Simplificando la biología para optimizar la reparación

TESIS DOCTORAL

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DECLARACIÓN

PROF. **DRA. LETICIA ALEJANDRA BLANCO ANTONA**, PROFESORA ASOCIADA DEL DEPARTAMENTO DE CIRUGÍA DE LA UNIVERSIDAD DE SALAMANCA, ÁREA DE ESTOMATOLOGÍA

CERTIFICA

Que la Tesis Doctoral que presenta al superior juicio del Tribuna, que designe la Universidad de Salamanca, Don **Eduardo Anitua Aldecoa**, titulada "**SIMPLIFICANDO LA BIOLOGIA PARA OPTIMIZAR LA REPARACIÓN**", ha sido realizada bajo mi supervisión, siendo expresión de la capacidad científica de su autor, que lo hacen acreedor del título de Doctor, siempre que así lo considere el citado tribunal.

Fdo: Prof. Dra. Leticia Alejandra Blanco Antona

Salamanca, a 19 de abril de 2023

Agradecimiento

En esta Tesis Doctoral, quiero por una parte, mostrar mi gratitud por la Universidad de Salamanca y el Departamento de Cirugía Bucal que me vio nacer, científicamente y clínicamente hablando y me marcó el camino en mis primeros pasos, pero que ante todo me mostró pasión e inquietud, por todo lo que estaba por llegar.
De la intuición a la evidencia se podría titular lo que han sido mis 44 años de vida profesional, donde comenzando haciéndome preguntas sobre por qué un alvéolo post-extracción cicatrizaba de manera adecuada, en qué casos se producían alveolitis y problemas que creaban una gran morbilidad para el paciente.

En esta tesis he querido mostrar como un camino que comenzó en el área de la cirugía oral, continuó en el área de la ortopedia, en el área de la dermatología, de la cirugía general… Hasta seguir en un campo tan apasionante como la oftalmología. Hemos introducido un primer capítulo sobre la evolución del sistema de coagulación para que sirva de hilo conductor para terminar utilizando el plasma rico en factores de crecimiento como colirio para tratar lesiones de la superficie ocular.

Un camino de la intuición a la evidencia, que puede ser un ejemplo inspirador para otros médicos que estén iniciando su carrera profesional. Tener la mente abierta es algo que debemos de enseñar a todos nuestros alumnos y por qué no soñar con nuevos tratamientos y nuevas aplicaciones terapéuticas.

Solo unas palabras para agradecer a todo el equipo que me lleva acompañando estos últimos 30 años de trabajo científico en el área de la investigación sobre hemoderivados y por supuesto a Paco Seirulo, a quien dedico de corazón esta tesis doctoral.

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Resumen

Todos los seres vivos, desde los organismos unicelulares hasta los complejos animales pluricelulares y multisistémicos, incluidos los vertebrados, poseen una variedad de barreras físicas y químicas, así como un repertorio de respuestas fisiológicas para asegurar la integridad, la supervivencia y la homeostasis, procesos que a menudo se agrupan como sistema inmunitario o de defensa del huésped. Un tema persistente en la evolución de los animales ha sido la prevención tanto de la pérdida de líquidos como de la invasión de patógenos a consecuencia de la alteración de las barreras anatómicas, físicas y químicas inducida por un traumatismo o una infección. Para hacer frente a estas dos emergencias vitales, se ha desarrollado un vasto repertorio de respuestas inmediatas y locales con funciones en la infamación y la coagulación, que consisten en el sellado de heridas y la eliminación de patógenos, lo que resulta en la restauración de la homeostasis y la curación de heridas.

La función inmunoreparadora de la inmunotrombosis se basa en biomoléculas que incluyen la trombina, el fibrinógeno, los factores de crecimiento, las citocinas y las micropartículas que se originan principalmente en el plasma, las plaquetas activadas y los macrófagos residentes en el tejido, todos los cuales son inductores y facilitadores de la reparación tisular. Varias proteínas multidominio intravasculares de los sistemas de coagulación y fibrinolítico, incluyendo la trombina, la fibrina(fibrinógeno), la plasmina(plasminógeno), el FXII y otras serinas proteasas filogenéticamente y estructuralmente están relacionadas, y con funciones importantes en la reparación de heridas. Además, el contenido de los gránulos de las plaquetas que incluyen factores de crecimiento y citoquinas son necesarios para la reconstrucción del tejido después de la lesión. De hecho, las proteínas del sistema de coagulación son proteínas con acción pleiotrópica con funciones que abarcan la coagulación, inflamación y la reparación de

lesiones. Así, el desarrollo de los concentrados plaquetarios se puede entender como una herramienta para beneficiarse de estos mecanismos de reparación.

La historia reciente del plasma rico en factores de crecimiento comenzó a principios del siglo XX con el uso de la fibrina y sus derivados como agentes hemostáticos en cirugía. Más recientemente, Tayapongsak et al. utilizaron clínicamente la fibrina autóloga como adyuvante en la cirugía maxilofacial. Estos primeros conceptos de combinar las propiedades de dos productos, los selladores de fibrina y los factores de crecimiento plaquetario, condujeron al desarrollo de las tecnologías de la matriz autóloga de fibrina. Siguiendo estos principios, Marx desarrolló en 1998 un método para producir plasma rico en plaquetas (PRP) a partir de un separador de gradiente de densidad y lo aplicó con éxito en la cirugía maxilofacial, aunque el gran inconveniente de esta técnica era la gran cantidad de sangre del paciente (400-450 mL) que se requería. Anitua dio un paso gigante al describir un método de preparación de plasma rico en plaquetas libre de leucocitos que requería de volumen pequeño de sangre y solo utilizó iones de calcio para la activación de las plaquetas. Este PRP, conocido hoy en día como el plasma rico en factores de crecimiento (PRGF), ha sido aplicado con éxito en el primer ensayo clínico del uso de concentrados plaquetarios en la preservación del reborde alveolar. La tecnología del PRGF se está utilizando actualmente para el tratamiento de diversas patologías de diferentes áreas de la medicina como la odontología e implantología oral, ortopedia, medicina del deporte y dermatología, entre otros, para promover la curación de heridas y la regeneración de tejidos. La aplicación de las diferentes formulaciones que se obtienen mediante la tecnología del PRGF es directa por parte del especialista en la zona a tratar del paciente. Dicho tratamiento puede ser ambulatorio o quirúrgico dependiendo de la patología a tratar. Sin embargo, en el caso del tratamiento de patologías oftálmicas, la mayoría de los trastornos de la superficie ocular son enfermedades crónicas que deben tratarse durante mucho tiempo para lograr resultados satisfactorios. Asimismo, es el propio paciente quien diariamente tiene que aplicarse el

tratamiento ya que es necesaria su aplicación en forma de colirio entre 2 y 8 veces al día. Por lo tanto, las terapias utilizadas para el manejo de estas enfermedades deben mantener su funcionalidad y estabilidad durante varios meses para ser utilizadas diariamente a lo largo de este tiempo.

Así, esta Tesis Doctoral fue desarrollada para dar respuesta a esta necesidad clínica de aplicar el potencial regenerativo del plasma rico en factores de crecimiento (PRGF) en una formulación segura y eficaz que se podría aplicar por el paciente en su casa. Para ello fue necesario desarrollar 5 trabajos de investigación que resultaron en 5 publicación científicas que forman esta Tesis.

El primer trabajo fue necesario para diseccionar el origen de la inmunotrombosis que articula los primeros instantes de la reparación tisular. Fruto de esta revisión, se ha descrito que el elemento tractor de la evolución fue la supervivencia mediante la restauración de la homeostasis y la reconstitución de los tejidos. Esta revisión también contextualizó tanto los aspectos universales de la reparación tisular como el uso terapéutico del plasma rico en plaquetas como enfoque de la biología como estrategia terapéutica en el contexto de los cambios evolutivos de la coagulación y la hemostasia. Así con este trabajo se ha sentado la base para explorar el efecto de la separación de la fibrina del PRGF en un formato de sobrenadante que sería apto para su uso como suplemento en los cultivos celulares o como colirio oftalmológico.

En el segundo trabajo se ha realizado una revisión sistemática para averiguar tanto el método de preparación como los resultados del uso el uso de plasma humano rico en plaquetas (en formato de sobrenadante) como sustituto de los sueros xenogénicos en las terapias celulares. Fruto de esta revisión, se ha demostrado que el uso de plasma rico en plaquetas empobrecido en leucocitos (en formato de sobrenadante) es una alternativa factible a los sueros xenogénicos. Así, se ha reconfirmado la idoneidad del PRGF (libre de leucocitos) para ser la base del desarrollo de un colirio para aplicaciones oftalmológicas.

En el tercer trabajo fue importante un estudio comparativo entre el colirio de suero autólogo (preparado desde la sangre) y el colirio PRGF para averiguar las diferencias entre las dos preparaciones. En este trabajo experimental se han preparado los dos tipos de colirio de cada donante y fueron añadido a cultivos celulares de los queratocitos del estroma corneal humano (HK). El análisis proteómico se llevó a cabo para analizar y caracterizar los perfiles proteicos diferenciales entre el PRGF y el suero autólogo, y las proteínas expresadas diferencialmente en las células HK. Los resultados obtenidos muestran que el suero autólogo sin diluir induce la activación de diferentes vías relacionadas con una respuesta inflamatoria, angiogénica, de estrés oxidativo y de cicatrización en las células HK respecto al PRGF. Estos resultados sugieren que el PRGF podría ser una mejor alternativa que el suero autólogo para el tratamiento de la superficie ocular.

En los siguientes dos trabajos se ha estudiado experimentalmente los diferentes métodos para la conservación del colirio PRGF. Los métodos de conservación ensayados fueron la liofilización y/o la conservación en frío. Así, en el cuatro trabajo, el colirio PRGF fue liofilizado solo o en combinación con un lioprotector (trehalosa), y luego se almacenaron las muestras durante 3 meses a temperatura ambiente o a 4°C. Se analizaron varios factores de crecimiento en cada tiempo y condición de almacenamiento. Además, se evaluó el potencial proliferativo y migratorio de los colirios liofilizados ricos en factores de crecimiento conservados durante 3 meses a diferentes condiciones de temperatura en queratocitos humanos primarios. Los resultados indicaron que el colirio liofilizado del PRGF ha conservado los principales factores de crecimiento y su actividad biológica tras su almacenamiento a temperatura ambiente o a 4°C durante los 3 meses del ensayo. Por lo tanto, el colirio liofilizado del PRGF conserva sus características biológicas incluso sin el uso de lioprotectores durante al menos 3 meses.

En el quinto trabajo, el objetivo fue analizar si los colirios del PRGF conservan su actividad y sus propiedades biológicas después de su almacenamiento durante 9 y 12 meses a -20°C, y a 4°C, y a temperatura ambiente (TA) durante 3 y 7 días en comparación con las muestras frescas (t0).

Los resultados indicaron la estabilidad biológica del colirio PRGF en todas las condiciones ensayadas. Todos los niveles de los principales factores de crecimiento se conservaron en cada tiempo y condición de almacenamiento. No se observaron diferencias en la proliferación de queratocitos humanos tras el tratamiento con el colirio del PRGF en ninguno de los tiempos o temperaturas estudiados. No se observó contaminación microbiana en ninguno de los colirios del PRGF. Así, el colirio del PRGF puede almacenarse hasta 12 meses sin que se reduzcan los principales factores de crecimiento y proteínas y sin que se produzca ninguna contaminación microbiana. Además, la actividad biológica del colirio del PRGF se mantiene después de almacenarlas durante 3 y 7 días a 4°C o a temperatura ambiente.

Estos resultados fueron confirmados en el sexto artículo dónde se ha realizado un estudio proteómico para ver el efecto de la liofilización del colirio del PRGF en la composición proteica del colirio y en las expresión de proteínas por queratocitos del estroma corneal humano (HK). Los resultados mostraron que se han detectado 280 proteínas, y sólo 8 de ellas alcanzaron diferencias significativas entre las dos formulaciones. Además, 101 de las 3213 proteínas detectadas en las células HK mostraron una desregulación estadísticamente significativa tras el tratamiento con PRGF o PRGF lyo. El análisis de ontología génica mostró que estas proteínas desreguladas estaban implicadas en 30 procesos funcionales. Sin embargo, el *Ingenuity Pathway* mostró que no se encontraron diferencias significativas en ninguno de los procesos identificados. Por lo tanto, el presente estudio muestra que no se encontraron diferencias significativas en el perfil proteómico o en la activación de vías de señalización en células HK entre PRGF y PRGF lyo

La presente investigación ha puesto de manifiesto la posibilidad de deferir la preparación del PRGF de su aplicación clínica. Ofreciendo la posibilidad de una aplicación crónica y repetida del PRGF, que ha sido preparado a partir de una única extracción de sangre. El hecho de que el colirio del PRGF no tiene fibrina abre el camino hacía su uso en otras áreas de la medicina, como por ejemplo la reproducción asistida dónde el tiempo entre la aplicación del PRGF y la transferencia del embrión es inferior al tiempo de la degradación de la fibrina. Así el uso del colirio del PRGF podría ser ventajoso para no interferir con la implantación del embrión en el endometrio. Esta investigación es una prueba como el hecho de simplificar la formulación del PRGF ha abierto el camino para optimizar la reparación en oftalmología y otras aplicaciones que quedan por descubrir.

Tabla de contenido

Lista de figuras

actúan como ligandos extracelulares al unirse a receptores transmembrana en la superficie de las células diana de los tejidos dañados, activando así vías intracelulares para inducir una amplia gama de especificaciones celulares durante la inflamación y el proceso de reparación: supervivencia celular, proliferación, migración, diferenciación, transdiferenciación, maduración y cambios en la síntesis de proteínas y el metabolismo. Estos productos biológicos se perfilan como un prometedor enfoque terapéutico con efectos antiálgicos, antiinflamatorios, tróficos, antifibróticos y angiogénicos en tejidos específicos. Se aplican en lesiones estéril-inflamatorias como la artrosis, tendinopatías, lesiones del cartílago, neuropatías periféricas, patologías del disco intervertebral, quemaduras y úlceras cutáneas, úlceras corneales y sequedad ocular, entre otras dolencias.

Figura 3. Gráfico que muestra el rango de concentración de proteínas en relación con otras 439 proteínas secretadas a la sangre (eje x), así como la concentración en una escala absoluta (eje y) con varias proteínas plasmáticas seleccionadas (marcadas en el gráfico). (Tomada de https://www.proteinatlas.org/humanproteome/blood+protein/proteins+detected+by+immunoassay).

Figura 4. Diagramas de violín que muestran la distribución de las concentraciones plasmáticas de 22 grupos diferentes según su función proteica principal. (Tomada de https://www.proteinatlas.org/humanproteome/blood+protein/proteins+detected+by+immunoassay).

Lista de Abreviaturas y Acrónimos

Abreviatura	Significado
b-FGF	Factor de crecimiento fibroblástico básico
CCR3	Receptor 3 de quimiocina tipo C-C
COX2	Ciclooxigenasa 2
CXCR4	Receptor de quimiocinas C-X-C tipo 4
EDTA	Ácido etilendiaminotetraacético
EGF	Factor de crecimiento epidérmico
FGF	Factor de crecimiento de fibroblastos
GMP	Buenas Prácticas de Fabricación
HGF	Factor de crecimiento hepatocitario
HGFA	Activador del Factor de crecimiento hepatocitario
IFN-γ	Interferón gamma
IGF-1	Factor de crecimiento similar a la insulina-1
IL	Interleuquina
IPA	Software privado para el análisis, integración e interpretación de los datos obtenidos de experimentos ómicos, como proteómica, y microarrays entre otros
MAPK	Vía de señalización dependiente de la proteíncinasa de activación mitogénica
MET	Proteína de transición epitelial-mesenquimal
MFA	Matriz de fibrina autóloga
MSC	Células madre mesenquimales
mTOR	Vía de señalización de diana de rapamicina en células de mamífero
NETosis	Formación de trampas extracelulares de neutrófilos
NF-κB	Factor de transcripción kappa-B
NGF	Factor de crecimiento nervioso
p70S6K	Vía de señalización de proteína cinasa S6 ribosomal beta 1
PDGF	Factor de crecimiento derivado de plaquetas
PF4	Factor plaquetario 4
PK	Precalicreína
PRGF	Plasma rico en factores de crecimiento
PRP	Plasma rico en plaquetas

RANTES	Quimiocina de regulación por activación expresada y secretada por los linfocitos T
ROS	Especies reactivas de oxígeno
SA	Suero autólogo
SBF	SBF
TA	Temperatura ambiente
TGF-β	Factor de crecimiento transformante beta
TNF-α	Factor de necrosis tumoral alfa
VE	Vesículas extracelulares
VEGF	Factor de crecimiento endotelial vascular

Capítulo 1: INTRODUCCIÓN

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1.1 Evolución del sistema de coagulación

Todos los seres vivos, desde los organismos unicelulares hasta los complejos animales pluricelulares y multisistémicos, incluidos los vertebrados, poseen una variedad de barreras físicas y químicas, así como un vasto repertorio de respuestas fisiológicas para asegurar la integridad, la supervivencia y la homeostasis, procesos que a menudo se agrupan como sistema inmunitario o de defensa del huésped (1-3). Un tema persistente en la evolución de los animales ha sido la prevención tanto de la pérdida de líquidos como de la invasión de patógenos a consecuencia de la alteración de las barreras anatómicas, físicas y químicas inducida por un traumatismo o una infección (4, 5). Para hacer frente a estas dos emergencias vitales, los invertebrados han desarrollado un vasto repertorio de respuestas inmediatas y locales con funciones en la inflamación y la coagulación, que consisten en el sellado de heridas y la eliminación de patógenos, lo que resulta en la restauración de la homeostasis y la curación de heridas (4). En los invertebrados marinos, como los erizos de mar, los celomocitos se agregan tras sufrir una lesión o alrededor de sustancias extrañas para formar un coágulo celular mediado por la amasina, una proteína plasmática cuyos multímeros unen los celomocitos entre sí (6). Sin embargo, en otros invertebrados como los cangrejos herradura, los hemocitos en presencia de moléculas extrañas como el lipopolisacárido liberan los depósitos de proteínas intracelulares y factores antibacterianos (4, 6, 7). Los primeros contienen factores de coagulación incluyendo el coagulógeno que se transforma muy rápidamente en el gel coagulina, que es la base de la coagulación de la hemolinfa y la eliminación de patógenos y cuerpos extraños (4, 6, 7). Los mamíferos, en cambio, han acoplado la inflamación y la coagulación a través de componentes de defensa del huésped entrelazados y cooperativos entre sí, cuyos protagonistas son las células sanguíneas

circulantes (células polimorfonucleares, monocitos y plaquetas), las células tisulares estacionarias y migratorias (nociceptores, células endoteliales, macrófagos y fibroblastos) y los sistemas de la cascada inmunitaria innata intravascular originada en el plasma (sistemas de complemento, de activación de la coagulación y de fibrinolísis), en una respuesta agrupada denominada imunotrombosis (8-10).

A pesar de la convergencia en función, sin embargo, las proteínas formadoras de immunocoágulos y los hemocitos de los invertebrados guardan pocas similitudes estructurales y filogenéticas con las proteínas hemostáticas y los trombocitos-plaquetas de la inmunotrombosis de los vertebrados (7, 11). Significativamente, la primera conversión catalizada por la trombina del fibrinógeno a la fibrina del sistema de coagulación de los vertebrados se ensambló a lo largo de una ventana de 50 a 100 millones de años (11) en los primeros vertebrados marinos sin mandíbula similares a los actuales pintarrojas y lampreas, que poseen protrombina, factor tisular, fibrinógeno, FVII y FX pero carecen de genes para el FVIII y FIX y el sistema de contacto (12, 13). Estos animales marinos surgieron al inicio del período cámbrico (11, 12) hace aproximadamente 540 millones (Figura 1).

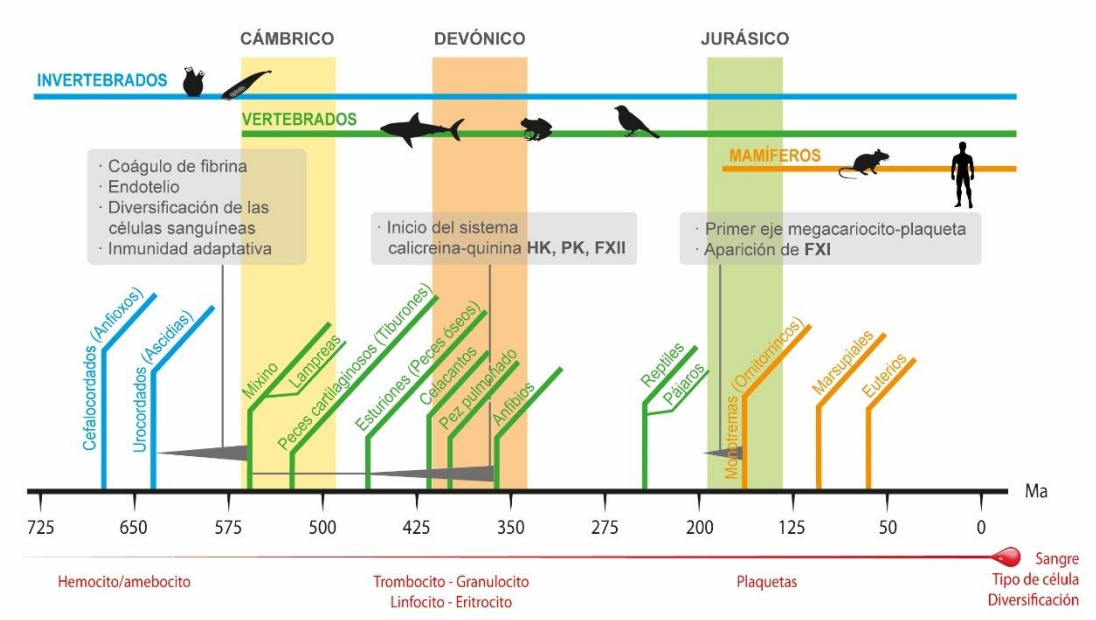

Figura 1. Una visión general de algunos de los principales eventos biológicos y ambientales en la evolución del sistema de coagulación de los vertebrados. La radiación cámbrica estuvo precedida por una duplicación de todo el genoma que, junto con variaciones genéticas en forma de duplicación de genes, combinación de exones y mutaciones simples, condujo a un periodo de grandes innovaciones, incluida la aparición de organismos (los primeros vertebrados agnatos sin mandíbula similares a las actuales pintarrojas y lampreas) con una versión simple del sistema de coagulación que incluía protrombina, factor tisular, fibrinógeno, FVII y X. La aparición del sistema de contacto y su integración en el sistema de coagulación de los vertebrados fue gradual, y se ha asociado a la transición del agua a la tierra de los vertebrados y a la aparición de los primeros anfibios hace unos 400-390 millones de años. El origen del eje megacariocito/plaqueta aún está por determinar, pero podría haber surgido con la aparición de los primeros mamíferos alrededor de hace unos 200-166 millones de años (el círculo rojo y el rombo negro representan los puntos de la evolución en los que se ha propuesto que se produjeron el primer y el segundo evento de duplicación del genoma completo).

Sin embargo, la radiación cámbrica, precedida por el evento de duplicación del genoma completo (14, 15) también fue testigo de la aparición de otras innovaciones fisiológicas, incluyendo un verdadero endotelio, un sistema circulatorio cerrado y cada vez más presurizado, la diversificación de las células sanguíneas y el sistema inmunitario adaptativo (15-18) (figura 1). Este significativo evento genómico, junto con la duplicación de genes, la combinación de exones, y la simple mutación de genes preexistentes, podrían haber proporcionado a los organismos marinos multicelulares las variaciones genéticas que condujeron a la expansión de los principales factores de transcripción y de las proteínas multidominio , ambas moléculas clave para la radiación de los organismos multicelulares en el período Cámbrico, incluyendo la materialización de la primera versión del sistema de coagulación en los vertebrados (18-20). Estos mecanismos genéticos dotaron a las proteínas mosaico de los sistemas del complemento, la coagulación, el contacto y la fibrinólisis, con algunas partes similares, mientras que otras son distintas, generando así afinidades intrínsecas especialmente útiles para crear módulos y redes moleculares cooperativos (21-23).

El precursor de la protrombina se separó de las serinas proteasas preexistentes ya en los deuterostomos hace 710-780 millones de años (genes Bf/C2 y MASP, C1r y C1s), que junto con el C3 ya operaban como un sistema de complemento primordial (21, 24, 25). Estas serina proteasas, a su vez, habían derivado de los dominios de serina proteasas similares a la quimotripsina con la tripsina como genes de origen (21, 25) por duplicación de genes, combinación aleatoria de exones y mutaciones simples que llevó a la sustitución de aminoácidos (11), de forma similar al origen de la trombina y otros factores de coagulación serina proteasas dependientes de la vitamina K (FVII, FIX, proteína C y FX) posteriormente (21-23). El otro sustrato esencial de los coágulos de los vertebrados, el fibrinógeno, no está presente en los invertebrados, pero moléculas que contienen algunos dominios de fibrinógeno se encuentran en los animales, desde las esponjas hasta los mamíferos, con funciones en el desarrollo embrionario, la aglutinación, el reconocimiento de patógenos, la lisis bacteriana, la defensa contra parásitos y la aloinmunidad (autoreconocimiento) (22, 26, 27).

Desde el Cámbrico y durante el Devónico, y coincidiendo con la puesta en marcha de las innovaciones fisiológicas antes mencionadas y que dieron lugar a la diversidad de vertebrados marinos multisistémicos, la versión más simple del sistema de coagulación evolucionó hacia la compleja maquinaria de coagulación de los mamíferos conservando, añadiendo e integrando otras serina proteasas en varias etapas (11). Este es el caso del sistema de activación de contacto/calicreína (CAS) de los humanos, que inicialmente evolucionó en los vertebrados marinos como un sistema inflamatorio de formación de quininógeno-quinina independientemente de los eventos de formación de fibrina, para finalmente converger con la coagulación asociado a la aparición de los tetrápodos en la transición agua-tierra en torno a 400-390 millones de años (11, 12) (Figura 1). El componente central del CAS humano, el gen FXII (f12), surgió antes de la aparición de las lampreas a través de la duplicación del segmento cromosómico que contiene el gen

activador pro-HGF (factor activador del factor de crecimiento hepatocítico, HGFAC) ya presente en los peces cartilaginosos (tiburones, por ejemplo) (12, 28).

Otro nivel de complejidad del sistema de coagulación de los mamíferos fue la aparición del eje megacariocitos/plaquetas (M/Pa). La comparación de los sistemas hemostáticos de los mamíferos más antiguos que se reproducen por huevos no muestra diferencias notables con el M/Pa de los euterios (mamíferos placentarios) (7), un hecho que sugiere que la aparición del M/Pa podría haber ocurrido alrededor de 166 millones de años (figura 1). Sin embargo, la serie de acontecimientos genéticos y ecológicos que dieron lugar al M/Pa sigue siendo un rompecabezas, al igual que la característica poliploide de los megacariocitos (7). Es probable que fuerzas selectivas hayan impulsado la selección del eje M/Pa debido a la generación de trombos arteriales resistentes que optimizan la hemostasia en las condiciones de alta presión y alto flujo del sistema arterial de los mamíferos, la compartimentación y la inhibición de la replicación y diseminación de patógenos mediante la detección de patógenos por los receptores tipo Toll (TLR) 2 y 4, y la posterior liberación de trombocidinas y kinocidinas microbicidas (29, 30). También se ha sugerido que el eje M/Pa podría haber favorecido la implantación del embrión así como haber evitado una hemorragia masiva en el momento del parto, eventos clave en la reproducción de mamíferos con placenta invasiva (31).

1.2 Roles del sistema de coagulación en la reparación de los tejidos

Pero vayamos a la historia reciente del plasma rico en factores de crecimiento (PRGF) y cómo surgió. La historia reciente del plasma rico en factores de crecimiento comenzó a principios del siglo XX con el uso de la fibrina y sus derivados como agentes hemostáticos en cirugía (32). Más tarde, Young y Medawar describieron el uso de fibrina-fibrina en la sutura de nervios periféricos en animales (33). Fue en 1944 cuando Cronkite describió por primera vez el uso de fibrinógeno y trombina como sellado biológico en los

trasplantes de piel (34). Sin embargo, los preparados utilizados hasta entonces carecían de la resistencia necesaria y no eran muy estables en el tiempo. Ya en los años setenta, Matras y sus colegas continuaron con el desarrollo de selladores de fibrina, concretamente intentando aumentar las propiedades adhesivas y curativas de la fibrina mediante la concentración de fibrinógeno por métodos químicos y de crioprecipitación (35). Una desventaja de estos métodos era que presentaban un riesgo de transmisión de enfermedades y la posibilidad de generar reacciones alérgicas debido al uso de trombina bovina. Mencionar que Knighton y col. publicaron en 1986, un trabajo de curación de heridas crónicas, trasladando a la terapia humana un método de utilización de plaquetas para curación de córnea en conejos (36, 37). Su procedimiento de obtención de plaquetas sin embargo se consideraría hoy toxico en humanos ya que las plaquetas eran lavadas con una solución tampón y su posterior mezcla con otro tipo de solución, para finalmente ser activadas con trombina bovina. Más recientemente, Tayapongsak et al. utilizaron clínicamente la fibrina autóloga como adyuvante en la cirugía maxilofacial (38). Estos primeros conceptos de combinar las propiedades de dos productos, los selladores de fibrina y los factores de crecimiento plaquetario, condujeron al desarrollo de las tecnologías de AFM. Siguiendo estos principios, Marx (39) desarrolló en 1998 un método para producir plasma rico en plaquetas (PRP) a partir de un separador de gradiente de densidad y lo aplicó con éxito en la cirugía maxilofacial, aunque el gran inconveniente de esta técnica era la gran cantidad de sangre del paciente (400-450 mL) que se requería. Resumiendo, podríamos indicar que los métodos desarrollados con anterioridad eran diferentes al método diseñado por Anitua (40) por distintos motivos: utilizaban volúmenes entorno a medio litro de sangre (450ml.), mediante centrifugaciones dobles y a velocidades elevadas (3000-5600rpm), largas y muy variadas que conducían a la activación plaquetaria en el propio procedimiento de obtención del plasma. Los productos terapéuticos obtenidos eran fibrinógeno o un producto rojo con las tres líneas celulares de la sangre: glóbulos rojos, leucocitos y plaquetas y para su aplicación

terapéutica se requería de un activador que era la trombina bovina (es decir, un producto de origen animal), asociada al cloruro cálcico.

En este escenario de los productos terapéuticos derivados de la sangre humana y sus métodos de obtención, Anitua (40) publica su trabajo para obtener el plasma rico en factores de crecimiento (PRGF) autólogo, partiendo de pequeños volúmenes de sangre (aproximadamente 40 mL) libres de eritrocitos y leucocitos y activándolo con cloruro cálcico en lugar de utilizar trombina bovina (40). Tras el desarrollo inicial en el campo de la cirugía oral y maxilofacial, los PRP se han utilizado en diversas especialidades médicas, como la traumatología, la cirugía ortopédica y medicina del deporte, dermatología, oftalmología, medicina reproductiva, regeneración nerviosa, cirugía general y cicatrización de heridas, entre otros (41) (figura 2).

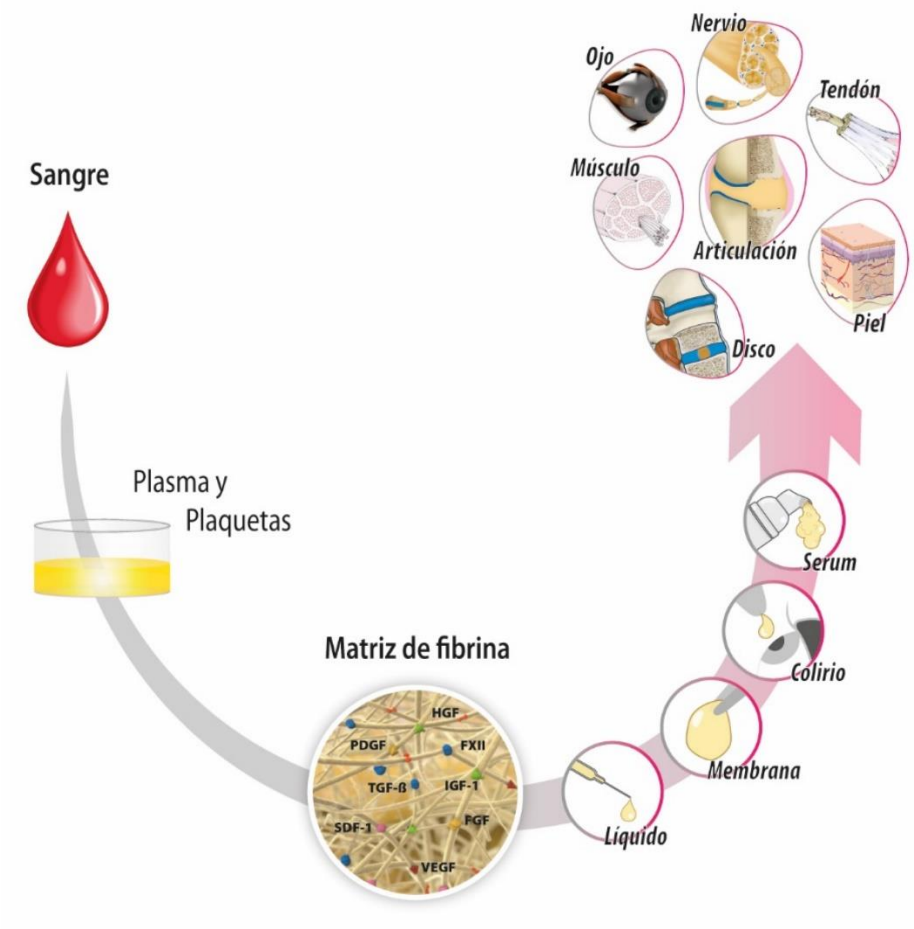

Figura 2. La matriz de fibrina autóloga (MFA) es un subproducto de los sistemas de defensa y reparación propios de la biología. Su obtención ex vivo implica la anticoagulación de la sangre y centrifugación moderada para la formación de fibrina mediante el restablecimiento de la producción de trombina. La fibrina aún en estado líquido en transición a gel es inyectado en el tejido dañado y funciona como un entramado dinámico de líquido a gel que transporta factores de crecimiento derivados del plasma y las plaquetas, como TGF-β1, PDGF, VEGF, HGF, FXII o IGF-1, como mediadores biológicos para la reparación tisular. La biodegradación gradual de la matriz de fibrina mediada por la serina proteasa plasmina liberará factores de crecimiento que actúan como ligandos extracelulares al unirse a receptores transmembrana en la superficie de las células diana de los tejidos dañados, activando así vías intracelulares para inducir una amplia gama de especificaciones celulares durante la inflamación y el proceso de reparación: supervivencia celular, proliferación, migración, diferenciación, transdiferenciación, maduración y cambios en la síntesis de proteínas y el metabolismo. Estos productos biológicos se perfilan como un prometedor enfoque terapéutico con efectos antiálgicos, antiinflamatorios, tróficos, antifibróticos y angiogénicos en tejidos específicos. Se aplican en lesiones estéril-inflamatorias como la artrosis, tendinopatías, lesiones del cartílago, neuropatías periféricas, patologías del disco intervertebral, quemaduras y úlceras cutáneas, úlceras corneales y sequedad ocular, entre otras dolencias.

Las nuevas aplicaciones no clínicas incluyen el uso de PRP como suplemento del medio de cultivo para la expansión ex vivo de células madre (42) o como biotinta específica para el paciente para la ingeniería de tejidos (43). Por último, es probable que las plaquetas en el AFM tengan un papel cada vez más importante en el desarrollo de nuevos enfoques de administración de fármacos. Como hemos comentado hasta ahora, estos pequeños fragmentos acelulares derivados de los megacariocitos son actores clave no sólo en la hemostasia y la trombosis, sino también en múltiples funciones como la reparación tisular, la inflamación y la inmunidad. Su particular estructura vinculada a estas funciones permite explotar todo su potencial como herramienta en el desarrollo de nuevos enfoques de ingeniería biomédica. Las nanopartículas, los nanorecubrimientos y las nanofibras recientemente desarrollados pueden permitir la administración selectiva de proteínas terapéuticas para luchar contra el cáncer, ayudar en las lesiones vasculares, combatir las infecciones, o reparar tejidos (44-46).

1.3 Uso de los PRPs en las células madre

La terapia celular representa un enfoque alternativo y prometedor para reparar tejidos dañados en muchas aplicaciones clínicas en las que el uso de biomateriales puede no ser suficiente (47). Las células madre mesenquimales (MSC) son con frecuencia, aunque no siempre, la fuente celular primaria en medicina regenerativa. Son buenas candidatas debido a su gran capacidad de autorrenovación y de diferenciación multilinaje junto con fuertes propiedades inmunosupresoras (48, 49). Las MSC humanas pueden aislarse de varios tejidos, principalmente de la médula ósea, del tejido adiposo y de la sangre del cordón umbilical. Sin embargo, su baja prevalencia las hace insuficientes para aplicaciones clínicas sin una expansión *ex vivo* previa (50).

En la actualidad, el suero bovino fetal (SBF), también denominado suero fetal de ternera es el suplemento de cultivo celular más utilizado tanto en el campo de la investigación como en el clínico (51). Sin embargo, el uso de productos xenogénicos conlleva muchos problemas de seguridad y normativos. El SBF es un suplemento mal definido con una gran variabilidad entre lotes. Su uso conlleva un riesgo de transmisión zoonótica de contaminantes, como virus o priones, y posibles reacciones inmunológicas adversas debidas a componentes xenogénicos. Además, la obtención de sangre animal implica ciertas cuestiones éticas y de bienestar (50, 52). Todas estas cuestiones exigen la búsqueda de alternativas adecuadas para desarrollar nuevos suplementos de cultivo para su aplicación clínica siguiendo las Buenas Prácticas de Fabricación (GMP). Anualmente se utilizan en todo el mundo más de 2 millones de fetos bovinos para producir aproximadamente 800.000 L de SBF para investigación biológica, ensayos clínicos y producción farmacéutica. Sin embargo, a la vez que existe una demanda creciente coexiste una oferta restringida debido al cambio climático y a la reducción de las reservas ganaderas. En consecuencia, los costos de la obtención de SBF se han incrementado significativamente (más del 300% desde el 2016) (51). En este desfavorable contexto, el PRP se ha convertido en una alternativa prometedora al SBF para la expansión celular, reduciendo tanto el riesgo de xenoinmunización y transmisión zoonótica, como el coste de su adquisición (53). El plasma rico en plaquetas proporciona una herramienta interesante para estimular a las células y desencadenar cambios que activan varios procesos fisiológicos que finalmente dan lugar a la reparación tisular (54, 55). De hecho, las plaquetas contribuyen a ello mediante la liberación de biomoléculas fisiológicamente equilibradas que pueden orquestar el comportamiento celular en términos de crecimiento, reclutamiento, diferenciación y morfogénesis. Estas biomoléculas proceden de los gránulos plaquetarios (gránulos alfa, delta y lambda) y del plasma (56). Las plaquetas interactúan con las células liberando factores de crecimiento al unirse a sus receptores en la superficie celular. Por ejemplo, el factor de crecimiento

derivado de plaquetas (PDGF) interactúa con células mesenquimales (como fibroblastos, osteoblastos y adipocitos) que expresan receptores de tipo α y β (57). Estos receptores participan en la transducción del estímulo proliferativo y los receptores de tipo β participan en la transducción de la quimiotaxis (58). Otro mediador importante de la comunicación celular es el factor de crecimiento transformante beta (TGF-β), que participa en todos los procesos fisiológicos (59). La mayoría de las células expresan receptores para este factor de crecimiento que induce a las células madre mesenquimales a proliferar y diferenciarse (60). Se trata de un factor angiogénico, sin embargo, tiene un efecto inhibidor sobre la formación de osteoclastos y la proliferación de células epiteliales (61). El factor de crecimiento epidérmico (EGF) induce el reclutamiento y la proliferación de células epiteliales y fibroblastos. Desempeña un papel importante en la síntesis del tejido de granulación. Por ejemplo, un elevado número de receptores de EGF son expresados por precondrocitos, fibroblastos y preosteoblastos (62). La fibronectina interactúa con las células, así como con los componentes de la matriz extracelular para favorecer la proliferación y la migración celular con el fin de sustituir el coágulo sanguíneo por la matriz provisional (63). El factor de crecimiento fibroblástico básico (b-FGF) es un factor mitogénico y angiogénico que orquesta la proliferación de células madre mesenquimales (64, 65). El factor de crecimiento similar a la insulina-1 (IGF-1) es pro-angiogénico e induce la proliferación de preosteoblastos y la formación de matriz extracelular por los osteoblastos (66-68). Influye en la proliferación y diferenciación de las células madre mesenquimales durante la generación de cartílago, tejido adiposo, músculos y neuronas (69, 70). La angiopoyetina-2 y el factor de crecimiento endotelial vascular (VEGF) trabajan juntos para promover la angiogénesis (71). Curiosamente, las plaquetas liberan, por ejemplo, el factor plaquetario 4 (PF4) que inhibe la angiogénesis probablemente para modular la misma (61). El VEGF es un factor mitogénico y estimula la diferenciación de distintas células (como fibroblastos, células epiteliales y células renales) (72). Los nucleótidos liberados por las

plaquetas (ATP y ADP) activan a otras plaquetas y participan en todas las fases de la cicatrización tisular. Esto último se ve respaldado por la expresión de receptores P2 (unen nucleótidos extracelulares) en casi todos los tipos celulares (73). Las plaquetas almacenan el 95% del neurotransmisor serotonina presente en la sangre. Es un factor mitogénico (por ejemplo, en células similares a los hepatocitos y los osteoblastos) que participa en la remodelación tisular (74-77). Además, las plaquetas liberan biomoléculas (trombocidinas, PF4, Quimiocina de regulación por activación expresada y secretada por los linfocitos T (RANTES), proteína básica plaquetaria y timosina beta 4) que median en su potencial antimicrobiano (78, 79). Otras moléculas, como la interleuquina 4 (IL-4), el factor de crecimiento hepatocitario (HGF) y el factor de necrosis tumoral alfa (TNF-α), podrían regular la inflamación mediante la inhibición de la activación del factor de transcripción kappa-B (NF-κB) y la expresión de la ciclooxigenasa 2 (COX2) y del receptor de quimiocinas C-X-C tipo 4 (CXCR4) (80).

Por otro lado, durante los procesos de activación y apoptosis, las plaquetas liberan vesículas extracelulares (VE). La Sociedad Internacional de Vesículas Extracelulares (ISEV) define los exosomas como las vesículas extracelulares más pequeñas, unidas por una bicapa lipídica y sin núcleo funcional, liberadas a través de un proceso endocítico (81). Las VE constituyen una población heterogénea de vesículas de membrana formada por exosomas (30-150 nm), microvesículas (100-1000 nm) y cuerpos apoptóticos (100-5000 nm). Estas vesículas transportan importantes moléculas bioactivas, como proteínas, lípidos y ADN mitocondrial, así como microARN, ARN no codificante largo y ARN mensajero. Estas vesículas pueden ser absorbidas por otras células, lo que introduce otro nivel de complejidad en términos de señalización intercelular (82). Cada tipo de VE tiene características únicas y su composición representa al tipo de célula de origen y su estado fisiológico. Este "marcador de origen" es responsable de su función y les concede propiedades organotrópicas que les confieren especificidad de acción (83, 84). Dado que los exosomas pueden penetrar en tejidos inaccesibles para las plaquetas,

como las articulaciones, la linfa y la médula ósea, la diseminación de componentes plaquetarios en tejidos y órganos más allá de la sangre puede ser una de sus funciones más importantes. Así, se ha descubierto que los exosomas plaquetarios participan en diversos procesos biológicos y patológicos importantes, como la coagulación, la angiogénesis, la inflamación, la inmunorregulación y la progresión tumoral (85).

Por todo ello, se han propuesto derivados de sangre humana como sustitutos de los suplementos xenogénicos (42, 53). De hecho, en los últimos años, el uso de plasma rico en plaquetas (PRP) ha experimentado un gran desarrollo en muchas aplicaciones del campo de la medicina regenerativa (54, 61, 86, 87). Su justificación radica en el papel fisiológico de las plaquetas, que, tras su activación, liberan factores de crecimiento y otras moléculas bioactivas, promoviendo así el proceso de cicatrización de heridas (86-92).

Sin embargo, el uso de PRP plantea varias cuestiones que deben abordarse para crear una estandarización en el complejo y cambiante uso del PRP. Estas cuestiones están relacionadas con la definición del plasma rico en plaquetas en términos de concentración de plaquetas, contenido leucocitario, tipo de formulación (activada o no activada), el tipo de activador cuando sea necesario (iones de calcio, trombina o métodos físicos) y los métodos de preparación, incluido el tipo y la concentración de anticoagulante y no menos importante los protocolos de aplicación. Para responder a esta pregunta, es necesario describir la preparación del PRP, de modo que pueda realizarse un análisis comparativo. Es necesario implementar la transparencia mediante el uso de un sistema de clasificación o algoritmo que describa las formulaciones de PRP. Una necesidad similar, pero en el ámbito clínico, ha motivado la sugerencia de varios sistemas para la clasificación y estandarización de los diferentes PRPs (93-97). Ítems como las características del donante de sangre, el dispositivo médico para la extracción de sangre, las características de la sangre, el procesamiento de la sangre para la preparación del PRP, la definición del

PRP, el sello distintivo del PRP, el procedimiento de activación del PRP cuando proceda, la nomenclatura de la formulación del PRP, el contenido de biomoléculas clave, el origen del PRP en relación con las células, la dosis del PRP y la inactivación microbiana cuando proceda, permitirían el análisis comparativo y la reproducción del PRP por otros investigadores.

El plasma rico en plaquetas se utiliza en diferentes formas (sobrenadantes derivados del PRP y PRP no activado) y a diferentes concentraciones. No se dispone de estudios comparativos para evaluar la concentración óptima de plaquetas del PRP para el cultivo de células madre. Sin embargo, existe un consenso entre todos los estudios en cuanto a la preparación de un PRP reducido en leucocitos. La proliferación celular es la prueba que se ha utilizado ampliamente para determinar la concentración más adecuada de PRP en el medio de cultivo. Así, la dosis óptima de PRP oscila entre el 0,5% y el 20%, siendo el 10% de PRP la más utilizada. En cuanto a la activación plaquetaria, los tres métodos más comunes incluyen el uso de cloruro cálcico, trombina y procedimiento de congelación. Sin embargo, no existen estudios comparativos que recomienden un método de activación óptimo. Los anticoagulantes se utilizan habitualmente para prevenir la coagulación de la sangre, ya sea neutralizando la trombina (heparina e hirudina) o quelando los iones de calcio (oxalato, EDTA y citrato) (98, 99). El calcio quelante ofrece las ventajas de recuperar la coagulación añadiendo posteriormente un exceso de iones de calcio. Es importante prestar atención al tipo y la concentración del anticoagulante para no alterar el tamaño, la morfología, el recuento y la actividad de las células sanguíneas (100, 101). Es más, se han descrito cambios en la diferenciación celular y la mitogénesis al alterar la concentración del anticoagulante (102, 103). El citrato sódico es el anticoagulante más utilizado en los artículos revisados, asociándose a una mayor recuperación plaquetaria y a la estabilidad genética de las células estromales mesenquimales (102). El uso de pool y su tamaño son parámetros importantes para reducir la variabilidad del lisado plaquetario humano en lo que

respecta a la concentración de factores de crecimiento y a la divergencia entre lotes (104). Sin embargo, el riesgo potencial de transmisión de enfermedades puede aumentar a medida que se incrementa el tamaño del pool (105).

En el contexto de la terapia celular, la identidad (estructura/composición molecular, propiedades biológicas, fisicoquímicas o inmunológicas) es un requisito para demostrar la singularidad de la materia prima (106). Sin embargo, esto se complica en el caso del plasma rico en plaquetas, ya que no existe consenso sobre la concentración óptima de plaquetas y, por tanto, sobre la concentración de biomoléculas. No obstante, todos los estudios incluidos en esta revisión utilizaron PRP reducido en leucocitos. Para el uso rutinario, se necesitan medios químicamente definidos para el cultivo estandarizado de MSC según las directrices de las GMP (107). Se necesitan más estudios comparativos bajo un proceso de fabricación conforme a las GMP utilizando PRP para definir los criterios de composición que deben cumplirse. Otra posibilidad es la realización de pruebas de rendimiento en relación con la contaminación, las proteínas totales, el pH y la osmolaridad (106). Desde el punto de vista de las buenas prácticas de fabricación (GMP), el PRP debe estar libre de riesgo de contaminación, no ser inmunogénico, no ser oncogénico, ser eficaz para aumentar la tasa de proliferación celular y ser eficaz para conservar sin modificaciones el fenotipo de las MSC y su capacidad de diferenciación (106).

El uso de PRP ha demostrado ser eficaz para aumentar, o al menos no disminuir, la tasa de proliferación celular, mantener sin modificar el fenotipo de las MSC (excepto en el caso de las células madre ectomesenquimales procedentes de dientes humanos exfoliados en pases altos), preservar su estabilidad genética y respaldar su capacidad de diferenciación. Por lo tanto, el uso de PRP leucodeplecionados es factible como alternativa a los sueros xenogénicos para el cultivo de células madre. Sin embargo, faltan descripciones completas de la preparación, caracterización y composición del PRP.

Además, es necesario establecer estudios comparativos entre diferentes composiciones de PRP para determinar parámetros de control de calidad y directrices que sean aceptadas universalmente. Todas estas limitaciones dificultan la obtención de recomendaciones sobre las características del PRP para el cultivo de células primarias en relación con las terapias celulares y en última instancia consenso sobre su efecto biológico.

1.4 Limitaciones del estado actual del PRGF

La tecnología del PRGF se está utilizando actualmente para el tratamiento de diversas patologías de diferentes áreas de la medicina como la odontología e implantología oral, ortopedia, medicina del deporte y dermatología, entre otros, para promover la curación de heridas y la regeneración de tejidos. La aplicación de las diferentes formulaciones que se obtienen mediante la tecnología del PRGF en dichas áreas médicas es directa por parte del especialista en la zona a tratar del paciente. Dicho tratamiento puede ser ambulatorio o quirúrgico dependiendo de la patología a tratar. Sin embargo, en el caso del tratamiento de patologías oftálmicas, la mayoría de los trastornos de la superficie ocular son enfermedades crónicas que deben tratarse durante mucho tiempo para lograr resultados satisfactorios. Asimismo, es el propio paciente quien diariamente tiene que aplicarse el tratamiento ya que es necesaria su aplicación en forma de colirio entre 2 y 8 veces al día. Por lo tanto, las terapias utilizadas para el manejo de estas enfermedades deben mantener su funcionalidad y estabilidad durante varios meses para ser utilizadas diariamente a lo largo de este tiempo.

Bajo el concepto "de usted y para usted", diversos grupos científicos han desarrollado protocolos y tecnologías para utilizar la propia sangre del paciente, y en especial el plasma y las proteínas derivadas de las plaquetas, para favorecer la reparación y regeneración de los tejidos. No obstante, este planteamiento no es totalmente nuevo. De

hecho, la sangre se mencionó en el primer documento médico conocido (el papiro Ebert) hace más de 3.000 años. En las últimas décadas, el uso de colirios de suero autólogo (SA) se ha extendido al tratamiento de diversas patologías de la superficie ocular. Los resultados obtenidos hasta la fecha son alentadores (108, 109), pero varios problemas relevantes limitan la extensión y versatilidad de este enfoque. Por ejemplo, no hay consenso sobre el protocolo y la dosificación de una preparación de colirio de suero autólogo (110); los colirios de suero contienen productos de la degranulación leucocitaria, incluidos agentes proinflamatorios como las metaloproteinasas y las hidrolasas ácidas, los cuales pueden desencadenar efectos destructivos en los tejidos (111). Además, hay pocos estudios que muestren la estabilidad biológica del suero autólogo relacionados con su conservación y almacenamiento. Recientemente, se han descrito las plaquetas como fuente principal de factores de crecimiento autólogos, muchos de los cuales son claves en el proceso de regeneración tisular (112). Al concentrar las plaquetas en el plasma humano y promoviendo la liberación de todos factores de crecimiento, es posible obtener entre otras formulaciones un sobrenadante rico en cientos de proteínas y factores de crecimiento implicados en la regeneración de la superficie ocular, como el factor de crecimiento epidérmico (EGF), factor de crecimiento derivado de plaquetas (PDGF), factor de crecimiento de fibroblastos (FGF) y factor de crecimiento nervioso (NGF) son secretados por los gránulos alfa de las plaquetas (113). La tecnología del plasma rico en factores de crecimiento (PRGF) es un enfoque estandarizado y optimizado que tiene por objeto la preparación y uso de varias formulaciones de y para un único paciente. La tecnología del PRGF está basada en el uso de un volumen limitado de plasma enriquecido en plaquetas, que se obtiene del paciente. Una vez activado el concentrado de plaquetas, se forma un andamio de fibrina tridimensional y biocompatible, y una miríada de factores de crecimiento y proteínas con un alto potencial terapéutico como PDGF, EGF, FGF, factor de crecimiento transformante beta (TGF-β), factor de crecimiento endotelial vascular (VEGF), factor de crecimiento

similar a la insulina-1 (IGF-1), factor de crecimiento hepatocitario (HGF), fibronectina, y trombospondina se liberan progresivamente obteniéndose finalmente un sobrenadante o colirio (114, 115). El colirio de PRGF es un hemoderivado autólogo obtenido mediante un sistema cerrado que incluye la activación plaquetaria, evitando leucocitos y moléculas proinflamatorias y con un mayor contenido en factores de crecimiento que el suero autólogo. Otras características importantes del colirio de PRGF en la regeneración tisular radican en su actividad bacteriostática/bactericida, su potencial antifibrótico y antiinflamatorio (116, 117). Diversos estudios *in vitro* han puesto de manifiesto los efectos beneficiosos de los colirios de PRGF sobre las células primarias de la superficie ocular humana, acelerando el cierre de la herida corneal y aumentando la proliferación y migración de las células epiteliales corneales en comparación con el SA (118, 119). Un estudio *in vivo* demostró que los colirios de PRGF reducían el tiempo de reepitelización en comparación con un plasma rico en plaquetas sin activación plaquetaria y en comparación con SA (119). Un estudio *in vitro* posterior demostró que los colirios de PRGF son capaces de ejercer un efecto antiinflamatorio mayor que el SA sobre los fibroblastos de la superficie ocular tratados con IL-1β y TNFα proinflamatorios (117). Asimismo, un estudio reciente demostró que los colirios de PRGF eran capaces de reducir significativamente el número de miofibroblastos inducidos por el factor de crecimiento transformante (TGF)-β1 en comparación con el SA, lo que sugiere que el PRGF puede promover la regeneración de heridas corneales, reduciendo la formación de cicatrices (120). Para intentar dilucidar las vías por las que el colirio de PRGF ejerce su potencial antifibrótico en comparación con el SA, se realizó un estudio proteómico en queratocitos corneales diferenciados a miofibroblastos, que posteriormente fueron tratados con PRGF o SA (121). Este estudio muestra que el colirio de PRGF redujo la activación de varias proteínas implicadas en las vías por las que el TGF-β1 ejerce su acción para inducir la formación de fibras de α-actina en queratocitos del estroma corneal humano, induciendo su transformación en miofibroblastos. Por otro lado, es importante

mencionar que todos los estudios descritos anteriormente se llevaron a cabo con colirios de SA diluidos al 20%, ya que suelen utilizarse en la práctica clínica con el fin de reducir la concentración de TGF-β1 para evitar su efecto potencialmente nocivo (110, 122). Sin embargo, recientes grupos y estudios abogan por el uso de SA al 100%, aumentando la concentración de otros factores beneficiosos implicados en la cicatrización de heridas oculares como el factor de crecimiento epidérmico (EGF) o la fibronectina para conseguir mejores resultados clínicos (123, 124).

Además de los diversos ensayos preclínicos realizados con el colirio de PRGF, éste ha sido ampliamente utilizado para el tratamiento de patologías de la superficie ocular como defectos epiteliales corneales, ojo seco derivado de diferentes etiopatologías, queratitis neurotrófica y enfermedad de injerto contra huésped, entre otras, obteniendo resultados clínicos alentadores (125). Para garantizar la conservación de las propiedades biológicas del colirio de PRGF durante el periodo de tratamiento, se indica a los pacientes que guarden los envases del colirio de PRGF en uso a 4 °C o a temperatura ambiente, mientras que el resto de los dispensadores oftálmicos deben almacenarse a -20 °C hasta que se necesiten. Diversos trabajos han demostrado que el colirio de PRGF puede almacenarse hasta 6 meses sin que se reduzca la concentración de las principales proteínas y factores de crecimiento implicados en la regeneración de la superficie ocular (115, 126). Además, en un estudio preclínico llevado a cabo con células de la superficie ocular se demostró que el colirio de PRGF puede conservar su composición y su actividad biológica tras ser conservado tanto a 4°C como a temperatura ambiente durante 3 días (126).

Algunos trastornos oculares necesitan un periodo de tratamiento corto, de días a unas pocas semanas, para lograr una restauración completa del tejido dañado de la superficie ocular. Sin embargo, los síntomas de la patología crónica subyacente pueden aparecer en unos meses, por lo que es necesario aplicar una nueva terapia. Por otro lado, en algunos países como Alemania, las autoridades competentes pueden exigir la cuarentena de los

preparados de colirio hasta que los resultados de las pruebas de contaminación bacteriana estén disponibles, lo que podría llevar de 1 a 2 semanas (110). Además, algunos trastornos oculares son unilaterales, que afectan sólo a un ojo, en cuyo caso, el colirio de PRGF en uso puede durar más de los 3 días de tratamiento establecidos actualmente para su estabilidad a corto plazo almacenados a temperatura ambiente o a 4 °C. A este respecto, parece muy interesante evaluar si un almacenamiento a largo plazo a -20 °C podría reducir la eficacia del tratamiento de colirio de PRGF e investigar cuánto tiempo permanece estable dicho colirio almacenado a diferentes condiciones.

Por otro lado, aunque habitualmente se utilizan colirios autólogos para el tratamiento de trastornos oculares, algunos pacientes no son aptos para ser donantes debido a enfermedades inflamatorias sistémicas, a la edad y a otros tipos de trastornos o comorbilidades. La posibilidad de disponer de un producto alogénico derivado de la sangre podría ser una alternativa interesante para tratar varias enfermedades de la superficie ocular en este tipo de pacientes (127).

En ambos casos, tanto productos autólogos como alogénicos, la liofilización de colirios podría presentarse como una excelente alternativa para conseguir una mayor vida útil de estos productos, evitando la dependencia de la cadena de frío. El proceso de liofilización suele modificar las estructuras de las proteínas debido a la baja temperatura y a la mayor concentración de solutos debida al procedimiento de congelación (128). Asimismo, se suelen añadir al producto algunos excipientes, como la trehalosa, para proteger las proteínas del estrés que se genera durante el procesamiento y el almacenamiento. La eficacia de la trehalosa está relacionada con su capacidad de reemplazar algunas moléculas de agua, impidiendo la deshidratación incontrolada y promover la estabilización de las proteínas (129). Sin embargo, un estudio reciente demostró que los colirios de PRGF se pueden liofilizar manteniendo sus propiedades biológicas incluso sin el uso de lioprotectores, como la trehalosa (130), cuyo papel en la protección de la superficie ocular está bien demostrado (131).

Capítulo 2: OBJETIVOS

HIPÓTESIS

La tecnología autóloga PRGF ofrece diferentes formulaciones (coágulo, membrana, líquido inyectable) que se adaptan a las diferentes necesidades clínicas. Estas formulaciones están globalmente compuestas por la fibrina y por otro lado por biomoléculas y minerales presentes en el plasma y las plaquetas. Además, la tecnología PRGF puede usarse como alternativa al suero bovino fetal en el cultivo de células madre y como un colirio oftalmológico al separar la fibrina de los demás componentes con el objetivo de favorecer su almacenamiento y uso de forma repetida sin necesidad de volver a realizar extracciones de sangre.

Capítulo 2: OBJETIVOS

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1. Enmarcar los aspectos universales de la reparación tisular como un subproducto de los mecanismos subyacentes al sistema de coagulación y defensa y al plasma rico en factores de crecimiento. Lo haremos vinculando una perspectiva evolutiva y otra clínico-terapéutica, con énfasis en el sistema de coagulación, las plaquetas y la hemostasia.
2. Recopilar las evidencias actuales sobre el uso del PRP como alternativa a los productos xenogénicos, basados en sueros animales, y como suplemento de cultivo celular destinado a terapias celulares, con el fin de establecer criterios sobre las características óptimas del PRP para dicha aplicación.
3. Caracterizar y cuantificar la composición proteica del colirio obtenido mediante la tecnología del PRGF en comparación con el suero autólogo (SA). Así como determinar la expresión proteica de los queratocitos corneales tratados con PRGF o SA y analizar las vías de señalización que se encuentren desreguladas.
4. Analizar la estabilidad de los colirios de PRGF almacenados a -20 °C durante 9 y 12 meses, y a 4°C y a temperatura ambiente durante 3 y 7 días evaluando tanto su contenido en proteínas y factores de crecimiento, como su potencial biológico en células de la superficie ocular.
5. Analizar las condiciones óptimas de almacenamiento del colirio de PRGF liofilizado para preservar la composición y la actividad biológica tras su almacenamiento durante 3 meses a temperatura ambiente o a +4°C.

6. Analizar el efecto de la liofilización del colirio del PRGF en su composición proteica y efecto biológico mediante estudios de proteómica.

Capítulo 3: PUBLICACIONES ORIGINALES

3.1. ARTÍCULO I

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RESUMEN

La evolución, desde los invertebrados hasta los mamíferos, ha dado forma a la inmunocoagulación como respuesta de defensa y reparación contra los traumatismos y las infecciones. Este mosaico de mecanismos inmediatos y localizados de eliminación de patógenos y reparación de heridas tiene como resultado la supervivencia, restauración de la homeostasis y la reconstitución de los tejidos. En los mamíferos, la inmunotrombosis se ha complementado con el sistema neuroendocrino, las plaquetas y el sistema de contacto, entre otros componentes, añadiendo capas de complejidad mediante la interconexión de cascadas proteolíticas sanguíneas, células sanguíneas y el sistema neuroendocrino. De este modo, la inmunotrombosis dota a los seres humanos de ventajas de supervivencia, pero conlleva vulnerabilidades en el mundo moderno con cambios drásticos sin precedentes y cada vez más desafiante. La inmunotrombosis y la reparación tisular parecen ir de la mano, con mecanismos comunes que median en ambos procesos, lo cual queda de manifiesto por los recientes avances que están descifrando los mecanismos del proceso de reparación y de las vías bioquímicas que sustentan la coagulación, la hemostasia y la trombosis. Esta revisión pretende

contextualizar tanto los aspectos universales de la reparación tisular como el uso terapéutico de la matriz de fibrina autóloga como enfoque de la biología como estrategia terapéutica en el contexto de los cambios evolutivos de la coagulación y la hemostasia. Además, intentaremos aportar algo de luz sobre los mecanismos moleculares que subyacen al uso de la matriz de fibrina autóloga como terapia biomimética inspirada en la biología, adaptada por la evolución y diseñada por el ser humano.

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Healing through the lens of immunothrombosis: Biology-inspired, evolution-tailored, and human-engineered biomimetic therapies

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ABSTRACT

Evolution, from invertebrates to mammals, has yielded and shaped immunoclotting as a defense and repair response against trauma and infection. This mosaic of immediate and local wound-sealing and pathogen-killing mechanisms results in survival, restoration of homeostasis, and tissue repair. In mammals, immunoclotting has been complemented with the neuroendocrine system, platelets, and contact system among other embellishments, adding layers of complexity through interconnecting blood-born proteolytic cascades, blood cells, and the neuroendocrine system. In doing so, immunothrombosis endows humans with survival advantages, but entails vulnerabilities in the current unprecedented and increasingly challenging environment. Immunothrombosis and tissue repair appear to go hand in hand with common mechanisms mediating both processes, a fact that is underlined by recent advances that are deciphering the mechanisms of the repair process and of the biochemical pathways that underpins coagulation, hemostasis and thrombosis. This review is intended to frame both the universal aspects of tissue repair and the therapeutic use of autologous fibrin matrix as a biology-as-a-drug approach in the context of the evolutionary changes in coagulation and hemostasis. In addition, we will try to shed some light on the molecular mechanisms underlying the use of the autologous fibrin matrix as a biology-inspired, evolution-tailored, and human-engineered biomimetic therapy.

1. Introduction

Every living being, from single-celled organisms to complex multicellular and multi-system animals including vertebrates, possesses a variety of physical and chemical barriers, and a vast repertoire of physiological responses in order to secure integrity, survival, and homeostasis, processes often grouped as the immune or host defense system [1–3]. Invertebrates and vertebrates, including mammals, have evolved a cluster of immediate and local responses known as immunoclotting and immunothrombosis respectively that function in a wound-sealing, microbial-killing and -clearing, and homeostasis-restoring manner resulting in tissue repair [4–9]. Pivotal players of immunothrombosis are blood circulating and tissue stationary and mobile resident cells, and the intravascular innate immune system [8,10,11]. The plasma proteins of the humoral intravascular innate immune cascade systems such as prothrombin-thrombin [12–14], fibrin (ogen) [15,16], FXII [17–20] and complement (C3, C5, and their active fragments C3a, C5a anaphylatoxins) [21–23] together with the payload released by activated platelets play a major role in tissue repair and remodelling [12,16,22,24,25]. Pathological changes in the interplay between the intravascular serine protease cascade systems and circulating blood cells as well as vascular and tissue cells, however, are involved in the pathogenesis of several systemic conditions such as sepsis, hemorrhagic shock, systemic inflammation, liver fibrosis, or rheumatic syndromes [8,11,26–28]. Recent work deciphering the roles of blood cells and of the biochemical intravascular innate immune machinery, specifically coagulation and hemostasis, has led to filter out platelets and coagulation proteins that create an autologous fibrin matrix (AFM). These blood-derived products have a healing potential as a local biology-as-a-drug approach, to repair musculoskeletal tissues, skin and corneal ulcers and burns with many other uses [11,29–31].

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Abbreviations

AFM	Autologous fibrin matrix
APC	Activated Protein C
BK	Bradykinin
CNS	Central nervous system
CP	Classical pathway
CRP	C-reactive protein
CS	Contact system
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DCO	Damage control orthopaedics
DC-SGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
ECs	Endothelial cells
ECM	Extracellular matrix
EPCR	Endothelial protein C receptor
FGF	Fibroblast growth factor
FXII	Factor XII
GAG	Glycosaminoglycans
GFs	Growth factors
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HMWK	High-molecular-weight kininogen
HPA	Hypothalamic-pituitary-adrenal
HUVEC	Human umbilical vein ECs
IGF-1	Insulin-like growth factor 1
IL	Interleukin
KKS	Kallikrein-kinin system
LP	Lectin pathway
LPS	Lipopolysaccharide
LXA4	Lipoxin A4
MAMPs	Microbe-associated molecular patterns
MASP-1	Mannose-associated serine protease 1
MBL	Mannose-binding lectin
MEP cell	Megakaryocyte/erythrocyte progenitor cell
MIF	macrophage migration inhibitory factor
MK	Megakaryocytes
MMP-2	Matrix metalloproteinase-2
MODS	Multiple organ dysfunction syndrome
Mya	Million years ago
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nod-like receptors
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin I_2
Piezo2	Piezo ion channel 2
PK	Prekallikrein
PMP	Platelet microparticles
PPO	Prophenoloxidase system
PRP	Platelet-rich plasma
PRRs	Pattern recognition receptors
RAGE	Receptors for advanced glycation end products
ROS	Reactive oxygen species
SASP	Senescence-associated secretory program
SDF-1	Stromal cell-derived factor 1
SIRS	Systemic inflammatory response syndrome
SMA	Smooth muscle actin
SMAD2	Mothers against decapentaplegic homolog 2
SPMs	Specialized pro-resolving mediators
STAT3	Signal transducer and activator of transcription 3
TFPI	Tissue factor pathway inhibitor
TFs	Transcription factors
TGF-β	Transforming growth factor beta
Th2	T helper 2 cells
TLR	Toll-like receptor
TM	Thrombomodulin
TRP	Transient receptor potential channels
VEGF	Vascular endothelial growth factor
VVEC	Vasa vasorum ECs
VWF	von Willebrand factor

This review is intended to frame both the universal aspects of tissue repair as a byproduct of the mechanisms underlying the host defense system and AFM. In addition, we will try to shed some light on the biological paradox of host defense mechanisms (immunothrombosis) as local tissue repair enhancer on the one hand while generating systemic pathogenic processes on the other. We will do so by linking an evolutionary and a clinical-therapeutic perspective, with an emphasis on the coagulation system, platelets and hemostasis.

2. Evolutionary perspective of intravascular innate immune cascade systems

Mammalian intravascular innate immune cascade systems (complement, coagulation, contact, and fibrinolysis systems) were not generated from scratch nor were they assembled all at once but, like mammalian cell types and their biochemical pathways, they derived from simpler versions originating in non-mammalian vertebrates and invertebrates [32–35]. At the molecular and cellular level, they evolved from precursor genes, proteins, and cell types that originally served other functions [32–34,36–38].

2.1. Innate immunity and coagulation: partners throughout evolution

To cope with trauma- and infection-induced disruption of physicochemical and anatomical barriers, invertebrates evolved a vast repertoire of immediate and local cell-and/or humoral-based responses, with dual roles in immunity and clotting. These responses consist of wound-sealing and pathogen-killing, resulting in restoration of homeostasis and wound healing [6,10,39–42]. Thus, in marine invertebrates like sea urchins, with low protein content in their plasma, the only type of cell present in the coelomic fluid, the coelomocyte, aggregates upon injury or in the presence of foreign substances to form a cellular clot mediated by amassin, a plasma protein whose multimers attach the coelomocytes to each other and ending up by sealing the wound [4]. However, in more highly evolved invertebrates like the american horseshoe crab (*Limulus polyphemus*), clotting defense responses involve cell and humoral components and are initiated by the hemocyte (also known as amoebocyte) that detects non-self molecules (PAMPs) such as lipopolysaccharide (LPS) through pattern recognition receptors (PRRs), leading to hemocyte activation [4–6,9]. This activation triggers hemocyte aggregation, hemocyte antibacterial- and hemolymph clotting-factor release, including coagulase and coagulogen, the latter rapidly transformed into the gel coagulin, and the activation of the primordial complement system [4,6,9,43]. These responses are the basis of hemolymph clotting, clearance-killing of pathogens and foreign bodies, and wound healing with dual defense and repair roles of immunoclotting [4,6,9,44]. In insect, immunoclotting evolved as a locally operative mechanism, even generating microclots to entrap bacteria, with a very low risk for

thrombosis due to the open circulatory system of invertebrates [6,43,45], but also as an integral part of the healing-regeneration process [6,46]. Mammals instead, have coupled inflammation and coagulation through a combination of cooperative host defense components and strategies, whose pivotal players are circulating blood cells (polymorphonuclear cells, monocytes, lymphocytes and platelets), tissue stationary and mobile resident cells (dendritic and mast cells, nociceptors, endothelial cells, macrophages and fibroblasts), and intravascular innate immune cascade systems (complement, coagulation, contact, and fibrinolysis systems) [3,8,10,11].

In the evolutionary journey of the vertebrate coagulation and hemostasis system (Fig. 1), the first vertebrate clotting component to emerge was a thrombin-like protein. The thrombin precursor diverged from the preexisting complement and mannan-binding protein associated serine proteases (C1r, C1s, and MASP-1, MASP-2, MASP-3) already present in deuterostomes 710–780 million years ago (Mya), that were already operating as a primordial complement system (C3, Bf, and MASP genes) [47–51]. These trypsin-like serine proteases in turn had derived by gene duplication, exon shuffling, and simple mutation, from the chymotrypsin-like serine protease domains with trypsin as the origin gene [34,51], leading to amino acid replacement [35], similar to the origin of thrombin and other vitamin K- dependent serine protease clotting factors (FVII, FIX, and FX) later on [7,33–35,52].

The aforementioned variations endowed the complement, coagulation, and fibrinolysis systems with complex modular allosteric serine proteases, sharing some domains and thereby generating intrinsic chemical affinities with one another or with other protein substrates or cofactors, being especially useful in creating local, cooperative, molecular networks as infection and injury recognition molecules [7,11,34,35,64]. The evolved genes and encoded proteins conferred to their bearers advantages for host survival, but also generated systemic vulnerabilities, as we will see in section 4 [3,11,28,35]. Accordingly, the allosteric and catalytic serine protease thrombin plays multiple roles as regulator of inflammation and in tissue repair, and is an example of pleiotropism that directly cross-links immunity and coagulation with the repair process as we will describe further in section 3.1 [13,39,65].

The second and essential substrate of vertebrate blood clots, fibrinogen, is not present in invertebrates [35], although they carried the raw material in the form of fibrinogen-like loci encoding fibrinogen-domain-containing proteins with no coagulation but rather an immune-parasite defense function [66]; an example is the urochordate sea squirt, that occupies a critical intermediary position between invertebrates and vertebrates (Fig. 1), and possesses three genes that encode a protofibrinogen with almost all the features of vertebrate fibrinogen [35]. In any event, the prototype of the coagulation cascade derived from these molecules in urochordates would not be effective in hemostasis but rather an immune enhancer with hemolymph cell agglutination as a cell-based wall-off mechanism simultaneously acting as a prophylactic mechanism in trapping soluble parasite-derived molecules, and in killing bacteria [35,67,68].

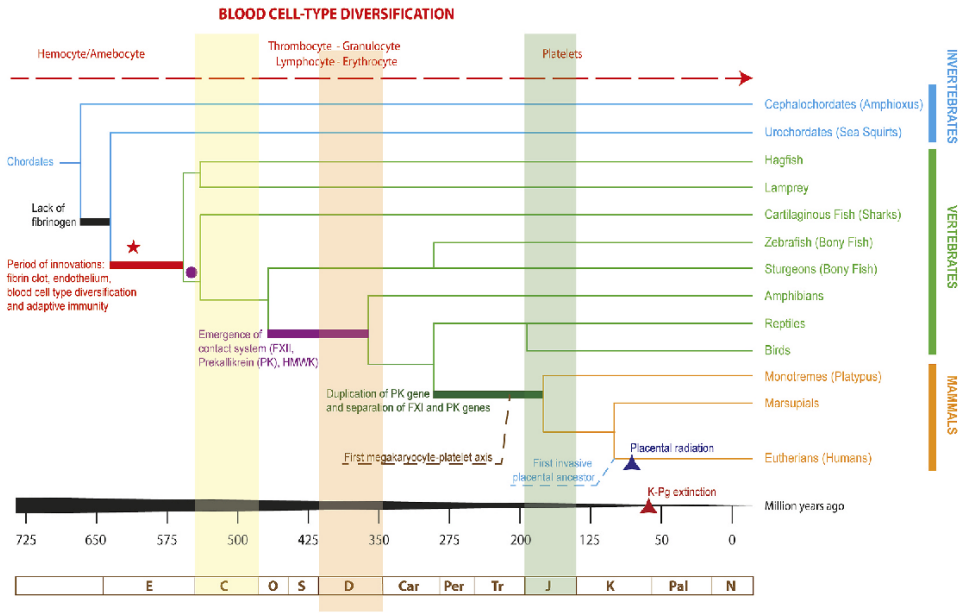

Fig. 1. An overview of some of the main events in the evolution of the vertebrate blood coagulation system. Phylogeny and the approximate times of evolution (as determined by fossil records and molecular clock framework analysis) of various chordates, highlighting significant evolutionary events and innovations in the emergence of the human coagulation and hemostatic systems. The red star and purple dot represent the points in evolution where the first and second whole-genome duplication events have been proposed to occur. The red bold line represents a period of innovations. This period spans the time that followed the divergence of urochordates and the lineage giving rise to the earliest jawless vertebrate (hagfish and lampreys, more than 500 Mya), which are marine animals that possess a simple coagulation version involving prothrombin, tissue factor, fibrinogen, FVII and X though they lack genes for coagulation factors VIII, and IX, and the entire contact system [51,53]. This very period of time of great inventions witnessed the emergence of a true endothelium, the blood cell-type diversification, and the adaptive immune system, and broadly coincides with the onset of Cambrian radiation (540 Mya) [54]. The purple bold line indicates the emergence of the contact system (FXII, HMWK, and PK), that is associated, somewhere along the Devonian period around 400-390 Mya, with the water-to-land transition of vertebrates, and with the appearance of first amphibians, and then reptiles [55]. The origin of the MK-platelet axis remains to be determined but has been suggested to occur in the 200-160 Mya window [56,57]. The blue triangle represents the time in evolution that includes the placental radiation of mammals, including rodentia and primates has been hypothesized to occur [58,59]. The red triangle represents the Cretaceous-Paleogene (K-Pg) mass extinction event, 66 Mya, where among the existing mammals only borrowing small animals survived (E Ediacaran, C cambrian, O ordovician, S silurian, D devonian, Car carboniferous, Per permian, Tr triassic, J jurassic, K cretaceous, Pal paleogene, N neogene) [35,55,56,58–62]. Adapted from Ref. [63] with permission. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Notwithstanding the superficial resemblances and functional common trends between invertebrate hemocyte aggregation, hemocyte clotting factor release, and hemolymph jelling on the one hand, and mammalian platelet aggregation and fibrin clotting on the other, there is little similarity between the primary structure of proteins involved in the invertebrate coagulation reactions (amassin, coagulogen, vitellogenin), and proteins necessary for the thrombin-catalyzed conversion of fibrinogen to fibrin in vertebrates (Prothrombin, fibrinogen, FVII); the similarities being an example of convergence in function [6,35,44,57,69]. Analysis of gene organization, the toolbox of protein domains, molecular cloning data, and comparative sequencing, supports the concept that the simplest version (genes and proteins for generating the thrombin-catalyzed conversion of fibrinogen to fibrin) of the complex vertebrate blood coagulation network evolved independently and was assembled more than 500 Mya, over a period of a 50–100 million year window (Fig. 1) [35,54,70,71]. Nevertheless, genetic variations (in the form of gene duplication, exon shuffling, or simple mutation) predate protein novelties and depend on environmental changes to be turned into biological innovations [57]. The relatively short window of time that followed the divergence of protochordates (cephalochordates and urochordates) and the lineage giving rise to earliest vertebrates known as jawless fish (agnathans similar to today's hagfish and lampreys) [35,36,53,70,71] also witnessed the emergence of a closed and increasingly pressurized circulatory system, blood cell specialization, the endothelium, and the adaptive immune system (Fig. 1) [35,54,70]. From the simplest version of the jawless coagulation system with tissue factor, prothrombin and fibrinogen as the starting point, to the sophisticated and embellished mammal coagulation system, the clotting machinery evolved in distinct stages [35], from downstream onwards by integrating enzymes upstream [72]. This is the case of the emergence and integration of the inflammatory arm of the contact system (CS) into the older tissue factor-triggered extrinsic coagulation pathway [35,52,73] (Fig. 1). Made up of serine proteases factor XII (FXII) and prekallikrein (PK), and the multidomain glycoprotein high-molecular-weight kininogen (HMWK), the kallikrein-kinin system (KKS) initially evolved as an inflammatory pathway by domain acquisition, chromosomal segment duplication, and exon shuffling parallel to the still independent of fibrin clot formation cascade [52,74]. Although present in all vertebrates, the non-protease HMWK expanded from lampreys to humans by domain acquisition and shuffling [35,52] as confirmed by a recent research that identified the presence of the PK gene (klkb1) and a simple version of the HMWK gene (kng1) in the coelacanth and lungfish, two lobe-finned fish ancestral to tetrapods [52,74]. On the other hand, the serine protease FXII, arose through duplication of a hepatocyte growth factor activator (HGFA) containing chromosomal segment [73–75]. The FXII gene (f12) is present in lungfish, amphibians, reptiles, and mammals but not cartilaginous or ray-finned fish, whereas the FXI gene (f11) is present in platypus and opossum, but not in amphibians, reptiles, or birds [74] somehow suggesting that the duplication of the PK gene (klkb1) giving rise to FXI gene (f11) occurred late in vertebrate evolution in a proto-mammalian ancestor (Fig. 1) [74]. Importantly, new research reveals that kallikrein (PK) may directly activate FIX [74,76] as does its parent molecule FXI, resulting in thrombin generation and fibrin formation independently of FXII and FXI [77] which suggest that the merger of inflammatory and hemostatic arms of CS contact/kallikrein system with the fibrin formation pathway might have occurred with the emergence of tetrapods [76,77] in the water-to-land transition of vertebrates around 400-390 Mya [35,52,55,74,75]. Overall, these new data confirm that the chain of genetic events necessary to lead to biological innovations with change in function predate and never come about during the great transitions that change in function are associated with (see Fig. 1 and section 3.2) [57].

2.2. Intravascular innate immune cascades, endothelium, and the circulatory system: a solution to multi-systemic organisms

Multicellularity and the progressive yet transitional spatially organized and regulated division of labor through cell type diversification involved segregation and divergence of function in the sister cell types [78–80]. These processes allowed primitive metazoans to perform different cellular functions at the same time, a significant evolutionary leap in sharp contrast with protozoans in which just one cell performed all of the physiological functions [1,2,78] yet with a complex life cycle with multiple temporarily regulated cell states [78]. Multicellularity however inexorably led animals to grow larger body size, and size matters. Both the earliest single-celled animals and today's 100-metric ton blue whale ultimately depend on diffusion to supply oxygen and nutrients to, and remove carbon dioxide and metabolic wastes from each cell in the body [54,81]. Moreover, growing bigger increased metabolic rates and generated large diffusion distances compromising the aforementioned functions (diffusion of oxygen, supply of nutrients, and elimination of metabolic wastes) [54,81]. In this evolutionary landscape of animals, cardiocirculatory systems have evolved to provide every cell in the body with a bulk flow delivery of gases and nutrients, and elimination of wastes to meet the metabolic requirements [54,81]. In this context, the proteolytic serine protease cascades underlying the immunoclotting defense system, which in invertebrates such as arthropods operates locally and immediately [6], faced new challenges imposed by the greater sophistication and the increasingly large bodies of many invertebrates in the Ediacaran (E 600 Mya), and in the Cambrian diversification (C 543 Mya) leading to emergence of vertebrates (Fig. 1) [82]. Initially, in lower vertebrates, the novel endothelial cells that lined an emerging closed and increasingly pressurized circulatory system together with thrombocytes were keeping the already irreplaceable commodity containing the precious red cells, namely the blood, in a fluid phase [35,54]. But this was not enough: the newly generated vascular and interstitial space had to be free from noxious biotic and abiotic particles [32,83], a challenge that in primitive vertebrates prompted blood cell type diversification, including the emergence not only of thrombocytes but of neutrophils, lymphocytes and other blood cells [84–86]. But by then, the diversification of soluble blood proteins of the complement and coagulation system was already underway and increasingly complex [32,34]. To understand this complexity, consider that humans possess approximately 100,000 km of vessels lined with 1.3 trillion of endothelial cells, covering a surface area of 4000 m^2–7000 m^2 [45,87,88], and the elbow to elbow time for an individual red blood cell has been estimated to be less than 30 s [35,83].

The challenge of integrating immunothrombosis can be interpreted as follows. Firstly, by conferring a significant survival advantage to the bearers, selection pressure conserved the local, immediate, and vigorous immunoclotting response that involved endothelial cells, thrombocytes, and negatively charged phospholipid membrane-mediated and Ca^{+2}-dependent activation of blood-borne proteolytic cascades [7,28,35,89]. In this way, proteolytic cascades maintained a compartmentalized defense response simultaneously limiting blood loss and avoiding both the replication and access of pathogens within a closed, endothelial cell-lined circulatory system [28,83,90]. Today, this is also so for lingering focal infections and/or the myriad breaches inflicted on vertebrate skin and organs such as lungs and gut by parasites, where immunothrombosis, aids both in killing intruders and in repairing the breaches, thereby preventing the infection from being systemic [7,40].

Secondly, the newly generated vascular and interstitial space had to be kept free from noxious biotic and abiotic particles as an additional survival requirement. This need likely exerted a strong selective pressure on the complement and coagulation systems as well as on blood cell type diversification, establishing an intense crosstalk among them [32,83]. Accordingly, the multi-arm mammalian complement system evolved in several steps through more than 1200 million years [32,47,48,50,91]. Starting as an intracellular C3-like multi-task protein with

metabolic, cell survival, and opsonic immune functions in single-celled organisms such as choanoflagellates (the precursors of sponges), the complement system of vertebrates evolved as a multicomponent network of foreign and altered host cell detectors, mediators, and effectors from trypsin as the precursor gene [32,34,38,51,91]. In doing so, the complement system, as well as other blood cascade systems, took on a novel role of guardian of the intravascular homeostasis by protecting and keeping the intracellular, interstitial, and intravascular space free from biotic, abiotic, and altered and non-self cells [32,47,48,50] thereby operating as a purging system [7,32,35,83,91].

Similar to blood cascade systems, blood cell types of mammals evolved from less specialized precursor cell types that originally were multifunctional, performing several steps of an ancient innate immune response as well as serving other digestive, metabolic, and homeostatic roles [9,79,84,92]. Despite this understanding, the overall picture and the precise evolutionary history of the astounding emergence and diversification of vertebrate blood cell types remain unresolved. Endothelial cells, erythrocytes, thrombocytes, granulocytes and monocytes, and lymphocytes all emerged following the divergence of protochordates and the appearance of the earliest vertebrates (period of innovations, see Fig. 1) [9,35,54,71,93–95]. An interesting evolution/developmental hypothesis in biology that articulates phylogenetic and ontogenetic approaches suggests that all the cell lineages that make up the cell phenotypes of the vertebral vascular system including endothelial cells, pericytes, and blood circulating cells, share a common phylogenetic and ontogenic ancestor. This could be the case of a free floating coelomic cell originated from a coelomic wall of the invertebrate hemal system, termed the hemocyte [94,96,97]. Significantly, endothelial cell heterogeneity has been conserved from the most basal vertebrate (Hagfish) to mammals [54], complementing the conserved adherent, migratory and secretory functions of their invertebrate coelomic amoebocyte ancestor with the new epithelial phenotype of vertebrate endothelial cells [54,70,94,97]. Accordingly, these functions of the mammalian endothelial cell as exploratory and migratory cells are at the core of angiogenesis and repair function. For example, the mobilization of bone marrow endothelial progenitor cells to the circulation in response to vascular endothelial growth factor (VEGF), and their accumulation within the damaged tissue [94,97]. Moreover, under physiological conditions, mammalian endothelial cell secretory function contributes to the blood fluidity by promoting anticoagulant properties and counteracting platelet activation. They do so by expressing a large range of proteins and metabolites including but not limited to thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), prostaglandin E_2 (PGE_2) and prostacyclin I_2 (PGI_2), and nitric oxide (NO), all acting as brakes. In this way they provide the luminal surface of the endothelium with anticoagulant antithrombotic, and anti-inflammatory properties. Noteworthy is their absence from artificial surfaces of medical devices in contact with blood [87,98–100].

Similar to the origin of endothelial cells, much of our knowledge about the cell type diversification of the myeloid lineage remains poorly understood. Sponges, one of the most basal multicellular metazoans, possess cells termed archaeocytes that bear structural and functional similarities with blood stem cells in vertebrates, and which serve as a stem cells that generate other cell types (gametes, sclerocytes) [84]. Similarly, interstitial cells in the phyla cnidarians or the neoblasts in platyhelminths (flatworms) perform stem cell functions [84]. On the other hand, molluscs and arthropods possess a hemolymph fluid with a variety of hemocytes (plasmatocytes or monocytes, granulocytes, and eleocytes) whose precursor is the immature hemolymph prohemocyte with multipotent capabilities, although in terrestrial arthropods hemocytes are interconvertible, unlike blood cells of vertebrates [85]. Invertebrate haemocytes store metabolic waste products, distribute nutrients, encapsulate and phagocytose eggs from parasites, and some contain densely packed granules similar to vertebrate neutrophils, eosinophils and basophils [9,84]. While plasmatocytes can best be compared with vertebrate monocyte/macrophages, coagulocytes may represent a specialized type of plasmatocyte-bearing granules that store hemolymph clotting and antibacterial factors similarly to vertebrate thrombocytes and mammalian platelets [6,9,84]. In addition, invertebrate granulocytes share some of their cargo with vertebrate neutrophils, eosinophils and basophils, with granules packed with enzyme-filled lysosomes [6,9,84]. These examples of circulating cell phenotypic diversity at the beginning of metazoan evolution reflect that the ancestral myeloid cells had already a high degree of phenotypic complexity, which was partially underpinned on a specific and independent genetic core regulatory complex that enabled and maintained the distinct gene expression program of a cell type within the organism [80]. Therefore, blood cell type diversification and specialization in vertebrates and mammals did not necessarily add complexity to an individual cell type but rather, and through segregation and divergence of primary and accessory or supportive functions, led to the emergence of many specialized cell types by partitioning and modifying the already existing functions of ancestral cell type into the new sister cell types [79,80,92]. Examples supporting this paradigm may be the professional macrophages [92], the red blood cells [95], or even the megakaryocytes (MKs) [101] of mammals where vertebrate thrombocytes are represented as anucleated platelets in mammals [101], and the nucleated erythrocytes of vertebrates undergo deletion of nucleus and organelles. Moreover, and indirectly supporting the hypothesis of the coelomic hemocyte as the common cell type ancestor of blood cells, erythrocytes are emerging as modulators of innate immunity in birds, amphibians, fishes, and mammals [95]. Their CD35 membrane receptor binds opsonized particles and transports them to liver and spleen where carried particles are removed [83]. At this point we must emphasize however that the developmental and evolutionary lineage of cell types are not necessarily the same, which could be the case of erythrocytes and megakaryocytes [101].

2.3. *The multicompetent platelet: the last newcomer in the immune continuum of mammals?*

Another layer of complexity in this evolutionary journey of coagulation and hemostasis (Fig. 1) in the context of the closed circulatory system of mammals is the emergence of platelets, as only mammals possess a megakaryocyte/platelet axis [9]. Despite remarkable phenotypic similarities including bactericidal, phagocytic, migratory, chemotactic, and hemostatic functions between invertebrate hemocytes, non-mammalian vertebrate nucleated thrombocyte and mammalian platelets [7,9], cell type homology is essentially only to be drawn between hemocytes, thrombocytes, and megakaryocytes [101] as only nucleated cells are considered units of evolution [9,80]. The blood cell type with primary hemostatic function in vertebrates is the thrombocyte that in non-mammalian vertebrates is nucleated, as is the erythrocyte, both derived from a thrombocyte-erythroid progenitor and sharing features of a nucleated, diploid oval-shaped phenotype [101]. Similarly, the mammalian unique enucleated erythrocytes and the polyploid megakaryocytes from which derive the cell fragments termed platelets, are generated from a common bipotent myeloid megakaryocyte/erythrocyte progenitor (MEP) cell [101,102].

Recent studies suggest that selective forces might have favored a more rapid and robust local immunothrombosis over systemic thrombosis risk in mammals. These consist of many survival advantages and trade-offs among defense mechanisms and hemodynamic consequences of the coagulation system and hemostasis [7,14,37,103,104]. A feature is the hemodynamic advantage of platelets that stems from their small size, which endows them with biophysically optimized features to act as the first of the circulating cells following vascular injury or pathogen aggression [105,106]. Given the diameter of capillaries, avian thrombocytes, and mammalian platelets (10–50 μm, 6–7 μm, and 1–3 μm respectively), platelets are optimized to fulfill their function under shear in flowing blood, aggregating to provide thrombus formation and then

promoting fibrin formation leading to stabilization of the initial hemostatic plug. The generation of resistant arterial plugs optimizes hemostasis in the high-pressure, high-flow conditions of mammalian arterial systems [16,103]. Furthermore, anucleated platelets possess a high surface-to-volume ratio, and a membrane with, for example, the αIIbβ3 integrin present at 50000–80000 copies per platelet. This feature renders them highly reactive, a key property to survey and repair any endothelial breach [107], which exposes collagen from the subendothelial matrix and binds to circulating von Willebrand factor (VWF) [87]. Once platelets sense endothelial damage they initially adhere through platelet surface GPIbα receptors to the A1 domain of VWF glycoprotein, which through the exposed A3 domain has already bound to the exposed subendothelial collagen I and III fibers [37]. Transient attachment is stabilized through α2β1 and GPVI [37,108,109] with platelet activation leading to platelet aggregation with principally fibrinogen, but also fibronectin, vitronectin, VWF and thrombospondin participating in the formation of platelet to platelet bridges [110]. Importantly, platelets activated by the tissue factor-induced thrombin offer their membrane surface as a gathering point for the zymogen activations involved in local generation of more thrombin, in the generation of fibrin and in controlling inflammation [35,111].

Significantly, in a quiescent manner, approximately 750 billion platelets patrol with manoeuvrability, massively occupying the external border near the vessel wall in flowing blood, a phenomenon known as margination [105,112,113]. In doing so, they survey and perform reparative labor on the vasculature, thereby acting as the first responders to endothelial disruption of multiple origins [105,106]. Roughly 100 billion new platelets (10% of the platelet count) released daily by megakaryocytes are used up in this maintenance work that, together with more than twenty plasma proteins that constitute the basis of the coagulation system and more than fifty plasma soluble and cell receptor proteins of the complement system, and endothelial-expressed and secreted thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), prostacyclin (PGI_2), and nitric oxide (NO), keep the intravascular space and blood both sterile and the later in a fluid state [11,87,114,115].

Platelets, besides the release of platelet microbicidal peptides (thrombocidins and kinocidins) [116,117] and through toll-like receptor (TLR) 2 and 4, receptor for advanced glycation end products (RAGE), and dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SGN), also sense and recognize damage-associated molecular patterns (DAMPs) and PAMPs, thereby contributing to tagging and eliminating pathogens [104–106,118].

Furthermore, in the case of injuries and pathogen invasion [9,102,119], there may be an explosive fragmentation of MK generating thousands of platelets on demand [14,101,102]. Thrombin-mediated activation of IL-1α feeds back to hemostasis by adding an extra boost of platelets but also supports local immunothrombosis and wound healing, a fact that again links immunothrombosis and healing [14,119]. In fact platelets can be considered as an innate immune and repair effector of MKs as secretory cells [102,111]. Finally, Martin and Wagner [120] suggested another significant biological advantage stemming from a compartmentalized, robust megakaryocyte/platelet-mediated hemostatic response, namely, to facilitate embryo implantation by allowing an invasive placentation and to avoid mortal hemorrhage during childbirth of eutherian mammals (Fig. 1). However, the origin of the megakaryocyte/platelet axis has been suggested to have occurred around 166 Mya [9,56], a date that goes further back compared to the appearance of eutherian mammals roughly 100 Mya [58,120] (Fig. 1). Moreover, comparisons of the hemostatic systems of non-placental (egg-laying monotremes, and marsupials) and placental mammals, do not show noticeable differences in megakaryocytes and platelets [9]. But these facts do not preclude Martin and Wagner's [120] suggested selective advantage (Fig. 1).

Overall, the series of events that eventually gave rise to the emergence of the megakaryocyte/platelet axis remain a puzzle [9] as is the polyploid feature of megakaryocytes. Some authors point to megakaryocyte's enormous genome as a means to support such massive synthetic activity that leads to a daily release of 100 billion new released platelets that carry a rich repertoire of messenger RNAs, microRNAs and even transcription factors. These endow platelets with a translational activity, and a copious amount of growth factors, adhesion molecules, and cytokines that contribute to their multifaceted roles in immunothrombosis and tissue repair [111,113,121,122].

3. Defense and repair: two sides of the same coin

Defense and repair functions are evolutionarily linked to the invertebrate and vertebrate responses to environmental challenges, and grouped as immunoclotting [3,9]. This response consists of both immediate and local wound-sealing and pathogen-killing mechanisms to restore homeostasis and effect tissue repair (Fig. 2) [3,9,35,69]. Despite

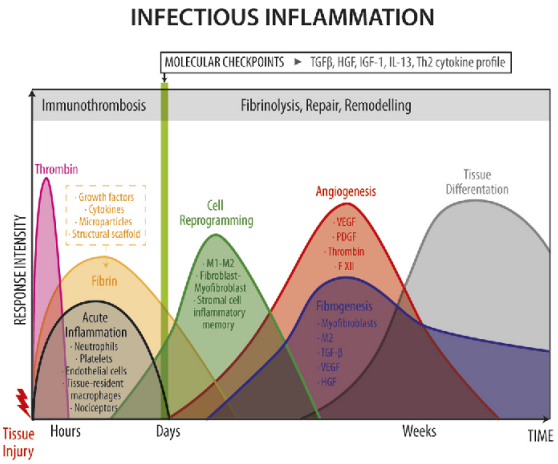

Fig. 2. Immunothrombosis: a mosaic of defense mechanisms assembled, selected, and evolved to act locally. Immunothrombosis is a highly conserved local and multitask cluster of defense responses made up of hemostasis and coagulation, the sensory nervous and innate immune systems, and fibrogenesis, whose key cell players are circulating blood cells (polymorphonuclear cells, monocytes, and platelets), and tissue stationary and mobile resident cells (nociceptor neurons, endothelial cells, macrophages, and fibroblasts) [8,10,11,27,28,39,89,90]. Tissue injury- and infection-derived DAMPs and PAMPs activate blood circulating cells, tissue stationary and mobile resident cells, and intravascular innate immune cascade systems [8,10,11]. Tissue factor expressed on damaged tissue, and activated FXIIa trigger the generation of thrombin which leads to stopping bleeding through the fibrin clot and platelet plug, and together with the activation of neutrophils, immunothrombosis will result in the containment, destruction, and expulsion of pathogens including metazoan parasites, as well as abiotic molecules [8,41,106]. After this early hemostatic-inflammatory process curtails bleeding and pathogen invasion, molecular checkpoints stemmed from local necrotic, hypoxic and acidic microenvironment including cytokines such as IL-6 and IL-13, and transcription and growth factors from platelets, nociceptors, leukocytes, and macrophages (TGF-β, PDGF, VEGF, IGF-1, specialized pro-resolving mediators such as LXA_4 and maresins) [3,7,27,126–128] will shut down inflammation, switching the process from killing and destroying mode to healing mode through cell reprogramming, angiogenesis, and fibrogenesis. Examples of the induced transient repair cell phenotypes through adaptive cellular reprogramming of adult differentiated cells [92,127,129,130] are fibroblast/myofibroblast differentiation, myelin Schwann cell/repair Bungner cell transdifferentiation, and macrophage M1, M2, M3, M4 and neutrophil polarization [92,127,129–132]. In addition, the proliferation of endothelial cells together with fibroblast/myofibroblast differentiation will lead to angiogenesis and fibrogenesis respectively [98,129,133–135], two key processes in tissue healing. Adapted from Ref. [136] with permission.

the highly conserved function of immunoclotting, however, the molecular and cellular components underlying this multistaged process differ significantly among diverse phyla [9,35,43,123–125].

3.1. Tissue repair process: past and priorities with precision but not perfection

In arthropods like butterflies, clot formation initiates with coagulocyte aggregation and degranulation, and activation of the prophenoloxidase system (PPO); the result is a hard clot that will attract plasmocytes late in clot formation. These events enable epidermal regeneration that grows across the wound and replaces the scab, and whose final outcome depends on the presence-absence of microbial invaders and the nature of foreign antigens [6].

Similarly, in insects like drosophila, wound healing relies mainly on plasma proteins such as fondue and hemocyte-released hemolectin, the latter a VWF domain-bearing protein that promotes hemolymph coagulation serving the formed clot as a defense against nematodes as well as a scaffold for the repair process [43,46]. In deers instead, the regeneration of antlers, a muscle and joint-free bony cranial appendage of approximately ten kg is completed in 55–60 days (up to 28 rounds in a life cycle) [137]. The process starts with bleeding on the cast plane of a pedicle stump immediately after casting of the antler, followed by a significant proliferation and differentiation of a single stem cell coming from the pedicle periostium, a process similar to human bone fracture healing [137]. Besides playing a central role in blood coagulation and NO release from ECs, thrombin and proteolytically inactive thrombin-derived peptides exhibit at low concentration anti-inflammatory, antiapoptotic, and growth factor-like activity by stimulating fibroblasts and endothelial cells similarly to activated Protein C (APC), and thrombin itself appears to be necessary for limb, heart, and lens regeneration in the salamander [12,65]. Moreover, thrombin activates the HGFA-HGF-MET signaling pathway [138], platelets through the cleavage and activation of the receptors PAR-1 and PAR-4 [109], and the proIL-1α, the latter favoring thrombopoiesis and wound repair after acute platelet loss and injury [14].

But mammals have evolved as well a Th2 response to deal with lingering focal infections and/or the myriad breaches inflicted by parasites on the epithelial layer of organs such as skin, lung and gut [41,133,134]. This is a local and immediate response which overlaps with the immunothrombotic response. Immunothrombosis in physiological conditions occurs only on demand and is dictated by microenvironmental cues coming from tissue injured. These include hypoxia, acidic pH, necrotic cell- and microbial-derived molecular signals (DAMPs, PAMPs), cytokines such as IL-6, and transcription (TFs) and growth factors (GFs) [3,7,27,127]. Furthermore, immunothrombosis shares several molecules with the senescence-associated secretory program (SASP) such as transforming growth factor beta (TGF-β), IL-6, VEGF, and Insulin-like growth factor 1 (IGF-1) [139,140]. Also to be considered are tissue injury-derived biochemical signals [127,141] such as subendothelial collagen, VWF deposited on collagen, and tissue factor that combine to activate the coagulation and hemostasis as well as the complement system [11,29,41,89]. In addition, other stimuli including but not limited to TGF-β, VEGF, NFκB, STAT3, SMAD2, will induce transient repair cell phenotypes through adaptive cellular reprogramming of adult differentiated cells [92,127,129,130,140] (Fig. 2). Some significant examples of this cell plasticity are fibroblast/myofibroblast differentiation, myelin Schwann cell/repair Bungner cell transdifferentiation, and macrophage M1, M2, M3, M4 and neutrophil polarization [92,127,129–132]. Both immunothrombosis and cell reprogramming also appear to be crucial to culminate in the tissue repair process [127,130,132,140,141] (Fig. 2). The early hemostatic-inflammatory period not only stops bleeding through the fibrin clot and platelet plug but also mediates the containment, destruction, and expulsion of pathogens and metazoan parasites, through Th2 lymphocytes. These cells will release IL-13, a cytokine that polarizes macrophages toward a profibrotic and reparative M2 phenotype thereby bridging the gap between sealing-killing and healing [41,133,134] (Fig. 2). In the absence of pathogen, as under sterile inflammatory conditions (severe trauma, ischemia, ischemia-reperfusion, intravascular artificial medical devices), activated intravascular serine protease cascades, platelets and endothelial cells together with polarized M2 macrophages release TGF-β, PDGF, VEGF, LXA4, IL-6, IL-13 and IGF-1 that operate as molecular checkpoints. In doing so, they rapidly shut down early inflammation including neutrophil infiltration and dendritic cell (DC) activation, switching the process from an inflammatory to a healing mode through the macrophage M1 to M2 phenotypic switch, differentiation of fibroblasts into myofibroblasts, and the proliferation of endothelial cells, leading to fibrogenesis and angiogenesis respectively [98,129,133–135,140,142] (Figs. 2 and 3).

This early time window appears to be determinant since the continued presence of neutrophils (necessary in infectious inflammation, but dispensable in sterile inflammation) may delay the healing process and generates fibrosis [17,23,125,140,142,148,152]. As primary effector cells in tissue repair and fibrosis together with macrophage M2, myofibroblasts synthesize and deposit extracellular matrix (ECM) components including fibrillar collagen types I to IV, fibronectin and release reactive oxygen species (ROS), thereby bridging the injury gap and replacing the transient fibrin scaffold [129]. Therefore, immunothrombosis as a multicomponent response has built in redundancy and robustness where signaling versatile proteins including thrombin and thrombin-derived peptides, FXII, VEGF, TGF-β, HGF, IGF-1, and PDGF among others, and some transcription factors rather than cells *per se*, are used interchangeably *in vivo* in a complex non-lineal manner during cell information transfer principle in the regulatory pathways which operate locally during defense and tissue repair [12,92,106,134,140,153]. It is highly likely that selection exerted a strong pressure on mechanisms underlying the early phases of immunothrombosis to operate quickly with biological precision by linking immunity and coagulation as a key effective survival factor. Evolution has left the immuno-reparative function of fibrogenesis and angiogenesis as open and condition-sensitive processes aimed at functional recovery rather than at structural perfection [3,39,41,134,154].

3.2. Tissue repair as a byproduct of immunothrombosis

The immunoreparative role of immunothrombosis relies on biomolecules including thrombin, fibrin (ogen), growth factors, cytokines, and microparticles primarily originating from plasma, activated platelets, and tissue resident macrophages, all of which are tissue repair enhancers [12,16,22,106,140]. In this respect, tissue healing might be conceptualized as a byproduct of immunothrombosis. And activated plasma-born intravascular innate immune cascade systems together with polymorphonuclear cells, monocytes, and platelets prevent the hemorrhaging and pathogen invasion caused by trauma- and infection-induced damage. Moreover, interaction with resident tissue cells including nociceptors, endothelial cells, macrophages, and fibroblasts will favor tissue repair [10,39,41,65,154] (Figs. 2–4). Although injury, defense, and repair appear to go hand in hand, tissue healing is developed in a manner that is not pre-specified by any genetic program, and it is dictated by microenvironmental cues stemmed from cell- and soluble factor-mediated circuits of immunothrombosis, as well as from pathogen and commensal microorganism products [92,135,140,147,148]. This is the case of fibrogenesis as a physiological process with a continuum spectrum that might be transformed into a pathological fibrotic condition [42,129,140,141], thus the repair process will not resolve with a unitary outcome (Fig. 2) [140,155]. For instance, the coexistence of commensals or pathogenic bacteria and their products (microbe-associated molecular pattern (MAMPs) and PAMPs) [147,148] or ECM-molecule debris (DAMPs) stemmed from tissue damage (sterile inflammation), elicit a persistent profibrotic M2 macrophage phenotype and the release of PDGF, IL-6, IL-13, and TGF-β [42,129,135].

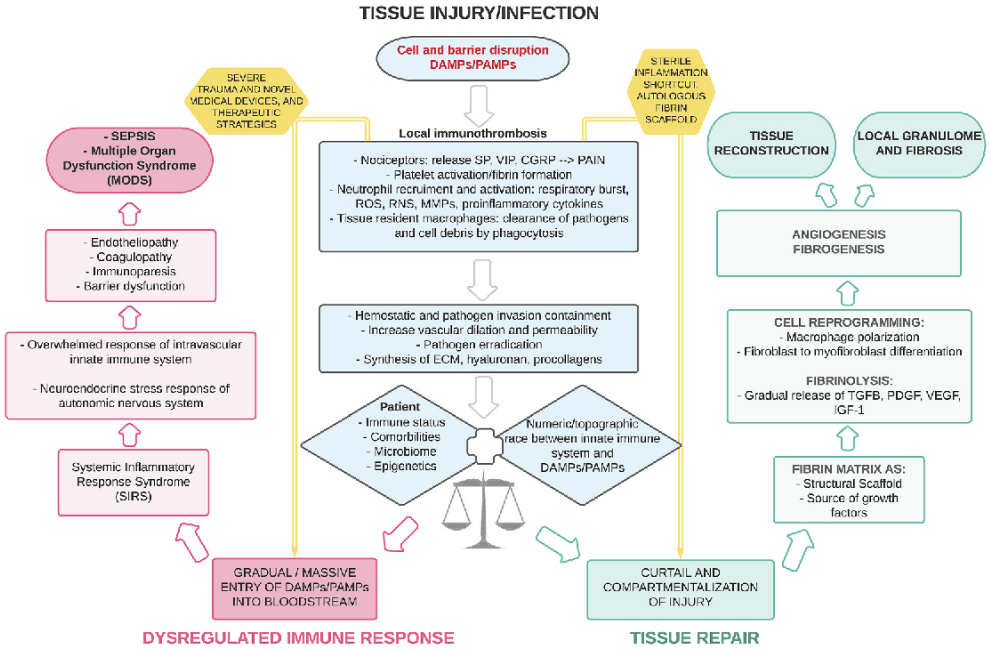

Fig. 3. Immunothrombosis: Challenges and therapeutic opportunities. By coupling inflammation and coagulation, immunothrombosis operates as a cooperative and amplifying strategy to cope with trauma and infection. The activation of nociceptors, endothelial cells, fibroblasts, tissue resident macrophages, circulating platelets and neutrophils, and plasma innate immune cascade systems [8,10,11] leads to curtail and compartmentalize the damage through wound-sealing, clearance-killing of pathogen and foreign bodies, and synthesis of ECM, thereby closing the damaged gap and giving way to tissue repair (see section 3). But, when the local inflammatory response is not resolved timely, often a persistent chronic inflammation leads to the formation of a nonfunctional secondary angioneurofibrotic scarring (local granulome and fibrosis) [129,143–145]. Autologous fibrin matrix may act as a shortcut therapeutic strategy in musculoskeletal sterile inflammatory pathologies by dampening inflammation and enhancing tissue repair and reducing pain [30,126,146]. However, when the triggering emergency cannot be handled in a tissue autonomous manner (due to the patient status, a severe polytrauma, and/or the use of cutting-edge medical strategies), the numeric and topographic race between DAMPs and PAMPs, and the well-developed local checks including intravascular innate immune cascade systems tips the balance towards a gradual or massive entry of biotic and abiotic molecules into the bloodstream [147,148]. In this new either sterile-inflammatory or infectious-inflammatory context, the complement cascade, the coagulation-contact and fibrinolytic systems may be locally and systemically dysregulated, thereby causing a SIRS [27,149,150]. Moreover, the neuroendocrine stress response mediated by the activation of the autonomic nervous system interacts with the endothelium and the innate immune system, resulting in a barrier dysfunction that may lead to an endotheliopathy, coagulopathy, immunoparesis, and the breakdown of protective cell barrier of the gut. Frequently, the non-lineal dynamic response of systemic immunothrombosis operates as a feedback loop with several arms leading to MODS and sepsis (see section 4) [27,151].

With dual fibrogenic and anti-inflammatory roles, TGF-β influences the number, activity, and/or life span of myofibroblasts and the generation of a fibrotic process [42,129]. Accordingly, sterile well-performed surgical techniques generate little or no inflammation and scar [42], whereas an excess of trauma- and surgery-derived DAMPs induces immune activation with local and systemic detrimental consequences. This has given rise to the concept of damage-control orthopaedic (DCO) surgery and the use of minimally invasive surgical approaches (Fig. 5) [27].

Supporting this idea, several studies in germ-free Swiss mice have shown that fetal skin wound healing and skin wound healing were accelerated, scarless, and associated with a reduced presence of neutrophils and increased content of anti-inflammatory M2 macrophages [125,148,152]. Similarly, early phases of wound healing on mice lacking the complement C3 component are accelerated, which is associated with a lessening mode of the inflammation intensity, significant reduction of neutrophils, important increase of mast cells, and accelerated angiogenesis at the injured sites [23].

Last but not least, FXII is an important plasma zymogen whose FXII/uPAR/pAkt 2 axis may drive the neutrophil-driven inflammation by promoting neutrophil trafficking and neutrophil extracellular trap (NET) formation resulting in impaired wound healing in a model of sterile inflammation, even though it favors further plasma FXIIa generation and blood coagulation [17]. In addition, FXII activation accelerates and strengthens fibrin-clotting formation in the presence of inert soil particles as a proxy of contaminated wounds in the plasma of terrestrial mammals like mice and humans but not in cetaceans (whales, dolphins) which lack of PK and possess an inactive pseudogene FXII, and in birds which have lost the FXII gene [18]. Moreover, a recent study showed that the fibrin biofilm formed in air-liquid interface in human and mice plasma, and in a murine dermal injury model, covers the formed blood clots and protects from bacterial proliferation and dissemination [160]. Overall, and taking into account these three studies and the aforementioned considerations, it is reasonable to hypothesize that FXII is necessary *in vivo* mainly to reinforce clotting in infectious conditions. Accordingly, recent research indicates that FXII operates as a pattern recognition molecule in infectious epithelial wounds by binding to both microbial walls or their products [19,161], and to infectious proxies such as soil particles, shortening the fibrin formation, attracting and promoting neutrophil activities and NETosis, as well as generating local bradykinin-mediated vasodilation [17–19,160,161]. In doing so, FXII may contribute to quickly compartmentalize and sterilize the wound thereby limiting the systemic spread of biotic elements in terrestrial animals [17,160], which are in contact with dirt-soil particles [18,162], as well as the systemic spread of small abiotic nanoparticles that escape from the multiarmed complement system [11,143], thereby promoting survival. Therefore, this FXII-mediated function in epithelial wounds (and likely in the epithelium of lungs and gut) might have played a strong selection pressure in animals in their transition from water to land. In fact, intertidal, mud,

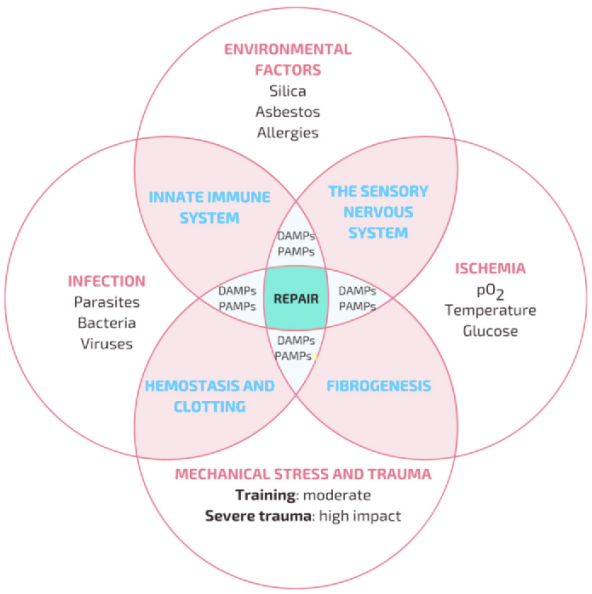

Fig. 4. Tissue repair as a byproduct of the biological defense process. The activation of the host biological defense processes and tissue repair appear to go hand in hand with mutual mechanisms mediating both immunothrombosis and healing. Several disruptors including infectious agents, environmental factors, ischemia, and mechanical stress may activate one or several arms of the host defense responses in an attempt to curtail the damage. In this context, the restoration of injured tissue might be conceptualized as a byproduct of the activated intravascular innate immune cascade systems, and tissue stationary and migratory cells including nociceptors, endothelial cells, fibroblasts, and resident macrophages [10,39,65,104,154]. Adapted from Ref. [30] with permission.

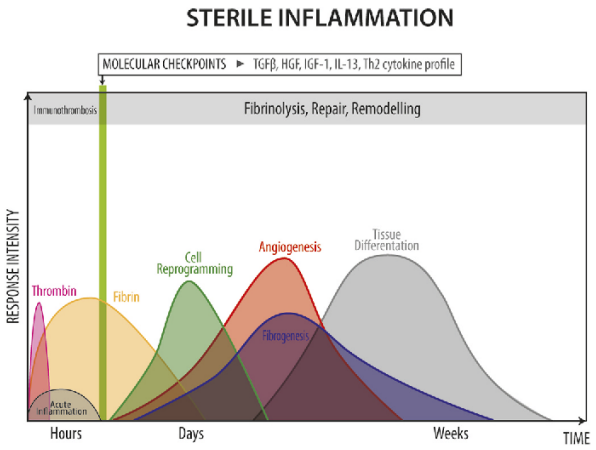

Fig. 5. Autologous fibrin scaffold: Mimicking the immunoreparative role of local thromboinflammation in sterile inflammatory conditions. This model is a representation of both the activation of host biological systems in sterile inflammatory conditions, and the scientific rationale behind the use of the autologous fibrin scaffold. The disruption of structural integrity of vascularized mammalian tissues by noxious agents, and affecting tissues such as skin, the central and peripheral nervous system, or musculoskeletal tissues, elicits the activation of platelets and nociceptor neurons, the formation of a fibrin clot, and the recruitment of neutrophils and monocytes to the affected site as a first stop-and-go check point to quickly stop bleeding, destroy bacteria, and avoid noxious stimuli created by behavioral changes [10,89]. However, in the absence of pathogen or their products, as in a sterile inflammation setting, molecular checkpoints rapidly shorten and dampen the early inflammatory window, switching the process from a killing and destroying mode to one that promotes healing [42,98,129,133–135] as suggested from how regenerative species react to injury [125], and from fetal scarless wound healing [156–158] (Fig.. 2 and 3). Early inflammation has been suggested to be an important therapeutic window, and its dampening might drastically reduce the fibrotic outcome of tissue injury in a cell-circuit framework for tissue repair and fibrosis [140]. Accordingly, in sterile inflammation defense responses are shorter and less intense than in infectious immunothrombosis (Fig. 2). Importantly, platelets may play an important role in the early resolution of sterile inflammation since they are a significant source of SPMs including maresins, resolvins, and LXA4 [126,128], the latter an endogenous arachidonic acid-derived mediator that has been reported to counter-regulate inflammatory processes and promote resolution responses in Achilles tendinopathic stromal cells [159]. Moreover, studies using leukocyte-free autologous fibrin matrix in musculoskeletal tissues, skin and corneal injuries reported a shorter repair time of tissue damage, a significant pain reduction, and expedite recovery function compared with the control group [24,30].

and dirty soil environments are abundant in white clay with silica-rich compounds like kaolin and Celite (key cofactors to activate FXII) [18, 163]. This microenvironment prevailed for the first amphibians, and latter for reptiles and other terrestrial vertebrates, including mammals [164]. In fact, marine mammals possess an inactive pseudogene FXII, and many fish lack FXII as do aerial animals like birds that have lost the FXII gene [73,74]. On the other hand, in the current cutting edge medical therapies, FXII as key zymogen of the contact system may impair wound healing in a model of sterile inflammation [17], as well as induce immunothrombosis on medical devices, both events to be considered as a trade-off of the contact system's activation [11,17,42].

4. Living with the past: emerging tradeoffs of intravascular innate immune system

From an evolutionary point of view, the integration of immunoclotting into an increasingly complex mammal bodies generated much interplay among the blood-born proteolytic cascades, blood cell types, and the neuroendocrine system changes that endowed enormous survival advantages. However, in our modern world, with developed cutting edge therapeutic strategies and technologies, this functional cross talk entails tradeoffs in a complex nonlinear manner [27,28,149, 165–167]. This is the current case of the exposure of blood to unscheduled and never anticipated medical devices and procedures, sedentary and prothrombotic life-style, and highly polluted environments so unlike those on which the integrated systems were evolved, tuned, and operated. The result has been increasing vulnerabilities at unprecedented levels in the form of new diseases, from thrombo-embolic events and ischemia/reperfusion injuries to systemic inflammatory syndrome and sepsis [11,27,106,167,168]. Sepsis, a deleterious, non-resolving inflammatory host response to infection, occurs in 1.5 million Americans annually and it causes more than 250000 deaths each year in the United States alone [169,170]. Moreover, the final common end-point of these infectious (sepsis) or severe sterile challenges (such as polytrauma, hemorrhagic shock, massive and repetitive blood transfusions, use of extracorporeal circulatory devices, organ and tissue transplantations, and intravascular artificial medical devices) is the increasingly seen multiple organ dysfunction syndrome (MODS) (Fig. 3) [27,149,171].

4.1. Immunothrombosis: a mosaic of defense mechanisms assembled to act locally

Immunothrombosis in humans is a multilayered nonlinear response where the local cell-to-cell and extracellular matrix-to-cell short range signaling pathways represented by complement, coagulation and contact systems, and fibrinolysis, have been complemented with and integrated into the long-range neuroendocrine-immune signaling pathways

[10,149,165,172,173]. Pathogen- and damage-recognition molecules including Toll-like receptors (TLRs), Nod-like receptors (NLRs), receptors for advanced glycation end products (RAGE), FXII, FVII, HMWK, C1q, Mannose-binding lectin (MBL), Ficolins, Properdin, C-reactive protein (CRP) are multidomain molecules present intracellularly, in the fluid phase and/or embedded in membranes of blood cells, endothelial cells, and nociceptors [11,174–176]. The serine proteases of the complement, coagulation and contact, and fibrinolysis system, whose protein domains possess intrinsic affinities, have created molecular networks that operate locally and extremely fast both in positive and amplifying or in negative and containment feedback loops. This is the case of the thromboinflammatory response to microbial pathogens, hemorrhagic shock, ischemia-reperfusion injuries, massive and repetitive blood transfusions, extracorporeal circulation, organ and tissue transplantations, intravascular artificial medical devices and massive trauma and burning injuries [11,27,28,149,171]. In order to compartmentalize the damage [3,7,33–35,177], blood circulating cells, nociceptor neurons, endothelial cells [166,178], tissue resident macrophages, dendritic and mast cells, and stromal fibroblasts are major effectors and players with dual roles in local neuroimmune surveillance and systemic body hemodynamic and organ homeostasis [166,178].

On the one hand, sensory neurons (Fig. 4) form networks within the primary physical barriers, namely, the different epithelial layers of the skin and mucosal surfaces of the respiratory and gastrointestinal tract, as well as joints and muscles. These nociceptor terminal branches form a receptive field which may include vascular endothelial and smooth muscle cells, tissue resident macrophages, and dendritic and mast cells among other immunocompetent cells [10,179,180]. The branches are endowed with receptors at the axon endings (transient receptor potential (TRP) ion channels VI (TRPVI) and subfamily M member 8 (TRPM8), piezo ion channel 2 (Piezo2), several TLRs, and receptors for TNF, IL-1β). As a result, nociceptors sense the noxious stimuli and generate action potentials that travel in an orthodromic fashion to neuronal bodies toward the central nervous system (CNS) but when action potentials reach branch points they are diverted to other peripheral endings of the same neuron in an antidromic manner [10,179,180]. This mechanism, known as axon reflex, together with the activated platelets results in the local release of histamine, serotonin, ATP, Ca^{+2}, substance P, calcitonin gene-related peptide, and matrix metalloproteinases, concomitantly with the activation of coagulation and hemostasis pathways. This leads to vasodilation and increased permeability of local vessels that allow the passage of plasma and leukocytes into the damaged tissue parenchyma [10,87,129,181]. Sensory neurons not only mediate in the local but also in the remote responses to injury and infection by local and systemic immunomodulatory axon reflexes that are at the origin of neurogenic inflammation and the anti-inflammatory response [10,27,178].

In contrast, endothelial cells in their quiescent state, offer their stationary yet dynamic luminal membrane in constant contact with circulating blood as an anti-thrombotic and complement-regulatory surface thanks to a layer of proteoglycans and heparan sulfate molecules covering the endothelium known as the glycocalyx. However, the opposing endothelial membrane in contact with the basement membrane is in close vicinity with nociceptor neurons, tissue resident macrophages, dendritic and mast cells, among other tissue resident cells [10,98,179]. Therefore sandwiched between the local intravascular innate immune system with its humoral and cellular arms and the systemic neuro-immuno-endocrine system, the heterogeneous endothelium is the interface between the environment and the internal milieu, acting as a gathering point, barrier, and target of host defense mechanisms [35,98]. The evolution-tailored hierarchical interplay of serine protease proteins of the complement and coagulation cascades and the contact system with immune and endothelial cells, and neurons, with their positive and negative molecular feedback mechanisms and neural axon reflexes, have evolved to operate extremely fast and precisely providing significant survival advantages. This is the case when local challenges are minor enough to be solved by the house keeping activity primarily carried out by resident macrophages and helped by ECs, platelets and stromal fibroblasts, the later using their inflammatory memory [3,27,182–184]. However, when the triggering processes, cannot be handled in a tissue autonomous manner, the insufficient local containment response gives way with increasing amounts of DAMPs and PAMPs gaining access to intravascular space. Importantly, and in its role of a purging system, the collaborative crosstalk between blood complement, coagulation and contact systems, with tissue stationary fibroblasts, dendritic and mast cells, tissue resident and migratory macrophages, endothelial cells and circulating polymorphonuclear cells, monocytes, and platelets becomes overwhelmed and dysregulated (Fig. 3) [7,11,177]. This new scenario may lead to a systemic, amplifying inflammatory syndrome as a zealous response and vicious cycle of intravascular innate immune cascades, endothelium, and blood cells, yet revealing the costs and vulnerabilities of host defense systems (Fig. 3) [3,11,27,90].

4.2. *Lifestyle and medical novelties with roles in emerging tradeoffs*

This is the case of major trauma and massive burns, hemorrhagic shock, ischemia-reperfusion injuries, sepsis or when patients in intensive care units, react badly to massive and repetitive blood transfusions, organ and tissue transplantations, and the use of blood-contacting medical devices including extracorporeal circulation [11,27,28,149,171,185]. Those patients, who would previously have died, now survive, but the danger is not finished once the "*talismanic*" values of the physiological homeostasis, including blood pressure and red blood cell count among other values, have returned to normal levels [7,27,28,149,166,169,186]. The physiological systems of these "*new patients*" may usher in systemic inflammatory syndrome and sepsis, frequently leading to MODS [27,149,166,169,187]. These novel complex-to-treat medical conditions bring out biological tradeoffs that our organism tolerated in other environments due to the major benefits conferred by the interconnectedness of immunothrombosis and neuroendocrine system [3,11,35,143]. In fact, the dysregulated nonlinear host response of patients suffering from systemic inflammatory syndrome and MODS (Fig. 3), makes it enormously difficult to therapeutically rebuild the broken physiological cardiorespiratory, digestive and renal networks in those patients suffering from MODS [27,149,165,188] as well as to rebalance the intravascular innate immune system due to the evolutionary molecular interconnectedness.

Our modern society has also brought vulnerabilities in the form of new diseases, from thrombotic events and ischemia/reperfusion injuries to autoimmune conditions and chronic inflammation and cancer [11,27,143,167,168,189]. This is the case with a sedentary and prothrombotic life-style, the exposure of blood and other body fluids to medical devices, procedures, and novel and toxic environmental nanoparticles stemming from food supplements, household materials, dental applications, cosmetics, vehicle exhaust and mechanical wear of the brakes (αFe_2O_3) or from smoke (cigarettes, environmentally-caused wild fires, cooking). For example, small nanoparticles of TiO_2 (present in paint, toothpaste, sunshield and cosmetics) are too small to activate the classical complement pathway through the multi-armed recognition proteins of complement (C1q, ficolins) and get rid of them in its role of purging system [143,190]. In contrast, the pattern-recognition protein FXII binds one to one with TiO_2 nanoparticles as well as with other particles of similar size and may mount an inflammatory and bradykinin-mediated inflammatory and angiogenic response that might aggravate chronic inflammatory pathologies [143,190]. Therefore, we should take into account our novel life-style model and environmental innovations as a source of elements that render our biology not imperfect or imprecise but unpredictable for some current human therapeutic purposes instead of blaming the blood-born proteolytic cascades, blood cell types, and neuroendocrine system for operating and fulfilling the roles for which they were crafted and tuned very precisely [3,10,11,35,124,143,167].

5. From tissue repair as byproduct to fibrin matrix as biology-as-a-drug approach

Blood is a multitask, fluid, connective tissue that besides coordinating and communicating between nonadjacent tissues, its circulating proteins and cells influence the repair and regenerative capacity of multiple tissues and organs having always been present in the equation of healing therapies [191–193]. Several lines of evidence derived either from systemic or local stem cell niche therapies, and represented by parabiosis or microfractures and tendon scarifications, respectively, support the concept that factors derived from platelets or plasmatic proteins are candidates for mammalian tissue rejuvenation and healing [24,192,193].

Autologous fibrin matrix (AFM) is emerging as a local biology-as-a-delivery system of GFs to expedite repair of sterile inflammatory injuries such as osteoarthritis, tendinopathies, cartilage injuries, peripheral neuropathies, intervertebral disc degeneration, skin burns and ulcers, corneal ulcers, and dry eyes among other conditions [24,29,30,

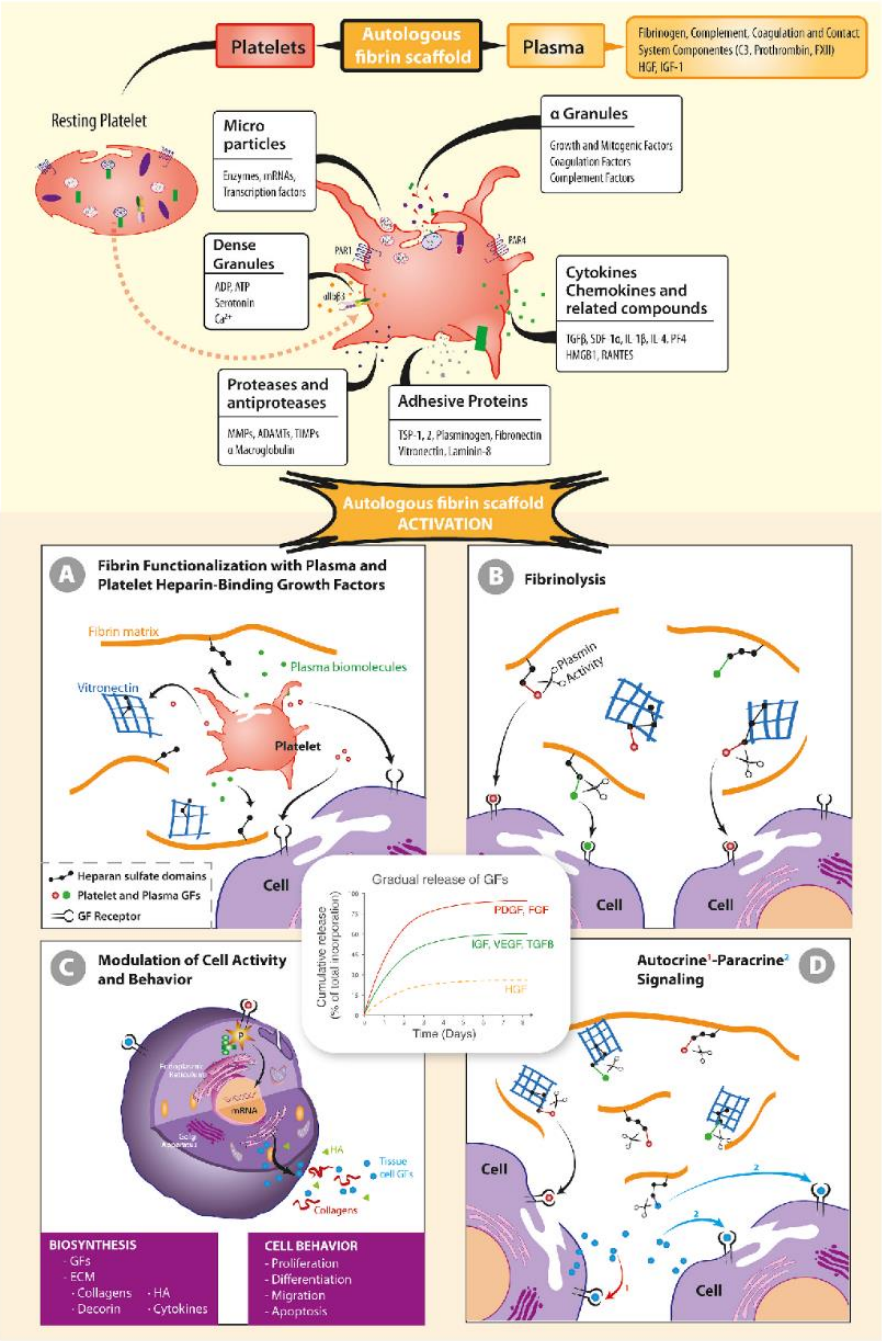

Fig. 6. Autologous fibrin scaffolds as a local biology-as-a-delivery system of growth factors (GFs) to enhance repair of sterile inflammatory injuries. Autologous fibrin matrix is a liquid-to-gel dynamic carrier of biological mediators for tissue repair. When the fibrin scaffold is formed, the three-dimensional matrix traps many of the plasma and platelet-released growth factors and cytokines. Several of these GFs are bound temporarily to heparin sulfate proteoglycan domains of fibrin matrix components (in a yuxtacrine or matricrine mode) (A). After its application to injured sites, the fibrinolysis mediated by plasmin that is generated through both the activation of plasminogen by a tissue plasminogen activator [87,219]. Immune and mesenchymal cells migrate into the clot and accompany fibrin degradation thereby matching the speed of the ingrowing repair tissue [220–223] (B). GFs bind to *trans*-membrane receptors present on the surface of target cells, thereby activating intracellular pathways that convey the signal to the nucleus, to eventually induce a wide range of genes that will translate into changes in cell survival, proliferation, migration, differentiation, transdifferentiation, and maturation (C). These effects include the synthesis and secretion of a new ste of GFs and cytokines, which also interact with their receptors in a diffusible manner (autocrine and paracrine pathways), the synthesis of ECM components such as collagens, decorin, hyaluronic acid and GAG, and also modify cell survival, proliferation, differentiation, and migration (C) [30]. Moreover, some GFs in a diffusion mode (autocrine and paracrine) will directly reach their cognate cell-surface receptor, thereby exerting an immediate cell-biosynthetic and cell-behaviour modification (D). Adapted from Ref. [224] with permission.

194].

The therapeutic potential of this liquid-to-gel dynamic scaffold relies on the biochemical machinery of coagulation and platelets, including but not limited to thrombin [12,13], fibrin [15,16], FXII, and the payload released by activated platelets from growth factors and cytokines to microparticles (Figs. 6 and 7) [16,24,29–31].

5.1. A brief history of the development of autologous fibrin scaffolds

The recent history of AFM began in the early twentieth century with the use of fibrin and its derivatives as hemostatic agents in surgery [195]. Later, Young and Medawar described the use of fibrinogen-fibrin in the suture of peripheral nerves in animals [196]. It was in 1944 when Cronkite first described the use of fibrinogen and thrombin as a biological glue in skin transplants [197]. However, the preparations used so far lacked the necessary strength and were not very stable over time. Already in the seventies, Matras and colleagues continued with the development of fibrin sealants, specifically trying to increase the adhesive and healing properties of fibrin by the concentration of fibrinogen by chemical and cryoprecipitation methods [198]. A disadvantage of these methods was that they present a risk of disease transmission and the possibility of generating allergic reactions due to the use of bovine thrombin. More recently, Tayapongsak et al. used autologous fibrin clinically as an adjuvant in maxillofacial surgery [199]. These early concepts of combining the properties of two products, fibrin sealants and platelet growth factors, led to the development of AFM technologies. Following these principles, Marx [200] developed in 1998 a method to produce platelet-rich plasma (PRP) from a density gradient separator and applied it successfully in maxillofacial surgery, however the great disadvantage of this technique was the large amount of patient blood (400–450 mL) required. In the same decade, Anitua described the use of autologous PRP, but starting from small volumes of blood (approximately 40 mL) and activating with calcium chloride instead of using bovine thrombin [201]. After the initial development in the field of oral and maxillofacial surgery, AFM have been used in various medical specialties, such as traumatology, orthopaedic surgery and sports medicine, dermatology, ophthalmology, reproductive medicine, nerve regeneration, general surgery and wound healing, among others [30, 202–205].

In addition, AFMs have been used alone or as an adjuvant in combination with different materials in order to increase their regenerative power, such as hyaluronic acid, bone, fat or dermal substitutes [206, 207].

New non-clinical applications include the use of PRP as a culture medium supplement for *ex vivo* expansion of stem cells [208] or as a patient-specific bio-ink for tissue engineering [209]. Finally, platelets in the AFM are likely to have an increasingly important role in the development of new drug delivery approaches. As we have discussed so far, these small acellular fragments derived from megakaryocytes act as key players not only in hemostasis and thrombosis but also in multiple roles such as angiogenesis, inflammation and immunity. Their particular structure linked to these functions enables to exploit their full potential as a tool in the development of new approaches of biomedical engineering. Recently developed platelet-mimetic nanoparticles, nanocoatings and nanofibers can enable the targeted delivery of therapeutic proteins to struggle against cancer, help in vascular injury or fight off infections [210–212].

5.2. The scientific rationale behind autologous fibrin scaffolds

Autologous fibrin scaffolds and related products derived from platelet-rich plasma (PRP), are a by-products of nature's own healing systems that involve blood anticoagulation and mild centrifugation steps followed for fibrin formation by restoring thrombin production. Initially this involves the separation of a fluid phase plasma where platelets, white cells, and red cells are suspended in different concentrations depending on the centrifugation and fractionation methods employed [29,30,213]. The *ex vivo* activation of PRP by adding CaCl2, at low concentrations to restore calcium homeostasis [214,215] generates low but yet efficient concentration of autologous thrombin [216]. This results in a slow, gradual, and dynamic liquid-to gel fibrin scaffold, via the already activated intrinsic pathway of coagulation downstream of FX

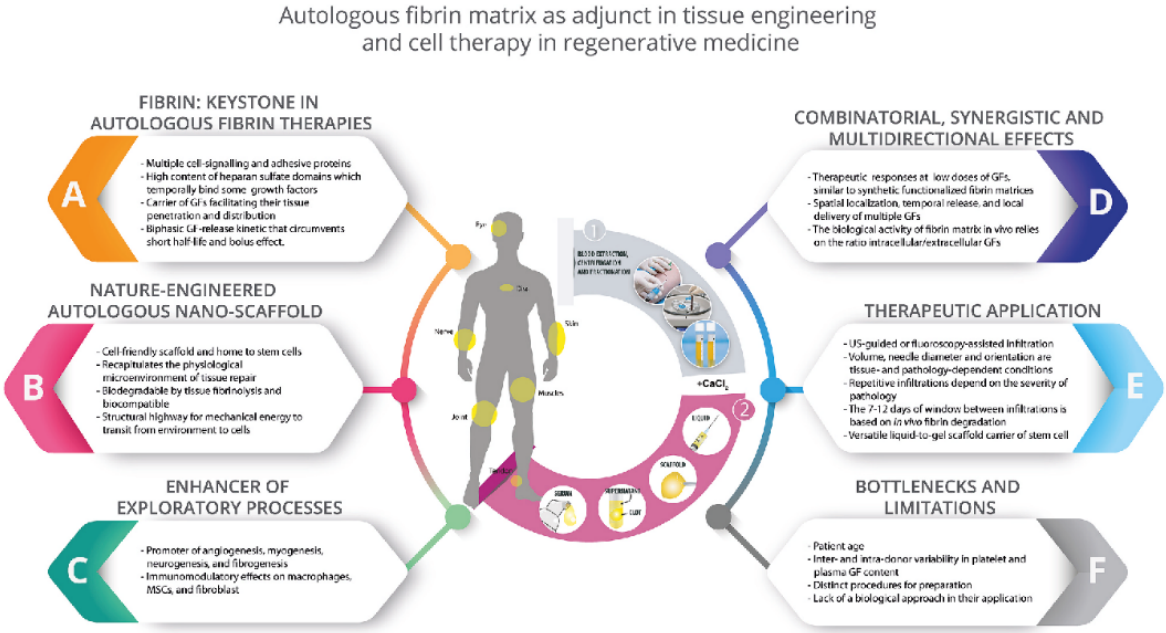

Fig. 7. Scheme summarizing chief roles played by the fibrin as a center piece in tissue repair when autologous fibrin scaffolds are applied into injured area [160,194,225,226]. Adapted from Ref. [224] with permission.

[162,217]. Moreover, the generated native thrombin simultaneously activates platelets and their degranulation allows the release of a panoply of biologically active proteins and metabolites; the polymerization of plasma fibrinogen into a progressively insoluble fibrin matrix provides the functional unit for tissue repair [29,31,218].

The scientific rationale behind AFM as a tissue repair enhancer in sterile inflammatory injuries is essentially provided by GFs and biomolecules stemmed from platelets and plasma, the fibrin matrix, and the interaction of native GFs with both the newly generating ECM and tissue cells where the liquid-dynamic matrix is injected or applied [29,31,221] (Fig. 6). The architectural keystone of the fibrin matrix is the high content of heparin sulfate domains that transiently bind growth factors protecting them from proteolysis thereby extending their short half-life, and placing them in the vicinity of cells facilitating tissue penetration, spatial localization, and *in situ* delivery of tissue enhancer biomolecules [30,194]. Ample evidence indicates that beside the antimicrobial and regenerative properties of the fibrin matrix and the biofilm itself [16,136,160,227,228], the biomolecules contained within the matrix including thrombin [12–14], fibrin (ogen) [15,16], complement C3a, C5a fragments [22,23] together with growth factors, cytokines, contact system zymogen FXII, and microparticles either released by activated platelets or derived from plasma, play a significant role as tissue repair enhancers [12,16,20,22,30]. In addition, platelet-released effector molecules influence the resolution of inflammation since they are a significant source of specialized pro-resolving mediators (SPMs) including maresins, resolvins and Lipoxin A4 (LXA4) [126,128], and therefore may contribute to shorten the early inflammatory process in sterile inflammatory context (Fig. 5). In doing so, fibrin matrix partially mimics the injured ECM by acting as a depot of regulatory factors (Figs. 6 and 7) [222,229–231].

AFM is injected as a liquid dynamic scaffold in the time window of 1–5 min when the fibrin matrix is still liquid macroscopically but undergoing a microscopic gelling process, which is marked by the gel point or clotting time, meaning the change from liquid to solid undergone by the matrix when 15–20% of the fibrinogen has been incorporated into the gel by branching points (approximately 4–5 min) [232]. The liquid-to gel dynamic scaffold extensively permeates through areas that surround the injection site, and anchors by way of the activated platelets conveyed by the fibrin clot to the collagen and other ECM proteins exposed in damaged tissue margins; the result is a 3-D fibrin-extracellular matrix-like malleable structure [30,87] (Fig. 7). In doing so, the injected fibrin bridges the gap of injured areas where tissue plasmin will initiate the fibrin biodegradation by tissue fibrinolysis, and during the next 7–10 days will be releasing both immediately and in a gradual and delayed manner the growth factors trapped during the fibrin formation, thereby operating as a biomimetic biphasic GF delivery system (Fig. 6) [16,30].

Several biological features might partially account for the safety and efficiency of fibrin matrix as a GF delivery system [30] (Figs. 6 and 7). The first is the autologous fibrin scaffold-mediated paracrine effect exerted on several cell phenotypes that induces the synthesis of additional amounts of HGF, VEGF and ECM compounds including hyaluronic acid that will amplify the initial cell response [233–236]. The second is the angiogenic effects of FXII, and thrombin, the latter being anti-inflammatory at low concentrations (Fig. 6) [12,13,20]. The third is that microparticles shed by platelets (PMPs) and present in the plasma may contribute to the resolution of inflammation [237–240], exert an immunosuppressive [237] and anti-inflammatory [237,241,242] effect by the immediate release of TGF-β, as well as providing an angiogenic and neurogenic effect [243–245]. The fourth is the locally limited spatial and temporal scale to which autologous fibrin matrix is exposed to cells by generating chemotactic GF gradients of differing durations (Fig. 7) [246,247]. Finally, the low amounts of growth factors released by this matrix while increasing the sensitivity of cells to changes at nanoscale level will also facilitate their fast clearance of them resulting in a rapid restoration of growth factor homeostatic conditions [30,248,249].

5.3. *Fibrin matrix: a complex nonlinear therapeutic growth factor system*

Initially, the use of AFM raised concerns about generating fibrosis and aberrant neovascularization due to the significant presence of TGF-β1 and VEGF, two master switches regulating fibrogenesis and angiogenesis respectively [129,250,251]. Experimental and clinical evidence coming from the application of AFM or its supernatant, however, indicate that biomolecules trapped within the fibrin matrix operate, with cell-instructive functions, in a complex nonlinear dynamic manner during tissue healing process [30]. Accordingly, GFs such as TGF-β, VEGF, HGF, IGF-1, stromal cell-derived factor 1 (SDF-1), and PDGF among others, generate biological information circuits whose redundancy and easily changeable regulatory linkage at the molecular level influence and accommodate fibrogenesis and angiogenesis [3,39,41,134,154], two key processes aimed at functional healing rather than at tissue structural perfection [3,39,41,134,154].

For example, the synthesis of ECM elements such as collagen type I [234–236,252–254], decorin and fibronectin [253,254], and hyaluronic acid (HA) [236] from healthy human tendon, skin, and synovial fibroblasts, primary human gingival fibroblasts, and tendinopathic cells cultured with fibrin matrix supernatant is the same regardless of whether low or high concentration of TGF-β1, PDGF, and VEGF are present [234–236,252–254]. In addition, primary human keratocytes and conjuctival fibroblast cultures stimulated with AFM supernatant whose concentration of TGF-β1 was 2.5 ng/mL did not induce any myofibroblast differentiation whereas when stimulated with 2.5 ng/mL of TGF-β1 alone they showed a significant increase in the number of myofibroblasts [255]. Similarly, primary human gingival fibroblasts cultured with 6 or 10 ng/mL of TGF-β1 in the AFM supernatant did not affect the fibroblast myodifferentiation rate [256]. Significantly, in primary human keratocyte, conjuctival fibroblast, and gingival fibroblast cultures, the concurrent presence of TGF-β1 and AFM surpernatant prevented the TGF-β1-stimulated fibroblast myodifferentiation with less than 0.2% α-smooth muscle actin (SMA) positive cells present in these cell cultures [255,256]. Likewise, for TGF-β1-pretreated primary human keratocyte, conjuctival fibroblast, and gingival fibroblast cultures there was a significant reduction in the number of α-SMA positive cells to below 12% when AFM supernatant was added [255,256]. These outcomes are consistent with a HGF-reduced α-SMA and type III collagen expression and elevated matrix metalloproteinase-2 (MMP-2) expression on TGF-β1 treated primary Achilles tendon fibroblasts [257]. Finally, results from healthy tendon and primary human gingival fibroblasts cultures indicate that the paracrine secretion of VEGF and HGF is independent of the low or high concentration of TGF-β1, PDGF, VEGF conveyed by fibrin matrix supernatant or fibrin matrices whose HGF and IGF-1 is similar [233,234,236].

In vivo studies stemmed from corneal wound healing in mice, rabbits [258,259] and human ocular surface pathologies (neurotrophic keratitis and Sjögren syndrome) where the opacification of the cornea by fibrotic scar formation leads to loss of visual acuity [259–265] corroborate that AFM or its supernatant operate as a complex nonlinear therapeutic growth factor provider with anti-fibrotic and tissue-specific angiogenic outcomes. The application of a fibrin-free eye drops formulation (with a similar composition to fibrin matrix supernatant) in either a photorefractive keratectomy mouse model or a cornea wounded rabbit model induced a faster re-epithelization and a mature and specialized corneal epithelium with a very low presence of α-SMA expressing cells and no haze formation, significantly reducing an eventual scar formation [258,259,266]. Importantly, several human studies have evaluated the biological and clinical effects of the fibrin-free eye drops formulation on persistent corneal epithelial defects and ocular surface pathologies (neurotrophic keratitis, Sjögren syndrome and autoimmune dry eye, evaporative dry eye, ocular rosacea, traumatic glaucoma, herpes and adenovirus keratitis) [260–265]. Results show a significant reduction of

pain, dryness, photophobia, and blurred vision, associated with high rates of corneal defect/ulcer resolution, and importantly, with no angiogenesis and scarring on the damaged ocular surfaces [260–265]. Successful corneal healing, an improvement or maintenance of visual acuity, a reduction of inflammation, and an antifibrotic effect in t complex ocular surface surgeries have also been reported using a fibrin membrane formed from autologous plasma rich in growth factors [202, 267]. Overall, the analysis of data resulting from these *in vitro* and *in vivo* research suggests that the presence of TGF-β1, a hallmark of fibrogenesis and potentially profibrotic cytokine [129,268] within AFM is counteracted by the simultaneous presence of HGF, IGF-1, fibroblast growth factor (FGF), VEGF, and PDGF as well as by PMPs concomitantly conveyed by AFM or its supernatant (eye drops).

Several mechanisms have been suggested to account for this complex nonlinear dynamic of GFs within AFM on fibrogenesis. HGF, a multidomain protein with pleiotropic and multipurpose functions, has been reported to attenuate decapentaplejic (SMAD) nuclear translocation [269] on renal fibroblasts stimulated by TGF-β1 leading to an antifibrotic effect as a downstream effector of the peroxisome proliferator-activated receptor-γ (PPAR-γ) [269], and to expedite re-epithelialization in skin wound healing by dedifferentiation of epidermal cells through the B1Integrin/ILK pathway [270]. Moreover, in primary tendon fibroblasts the TGF-β1 induced myofibroblast differentiation is inhibited by HGF through the activation of AMPK signaling [271] or by activating the inhibitory protein SMAD7 leading to the inhibition of TGF-β1 signaling pathways [272–274]. Furthermore, HGF promotes myofibroblast apoptosis [275] and matrix degradation by increasing collagenase expression (MMP-1 MMP-2 and MMP-3) and decreasing their inhibitor TIMP-1 expression [257,276]. Accordingly, HGF reverses fibroproliferative disorders such as hypertrophic scars [277] and keloids [278]. Another significant morphogen within AFM is IGF-I which may contribute to the non-scarring cornea healing by inhibiting the TGF-β/SMAD pathway of fibrosis in human keratocytes *in vitro* [279]. In addition, IGF-1 is known to accelerate muscle and nerve regeneration by promoting a balance between inflammation and fibrogenesis through the suppression and activity of macrophage migration inhibitory factor (MIF) and NFκB, both implicated in macrophage type I polarization leading to non-resolving inflammation and fibrosis [280–282]. Supporting the aforementioned results, the treatment with IGF-1 of injured muscles in an IGF-IR ± heterozygous mouse model, inhibited TGF-β-stimulated Smad3 phosphorylation and increased the *p*-Akt and Smad3 interaction in myoblasts. In doing so, IGF-1 interrupts TGF-β signaling leading to a stimulation of myogenesis and preventing fibrosis [283–285]. However, a decrease in IGF-I has been associated with inflammation and fibrosis [285] in patients with non-alcoholic fatty liver disease, while an increased expression of IGF-I limits fibrosis through an antiapoptotic, anti-inflammatory, and antioxidant effect [285–287]. Another growth factor within AFM, namely FGF, is a further potential candidate to attenuate the fibrotic effect of TGFβ-1 by triggering the dedifferentiation of myofibroblasts and favouring the fibroblast phenotype [288,289].

Angiogenesis is a multistep process driven by the spatial and temporal cooperative interplay of endothelial cells (ECs), pericytes, macrophages, and fibroblasts and angiogenic factors including, VEGF, HGF, IGF-1, TGF-β1, PDGF, SDF-1,FXII, and Ang 1, and PMPs. It is another example of the complex nonlinear effect of AFM in promoting tissue-specific angiogenic outcomes [233,234,244,290–301]. For example, the *in situ* application of VEGF/PDGF [302,303], FGF2/PDGF [304,305] or VEGF/IGF-1 [292,304] in a hindlimb ischemia mouse model promotes a therapeutic angiogenesis indicating that a combination of angiogenic factors rather than a single growth factor is more effective in generating a mature functional vascular network [306]. Moreover, a bolus application of VEGF and IGF-1 did not show benefits in terms of vascularization and perfusion [292], whereas the sustained delivery of the same GFs within an alginate gel stimulated angiogenesis and perfusion, enhanced neuromuscular junction and muscular regeneration, and reduced fibrosis [292]. Consistent with these results, AFM supernatant and platelet-derived extract promote the proliferation of primary human umbilical vein ECs (HUVEC) and pulmonary artery vasa vasorum EC (VVEC), and induce the formation of new capillaries in the *in vivo* implanted matrigel plugs as indicated by a double immunohistochemical staining for CD31/α-SMA and for CD31/PECAM-1 respectively [307,308]. Importantly, the absence of TGF-β does not affect *in vivo* angiogenesis as demonstrated in patients with defective α-granule biogenesis in megakaryocytes, linked to a Gray platelet syndrome in humans or TGF-β neutralization with antibodies [268,309]. Significantly, TGF-β1, HGF, IGF-1, and FXII are GFs that are present in a latent form and whose cell signaling requires proteolytic activation, a fact that contribute to why AFM acts with a complex nonlinear dynamic.

Significantly, and though indirect evidence in support of AFM as complex nonlinear therapeutic growth factor provider, human therapeutic clinical doses of autologous GFs conveyed by AFM for an absolute larger skin, cartilage, nerve, and muscle injured area are significantly lower compared with the single or combinatorial recombinant GF application used for structure-modifying regenerative purposes in animal and human research [30,221,292,310–316]. Several factors might account for this observation [30]. The first is the paracrine effect exerted on several cell phenotypes that adds an amount of HGF, VEGF and ECM compounds thereby amplifying the initial response [233–235]. The second is the angiogenic effect of FXII and thrombin, the latter being anti-inflammatory at a low concentration [13,20]. The third is that platelet and plasma microparticles provide an additional source of TGF-β and other biomolecules with anti-inflammatory, immunomodulatory, angiogenic, and neurogenic effects [243–245]. The fourth is that fibrin operates as a carrier of GFs to the close vicinity of target cells somehow filling the spatial gap in the damage area. This fact is key as GFs are biochemical signals that operate for limited length and time scales in an intracrine, autocrine and paracrine modes diffusing over distances of just a few micrometers. Thus, the intensity of the signal decays more than linearly with the distance from the source roughly $1/r^2$, being in 3-D even more rapid [249].

Overall, such *in vitro*, *in vivo*, and clinical studies suggest that these blood-derived products operate in a complex nonlinear dynamic, with a robust antifibrotic and tissue-specific angiogenesis effects as emergent properties that cannot be predicted by the concentrations of TGF-β1, VEGF, HGF, or IGF-1 on their own, and where the simultaneous presence of several growth factors appears to be pivotal for the therapeutic effects [30,165,317].

5.4. *Technical ins and outs of the use of the autologous fibrin matrix with biological repercussions*

There exist several pitfalls derived from the many preparation methods (type of anticoagulant, activation or not, centrifugation speed to separate platelets from other blood cells, pH), which in conjunction with the poor standardization in the way that it is applied can lead to misleading conclusions regarding their clinical efficacy derived from the very diverse biological composition and effects [224]. Importantly, this localized therapy does not appear to entail any thrombo-inflammatory reaction [315,318,319] as might be expected by both the activation of the contact system and the interplay between intravascular innate immune cascade systems [8,11,224].

The uncoupling of thrombotic and inflammatory responses of this biologic together with the antifibrotic, antialgic, and immunomodulatory actions are the hallmark of AFM [30]. Although the mechanisms that drive the *ex vivo* uncoupling of FXII-driven thrombosis and inflammation remains to be determined, several factors might account for this dissociation [320]. Among them, is the absence of leukocytes, the *ex vivo* activation of the intrinsic coagulation pathway with calcium chloride ($CaCl_2$), and the citrate-dependent inhibition of activation of the complement system [11,16,30]. The incorporation of leukocytes (and inevitably erythrocytes) into the fibrin matrix, primarily

neutrophils, promotes local pathophysiological reactions such as inflammation [321], a fast and early ECM and fibrin degradation, and proinflammatory cytokine release [322]. In *in vitro* and *in vivo* experimental models, leukocytes in the fibrin matrix through their incomplete separation when preparing PRP will allow their interaction with already primed and inflamed stromal fibroblasts, trigger fibroblast inflammatory memory and favor the release of pro-inflammatory cytokines [182,183], a fact that is not observed when the PRP preparations are leukocyte-free [254,323–327]. Moreover, there may be other detrimental effects stemmed from an erythrocyte-derived heme-iron induction of the pro-inflammatory macrophage phenotypic switch [182,328–331] that together with neutrophil activities and NETosis may operate as non-resolving inflammation and profibrotic agents respectively [17,140,159,332,333]. This can even exacerbate the inflammation-driven pathology that the applied fibrin based network is intended to treat [334,335]. Importantly, *ex vivo* activation of PRP with $CaCl_2$, yields low concentrations of thrombin, which itself exhibits growth factor-like fibroblast and endothelial cell proliferation, migration, antiapoptotic, and inflammatory modulation activity. In contrast, alternative modes of PRP activation can generate high doses of thrombin, which would then also operate as an inflammatory mediator to recruits monocytes, activates the NF-κB of endothelial cells, and triggers the release of cytokines from mast cells [13,216]. The *ex vivo* activation of PRP, in the absence of leukocytes, will circumvent the NF-κB-dependent inflammatory gene expression and the generation of proinflammatory cytokines by monocytes [336]. Not to be forgotten is that, when directly injected non-activated PRP, platelets may activate the complement system, as well as promote the formation of platelet-leukocyte complexes and neutrophil extracellular traps (NETs) *in situ* [33,336,337]. The growth factor HGF is secreted as an inactive precursor (proHGF) and its proteolytic activation by HGFA is promoted by thrombin and kallikrein enzymes, thereby producing pleiotropic HGF regenerative activities in damaged areas provided that the coagulation cascade has been activated [138,338]. Exogenous activation with commercially available bovine thrombin runs the risk of adverse effects, which include immune reactions, thrombosis, and hemorrhage [339]. The use of citrate as anticoagulant has a significant biological impact on PRP dynamics as this anticoagulant inhibits both coagulation and complement activation by chelating and removing ion Ca^{2+} from plasma but not FXII and therefore the inflammatory arm of the contact system [217], which may contribute to the uncoupling between thrombotic and inflammatory responses of this biologic. In fact, while the addition of $CaCl_2$ initiates the intrinsic arm of the coagulation cascade, the classical (CP) and lectin (LP) pathways of complement continue to be disturbed [11]; citrate is a potent complement inhibitor already at a low 0.25 mM concentration and reduces the granulocyte activation [217,340]. Significantly, since sodium citrate does not inhibit the activation of FXII there is a generation of bradykinin (BK) [217]. This major proinflammatory mediator not only recruits neutrophils to the injured site and activates resident macrophages [341] *in vivo* it also induces a second-generation of mediators such as nitric oxide, prostaglandins, and leukotrienes [320,342]. However, and due to the very short half-life of the BK in plasma and serum (<30 s) [343], by the time the fractionated plasma is first activated *ex vivo* and then injected as an AFM (at least 10–15 min after the activation of the inflammatory arm of the contact system), most of generated BK has been degraded by plasma kininases and by blood clotting enzymes [343,344]. This physiological BK degradation in AFM is indirectly confirmed by the absence of pain or inflammation at the site of the autologous fibrin scaffold injection [315,319,345]. Last but not least, the use of citrate as anticoagulant does not modify the growth factor kinetic nor the major biological properties of AFM [231].

6. Future perspectives

Evolution, from invertebrates to mammals, has yielded and shaped immunothrombosis as a highly conserved defense and repair response. The constitutive molecular pathways are a goldmine from where to learn to design and optimize new bioengineering and synthetic biologically-based therapies. Tissue repair as a by-product of the processes underlying immunothrombosis highlights potential therapeutic interventions to enhance the repair of musculoskeletal injuries associated with chronic inflammation. This is the case for blood-derived autologous fibrin scaffolds that have a significant repair and regenerative potential and which harness the healing properties of molecular and cellular components of coagulation and hemostasis. Nonetheless, further work is required to better address and overcome some of the challenges that have arisen from its use, the myriad of methods for AFM preparation often with incomplete characterization and with poor standardization in the way they are applied. Moreover, the precise molecular mechanisms underlying this versatile, biology-inspired, evolution-tailored, and human-engineered biomimetic therapy remain insufficiently understood, as is the paradox of host defense mechanisms acting as local tissue repair enhancers in man while in other situations generating systemic pathogenic processes.

7. Conclusions

1. Evolution from invertebrates to mammals has yielded and shaped a mosaic of mechanisms with roles in inflammation, clotting, and healing, that has led to consider tissue healing being considered as a byproduct of the mechanisms underlying immunothrombosis.
2. Despite the superficial resemblances and common functional trends between invertebrate immunoclotting and mammalian immunothrombosis, the complex vertebrate blood coagulation network emerged independently, together with a closed circulatory system, blood cell specialization, the endothelium, and the adaptive immune system, more than 500 Mya, over a period of a 50–100 million year window.
3. The emergence of multi-systemic organisms, through gene and whole genome duplication, exon shuffling, and mutations, has led to sophisticated host defenses by the progressive addition of complexity in the form of the contact system, the embellishment of the neuroendocrine system, blood cell type diversification and endothelium development, the expansion of the proteins of the complement pathway, and the emergence of the megakaryocyte-platelet axis among others.
4. The systemic interplay among blood proteolytic cascades, blood cell types and endothelium, and neuroendocrine system endowed their bearers with survival advantages. But in the current world, that has provided unprecedented cutting-edge therapies, the trade off is that disruption of physiological homeostasis has led to thrombotic and inflammatory diseases largely influenced by lifestyle and posing a heavy economic burden.
5. Harnessing the healing potential of fibrin matrix, an autologous liquid-to-gel scaffold operates as a local and inexpensive shortcut therapeutic strategy in sterile inflammatory conditions by mimicking the immunoreparative role of growth factors and other biomolecules trapped in the fibrin fabric. Once applied to damaged area, fibrinolysis will gradually release its cargo, including growth factors and other biomolecules with antialgic, anti-inflammatory, and trophic effects.
6. This versatile, biology-inspired, evolution-tailored, and human-engineered biomimetic therapy is in its naissance, and offers much hope for the future particularly as it is well adapted for third world countries.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare that E.A. is the Scientific Director of and S.P. and R.

P. are scientists at BTI Biotechnology Institute, a biomedical company that investigates in the fields of regenerative medicine and PRGF-Endoret technology. The rest of the authors state that that they have no conflicts of interest that are relevant to the content of this article.

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3.2. ARTÍCULO II

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RESUMEN

Se ha producido una oleada de interés científico por el uso de plasma humano rico en plaquetas (PRP) como sustituto de los sueros xenogénicos en las terapias celulares. Sin embargo, es necesario crear una estandarización en este campo. Esta revisión sistemática se basa en búsquedas bibliográficas en las bases de datos PubMed y Web of Science hasta junio de 2021. Cuarenta y un estudios completaron los criterios de selección. La composición del PRP se informó completamente en menos del 30% de los estudios. El PRP se ha utilizado como sobrenadante derivado del PRP o como PRP no activado. Se pudieron identificar dos rangos para la concentración de plaquetas, el primero entre 0,14 × 106 y 0,80 × 106 plaquetas/µL y el segundo entre 1,086 × 106 y 10 × 106 plaquetas/µL. Varios estudios han combinado el PRP con un tamaño de grupo que varía entre cuatro y nueve donantes. La dosis óptima de PRP o sobrenadante de PRP es del 10%. El PRP o los sobrenadantes derivados del PRP tienen un efecto positivo sobre el número y el tamaño de las colonias de MSC, la proliferación celular, la diferenciación celular y la estabilidad genética. Se ha demostrado que el uso de PRP empobrecido en leucocitos es una alternativa factible a los sueros xenogénicos. Sin embargo, es necesario mejorar la descripción de la metodología de preparación del PRP, así como su

composición. Se han identificado y presentado varios argumentos con el fin de crear directrices para futuras investigaciones.

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Review

Platelet-Rich Plasma as an Alternative to Xenogeneic Sera in Cell-Based Therapies: A Need for Standardization

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Abstract: There has been an explosion in scientific interest in using human-platelet-rich plasma (PRP) as a substitute of xenogeneic sera in cell-based therapies. However, there is a need to create standardization in this field. This systematic review is based on literature searches in PubMed and Web of Science databases until June 2021. Forty-one studies completed the selection criteria. The composition of PRP was completely reported in less than 30% of the studies. PRP has been used as PRP-derived supernatant or non-activated PRP. Two ranges could be identified for platelet concentration, the first between 0.14×10^6 and 0.80×10^6 platelets/µL and the second between 1.086×10^6 and 10×10^6 platelets/µL. Several studies have pooled PRP with a pool size varying from four to nine donors. The optimal dose for the PRP or PRP supernatant is 10%. PRP or PRP-derived supernatants a have positive effect on MSC colony number and size, cell proliferation, cell differentiation and genetic stability. The use of leukocyte-depleted PRP has been demonstrated to be a feasible alternative to xenogeneic sera. However, there is a need to improve the description of the PRP preparation methodology as well as its composition. Several items are identified and reported to create guidelines for future research.

Keywords: platelet-rich plasma; cell therapy; PRGF; stem cells; xenogeneic supplements; regenerative medicine; cell culture

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1. Introduction

Cell therapy represents a promising alternative approach to repair damaged tissues in many clinical applications where the use of biomaterials may not be sufficient [1]. Mesenchymal stem cells (MSCs) are frequently, but not always, the primary cell source in regenerative medicine. They are good candidates due to their great ability of self-renewal and multilineage differentiation along with strong immunosuppressive properties [2,3]. Human MSCs can be isolated from several tissues, mainly bone marrow, adipose tissue and umbilical cord blood. However, their low prevalence makes them insufficient for clinical applications without prior ex vivo expansion [4].

Currently, fetal bovine serum (FBS), also referred to as fetal calf serum (FCS), is the most widely used cell culture supplement in both the research and clinical fields [5]. However, the use of xenogeneic products involves many safety and regulatory concerns. FBS is an ill-defined supplement with great variability among batches. Its use entails a risk of zoonotic transmission of contaminants, such as viruses or prions, and possible adverse immunological reactions due to xenogeneic components. Additionally, obtaining

blood from the animals involves certain ethical and welfare issues [4,6]. All these concerns demand suitable alternatives to develop new culture supplements for clinical application following the Good Manufacturing Practice (GMP). In this sense, from 1979 until the present date, an increasing amount of experimental works have been caried out using FBS substitutes as culture medium supplements, with the majority of the articles having been published by researchers in USA, China and Italy [7]. On the other hand, annually, more than 2 million bovine fetuses are used worldwide to produce approximately 800,000 L of FBS for biological research, clinical trials, and pharmaceutical production. However, there is a simultaneous increasing demand with a restricted supply due to climate change and the reduction in livestock reserves. Consequently, FBS costs have been significantly increased (over 300% in recent years as of 2016) as well as questionable practices in production having been adopted [5]. In this unfavorable context, PRP becomes a promising alternative to FBS for cell expansion, reducing the risk of xenoimmunization and zoonotic transmission as well as the cost of its acquisition [8].

Platelet-rich plasma provides an interesting tool to influence the cells and trigger changes that activate several physiological processes that conclude in tissue healing [9,10]. Indeed, platelets contribute to this by the release of physiologically balanced biomolecules that can orchestrate cell behavior in terms of growth, recruitment, differentiation and morphogenesis. These biomolecules are sourced from platelet granules (alpha, delta and lambda granules) and plasma [11]. Platelets interact with cells by the release of growth factors upon binding to their receptors on the cell surface. For example, platelet-derived growth factor (PDGF) interacts with mesenchymal cells (such as fibroblasts, osteoblasts and adipocytes) that express α- and β-type receptors [12]. These receptors participate in the transduction of proliferative stimulus and β-type receptors participate in the transduction of chemotaxis [13]. Another important mediator of cell communication is beta-transforming growth factor (β-TGF), which participates in all physiological processes [14]. Most of the cells express receptors for this growth factor that induces mesenchymal stem cells to proliferate and differentiate [15]. It is an angiogenic factor. However, it has an inhibitory effect on osteoclast formation and epithelial cell proliferation [16]. The epidermal growth factor (EGF) induces epithelial cell and fibroblast recruitment and proliferation. It plays an important role in the synthesis of the granulation tissue. For example, a high number of EGF receptors are expressed by pre-chondrocytes, fibroblasts and pre-osteoblasts [17]. In this regard, fibronectin interacts with cells as well as components of the extracellular matrix to promote cell proliferation and migration in order to replace the blood clot by the provisional matrix [18]. Basic-fibroblastic growth factor (b-FGF or FGF-2) is a mitogenic and angiogenic factor that orchestrates the proliferation of mesenchymal stem cells [19,20]. Insulin-like growth factor-I (IGF-I) is pro-angiogenic and induces the proliferation of pre-osteoblasts and the extracellular matrix formation by osteoblasts [21–23]. It influences mesenchymal stem cell proliferation and differentiation during the generation of cartilage, adipose tissue, muscles and neurons [24,25]. Angiopoietin-2 and vascular endothelial growth factor (VEGF) work together to promote angiogenesis [26]. Interestingly, platelets release, for example, platelet factor 4 (PF4) that inhibits angiogenesis probably to control angiogenesis [16]. VEGF is a mitogenic factor and stimulates the differentiation of different cells (such as fibroblasts, epithelial cells and renal cells) [27]. Platelet-released nucleotides (ATP and ADP) activate other platelets and participate in all phases of tissue healing. The latter is supported by the expression of P2 receptors (binds extracellular nucleotides) in almost all cell types [28]. Platelets store 95% of the neurotransmitter serotonin present in the blood. It is a mitogenic factor (for example, hepatocyte- and osteoblast-like cells) that participates in tissue remodeling [29–32]. Moreover, platelets release biomolecules (thrombocidins, PF4, RANTES, platelet basic protein and thymosin beta 4) that mediate their anti-microbial potential [33,34]. Other molecules, such as interleukin 4 (IL-4), hepatocyte growth factor and tumour necrosis factor alpha (TNF-α), could regulate inflammation through the inhibition of the activation of transcription factor kappa-B (NF-κB) and the expression of cyclooxygenase 2 (COX2) and C-X-C chemokine receptor type 4 (CXCR4) [35].

During the processes of activation and apoptosis, platelets release extracellular vesicles (EVs). The International Society for Extracellular Vesicles (ISEV) defines exosomes as the smallest extracellular vesicles, bound by a lipid bilayer and without a functional core, released through an endocytic process [36]. EVs constitute a heterogeneous population of membrane vesicles consisting of exosomes (30–150 nm), microvesicles (100–1000 nm) and apoptotic bodies (100–5000 nm). These vesicles carry important bioactive molecules, including proteins, lipids and mitochondrial DNA, as well as miRNA, long non-coding RNA and mRNA. These vesicles can be taken up by other cells, which introduces another level of complexity in terms of intercellular signaling [37]. Each type of EV has unique characteristics, and its composition represents the cell type of origin and its physiological state. This "origin marker" is responsible for their function and confers organotropic properties that give them specificity of action [38,39]. Since exosomes can penetrate tissues inaccessible to platelets, such as joints, lymph and bone marrow, the dissemination of platelet components in tissues and organs beyond the blood may be one of their most important functions. Thus, platelet exosomes have been found to participate in a variety of important biological and pathological processes, including coagulation, angiogenesis, inflammation, immunoregulation and tumor progression [40].

In this sense, human blood derivatives have been proposed as replacements for xenogeneic supplements [8,41]. In recent years, the use of platelet-rich plasma (PRP) has undergone a major development in many applications of the regenerative medicine field [9,16,42,43]. Its rationale for use lies in the physiological role of platelets, which, upon activation, release growth factors and other bioactive molecules, thus promoting the wound healing process [42–48]. Still, depending on the method to obtain PRP, the composition and concentration of its components may be affected and, ultimately, its biological effect [9,11,48–50].

Thus, the aim of this review is to gather the current evidence on the use of PRP as an alternative to the widely used xenogeneic products, based on animal sera, and as a cell culture supplement aimed at cell therapies in order to establish criteria for the optimal characteristics of PRP for this application.

2. Methods

This systematic review was performed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Figure 1). It was based on literature searches performed in PubMed and the Web of Science database until 4 June 2021 using ((PRP) OR (platelet rich plasma) OR (plasma rich in growth factors) OR (PRGF)) AND (cell culture) AND (cell culture supplement) as the search strategy. The inclusion criteria were (1) the use of PRP or PRP derivatives as supplement in the culture medium and (2) the presence of a control group (xenogenic supplement). Papers that met the following criteria were excluded from the analysis: (1) the language of the article was any other than English or Spanish; (2) out of scope; (3) non-human origin of PRP or cells; (4) being prepared by aphaeresis or not PRP; (5) clinical studies; (6) reviews, thesis, book chapters or communications at conferences; (7) no full-text available; and (8) duplicates. The protocol of this systematic review was not registered.

Data extraction: The articles in each database were evaluated for inclusion in this review by two independent reviewers (MZ and MT) according to the selection criteria. First, the articles were screened by reading the title and the abstract. Then, the full text of those articles that could be eligible or were doubtful for inclusion were consulted. The reviewers resolved possible discrepancies throughout the entire process by consensus. For data extraction, a template was created as a file in Microsoft Excel to include the following data: author and year of publication, cell phenotype, type of blood-derived supplement, PRP preparation method (type of anticoagulant, number of centrifugations, PRP obtaining and activation method) and composition, xenogeneic product tested, dose of PRP and xenogeneic supplementation in the culture medium, type of assays and results.

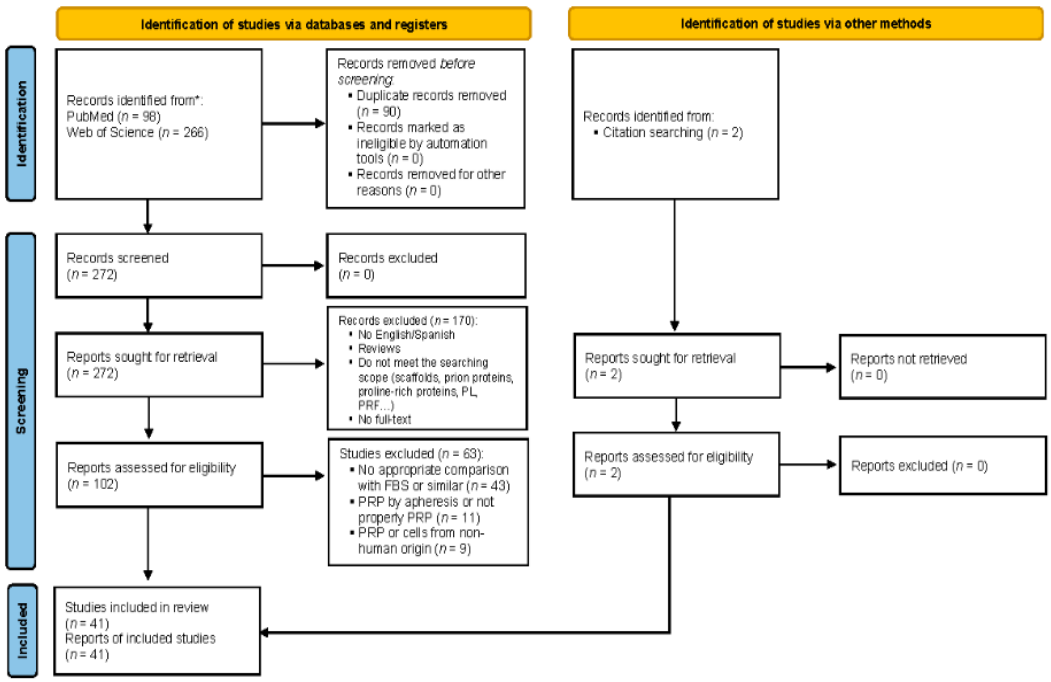

Figure 1. Flowchart summarizing the identification, screening, eligibility and inclusion of the studies in this review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Assessment of the reporting quality and risk of bias: The criteria reported by Golbach et al. was applied to assess the quality and the risk of bias [51]. The presence ("yes/partly") or absence ("no") of essential information determined the reporting quality of the article. Three grades for the risk of bias were used—low, moderate, or high—depending on whether the answers were "yes", "partly" or "no", respectively. If details were not provided, then the risk of bias was judged as unknown.

3. Results

The search strategy produced a total of 366 articles to which 2 papers were added from other sources (Figure 1). However, 92 articles were removed as duplicates. Consequently, the number of papers to be more exhaustively screened was 274, of which 170 met the exclusion criteria and were also removed. Finally, 41 papers, as shown in Table 1, were included for the analysis.

Table 1. Detailed information of the studies included in this review.

Reference	Phenotype	Comparison Groups	Assays	Conclusion
Amable et al., 2014 [52]	BM-MSC, AT-MSCs and WJ-MSC	10% FBS vs. 1%, 2.5%, 5%, 10%, 20%, 30%, 40%, 50% PRP	Proliferation, trilineage differentiation, gene expression, and cytokine, growth factor and extracellular matrix quantification	✓
Anitua et al., 2019 [49]	hDPSCs	10% FBS vs. 10% PRGF	Isolation, migration, proliferation, osteogenic and adipogenic differentiation, senescence and cryopreservation	✓
Atashi et al., 2015 [50]	AT-MSCs	10% FBS vs. 1%, 5%, 10%, 20%, 40%, 60% of either nPRP or tPRP	Cell viability, cell proliferation, cell phenotype, trilineage differentiation, chromosome stability cytogenetic analysis	✓
Barlian et al., 2018 [53]	ADSCs	10% FBS (control) vs. 5%, 10%, 20% PRP	Chondrogenic differentiation	✓
Barlian et al., 2020 [54]	WJ-MSCs	10% FBS vs. 10% PRP	Chondrogenic differentiation (collagen type II, GAG accumulation).	✓
Beccia et al., 2021 [55]	ASCs	10% FBS vs. 2% PRP	Morphology and proliferation	✘
Berndt et al., 2019 [56]	NHDF	10% FBS vs. 1%, 5%, 10%, 20%, 30%, 40%, 50% PRP	Cell proliferation, cell cycle analysis, cell morphology, alpha-SMA and vimentin expression, metabolic activity assessment, cell adhesion, wound healing, genomic stability	✓
Berndt et al., 2021 [57]	NHDF	10% FBS vs. 1%, 5%, 10%, 20%, 30%, 40%, 50% PRP	NHDF proliferation and activation.	✓
Bindal et al., 2019 [58]	hiDPSCs	10% FBS vs. 10%, 20% PRP	Viability, proliferation, proangiogenic gene expression, proangiogenic growth factor release	✓
Brini et al., 2016 [59]	hDFs / hObs	10% FBS vs. 5% PRGF (cell proliferation and viability) 10% FBS vs. 2.5% PRGF (osteoblast differentiation)	Cell proliferation and viability, osteogenic differentiation	✓
Chieregato et al., 2011 [60]	ADSCs	10% FBS vs. 10% hPRP	Morphology, CFU, proliferation and MEK-1/2 role, multiple differentiation capacity, immunophenotype	✓

Table 1. *Cont.*

Reference	Phenotype	Comparison Groups	Assays	Conclusion
do Amaral et al., 2015 [61]	NCCs and MSCs from bone marrow	10% FBS vs. 1%, 2.5%, 5%, 10% PRPr	Cell proliferation, GAGs, pellet area measurement, chondrogenic genes quantification, sGAG quantification	✓
Gonzales et al., 2013 [62]	MFCs	10% FBS vs. 5%, 10%, 20% PRP	DNA quantification, gene expression (col I, col II and aggrecan), histology (H&E)	✓
Hernáez-Moya et al., 2020 [63]	LESCs	5% FBS vs. 10% s-PRGF	Cell growth, cell size and gene expression of stem/progenitor limbal cells markers and K12 marker for corneal epithelial differentiation	✓
Hosseini et al., 2017 [64]	Human ovarian cells	10% FBS vs. 10% PRP	Follicle growth and viability assessment. Histological analysis.	✓
Ismail et al., 2020 [65]	SVF cells	10% FBS vs. 10% tPRP	Cell number and clonogenicity	✓
Kazemneja et al., 2014 [66]	MenSCs	10% FBS vs. PGS vs. PRP vs. HPR (proliferation assays) 15% FBS vs. PGS vs. PRP vs. HPR (differentiation assays)	Cell proliferation and osteogenic differentiation (Alizarin Red, ALP activity, OCN level)	✓
Kinzebach et al., 2013 [67]	LA-MSC and BM-MSC	2.5%, 5%, 7.5%, 10% FBS vs. 2.5%, 5%, 7.5%, 10% tPRP	Expansion of MSCs and differential proteomics, proliferation and stimulation assays. Growth factors quantification and cytokine receptors expression. Adipo- and osteogenic differentiation.	✓
Kishimoto et al., 2013 [68]	ASCs and BMSCs	0.125%, 0.25%, 0.5%, 1%, 2%, 4% FBS vs. 0.125%, 0.25%, 0.5%, 1%, 2%, 4% PRP (optimal concentration) 2%, 10% FBS vs. 0.5% PRP (proliferation)	Determination of the supplement optimal concentration, proliferation	✓
Kokaoemer et al., 2007 [69]	AT-MSCs	10% FCS vs. 10% tPRP	Morphology, adhesion, CFU, cumulative population doubling rates, adipogenic and osteogenic differentiation, immunophenotype	✓
Lang et al., 2017 [70]	ASCs	20% FCS vs. 10%, 20% PRP	Cell cycle analysis, expression of PDGF receptorβ, c-MYC, and MEK-1, PDGF receptor β Inhibition	✓
Loibl et al., 2016 [71]	ASCs	20% FCS vs. 10%, 20% ACS	Cell cycle analysis	✓
Loibl et al., 2016 [72]	ASCs	20% FCS vs. 10%, 20% ACS	Cell cycle analysis and proteomic profile	✓

Table 1. *Cont.*

Reference	Phenotype	Comparison Groups	Assays	Conclusion
Martínez et al., 2019 [73]	PDL cells	10% FBS vs. 2.5%, 5%,10% PRP	Cell proliferation and clonogenic proliferation	✓
McLaughlin et al., 2016 [74]	ASCs	10% FBS vs. 10% tPR	Morphology, growth rate, gene expression (BMP-2, BMP-4, VEGF, TGF-beta, PDGF-B and FGF-2)	✓
Muraglia et al., 2014 [75]	MSCs from bone marrow/human skin fibroblasts/hObs/human articular chondrocyte	10% FCS vs. 5% PRP	Clonogenic assay (MSCs) and cell viability of primary cultures	✓
Okada et al., 2016 [76]	hDFCs	10% FBS vs. 1%, 5%, 10%, 20% PRGF	Osteogenic differentiation, cell proliferation, cell migration and osteogenic gene expression	✓
Phetfong et al., 2017 [77]	ADMSCs	FBS vs. Hplasma	Cell morphology, proliferation, CFU, immnuphenotyping, osteogenic and adipogenic differentiation, senescence	✓
Ramos-Torrecillas et al., 2014 [78]	Human gingival fibroblasts	10% FBS vs. 10% PRP	Cell growth rate, cell morphology and antigenic expression	✓
Riestra et al., 2017 [79]	LESCs	10% FBS vs. 10% PRGF	Measurement of the extent of outgrowths of cultures of LEPCs, number of cells and colony forming efficiency. Morphological analysis and immunocytochemistry and quantification of p63-α IHCE.	✓
Rosadi et al., 2019 [80]	ADSCs	10% FBS vs. 10%PRP	Cell proliferation, differentiation assays (GAG levels and mineralization, secretion of TGF-β1, expression of specific stem cell surface protein markers, gene expression)	✓
Simon et al., 2018 [81]	MSCs	10% FCS vs. 10% FCS+bFGF vs. 10% PRP	Cell proliferation, lineage-specific markers, gene expression	✓
Suchánek et al., 2016 [82]	SHED	2% FCS + GFs (FCS+) vs. 2% PRP + GFs (PRP+)	Proliferative capacity, cumulative population doubling, morphology, viability, expressing cluster of differentiation	✓
Suchánková et al., 2014 [83]	hDPSC	2% FCS vs. 2% PRP	Proliferation, population doublings, viability, phenotypic analysis	✓
Sun, Xiaojiang et al., 2008 [84]	MSCs from bone marrow	10% FBS vs. 10% APM	Cell morphology, proliferation, surface markers, growth cycle, and apoptosis; osteogenic differentiation; number and area of ALP$^+$CFU-Fs; adipogenic differentiation.	✓
Talebi et al., 2021 [85]	CCRF-CEM	10% FBS vs. 2%, 5%, 10%, 15% PRP	Cell viability and YKL-40 mRNA and protein levels.	✓

Table 1. *Cont.*

Reference	Phenotype	Comparison Groups	Assays	Conclusion
Tavakolinejad et al., 2014 [86]	ADSCs	10% FBS vs. 10%, 15% hPRP	Proliferation and osteogenesis	✓
Tchang et al., 2017 [87]	SVF cells	10% FBS vs. 10% tPRP	2D mineralization assay and 3D angiogenesis	✓
Van Pham et al., 2014 [88]	UCB-MSCs	10% FBS vs. 2, 5, 7, 10% PRP	Number of adherent cells and their expansion, percentage of successfully isolated cells in the primary culture, surface marker expression, in vitro differentiation potential following expansion	✓
Vogel et al., 2006 [89]	BM-MSC	2% FCS vs. 3% PRP	Growth rate; osteogenic, adipogenic and chondrogenic differentiation capacity	✓
Xian et al., 2015 [90]	Human keratinocytes and fibroblasts from skin	5% FBS vs. 10%, 20% PRP	Extracellular matrix gene expression, proliferation, migratory property, soluble factors secretion	✓

ACS: autologous conditioned plasma; **ADMSCs:** human adipose-derived mesenchymal stem cells; **ADSCs:** human adipose-derived mesenchymal stem cells; **ADSC-SS:** ADSCs cultured on the scaffold; **ALP:** alkaline phosphatase; **ALP$^+$CFU-Fs:** alkaline phosphatase–positive (ALP$^+$) fibroblast colony-forming units (CFU-Fs) under osteogenic conditions; **APM:** autologous plasma derived from bone marrow; **ASCs:** human adipose-derived stem cells; **AT-MSCs:** human adipose-derived mesenchymal stem cells; **BM-MSC:** human mesenchymal stromal cells from bone marrow; **BMP-2:** bone morphogenetic protein 2; **BMP-4:** bone morphogenetic protein 4; **BMSCs:** bone marrow-derived mesenchymal stem cells; **CCRF-CEM:** human T lymphoblasts from acute lymphoblastic leukemia; **CFE:** colony-forming efficiency; **CFU:** colony-forming units; **FBS:** fetal bovine serum; **FCS:** fetal calf serum; **FGF-2:** Fibroblast growth factor 2 (basic); **GAG:** glycosaminoglycans; **hDFs:** human dermal fibroblasts; **hDFCs:** human dental follicle cells; **hDPSCs:** human dental pulp stem cells; **hiDPSCs:** human inflamed dental pulp stem cells; **HAS:** human serum albumin; **HPR:** human platelet releasate; **hObs:** human osteoblasts; **Hplasma:** human plasma; **hPRP:** human platelet-rich plasma; **LA-MSC:** lipoaspirate-derived MSC; **LESCs:** Human limbal epithelial stem/progenitor cells; **MenSCs:** menstrual-blood-derived stem cells; **MFCs:** human meniscal fibrochondrocytes; **MSCs:** mesenchymal stem cells; **NCCs:** human nasoseptal chondrogenic cells; **NHDF:** Normal human dermal fibroblasts; **nPRP:** non-activated PRP; **OCN:** osteocalcin; **OIM:** osteogenic induction medium; **PDGF-B:** platelet-derived growth factor subunit B; **PDL:** periodontal ligament; **PGS:** platelet gel supernatant; **PRGF:** plasma rich in growth factors; **PRPr:** platelet-rich plasma releasate; **TGF-beta:** transforming growth factor beta; **SHED:** ecto-mesenchymal stem cells from human exfoliated deciduous teeth; **s-PRGF:** Serum derived from plasma rich in growth factors; **SVF:** stromal vascular fraction; **tPRP:** thrombin-activated PRP; **UCB-MSCs:** human umbilical–cord blood-derived MSCs; **VEGF:** vascular endothelial growth factor; **WJ-MSC:** Wharton's Jelly-derived MSC; ✓: experimental results supporting the replacement of xenogeneic supplements with PRP; ✘: best experimental results for the FBS supplement.

3.1. Reporting Quality and Risk of Bias

The assessment of the reporting quality indicated that the aspects on which the studies focused the most were cell origin and the statements regarding the conflict of interest or ethical aspects (Figure 2). The composition of the PRP was the least considered, being completely reported in less than 30% of the included articles. Less than 50% of the included studies completely reported on cell characteristics. Sample size was adequately reported in almost 60% of the studies. Low performance bias was judged in most of the articles, and it was more favorable for the item regarding duration of treatment. The method used for measuring the outcomes was considered appropriate in all the reviewed articles.

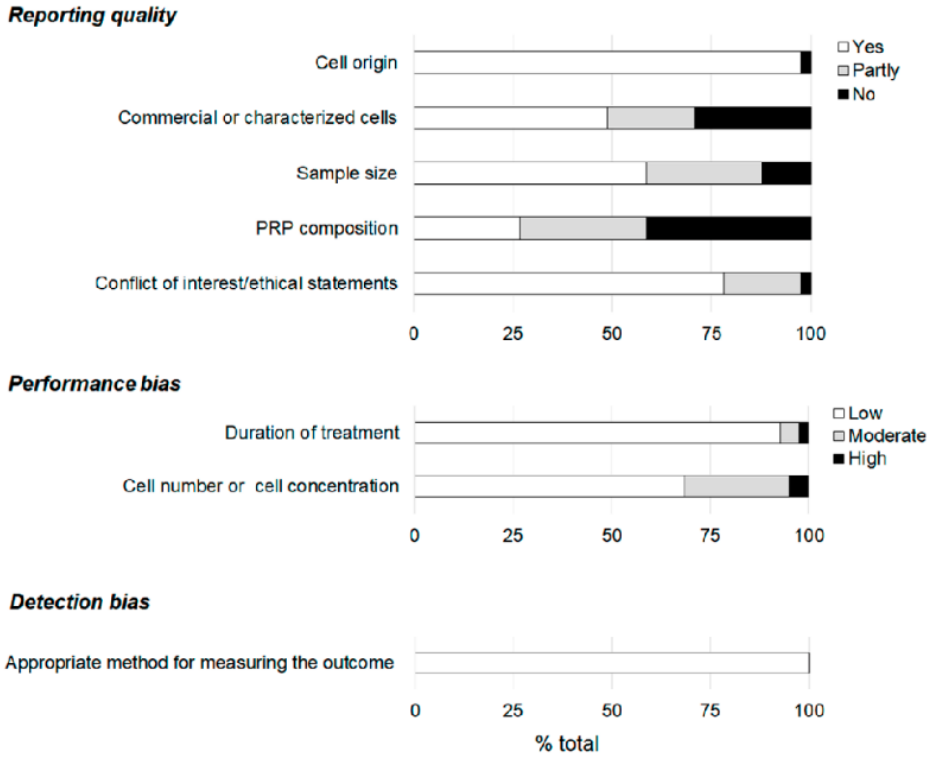

Figure 2. Assessment of the quality and risk of bias of the included studies according to the criteria reported by Golbach et al. [51].

3.2. Human Versus Xenogeneic Cullture Medium Supplement

Regarding the xenogeneic culture medium supplement, FBS (also known as FCS) was the only animal origin supplement used in the reviewed papers. Table 2 describes the methods used for the preparation of the PRP.

Table 2. Description of the PRP acquisition process in the reviewed articles.

Reference	Type of Anticoagulant	Comparison Groups	Number of Centrifugations	PRP Acquisition	Activation Method
Amable et al., 2014 [52]	ACD	10% FBS vs. 1%, 2.5%, 5%, 10%, 20%, 30%, 40%, 50% PRP	Two	Platelet-containing plasma above the buffy coat. Platelets concentrated and suspended in a smaller volume of plasma.	Calcium chloride
Anitua et al., 2019 [49]	Sodium citrate	10% FBS vs. 10% PRGF	One	PRGF: plasma column just above the buffy coat.	Calcium chloride
Atashi et al., 2015 [50]	Sodium citrate	10% FBS vs. 1%, 5%, 10%, 20%, 40%, 60% of either nPRP or tPRP	One	Regenkit: plasma containing platelets above the white and red blood cells.	nPRP: non-activated
	W/o	10% FBS (control) vs. 5%, 10%, 20% PRP		Regenkit: plasma over the red and most of the white blood cells formed a clot. The serum extracted from the clot was added 1:10 to PRP to activate the platelets and obtain tPRP.	tPRP: thrombin
Barlian et al., 2018 [53]	NA	10% FBS vs. 10% PRP	NA	NA	NA
Barlian et al., 2020 [54]	NA	10% FBS vs. 2% PRP	NA	NA	NA
Beccia et al., 2021 [55]	Buffered solution of sodium citrate, theophylline, adenosine and dipyridamole	10% FBS vs. 1%, 5%, 10%, 20%, 30%, 40%, 50% PRP	Two	Plasma portion separated from cells.	Non-activated
Berndt et al., 2019 [56]	NA	10% FBS vs. 1%, 5%, 10%, 20%, 30%, 40%, 50% PRP	One	Plasma containing platelets, remained above the gel layer, was homogenized by turning the tube five times.	Non-activated
Berndt et al., 2021 [57]	NA	10% FBS vs. 10%, 20% PRP	One	The red and white blood cells are trapped under the gel, and platelets settled on the surface of the gel are resuspended by inverting the tube.	Non-activated
Bindal et al., 2019 [58]	NA	10% FBS vs. 5% PRGF (cell proliferation and viability) 10% FBS vs. 2.5% PRGF (osteoblasts differentiation)	NA	NA	Freezing

Table 2. *Cont.*

Reference	Type of Anticoagulant	Comparison Groups	Number of Centrifugations	PRP Acquisition	Activation Method
Brini et al., 2016 [59]	Sodium citrate	10% FBS vs. 10% hPRP	One	PRGF, the 2 mL plasma just above the buffy coat containing the highest platelets concentration, was collected.	Calcium chloride
Chieregato et al., 2011 [60]	Heparin	10% FBS vs. 1%, 2.5%, 5%, 10% PRPr	One	NA	Freezing and sonication
Do Amaral et al., 2015 [61]	Citrate sodium	10% FBS vs. 5%, 10%, 20% PRP	Two	PRP from protocol 2: The upper plasma fraction without leukocyte and red cells was centrifuged. Platelets pellet was resuspended with the supernatant (platelet-poor plasma).	Calcium chloride
Gonzales et al., 2013 [62]	Hirudin	5% FBS vs. 10% s-PRGF	One	The upper phase containing PRP.	Thrombin??
Hernáez-Moya et al., 2020 [63]	Sodium citrate	10% FBS vs. 10% PRP	One	The complete supernatant fraction without red and white blood cells.	Calcium chloride
Hosseini et al., 2017 [64]	Acid citrate solution	10% FBS vs. 10% tPRP	Two	The top and middle layers after the first centrifugation were centrifuged again and the remaining 0.5 mL of plasma containing precipitated platelets was mixed evenly and considered to be PRP.	Thrombin
Ismail et al., 2020 [65]	NA	10% FBS vs. 10% PGS vs. 10% PRP vs. 10% HPR (proliferation assays) 15% FBS vs. 15% PGS vs. 15% PRP vs. 15% HPR (differentiation assays)	NA	Two platelet concentrates from buffy coats extracted from whole-blood donations of four AB blood group-typed donors were pooled and suspended in the plasma of one AB donor.	Thrombin
Kazemnejad et al., 2014 [66]	NA	2.5%, 5%, 7.5%, 10% FBS vs. 2.5%, 5%, 7.5%, 10% tPRP	NA	NA	PRP: freezing PGS: thrombin HPR: thrombin
Kinzebach et al., 2013 [67]	NA	0.125%, 0.25%, 0.5%, 1%, 2%, 4% FBS vs. 0.125%, 0.25%, 0.5%, 1%, 2%, 4% PRP (optimal concentration) 2%, 10% FBS vs. 0.5% PRP (proliferation)	NA	Buffy coat-derived pooled platelet concentrates.	Thrombin
Kishimoto et al., 2013 [68]	Sodium citrate	10% FCS vs. 10% tPRP	Two	The upper 1 cm of the erythrocyte layer was collected as the PRP layer.	Freezing

Table 2. *Cont.*

Reference	Type of Anticoagulant	Comparison Groups	Number of Centrifugations	PRP Acquisition	Activation Method
Kokaoemer et al., 2007 [69]	NA	20% FCS vs. 10%, 20% PRP	NA	Pooled platelet concentrate out of buffy coats.	Thrombin
Lang et al., 2017 [70]	W/o	20% FCS vs. 10%, 20% ACS	One	Arthrex: a plasma layer appeared on the top and the red/white blood cell layer was apparent at the bottom. The plasma containing the platelets was isolated.	Freezing
Loibl et al., 2016 [72]	NA	20% FCS vs. 10%, 20% ACS	One	Arthrex	NA
Loibl et al., 2016 [71]	NA	10% FBS vs. 2.5%, 5%, 10% PRP	One	Arthrex: a plasma layer appeared on the top and the red/white blood cell layer was apparent on the bottom. The plasma, containing the platelets, was isolated.	Freezing
Martínez et al., 2019 [73]	NA	10% FBS vs. 10% tPR	One	GPS III	Calcium chloride and thrombin
McLaughlin et al., 2016 [74]	NA	10% FCS vs. 5% PRP	NA	Harvest SmartPrep System	Thrombin
Muraglia et al., 2014 [75]	NA	10% FBS vs. 1%, 5%, 10%, 20% PRGF	Multiple	Buffy coat samples. The platelet pellet was brought to a final volume with PPP to obtain a concentration of 10×10^6 platelets/µL.	Freezing
Okada et al., 2016 [76]	Sodium citrate	10% FBS vs. 10% Hplasma	One	PRGF: the plasma fraction (1 mL over the buffy coat) was collected as F2.	Calcium chloride
Pham et al., 2013 [88]	NA	10% FBS vs. 10% PRP	Two	After the second centrifugation, the platelet pellet was resuspended with the third of the plasma volume.	Calcium chloride
Phetfong et al., 2017 [77]	NA	10% FBS vs. 10% PRGF	NA	It was prepared from FFP.	Calcium chloride
Ramos-Torrecillas et al., 2014 [78]	Lithium heparin	10% FBS vs. 10% PRP	Two	The whole plasma portion and top layer of red blood cells. After a new centrifugation, the upper portion of the plasma was discarded, and the remainder was the PRP.	NA
Riestra et al., 2017 [79]	Sodium citrate	10% FCS vs. 10% FCS + bFGF vs. 10% PRP	One	Liquid PRGF: the lower 2 mL of the plasma column (F2) was discarded. The rest of the plasma column (F1) was drawn off avoiding the buffy coat.	Calcium chloride

Table 2. *Cont.*

Reference	Type of Anticoagulant	Comparison Groups	Number of Centrifugations	PRP Acquisition	Activation Method
Rosadi et al., 2019 [80]	NA	2% FCS + GFs (FCS+) vs. 2% PRP + GFs (PRP+)	NA	NA	NA
Simon et al., 2018 [81]	NA	2% FCS vs. 2% PRP	Two	The platelet-rich layer above the buffy coat was centrifuged and the resulting platelet pellet was resuspended to obtain PRP.	Non-activated
Suchánek et al., 2016 [82]	NA	10% FBS vs. 10% APM	NA	NA	NA
Suchánková et al., 2014 [83]	NA	10% FBS vs. 2%, 5%, 10%, 15% PRP	NA	NA	NA
Sun, Xiaojiang et al., 2008 [84]	NA	10% FBS vs. 10%, 15% hPRP	Two	Bone marrow was concentrated through density gradient centrifugation. After removing the remaining red blood cells and fatty droplets by centrifugation, the APM was collected.	NA
Talebi et al., 2021 [85]	Sodium citrate	10% FBS vs. 10% tPRP	Two	Supernatant from the first centrifugation, including PRP, was again centrifuged and the lower half part, which contains a large number of platelets in the form of the platelet plug, was considered as PRP.	Shaking at 22 °C for nine days.
Tavakolinejad et al., 2014 [86]	NA	10% FBS vs. 2%, 5%, 7%, 10% PRP	Two	The platelets were precipitated and the plasma was removed; then, the platelets were resuspended in 50 mL plasma	Freezing
Tchang et al., 2017 [87]	NA	2% FCS vs. 3% PRP	NA	Platelet concentrates from buffy coats extracted from whole blood.	Thrombin
Vogel et al., 2006 [89]	Citrate–phosphate–dextrose	5% FBS vs. 10%, 20% PRP	One	Allogenic leukocyte-depleted PRP was obtained from a blood bank. Pooled buffy coats were centrifuged and leukocyte-depleted by filtration.	NA
Xian et al., 2015 [90]	Sodium citrate	5% FBS vs. 10%, 20% PRP	Two	Harvested PRP without red cells was concentrated by discarding 2 mL of plasma after the second centrifugation.	Non-activated

ACD: citrate dextrose solution A; **APM:** autologous plasma derived from bone marrow; **F2:** fraction 2; **FFP:** fresh frozen plasma; **HPR:** human platelet releasate; **NA:** not available; **nPRP:** non-activated PRP; **PGS:** platelet gel supernatant; **PPP:** platelet-poor plasma; **PRGF:** plasma rich in growth factors; **PRP:** platelet-rich plasma; **tPRP:** thrombin-activated PRP; **W/o:** without.

A complete description of the cell composition of PRP was provided only in two articles, and commercial systems were used in another nine articles (PRGF [55,80,83], Regenkit [50], Arthrex [70–72], GPS III [73] and Harvest SmartPrep System [74]). The platelet concentration was lower than 1×10^6 platelets/µL in 13 studies with a range from 0.141×10^6 to 0.8×10^6 platelets/µL [50,56,57,59,60,62,68,70–72,76,77,80]. Most of these studies had a platelet concentration of $\leq 0.5 \times 10^6$ platelets/µL [50,56,57,59,62,70–72,76,80]. In 10 studies, the platelet concentration was higher than 1×10^6 platelets/µL (range from 1.086×10^6 to 10×10^6 platelets/µL) [54,57,65,69,70,73,78,79,91,92]. Most of these studies had a concentration between 1×10^6 and 3×10^6 platelets/µL [54,57,65,69,70,73,91,92]. However, there was a consensus among all the studies included in the review in preparing a leukocyte-reduced PRP. Activated PRP versus its inactivated form was the most used option (84%), whereas no information concerning this issue was available in a considerable number of articles (22%). For the activation of the platelets, different agents were used: calcium chloride (31% PRP products), thrombin (34% PRP products) and calcium chloride + thrombin (3% PRP products) (Table 2). Physical methods of activation were also utilized: freezing (24% PRP), freezing + sonication (3% PRP products) and shaking for 9 days (3% PRP products). The PRP or the PRP-derived supernatant were stored frozen at −80 °C, cold at 4 °C, or lyophilized and stored at different temperatures.

3.2.1. Screening for the Optimal Dose of PRP in the Culture Medium

The screening for the optimal dose was not performed in all the included studies. For BM-MSCs, two studies screened the optimum dose of PRP-derived supernatants and a value of 10% was reached [52,67]. However, do Amaral et al. showed similar results to 10% FBS at a PRP concentration of 2.5% rather than 10% [61]. In another study, low concentrations (≤2%) of PRP were screened to prevent culture medium hardening to gel and 0.5% was selected as the optimum dose [68]. In the case of AT-MSCs, there are five studies that screened PRP-derived supernatants as an optimal dose against 10% FBS [54,56,57,71,90]. All these studies agree that a 10% PRP supplement would be the optimal dose. For the same cell phenotype, similar positive results were obtained using a 10% concentration [64,73,78,81], although 20% produced an optimal concentration in another three studies [70–72]. In the same way, the non-activated PRP was screened [56,59,72]. To prevent culture medium gelation, Beccia et al. selected a dose of 2%, whereas a dose of 0.5% was chosen by Kishimoto [55]. For Atashi et al., the optimal dose was 20% and did not report any gelling of the culture medium [50], while Rosadi et al. used a concentration of 10% PRP [80]. Only the study of Beccia et al. showed a lower cell proliferation for PRP with respect to the xenogeneic supplement [55]. It is worth mentioning that the use of non-activated PRP was more effective in inducing cell proliferation than PRP-derived supernatants in the study by Atashi et al. [50].

For Wharton's Jelly-derived MSCs, two experimental works found the optimal concentration of PRP supernatants at 10% [52,54]. In the case of dental pulp stem cells, PRP and human platelet lysate (supernatant) were screened at concentrations of 10% and 20% against 10% of FBS [58]. Both concentrations significantly increased the cell proliferation, with the highest being for the human platelet lysate at 20%. Better results for 10% of PRGF supernatant than 10% FBS were obtained in the isolation, migration, proliferation and differentiation (osteogenesis and adipogenesis) of DPSCs [49]. Similarly, 10% PRP produced the highest proliferation against 2% FCS [83]. Regarding human dermal fibroblasts, Berndt et al. screened different concentrations of PRP (1–50%) and the maximum response in cell proliferation and metabolic activity was reported at a concentration of 20% [56,57]. Xian et al. co-cultured dermal keratinocytes and fibroblasts to compare 5% FBS with 10% and 20% of PRP [90] as culture medium supplements, producing an increased tissue remodeling promoted by 10% PRP and encouraging inflammation and collagen deposition by 20% PRP. Human meniscal fibrochondrocytes showed a higher proliferation with 10 and 20% PRP as compared to 10% FBS, with the mRNA expression of collagen type I + being significantly lower at 3 days, but not at 7 days, for the PRP [62]. Human limbal progenitor cells were

cultured with 10% of PRGF supernatant and achieved a faster growth while maintaining the stem/progenitor phenotype in comparison with FBS (5% or 10%) [63,79]. Moreover, in addition to enhancing the colony-forming efficiency, PRGF could provide a fibrin scaffold for culturing human limbal progenitor cells [79]. Regarding the stromal vascular fraction, 10% of t-PRP enhanced the total number of cells without altering their clonogenicity as compared to 10% FBS [65,87]. Kazemnejad et al. compared different blood derivatives for the culture of human menstrual-blood-derived stem cells [66]. They found a similar effect for 10% PRP and 10% FBS on promoting the cell proliferation and mineralization process. However, osteogenic markers, such as osteocalcin and alkaline phosphatase, were higher in cells cultured with 15% PRP and 15% platelet gel supernatant compared to 15% FBS, respectively, although the human platelet releasate (no plasma proteins) was the one that scored more differences with FBS. Regarding periodontal ligament cells, 5% PRP, 10% PRP and 5% PPP enhanced more cell proliferation than 10% FBS [73]. Okada et al. observed that 10% and 20% of PRGF supernatants increased human dental follicle cells proliferation in comparison to 10% FBS and upregulated the gene expression related to bone regeneration [76]. Furthermore, the cell proliferation of ectomesenchymal stem cells from human exfoliated teeth was reported to be more enhanced after being cultured with 10% PRP (without growth factor supplements) than the results that were obtained with 2% FCS (supplemented with growth factors) [82]. However, at higher passages, it induced changes in the cell phenotype. Talebi et al. reported that CCRF-CEM proliferation increased at 10% and 15% PRP as compared to 10% FBS [85]. Human umbilical-cord-blood-derived MSCs cultured with different concentrations of PRP achieved the highest proliferation with 10% PRP [88]. Moreover, in other cell phenotypes (human ovarian cells and human gingival fibroblasts), better results were obtained by adding 10% PRP to the culture medium, as compared to 10% FBS [64,78]. Finally, human articular chondrocytes also showed a higher proliferation with 5% regenerated freeze-dried PRP compared to 10% FCS [75].

Most of the reviewed articles included stem cells, since those that assessed the xenogeneic supplement substitution for PRP in completely differentiated primary cells were only about a quarter. With regard to the origin of the cell type assessed, despite finding a high variability, cells from the adipose tissue were the most analyzed phenotype (17 out of the 41 articles), followed by those from dental origin. However, there was a high number of articles where minority phenotypes were found and, on the contrary, others in which cells from different origins were included (Table 1). Nonetheless, in order to make the comprehension of the results easier, the assessment of xenogeneic supplement substitution for PRP was performed according to the cell phenotype origin instead of referring to each revised article.

3.2.2. Adipose Tissue

Most of the studies included in this section evaluated the replacement of FBS/FCS at 10 or 20% concentrations. Although the PRP was screened over a wider range of concentrations, most of the articles (94%) did show that PRP can replace the xenogeneic culture supplements.

In all [50,52,53,55,60,65,67–72,74,77,80,86] but one paper [87], the ability of PRP to replace FBS was assessed in cell proliferation, cell growth or cell cycle processes. All authors [50,52,53,60,65,67–72,74,77,80,86] except Beccia et al. [55] showed similar or even statistically superior results of PRP when compared to FBS. Some of them pointed out that the cell response to PRP was dose-dependent [56,57,75,76,90]. However, there was a disparity of conclusions regarding the percentage that stimulates the highest proliferation rate. Amable et al. [52] claimed that PRP inhibits cell growth above 10%. In addition, Atashi et al. [50] reported less effective results in the case of activated PRP (tPRP) compared to non-activated PRP (nPRP), although in both cases they were superior to FBS. Lang et al. [70] and Lobil et al. [72] went even further by determining the mechanisms underlying PRP stimulation of proliferation. This effect could be mediated by the inactivation of Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase (PTEN) that might then activate

the PKB/AKT pathway. In fact, Chieregato et al. [60] reported the involvement of other signaling pathways, such as MEK-1/2. As it was mentioned previously, there was one paper whose conclusions were the opposite [55]. That is, PRP promoted cell proliferation, but at significantly lower level than FBS. This, however, could be related to the lower tested concentration of PRP. In this case, PRP was applied at 2%, whereas FBS was applied at 10%.

Cell morphology was also the subject of study in five studies [59,64,73,78,81]. All articles but Beccia et al. [55] agreed that the cells cultured with PRP were smaller and more spindle-shaped than those cultured with the xenogeneic supplements. The cell phenotype remained unchanged regardless of the supplement used [56,64,71,73]. Moreover, the use of PRP reduced cellular senescence [77] and did not alter chromosomal stability as revealed by cytogenetic analysis [50]. The colony-forming capacity of these stem cells was also evaluated [64,69,73,81], with the clonogenicity with both supplements not being different. In fact, Chieregato et al. [60] and Phetfong et al. [77] reported even more colony-forming units when the cells were cultured with PRP, maintaining the self-renewal of human adipose-derived stem cells after long-term culture.

The effect of PRP addition on cell differentiation was also widely studied. Two studies evaluated the trilineage differentiation [50,52]. Atashi et al. [50] observed that the differentiation potential was not affected regardless of the supplement used. In turn, Amable et al. [52] reported different results depending on the type of differentiation. PRP reduced adipogenic capacity, but increased osteogenic and chondrogenic differentiation. Interestingly, these authors also assessed the gene expression of pluripotent, adipogenic, osteogenic and chondrogenic markers. The use of PRP stimulated the expression of pluripotent genes and thereby downregulated differentiation markers, except for one of the quantified osteogenic markers (BMP2). Four studies assessed the effect of the PRP only on osteogenic and adipogenic differentiation [64,71,73,81]. The results showed that human adipose-derived stem cells were able to similarly differentiate with both culture supplements (PRP or FBS). Furthermore, Phetfong et al. [77] demonstrated a more robust osteogenic differentiation in the presence of the PRP. This was in accordance with the results of Chieregato et al. [60]. However, not only did they report an increase in osteogenesis, but also in adipogenic differentiation after cell exposure to PRP. Another two studies assessed only the chondrogenic differentiation and the results were, again, in favor of the use of the PRP supplement [53,80].

Regarding protein synthesis, Kocaoemer et al. showed a slightly higher total protein content in the culture medium of the PRP-treated cells (97.2 mg/mL and 87 mg/mL for tPRP and FCS, respectively) [69]. Moreover, Amable et al. showed that the protein secretion of adipose-tissue derived stem cells was altered by PRP supplementation [52], which is opposite (upregulated or downregulated) to that induced by FBS for most of the analyzed proteins (cytokines, growth factors, extracellular matrix and metalloproteinases). At the genetic level, the use of PRP, instead of FBS, upregulated the expression of BMP-2 and BMP-4 genes, while downregulating the expression of PDGF-B and FGF-2. The genetic expression of TGF-beta and VEGF was not significantly altered.

3.2.3. Oral Tissue

Seven articles were included in this category (Table 1). In all of them, the results were in favor of PRP as a suitable substitute of FBS/FCS to supplement the culture medium. There was no consensus on the optimal concentration of PRP. In fact, different percentages of PRP were studied, while the percentages of FBS/FCS remained unchanged, at 10% or 2%. Only in three of the articles was PRP composition partially detailed or a reference was made to the trademark that was already associated with the pre-defined parameters. Oral cells from different origins were used, but human dental pulp stem cells (hDPSCs) were predominant as they were used in 43% of the articles [55,62,87]. In the remaining studies, human dental follicle cells (hDFCs) [76], gingival fibroblasts [78], ecto-mesenchymal stem cells from human exfoliated deciduous teeth (SHED) [82] and periodontal ligament cells [73] were employed.

The comparison of PRP and FBS in the cell isolation process was only studied by Anitua et al. [49] who reported a significantly higher number of cells per explant in the PRP group.

Cell viability was also evaluated in three articles [62,86,87]. The authors reported that this parameter was not altered by PRP supplementation; in fact, it was comparable to that obtained with the xenogeneic supplement or even higher. Similar results were observed for cell morphology. This remained unchanged in two out of three articles [62,82,86]. Only Ramos-Torrecillas et al. [78] reported that, in the long-term culture (10 passages), two different populations were observed after culturing with PRP, while the culture with FBS yielded only one cell population, which was also corroborated by the antigenic expression of α-actin. These results did not occur in the short-term culture, where the morphology of gingival fibroblasts remained unchanged. The effect of FBS substitution on clonogenic ability was studied in only one article. Martínez et al. [73] reported that PRP stimulated the clonogenic ability of PDL cells. Finally, the effect of FBS substitution on cell senescence and cryopreservation was also evaluated [49]. The authors reported the same behavior for PRP as for the gold standard regarding these two processes.

The effect of replacing xenogeneic supplements with PRP on the stimulation of proliferation was evaluated in all the articles of this category. In the 71% of the studies, cell proliferation was found to be significantly higher in the PRP than in FBS groups. In the remaining 29%, the results were similar for both supplements [82,83]. Anitua et al. [49] and Okada et al. [76] also assessed the effect on cell migration. Both stated an increase after PRP addition, although the differences were only statistically significant in Anitua et al.'s article.

Two studies also evaluated the effect on osteogenic differentiation in hDPSCs and hDFCs [49,76]. The results showed that PRP led to a significant improvement in osteogenesis through an increase in hDPSCs mineralization [49] or through the upregulation of genes such as type I collagen, osteomodulin, alkaline phosphatase, bone morphogenic protein-4 and transforming growth factor-β in hDFCs [76] after culturing with the osteogenic medium supplemented with PRP. However, the adiogenic differentiation of oral cells was assessed in one study. [49] The adipogenesis of hDPSCs was found to be significantly higher in the PRP group than FBS group.

Regarding angiogenesis, Bindal et al. [58] observed that 8 out of the 12 selected pro-angiogenic genes (ANGPT1, EREG, FGF-2, VEGF-A, IGF-1, JAG-1, NPR2 and PLDXC1) were significantly augmented when lipopolysaccharide (LPS)-induced inflamed dental-pulp-derived stem cells (iDPSCs) were treated with 20% PRP-supplemented media [58]. In addition, the expression of genes related to adhesion molecules was also determined. The higher expression of BAI, NRP2, CCL11 and CDH5 and the downregulation of CCl2 and TGFβ3 were observed in 20% of the PRP-treated cells. Cytokine CXCL1, an inducer of microvascular endothelial migration and tube formation in vitro, was significantly expressed in cells treated with 20% PRP compared to FBS. Conversely, the expression of IFNA1 that inhibits angiogenesis during blood vessel remodeling was reported to be significantly increased in the FBS group.

3.2.4. Cartilage Tissue

The possibility of replacing xenogeneic culture supplements with PRP was also evaluated in cartilage cell cultures. Human nasoseptal chondrogenic cells (NCCs) [61], human meniscal fibrochondrocytes (MFCs) [62] and human articular chondrocytes [75] were the phenotypes used in three of the reviewed articles. Different percentages of PRP were also studied from 1% to 20%, all compared to a reference percentage of 10% FBS/FCS. All these studies found that PRP can also replace the xenogeneic cell culture supplement. However, there was also no consensus on the optimal PRP percentage. Do Amaral et al. [61] reported a greater enhancement of chondrogenic phenotype under a 2.5% PRP treatment rather than 10% PRP. In fact, this lower percentage (2.5%) also induced cell proliferation similarly to 10% FBS, while the proliferation rate increased when cells were cultured in 5% and 10% of PRP. These outcomes may in fact be interrelated. Gonzales et al. [62], on the other hand, detected an equal DNA quantity in MFCs cultured in 10% and 20% PRP in comparison

with 5% FBS. No differences in collagen type I expression were also detected for these percentages of PRP compared to FBS. So, they concluded that FBS could be replaced by 10% PRP or 20% PRP without altering proliferation and gene expression of human MFCs. Muraglia et al. [75] only tested one percentage of PRP (5%), as they reported, but did not show, that higher concentrations were less effective or even cytotoxic. However, this percentage was enough to stimulate a significantly higher cell proliferation after 6 and 8 days of culture in comparison with 10% FCS.

3.2.5. Bone Marrow Stem Cells (BM-MSCs)

The capability of PRP to expand hMSCs in vitro, comparing to FBS, was tested by Sun et al. in 2008 [84], confirming the similar spindle-shaped fibroblast-like morphology of cells isolated and cultured in media with either of both supplements. No differences regarding the surface markers were described.

Cell proliferation and differentiation were the most analyzed processes. In this sense, the higher proliferation rate sustained by 10% PRP compared to 10% FBS was confirmed [52,84] together with a shorter population doubling time and a lesser amount of early apoptotic cells, while comparable results between both supplements were reported by Kinzebah et al. [67]. What is more remarkable is the increased cell number yielded by the expansion medium supplemented with much lower percentages of PRPs compared to 10% xenogeneic serum [61,68]. Moreover, the effect of different storage conditions was tested by Muraglia et al. [91]; thus, increased MSC colony numbers and average size after culturing with PRP stored at 4 °C or −80 °C and a raised proliferation rate, even if in the case of the regenerated freeze-dried PRP, were described. Regarding the support of BM-MSCs' differentiation potential, the cells cultured with PRP retained a similar capacity to differentiate towards the osteogenic, chondrogenic and adipogenic lineages in two out of the five experimental works [67,89]. However, other experimental works described a significantly enhanced osteogenic differentiation with a higher ALP activity, increased calcium deposits and bone gene expression promoted by PRP compared to the same percentage of FBS, as well as a reduction in BM-MSCs adipogenic potential [52,84]. Moreover, a higher chondrogenic gene expression and glycosaminoglycans production, when FBS was replaced by PRP in the differentiation medium, producing a higher biochemical and biomechanical improvement, was observed by Do Amaral et al. [61].

3.2.6. Cells from Skin Tissues

Human dermal fibroblasts and keratinocytes are the two skin cell phenotypes used for assessing the xenogeneic culture medium supplement replacement by PRP, with the former being the most analyzed. Regarding the PRP acquisition method, non-activated PRP was used in the majority of the reviewed articles (60%). However, a multitude of favorable arguments were presented for the FBS substitution by PRP regardless of whether PRP is activated or not.

hDFs proliferation assays confirmed the higher promitogenic potential of several PRP percentages versus animal origin supplements [60,61,63,79], showing a dose-dependent increase in proliferation together with an enhancement in cell metabolic activity [56]. Only in one of the reviewed articles were no differences regarding the proliferative effect of PRP compared to FBS reported [90]. Moreover, the hDFs viability increase after 12 days was significantly enhanced by PRP addition comparing to that was obtained using a higher percentage of FBS [59]. In addition, a spindle-shaped morphology through cytoskeleton rearrangements and changes in alpha SMA, and vimentin expression reminding 3D matrix cultures were also described by Berndt et al. [56]. Regarding the effect on the matrix extracellular gene expression, collagen I and III, and fibronectin genes were upregulated when FBS was replaced by PRP, with the collagen differences being statistically significant [90]. When the fibroblast migration rate was assayed, conflicting results showed a decrease in cell motility in the presence of PRP [90], but also a faster beginning of migration in the wound healing assays [56].

With respect to keratinocytes, similar positive outcomes were reported. Thus, an increase in the percentage of proliferating cells along with a fastest cell migration induced by PRP compared to FBS was also described [90].

In addition to all the improvements achieved by the substitution of FBS by PRP being statistically significant, no genomic instability was reported regarding its use.

3.2.7. Umbilical Cord Tissue

Three articles assessed the effect of PRP supplementation on stems cells from the umbilical cord tissue [54,58,93]. Two studies used non-activated PRP, while only one study employed an activated PRP (Table 2).

One study was interested in evaluating the use of aPRP in the isolation of UCB-MSCs in comparison to 10% FBS [88]. The results showed that primary cultures with a complete medium containing 10% aPRP exhibited the highest success, whereas expansion in complete medium containing 5% aPRP was suitable. UCB-MSCs isolated using aPRP maintained their immunophenotypes and multilineage differentiation potential.

Another study evaluated the effect of PRP on the chondrogenic differentiation of hWJ-MSCs [54]. The results showed that 10% PRP was the optimal supplement to support the chondrogenesis of hWJMSCs. It induced the synthesis of the highest quantity of collagen II and also in a faster way. Amable et al. reported that 10% PRP induced the highest proliferation rate and the shortest population doubling time [52]. Wharton's Jelly-derived mesenchymal stromal cells secreted higher concentrations of chemokines and growth factors than other mesenchymal stromal cells (bone marrow stem cells and adipose tissue-derived stem cells) when cultured in PRP-supplemented media.

3.2.8. Miscellaneous

Two studies used human limbal epithelial stem cells (LESCs) where serum was obtained after the activation and clotting of plasma rich in growth factors (PRGF) [63,79]. Both studies supplemented the medium with 10% sPRGF. However, the FBS dose was different; one study used it at 5% and another study at 10% (Table 1). Hernaez-Moya et al. did not report significant differences regarding the size, stemness and proliferation of genes [63]. However, a lower number of k12 positive cells was observed in cultures maintained with s-PRGF, thus maintaining the stem/progenitor phenotype of LESCs. Riestra et al. observed that sPRGF induced a significantly greater growth area and higher number of cells [79]. Colony-forming efficiency was found to be also higher in the PRGF group. No significant differences in p63-α expression were found.

Hosseini et al. included human ovarian cells in their comparison between PRP and FBS as cell culture supplements [64]. The results showed that PRP better supported the viability and the growth of encapsulated/isolated human primordial and primary follicles. In another study, the PRP effect on the osteogenic differentiation potential of menstrual-blood-derived stem cells (MenSCs) was tested [66]. In this experimental work, FBS was compared with PRP, platelet gel supernatant (PGS) and human platelet releasate (HPR). There was no significant difference between the growth curves of neither the cultured MenSCs in the presence of different human platelet derivatives nor those in FBS. However, osteogenic differentiation was enhanced by PGS, PRP and HPR. Brini et al. compared activated PRGF to FBS in the osteogenic differentiation of human osteoblast cells [59]. The activated PRGF simultaneously enhanced both cell proliferation and osteo-differentiation, suggesting it as a valid alternative to FBS. Using the same cell type, Muraglia et al. compared PRP to FCS [75]. The study showed that PRP enhanced cell proliferation by more than four times.

Talebi showed that human T lymphoblasts from acute lymphoblastic leukemia CCRF-CEM treated with PRP not only were morphologically comparable to those treated by FBS, but also showed a greater viability at the concentrations of 10 and 15% [85]{Talebi, 2021 #3}. PRP supported cell culture, at least in part, via inducing YKL-40 expression at both mRNA and protein levels in a time- and dose-dependent manner.

The enhanced viability and similar proliferation rate of human mesenchymal stem cells (MSCs) of unknown origin, along with typical MSC morphology preservation promoted by PRP supplementation, was reported by Simon et al. [81]. The maintenance of specific antigen expression into the desired ranges, the prevention of cell differentiation and the lack of alteration of the apoptotic and antiapoptotic genes ratio were also observed.

4. Discussion

The current systematic review and previously published reviews could be an indicator for the increase in interest in using human PRP as a substitute of xengoneic serum in cell therapy [5,9,10,45,92–94]. However, the use of PRP raises several open questions that need to be addressed in order to create standardization in the complex and evolving use of PRP. These questions are related to the definition of the platelet-rich plasma in terms of platelet concentration, leukocyte content, formulation type (activated or non-activated), the activator type where required (calcium ions, thrombin or physical methods) and preparation methods, including the anticoagulant type and concentration.

To answer this question, the PRP preparation needs to be described, so a comparative analysis could be performed. PRP composition was the least considered item, being completely described in less than 30% of the included studies (Table 1). This finding has also been reported by previous systematic reviews [10,94]. Transparency by using a classification system or algorithm that describes the PRP formulations has to be implemented. A similar need, but in the clinical field, has motivated the suggestion of several systems for the classification and standardization of reporting on PRP [95–99]. Based on the results of this review and the classification systems of PRP in the clinical field, several items were identified in order to have a transparent description of PRP from the perspective of cell therapy. These items would allow the comparative analysis and reproduction of PRP by other researchers (Table 3). These items are the features of the blood donor, the medical device for blood extraction, the characteristics of the blood, blood processing for PRP preparation, the definition of the PRP, PRP hallmark, the PRP activation procedure where appropriate, the nomenclature of PRP formulation, the content of key biomolecules, the origin of the PRP relative to the cells, the dose of the PRP and the microbial inactivation where applicable.

This review demonstrated the considerable research that has been dedicated to defining the ideal medium that can substitute xenogenic systems. Platelet-rich plasma is used in different forms (PRP-derived supernatants and non-activated PRP) and at different concentrations. No comparative studies are available to assess the optimal platelet concentration of the PRP for stem cells culturing. Two ranges of platelet concentration could be considered: the first one between 0.14×10^6 and 0.80×10^6 platelets/μL ($<1.0 \times 10^6$ platelets/μL) [50,56,57,59,60,62,68,70–72,76,77,80] and the second between 1.086×10^6 and 10×10^6 platelets/μL ($>1.0 \times 10^6$ platelets/μL) [54,57,65,69,70,73,78,79,91,92]. However, there is a consensus among all the studies regarding the preparation of a leukocyte-reduced PRP. Cell proliferation is the test that has been widely used to determine the most successful PRP concentration in the culture medium. Thus, the optimal dose of PRP is ranged between 0.5% and 20%, with 10% PRP being the most widely used, which is in agreement with previous systematic reviews [92–94]. Regarding platelet activation, the three most common methods include the use of calcium chloride, thrombin and freezing procedure (Table 2). However, there are no comparative studies to recommend an optimal activation method.

Table 3. Items for the transparent description of PRPs in research for cell therapy applications.

Item	Description
Blood donor features	Age, sex and systemic health status (ASA)
Medical device for blood extraction	Type of medical devise (bags, vacutainer, syringe) and commercial information. Type of additives and their concentration Blood to anticoagulant ratio
Blood characteristics	Pooled or individually processed Hematocrit and concentration of platelets, leukocytes and red blood cells (RBCs) Blood group (ABO and Rh systems)
Blood processing for PRP preparation	Procedure: centrifugation, aphaeresis, microfluidic system Equipment used: commercial information Processing parameters: number of cycles, centrifugation force and time
PRP definition	Which specific part of the fractioned blood (plasma and/or buffy coat) is considered PRP?
PRP hallmark	Pooled or individually characterized Volume Concentration of platelets, leukocytes and RBCs Post-processing of PRP (lyophilization and freezing) Use of additives (type and concentration) Storage conditions
Activation	Yes/no Method of activation: calcium ions, thrombin, light, agitation Concentration of activator solution and ratio to PRP
Nomenclature of PRP formulation	Non-activated PRP Activated liquid PRP PRP serum PRP fibrin
Storage conditions	Lyophilized or not Temperature
Key biomolecules content	Biomolecules identification and kits used for quantifications
Origin of PRP relative to the cells	Autologous, allogenic or xenogenic
Pathogen detection	Yes/No; if yes, specify microorganism and the assay used
Microbial inactivation	Yes/No; if yes, specify the procedure
Dose of PRP	Percentage to the volume of cell culture medium

Regarding the type of anticoagulant, 23 studies have not specified it. Anticoagulants are commonly used to prevent the coagulation of blood by either neutralizing thrombin (heparin and hirudin) or chelating calcium ions (oxalate, EDTA and citrate) [100,101]. Chelating calcium offers the advantages of recovering the coagulation by adding an excess of calcium ions later. It is important to pay attention to the type and concentration of the anticoagulant in order not to disrupt the size, morphology, counting and activity of the blood cells [102,103]. Even more, changes in cell differentiation and mitogenesis have been reported by altering the concentration of the anticoagulant [104,105]. Sodium citrate is the most common anticoagulant utilized in the reviewed articles (10 studies), being associated with higher platelet recovery and the genetic stability of mesenchymal stromal cells [105].

Pooling and pool size are important parameters to reduce the variability in pooled human platelet lysate regarding the concentration of growth factors and batch-to-batch divergence [106]. However, the potential risk of transmitting diseases may rise as the pool size increases [107]. In this review, 14 studies pooled PRP with a pool size varying between four and nine donors. The most frequent pool sizes were four and eight donors. In two studies, quality control tests were performed [52,67]. Amable et al. assessed the growth factor concentration and compared it with values that had been previously determined [52], while cell

proliferation was employed by Kinzebach et al. [67]. In the context of cell-based therapy, the identity (molecular structure/composition, biological, physico-chemical or immunological properties) is a requisite to demonstrate the uniqueness of the raw material [108]. However, this is complicated in the case of platelet-rich plasma as there is no consensus on the optimal platelet concentration and, thus, the concentration of biomolecules. However, all the studies in the review used leukocyte-reduced PRP. For routine use, chemically defined media are necessary for the standardized culturing of MSCs under GMP guidelines [93]. There is a need for more comparative studies under a GMP-compliant manufacturing process using PRP to define the composition criteria that need to be fulfilled. Another possibility is the implementation of performance testing regarding contamination, total proteins, pH and osmolarity [108]. From the standpoint of good manufacturing practice (GMP), PRP should be free from contamination risk, non-immunogenic, non-oncogenic, effective in increasing the cell proliferation rate and effective in retaining unmodified the MSC phenotype and their differentiation capacity [108]. The results of this review support the use of PRP as it was reported to be effective in increasing, or at least not lowering, the cell proliferation rate, maintaining unmodified the MSC phenotype (except for ectomesenchymal stem cells from human exfoliated teeth at higher passages), preserving their genetic stability and supporting their differentiation capacity (Table 2). Moreover, PRP or PRP-derived supernatants have a positive effect on BM-MSCs culturing and also on MSC colony numbers and average size [75], cell proliferation [54,71,72,79,88] and osteogenic [52,84] and chondrogenic differentiation [52], but inhibit adipogenic differentiation [52,84]. In AT-MSCs, PRP treatment promotes cell proliferation [50,52,53,60,67–72,74,77,80,86] and chondrogenic [54,56,57,64,78,81,84], osteogenic [54,56,64,73,78,81] and adipogenic differentiation [56,64,73,78,81]. However, studies showed a lower osteogenic differentiation capacity [86], lower proliferation [55] and lower adipogenic differentiation capacity [52] in this cell phenotype. In the case of WJ-MSC culturing, the PRP or PRP-derived supernatants have a positive effect on cell proliferation [52,54] and osteogenic [52] and chondrogenic differentiation [52,54], but inhibit adipogenic differentiation [52]. Similar results have been observed for UCB-MSCs [88]. The PRP supernatant enhanced HDPS cell isolation, migration, proliferation and osteogenic and adipogenic differentiation, but without altering the adipogenic differentiation, senescence or their cryopreservation [49].

However, PRP induced molecular differences in cell culturing when compared to FBS. In stem cells from adipose tissues, Lang et al. [70] and Lobil et al. [72] suggested that enhanced cell proliferation by the PRP could be mediated by the inactivation of PTEN that might then activate the PKB/AKT pathway. Chieregato et al. [60] reported the involvement of other signaling pathways, such as MEK-1/2. Furthermore, Amable et al. [52] showed that the use of PRP stimulated the expression of pluripotent genes and thereby downregulated differentiation markers, except for one of the quantified osteogenic markers (BMP2). Regarding protein synthesis, Kocaoemer et al. showed a slightly higher total protein content in the culture medium of the PRP-treated cells [69]. Moreover, Amable et al. showed that the protein secretion of adipose-tissue-derived stem cells was altered by PRP supplementation [52], being opposite direction (upregulated or downregulated) to that induced by FBS for most of the analyzed proteins (cytokines, growth factors, extracellular matrix and metalloproteinases). At the genetic level, the use of PRP, instead of FBS, upregulated the expression of BMP-2 and BMP-4 genes, while downregulating the expression of PDGF-B and FGF-2. The genetic expression of TGF-beta and VEGF was not significantly altered [52]. In gingival fibroblasts, the use of PRP in long-term culture (10 passages) resulted in changes in cell population (two different posulations in PRP vs one population in the FBS) as corroborated by the antigenic expression of α-actin [78]. These differences were not observed in the short-term culture where the morphology of gingival fibroblasts remained unchanged. For hDPSCs, the use of PRP led to a significant improvement in osteogenesis through an increase in hDPSC mineralization [49] or through the upregulation of genes such as type I collagen, osteomodulin, alkaline phosphatase, bone morphogenic protein-4 and transforming growth factor-β in hDFCs [76]. In an inflammatory model,

Bindal et al. [58] observed that 8 out of 12 selected pro-angiogenic genes (ANGPT1, EREG, FGF-2, VEGF-A, IGF-1, JAG-1, NPR2 and PLDXC1) were significantly augmented when lipopolysaccharide (LPS)-induced inflamed dental pulp-derived stem cells (iDPSCs) were treated with 20% PRP-supplemented media [58]. The higher expression of BAI, NRP2, CCL11 and CDH5 and the downregulation of CCl2 and TGFβ3 were observed in 20% of the PRP-treated cells. Cytokine CXCL1, an inducer of microvascular endothelial migration and tube formation in vitro, was significantly expressed in cells treated with 20% PRP compared to FBS. Conversely, the expression of IFNA1 that inhibits angiogenesis during blood vessel remodeling was significantly increased in the FBS group. In BM-MSCs, the use of PRP led to a significantly enhanced osteogenic differentiation with higher ALP activity, increased calcium deposits and bone gene expression [52,84]. Moreover, a higher chondrogenic gene expression and glycosaminoglycan production was observed, resulting in a higher biochemical and biomechanical improvement [61]. For Wharton's Jelly-derived stem cells, the use of PRP induced the synthesis of the highest quantity of collagen II and also in a faster way. Amable et al. observed that mesenchymal stromal cells secreted higher concentrations of chemokines and growth factors than other mesenchymal stromal cells (bone marrow stem cells and adipose-tissue-derived stem cells) when cultured in PRP-supplemented media [52].

In a previous systematic review of pre-clinical studies, the combination of PRP and stem cells improved osteogenic and cartilage regeneration, but conflicting results have been reported for periodontal regeneration [10]. Guiotto et al. performed a systematic review similar to the one reported in this article [94]. Improved cell proliferation and differentiation supported the use of human platelet lysate as an alternative to xenogenic sera [92–94]. However, that systematic review lacks the assessment of risk of bias and was limited to human plasma lysate.

This review has several limitations. Firstly, there is a lack of complete descriptions of the preparation and characterization of PRP. Furthermore, there is an absence of comparative studies among different PRP. All these limitations make it difficult to derive recommendations regarding PRP characteristics for the culture of primary cells regarding cell therapies.

5. Conclusions

The use of leukocyte-depleted PRP as an alternative to xenogeneic sera for the culturing of stem cells was demonstrated to be feasible by this review. However, there is a need to improve the description of the PRP preparation methodology as well as its composition. Furthermore, there is a need to establish a potency/performance test and comparative studies among different PRP compositions to determine quality control parameters and universally accepted guidelines.

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3.3. ARTÍCULO III

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RESUMEN

En las últimas tres décadas, ha habido un interés especial en el desarrollo de fármacos que imitan las características de las lágrimas naturales para su uso en el tratamiento de varios trastornos de la superficie ocular.

En este sentido, la composición del plasma sanguíneo es muy similar a la de las lágrimas. Por lo tanto, se han desarrollado diferentes productos derivados de la sangre como el suero autólogo (AS) y el plasma rico en factores de crecimiento (PRGF) para el tratamiento de diversas patologías oculares. Sin embargo, se han realizado escasos estudios para analizar las diferencias entre ambos tipos de productos derivados de la sangre. En el presente estudio se extrajo sangre de tres donantes sanos y se procesó para obtener colirios de AS y PRGF. Posteriormente, los queratocitos del estroma corneal humano (HK) se trataron con PRGF o con AS sin diluir. El análisis proteómico se llevó a cabo para analizar y caracterizar los perfiles proteicos diferenciales entre el PRGF y el AS, y las proteínas expresadas diferencialmente en las células HK tras el tratamiento con PRGF y AS. Los resultados obtenidos en el presente estudio muestran que el AS sin diluir induce la activación de diferentes vías relacionadas con una respuesta inflamatoria, angiogénica, de estrés oxidativo y de cicatrización en las células HK respecto al PRGF.

Estos resultados sugieren que el PRGF podría ser una mejor alternativa que el AS para el tratamiento de la superficie ocular.

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Article

Proteomic Characterization of Plasma Rich in Growth Factors and Undiluted Autologous Serum

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Abstract: Over the last three decades, there has been special interest in developing drugs that mimic the characteristics of natural tears for use it in the treatment of several ocular surface disorders. Interestingly, the composition of blood plasma is very similar to tears. Therefore, different blood-derived products like autologous serum (AS) and plasma rich in growth factors (PRGF) have been developed for the treatment of diverse ocular pathologies. However, scarce studies have been carried out to analyze the differences between both types of blood-derived products. In the present study, blood from three healthy donors was drawn and processed to obtain AS and PRGF eye drops. Then, human corneal stromal keratocytes (HK) were treated with PRGF or undiluted AS. Proteomic analysis was carried out to analyze and characterize the differential protein profiles between PRGF and AS, and the differentially expressed proteins in HK cells after PRGF and AS treatment. The results obtained in the present study show that undiluted AS induces the activation of different pathways related to an inflammatory, angiogenic, oxidative stress and scarring response in HK cells regarding PRGF. These results suggest that PRGF could be a better alternative than AS for the treatment of ocular surface disorders.

Keywords: platelet-rich plasma; PRP; plasma rich in growth factors; PRGF; autologous serum; AS; proteomic; keratocytes; cornea; ocular surface

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1. Introduction

The increase in life expectancy across the last few decades is associated with the higher incidence of age-related diseases, including those pathologies affecting all tissues composing the eye from the ocular surface to the eye fundus.

Artificial tears are commonly used for the topical treatment of several ocular surface disorders. However, the physico-chemical properties of natural tears are by far more complex than artificial tears including pH I, osmolarity and their complex composition of water, lipids, proteins or salts among other components [1]. Several other treatments, like anti-inflammatory substances (corticoids or cyclosporine or secretagogues), have shown an improvement in symptoms, but it has been demonstrated that their use could induce side effects, such as ocular burning or increased ocular pressure [2–4]. New and interesting regenerative therapies like amniotic membrane transplantation (AMT) and recombinant growth factors have been used for the treatment of several ocular surface pathologies in the ophthalmology field [5]. However, the use of these treatments presents some limitations, such as the uncertain results, the elevated manufacturing costs, and

the risk of disease transmission in the case of AMT, making it necessary to explore other therapeutic approaches for ocular surface tissue regeneration [6–8].

Over the last thirty years, there has been a special interest in developing drugs that mimic the characteristics of natural tears such as the regenerative, lubrication, antimicrobial and anti-inflammatory properties. Interestingly, the composition of blood plasma is very similar to tears in terms of osmolarity, pH and many proteins related to tissue regeneration [9–11]. In this sense, different blood-derived products like autologous serum (AS) and platelet-rich plasma (PRP) have been developed for the treatment of diverse ocular pathologies like dry eye disease, persistent epithelial defects, or corneal ulcers with satisfactory results [12]. Nonetheless, the manufacturing protocol and, as a consequence, the composition of AS products, has been changing over time, mainly in relation to their preparation, such as centrifugation, coagulation time or dilution. This lack of protocol standardization, together with its high content in pro-inflammatory molecules like metalloproteinases and hydrolases, has provided controversial AS clinical outcomes [11,13]. In order to avoid these limitations, a protocolized and standardized technology has been developed: plasma rich in growth factor (PRGF) eye drops. This is a PRP with specific features that include platelet activation, avoiding leukocytes and pro-inflammatory molecules, and which contain a higher content of growth factors than AS [14–16]. These differences have been widely observed in several clinical studies where PRGF advantages have been shown in corneal epithelial defects, dry eye, neurotrophic keratitis and graft versus host diseases that were refractory to previous treatment with AS [17–19].

Furthermore, several pre-clinical studies have demonstrated the different capabilities of AS and PRGF to induce differential biological activities in ocular surface cells. An in vitro study carried out by Freire et al. showed that PRGF eye drops significantly increase corneal epithelial cell (HCE) proliferation compared to AS [20]. These results were corroborated in a posterior study in which PRGF eye drops reduced the corneal re-epithelialization time in an in vivo mechanical de-epithelialization model in rabbits regarding AS treatment [21]. A subsequent in vitro study demonstrated that PRGF eye drops are capable of exerting a higher anti-inflammatory effect than AS on ocular surface fibroblasts treated with proinflammatory IL-1β and TNFα [22]. Finally, a recent study showed that PRGF eye drops were able to produce a significant reduction in the number of transforming growth factor (TGF)-β1-induced myofibroblasts in comparison to AS, suggesting that PRGF may promote corneal wound healing regeneration, reducing scar formation [15]. To understand the pathways by which PRGF exerts its antifibrotic potential in comparison to AS, a proteomic study was performed in HK cells differentiated to myofibroblasts, which previously were to be treated with PRGF or AS [23]. This study shows that PRGF treatment inactivated or reduced the activation of several proteins involved in the pathways, whereby TGF-β1 exerts its action to induce the formation of α-actin fibers on human corneal stromal keratocytes (HK), inducing their transformation to myofibroblasts.

It is important to mention that all of the studies described above were carried out with 20% diluted AS eye drops as they are usually used in clinical practice in order to reduce the concentration of TGF-β1 to prevent its potentially harmful effect [12,13]. However, recent groups and studies advocate the use of 100% AS, increasing the concentration of other beneficial factors involved in ocular wound healing like epidermal growth factor (EGF) or fibronectin to achieve better clinical outcomes [24,25].

The purpose of the present study has been to characterize and quantify the protein composition of 100% PRGF in comparison to 100% AS. In addition, the differential protein expression of HK cells after treatment with PRGF or AS was also determined.

2. Results

2.1. Hematological Characterization of PRGF

PRGF preparations had a mean platelet enrichment of 2.0 ± 0.4-fold (431 ± 133 × 10^3 platelets/μL) over the platelet concentration in peripheral blood (215 ± 39 × 10^3 platelets/μL). In addition, minimum levels of leucocytes (0.2 ± 0.1 × 10^3 leucocytes/μL) were observed

in the PRGF preparations. PRGF is classified depending on the type of PRP classification, as is described in Table 1.

Table 1. PRGF-Endoret system (BTI Biotechnology Institute) classification.

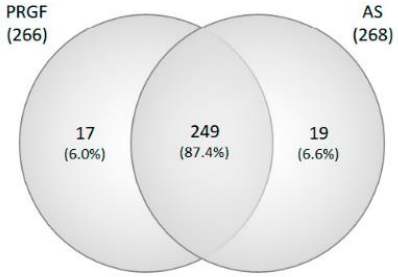
Classification	Reference	PRGF-Endoret
MISHRA	[26]	4 (B)
PAW	[27]	P2-x-Bβ
PLRA	[28]	P0.5 L-R-A+
DEPA	[29]	CCA
ISTH	[30]	PRP IIA1
MARSPILL	[31]	M A+ RBC-P Sp1 PL2-3 Lc-P A-
Consensus Experts	[32]	24-00-11

2.2. *Proteomic Characterization of Blood-Derived Products*

Protein samples coming from the two conditions (PRGF and AS) and obtained from the three donors were analyzed independently for differential protein expression. About 285 different proteins were detected in total between both types of formulations. For the complete list of proteins in these formulations and their relative expression see Supplementary File S1. The Venn diagram in Figure 1 summarizes the intersection of the total proteins in both blood-derived products (PRGF and AS). In summary, the number of proteins identified in each formulation was 266 and 268, respectively. The Venn diagram shows that 249 proteins (87.4% of all proteins) were shared by both formulations, while 17 proteins (6.0% of the total) were only identified in the PRGF formulation, and 19 (6.6%) were specific to the AS product. The lists of proteins shared or specific to each formulation are included in Supplementary File S1.

PRGF

Gene name	Protein description
ITA2B	Integrin alpha-IIb
AGRF5	Adhesion G protein-coupled receptor F5
CA2D1	Voltage-dependent calcium channel subunit alpha-2/delta-1
G6PE	GDH/6PGL endoplasmic bifunctional protein
F13A	Coagulation factor XIII A chain
ITB3	Integrin beta-3
LAMP2	Lysosome-associated membrane glycoprotein 2
TAGL2	Transgelin-2
PAFA	Platelet-activating factor acetylhydrolase
URP2	Fermitin family homolog 3
HV307	Immunoglobulin heavy variable 3-7
RP1BL	Ras-related protein Rap-1b-like protein
RA1L2	Heterogeneous nuclear ribonucleoprotein A1-like 2
ANAG	Alpha-N-acetylglucosaminidase
AGRG6	Adhesion G-protein coupled receptor G6
R4RL2	Reticulon-4 receptor-like 2
NOE1	Noelin



AS

Gene name	Protein description
ENOA	Alpha-enolase
CAH1	Carbonic anhydrase 1
MRC1	Macrophage mannose receptor 1
IL6RB	Interleukin-6 receptor subunit beta
TKT	Transketolase
TYB4	Thymosin beta-4
TRFL	Lactotransferrin
MBL2	Mannose-binding protein C
ICOSL	ICOS ligand
LRP1	Prolow-density lipoprotein receptor-related protein 1
CNTN1	Contactin-1
POSTN	Periostin
CD109	CD109 antigen
SRCRL	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D
IGF2	Insulin-like growth factor II
HVC05	Immunoglobulin heavy variable 3-30-5
HSP72	Heat shock-related 70 kDa protein 2
EGLN	Endoglin
LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1

Figure 1. Venn diagram comparison of two blood-derived fractions and the list of proteins specific for PRGF and AS.

A GO analysis was carried out with the aim of characterizing the functional processes these proteins are involved in. A total of 259 different GO terms were found between the two blood-derived products (PRGF and AS). The lists of all GO terms corresponding to each formulation can be found in Supplementary File S1. Figure 2 shows the twelve most

abundant GO terms found between the two formulations (PRGF and AS). The first ten GO terms shared by all formulations may be grouped into 3 main processes: (i) Cellular activity, consisting on proteolysis, negative regulation of endopeptidase activity and cell adhesion GO terms; (ii) Immune response combining the GO terms of innate immune response, complement activation (classical pathway), complement activation, inflammatory response and regulation of complement activation; and (iii) Platelet function, which comprises the GO terms of platelet degranulation and blood coagulation. Although the percentage of differentially expressed proteins involved in all these GOs are similar for every blood-derived product, there are at least two processes for each, such as extracellular matrix organization and immune response, meaning that some proteins were found in AS but not in PRGF.

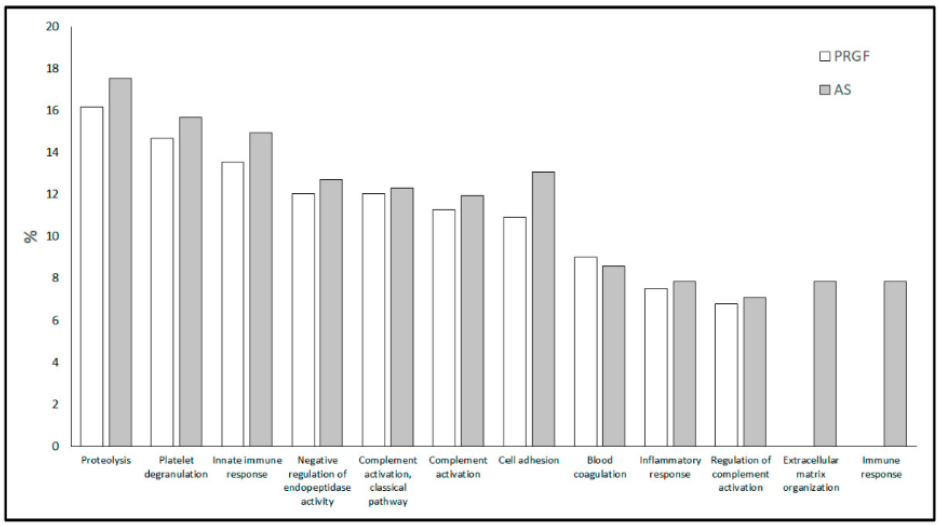


Figure 2. The ten most abundant Gene Ontology terms identified in PRGF and AS formulations, and two of the processes that were only found in AS formulation, which were related to extracellular matrix organization and immune response.

PRGF and AS protein composition was compared by a relative quantitative proteomics analysis. A total of 257 proteins were quantified with at least two different peptides (Supplementary File S2), of which 13 had a p value < 0.05 and a ratio > 1.5 in either direction. These proteins were selected for further analysis (Table 2).

Table 2. Statistically significant de-regulated proteins between AS vs. PRGF eye drops.

Protein Accession Number	Gene Name	Protein Description	Fold Change	*p*-Value
Q9UK55	ZPI	Protein Z-dependent protease inhibitor	1.2	0.0001
P68871	HBB	Hemoglobin subunit beta	31.4	0.0098
P02788	TRFL	Lactotransferrin	2.8	0.0156
P03952	KLKB1	Plasma kallikrein	1.1	0.0168
P14151	LYAM1	L-selectin	1.1	0.0192
P01024	CO3	Complement C3	1.0	0.0193
P08603	CFAH	Complement factor H	1.1	0.0253
P62328	TYB4	Thymosin beta-4	3.2	0.0255
P00915	CAH1	Carbonic anhydrase 1	13.2	0.0301
Q92496	FHR4	Complement factor H-related protein 4	1.7	0.0329
P08709	FA7	Coagulation factor VII	1.4	0.0428
P22105	TENX	Tenascin-X	0.9	0.0474
P01860	IGHG3	Immunoglobulin heavy constant gamma 3	1.2	0.0491

The GO analysis showed that these differentially expressed proteins could be involved in different biological processes (Table 3). However, when IPA analysis was performed to characterize the functional processes in which the differentially expressed proteins are involved, no significant differences were found in any of the processes identified (Supplementary File S3).

Table 3. Gene Ontology analysis of de-regulated proteins with statistically significant differences between AS vs. PRGF.

GO Term	GO Definition	Genes	%	*p*-Value
0006956	Complement activation	P08603, P01860, P01024	23.1	0.0017
0006508	Proteolysis	P03952, P02788, P01860, P01024	30.8	0.0047
0007596	Blood coagulation	P68871, P08709, Q9UK55	23.1	0.0073
0006957	Complement activation, alternative pathway	P08603, P01024	15.4	0.0093
0030449	Regulation of complement activation	P08603, P01024	15.4	0.0212
0001895	Retina homeostasis	P02788, P01860	15.4	0.0282
0015701	Bicarbonate transport	P00915, P68871	15.4	0.0310
0006958	Complement activation, classical pathway	P01860, P01024	15.4	0.068
0010951	Negative regulation of endopeptidase activity	Q9UK55, P01024	15.4	0.0831
0030036	Actin cytoskeleton organization	P62328, P22105	15.4	0.0891

2.3. Proteomic Characterization of HK Cells Treated with Blood-Derived Products

Protein samples coming from HK cells treated with both conditions (PRGF and AS) obtained from three different donors were analyzed for differential expression. A total of 3236 proteins were quantified, and 352 of them showed statistically significant differences (Supplementary File S4). Further analyses were carried out to study these 352 differentially expressed proteins in HK cells after PRGF and AS treatment. A Gene Ontology (GO) analysis was carried out to initially characterize the functional processes that these significantly differential expressed proteins are involved in, and a total of 166 GO terms were significantly enriched (Supplementary File S4).

Ingenuity pathways analysis (IPA) was accomplished for further characterization of the functional processes in which the proteins with significant differential expression were involved. The comparison of protein expression in HK cells treated with PRGF and AS showed that several pathways were significantly deregulated. Figure 3 summarizes the canonical pathways that were most enriched among PRGF vs. AS differentially expressed proteins. This analysis revealed that these canonical pathways were distributed mainly between six relevant biological functions: (A) Inflammation; (B) EGF pathway; (C) Actin cytoskeleton signaling; (D) Protein synthesis, cell proliferation and motility; (E) Angiogenesis; and (F) Oxidative stress. The IPA Z-score, which provides an estimation of the expected activation/inhibition of these pathways based on the expression pattern of the differentially regulated proteins, suggests an increased activity for these functions in AS-mediated response in comparison to PRGF-mediated response (Supplementary File S5). Six of the 17 canonical pathways are related to signaling pathways activated by inflammatory mediators such as Acute Phase Response Signaling, LPS-stimulated MAPK Signaling, CCR3 Signaling in Eosinophils, IL-6 Signaling, IL-15 Signaling and CXCR4 Signaling (Figure 3).

The significant association between the differentially expressed proteins in HK cells after AS treatment with the inflammation-related terms is higher than with PRGF, suggesting a great correlation between AS and inflammatory induction. Furthermore, the IPA upstream regulator analysis suggests that several differentially expressed proteins after AS treatment are related to the activation of HK cells by some inflammatory cytokines like IFN-γ (Figure 4A). In addition, the IPA disease and biofunctions analyses showed that several proteins enriched in HK cells treated with AS in comparison to those treated with PRGF are related to the development of a cellular immune response (Figure 4B). Finally, when upstream regulators are related with disease and biofunctions, the IPA analysis showed that some differentially expressed proteins after AS treatment correlate the IFN-γ activation of HK cells with the immune response of these cells (Figure 4C). All the above

results demonstrate that AS treatment induce a significantly higher inflammatory response in HK cells when compared to PRGF treatment.

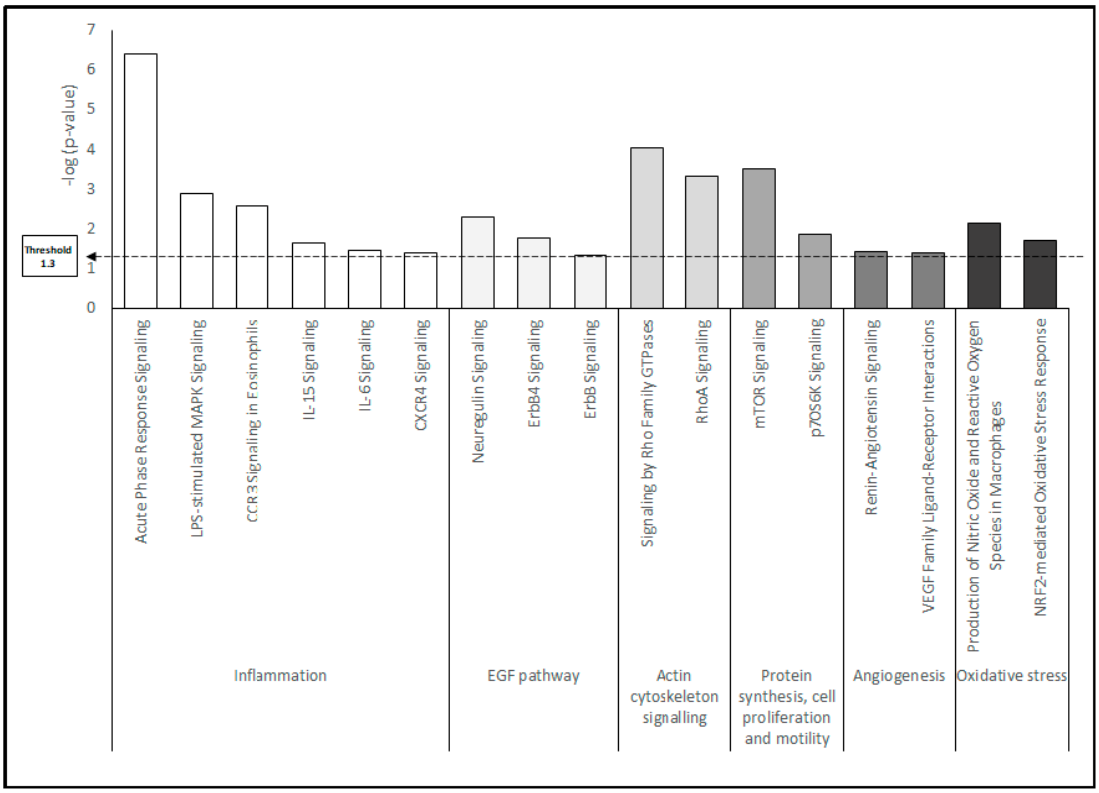


Figure 3. Canonical pathway analysis of the differentially expressed proteins in HK cells after treatment with PRGF or AS. The most significantly enriched canonical pathways (−log *p* values in the y axis) are displayed. The results are clustered in functionally related groups of processes: Inflammation; EGF pathway; Actin cytoskeleton signaling; Protein synthesis, cell proliferation and motility; Angiogenesis; and Oxidative stress.

Three additional canonical pathways (Neuregulin Signaling, ErbB4 Signaling, and ErbB Signaling) are related to EGF response (Figure 3). The EGF pathway activation seems to be more highly correlated to AS treatment than to PRGF.

Two additional canonical pathways, like signaling by Rho Family GTPases and RhoA Signaling, related to actin cytoskeleton signaling, were significantly more activated in HK cells treated with AS compared to PRGF (see Figure 3, Supplementary Figure S1 and Figure 5A). Furthermore, the IPA upstream regulator analysis revealed that some differentially expressed proteins with a significant difference between AS and PRGF treatment were related to TGF-β1 activation (Supplementary Figure S2). In addition, the IPA downstream analyses showed that many of these differentially expressed proteins in HK cells treated with AS were related to the activation of the organization of cytoskeleton (Supplementary Figure S3). Finally, a combination of upstream and downstream pathways showed that some differentially expressed proteins after AS treatment correlate the possible activation of HK cells with TGF-β1 and the activation of the organization of the cytoskeleton (Figure 5B). The significant association between the differentially expressed proteins in HK cells after AS treatment with the cytoskeleton-related terms is higher than with PRGF, suggesting a tight correlation between AS and cytoskeletal functions.

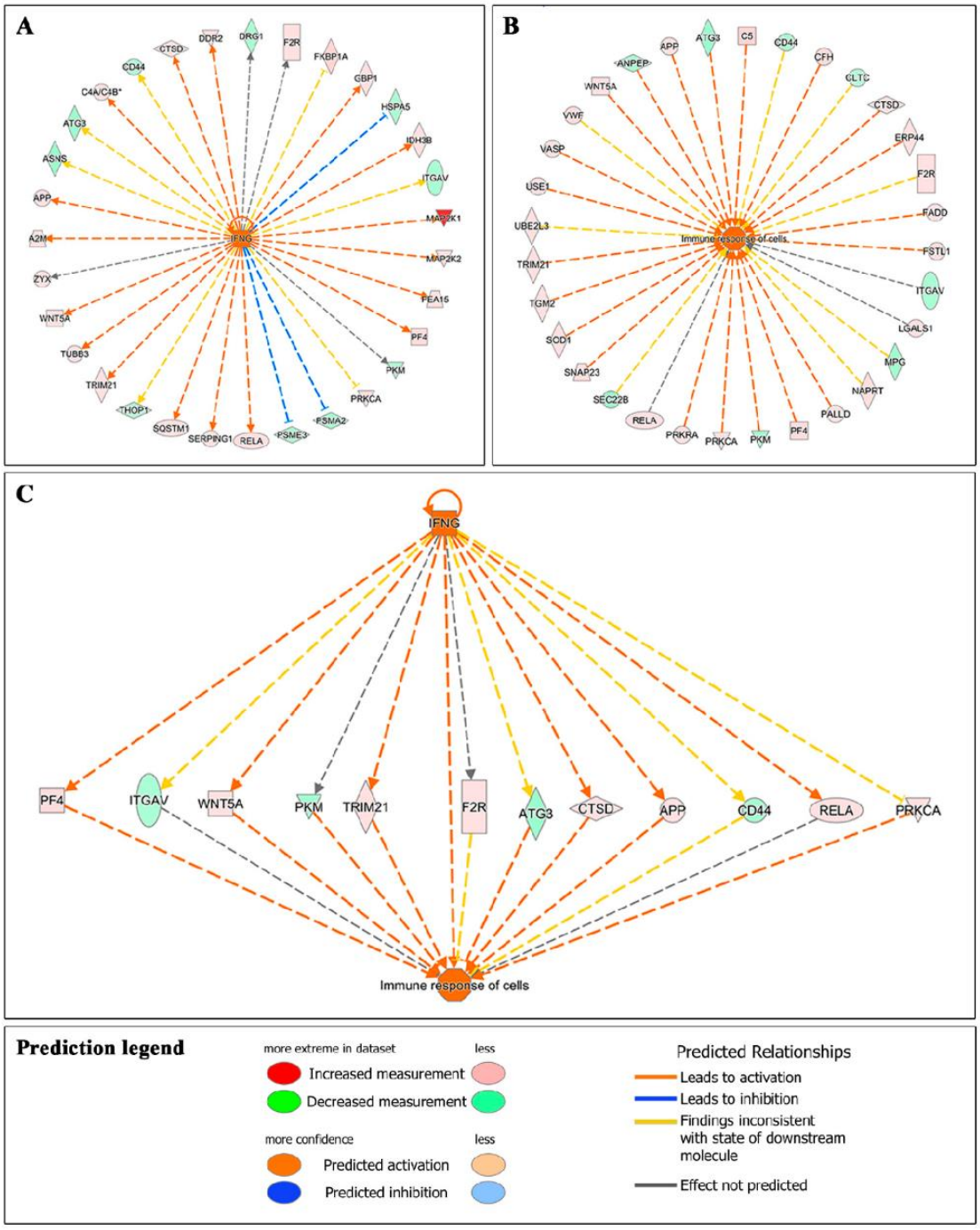


Figure 4. IPA analysis of differentially expressed proteins related to an immune response. (**A**) the IPA upstream regulators analysis suggests that several proteins differentially expressed with AS treatment are related to the activation of HK by IFN-γ. (**B**) IPA downstream analysis also showed that numerous differentially expressed proteins are associated with an immune response of HK cells treated with undiluted AS. (**C**) Finally, IPA upstream/downstream analysis link some differentially expressed proteins with the activation of HK cells by IFN-γ and the induction of an immune response.

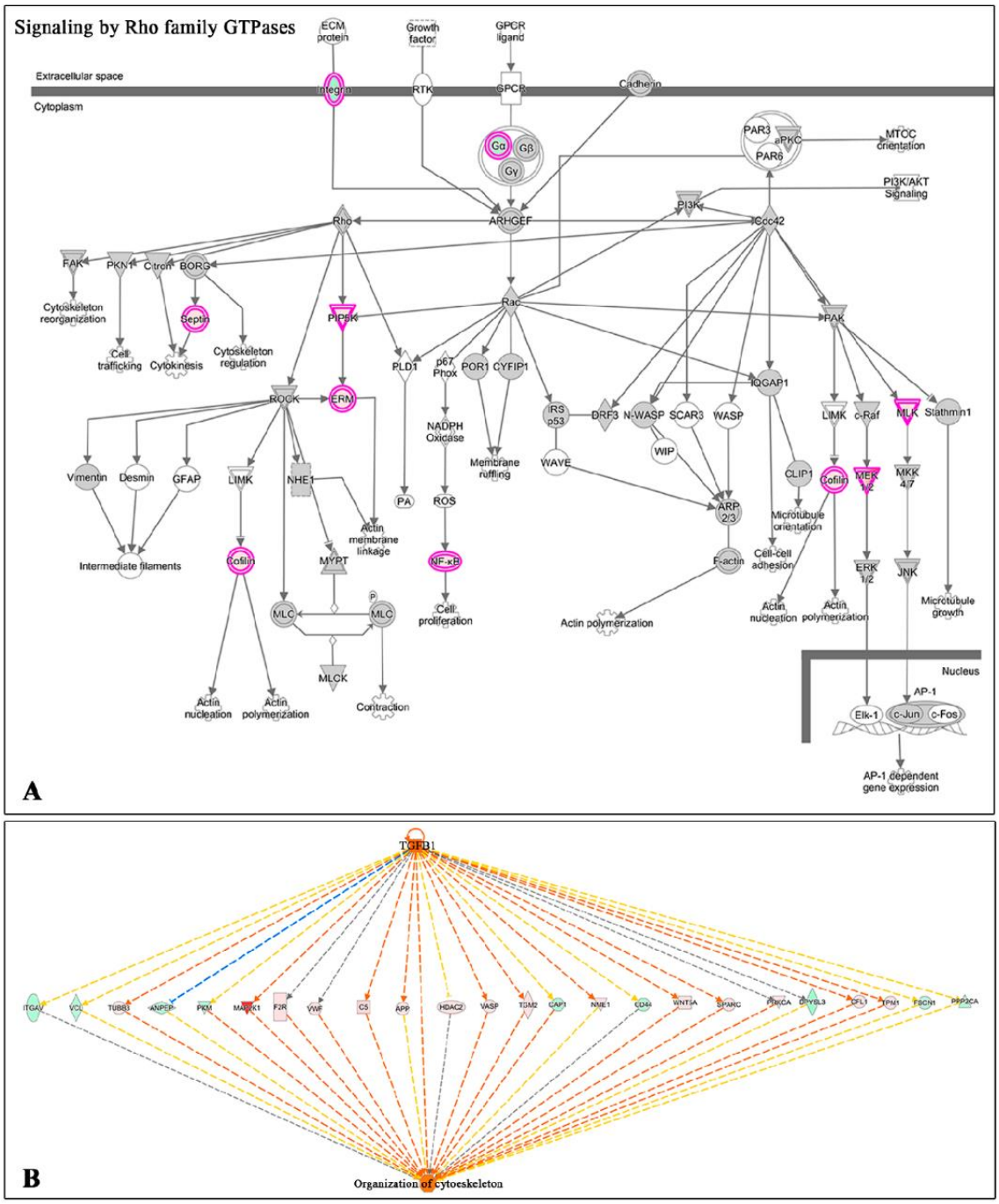


Figure 5. IPA analysis of differentially expressed proteins related to actin cytoskeleton activation. (**A**) Signaling cascade of Rho Family GTPases pathway. Red circles represent over-expression of proteins in HK ells treated with undiluted AS. (**B**) IPA upstream/downstream analysis link some differentially expressed proteins with the activation of HK cells by TGF-β1 and the activation of the cytoskeleton organization.

Other important processes related to wound healing, such as angiogenesis and protein synthesis, cell proliferation and motility, were also represented with two canonical pathways, each one like Renin-Angiotensin Signaling and VEGF Family Ligand-Receptor and Interactions mTOR signaling and p70S6K signaling, respectively (Figure 3). In both cases, AS treatment significantly increased the number of differentially expressed proteins in HK cells in comparison to PRGF.

Finally, two more regulatory pathways related to oxidative stress (Production of Nitric Oxide and Reactive Oxygen Species in Macrophages and NRF2-mediated Oxidative Stress Response) were also significantly activated in AS compared to PRGF (Figure 3 and Supplementary Figure S4). In addition, the IPA upstream analysis revealed that some differentially expressed proteins were related to the activation of HK cells by oxidative stress, similar to the induction of HK cells by the addition of hydrogen peroxide (Figure 6). These results suggest that oxidative stress is significantly increased in HK cells after AS treatment in comparison to PRGF.

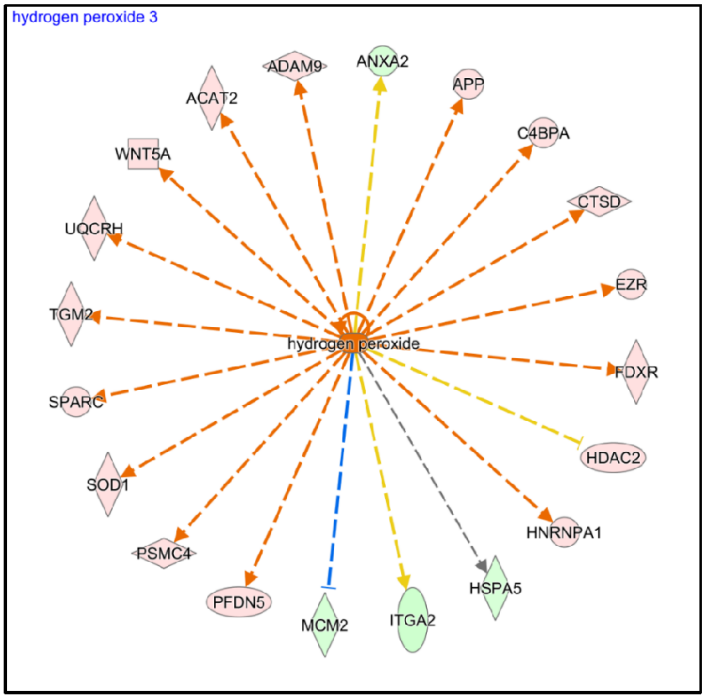


Figure 6. IPA Comparison Analysis predicted hydrogen peroxide as a top upstream regulator for some protein differentially expressed in HK cells after AS treatment.

3. Discussion

Over the past four decades, an increasing number of blood-derived products have been developed to improve tissue regeneration in several ocular surface diseases. There are several protocols and processes that have been established to produce this type of blood derivative, obtaining different products which contain different protein compositions, leading to a wide variety of clinical outcomes for the treatment of the same ocular surface disorders. The first blood-derived product used for the treatment of ocular surface pathologies was autologous serum (AS) diluted at 20%. However, when platelets were found to be one of the most important sources of protein and growth factors with regenerative properties, blood-derived products enriched in platelets were increasing their popularity in their use for the treatment of ocular pathologies due to their higher content in growth factors regarding AS [9].

Apart from this, autologous serum was empirically used from the outset diluted at 20% because the concentration of several growth factors with antiproliferative and profibrotic properties like TGF-β were observed that were 5 times higher in AS than in tears. Since then, autologous serum eye drops were prepared at 20% dilution to prevent the potentially harmful effect [13,33]. However, AS dilution may reduce the concentration of several beneficial factors proven to support proliferation and migration of corneal epithelial cells [13]. Nonetheless, in the last few years, several groups and studies have supported the use of AS at higher concentrations of 50–100% to increase the concentration of growth factors with regenerative potential in touch with the damaged tissue, demonstrating good results in terms of both efficacy and safety [24,34,35].

We used sets of samples (PRGF and AS) from three donors. The first step of this study was to conduct a characterization of blood-derived products (undiluted PRGF and AS), which showed that PRGF contains two-fold higher platelet concentrations than platelets contained in peripheral blood used to obtain AS. Similar results were also observed in a comparative study between PRGF and AS, where the concentration of platelets in PRGF samples was almost twice that of AS [15]. In addition, in the present study, we present a proteomic analysis of the eye drops derived from PRGF in comparison with undiluted AS. Furthermore, the comparative proteomic expression between HK cultured cells treated with PRGF or AS was also determined.

When the proteomic analyses of the different blood-derived products were carried out, the results showed that only 285 proteins were identified between both formulations (PRGF and AS). Of all the proteins identified between both blood-derived formulations, significant differences between PRGF and AS were only found in 13 of them. However, these differences were not related to the activation of any biological pathways. These results suggest that there would be no differences between PRGF and AS. However, in spite of the depletion of the most abundant proteins, the large dynamic range of protein concentrations in blood-derived products, more than 10 orders of magnitude could mask the proteins with a lower concentration in contrast to high abundance proteins [36,37]. Therefore, an analysis of scarce proteins using further proteomic methods to unravel the differences in the protein composition of these two products (PRGF and AS) could be necessary.

In contrast, 3236 differentially expressed proteins were found when the proteome obtained from HK cells treated with PRGF or AS were compared, of which 352 were shown to have statistically significant differences. The analysis of the processes which these proteins are involved in revealed that these processes could be summarized as having six main biological functions: (A) Inflammation; (B) Angiogenesis; (C) Oxidative stress; (D) EGF pathway; (E) Protein synthesis, cell proliferation and motility; and (F) Actin cytoskeleton signaling. All these biological functions were significantly increased in HK cells treated with AS rather than in those cells treated with PRGF.

These results suggest a close relationship between AS-treated cells and the activation of different pathways in HK cells related to an inflammatory response, which was mainly related to Acute Phase Response Signaling, LPS-stimulated MAPK Signaling, CCR3 Signaling in Eosinophils, IL-6 Signaling, IL-15 Signaling and CXCR4 Signaling. These results were confirmed with an IPA analyses, which showed that several proteins, differentially expressed in a statistically significant form in HK cells treated with AS, may be associated with a cellular activation by some inflammatory cytokines such as IFN-γ, which leads to an immune response in the HK cells (see Figure 4). All these pathways associated with an immune response in HK cells after AS treatment could be related to the presence of inflammatory cytokines like IL-1, IL-6, IL-15, TNFα or IFN-γ in AS samples derived from the presence of macrophages and leukocytes during the preparation of this type of blood-derived product [38,39]. The etiopathology of several ocular diseases has an immunological component, or is secondary to systemic inflammatory diseases such as Sjögren's syndrome, rheumatoid arthritis, diabetes, graft versus host disease, among others [40–42]. Therefore, these ocular diseases should be treated with a therapy with a low content in inflammatory cytokines, which us able to regenerate the damaged tissue,

exerting at the same time an anti-inflammatory effect. A recent study showed that PRGF exerts a higher anti-inflammatory effect than AS diluted at 20% in an in vitro model of ocular surface fibroblasts treated with proinflammatory IL-1β and TNFα [22]. The present results suggest that undiluted AS could induce a higher inflammatory response in HK cells than PRGF eye drops.

In addition, inflammatory processes in pathologic cornea may stimulate the production of angiogenic factors by different ocular surface cells like epithelial cells or keratocytes. Some of these factors, such as vascular endothelial growth factor (VEGF), have been identified and isolated from the cornea [43]. The cornea is a unique avascular tissue, which gives it the characteristics of transparency and regularity that are essential for maintaining the optical function of the eyes. Many corneal disorders, such as infection, injury, and autoimmune reactions, lead to corneal angiogenesis. Corneal invasion by vessels induces its opacification, reducing patient visibility. Therefore, it is essential to reduce the possibility of inducing an angiogenic effect from drugs used to treat ocular surface pathologies. The present study shows that AS treatment induces a higher activation of proteins related to Renin-Angiotensin Signaling and VEGF Family Ligand-Receptor pathways than PRGF, suggesting that AS treatment may induce a significant higher activation of an angiogenic response in HK cells rather than PRGF eye drops.

On the other hand, several studies have demonstrated that inflammation has a strong association with oxidative stress [44]. Oxidative stress is characterized by the production of reactive oxygen species (ROS), like the superoxide anions, hydrogen peroxide, and hydroxyl radicals, which are related to cell damage inducing lipid peroxidation of membranes, oxidative changes in proteins, and oxidative damage to DNA [45]. An inflammatory process could induce an increase in ROS levels due to fueled oxygen consumption or reduced antioxidative defense in the affected tissue [46]. Several ocular disorders, like corneal inflammation, dry eye disease, keratoconus, and Fuchs' endothelial dystrophy, are associated with oxidative stress [47,48]. Several antioxidant treatments have been proposed to reduce the inflammatory reaction in several ocular diseases trying to induce ocular tissue healing [47,49,50]. Recent studies showed that PRGF treatment reduced the cytotoxic effects induced in retinal pigment epithelial cells exposed to an oxidative stress environment modulating the antioxidant pathways [51,52]. The present study shows that undiluted AS treatment induces an activation of oxidative stress pathways in HK cells similar to induction by the addition of hydrogen peroxide. The proteins related to oxidative stress were differentially expressed in a statistically significant form in HK cells treated with AS in comparison to those treated with PRGF, as is shown in Figure 6.

In addition, the present results revealed that proteins which are more abundant in AS-treated cells were clustered into an additional canonical pathway group related to EGF pathway activation. EGF, through binding to the EGF receptor (EGF-R), stimulates the proliferation of corneal epithelial and endothelial cells and accelerates epithelial wound healing [53,54]. Furthermore, EGF promotes cell motility through its receptor phosphorylation, leading to an actin cytoskeletal rearrangement [55]. However, in the case of keratocytes, EGF induces cell differentiation to myofibroblasts through the stimulation of the EGF-R signaling pathway [56]. Myofibroblasts are responsible for wound contraction and extracellular matrix deposition and organization during injury repair. However, the continued presence of myofibroblasts after wound healing has been found to be the primary biological episode responsible for the development of scarring tissue [57]. Therefore, the present results suggest that the activation of EGF pathways in HK cells after AS treatment may promote scar tissue formation compromising corneal transparency.

Furthermore, AS-treated cells reveal a greater association with processes such as protein synthesis, proliferation, and cellular motility, mainly related to mTOR (mammalian target of rapamycin) signaling and p70S6K (Ribosomal protein S6 kinase beta-1) signaling. The p70S6K is a downstream target of mTOR signaling and the mTOR is a serine/threonine protein kinase that has been found to affect many cellular functions, including cell growth, proliferation, and metabolism [58]. In addition, in a recent study of a rabbit alkali burn

model, it was shown that the inhibition of the mTOR pathway promoted the autophagy and inhibited the proliferation, invasion, and migration of corneal stromal cells, promoting corneal wound healing [59]. Furthermore, it has been shown that mTOR signaling may induce scarring, neovascularization, and inflammation in the cornea [60,61]. An additional study showed that TGF-β activated the mTOR pathway in corneal stromal fibroblasts, and that rapamycin (a mTOR inhibitor) inhibited corneal stromal fibroblasts proliferation and modulated their transformation into myofibroblasts [62]. Hence, the mTOR signaling pathway activation after undiluted AS treatment could induce a higher tissue fibrosis than PRGF. In the same way, significant pathway activation associated with actin cytoskeleton signaling (Rho Family GTPases and RhoA Signaling) has been showed in HK cells treated with undiluted AS in comparison to PRGF. Rho family GTPases control diverse signal transduction pathways, one of the main functions of which is to control the actin cytoskeleton. Members of the Rho family GTPases include RhoA, -B, and -C, Rac1 and -2, and Cdc42. RhoA regulates actin polymerization, inducing the formation of stress fibers and the assembly of focal adhesion complex [63]. Several growth factors, like TGF-β and basic fibroblast growth factor (FGF)-2, induce the activation of Rho signaling pathways; however, TGF-β seems to be the main factor which activates these pathways and therefore is the main inductor to keratocyte differentiation to myofibroblasts, leading to the expression of α-SMA [64,65]. In the present study, the results observed after IPA analyses showed that a large number of proteins differentially expressed in a statistically significant manner in HK cells treated with AS were related to the activation of the organization of cytoskeleton similar to the stimulation of HK cells by TGF-β1, as is shown in Figure 5. Our results come along with previous proteomic study in HK myodifferentiated cells after TGF-β1 incubation and treatment with PRGF or 20% AS [23]. This study showed that PRGF treatment inactivated or reduced the activation of several proteins involved in the pathways whereby TGF-β1 exerts its action to induce the formation of α-actin fibers on HK cells, inducing their transformation to myofibroblasts. However, AS treatment was not able to reduce TGF-β1 action in myodifferentiated HK cells. According to this, previous studies have also demonstrated that PRGF exerts an anti-fibrotic effect by reducing the transformation of TGF-β1-treated stromal fibroblasts to myofibroblasts [66], minimizing scar formation while improving corneal tissue regeneration [67]. Furthermore, it has been shown that this anti-fibrotic effect of PRGF is significantly higher than AS diluted at 20% in HK cells differentiated to myofibroblasts by TGF-β1 [15]. The higher content of fibroblast growth factor (FGF) in PRGF formulations rather than in AS may be a possible mechanism by which PRGF induces less activation of the actin cytoskeleton pathways. Some studies have demonstrated that FGF-1 and -2 promote the fibroblast phenotype and reverse the myofibroblast phenotype [68].

The present study suggests that undiluted AS induces the activation of different pathways in HK cells related to an inflammatory, angiogenic, oxidative stress and scarring response in comparison to PRGF. Thus, PRGF could be a better alternative than AS for the treatment of ocular surface disorders. However, blood-derived products are composed of a wide variety of proteins and growth factors; hence, further studies are needed to unravel the proteins involved in the mechanisms underlying the differentially regulated pathways between PRGF and AS.

4. Materials and Methods

4.1. PRGF and Autologous Serum (AS) Preparations

Blood from three healthy male donors was drawn off after obtaining informed consent into 9 mL tubes with 3.8% (w/v) sodium citrate or in serum collection tubes (Z Serum Clot activator, Vacuette, GmbH, Kremsmünster, Austria) for PRGF and AS preparation respectively (Figure 7). The study was performed following the principles of the Declaration of Helsinki. Blood sample for PRGF was centrifuged at 580× *g* for 8 min at room temperature in an Endoret System centrifuge (BTI Biotechnology Institute, S.L., Vitoria, Álava, Spain); the whole plasma column was collected using Endoret ophthalmology kit (BTI Biotechnol-

ogy Institute, S.L., Vitoria, Álava, Spain) avoiding the buffy coat collection. Platelets and leukocytes counts were performed with a hematology analyzer (Pentra ES 60, Horiba ABX SAS, Montpelier, France). The whole plasma was activated with 10% calcium chloride at a rate of 20 µL per milliliter of PRGF, and the obtained PRGF supernatants were filtered, aliquoted and stored at −80 °C (the undiluted PRGF was termed PRGF). Blood sample for autologous serum preparation was allowed to clot at room temperature for 40 min and then it was centrifuged for 10 min at 1000× *g*; after that, serum was collected and filtered by 0.22 µm PVDF filters, aliquoted and stored at −80 °C until use (the undiluted AS was termed AS).

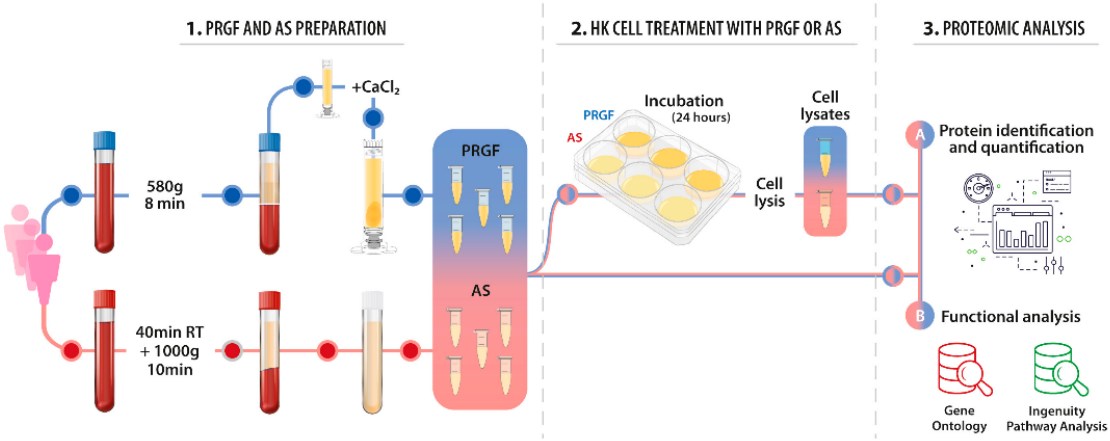


Figure 7. The study design is shown schematically. First, the two blood derivatives, PRGF and AS, were obtained. On the one hand, HK cells were incubated with these formulations to assess their response to each blood derived product by proteomic techniques. On the other hand, both PRGF and AS preparations were characterized by the same proteomic techniques.

4.2. Cells

Cells involved in assays were human corneal stromal keratocytes (HK) (ScienCell Research Laboratories, San Diego, CA, USA) that were cultured according to the manufacturer's instructions. Briefly, cells were maintained in culture until confluence in Fibroblast medium supplemented with Fibroblast Growth Supplement (FGS), 2% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin) (ScienCell Research Laboratories, San Diego, CA, USA) and then were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion.

HK cells were seeded in a 6-well plate at a density of 50,000 cells per cm^2 in serum-free medium supplemented with 20% (*v*/*v*) of the different treatment samples (PRGF and AS) obtained from the three donors (Figure 7). The HK cells were incubated with each treatment for 24 h. Then, culture media were discarded and the wells were rinsed with PBS. In order to obtain the proteins from cells, 400 µL of cell lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS was added to each well. Samples were incubated for 30 min at room temperature under agitation and digested following the filter-aided FASP protocol described by Wisniewski et al. [69] with minor modifications. Trypsin was added to a trypsin:protein ratio of 1:10, and the mixture was incubated overnight at 37 °C, dried out in a RVC2 25 speedvac concentrator (Christ), and resuspended in 0.1% FA.

4.3. Proteomic Analysis

Samples were submitted to LC-MS label-free analysis using a novel hybrid trapped ion mobility spectrometry–quadrupole time of flight mass spectrometer (timsTOF Pro with PASEF, Bruker Daltonics, Billerica, MA, USA) coupled online to a nanoElute liquid

chromatograph (Bruker. Sample (200 ng) was directly loaded in a 15 cm Bruker nanelute FIFTEEN C18 analytical column (Bruker) and resolved at 400 nL/min with a 30 min gradient. Column was heated to 50 °C using an oven.

Protein identification and quantification was carried out using MaxQuant software using default settings [70]. Searches were carried out against a database consisting of human protein entries (Uniprot/Swissprot), with precursor and fragment tolerances of 20ppm and 0.05 Da. Only proteins identified with at least two peptides at FDR < 1% were considered for further analysis. Data (LFQ intensities) were loaded onto Perseus platform [71] and further processed (log2 transformation, imputation) before statistical analysis (Student's *t*-test).

4.4. Functional Analysis

Gene Ontology (GO) enrichment analysis was carried out using the DAVID online tool (http://david.abcc.ncifcrf.gov/summary.jsp accessed 7 October 2020) [72,73]. DAVID is a GO Term annotation and enrichment analysis tool used to highlight the most relevant GO terms associated with a given gene list. A Fisher's Exact test is used in order to determine whether the proportion of genes considered into certain GO term or categories differ significantly between the dataset and the background. Biological Process (BP), Molecular Function (MF) and Cellular Component (CC) categories were assessed, and only GO Terms enriched with a p value < 0.05 were considered for comparison and discussion.

Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity accessed on 7 October 2020) was used for the functional analysis of the proteins identified. The calculated p-values determine the probability that the association between proteins in the dataset and a given canonical pathway, functional network or upstream regulator is explained by chance alone, based on a Fisher's exact test (p value < 0.05 considered as significant). Activation z-score represents the bias in gene regulation that predicts whether the upstream regulator exists in an activated (positive values) or inactivated (negative values) state, based on a knowledge of the relationship between the effectors and their target molecules.

5. Conclusions

Although further studies are needed to find the possible proteomic differences between PRGF and AS due to the large dynamic range of protein concentrations in these types of blood-derived products, the present study showed that PRGF and AS induce a significantly difference response in HK cells. Undiluted AS induces the activation of different pathways in HK cells related to an inflammatory, angiogenic, oxidative stress and scarring response in comparison to PRGF. These results suggest that PRGF could be a better alternative to AS for the treatment of ocular surface disorders.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222212176/s1.

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Data Availability Statement: All the obtained data used to support the findings of this study are available from the corresponding author upon reasonable request.

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3.4. ARTÍCULO IV

Anitua E, de la Fuente M, Muruzábal F, Merayo-Lloves J. **Stability of freeze-dried plasma rich in growth factors eye drops stored for 3 months at different temperature conditions. Eur J Ophthalmol. 2021 Mar;31(2):354-360**.

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RESUMEN

El objetivo de este estudio fue analizar el contenido biológico y la actividad de los colirios de plasma liofilizado rico en factores de crecimiento tras su almacenamiento a 4°C y a temperatura ambiente durante 3 meses con respecto a las muestras frescas (tiempo 0).

El plasma rico en factores de crecimiento se obtuvo tras la centrifugación de la sangre de tres donantes sanos. Tras la preparación del colirio del plasma rico en factores de crecimiento (PRGF) se liofilizaron las muestras solas o en combinación con un lioprotector (trehalosa), y luego se almacenaron durante 3 meses a temperatura ambiente o a 4°C. Se analizaron varios factores de crecimiento en cada tiempo y condición de almacenamiento. Además, se evaluó el potencial proliferativo y migratorio de los colirios liofilizados ricos en factores de crecimiento conservados durante 3 meses a diferentes condiciones de temperatura en queratocitos humanos primarios.

Los diferentes factores de crecimiento analizados mantuvieron sus niveles en cada tiempo y condición de almacenamiento. Los colirios de plasma liofilizado rico en factores de crecimiento almacenados a temperatura ambiente o a 4°C durante 3 meses no mostraron diferencias significativas en la actividad proliferativa de los queratocitos en comparación con las muestras frescas. Sin embargo, se ha mejorado significativamente

la migración de estas células tras el tratamiento con colirio de plasma liofilizado rico en factores de crecimiento conservado durante 3 meses en comparación con los obtenidos a tiempo 0. No se observaron diferencias significativas entre el colirio de plasma liofilizado rico en factores de crecimiento mezclado o no con lioprotector.

El colirio de plasma liofilizado rico en factores de crecimiento conserva los principales factores de crecimiento y su actividad biológica tras su almacenamiento a temperatura ambiente o a 4°C durante al menos 3 meses. El colirio de plasma liofilizado rico en factores de crecimiento conserva sus características biológicas incluso sin el uso de lioprotectores durante al menos 3 meses.

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Stability of freeze-dried plasma rich in growth factors eye drops stored for 3 months at different temperature conditions

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Abstract

Purpose: The purpose of this study was to analyze the biological content and activity of freeze-dried plasma rich in growth factors eye drops after their storage at 4°C and at room temperature for 3 months with respect to fresh samples (time 0).

Methods: Plasma rich in growth factors was obtained after blood centrifugation from three healthy donors. After platelet activation, the obtained plasma rich in growth factors eye drops were lyophilized alone or in combination with lyoprotectant (trehalose), then they were stored for 3 months at room temperature or at 4°C. Several growth factors were analyzed at each storage time and condition. Furthermore, the proliferative and migratory potential of freeze-dried plasma rich in growth factors eye drops kept for 3 months at different temperature conditions was evaluated on primary human keratocytes.

Results: The different growth factors analyzed maintained their levels at each time and storage condition. Freeze-dried plasma rich in growth factors eye drops stored at room temperature or 4°C for 3 months showed no significant differences on the proliferative activity of keratocytes in comparison with fresh samples. However, the number of migratory human keratocytes increased significantly after treatment with lyophilized plasma rich in growth factors eye drops kept for 3 months compared to those obtained at time 0. No significant differences were observed between the freeze-dried plasma rich in growth factors eye drops whether mixed or not with lyoprotectant.

Conclusion: Freeze-dried plasma rich in growth factors eye drops preserve the main growth factors and their biological activity after storage at room temperature or 4°C for up to 3 months. Lyophilized plasma rich in growth factors eye drops conserve their biological features even without the use of lyoprotectants for at least 3 months.

Keywords

Plasma rich in growth factors, stability, platelet growth factors, freeze-dried, platelet-rich plasma

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Introduction

Ocular surface injuries include epithelial and stromal tissue disorders. The effective healing of these disorders is crucial to recover the main functions of the damaged ocular tissues. Healing is the net outcome of complex cellular and extracellular processes that are coordinated by different proteins including various growth factors such as epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor

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(VEGF), and platelet-derived growth factor (PDGF), among others.[1,2]

In recent years, a type of platelet-rich plasma (PRP), denominated as plasma rich in growth factors (PRGF),[3] has been widely used in its eye drops formulations for the treatment of several ocular surface diseases such as dry eye, corneal ulcers, or persistent epithelial defects.[4–10] The beneficial effects obtained from the use of PRGF eye drops in the treatment of ocular pathologies are referred to their biological and biophysical properties, which are similar to the artificial tears, including pH, osmolarity, protein content, growth factors, as well as their anti-microbial and anti-inflammatory effects.[11,12]

Ocular surface disorders are chronic pathologies that require medium- or long-term treatment. Hence, it is essential that the stability and the biological activity of the treatments can be maintained for long periods of time in order to be used daily for months. In the case of eye drops derived from blood derivatives, several studies have demonstrated their safety and stability over several months, although their long-term storage and their maintenance during the period of application require the use and dependence of a cold chain (storage at −20°C and +4°C during their use).[13]

However, some patients are not suitable to be donors to obtain autologous hemoderivative products due to systemic inflammatory diseases, age, and other types of disorders or comorbidities. In these types of patients, an allogeneic blood-derived product could be an alternative to treat several ocular surface diseases, such as severe dry eye in patients with graft versus host diseases.[14] In these cases, the possibility of off-the-shelf storage would be advantageous for the use of blood-derived eye drops.[15]

Recently, our group demonstrated that PRGF eye drops can be lyophilized by maintaining their biological properties even without the use of lyoprotectants, such as trehalose (unpublished results), whose role in the protection of the ocular surface is well demonstrated.[16]

The purpose of this study was to analyze the optimal storage conditions of freeze-dried PRGF eye drops to preserve the composition and the biological activity after storage for 3 months at room temperature (RT) or +4°C in comparison with the freshly prepared eye drops.

Materials and methods

PRGF sample preparation

This study was conducted according to the principles of the Declaration of Helsinki following approval by the corresponding ethical committee. After collection of the informed consent, blood from three healthy donors was drawn-off into 9 mL tubes with 3.8% (wt/v) sodium citrate. Then, blood was centrifuged at 580 *g* for 8 min at RT in an Endoret system centrifuge (BTI Biotechnology Institute, Vitoria-Gasteiz, Spain). The whole plasma column was collected avoiding the layer containing leukocytes using Endoret ophthalmology kit (BTI Biotechnology Institute). Hematology analyzer (ABX Micros 60; Horiba, Montpelier, France) was used to analyze platelet and leukocyte concentration in each sample. Whole PRP volume was activated with calcium chloride and incubated for 1 h at 37°C. The supernatant enriched in growth factor was collected, filtered, and aliquoted in glass vials for lyophilization and divided into three groups: (1) PRGF: fresh PRGF supernatant (used as a control), (2) PRGF lyo: pure PRGF supernatant frozen at −80°C, and (3) PRGF lyo + 2.5T: PRGF supernatant was mixed with 2.5% trehalose as lyoprotectant and frozen at −80°C. Samples belonging to Groups 2 and 3 were introduced in the lyophilizer (LyoBeta; Telstar, Terrassa, Spain). The primary drying phase was carried out at −50°C and 0.1 mBar for 24 h. Finally, secondary drying phase was performed at +20°C and 0.1 mBar for 12 h. After lyophilization, one part of the samples was used immediately and the other part was divided into two halves: one half was stored for 3 months at +4°C and the other half was stored at RT. The freeze-dried samples were reconstituted before their use with sterile water at the same volume used prior to the lyophilization process.

Growth factor levels in PRGF eye drops

The concentrations of several growth factors, such as EGF, PDGF, TGF-β1, and VEGF, were analyzed to assay the stability of freeze-dried PRGF eye drops at each time and storage condition. These growth factors were analyzed using the commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA).

Cell culture

The biological activity of the different eye drops stored at different times and conditions was studied on primary corneal keratocytes (called HK; ScienCell Research Laboratories, San Diego, CA, USA). HK were cultured following the manufacturer's instructions. Briefly, cells were cultured in fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories), antibiotics, and 2% fetal bovine serum (FBS; termed Complete FM) at 37°C and 5% CO_2 atmosphere until confluence.

Upon reaching confluence, the cells were detached using a commercial enzyme free of animal traces (TrypLE Select; Gibco–Invitrogen, Grand Island, NE, USA). Trypan blue dye was used to check the viability of the cells by exclusion method, based on the principle that non-viable cells take up the dye while viable cells do not.

Cell proliferation

Human keratocytes (HK) were seeded in a 96-well dark-bottom plate at a density of 10,000 cells/cm^2 in serum-free medium supplemented with 20% (v/v) of the different PRGF samples kept at different storage times and conditions. At each time of the study, cells incubated with FBS diluted at 0.1% (v/v) were used as a control of cell growth and to normalize the results obtained at each study time. The study period was 72 h. CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA, USA) was used to analyze the cell density obtained with each PRGF sample. Briefly, the wells were washed carefully with phosphate-buffered saline (PBS) after removing the culture medium. Subsequently, to improve the cell lysis efficiency in the CyQUANT assay, the plate was frozen at −80°C. Then, the plate was thawed at RT and the samples were incubated with RNase A (1.35 kU/mL) diluted in cell lysis buffer for 1 h at RT. Before, the 2× CyQUANT GR dye/cell lysis solution was added to each well of the plate, mixed gently, incubated for 5 min at RT, and protected from light. A fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany) was used to measure the fluorescence of the sample. After that, the fluorescence values obtained for each well were divided by the value obtained for the cells cultured with 0.1% FBS to normalize the results obtained at each time of the study.

Cell migration

In order to quantify the migratory/chemotactic potential of the different PRGF eye drop samples on HK, the cells were seeded at high density inside the culture inserts (ibidi GmbH, Martinsried, Germany) placed in a 24-well plate and were grown with Complete FM until confluence. The inserts were then carefully removed obtaining two separate cell monolayers leaving a cell-free gap of approximately 500 µm thickness. The cells were washed with PBS and incubated in quintuplicate with the different PRGF eye drops obtained in the study for 24 h. Like cell proliferation assay, some wells were also incubated with 0.1% FBS as a control of cell migration and to normalize the results obtained at each time of the study. After this period, the different culture media were removed and the cells were incubated with Hoechst 33342 (Invitrogen, Carlsbad, CA) for 10 min. To quantify the number of migrated cells, phase-contrast images were taken from the central part of the gap before treatment, and phase contrast and fluorescence after 24 h of treatment using a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB; Leica Microsystems, Barcelona, Spain). ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA) was used to measure the gap area taken in each image and the number of migrated cells after 24 h. After that, the number of migratory cells obtained for each well treated with PRGF samples was divided by the value obtained for cells cultured with 0.1% FBS to normalize the results obtained at each time of the study.

Statistical analysis

Data are expressed as mean ± SD (see Supplementary Table). To evaluate the differences between the variables analyzed at the different storage temperatures (RT and 4°C) and times points (t0 and t3), non-parametric Friedman procedure was carried out followed by Wilcoxon test to discriminate among the statistical means. The significance level was established at $p=0.05$. SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA) was used to perform the statistical analyses.

Results

Endoret preparations had a mean platelet enrichment of 2.14-fold over the platelet concentration in peripheral blood. No detectable levels of leucocytes were observed in any of the PRGF preparations.

Protein levels in PRGF eye drop storage at different time and conditions

The characteristics of the different samples of PRGF eye drops used for this study were analyzed at the day of sample collection (time 0, t0), and after storage in freeze-dried form (PRGF lyo and PRGF lyo + 2.5T) at +4°C and at RT for 3 months (t3).

The results revealed that all concentration levels of the different growth factors analyzed in the study showed no significant changes after 3 months of storage at +4°C or RT in a freeze-dried state in comparison with the fresh samples (samples obtained at t0; Figure 1). Furthermore, no significant differences ($p>0.05$) were observed between the freeze-dried PRGF eye drops kept at 4°C or RT for 3 months.

PRGF eye drop effect over cell proliferation

Figure 2 shows the representative images of cells treated with freshly prepared PRGF eye drops (t0) and with freeze-dried PRGF eye drops stored at RT and 4°C for 3 months (t3). The HK proliferation index was not modified after treatment with PRGF eye drops obtained at t0 (fresh or freeze-dried samples evaluated at the obtaining time) or after storage at +4°C or RT for 3 months in lyophilized form (see Figure 2). Similarly, no significant differences were observed in the proliferative potential between freeze-dried PRGF eye drops stored at 4°C or RT for 3 months.

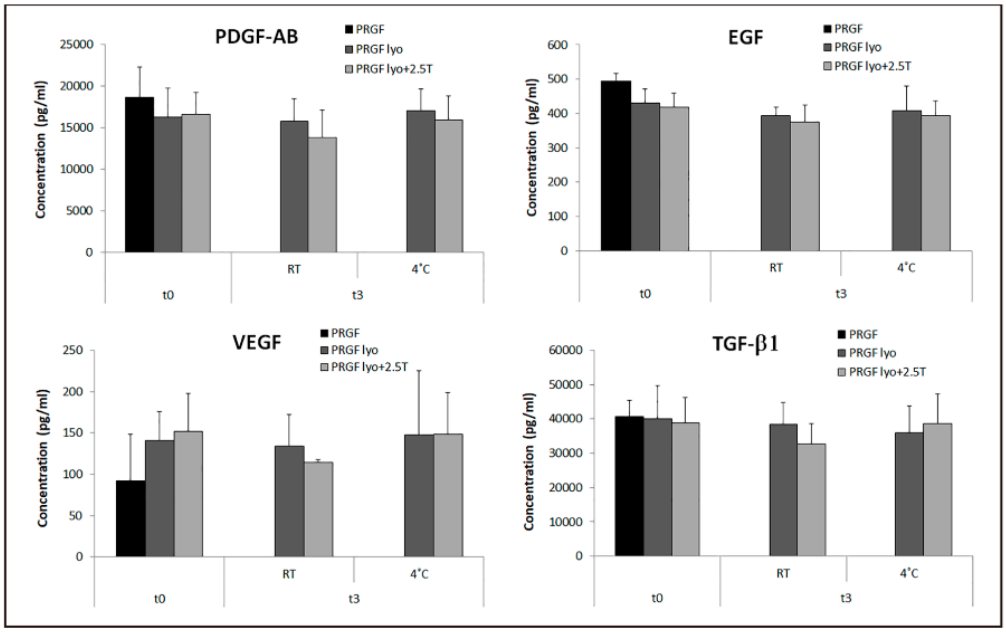

Figure 1. Several growth factors involved in the ocular surface tissue regeneration were analyzed in the different study samples. No significant differences ($p > 0.05$) were observed among the freeze-dried PRGF eye drop samples stored for 3 months at 4°C or RT and the PRGF eye drop samples obtained at time 0 (fresh samples) in any of the analyzed factors.

Migratory potential of freeze-dried PRGF eye drops

Figure 3 shows the representative images of HK treated with PRGF eye drop samples obtained at t0 and after storage for 3 months at RT and 4°C in a freeze-dried manner. Migratory activity of HK increased significantly ($p < 0.05$) after treatment with freeze-dried PRGF eye drop samples stored at RT or 4°C for 3 months with respect to t0 (Figure 3). These differences were observed among the different freeze-dried samples (PRGF lyo and PRGF lyo + 2.5T) stored for 3 months at RT and 4°C with respect to fresh PRGF samples. Significant differences were also detected among pure lyophilized PRGF eye drop samples (PRGF lyo) or mixed with 2.5% trehalose (PRGF lyo + 2.5T) stored for 3 months at +4°C or RT with respect to their freeze-dried sample obtained at t0 (see Figure 3). No differences ($p > 0.05$) were observed in the HK migratory potential between the freeze-dried PRGF samples mixed with or without trehalose (PRGF lyo + 2.5T and PRGF lyo, respectively).

Discussion

In recent years, hemoderivative products have been widely used for the treatment of different ocular surface diseases such as dry eye, persistent epithelial defects, and ocular ulcers.[17–19] The benefits of these type of products are mainly attributed to their content in growth factors that are involved in the regeneration of the ocular surface tissues such as EGF, TGF-β1, VEGF, or PDGF, levels of which are similar to those observed in the natural tears.[1,2,20] It is very common that the diseases mentioned above need long-term treatments, being necessary to store these blood derivative products at low temperatures in order to maintain their biological characteristics during this period of application.[21] However, the long-term storage of blood-derived products and their application during the period of use requires the dependence of a cold chain (storage at −20°C to keep it during a large period of time and +4°C during its use).[13] In this study, we have demonstrated that freeze-dried PRGF eye drops maintain the levels of different growth factors involved in ocular surface tissue regeneration as well as their biological activity after their storage at RT or at 4°C for at least 3 months.

The cause of a significant increase in the migratory activity of keratocytes after treatment with freeze-dried PRGF eye drops stored for 3 months in contrast to the PRGF eye drops obtained at t0 remains unknown. One possible explanation is that some proteins or growth factors that could be involved in the control/inhibition of cell migration could be partially or totally denaturalized during the storage period. Although in previous studies a slight increase in migratory capacity of HK was observed after treatment with PRGF eye drops stored at −20°C for 3 months,[13] these changes did not become significant, hence further investigations will be needed to evaluate the results obtained at this point in this study.

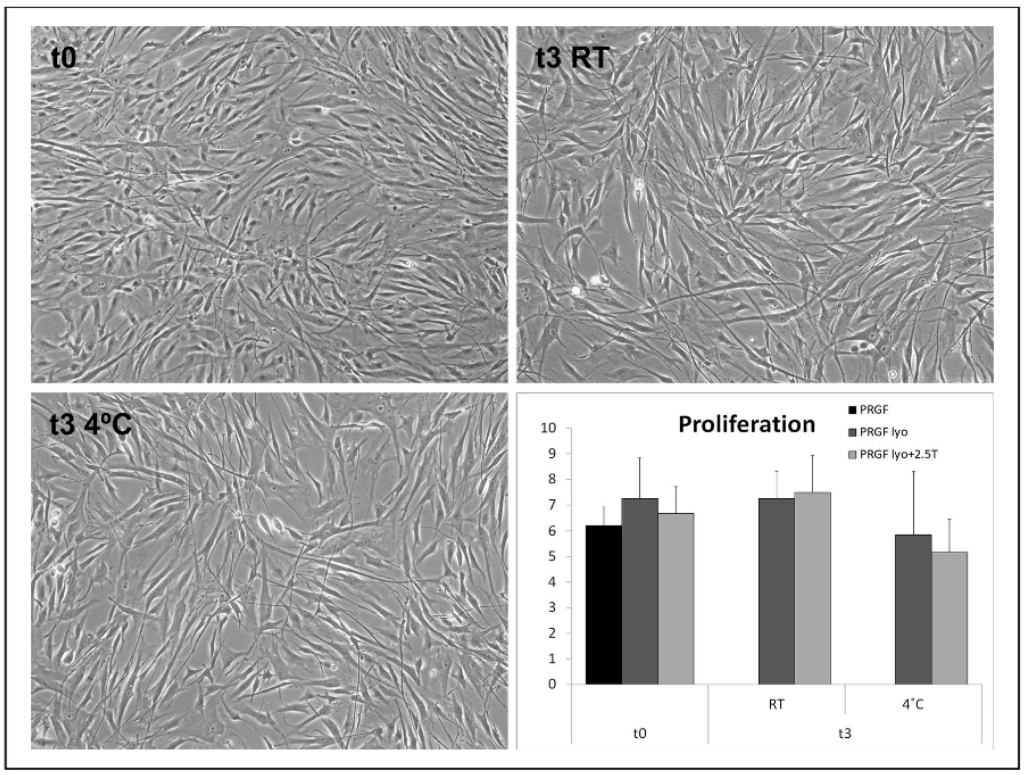

Figure 2. Proliferation of HK after treatment with freeze-dried PRGF eye drops. Representative phase-contrast photomicrographs showing human primary keratocytes cultured with fresh PRGF-Endoret samples (t0) and freeze-dried PRGF-Endoret eye drops after storage for 3 months at room temperature (t3 RT) and at 4°C (t3 4°C). No significant differences ($p > 0.05$) were observed among the proliferation induced by the PRGF eye drops at any time and condition of storage (with or without the use of trehalose).

In recent years, several studies have been carried out to evaluate the stability of freeze-dried hemoderivative products.[22,23] Although remarkable results were observed in these studies, the lyophilized products were stored at temperatures under 4°C for their maintenance. In this study, it has been shown that lyophilized PRGF eye drops can be stored at RT for up to 3 months preserving their biological features like those of fresh PRGF eye drops. Hence, it is necessary to highlight that, one important benefit of the freeze-dried PRGF eye drops is their easy storage, allowing to keep this product at RT for at least 3 months avoiding the dependence on cold chain.

The freeze-drying process could alter protein structures due to the decrease in temperature and as a consequence of solute concentration increase during the freezing procedure.[24] The low temperatures encourage protein denaturation by disrupting the interactions between proteins in a similar way as thermal denaturation.[25,26] Multiple lyophilized products contain cryoprotectants or lyoprotectants to avoid protein denaturation during the freeze-drying process. The most common protectants used in freeze-dried protein formulations are disaccharides, such as sucrose or trehalose, due to their capability to substitute water molecules undergoing protein-stabilizing effects.[27] However, the use of trehalose could cause detrimental results in ocular surface fibroblast proliferation, thus reducing their regenerative capability.[28] In a recent work sent for publication, we have demonstrated that the developed freeze-dried PRGF eye drops without lyoprotectants maintain the growth factor levels and the biological activity in a similar way to the lyophilized PRGF eye drops mixed with 2.5% or 5% trehalose (unpublished results). In the present work, we have used the PRGF combined with 2.5% trehalose to evaluate whether freeze-dried PRGF eye drops alone preserve their biological potential in a similar manner as the first ones for 3 months of storage at 4°C or RT. The results obtained showed that freeze-dried PRGF eye drops without trehalose kept the concentrations of different growth factors and their biological potential in similar levels to those lyophilized PRGF samples mixed with trehalose. Thus, PRGF eye drops maintain their endogenous origin avoiding exogenous preservatives which can increase the risk of chemical toxicity.[29] Furthermore, the lyophilization process would permit off-the-shelf storage of the PRGF eye drops, facilitating the accessibility to this therapy to those patients who need several applications for a long period of time or for those who require an allogeneic application due to the unsuitability to obtain autologous hemoderivative products.[30]

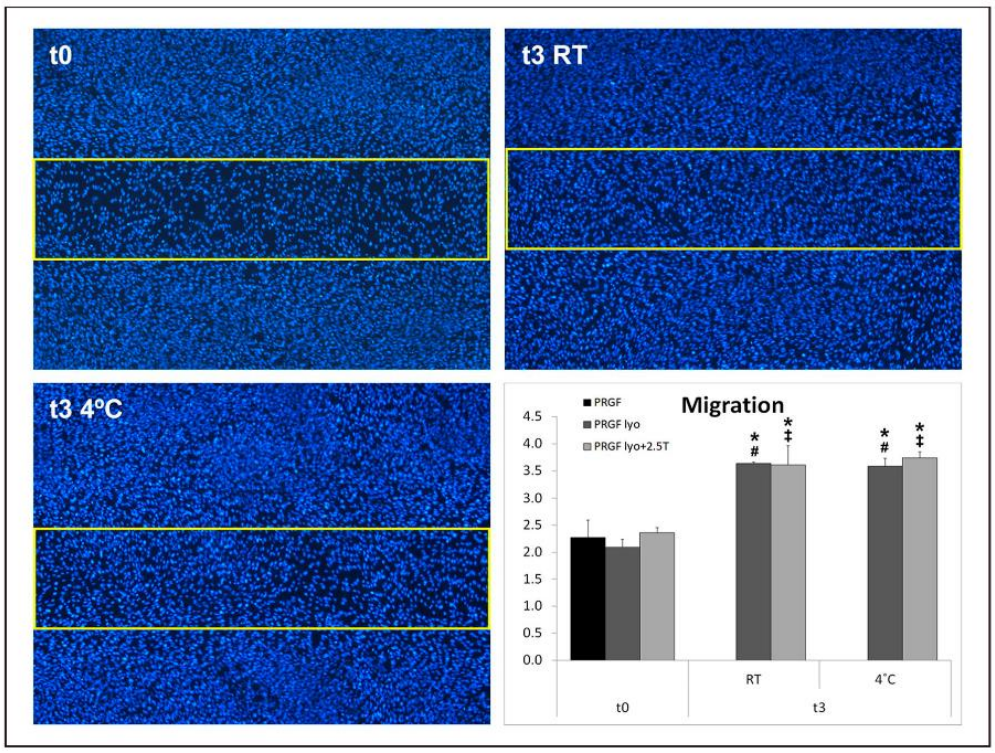

Figure 3. Migratory potential of lyophilized PRGF eye drops on HK. Representative fluorescence Hoechst images of HK after treatment with fresh PRGF-Endoret samples (t0) and with freeze-dried PRGF-Endoret eye drops stored for 3 months at room temperature (t3 RT) and at 4°C (t3 4°C). Yellow rectangle included in each image identifies the migration gap area. The graph shows that cell migration was significantly higher when cells were cultured with freeze-dried PRGF eye drop samples (with or without trehalose, PRGF lyo, and PRGF lyo + 2.5T, respectively) stored at RT or 4°C for 3 months in comparison with time 0. Furthermore, freeze-dried PRGF eye drops without (PRGF lyo) or with trehalose (PRGF lyo + 2.5T) stored for 3 months increased significantly the migratory potential of HK regarding their respective freeze-dried PRGF eye drops obtained at time 0.
*Statistically significant differences among the different freeze-dried PRGF samples stored for 3 months and the fresh PRGF eye drops ($p < 0.05$).
#Statistical significance of PRGF lyo eye drops stored for 3 months with respect to PRGF lyo obtained at time 0 ($p < 0.05$).
‡Statistically significant differences of PRGF lyo + 2.5T samples stored for 3 months regarding PRGF lyo + 2.5T obtained at time 0 ($p < 0.05$).

In summary, this study suggests that freeze-dried PRGF eye drops stored for up to 3 months at RT or 4°C preserve the principal growth factors and proteins involved in ocular surface tissue regeneration. Furthermore, lyophilized PRGF eye drops maintain their biological activity for 3 months stored at 4°C or at RT. Finally, it is important to highlight that freeze-dried PRGF eye drops conserve their biological characteristics even without the use of lyoprotectants for at least 3 months.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: E.A. is the Scientific Director and M.d.l.F. and F.M. are scientists at BTI Biotechnology Institute, a company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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Supplemental material

Supplemental material for this article is available online.

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3.5. ARTÍCULO V

Anitua E, de la Fuente M, Muruzábal F, Merayo-Lloves, J. **Short – and long – term stability of plasma rich in growth factors eye drops. Cornea 2021 Jan;40(1):107-112.**

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El objetivo de este estudio fue analizar si los colirios ricos en factores de crecimiento (PRGF) conservan su actividad y sus propiedades biológicas tras su almacenamiento durante 9 y 12 meses a -20°C, y a 4°C, y a temperatura ambiente (TA) durante 3 y 7 días en comparación con las muestras frescas (t0).

Los colirios de PRGF se obtuvieron de 6 donantes sanos. A continuación, se almacenaron durante 9 y 12 meses a -20°C. En cada momento, se descongelaron diferentes muestras de colirio de PRGF y se mantuvieron a TA o a 4°C durante 3 y 7 días. Se analizaron el factor de crecimiento derivado de las plaquetas-AB, el factor de crecimiento epidérmico, el factor de crecimiento transformante-β1, el factor de crecimiento endotelial vascular, la angiopoyetina-1 y la trombospondina-1 en cada momento y temperatura de almacenamiento. Además, también se evaluaron el nivel de pH, la contaminación microbiana y el potencial proliferativo sobre los fibroblastos estromales humanos primarios de la córnea y los queratocitos humanos de cada colirio PRGF obtenido.

Todos los niveles de los principales factores de crecimiento se conservaron en cada tiempo y condición de almacenamiento. No se observaron diferencias en la proliferación de queratocitos humanos tras el tratamiento con colirios de PRGF en ninguno de los tiempos o temperaturas estudiados. No se observó contaminación microbiana en

ninguno de los colirios de PRGF. Por último, los niveles de pH aumentaron significativamente tras 9 y 12 meses de almacenamiento a -20°C en comparación con t0. El colirio PRGF puede almacenarse hasta 12 meses sin que se reduzcan los principales factores de crecimiento y proteínas y sin que se produzca ninguna contaminación microbiana. Además, la actividad biológica del colirio PRGF se mantiene después de almacenarlas durante 3 y 7 días a 4°C o a temperatura ambiente.

BASIC INVESTIGATION

Short- and Long-Term Stability of Plasma Rich in Growth Factors Eye Drops

Eduardo Anitua, MD, DDS,† María de la Fuente, MSc,*† Francisco Muruzábal, PhD,*† and Jesús Merayo-Lloves, MD, PhD‡§*

Purpose: To analyze whether plasma rich in growth factors (PRGFs) eye drops preserve their activity and biological properties after storage for 9 and 12 months at −20°C, and at 4°C, and at room temperature (RT) for 3 and 7 days in comparison to fresh samples (t0).

Methods: PRGF eye drops were obtained from 6 healthy donors. Then, they were stored for 9 and 12 months at −20°C. At each time, different PRGF eye drops samples were thawed and maintained at RT or at 4°C for 3 and 7 days. Platelet-derived growth factor-AB, epidermal growth factor, transforming growth factor-β1, vascular endothelial growth factor, angiopoietin-1, and thrombospondin-1 were analyzed at each time and temperature of storage. In addition, the pH level, the microbial contamination, and the proliferative potential on primary human corneal stromal fibroblasts human keratocytes of each obtained PRGF eye drops were also evaluated.

Results: All growth factor levels were preserved at each time and storage condition. No differences were observed on the human keratocytes proliferation after treatment with PRGF eye drops at any studied time or temperature. No microbial contamination was observed in any of the PRGF eye drops. Finally, the pH levels increased significantly after 9 and 12 months of storage at −20°C compared with t0.

Conclusions: PRGF eye drops can be stored for up to 12 months without reduction of the main growth factors and proteins and without any microbial contamination. Furthermore, the biological activity of the PRGF eye drops is maintained after storing for 3 and 7 days at 4°C or at RT.

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E. Anitua is the Scientific Director, and M. de la Fuente and F. Muruzábal are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology. The remaining author has no conflicts of interest to disclose.

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Key Words: plasma rich in growth factors, stability, growth factors, eye drops, platelet-rich plasma

(*Cornea* 2020;00:1 6)

Plasma rich in growth factors (PRGFs) eye drops has been successfully used for the treatment of several ocular surface disorders, such as dry eye, persistent epithelial defects, Sjögren syndrome, and neurotrophic keratitis, among others.[1–6] These fruitful results could be attributed to the high similarities found between the characteristics of PRGF eye drops and the tear film. Both contain a wide range of proteins and growth factors, such as platelet-derived growth factor-AB (PDGF-AB), epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), fibronectin, and vitamin A, among others, that are involved in the different biological processes of the ocular tissue regeneration.[7–9] Other important features of PRGF eye drops shared with the tear films are their antimicrobial, antifibrotic, and antiinflammatory properties.[10–12]

In addition, it is necessary to highlight that the PRGF eye drops therapy used for the treatment of eye disorders is produced under a standardized protocol in comparison to other blood-derived products.[13] Furthermore, PRGF eye drops manufacturing follows the regulatory framework published on May 23, 2013, by the Spanish Agency for Medicines and Health Products in which the classification of the nonsubstitute therapeutic use of autologous plasma and its fractions, components, or derivatives as drugs for human use to attend special needs was established.[14]

Most of the ocular surface disorders are chronic diseases that must to be treated for a long time to achieve successful outcomes. Therefore, therapies used for the management of these diseases should maintain their functionality and stability for several months to be used daily along this time. To ensure the preservation of the biological properties of PRGF eye drops in clinical practice, patients are instructed to store the PRGF eye drops containers in use at 4°C or at room temperature (RT), whereas the rest of the droppers should be stored at −20°C until required. Previous works have shown that PRGF eye drops can be stored for up to 6 months without reduction of the main proteins and growth factors implicated in ocular surface wound healing.[15,16] Furthermore, it was demonstrated that PRGF eye drops can preserve their composition and biological activity both at 4°C and at RT for 3 days.

However, some ocular disorders need a short treatment period, from days to a few weeks, to achieve a complete restoration of the ocular surface tissue. However, the symptoms

of the underlying chronic pathology may appear in a few months, thus requiring a new therapy application. On the other hand, in some countries such as Germany, competent authorities might require the quarantine of the eye drops preparations until the results of the bacterial contamination tests are available, which could take up to 1 to 2 weeks.[17] In this sense, the storage time of the blood derivative eye drops is already progressing. In addition, some ocular disorders are unilateral, affecting only one eye, in which case, the PRGF eye drops in use may last longer than the current 3 days treatment established for their short-term stability stored at RT or at 4°C. In this regard, it seems very interesting to evaluate whether long-term storage at −20°C could reduce the efficacy of the PRGF eye drops therapy and to investigate how long PRGF eye drops remain stable under different storage conditions.

The purpose of this study was to analyze whether PRGF eye drops maintain their content in proteins and growth factors as well as their biological potential after preservation at −20°C for 9 and 12 months compared with the freshly prepared eye drops. The conservation of PRGF eye drops after 3 and 7 days both at 4°C and at RT was also determined. For these purposes, the concentration of different growth factors, the pH level, the proliferative potential, and the microbial contamination against the freshly obtained eye drops were evaluated at each time and storage condition.

MATERIAL AND METHODS

PRGF Preparation

The study was approved by the local clinical research ethics committee (number of protocol BTI-04-IV/18/2018-7). The study was performed following all the declaration of Helsinki principles. Blood from 6 healthy donors was collected after they signed the informed consent. PRGF eye drops from each donor were performed using the Endoret Ophthalmology kit (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). After blood centrifugation, the whole plasma column was drawn off, avoiding buffy coat collection containing the leukocytes. Platelet concentration was measured with a hematology analyzer (Micros 60; Horiba ABX, Montpelier, France). The harvested platelet-rich plasma was activated with calcium chloride and incubated until complete clot retraction. Finally, the obtained supernatant was filtered with a filter pore size of 0.2 μm (Merk Millipore, Carrigtwohill, Ireland) and aliquoted into the BTI eye dropper to be used as fresh samples (t0) or to be stored at −20°C for 9 (t9) months and 12 (t12) months. After this time, 5 aliquots from each donor and each storage time (t9 and t12) were removed from the freezer and thawed, and then, one of them was immediately used and the rest were stored for 3 (3 d) and 7 (7 d) days at 4°C or at RT until their use.

Sterility Analysis

Sterility analysis were carried out after the European Pharmacopoeia principles for the sterility test. One milliliter from each PRGF samples stored at different times and temperatures was collected to check the sterility. Briefly, thioglycollate broth for the culture of anaerobic bacteria and tryptic soy broth for the culture of both fungi and aerobic bacteria were used for qualitative determination of microbial noncontamination of the different PRGF samples. After inoculation, culture vials were incubated at 32°C for thioglycollate broth and at 22°C for tryptic soy broth for 14 days and monitored for the growth of microorganisms. Increasing of broth turbidity was considered as a positive indicator of microbial contamination. Sterility was considered when no contamination of microorganisms occurred.

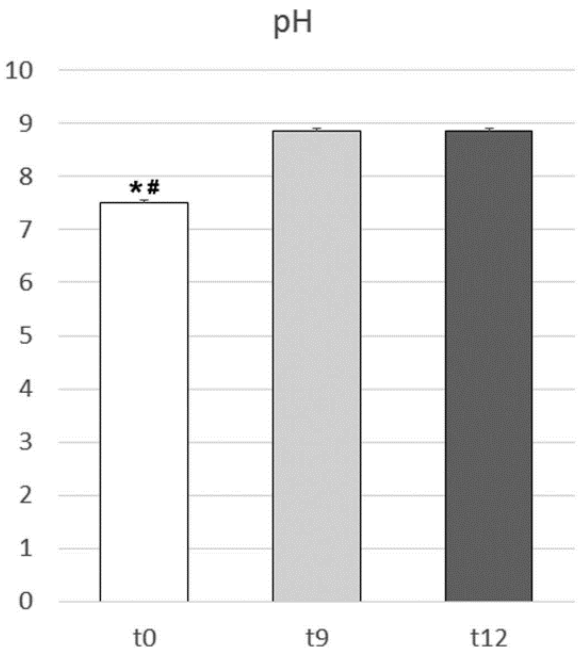

FIGURE 1. Levels of pH measured in fresh PRGF-Endoret samples (time 0) and in PRGF-Endoret eye drops after storage at −20°C for 9 (t9) and 12 (t12) months. Significant differences were observed among pH values obtained in t0 samples in comparison to t9 (*$P < 0.05$) and t12 (#$P < 0.05$).

PRGF Eye Drops Characterization

The stability of PRGF eye drops at each time and storage temperature was evaluated by the quantification of several growth factors involved in ocular surface tissue regeneration such as EGF, PDGF-AB, TGF-β1, VEGF, angiopoietin-1 (ANG-1), and thrombospondin-1 (TSP-1). They were measured using commercially available Quantikine Colorimetric Sandwich enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The pH was analyzed with a pH meter (Thermo Scientific, Madrid, Spain) on the fresh samples (t0) and on those that have been stored at −20°C for 9 and 12 months obtained from each donor.

Cell Culture

Primary human corneal stromal fibroblasts [termed human keratocytes (HK)] (ScienCell Research Laboratories, San Diego,

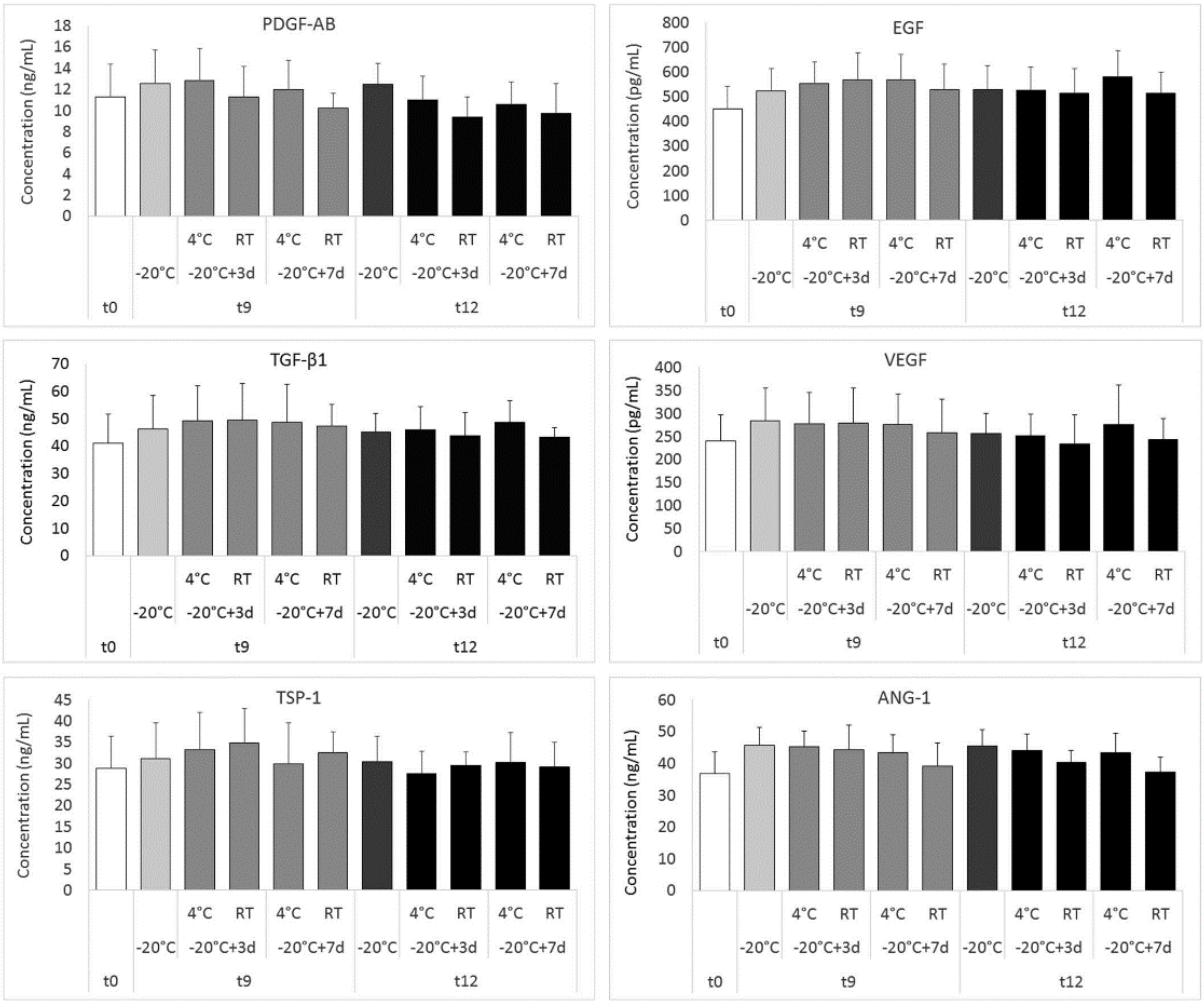

FIGURE 2. Concentration of the different growth factors analyzed in the study PDGF-AB, EGF, TGF-β1, VEGF, TSP-1, and ANG-1 in the PRGF eye drops samples stored at different times and temperature conditions. No significant differences ($P > 0.05$) were observed among the distinct time and storage conditions of the growth factors analyzed in the PRGF eye drops.

CA) were cultured following the manufacturer's instructions. Briefly, the cells were cultured at 37°C and 5% CO_2 atmosphere in a fibroblast medium supplemented with Fibroblast Growth Supplement (ScienCell Research Laboratories), 2% fetal bovine serum, and antibiotics until confluence. Then, animal origin-free trypsin-like enzyme (TrypLE Select; Gibco-Invitrogen, Grand Island, NY) was used to detach the cells, and trypan blue dye exclusion method was carried out to assess the cell viability. Finally, these cells were used in proliferation assays.

Cell Proliferation

Cells were seeded at a density of 5000 cells/cm^2 on 96-Well Optical-Bottom black microplates in serum-free medium supplemented with 20% (vol/vol) of PRGF eye drops obtained from each donor and kept at each time and temperature condition mentioned above. Then, the cells were incubated with each PRGF sample for 96 hours. The cell density was analyzed using the CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA). Briefly, the culture medium was removed, and the wells were carefully washed with phosphate-buffered saline for not disrupting the cellular monolayer. Later, the plate was frozen at −80°C to improve the cell lysis efficiency. After that, the plate was thawed at RT by performing the CyQUANT assay after leaflet instructions and including a RNase A treatment. The fluorescence of the sample was measured using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany). A control group (control) was included on each plate as an internal control of basal cell growth and as a

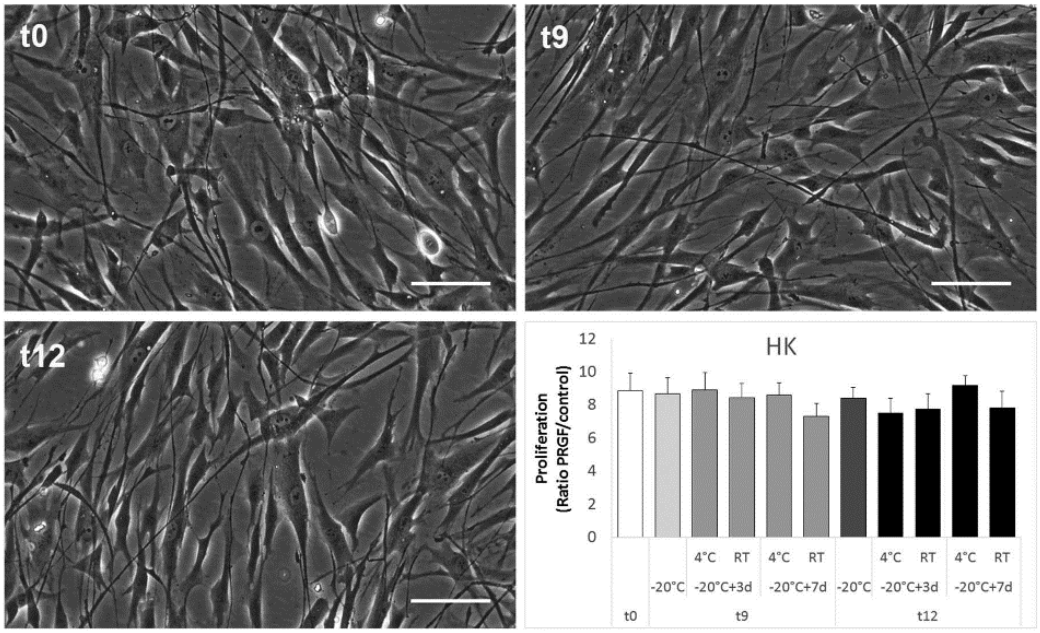

FIGURE 3. Representative phase contrast photomicrographs showing the proliferation of HK cells treated with fresh PRGF samples (t0) and with samples stored at −20°C for 9 (t9) and 12 months (t12). There were no significant differences ($P > 0.05$) among the proliferation rate induced by PRGF eye drops stored at any time and temperature condition.

reference control of cell growth between the different plates used in the study. For this control, the cells were incubated with basal fibroblast medium and 0.1% fetal bovine serum and antibiotics. To normalize the cell growth of the samples incubated in different plates, the fluorescence value obtained in each PRGF sample well was divided by the mean value of the control obtained on the corresponding plate (ratio = PRGF sample/control).

Statistical Analysis

Data are expressed as mean ± SD. After the analysis of the normal distribution and homoscedasticity of the results, one-way analysis of variance was used to assess the differences among the variables at the 3 different times points (t0, t9, and t12) and at distinct temperatures of storage (−20°C, RT, and 4°C), whereas the nonparametric Kruskal–Wallis test with a subsequent Mann–Whitney analysis test for multiple comparisons between groups were used in cases where no normality was detected. The level of significance was set at $P = 0.05$. Statistical analyses were performed using SPSS software (version 15.0; SPSS Inc, Chicago, IL).

RESULTS

Characterization of PRGFs Samples

The different samples of PRGF eye drops were obtained from 6 healthy donors (3 women and 3 men) with a mean age of 46 years (SD = 8), ranging from 31 to 52 years. PRGF samples showed a 1.95-fold increase on platelet concentration over peripheral blood. PRGF contained no detectable levels of leukocytes.

Sterility Analysis

No microbiological contamination was detected in any of the PRGF eye drops stored at different times and temperature conditions. Furthermore, no sign of microorganism growth was observed in any of the cell cultures assayed for proliferation with the different PRGF eye drops.

pH Levels

The pH levels of PRGF eye drops obtained on the same day of the assay (fresh samples, t0) or those samples stored at low temperatures (−20°C) for 9 (t9) or 12 (t12) months are represented in Figure 1. The pH levels (mean ± SD) increased significantly from the t0 (7.51 ± 0.04) to t9 (8.85 ± 0.06) and t12 (8.87 ± 0.04).

Levels of Growth Factors at Different Time and Temperature of Storage

The content of different growth factors related to the ocular tissue regeneration were measured in different PRGF eye drops samples on the day of collection (fresh samples, t0) and after storage at −20°C for 9 (t9) and 12 (t12) months. The different growth factor concentrations measured at each storage time is represented in Figure 2. None of the growth factors analyzed in the study showed a significant change ($P > 0.05$) after PRGF eye drops storage for 9 and 12 months

in comparison to the fresh samples (t0). In fact, the concentration of all growth factors analyzed in this study remained unchanged at each studied time.

At each study time (9 and 12 mo), 4 samples from each donor were thawed from the freezer and 2 of them were stored for 3 days at 4°C and RT and the other 2 were kept for 7 days at 4°C and RT. The levels of several growth factors (PDGF-AB, TGF-β1, VEGF, EGF, ANG-1, and TSP-1) analyzed in the eye drops stored during 3 and 7 days at 4°C or RT are presented in the Figure 2. No significant differences ($P > 0.05$) were observed in the content of the different growth factors among the different times and storage conditions evaluated in this study.

Proliferation Assay

Representative images of HK cells incubated for 96 hours with fresh PRGF eye drops (t0) and with PRGF eye drops stored at −20°C for 9 and 12 months are shown in Figure 3. The proliferation rate of HK cells showed no significant differences ($P > 0.05$) after treatment with the PRGF eye drops maintained at different time and temperature conditions.

DISCUSSION

In this study, the stability of PRGF eye drops stored at −20°C for 9 and 12 months and for 3 and 7 days at RT or at 4°C was evaluated. Recent studies have demonstrated the efficacy of PRGF eye drops for the treatment of several ocular surface disorders.[2,18,19] However, the storage of the PRGF eye drops for long- and short-term use could increase, assuming that some clinical doses are based on a daily use of the eye drops from 2 to 4 instillations and that the treatment time could last more than 9 months.[2,20] In this sense, we decided to evaluate the composition and biological activity of PRGF eye drops after different storage conditions.

Our results show that no significant differences ($P > 0.05$) were found in the levels of different growth factors and cytokines involved in ocular surface tissue regeneration such as TGF-β1, EGF, VEGF, PDGF-AB, TSP-1, and ANG-1 in the PRGF eye drops samples after their storage both at 9 and 12 months at −20°C. In addition, the levels of these proteins remain similar in comparison to the PRGF eye drops obtained at time 0 from the different donors (fresh samples). Current results could also simplify the logistic for patients depending on the procedure for chronic disorders treatment over long-term periods. For instance, this could allow patients to initiate the treatment again when symptoms reappeared without further blood collection and eye drop manufacturing.

Some stability studies of blood derivative products have shown that several growth factors and proteins analyzed at different storage temperatures for up to 24 hours were reduced in their concentration levels after storage at RT or 4°C.[21] In consequence, patients are specifically instructed to avoid keeping the eye droppers in their pocket or near to a warm place for more than a few moments and to discard those dispensers that have been left unrefrigerated for more than 2 to 3 hours. However, no differences in the concentration of the main protein levels were observed in our study among the PRGF eye drops obtained at time 0 (fresh samples) or stored at −20°C for 9 and 12 months and the eye drops stored at RT or 4°C for 3 and 7 days. In addition, no significant differences ($P > 0.05$) were observed in corneal stromal fibroblast proliferation after treatment either with the PRGF obtained at time 0 (fresh samples) or stored up to 9 and 12 months at −20°C and maintained at 4°C or RT for 3 and 7 days. The latter results need to be highlighted because they significantly improve the dosage of autologous eye drops, allowing the consumption of the PRGF eye drops in use for up to 7 days without dependence on a cold chain.

The pH of the tear films has similar values to the physiological levels (pH = 7.4). Likewise, eye drops obtained from blood-derived products also show comparable pH values as the tear film.[17,22] However, the eye is able to tolerate pH values ranging from 3.5 to 9, thanks to the buffering capacity of the tears.[23] In this work, the pH levels increased significantly in PRGF eye drops from mean values of 7.5 in fresh samples to values approximately 8.8 in PRGF eye drops stored at −20°C for 9 and 12 months. Despite that, our results show that pH levels remained in values below 9 during the entire study period; hence, PRGF eye drops stored at −20°C for up to 12 months should be perfectly tolerated by the ocular tissues.

To avoid the risk of chemical toxicity, blood-derived eye drops are commonly used without preservatives.[24] Recent studies have demonstrated the natural antimicrobial properties of the blood-derived products.[10,25] However, special care is taken to avoid the potential microbial contamination of the eye drop dispenser related to its long-term use.[26,27] As a consequence, it is recommended to keep the eye drops dispenser in use at 4°C for 5 to 7 days.[28] Despite this recommendation, microbial contamination was found in up to 25% of the eye drops dispensers analyzed.[24,29,30] In this study, no microbial contamination was detected in any of the eye droppers containing PRGF eye drops from each donors examined at each time point and at any of the evaluated temperature conditions. In addition, no microbial contamination was observed in the corneal stromal fibroblast cultures treated with PRGF eye drops stored at different times and temperatures.

In summary, this study shows that PRGF eye drops can be stored for up to 12 months without reduction of the main growth factors and proteins implicated in ocular surface tissue regeneration and without any microbial contamination. Furthermore, the biological activity of the PRGF eye drops is maintained when they are stored for 3 and 7 days at 4°C or at RT.

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3.6. ARTÍCULO VI

Anitua E, Pino A, Azkargorta M, Elortza F, Merayo-Lloves J, Muruzabal F. **Differential Protein Content between Fresh and Freeze-Dried Plasma Rich in Growth Factors Eye Drops**. **Biomolecules. 2022 Sep 1;12(9):1215.**

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El objetivo de este estudio fue analizar el efecto de la liofilización del colirio del plasma rico en factores de crecimiento (PRGF) en su composición proteómica y su efecto sobre cultivos celulares de queratocitos del estroma corneal humano (HK).

Después de la extracción de sangre, se preparó el colirio PRGF de 3 donantes sanos. Parte del colirio fue liofilizada (PRGF lyo). A continuación, las células HK se trataron con ambas formulaciones. Se realizó un análisis proteómico para evaluar el perfil proteómico diferencial entre el PRGF y el PRGF lyo, y la expresión proteínica diferencial de las células HK tratadas con estas dos formulaciones. Los resultados mostraron que se han detectado 280 proteínas, y sólo 8 de ellas alcanzaron diferencias significativas entre las dos formulaciones. Además, 101 de las 3213 proteínas detectadas en las células HK mostraron una desregulación estadísticamente significativa tras el tratamiento con PRGF o PRGF lyo. El análisis de ontología génica mostró que estas proteínas desreguladas estaban implicadas en 30 procesos funcionales. Sin embargo, el *Ingenuity Pathway* mostró que no se encontraron diferencias significativas en ninguno de los procesos identificados. Por lo tanto, el presente estudio muestra que no se encontraron diferencias

significativas en el perfil proteómico o en la activación de vías de señalización en células HK entre PRGF y PRGF lyo

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Differential Protein Content between Fresh and Freeze-Dried Plasma Rich in Growth Factors Eye Drops

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Abstract: The purpose of this study was to analyze the proteomic composition of plasma rich in growth factors eye drops (PRGF) in comparison to lyophilized PRGF eye drops (PRGF lyo). The differential protein expression of keratocyte (HK) cells after PRGF or PRGF lyo treatment was also determined. Blood from different donors was collected and processed to obtain PRGF and PRGF lyo eye drops. Then, HK cells were treated with both formulations. A proteomic analysis was performed to evaluate the differential proteomic profile between PRGF and PRGF lyo, and the differential protein expression by HK cells after treatment with both blood-derived products. About 280 proteins were detected between both blood-derived formulation, with only 8 of them reaching significant differences. Furthermore, 101 out of 3213 proteins showed statistically significant deregulation in HK cells after treatment with PRGF or PRGF lyo. Gene Ontology analysis showed that these significant deregulated proteins were involved in 30 functional processes. However, the Ingenuity Pathway Analysis showed that no significant differences were found in any of the identified processes. In summary, the present study show that no significant differences were found in the proteomic profile or in the signaling pathways activation in HK cells between PRGF and PRGF lyo.

Keywords: plasma rich in growth factors; PRGF; freeze-dried; lyophilization; proteomic; ocular surface; eye-drops; ocular disorders

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1. Introduction

The incidence of the different ocular surface disorders has been increasing along the last years [1]. The functional regeneration of the damaged ocular tissue is necessary and essential to recover the complete eye function and improve the patient life quality [2]. Most of the ocular surface disorders are commonly treated with artificial tears, but these treatments lack the characteristics of the natural tears [3]. Furthermore, they often contain additives that can potentially induce toxic or allergic reactions [4]. Several other therapies including anti-inflammatory drugs are frequently used for the treatment of ocular surface disorders showing symptom improvements, but their continued use could induce some drawbacks like ocular burning or increased ocular pressure [5–7].

Over the last three decades, blood derivative products like autologous serum (AS) and platelet-rich plasmas (PRP) have been developed for the treatment of different ocular pathologies [8–10]. The features of these products are similar to the natural tears such as their regenerative, lubrication, antimicrobial and anti-inflammatory properties. In addition, the characteristics of the blood-derivative products is very similar to the natural tears in terms of osmolarity, pH and many proteins which are involved in tissue regeneration [6,11,12]. These blood-derivative products have been successfully used for the

treatment of several ocular surface disease like dry eye disease, persistent epithelial defects, or corneal ulcers [8]. Nonetheless, the differential composition between both types of blood-derived products due to their different manufacturing protocol has been widely demonstrated [13,14]. As a consequence, different preclinical and clinical results have been obtained when both products have been used [15–19].

Plasma rich in growth factors (PRGF) eye drops is a type of PRP with specific characteristics including moderate platelet concentration, platelet activation, and lack of leukocytes [15,20]. PRGF eye drops have been used for the treatment of several ocular surface diseases such as corneal epithelial defects, dry eye, neurotrophic keratitis and graft versus host diseases among others, obtaining encouraging clinical outcomes [21].

Ocular surface disorders require medium- or long-term treatment because of their usual chronic condition. Therefore, it is necessary that treatments maintain their stability for long periods of time in order to be used daily for months. Several studies have demonstrated the safety and stability of PRGF eye drops stored for up to twelve months under low temperatures [22]. However, it implies that their long-term storage is dependent on a cold chain (−20 °C storage to keep it for a long period of time and +4 °C or room temperature during its use) [22–24].

On the other hand, although PRGF eye drops are commonly used in an autologous manner for the treatment of ocular pathologies, allogeneic products could be an interesting alternative to be used in those patients who are not suitable to be donors due to systemic inflammatory diseases, age, and other types of disorders or comorbidities [25].

Lyophilization of both types of products, autologous and allogeneic eye drops, could be an alternative to achieve longer shelf-life for both products avoiding a cold chain dependence. Recent preclinical studies have shown that PRGF eye drops maintain their biological properties after undergoing a freeze-drying process without the use of lyoprotectans like trehalose [26]. Furthermore, freeze-dried PRGF eye drops preserve the main growth factors and their biological activity for up to 3 months after storage at room temperature or 4 °C [27].

Although the stability of the main growth factors involved in ocular tissue regeneration has been studied after PRGF lyophilization, PRGF eye drops contain a much wider range of proteins and growth factors [13], then a deeper study is required to analyze the protein content of PRGF eye drops after a freeze-drying process. In addition, the freeze-drying process could modify the protein structures due to the low temperature and the higher concentration to which the sample is subjected during the freezing time [28]. And then, some lyoprotectants like trehalose are usually added to the product to avoid protein modifications [29]. However, the PRGF lyo used for the present study was obtained without the addition of any lyoprotectant. Therefore, this highlights the desirability of carrying out further PRGF lyo proteomic analysis to assess possible proteomic modifications occurred during the freeze-drying process. Then, the purpose of the present work was to characterize the proteomic composition of plasma-rich in growth factors after a freeze-drying process (PRGF lyo) regarding fresh PRGF (PRGF). In addition, the protein deregulation of HK cells after treatment with PRGF or PRGF lyo has been also determined.

2. Materials and Methods

2.1. Plasma Rich in Growth Factors Preparations

Blood from three healthy male donors was drawn off after informed consent into 9-mL tubes with 3.8% (*wt*/*v*) sodium citrate collection tubes. The study was performed following the principles of the Declaration of Helsinki. The process to obtain plasma rich in growth factors (PRGF) and the freeze-dried PRGF eye drops (PRGF lyo) was analogous to that described previously by our group [26]. PRGF from different donors was individually analyzed (not pooled). Figure 1 shows the whole workflow of the present study.

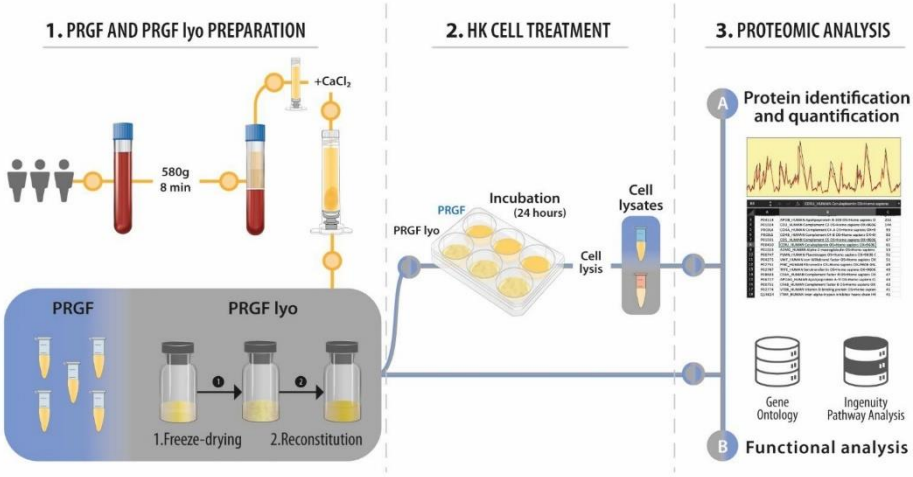


Figure 1. Schematic workflow of the proteomic study of the PRGF and PRGF lyo formulations. The study was divided into three parts; in the first part, the two blood derivatives, PRGF and PRGF lyo, were obtained. In the second part, HK cells were incubated with both blood derived products to analyze by proteomic techniques their response to each formulation. Finally, both PRGF and PRGF lyo were analyzed by the same proteomic techniques.

2.2. Cells

Human corneal keratocytes (HK) (ScienCell Research Laboratories, San Diego, CA, USA) was used to carry out the different assays of the present study. HK cells were cultured as described in previously published studies [30].

Keratocyte cells were seeded in a 6-well plate with serum-free medium supplemented with 20% (*v*/*v*) of the different treatments (PRGF and PRGF lyo) obtained from the three donors. Then, HK cells were incubated for 24 h. After that, culture media were removed, and cells were rinsed with PBS. Then, 400 µL of cell lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS was added to each well to obtain the cellular proteins. Samples were incubated for 30 min at room temperature under agitation and digested following the filter-aided FASP protocol described by Wisniewski et al. [31] with minor modifications. Trypsin was added to a trypsin:protein ratio of 1:10, and the mixture was incubated overnight at 37 °C, dried out in a RVC2 25 speedvac concentrator (Christ, Osterode am Harz, Germany), and resuspended in 0.1% FA.

2.3. Proteomic Analysis

The dataset of PRGF samples used for the comparison with PRGF lyo were previously employed to obtain the results of a study published previously, in which the PRGF dataset were compared with the dataset obtained using an autologous serum formulation [13].

The process by which proteomic analysis was carried out was essentially as described in detail previously [13].

MaxQuant software (Max-Planck Institute for Biochemistry, Martinsried, Germany, 1.6.5.0 version) using default settings was utilized for protein identification and quantification [32]. Searches were carried out against a database consisting of human protein entries (Uniprot/Swissprot), with precursor and fragment tolerances of 20 ppm and 0.05 Da. Only proteins identified with at least two peptides at FDR < 1% were considered for further analysis. Data (LFQ intensities) was loaded onto Perseus platform (Max-Planck Institute

for Biochemistry, Martinsried, Germany, 1.5.1.5 version) [33] and further processed (log2 transformation, imputation) before statistical analysis (Student's *t*-test).

2.4. Functional Analysis

DAVID online tool (http://david.abcc.ncifcrf.gov/summary.jsp (accessed on 7 October 2020)) was used to analyze the enrichment of Gene Ontology (GO) terms [34,35]. DAVID is a GO Term annotation and enrichment analysis tool used to highlight the most relevant GO terms associated with a given gene list. Fisher exact test was used to analyze whether the proportion of genes related to certain GO terms or categories differed significantly between the dataset and the background. Only were considered for comparison and discussion those GO Terms enriched with a p value < 0.05.

Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity (accessed on 7 October 2020) was used to analyze the functions in which the different identified proteins were related. A Fisher's exact test (p-value < 0.05 was considered significant) was used to calculate the probability by which proteins in the data set associated with a particular canonical pathway, functional network or upstream regulator was not due to chance alone. Activation z-score reflects whether a biological function is in an activated (positive values) or inactivated (negative values) state, based on the knowledge of the relation between the effectors and their target molecules.

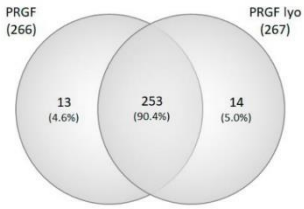
3. Results

3.1. Proteomic Characterization of Blood-Derivative Products

PRGF preparations showed a mean platelet enrichment of 2.2-fold over the platelet concentration in peripheral blood. In addition, no detectable levels of leucocytes were observed in any of the PRGF preparations. PRGF and PRGF lyo samples obtained from the three different donors were analyzed for differential protein expression. A total of 280 different proteins were detected between both types of formulations. Supplementary File S1 contains the complete list of proteins detected in these formulations, and their relative expression. Figure 2 shows the Venn diagram where the intersection of the total proteins from the two blood-derived products (PRGF and PRGF lyo) can be observed. In summary, the number of proteins identified was 266 in PRGF eye drops and 267 in PRGF lyo eye drops. Of the total proteins identified in each formulation, 253 proteins (90.4%) were shared by both blood-derived products, while 13 (4.6%) were only related to the PRGF formulation and 14 (5.0%) to the PRGF lyo. Supplementary File S1 contains the lists of shared or formulation-specific proteins.

PRGF

Gene name	Protein description
AOC3	Membrane primary amine oxidase
PCSK9	Proprotein convertase subtilisin/kexin type 9
HBB	Hemoglobin subunit beta
PDIA1	Protein disulfide-isomerase
PAFA	Platelet-activating factor acetylhydrolase
CD166	CD166 antigen
HV307	Immunoglobulin heavy variable 3-7
RA1L2	Heterogeneous nuclear ribonucleoprotein A1-like 2
CAMP	Cathelicidin antimicrobial peptide
ANAG	Alpha-N-acetylglucosaminidase
INHBC	Inhibin beta C chain
FCN2	Ficolin-2
NOE1	Noelin



PRGF lyo

Gene name	Protein description
IL6RB	Interleukin-6 receptor subunit beta
CD109	CD109 antigen
TBB1	Tubulin beta-1 chain
LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1
TYB4	Thymosin beta-4
ENOA	Alpha-enolase
MBL2	Mannose-binding protein C
PLST	Plastin-3
NID1	Nidogen-1
ICOSL	ICOS ligand
STOM	Erythrocyte band 7 integral membrane protein
SRCRL	Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D
HVC05	Immunoglobulin heavy variable 3-30-5
RL40	Ubiquitin-60S ribosomal protein L40

Figure 2. Venn diagram comparison of two blood–derived formulations and the list of proteins specific for PRGF and PRGF lyo.

A Gene Ontology (GO) analysis was performed to evaluate the functional processes in which these proteins participate. The results of the GO analysis showed that between both blood-derived products (PRGF and PRGF lyo) a total of 236 different GO terms were revealed. Supplementary File S1 contains the lists of all GO terms obtained from each formulation. The ten most abundant GO terms found between both formulations are represented in the Figure 3. They may be grouped in 3 main processes: (i) 3 of them like proteolysis, negative regulation of endopeptidase activity and cell adhesion GO terms may be related to the cellular activity; (ii) 5 GO terms out of 10 such as innate immune response, complement activation (classical pathway), complement activation, inflammatory response and regulation of complement activation are likely related with the immune response; and finally, (iii) the last 2 GO terms: platelet degranulation and blood coagulation are related to the platelet function. As it is shown in Figure 2, the percentage of deregulated proteins involved in all this GOs are similar for both blood-derivative products.

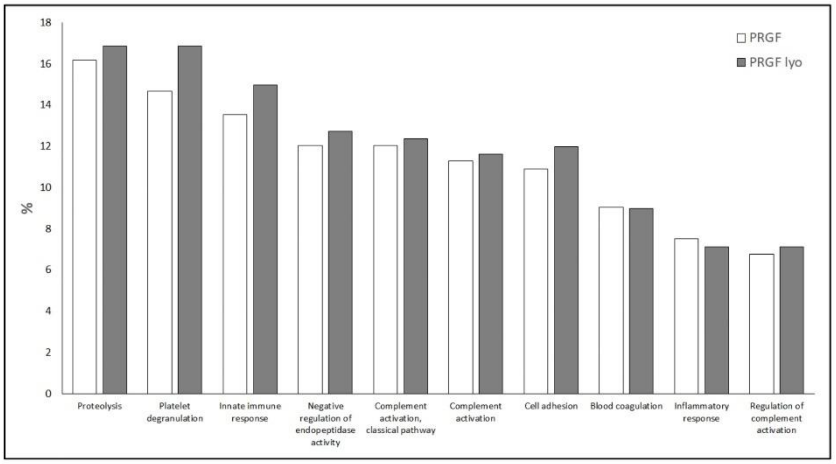


Figure 3. The ten most abundant Gene Ontology terms identified in PRGF and PRGF lyo formulations.

The relative quantitative proteomics analysis carried out to compare the protein composition of PRGF and PRGF lyo showed that a total of 257 proteins were identified with at least two different peptides, of which 8 showed significant differences ($p < 0.05$ and a ratio > 1.5 in either direction) between both formulations (Supplementary File S2). These proteins were selected for further analysis (Table 1).

Table 1. Statistically significant de-regulated proteins between PRGF vs PRGF lyo.

Protein Accession Number	Gene Name	Protein Description	Fold Change	*p*-Value
P22352	GPX3	Glutathione peroxidase 3	0.7	0.0077
P08603	CFAH	Complement factor H	0.9	0.0142
P04196	HRG	Histidine-rich glycoprotein	1.1	0.0245
Q9BYE9	CDHR2	Cadherin-related family member 2	0.6	0.0252
P02749	APOH	Beta-2-glycoprotein 1	0.8	0.0336
P37802	TAGL2	Transgelin-2	0.5	0.0436
P02649	APOE	Apolipoprotein E	0.6	0.0464
Q9UHG3	PCYOX	Prenylcysteine oxidase 1	0.8	0.0488

The GO analysis carried out with these significantly deregulated proteins showed that they could be involved in different biological processes (Table 2). However, IPA analysis showed no significant differences in any of the processes identified (Supplementary File S3).

Table 2. Gene Ontology analysis of de-regulated proteins with statistically significant differences between PRGF vs PRGF lyo.

GO Term	GO Definition	Genes	%	*p*-Value
0051918	Negative regulation of fibrinolysis	P02749, P04196	25	0.0042
0030195	Negative regulation of blood coagulation	P02749, P02649	25	0.0050
0043537	Negative regulation of blood vessel endothelial cell migration	P02649, P04196	25	0.0058
0001937	Negative regulation of endothelial cell proliferation	P02749, P02649	25	0.0120
0006641	Triglyceride metabolic process	P02749, P02649	25	0.0145
0000302	Response to reactive oxygen species	P02649, P22352	25	0.0161
0016525	Negative regulation of angiogenesis	P02749, P04196	25	0.0256
0098869	Cellular oxidant detoxification	P02649, P22352	25	0.0288
0030855	Epithelial cell differentiation	Q9BYE9, P37802	25	0.0288
0010468	Regulation of gene expression	P02649, P04196	25	0.0409
0002576	Platelet degranulation	P02749, P04196	25	0.0421
0006979	Response to oxidative stress	P02649, P22352	25	0.0449

3.2. Proteomic Characterization of HK Cells Treated with Blood-Derivative Products

Protein samples isolated from HK cells treated with both types of eye drops (PRGF and PRGF lyo) obtained from the three different donors were analyzed for differential expression. The results obtained showed that out of a total of 3215 proteins identified, 101 showed statistically significant differences (Supplementary File S4). These significant deregulated proteins were used to a Gene Ontology (GO) analysis to characterize the functional processes where these proteins are involved in. GO term analysis showed that 30 out of 41 GO terms identified were significantly enriched (Supplementary File S4).

Ingenuity pathways analysis (IPA) was carried out to evaluate the functional processes in which the deregulated proteins are involved. IPA showed that no significant differences were observed between the proteins deregulated in the HK cells after treatment with PRGF or PRGF lyo (Supplementary File S5).

4. Discussion

Several blood-derivative products have been developed along the last three decades to enhance tissue regeneration in different ocular surface diseases. Diverse protocols and procedures have been developed to obtain these type of blood derivatives. One of the common steps to obtain any of these blood-derived products is the withdrawal of a small volume of the patient own-blood, thus obtaining an autologous therapy [21]. However, in some cases, it is not possible to obtain an autologous product because some patients are not suitable to be donors due to certain health or physical conditions like systemic inflammatory diseases, age, and other types of disorders or comorbidities [25]. Then, allogeneic products could be an interesting alternative to be used in these patients. In the present study, PRGF and PRGF lyo were treated as independent samples to analyze not only differences between treatments, but also interindividual alterations. However, when working with allogenic therapies, pooled PRP should be considered. As previously reported by other authors, PRP preparation techniques for allogenic applications such as apheresis may also be used for the standardization and use of platelet derived products in tissue regeneration [36]. PRP obtained by these techniques can be collected in large amounts in donor centers and has the advantage of being technically standardized and reducing the inherent biological variability among donors.

On the other hand, different ocular surface disorders are usually chronic pathologies, and it makes necessary that the therapies used for their treatment must be stored for

long periods of time. Until recently, blood-derived products like PRGF eye drops had to be stored at low temperatures (−20 °C) for long periods of time to be used for the treatment of ocular surface diseases. So, this made the patient dependent on the cold chain for the storage of this type of treatment. However, recent studies have demonstrated that PRGF eye drops can be freeze-dried maintaining the main growth factors that are involved in ocular surface tissue regeneration [26]. In the present study, the proteomic profile of fresh and freeze-dried PRGF eye drops were analyzed to evaluate the differential composition between both types of formulations. Furthermore, the proteomic expression of human keratocytes cells after treatment with PRGF or PRGF lyo were also analyzed to evaluate whether both formulations could induce the expression of different proteins and the differential activation of diverse signalling pathways in cells from ocular surface. Results show that, excepting a small number of proteins, the most proteins detected in PRGF and PRGF lyo are shared by both preparations (253 proteins out of 280). Furthermore, although statistical differences were observed in some deregulated proteins between PRGF and PRGF lyo (8 proteins), IPA analysis showed that these differences were not associated with the deregulation of any biological pathways. Hence, these results suggest that there are no differences between both blood-derivative products (PRGF and PRGF lyo). However, although the depletion of the most abundant proteins was carried out, the wide dynamic range of protein concentrations in blood-derived products could mask the proteins with lower concentration with respect to the most abundant proteins [37,38]. Therefore, it would be necessary to carry out a deeper analysis of scarce proteins using proteomic techniques with higher definition to unravel the possible differences in the protein composition of both products (PRGF and PRGF lyo). In fact, other authors have also revealed that metabolites within these types of formulations may present variations depending on the preparation method and the donor [39]. Therefore, the metabolome of eye drops to be used in the ocular surface should also be taken into account for future studies as these organic compounds play a mayor role in human tears.

In the same vein, when proteomic analysis was carried out in HK cells treated with PRGF or PRGF lyo, 3213 deregulated proteins were found, of which 101 showed statistically significant differences. These significantly deregulated proteins were used to analyze the different functional processes which these proteins are involved in, identifying 41 significantly enriched GO terms. However, when IPA analysis was performed, no activation or inactivation of any cell signaling pathway was detected in relation to the significantly deregulated proteins identified among PRGF or PRGF lyo-treated HK cells. These results are in line with a previous study published by our group, where no differences in the biological activity of HK cells were observed after treatment with PRGF and PRGF lyo [26].

In recent years, several studies have suggested that some lyoprotectants, such as trehalose, should be added to different blood-derived products before undergoing a freeze-drying process in order to maintain their biological properties [40,41]. However, the results obtained in the present study have shown that there were no changes either in the proteomic profile of PRGF eye drops after lyophilization without the use of lyoprotectants or in the activation of different signaling pathways in HK cells compared with PRGF eye drops. These results are in accordance with those obtained in recent studies in which PRGF lyo without the use of any lyoprotectant showed no significant differences in the concentration of several growth factors and proteins and in their biological activity regarding fresh PRGF eye drops [26,27].

5. Conclusions

In summary, although further studies could be necessary to establish the possible proteomic differences between PRGF and PRGF lyo due to the high dynamic range of protein concentrations in these types of formulations, the present study shows that no significant differences were found in the proteomic profile between PRGF and PRGF lyo. Furthermore, these results suggest that PRGF and PRGF lyo induce similar signaling pathways activation in HK cells. All in all, the results observed in the present study suggest

that no modifications are suffered by PRGF eye drops after undergoing a freeze-drying process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12091215/s1, Supplementary Files S1–S5.

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Capítulo 4: DISCUSIÓN

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La función inmunoreparadora de la inmunotrombosis se basa en biomoléculas que incluyen la trombina, el fibrinógeno, los factores de crecimiento, las citocinas y las micropartículas que se originan principalmente en el plasma, las plaquetas activadas y los macrófagos residentes en el tejido, todos los cuales son inductores y facilitadores de la reparación tisular (30). En este sentido, la reparación tisular podría conceptualizarse como un subproducto de la inmunotrombosis. Varias proteínas multidominio intravasculares de los sistemas de coagulación y fibrinolítico, incluyendo la trombina, la fibrina(fibrinógeno), la plasmina(plasminógeno), el FXII y otras serinas proteasas filogenéticamente y estructuralmente relacionadas, como el activador del Factor de crecimiento hepatocitario (HGFA) y el HGF, están surgiendo como proteínas pleiotrópicas con funciones importantes en la reparación de heridas (132-142). Además, el contenido de los gránulos de las plaquetas que incluyen factores de crecimiento y citoquinas son necesarios para la reconstrucción del tejido después de la lesión (41, 143, 144). De hecho, las proteínas del sistema de coagulación son proteínas con acción pleiotrópica con funciones que abarcan la coagulación, inflamación y la reparación de lesiones. Este es el caso de la trombina que, además de su papel central en la coagulación, su señalización es necesaria para la regeneración de las extremidades, del corazón y del cristalino en la salamandra. En la reparación de heridas, la trombina activa las plaquetas, la IL-1α, y las vías de señalización HGFA-HGF-MET, estas últimas también activadas por la precalicreína (PK) y FXII, funcionando como un vínculo entre la lesión y la reparación (137, 138, 145, 146). Además, la trombina de mamíferos a baja concentración induce actividades anti-inflamatorias, antiapoptóticas, y de factor de crecimiento (147). Así mismo, el fibrinógeno de los vertebrados ejecuta múltiples funciones a parte de la formación del coágulo, desde la modulación inmune como un reactivo agudo de defensa contra la proliferación y diseminación bacteriana mediante la generación de un biofilm en la interfase aire-líquido del coágulo (148). Además, la matriz de fibrina desempeña un papel crucial en la reconstrucción tisular como un molde-soporte biodegradable y

alberga células y moléculas de adhesión. Además, la matriz de fibrina actúa como un depósito de morfógenos y citoquinas las cuales se unen a los dominios de sulfato de heparina y una vez liberados operan como moléculas instructoras de la actividad celular (41). El FXII es otro ejemplo de proteína multifuncional con labores de reconocimiento de patrones y de factor de crecimiento en la inflamación, la coagulación y la reparación de heridas (135). Localmente, la activación del FXII en las heridas en presencia de partículas del suelo abundantes en silicatos acelera y refuerza la formación de fibrina y atrae y promueve la actividad de los neutrófilos y la formación de trampas extracelulares de neutrófilos (NETosis), contribuyendo a compartimentalizar y esterilizar rápidamente las heridas epiteliales (135, 136). Esta respuesta previene la propagación sistémica de elementos bióticos en los vertebrados terrestres superiores priorizándola sobre la rápida curación de las heridas, con funciones inflamatorias, neoangiogénicas y profibróticas (135, 136). Estas funciones mediadas por el FXII en las heridas epiteliales de la piel (y probablemente en el epitelio de los pulmones y el intestino) podrían haber ejercido una fuerte presión de selección, primero en los anfibios y luego en los reptiles y otros vertebrados terrestres, incluidos los mamíferos que viven en entornos fangosos y de estuarios abundantes en silicatos (136). Este no es el caso de algunos vertebrados que apenas tienen contacto con el suelo, como los cetáceos (ballenas, delfines), que perdieron el gen klkb1 y poseen un pseudogen f12 inactivo, o las aves, que perdieron el gen f12 (149). Otras dos proteínas intravasculares, a saber, el HGFA y el HGF, podrían haber desempeñado funciones inmunotrombóticas y de reparación en la transición de los vertebrados del agua a la tierra, como ocurre en los mamíferos. El HGFA funciona como enlace molecular entre la lesión tisular y la reparación a través de la vía de señalización HGFA-HGF-MET, cuya activación es desencadenada por la trombina y las enzimas del sistema de contacto (PKa, FXII) (137, 138). La serina proteasa HGFA activa proteolíticamente el HGF, lo que da lugar a funciones pleiotrópicas mediadas por el HGF, desde la proliferación y diferenciación celular hasta actividades antifibróticas,

angiogénicas, anti-inflamatorias y regenerativas (41, 137, 138). Así mismo, el plasminógeno es otra proteína multidominio de circulación plasmática que junto con los moduladores de la vía de activación del plasminógeno presenta una diversidad de funciones biológicas además del papel básico y original de escindir la fibrina en diferentes fragmentos (fibrinilisis) (150, 151). Destacar que el eje plasminógeno-plasmina promueve la fagocitosis del fragmento de fibrina y de los neutrófilos apoptóticos mediante la estimulación de los macrófagos y las células dendríticas, un paso clave para la eliminación completa de la fibrina, la polarización de los macrófagos y la resolución de la inflamación (152). Por último, las plaquetas activadas liberan proteínas biológicamente activas con funciones importantes en la angiogénesis, la fibrogénesis, la proliferación, la migración, la reprogramación celular, y la resolución de la inflamación, todos ellos procesos clave en la reparación y regeneración de tejidos (30, 143, 153).

En las últimas cuatro décadas, se han ido desarrollando un número cada vez más elevado de productos derivados de la sangre para mejorar la regeneración tisular en diversas enfermedades de la superficie ocular. Son varios los protocolos y procesos que se han establecido para producir este tipo de hemoderivados, obteniéndose diferentes productos que contienen distintas composiciones proteicas, dando lugar como consecuencia a una gran variedad de resultados clínicos para el tratamiento de los mismos trastornos de la superficie ocular. El primer producto derivado de la sangre utilizado para el tratamiento de patologías de la superficie ocular fue el suero autólogo (SA) diluido al 20%. Sin embargo, cuando se descubrió que las plaquetas eran una de las fuentes más importantes de proteínas y factores de crecimiento con propiedades regenerativas, los productos derivados de la sangre enriquecidos en plaquetas fueron aumentando su popularidad en su uso para el tratamiento de patologías oculares debido a su mayor contenido en factores de crecimiento respecto al SA.

Aparte de esto, desde el principio se utilizó empíricamente el suero autólogo diluido al 20% porque se observó que la concentración de varios factores de crecimiento con propiedades antiproliferativas y profibróticas como el TGF-β era 5 veces mayor en el SA que en las lágrimas. Desde entonces, los colirios de suero autólogo se prepararon diluidos al 20% para evitar el efecto potencialmente nocivo (110). Sin embargo, la dilución de SA puede reducir la concentración de varios factores beneficiosos que se ha demostrado que favorecen la proliferación y migración de las células epiteliales de la córnea (110). No obstante, en los últimos años, varios grupos y estudios han apoyado el uso de SA a concentraciones superiores (entre el 50-100%) para aumentar la concentración de factores de crecimiento con potencial regenerativo en contacto con el tejido dañado, demostrando buenos resultados tanto en términos de eficacia como de seguridad (108, 123, 154).

En el presente trabajo se utilizaron conjuntos de muestras (PRGF y SA) procedentes de tres donantes con el fin de caracterizar los distintos productos derivados de la sangre (PRGF y SA sin diluir). Los resultados obtenidos en el presente trabajo muestran que el PRGF contiene concentraciones de plaquetas dos veces superiores a las plaquetas contenidas en la sangre periférica utilizada para obtener SA. Resultados similares se observaron en un estudio comparativo entre el PRGF y el SA en el que la concentración de plaquetas en las muestras de PRGF era casi el doble que la del SA (120). Además, en el presente trabajo, presentamos un análisis proteómico de los colirios derivados del PRGF en comparación con el SA sin diluir.

Asimismo, se ha analizado la expresión proteómica comparativa entre queratocitos corneales (HK) tratados con PRGF o SA. Cuando se llevaron a cabo los análisis proteómicos de los diferentes productos derivados de la sangre, los resultados mostraron que sólo se identificaron 285 proteínas entre ambas formulaciones (PRGF y SA). De todas las proteínas identificadas entre ambas formulaciones derivadas de la sangre sólo se encontraron diferencias significativas entre el PRGF y el SA en 13 de ellas.

Sin embargo, estas diferencias no estaban relacionadas con la activación de ninguna vía biológica. Estos resultados sugieren que no habría diferencias entre el PRGF y el SA. Sin embargo, a pesar de la depleción de las proteínas más abundantes llevada a cabo en ambos hemoderivados previamente a su análisis proteico, el gran rango dinámico de concentraciones de proteínas en los productos derivados de la sangre, más de 10 órdenes de magnitud, podría enmascarar las proteínas con una concentración más baja en contraste con las proteínas de mayor abundancia (155, 156).

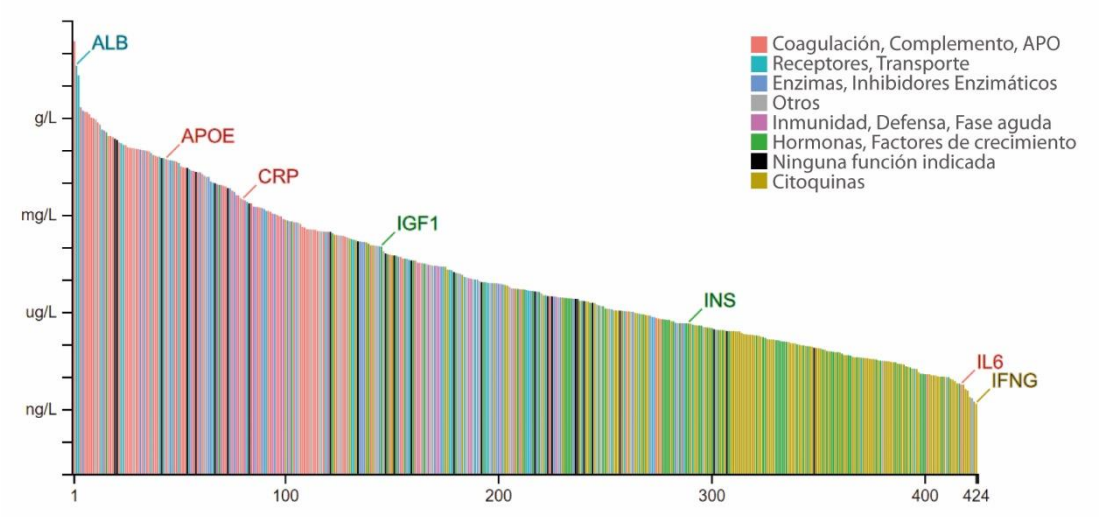

Figura 3. Gráfico que muestra el rango de concentración de proteínas en relación con otras 439 proteínas secretadas a la sangre (eje x), así como la concentración en una escala absoluta (eje y) con varias proteínas plasmáticas seleccionadas (marcadas en el gráfico). (Tomada de https://www.proteinatlas.org/humanproteome/blood+protein/proteins+detected+by+immunoassay).

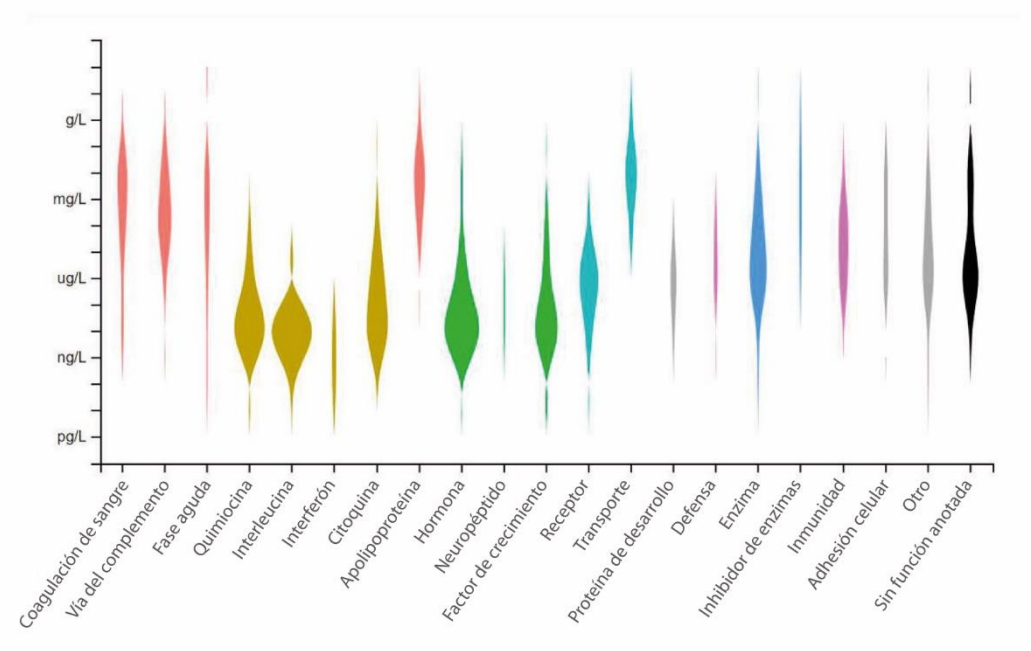

Figura 4. Diagramas de Violin que muestran la distribución de las concentraciones plasmáticas de 22 grupos diferentes según su función proteica principal. (Tomada de https://www.proteinatlas.org/humanproteome/blood+protein/proteins+detected+by+immunoassay).

Por lo tanto, podría ser interesante realizar en un futuro próximo un análisis de las proteínas que se encuentran en menor abundancia en el plasma sanguíneo mediante otros métodos proteómicos para desentrañar las diferencias en la composición proteica de estos dos productos (PRGF y SA).

Por el contrario, se encontraron 3236 proteínas expresadas diferencialmente cuando se analizó el proteoma de células HK tratadas con PRGF o SA, de las cuales 352 mostraron diferencias estadísticamente significativas. El análisis de los procesos en los que estas proteínas podrían estar involucradas se puede resumir en seis funciones biológicas principales: (A) Inflamación; (B) Angiogénesis; (C) Estrés oxidativo; (D) Vía del EGF; (E) Síntesis de proteínas, proliferación y motilidad celular; y (F) Señalización del citoesqueleto de actina. Todas estas funciones biológicas aumentaron significativamente en las células HK tratadas con SA en comparación con las tratadas con PRGF. Estos resultados sugieren una estrecha relación entre las células tratadas con SA y la activación de diferentes vías en las células HK relacionadas con una respuesta inflamatoria, que estaba relacionada principalmente con la señalización de la respuesta de fase aguda, la señalización MAPK estimulada por LPS, la señalización CCR3 en los eosinófilos, la

señalización de IL-6, la señalización de IL-15 y la señalización de CXCR4. Estos resultados se confirmaron con un análisis IPA, que demostró que varias proteínas, expresadas de forma diferencial y estadísticamente significativas en las células HK tratadas con SA, pueden estar asociadas con una activación celular por algunas citoquinas inflamatorias como el interferón gamma (IFN-γ), lo que conduce a una respuesta inmunitaria en las células HK. Todas estas vías asociadas a una respuesta inmunitaria en las células HK tras el tratamiento con SA podrían estar relacionadas con la presencia de citocinas inflamatorias como IL-1, IL-6, IL-15, TNFα o IFN-γ en muestras de SA derivados de la presencia de macrófagos y leucocitos durante la preparación de este tipo de producto derivado de la sangre (157, 158). La etiopatología de varias enfermedades oculares tiene un componente inmunológico, o es secundaria a enfermedades inflamatorias sistémicas como el síndrome de Sjögren, la artritis reumatoide, la diabetes o la enfermedad injerto contra huésped entre otras (159-161). Por lo tanto, estas enfermedades oculares deben tratarse con una terapia con un bajo contenido en citoquinas inflamatorias, que permita regenerar el tejido dañado, ejerciendo al mismo tiempo un efecto antiinflamatorio. Un estudio reciente demostró que el PRGF ejerce un efecto antiinflamatorio superior al SA diluido al 20% en un modelo *in vitro* de fibroblastos de la superficie ocular tratados con citoquinas proinflamatorias como la IL-1β y TNFα (117). Los presentes resultados sugieren que el SA sin diluir podría inducir una mayor respuesta inflamatoria en las células HK que el colirio de PRGF.

Además, los procesos inflamatorios en la córnea patológica pueden estimular la producción de factores angiogénicos por diferentes células de la superficie ocular como las células epiteliales o los queratocitos. Algunos de estos factores, como en el caso del factor de crecimiento endotelial vascular (VEGF), se han identificado y aislado de la córnea (162). La córnea es un tejido avascular único, que le otorga las características de transparencia y regularidad esenciales para mantener la función óptica de los ojos. Muchos trastornos de la córnea, como infecciones, lesiones y reacciones autoinmunes, conducen a la angiogénesis corneal. La invasión de la córnea por los vasos induce su opacificación, reduciendo la visibilidad del paciente. Por lo tanto, es esencial reducir la posibilidad de inducir un efecto angiogénico por parte de los fármacos utilizados para tratar las patologías de la superficie ocular. El presente estudio muestra que el tratamiento con SA induce una mayor activación de proteínas relacionadas con las vías de señalización renina-angiotensina y ligando-receptor de la familia VEGF que el colirio de PRGF, lo que sugiere que el tratamiento con SA puede inducir una activación significativamente mayor de una respuesta angiogénica en las células HK que el colirio de PRGF.

Por otra parte, varios estudios han demostrado que la inflamación tiene una fuerte asociación con el estrés oxidativo (163). El estrés oxidativo se caracteriza por la producción de especies reactivas de oxígeno (ROS), como los aniones superóxido, el peróxido de hidrógeno y los radicales hidroxilo, que están relacionados con el daño celular induciendo la peroxidación lipídica de las membranas, cambios oxidativos en las proteínas y daño oxidativo en el ADN (164). Un proceso inflamatorio podría inducir un aumento de los niveles de ROS debido a un mayor consumo de oxígeno o a una menor defensa antioxidante en el tejido afectado (165). Varios trastornos oculares, como la inflamación corneal, la enfermedad del ojo seco, el queratocono y la distrofia endotelial de Fuchs, están asociados al estrés oxidativo (166, 167). Se han propuesto varios tratamientos antioxidantes para reducir la reacción inflamatoria en varias enfermedades oculares tratando de inducir la curación del tejido ocular (166, 168, 169). Estudios recientes demostraron que el tratamiento con PRGF reducía los efectos citotóxicos inducidos en células epiteliales pigmentarias de la retina expuestas a un entorno de estrés oxidativo modulando las vías antioxidantes (170, 171). El presente estudio muestra que el tratamiento con SA sin diluir induce una activación de las vías del estrés oxidativo en las células HK similar a la inducción por la adición de peróxido de hidrógeno. Las proteínas relacionadas con el estrés oxidativo se expresaron de forma estadísticamente significativa en las células HK tratadas con SA en comparación con las tratadas con PRGF.

Además, los presentes resultados revelaron que las proteínas que son más abundantes en las células tratadas con SA se agrupaban en un grupo adicional de vías canónicas relacionadas con la activación de la vía del EGF. El EGF, a través de su unión al receptor del EGF (EGF-R), estimula las células epiteliales y endoteliales de la córnea y acelera la regeneración de heridas epiteliales (172, 173). Además, el EGF promueve la motilidad celular a través de la fosforilación de su receptor, lo que conduce a una reorganización del citoesqueleto de actina (174). Sin embargo, en el caso de los queratocitos, el EGF induce la diferenciación celular a miofibroblastos a través de la estimulación de la vía de señalización del EGF-R (175). Los miofibroblastos son responsables de la contracción de la herida y la deposición y organización de la matriz extracelular durante la reparación de lesiones. Sin embargo, se ha descubierto que la presencia continuada de miofibroblastos tras la cicatrización de la herida es el principal episodio biológico responsable del desarrollo de tejido cicatricial (176). Por lo tanto, los presentes resultados sugieren que la activación de las vías del EGF en las células HK tras el tratamiento con SA puede promover la formación de tejido cicatricial que comprometa la transparencia corneal.

Además, las células tratadas con SA revelan una mayor asociación con procesos como la síntesis de proteínas, la proliferación y la motilidad celular, relacionados principalmente con la señalización mTOR (diana de rapamicina en células de mamífero) y la señalización p70S6K (proteína cinasa S6 ribosomal beta 1). La p70S6K es una diana de la señalización mTOR y la mTOR es una proteína quinasa serina/treonina que afecta a muchas funciones celulares, como el crecimiento, la proliferación y el metabolismo celular (177). Además, en un estudio reciente de un modelo de quemadura alcalina de conejo, se demostró que la inhibición de la vía mTOR promovía la autofagia e inhibía la proliferación, invasión y migración de las células del estroma corneal, favoreciendo la cicatrización de las heridas corneales (178). Además, se ha demostrado que la señalización mTOR puede inducir cicatrices, neovascularización e inflamación en la córnea (179, 180). Otro estudio demostró que el TGF-β activaba la vía mTOR en los fibroblastos del estroma corneal y que la rapamicina (un inhibidor de mTOR) inhibía la proliferación de los fibroblastos del estroma corneal y moduló su transformación en miofibroblastos (181). Por lo tanto, la activación de la vía mTOR tras el tratamiento con SA sin diluir podría inducir una mayor fibrosis tisular que tras el tratamiento con colirio de PRGF. Del mismo modo, se ha demostrado una activación significativa de la vía asociada a la señalización del citoesqueleto de actina (GTPasas de la familia Rho y señalización RhoA) en células HK tratadas con SA sin diluir en comparación con el PRGF. Las GTPasas de la familia Rho controlan diversas vías de transducción de señales, una de cuyas funciones principales es controlar el citoesqueleto de actina. Entre los miembros de la familia Rho GTPasas se encuentran RhoA, -B y -C, Rac1 y -2 y Cdc42. RhoA regula la polimerización de actina, induciendo la formación de fibras de estrés y el ensamblaje del complejo de adhesión focal (182). Varios factores de crecimiento, como el TGF-β y el factor de crecimiento fibroblástico básico (FGF)-2, inducen la activación de las vías de señalización de Rho; sin embargo, el TGF-β parece ser el principal factor que activa estas vías y, por lo tanto, es el principal inductor de la diferenciación de queratocitos a miofibroblastos, lo que conduce a la expresión de α-SMA (183, 184). En el presente estudio, los resultados observados tras los análisis IPA mostraron que un gran número de proteínas expresadas diferencialmente y de manera estadísticamente significativa en las células HK tratadas con SA estaban relacionadas con la activación de la organización del citoesqueleto similar a la estimulación de las células HK por TGF-β1. Nuestros resultados coinciden con un estudio proteómico previo en células HK miodiferenciadas tras la incubación con TGF-β1 y el tratamiento con PRGF o 20% de SA (121). Este estudio mostró que el tratamiento con PRGF inactivaba o reducía la activación de varias proteínas implicadas en las vías por las que el TGF-β1 ejerce su acción para inducir la formación de fibras de

α-actina en las células HK, induciendo su transformación a miofibroblastos. Sin embargo, el tratamiento con SA no fue capaz de reducir la acción del TGF-β1 en las células HK miodiferenciadas. De acuerdo con esto, estudios previos también han demostrado que el PRGF ejerce un efecto antifibrótico al reducir la transformación de los fibroblastos estromales tratados con TGF-β1 en miofibroblastos (114), minimizando la formación de cicatrices y mejorando al mismo tiempo la regeneración del tejido corneal (185). Además, se ha demostrado que este efecto antifibrótico del PRGF es significativamente mayor que el del SA diluido al 20% en células HK diferenciadas a miofibroblastos por TGF-β1 (120). El mayor contenido de factor de crecimiento de fibroblastos (FGF) en las formulaciones de PRGF que en las de SA puede ser un posible mecanismo por el cual el PRGF induce una menor activación de las vías del citoesqueleto de actina. Algunos estudios han demostrado que el FGF-1 y -2 promueven el fenotipo fibroblástico y revierten el fenotipo miofibroblástico (186).

El presente estudio sugiere que el SA sin diluir induce la activación de diferentes vías en las células HK relacionadas con una respuesta inflamatoria, angiogénica, de estrés oxidativo y de fibrosis en comparación con el PRGF. Así pues, el PRGF podría ser una alternativa mejor que el SA para el tratamiento de los trastornos de la superficie ocular. Sin embargo, los productos derivados de la sangre se componen de una gran variedad de proteínas y factores de crecimiento, por lo que serán necesarios más estudios para desentrañar las proteínas que se encuentran implicadas en los mecanismos que subyacen a las vías reguladas diferencialmente entre el PRGF y el SA.

Por otro lado, en el presente trabajo también se ha evaluado la estabilidad del colirio de PRGF almacenado a -20 °C durante 9 y 12 meses y durante 3 y 7 días a RT o a 4 °C. Los resultados obtenidos en este estudio muestran que no se encontraron diferencias significativas ($P > 0,05$) en los niveles de diferentes factores de crecimiento y citoquinas implicados en la regeneración tisular de la superficie ocular, como TGF-b1, EGF, VEGF, PDGF-AB, TSP-1 y ANG-1 en las muestras de colirio de PRGF tras su almacenamiento tanto a 9 como a 12 meses a -20 °C. Además, los niveles de estas proteínas siguen siendo similares en comparación con los colirios de PRGF obtenidos a tiempo 0 (muestras frescas) de los distintos donantes. Dichos resultados también podrían simplificar la logística de los pacientes en función del procedimiento para el tratamiento de trastornos crónicos durante periodos prolongados. Por ejemplo, esto podría permitir a los pacientes iniciar de nuevo el tratamiento cuando reaparecieran los síntomas sin necesidad de nuevas extracciones de sangre y producción de colirios.

Capítulo 4: DISCUSIÓN

Algunos estudios de estabilidad de productos derivados de la sangre han demostrado que varios factores de crecimiento y proteínas analizados a diferentes temperaturas de almacenamiento durante un máximo de 24 horas vieron reducidos sus niveles de concentración tras el almacenamiento a temperatura ambiente o a 4 °C (187). En estos casos se indica específicamente a los pacientes que eviten guardar los dispensadores de colirio en el bolsillo o cerca de una fuente de calor y que desechen aquellos dispensadores que hayan permanecido sin refrigerar durante más de 2 o 3 horas. Sin embargo, en nuestro estudio no se observaron diferencias en la concentración de los principales niveles de proteínas entre los colirios de PRGF obtenidos a tiempo 0 (muestras frescas) o almacenados a -20 °C durante 9 y 12 meses y los colirios almacenados a temperatura ambiente o 4 °C durante 3 y 7 días. Además, no se observaron diferencias significativas ($P > 0,05$) en la proliferación de fibroblastos del estroma corneal tras el tratamiento con el PRGF obtenido a tiempo 0 (muestras frescas) o almacenado hasta 9 y 12 meses a -20 °C y mantenido a 4 °C o temperatura ambiente durante 3 y 7 días. Estos últimos resultados deben destacarse porque mejoran significativamente la dosificación de los colirios autólogos, permitiendo el consumo de los colirios de PRGF en uso hasta 7 días sin depender de una cadena de frío.

El pH de las películas lagrimales presenta valores similares a los fisiológicos (pH = 7,4). Asimismo, los colirios obtenidos a partir de productos derivados de la sangre también muestran valores de pH comparables a los de la película lagrimal (110, 188). Sin embargo, el ojo es capaz de tolerar valores de pH que oscilan entre 3,5 y 9 gracias a la capacidad amortiguadora de las lágrimas (189). En el presente trabajo, los niveles de pH aumentaron significativamente en los colirios de PRGF desde valores medios de 7,5 en las muestras frescas hasta valores aproximados de 8,8 en los colirios de PRGF almacenados a -20 °C durante 9 y 12 meses. A pesar de ello, estos resultados muestran que los niveles de pH se mantuvieron en valores inferiores a 9 durante todo el periodo de estudio; por lo tanto, los colirios de PRGF almacenados a -20 °C durante un máximo de 12 meses deberían ser perfectamente tolerados por los tejidos oculares.

Para evitar el riesgo de toxicidad química, los colirios derivados de la sangre se utilizan habitualmente sin conservantes (190). Además, estudios recientes han demostrado las propiedades antimicrobianas naturales de los productos derivados de la sangre (116, 191). Sin embargo, se debe tener especial cuidado para evitar la posible contaminación microbiana del dispensador de colirio relacionada con su uso a largo plazo (192, 193). En consecuencia, se recomienda mantener el dispensador de colirio en uso a 4 °C durante 5 a 7 días (194). A pesar de esta recomendación, se ha llegado a encontrar contaminación microbiana hasta en el 25% de los dispensadores de colirio analizados (190, 195, 196).

Capítulo 4: DISCUSIÓN

En el estudio de estabilidad biológica del colirio de PRGF mostrado en la presente tesis, no se detectó contaminación microbiana en ninguno de los dispensadores que contenían colirio de PRGF de cada uno de los donantes examinados en cada punto temporal y en ninguna de las condiciones de temperatura evaluadas. Además, no se observó contaminación microbiana en los cultivos de fibroblastos del estroma corneal tratados con colirios de PRGF almacenados a diferentes tiempos y temperaturas.

Como se ha mencionado anteriormente, en los últimos años, los productos de hemoderivados han sido ampliamente utilizados para el tratamiento de diferentes enfermedades de la superficie ocular como el ojo seco, los defectos epiteliales persistentes y las úlceras oculares (197-199). Los beneficios de este tipo de productos se atribuyen principalmente a su contenido en factores de crecimiento que intervienen en la regeneración de los tejidos de la superficie ocular como EGF, TGF-β1, VEGF o PDGF, cuyos niveles son similares a los observados en las lágrimas naturales (199-201). Es muy común que las enfermedades anteriormente mencionadas necesiten tratamientos a largo plazo, siendo necesario almacenar estos hemoderivados a bajas temperaturas para mantener sus características biológicas durante este periodo de aplicación (187). Sin embargo, el almacenamiento a largo plazo de productos derivados de la sangre y su aplicación durante el periodo de uso requiere la dependencia de una cadena de frío (almacenamiento a -20 °C para conservarlo durante un largo periodo de tiempo y a +4 °C durante su uso) (115). En el último trabajo presentado en esta tesis, se ha demostrado que los colirios liofilizados de PRGF mantienen los niveles de diferentes factores de crecimiento implicados en la regeneración tisular de la superficie ocular, así como su actividad biológica tras su almacenamiento a temperatura ambiente o a 4 °C durante al menos 3 meses.

La causa de un aumento significativo de la actividad migratoria de los queratocitos tras el tratamiento con colirios liofilizados de PRGF almacenados durante 3 meses en contraste con los colirios de PRGF obtenidos a tiempo 0 sigue siendo desconocida. Una posible explicación es que algunas proteínas o factores de crecimiento que podrían estar implicados en el control/inhibición de la migración celular podrían desnaturalizarse parcial o totalmente durante el periodo de almacenamiento. Aunque en estudios anteriores se observó un ligero aumento de la capacidad migratoria de las células de queratocitos corneales tras el tratamiento con colirios de PRGF almacenados a -20°C durante 3 meses (115), estos cambios no llegaron a ser significativos, por lo que serán necesarias nuevas investigaciones para evaluar los resultados obtenidos en este punto del estudio.

Capítulo 4: DISCUSIÓN

En los últimos años, se han realizado varios estudios para valorar la estabilidad de distintos productos hemoderivados liofilizados (202, 203). Aunque se observaron resultados notables en estos estudios, los productos liofilizados se almacenaron a temperaturas inferiores a 4 °C para su mantenimiento. En el estudio de estabilidad del colirio de PRGF liofilizado mostrado en la presente tesis, se ha demostrado que el colirio de PRGF liofilizado se puede almacenar a temperatura ambiente hasta 3 meses conservando sus propiedades biológicas similares a las de los colirios frescos de PRGF. Por lo tanto, es necesario destacar que, un beneficio importante del liofilizado del PRGF es su fácil almacenamiento, que permite mantener este producto a temperatura ambiente durante al menos 3 meses, evitando la dependencia de la cadena de frío.

El proceso de liofilización podría alterar las estructuras de las proteínas debido al descenso de la temperatura y como consecuencia del aumento de la concentración de soluto durante el procedimiento de congelación (128). Las bajas temperaturas favorecen la desnaturalización de las proteínas al alterar las interacciones entre ellas de forma similar a la desnaturalización térmica (204, 205). Muchos productos liofilizados contienen crioprotectores o lioprotectores para evitar la desnaturalización de las proteínas durante el proceso de liofilización. Los protectores más comunes utilizados en formulaciones de proteínas liofilizadas son los disacáridos, como la sacarosa o la trehalosa, debido a su capacidad para sustituir las moléculas de agua favoreciendo la estabilización de las proteínas (129). Sin embargo, el uso de trehalosa podría causar resultados perjudiciales en la proliferación de fibroblastos de la superficie ocular, reduciendo así su capacidad regenerativa (206). En un trabajo reciente, nuestro grupo de investigación ha demostrado que los colirios liofilizados de PRGF sin la adición de lioprotectores mantienen los niveles de factores de crecimiento y la actividad biológica de forma similar a los colirios liofilizados de PRGF mezcladas con trehalosa al 2,5% o al 5% (130). En el presente trabajo, se ha utilizado el PRGF combinado con trehalosa al 2,5% para evaluar si el colirio liofilizado de PRGF sin lioprotectores conservan su potencial biológico de forma similar a los que se les ha adicionado lioprotectores durante 3 meses de almacenamiento a 4 °C o temperatura ambiente. Los resultados obtenidos mostraron que el colirio de PRGF liofilizado sin trehalosa mantenía las concentraciones de los diferentes factores de crecimiento y su potencial biológico en niveles similares a los de las muestras de PRGF liofilizadas mezcladas con trehalosa. De esta manera, los colirios de PRGF liofilizados mantienen su origen endógeno evitando conservantes exógenos que pueden aumentar el riesgo de toxicidad química (190). Además, el proceso de liofilización permitiría el almacenamiento del colirio de PRGF, facilitando la accesibilidad de esta terapia a aquellos pacientes que necesiten varias aplicaciones

durante un largo período de tiempo debido a una enfermedad crónica o a aquellos que necesiten una aplicación alogénica debido a la dificultad o imposibilidad para obtener de estos pacientes con el fin de obtener productos derivados de la sangre que sean autólogos (207). La ausencia de efecto significativo de la liofilización del colirio del PRGF en su composición poética y efecto biológico es un argumento más que apoya esta formulación del PRGF.

La relevancia clínica de los resultados presentados en esta tesis también radica en su posible extrapolación al campo de la terapia celular. Las células progenitoras mesenquimales se producen con baja frecuencia en los tejidos y deben propagarse *ex vivo* para lograr una dosis adecuada para su aplicación clínica. Como ya se ha mencionado anteriormente, el uso de PRP ha demostrado ser eficaz para aumentar, o al menos no disminuir, la tasa de proliferación celular, mantener sin modificar el fenotipo de las MSC, preservar su estabilidad genética y respaldar su capacidad de diferenciación. De este modo, el sobrenadante de PRGF puede ser utilizado no sólo como colirio convencional para el tratamiento de patologías oftalmológicas sino como alternativa a los suplementos xenogénicos para el cultivo de células madre. Por lo tanto, las propiedades de estabilidad aquí descritas, así como la posibilidad de mantener el producto liofilizado facilitaría la logística, disminuyendo el número de extracciones por paciente, pero manteniendo suficientes lotes de suplemento con el mismo potencial biológico que permitiese el cultivo y diferenciación a largo plazo.

Capítulo 5: CONCLUSIONES

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1. A lo largo de millones de años, la inmunotrombosis de los mamíferos evolucionó de forma independiente a la de los invertebrados, desarrollando una compleja red de proteinas mosaico como parte de la coagulación sanguínea, junto con un sistema circulatorio cerrado, la especialización de las células sanguíneas, el endotelio y el sistema inmunitario adaptativo. Gracias a dicha evolución, se ha podido desarrollar en el presente la tecnología del PRGF, que funciona como una estrategia terapéutica al imitar el papel inmunoreparador de los factores de crecimiento y otras biomoléculas atrapadas en la red de fibrina. Esta terapia biomimética, versátil, inspirada en la biología, diseñada y seleccionada por la evolución y optimizada por el ser humano, está en su nacimiento y ofrece muchas esperanzas para el futuro.
2. Los trabajos recopilados en la revisión sistemática demostraron que el uso de PRP leucodeplecionado es factible como alternativa a los sueros xenogénicos para el cultivo de células madre. Sin embargo, es necesario mejorar la descripción de la metodología de preparación del PRP, así como su composición. Además, es necesario establecer una prueba de potencia/rendimiento y estudios comparativos entre diferentes composiciones de PRP para determinar parámetros de control de calidad y directrices universalmente aceptadas.
3. No se encontraron diferencias significativas en el contenido proteico entre el colirio de PRGF y suero autólogo (SA). Sin embargo, esto pudo deberse al gran rango dinámico de concentraciones de proteínas en estos tipos de derivados de la sangre, y a las limitaciones actuales en las técnicas de análisis proteómico de este tipo de muestras.
4. El colirio de PRGF y el SA indujeron una respuesta significativamente diferente en los queratinocitos corneales. El SA sin diluir indujo la activación de diferentes vías de señalización en los queratocitos corneales en comparación con el PRGF.

Dichas vías están relacionadas con el incremento de la respuesta inflamatoria, angiogénica, de estrés oxidativo y de fibrosis.

5. El colirio de PRGF pudo almacenarse hasta 12 meses a -20 ºC sin que se redujese la concentración de los principales factores de crecimiento y proteínas implicados en la regeneración de la superficie ocular y sin contaminación microbiana. Además, la actividad biológica del colirio de PRGF se mantuvo tras su almacenamiento durante 3 y 7 días a 4°C o a temperatura ambiente.
6. El colirio de PRGF liofilizado sin lioprotectores conservó los principales factores de crecimiento y proteínas tras su almacenamiento durante al menos 3 meses a temperatura ambiente o 4 °C. Además, el colirio de PRGF liofilizado sin lioprotectores mantuvo su actividad biológica durante 3 meses almacenado a 4 °C o a temperatura ambiente.
7. La liofilización del colirio del PRGF no afecta negativamente su composición proteica ni efecto biológico en modelo de cultivo celular.

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