamación en los modelos patológicos mejoran tras el tratamiento con PRGF, mientras que el tejido necrótico y el daño celular disminuyen significativamente con respecto al control positivo. Otros estudios han analizado el mecanismo protector del plasma rico en plaquetas en microambientes hostiles y concluyen que cuando algunos factores de crecimiento como el PDGF se unen a sus respectivos receptores transmembrana, se activa una cascada intracelular que activa genes involucrados en la proliferación, diferenciación y supervivencia celular [191]. En consecuencia, las PI3 kinasas, fosforilan PIP2 en PIP3, que actúa como mensajero secundario de la activación anti-apoptótica de AKT. A parte de promover el crecimiento celular, el metabolismo y la angiogénesis, AKT inhibe la muerte celular programada mediante la supresión de mediadores pro-apoptóticos como BAD (Bcl2 associated death promoter) y BAX (Bcl2 associated X protein) y la sobre-expresión de proteínas anti-apoptóticas como Bcl-2 (B cell lymphoma-2) [192,193]. Como se ha observado, el sello de identidad del estrés foto-oxidativo cutáneo, la formación de ROS, también decrece en respuesta al tratamiento intradérmico con PRGF. Estos resultados sugieren que el plasma rico en factores de

crecimiento podría potenciar la actividad antioxidante endogena de la piel mediante la reducción de niveles intracelulares de radicales libres. De hecho, otros estudios han demostrado recientemente que el PRGF es capaz de activar enzimas detoxificantes derivadas de la intervención del factor nuclear Nrf2 en la sobreexpresión del elemento de respuesta antioxidante celular (ARE) [194]. También se ha observado que el EGF juega un papel clave en la prevención del daño foto-oxidativo sobre fibroblastos dérmicos sometidos a radiación UV. Se ha demostrado que el EGF reduce los niveles intracelulares de ROS y que incrementa la expresión de la catalasa y superoxido dismutasa-1 (SOD-1), dos de las enzimas antioxidantes más relevantes de la respuesta celular. Además, este factor de crecimiento parece proteger la degradación de los extremos cromosómicos del ADN producida por la radiación UV y atenuar la creación de fotoproductos derivados del daño genético como CPD (cyclobutane pyrimidine dimers), 6-4PP (pyrimidine photoproduct) y 8-OHdG (oxo-deoxyguanosine) [195].

El análisis histológico de los explantes 3D irradiados muestra un efecto protector del PRGF en la integridad estructural de la piel basado en la reducción del edema intersticial y la preservación del grosor epidérmico. Además, se detectan fibras elásticas y colágenas mejor organizadas y menos deterioradas en aquellos modelos tratados con factores de crecimiento. Estos resultados son trasladables a estudios clínicos en los que pacientes con piel fotoenvejecida y tratados con PRGF desarrollaron un engrosamiento de la capa dermo/epidérmica junto con una reducción del área de foto-elastosis y un incremento del número de dendrocitos CD34+ profundos [196]. La matriz de fibrina intradérmica generada tras la infiltración de PRGF actúa a modo de andamiaje provisional para el crecimiento celular, como así lo demuestra la prevalencia de células Ki67+ proliferativas detectadas en nuestros explantes irradiados. Por lo tanto, incluso bajo condiciones de radiación solar prolongada las células de la dermis reticular podrían contraponerse al desequilibrio MMP/TIMP, lo que se traduciría en la regeneración del entramado fibrilar de colágeno y la disminución de la elastosis solar. De hecho, tras una radiación UV algunos factores de crecimiento inhiben la señal NF-κB que participa en la liberación de citoquinas pro-inflamatorias y que estimula la sobreproducción de colagenasas [197]. Recientes análisis histomorfométricos también ponen de manifiesto la capacidad del PRGF a la hora de remodelar las fibras colágenas, la elastina o el ácido hialurónico de la dermis. Dichos estudios revelan no solo una disminución de la fibrosis perifolicular y una restitución del entramado elástico de la unión dermo-epidérmica, sino que se observa también una reducción del infiltrado inflamatorio perivascular y una mejora en la involución del plexo vascular de los

folículos pilosos [198]. Por lo tanto, el PRGF se postula como una terapia interesante para la regeneración de la ECM dañada tras estrés foto-oxidativo.

5.4. Desarrollo de nuevas formulaciones terapéuticas basadas en la tecnología PRGF

Uno de los objetivos del presente trabajo de investigación ha sido testar la versatilidad de la tecnología PRGF en el diseño de nuevas formulaciones biofarmacéuticas que cumplan algunas de las necesidades terapéuticas aun no cubiertas en dermatología regenerativa. En este sentido, se ha logrado desarrollar un hidrogel tridimensional estable (PRGF-Gel o PG) y una formulación tópica almacenable (PRGF-Serum o PS) a partir única y exclusivamente de la sangre del propio paciente. Como resultado, las técnicas y el procedimiento de elaboración *in situ* de los biomateriales han sido susceptibles de ser protegidos por derecho de propiedad intelectual a través de la patente WO2015015037A1.

5.4.1. PRGF-Gel (PG)

La ingeniería de tejidos promociona el uso de andamiajes 3D a modo de vehículo de liberación de células y fármacos como posible solución a la escasez de órganos trasplantables. De manera ideal, los biomateriales deberían asemejarse fielmente a las características y funciones de la ECM nativa que no solo proporciona soporte mecánico a las células sino que suministra señales que dirigen diversos eventos biológicos incluyendo la adhesión celular, proliferación, diferenciación y supervivencia [199]. Las características y funciones específicas de la piel demandan biomateriales versátiles y adaptables para su uso en diferentes situaciones terapéuticas. Las materias primas que pueden emplearse en la elaboración de una matriz para ingeniería tisular deberían cumplir una serie de requerimientos como la no-toxicidad, biocompatibilidad, biodegradación controlada y propiedades mecánicas que imiten el tejido a reemplazar [200]. Los biomateriales de origen natural cumplen la mayoría de estos criterios con la ventaja añadida de minimizar la respuesta inmune e inflamatoria. Las interacciones célula-matriz son cruciales para la supervivencia del tejido y durante la adhesión y proliferación, se generan fuerzas mecánicas y contráctiles que se transmiten al andamiaje celular [201]. Por lo tanto, las propiedades mecánicas y reológicas de los biomateriales son de gran importancia ya que deben imitar el ambiente mecánico nativo para resistir y apoyar las fuerzas fisiológicas derivadas de la actividad celular. Para ser clínicamente relevantes, dichas matrices deben a su vez proporcionar la liberación de moléculas bioactivas como los factores de crecimiento que son proteínas señalizadoras clave en la regeneración de tejidos [202].

Estos “scaffolds” o andamiajes pueden ser utilizados como implantes trasplantables o a modo de hidrogeles inyectables. Los biomateriales inyectables son cada vez más populares ya que ofrecen la posibilidad de rellenar defectos tisulares complicados y heteromorfos de una forma mínimamente invasiva [203-205]. La caracterización biomecánica de estos constructos es de gran importancia ya que la viscoelasticidad es un prerrequisito. Deben adaptarse reologicamente a los dispositivos médicos durante la inyección, pero una vez infiltrados deben ser lo suficientemente elásticos y rígidos para resistir presiones y deformaciones por fuerza de cizalla [206,207]. En este sentido, el coágulo de fibrina derivado de la tecnología PRGF presenta una degradación relativamente rápida y un proceso de retracción que aunque es beneficiosa en ciertas aplicaciones quirúrgicas, puede resultar una limitación en otros ámbitos donde se requieran matrices estructurales con propiedades biomecánicas estables [208]. Para superar las limitaciones asociadas al proceso natural de retracción de la fibrina, en la presente tesis se ha desarrollado un “scaffold” proteico inyectable basado en la tecnología PRGF, denominado PRGF-Gel (PG). Este nuevo biomaterial es una formulación autóloga y biodegradable con la capacidad de liberar factores de crecimiento y actuar a modo de plataforma 3D para el crecimiento celular y por lo tanto ayudar en la regeneración del tejido cutáneo. Algunos grupos de investigación trabajan esta línea de desarrollo con la intención de obtener geles tridimensionales de larga duración basados en la combinación de derivados plaquetarios con otros materiales que prolonguen la naturaleza efímera de la fibrina. Dichos constructos incluyen geles PLEL(polilactido-etilenglicol)/plasma rico en plaquetas, esponjas de quitosano cargadas con plasma rico en plaquetas, hidrogeles de fibrina funcionalizados con ECM cartilaginosa o combinaciones de plasma y ácido hialurónico reticulado [209-212]. Sin embargo, estas matrices requieren el uso de elementos exógenos al paciente y agentes químicos reticulantes que pueden entorpecer la acción regenerativa de los factores de crecimiento autólogos y aumentar el riesgo de reacciones inmunes.

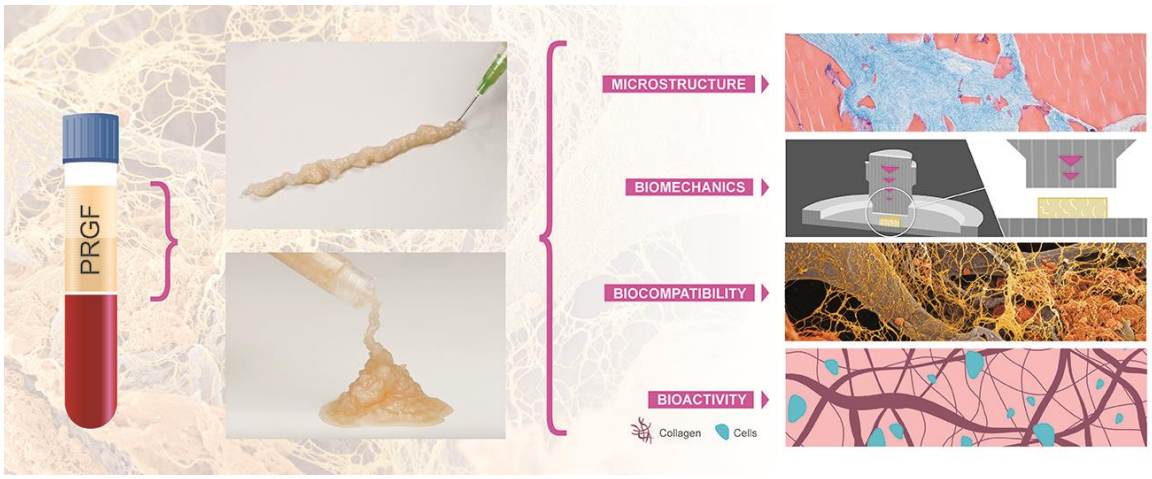


Figura 25: Diagrama ilustrativo de la naturaleza y caracterización biológica y biomecánica de PG

PG está basado en la polimerización térmica controlada de proteínas plasmáticas asociado al entrecruzamiento biológico de hebras de fibrina. La agregación por calor de las cadenas proteicas da lugar a geles semisólidos de elevada rigidez gracias a la formación de enlaces de hidrógeno entre las láminas β, la transformación de las hélices alfa y el establecimiento de puentes disulfuro entre grupos de cisteína expuestos [213-215]. Tras la activación plasmática, las plaquetas se agregan y el fibrinógeno plasmático polimeriza progresivamente en una red de anclaje organizada. El análisis microestructural de PG evidencia la presencia de depósitos proteicos gelificados que están interconectados mediante numerosas hebras de fibrina. Se observan también espacios microporosos de tamaño irregular que proporcionan una adecuada superficie de adhesión para potenciales nichos de colonización celular [216,217]. La fijación de fibroblastos dérmicos, proliferación y adaptación a largo plazo demuestra que PG sostiene el crecimiento celular manteniendo su actividad metabólica y viabilidad debido a la liberación de factores de crecimiento y al soporte estructural del andamiaje proteico. Otros estudios han destacado la capacidad de otros biomateriales inyectables de actuar a modo de sistemas de liberación localizada de poblaciones celulares precargadas mediante procesos similares de solidificación *in situ* [218]. Nuestros resultados sugieren que los espacios porosos de la matriz de PG, podrían incrementar la absorción de nutrientes generando un microambiente óptimo para la regeneración tisular [219]. Por otro, hemos observado que incluso tras repetidos días de biodegradación enzimática, PG podría funcionar como un scaffold prolongado una vez implantado en el tejido diana. Este tipo de biomateriales son adecuados para terapias que requieran una liberación de moléculas bioactivas y necesiten una estabilidad espacial y volumétrica prolongada. La biocompatibilidad de PG supone además una

ventaja biológica frente a otros materiales exógenos, debido a la ausencia de moléculas de deshecho citotóxicas que suelen liberarse al tejido adyacente [220].

Los productos que son inyectados de forma intradérmica o subdérmica en la piel, están sometidos a la suma e interacciones de estrés por cizalla, compresión vertical y fuerzas de tensión [5]. Con el fin de determinar las propiedades biomecánicas de PG se han determinado parámetros clave como G´ (módulo elástico), G´´ (módulo viscoso), G* (módulo complejo), elasticidad (tan δ), η (viscosidad), módulo de Young y fuerza de extrusión. Desde un punto de vista reológico, estos biomateriales deben ser lo suficientemente viscosos bajo fuerzas de cizalla elevadas para poder ser infiltrados de forma precisa a través de una aguja hipodérmica, mientras que deben mantener la suficiente elasticidad para sostener una estructura tridimensional durante la regeneración tisular [221]. Nuestros resultados indican que en el caso de PG existe una prevalencia de G´sobre G´´ lo cual podría estar relacionado con el entrecruzamiento de la fibrina a nivel molecular, dando lugar a un material estático con tendencia a permanecer en el lugar de aplicación en vez de deslizarse y extenderse como un fluido viscoso [222]. Fenómenos observados de estrechamiento por cizalla demuestran que PG posee un perfil reológico óptimo para la viscosuplementación de defectos cutáneos que podría explicarse por la reorganización progresiva de las cadenas poliméricas y su alineamiento a lo largo del eje de estrés mecánico [212]. Adicionalmente, la independencia de la viscosidad angular frente a barridos de frecuencia confirman que el componente elástico del biomaterial permite que se comporte como un deposito del gel estable evitando que fluya fuera del tejido diana [223]. Los resultados reológicos evidencian la naturaleza viscoelástica del gel y su capacidad para recuperar parcialmente su forma original tras deformación mecánica [224]. PG muestra una elevada rigidez y tiene un comportamiento pseudoplástico asociado a una alta cohesividad. Esto se debe al espesor natural del andamiaje y también a las fuerzas internas que evitan la disrupción de partículas del depósito original [207]. Finalmente, los datos de extrusión demuestran su óptima inyectabilidad y su potencial a la hora de formar un gel estable *in situ* partiendo de una dosificación precisa del producto [209].

Este nuevo biomaterial permite la liberación de proteínas plasmáticas, plaquetarias, citoquinas y morfógenos que promueven la regeneración del tejido local. Los poros de la red de fibrina presentes entre los depósitos de gel, secuestran factores de crecimiento como el EGF, IGF-I, PDGF-AB y TGF-β1 vía dominios heparán sulfato, permitiendo así el aporte gradual de moléculas bioactivas al microambiente local [82]. Estas proteínas están involucradas en procesos de reepitelización y estimulación de la migración y mitogénesis celular [225]. Los resultados del presente trabajo de

investigación demuestran que el extracto de PG promueve la proliferación celular y la deposición de ECM local a través de la síntesis de colágeno tipo I y ácido hialurónico. Además, se han realizado ensayos de encapsulación de fibroblastos dérmicos y co-cultivo celular en disposición tridimensional observandose que PG se comporta como un andamiaje 3D biocompatible ya que las células son capaces de adaptarse, establecerse y multiplicarse en su interior. Los fibroblastos muestran una dispersión uniforme a lo largo de la superficie porosa de la matriz manteniendo su morfología funcional y viabilidad, lo que permite en última instancia una óptima colonización celular.

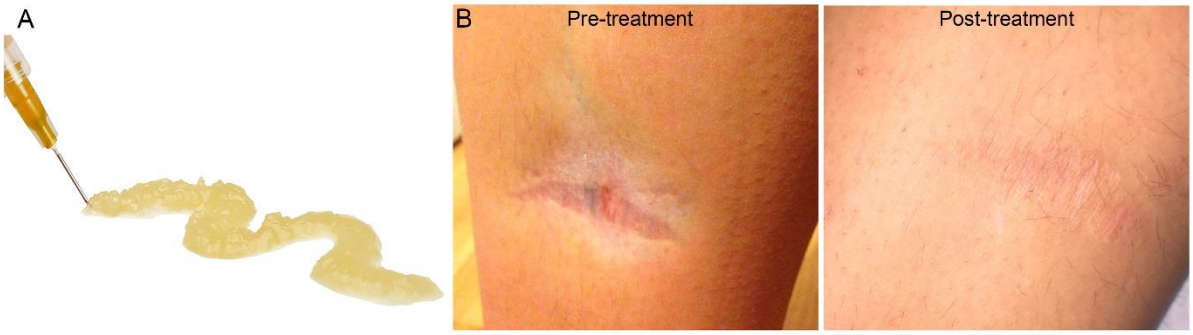


Figura 26: (A) Imagen macroscópica de PRGF-Gel y (B) su potencial para viscosuplementar defectos cutáneos volumétricos como cicatrices atróficas post-traumáticas. Adaptado de [226].

Aunque existen otros biomateriales que han sido utilizados para ingeniería de tejidos, hasta donde sabemos PG es el primer scaffold inyectable 100% autólogo basado en un gel de proteínas endógeno y enriquecido en factores de crecimiento con potencial terapéutico. De hecho, algunos estudios han comenzado a aportar evidencias clínicas a cerca de la eficacia y seguridad de PG y su potencial a la hora de viscosuplementar defectos cutáneos volumétricos como invaginaciones epidérmicas y cicatrices atróficas post-traumáticas que comprometen la funcionalidad de la piel [226-228].

5.4.2. PRGF-Serum (PS)

Tal y como se ha mencionado anteriormente, el deterioro paulatino de la piel genera un estrés oxidativo tisular que induce cambios fisiológicos y estructurales a largo plazo. Estos cambios afectan a la permeabilidad epidérmica, a la capacidad de retención hídrica y a la elasticidad cutánea provocando xerosis, renovación cutánea decelerada y atrofia [229]. Además, la vasculatura degenera progresivamente y la ECM de la dermis se vuelve más frágil debido a la degradación de las fibras colágenas y elásticas. La consecuencia de este deterioro es el desarrollo de una vulnerabilidad tisular y la aparición de zonas susceptibles a condiciones dermatológicas que en ocasiones están

asociadas a una morbilidad significativa como el eczema, dermatitis, enfermedades autoinmunes y neoplasia cutánea [230]. Además, muchas de estas patologías se cronifican y suele ser necesario establecer una posología a largo plazo que incluyan tratamientos crónicos. Recientemente, el uso de factores de crecimiento tópicos recombinantes ha surgido como una opción terapéutica atractiva para el tratamiento de estas afecciones [231-233]. Estos preparados suelen estar formados por proteínas dirigidas a mantener un equilibrio fisiológico mediante su integración en una emulsión farmacéutica que permita una aplicación tópica del ingrediente activo [234]. Sin embargo, hasta la fecha no existe ningún preparado 100% autólogo rico en factores de crecimiento que pueda ser utilizado como una formulación tópica adherente con un balance proteico óptimo para la regeneración cutánea.

En el presente trabajo de investigación se ha logrado desarrollar una nueva formulación personalizada a partir de la tecnología PRGF que ha sido específicamente diseñada a modo de serum de aplicación tópica (PS). Por un lado, se ha realizado una caracterización completa del producto incluyendo parámetros físico-químicos, biológicos y reológicos con la finalidad de evaluar su potencial regenerador. PS es inodoro, presenta una apariencia amarillenta/opaca y tiene una textura de hidro-loción viscosa y homogénea. Al no incluir ningún tipo de agente exógeno a modo de excipiente o conservante, esta formulación mantiene un pH de 7,6±0,2 que se encuentra en el rango de pH neutro, evitando cualquier tipo de irritación cutánea. Normalmente, las preparaciones farmacéuticas semisólidas sirven como vehículo para moléculas bioactivas de aplicación tópica. Las propiedades reológicas de estas formulaciones juegan un papel clave en su comportamiento final y en la biodisponibilidad del principio activo [235,236]. De hecho, la extensibilidad del producto afecta directamente a la biodisponibilidad del fármaco y en consecuencia de la eficacia del tratamiento [237]. La extensibilidad de PS muestra un amplio ratio de superficie cubierta por gramo de producto aplicado. Esto es debido en gran parte al comportamiento pseudoplástico que presenta, característico de fluidos no newtonianos. Estos productos son inicialmente viscosos y tras aplicarles un estrés de cizalla como la extensión sobre la superficie cutánea se promueve una mayor fluidez que favorece una dosificación sencilla y un aumento de la penetración percutánea del principio activo [238].

PS deriva de la tecnología PRGF y por lo tanto presenta un contenido elevado de proteínas, factores de crecimiento y citoquinas. Los resultados obtenidos indican que el nuevo serum es capaz de liberar algunos de los factores de crecimiento más relevantes terapeuticamente (EGF, TGF-β1, PDGF-AB o IGF-I), los cuales participan en procesos de

migración celular, diferenciación, formación de tejido de granulación y re-epitelización de defectos cutáneos [69,239,240]. Un aspecto interesante es que se ha determinado el grado de absorción percutánea de alguna de estas moléculas utilizando modelos *ex vivo* de piel 3D. La principal capa con función barrera de la piel es el estrato corneo ya que aporta protección frente a daños físicos, microbiológicos y de radiación y mantiene la homeostasis cutánea controlando la pérdida de agua transepidérmica [241]. Los principios activos tópicos deben penetrar esta primera capa para interactuar con sus receptores específicos e iniciar así la cascada de señalización que active una respuesta por parte de fibroblastos y otros tipos celulares [242]. Los factores de crecimiento y las citoquinas son moléculas hidrófilas y de un alto peso molecular que limita su capacidad de absorción superficial [231]. Sin embargo existen rutas alternativas que incluyen apéndices cutáneos como folículos pilosos y glándulas sudoríparas o zonas de piel comprometidas [243]. Para ello, la evaluación de la absorción percutánea mediante el uso de celdas de difusión Franz y explantes de piel 3D permite una visión más detallada de las posibilidades terapéuticas de estas formulaciones. Nuestros estudios sobre cultivos organotípicos cutáneos indican que a las 24 horas, tanto el EGF como el PDGF-AB son capaces de penetrar el estrato córneo en un 29% y 12% respectivamente a partir de una sola aplicación. Hay que tener en cuenta que la administración repetida de PS sobre la piel en tratamientos diarios prolongados, aumentaría la concentración final de dichas proteínas, incrementando su potencial regenerativo. La albúmina es la proteína mayoritaria del plasma humano y es una molécula de transporte natural con alta capacidad de unión ligando-específica [244]. De hecho, ha sido utilizada en numerosos estudios como proteína portadora de fármacos terapéuticos con el fin de aumentar su absorción percutánea gracias también a su alta solubilidad, estabilidad y biocompatibilidad [245,246]. Por lo tanto, puede que la alta concentración albúmina presente en un derivado plasmático como PS influya positivamente a la hora de facilitar la penetración de los factores de crecimiento autólogos en las capas profundas de la piel y promover así la regeneración tisular. De hecho, nuestros resultados también han demostrado que el extracto de PS es capaz de inducir la actividad metabólica de fibroblastos dérmicos humanos gracias a la estimulación de la mitogénesis, quimiotaxis y síntesis de componentes de la ECM como el colágeno o el ácido hialurónico.

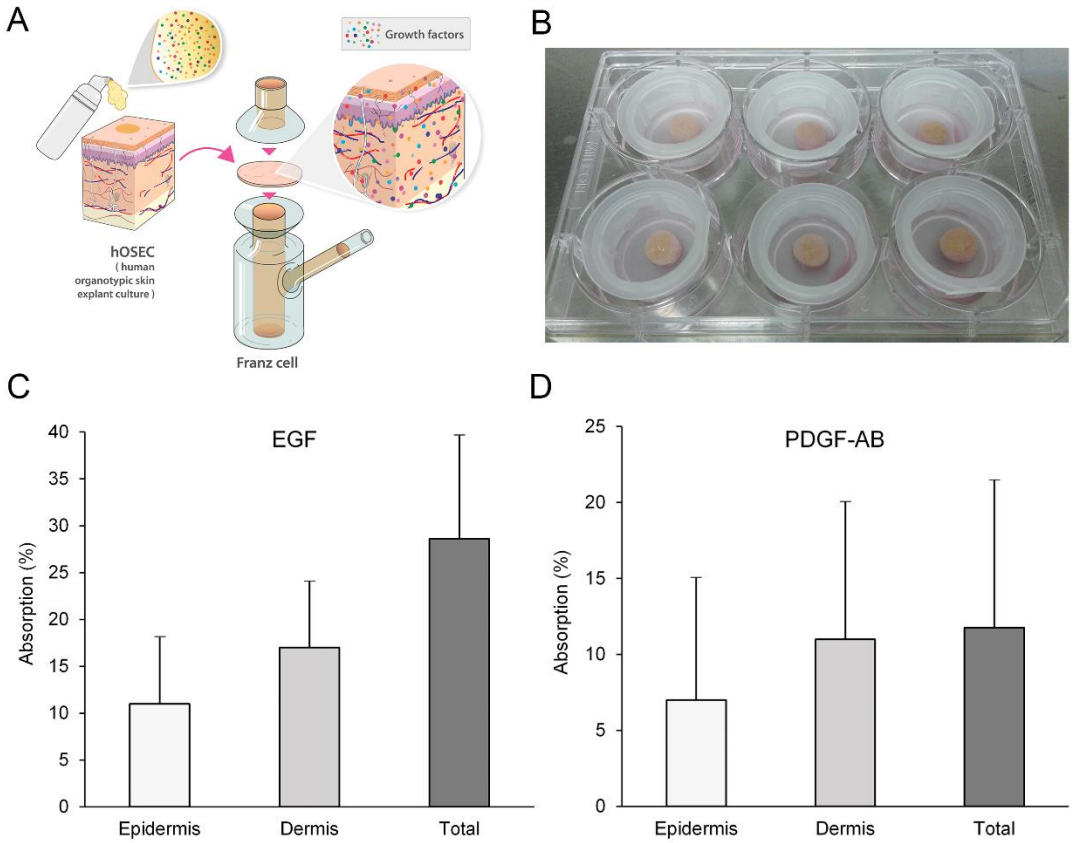


Figura 27: (A) Ilustración representativa del sistema de celdas Franz y modelos organotípicos de piel 3D humana. (B) Macrofotografías de los explantes cutáneos utilizados en los ensayos de absorción percutánea. (C-D) Penetración, epidérmica, dérmica y global de EGF y PDGF-AB tras 24 horas de aplicación tópica de PS.

El objetivo de administrar de forma tópica este cóctel proteico es revertir las manifestaciones del deterioro de la piel actuando sobre las células responsables de la remodelación dérmica de una forma mínimamente invasiva. Hasta la fecha, la vía de administración de derivados plaquetarios a nivel dermatológico estaba restringida a infiltraciones cutáneas o a la inserción de un coágulo de fibrina en un lecho ulceroso. Aunque dichas vías de aplicación han demostrado ser seguras y eficaces para el tratamiento de numerosas patologías, necesitan de un profesional sanitario para su administración y conllevan ligeras molestias asociadas a la venopunción periódica y a la aplicación del producto. Por lo tanto, disponer de un producto autólogo, de fácil aplicación (autoadministrable) y capaz de ser almacenado en condiciones refrigeradas supone una ventaja terapéutica significativa. De hecho, al igual que ocurre con otras formulaciones derivadas del PRGF como el colirio oftamológico, esta posibilidad minimiza el número de extracciones sanguíneas necesarias para disponer de un tratamiento diario y reduce las visitas del paciente a la consulta clínica [101]. Debido a la forma de aplicación del producto y a su régimen de dosificación en hipotéticas afecciones crónicas de la piel, es vital determinar la bioestabilidad y esterilidad de PS a

largo plazo. Para ello, trabajando bajo normativa de buenas prácticas de laboratorio (BPL) y bajo la supervisión de la Agencia Española del Medicamentos y Productos Sanitarios (AEMPS) se ha estudiado la estabilidad funcional de PS en condiciones de almacenamiento refrigeradas (5°C) durante 3 meses (Estudio BTI/BPL-2017/2). Con el ánimo de mantener la naturaleza 100% autóloga del producto, el protocolo de obtención de PS no incluye en ningún momento el uso o adición de excipientes o conservantes exógenos. Atendiendo a los resultados, es posible concluir que PS es físicamente estable y no muestra cambios organolépticos en su textura, olor, adherencia o color. El pH se mantiene en valores de neutralidad a lo largo de los 3 meses de almacenamiento refrigerado. El índice de extensibilidad superficial y viscosidad también permanecen constantes. Por otro lado, el potencial biológico de PS no se ve alterado en los tres meses de estudio tal y como indica la ausencia de diferencias estadísticamente significativas en los niveles de factores de crecimiento analizados. De hecho, las muestras frescas del producto y aquellas almacenadas durante 3 meses mostraron niveles similares de inducción mitogénica celular al aplicarse sobre cultivos de fibroblastos dérmicos. Finalmente se realizaron análisis microbiológicos para garantizar la esterilidad del PS almacenado y los resultados evidencian la ausencia de diferentes cepas bacterianas y fúngicas tanto de origen anaerobio como aerobio.

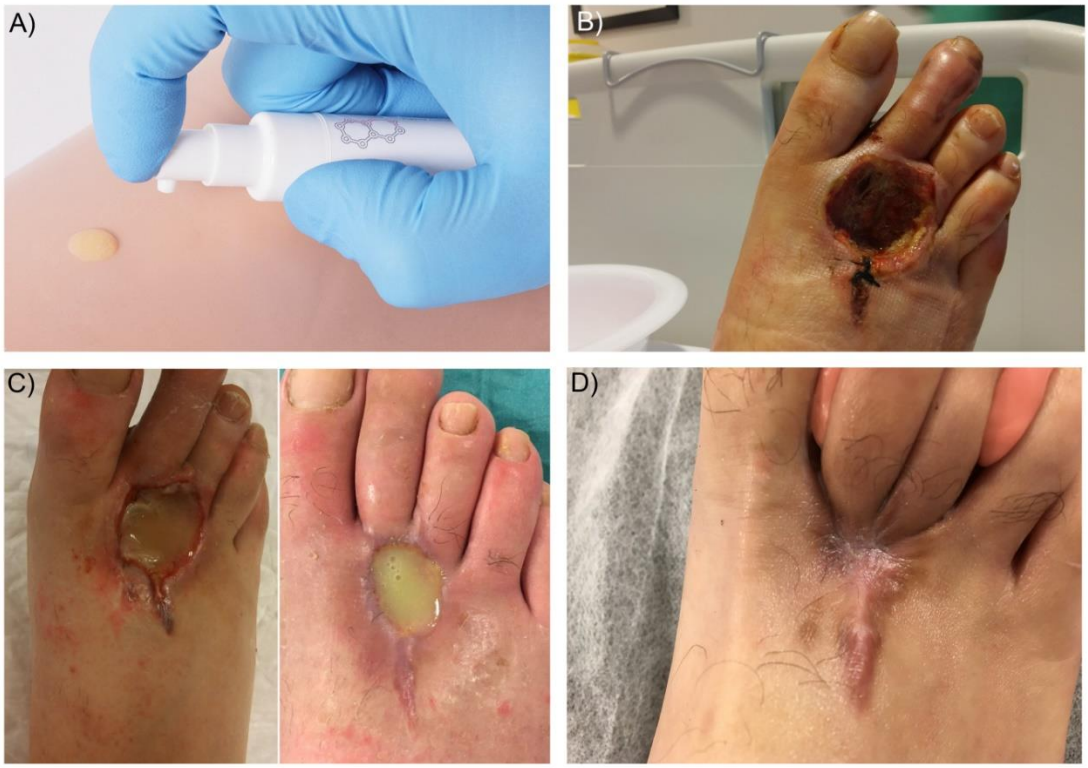


Figura 28: (A) Imagen macroscópica de PRGF-Serum y (B-D) su potencial para promover el cierre de heridas traumáticas. Adaptado de [247].

La formulación PS es posiblemente la primera formulación tópica 100% autóloga y almacenable obtenida a partir de la sangre del propio paciente. Esta formulación posee diversas propiedades biológicas y físico-químicas que la convierten en una herramienta útil en dermatología regenerativa. De hecho, algunos estudios han comenzado a aportar evidencias clínicas a cerca de la eficacia y seguridad de PS y su potencial a la hora de promover el cierre de heridas traumáticas de mal pronóstico [247].

5.5. Perspectivas y retos de futuro

El campo de las terapias autólogas basadas en derivados hemáticos y su aplicación en dermatología regenerativa se está expandiendo con rapidez. En consecuencia el desarrollo de nuevas tecnologías derivadas del uso de concentrados plaquetarios sigue siendo una de las líneas de investigación más prometedoras a día de hoy [107].

Las terapias avanzadas por ejemplo, están comenzando a utilizar este tipo de formulaciones a modo de sistemas de liberación de células madre. Las células progenitoras del tejido adiposo (ASC) han sido descritas como células con potencial multilinaje y capacidad regenerativa derivada de los factores solubles que liberan tras su implantación. Sin embargo, la pluripotencialidad de las ASC se ve severamente limitada debido a patologías sistémicas como la diabetes o los microambientes adversos presentes en afecciones crónicas donde la hipoxia local, el estrés oxidativo, la inflamación y la avascularidad reduce drásticamente su efecto terapéutico. Estas situaciones alteran la supervivencia celular de las ACS y aumentan su susceptibilidad a procesos apoptóticos que afectan a la cinética de liberación de moléculas clave en la regeneración de la piel [248]. Sin embargo, algunos estudios indican que la co-administración de células madre y PRP previene la muerte tisular y mejora la regeneración de la piel en comparación con el tratamiento celular aislado [249]. Aquellos defectos cutáneos tratados con esta terapia sinérgica muestran un ratio de re-epitelización superior junto con una mayor síntesis paracrina de factores de crecimiento como VEGF, HGF y FGF-2. La mejora en la supervivencia celular de las ACS administradas se traduce en una mayor proliferación y síntesis de moléculas señalizadoras que dirigen la migración de células mesenquimales y epiteliales locales a la zona afectada promoviendo la producción de elementos estructurales del tejido de granulación. Además, administrar células madre libres de vehiculización puede afectar negativamente a la biodisponibilidad de las mismas, por lo que el plasma rico en plaquetas podría ayudar a mejorar su localización en el lugar de inserción. Adicionalmente existe un aumento del reclutamiento de pericitos alrededor de los

capilares, los cuales desencadenan la formación de nuevos vasos sanguíneos y la estabilización de la microvasculatura cutánea [249].

Otra de las aplicaciones más prometedoras del plasma rico en factores de crecimiento es su uso a modo de suplemento de cultivo para la expansión *ex vivo* de células madre previo a su implantación en el tejido receptor [250]. La elección del tipo de célula progenitora, la ruta de administración, la cantidad óptima y el vehículo para el trasplante depende de la patología a tratar, pero también del protocolo de manipulación y expansión *ex vivo* elegido. El número de células madre que pueden ser aisladas de un tejido es limitado, y para un trasplante es necesario alcanzar una población celular adecuada que alcance una dosis clínica relevante a través de su expansión *ex vivo*. Para tratar superficies extensas de piel, por ejemplo, en grandes quemados, se necesita de una gran cantidad de células madre con potencial regenerativo. Esta producción a gran escala requiere la optimización de protocolos de cultivo en la búsqueda de un doble objetivo: garantizar la seguridad del tratamiento y obtener el mayor número posible de células madre en el menor tiempo posible [251]. Las técnicas de cultivo de células madre convencionales han evolucionado gradualmente para adaptarse a los nuevos requerimientos de las terapias celulares. Este progreso vertiginoso ha generado diferentes protocolos de cultivo y la ausencia de un consenso que aborde en particular el tipo de suplementos de cultivo utilizados. Existe una amplia variedad de suplementos disponibles, aunque el suplemento de elección es un producto de origen animal denominado suero bovino fetal (FBS). Sin embargo, existen ciertas limitaciones y contraindicaciones asociadas al uso de este compuesto xenogénico como su alta variabilidad entre lotes de producción, el riesgo de transmisión de enfermedades virales, la posibilidad de desencadenar una respuesta inmune xenogénica, el desarrollo de alergias, etc [252]. De hecho diversas agencias reguladoras como la FDA (Food and Drug Administration) y la Agencia Europea del Medicamento están promoviendo el reemplazo del FBS y otros productos de origen animal que puedan utilizarse en las terapias celulares [253]. En este sentido, existen múltiples estudios que avalan la eficacia y seguridad del plasma rico en plaquetas a modo de suplemento de cultivo de células madre para procedimientos de expansión *ex vivo* frente al FBS [254-258]. Las células madre mesenquimales poseen receptores superficiales de membrana que interaccionan con la gran mayoría de factores de crecimiento presentes en el PRGF, estimulando diferentes cascadas de señalización involucradas tanto en la proliferación como en la supervivencia celular [259].

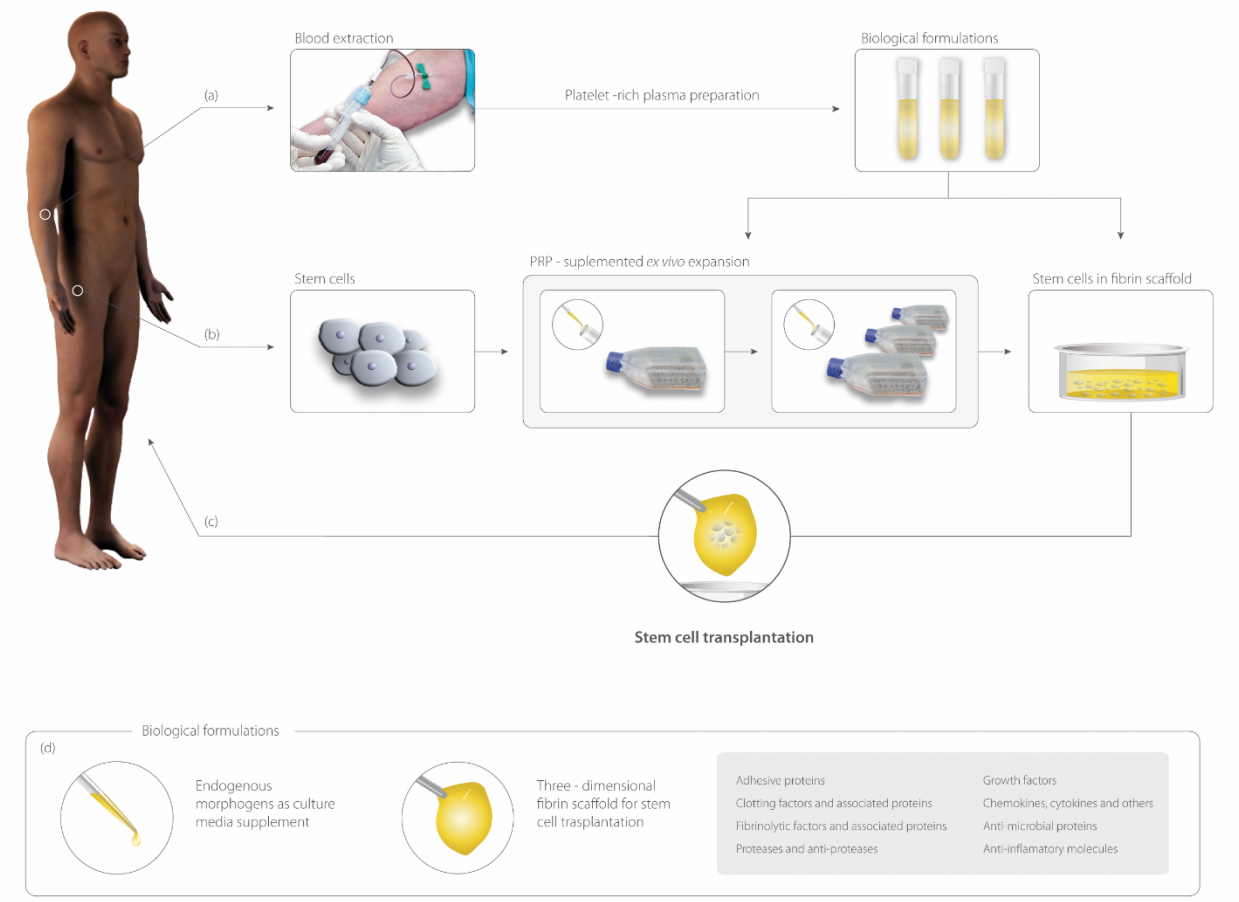


Figura 29: Visión general de un enfoque autólogo para terapias celulares. (A) Primero se obtiene una muestra sanguínea para elaborar diferentes formulaciones biológicas derivadas del PRGF ricas en morfógenos y matriz de fibrina. (B) Aislamiento de células madre del paciente que pueden ser fácilmente expandidas utilizando PRGF como suplemento de cultivo. Tras la expansión libre de sustancias xenogénicas, las células madre son incluidas en PRGF y se activa la cascada de coagulación para generar un andamiaje 3D cargado de células en un microambiente biomimético. (C) Finalmente, las células madre son retrasplantadas en el paciente. (D) Las formulaciones biológicas derivadas del PRGF contienen factores de crecimiento endógenos que promueven una expansión segura y eficaz de las células madre y aumenta su supervivencia en el órgano recipiente. Adaptado de [83].

Estas poblaciones cultivadas con plasma rico en plaquetas, mantienen su morfología e inmunofenotipo así como la eficiencia clonogénica, potencial de diferenciación y estabilidad cromosómica a lo largo de las subsecuentes divisiones celulares. Además, el uso de derivados plasmáticos autólogos favorece la bioseguridad del tratamiento, por lo que la tendencia actual parece indicar que su uso como suplemento de cultivo se generalizará e implementará en los protocolos de terapia celular [260].

Por último se están empezando establecer sinergias terapéuticas entre la tecnología de los derivados plaquetarios y el desarrollo de biotintas compatibles para técnicas de bioimpresión 3D de piel [261]. La bioimpresión es una tecnología de manufacturación

aditiva que ha evolucionado a partir de la impresión 3D y que permite la deposición simultánea y dirigida de un andamiaje tridimensional biocompatible junto con células viables para producir equivalentes tisulares funcionales. En el caso de la piel, estas matrices poseen diferentes tipos celulares y estructuras complejas que forman constructos finales que imitan el tejido nativo. Los principales pasos del proceso de bioimpresión de la piel son el visualizado y diseño de la arquitectura del tejido a elaborar, la selección de la biotinta y células apropiadas a utilizar y el proceso de impresión del andamiaje final [262]. Existe una amplia variedad de polímeros naturales y sintéticos que se utilizan a modo de biotintas para el desarrollo de órganos funcionales y estructuras tisulares. Estas materias primas requieren de numerosas propiedades que son vitales para el correcto funcionamiento del constructo final como la viscoelasticidad, la gelificación *in situ*, la biocompatibilidad, la biodegradabilidad, el estrechamiento por cizalla, la permeabilidad a nutrientes y oxígeno, la inyectabilidad, etc [263]. Algunas biotintas de origen natural están basadas en biomateriales presentes en la ECM como el colágeno, hidrogeles de gelatina/quitosano o también derivados de agregados celulares y esferoides. Interesantemente cada vez se está investigando más el uso de biotintas adaptables que sean compatibles con el crecimiento celular y que sean imprimibles y mecánicamente estables tras su extrusión [264]. En esta línea, el uso de los concentrados plaquetarios ricos en fibrinógeno polimerizable para la elaboración *in vitro* de extensas superficies cutáneas se está valorando como una biotinta más que aceptable. A medida que la importancia de la medicina personalizada es cada vez más clara, la necesidad de desarrollar biotintas autólogas que contengan factores biológicos específicos del paciente es un paso evidente y necesario. Algunos estudios ya están incorporando células cutáneas y plasma rico en plaquetas en hidrogeles para imprimir andamiajes 3D auto-polimerizables con iones de calcio nativos [265]. Los resultados indican que el tejido resultante promueve la angiogénesis y el reclutamiento de células madre, lo que lo convierte en un constructo ideal para la regeneración tisular. Estas bioimpresiones aumentan ligeramente el módulo compresivo de la biotinta que puede depositarse mediante una bioimpresora de extrusión y son capaces de liberar de forma gradual una serie de factores de crecimiento que afectan positivamente a diferentes poblaciones celulares nativas. La bioimpresión automatizada de plasma humano en combinación con fibroblastos dérmicos y queratinocitos epidérmicos puede dar lugar a equivalentes cutáneos de aldededor de 100cm^2 de superficie. Estos constructos multicapa han demostrado ser totalmente funcionales una vez trasplantados en un organismo vivo mostrando gran parte de las estructuras características del tejido original [266]. Este sistema de producción de piel abre la posibilidad al desarrollo de sistemas estandarizados y

automáticos que permitan reducir los costes y tiempos asociados a la aplicación y puedan ser utilizados para tratar de forma rápida y segura numerosas afecciones dermatológicas de gran extensión superficial como grandes quemados y úlceras crónicas. Gracias a los últimos avances en microfluídica y el uso de fibrinógeno polimerizable también se están desarrollando dispositivos médicos portátiles que permitan la biompresión de piel *in situ* directamente sobre el tejido del paciente sin necesidad de elaborar previamente los constructos en cámaras de pre-implantación [267].

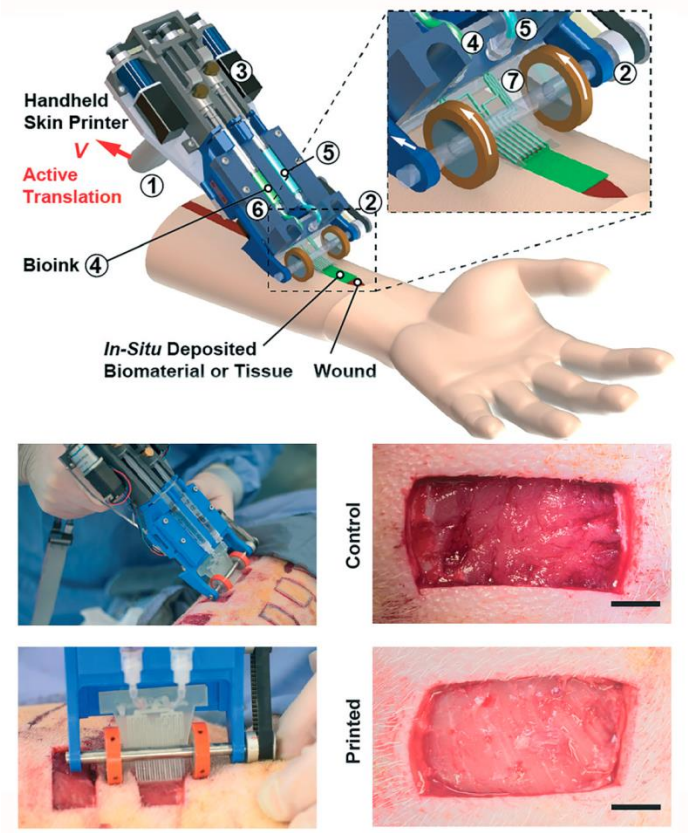
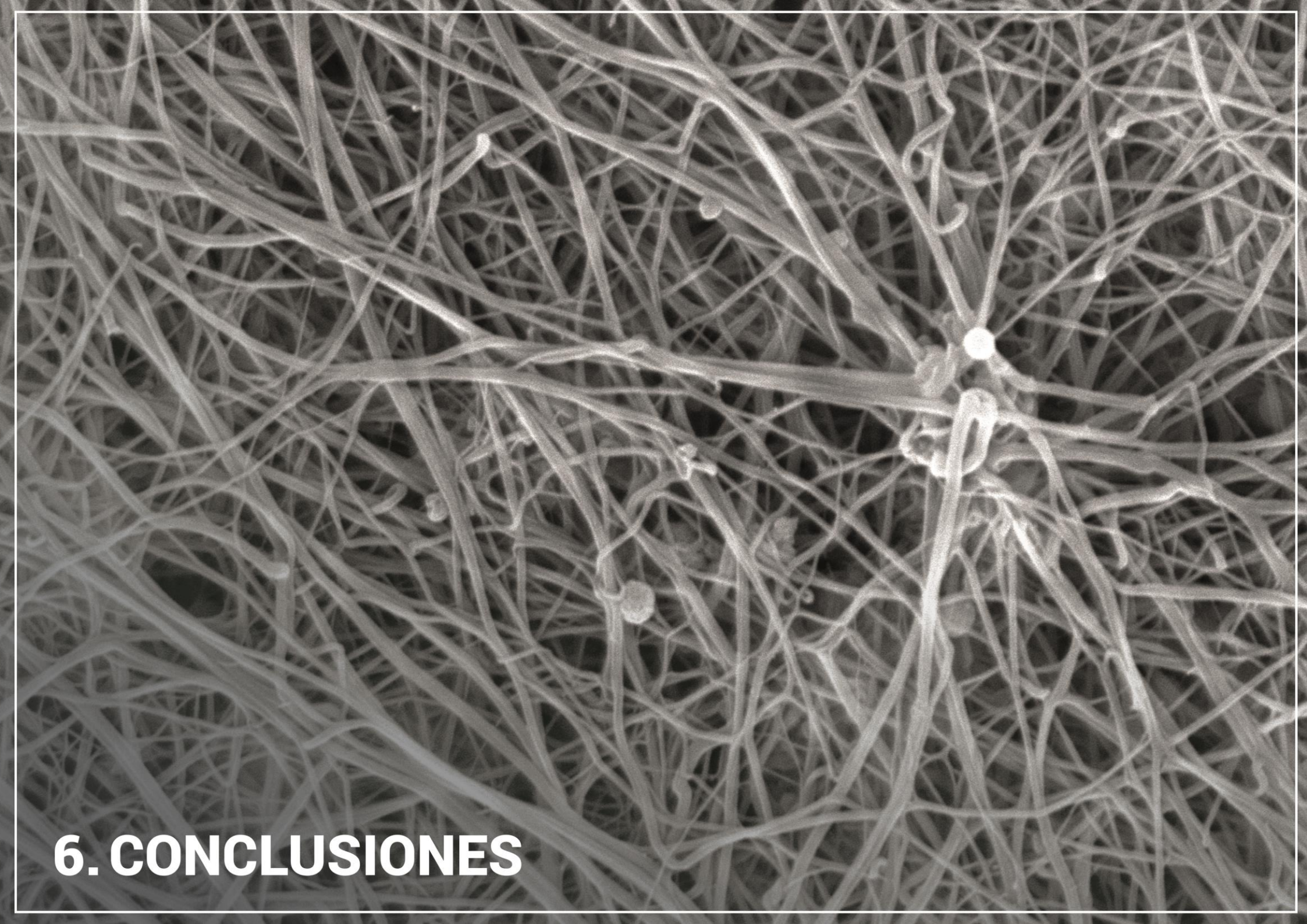
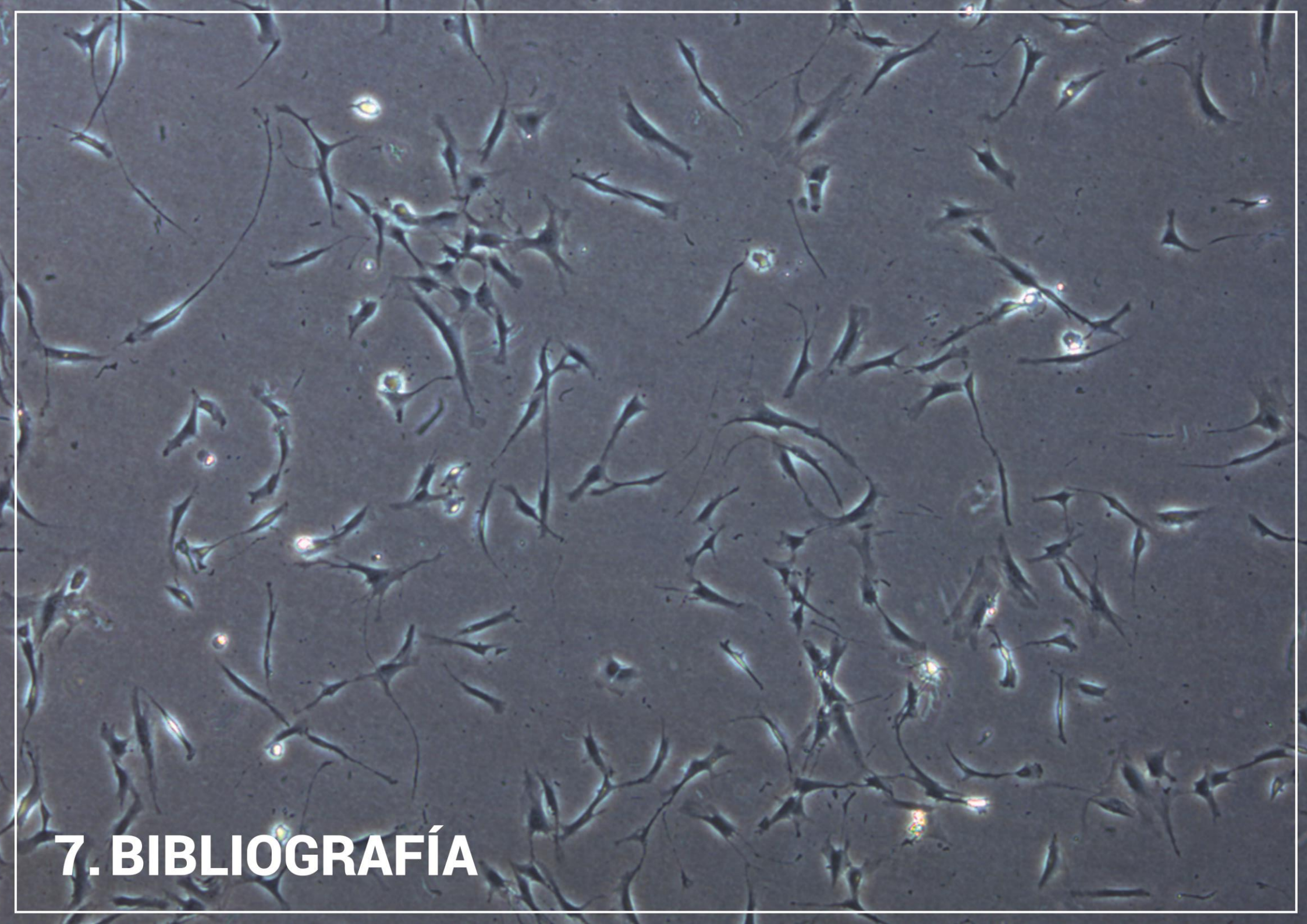


Figura 30: Dispositivo médico diseñado para bioimprimir piel directamente sobre la zona dañada basado en los últimos avances en microfluídica y biotintas ricas en fibrinógeno polimerizable. Adaptado de [267].



6. CONCLUSIONES

- El plasma rico en factores de crecimiento (PRGF) es un suplemento de cultivo idóneo para el estudio de modelos monocapa de fibroblastos dérmicos humanos ya que estimula procesos clave de la actividad y el metabolismo celular como la proliferación, migración, quimiotaxis, síntesis de factores de crecimiento y deposición de matriz extracelular.
- Aunque la naturaleza del PRGF es 100% autóloga, el contenido celular y proteico no presenta variaciones significativas en función del donante. De hecho, el potencial biológico del tratamiento ha sido testado sobre fibroblastos dérmicos y los resultados indican que se alcanzan niveles similares en cuanto a mitogénesis, motilidad, actividad antifibrótica y estimulación pro-contráctil.
- El PRGF es capaz de ejercer un efecto protector y regenerador frente a condiciones de estrés celular aumentando la supervivencia celular, reduciendo la apoptosis, disminuyendo los niveles intracelulares de radicales libre y manteniendo la síntesis de proteínas estructurales de la piel.
- El PRGF atenúa los efectos derivados del estrés foto-oxidativo por radiación UV en modelos de piel 3D. El tratamiento intradérmico de factores de crecimiento preserva la actividad metabólica de los explantes cutáneos, limita la necrosis tisular y reduce los niveles de especies reactivas del oxígeno (ROS). La integridad estructural del tejido conectivo formado por fibras de colágeno y fibras elásticas también se ve significativamente protegido frente al fotodaño por exposición solar.
- La nueva formulación tipo gel tridimensional estable obtenida a partir de la tecnología PRGF, es un biomaterial de elaboración *in situ* que presenta propiedades biomecánicas y biológicas idóneas para la viscosuplementacion prolongada de defectos cutáneos y la regeneración del tejido adyacente.
- Se ha desarrollado un serum tópico basado en las proteínas plasmáticas del propio paciente con características reológicas óptimas para su extensión superficial y absorción percutánea. La caracterización biológica y físico-química de la nueva formulación demuestra que su potencial regenerativo es estable durante 3 meses.



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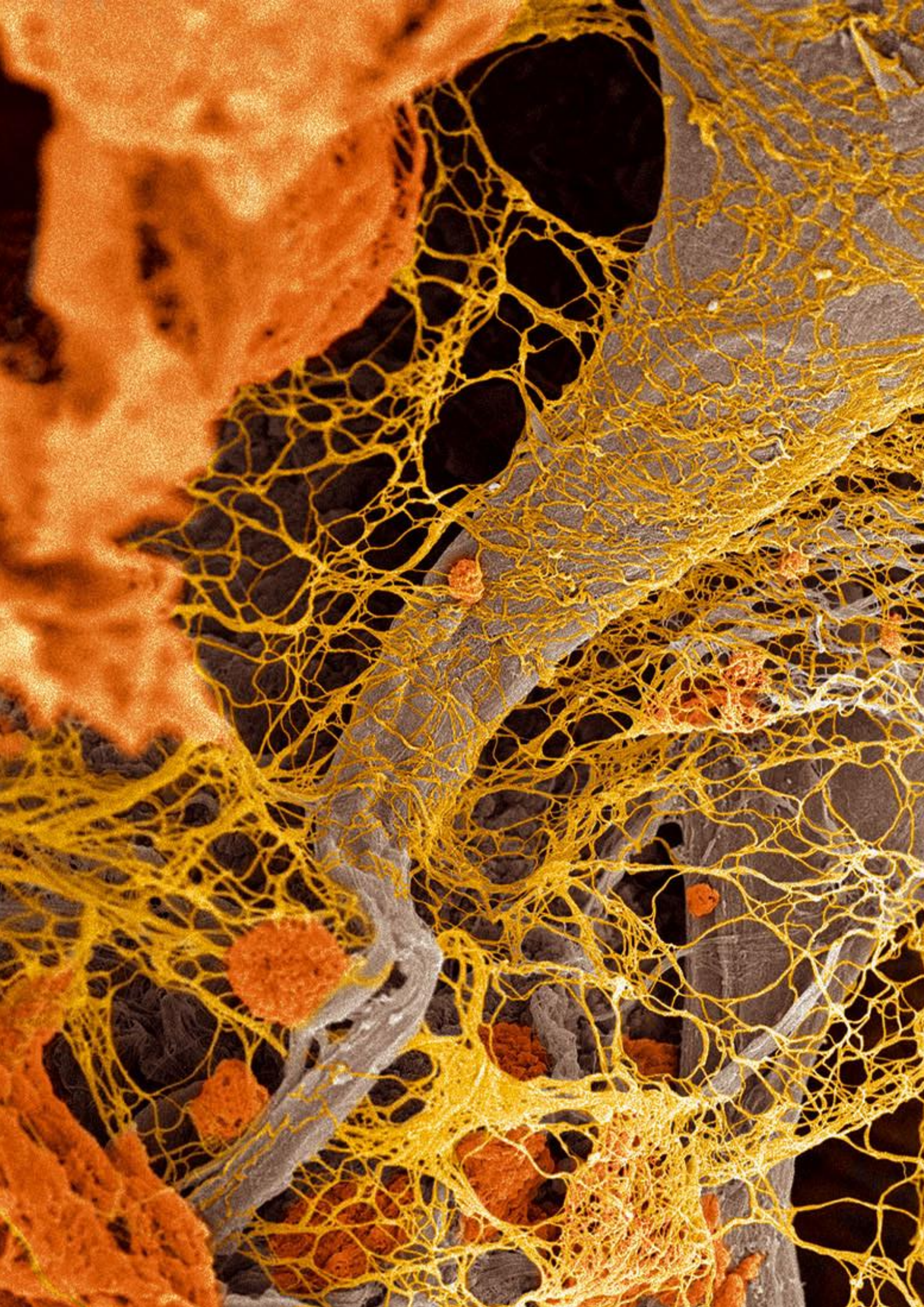


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