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Desarrollo de formulaciones oculares de progesterona para el tratamiento de la retinosis pigmentaria

Development of ocular formulations of progesterone for the treatment of retinitis pigmentosa

TESIS DOCTORAL
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“Todo hombre puede ser, si se lo propone, escultor de su propio cerebro”

Santiago Ramón y Cajal (1852-1934)

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Índice

Abreviaturas

Age-related macular degeneration	(AMD)
Acetonitrile	(ACN)
Alcohol polivinílico	(PVA)
Alginato de sodio	(AS)
Apparent permeability coefficient (condiciones Sink y no Sink)	(P_{eff})
Apparent permeation coefficient (condición Sink)	(P_{app})
β-cyclodextrins	(β-CD)
Bovine Cornea Opacity/Permeability test	(BCOP)
Central nervous system	(CNS)
Chitosan	(CS)
Chorioallantoic membrane	(CAM)
Ciclodextrinas	(CD)
Confidence interval	(CI)
Corneal and scleral insert	(C/S-I)
Critical micelle concentration	(CMC)
Cytomegalovirus retinitis	(CMV)
Degeneración macular asociada a la edad	(DMAE)
Diabetic retinopathy	(DR)
Egg-chorioallantoic membrane test	(HET-CAM)
Especies reactivas de oxígeno	(ERO)
Estrés oxidativo	(EO)
Etilcelulosa	(EC)
Field Emission Gun	(FEG)
Formic acid	(FA)
Glial fibrillar acidic protein	(GFAP)
Glutamate cysteine ligase C subunit enzyme	(GCLC)
Glycerine	(GL)
Hidroxipropilmetilcelulosa	(HPMC)
High hydrophilic-lipophilic balance	(HLB)
High-performance liquid chromatography	(HPLC)
Irritation score	(IS)
Lag time	(T_0)
Limit of detection	(LOD)
Limit of quantification	(LOQ)
Malondialdehyde	(MDA)

Metilcelulosa	(MC)
Multiple reaction monitoring	(MRM)
Outer nuclear layer	(ONL)
Phosphate saline buffer	(PBS)
Polivinilpirrolidona K30	(PVP-K30)
Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)	(PEO-PPO-PEO)
Polydispersion index	(PDI)
Polyvinyl caprolactam-polyvinyl acetate-Polyethylene glycol graft copolymer	(PCL-PVAc-PEG)
Postnatal	(PN)
Progesterone	(PG)
Propylenglycol	(PGL)
Quitosano	(QS)
Reactive oxygen species	(ROS)
Relative standard deviation	(RSD)
Retinal vein occlusion	(RVO)
Retinosis pigmentaria	(RP)
Scanning Electron Microscopy	(SEM)
Scleral inserts	(S-I)
Sistema nervioso central	(SNC)
Sodium alginate	(SA)
Steady state flow	(J)
Temperature of storage	(G′)
Temperature of loss	(G″)
Terminal Deoxinucleotidyl Transferase Assay	(TUNEL)

Índice de figuras y tablas

Figuras:

Tablas:

Introducción y fundamentación teórica

1. Estructura ocular

El ojo de un adulto estándar mide unos 25 mm de diámetro. De su área total, solo una sexta parte se encuentra expuesta fuera de la órbita y es esta desde la que se tiene acceso para la administración tópica de medicamentos. El resto se encuentra en el interior de la cuenca ocular. A continuación, se detallan las partes que constituyen el ojo [1]:

Película lacrimal

La película lagrimal es la primera capa de la córnea con la que entra en contacto la luz. Produce lubricación e hidratación a la superficie ocular. También es una fuente de oxígeno, inmunoglobulinas, lisozimas, lactoferrina y defensinas α y β.

Córnea

La córnea forma la porción delantera de la cubierta exterior del globo ocular. Su función es doble, proteger el interior del ojo y proporcionar dos tercios del poder refractivo ocular. Está compuesta por cinco capas:

El epitelio se encuentra en la parte más externa, compuesto por 5-7 capas de células, con un espesor aproximado de 50 µm. El epitelio es uniforme y presenta una superficie lisa y regular, formada por epitelio escamoso estratificado no queratinizado. Existe una relación simbiótica entre la película lagrimal y el epitelio, ya que las mucinas de la película lagrimal que se encuentran en contacto directo con el epitelio corneal interactúan estrechamente con el glucocálix de las células epiteliales de la córnea para permitir la propagación hidrófila de la película lagrimal con cada parpadeo [2].

La capa de Bowman se encuentra inmediatamente debajo del epitelio corneal, con un espesor de 12 µm, y está formada por la condensación de colágeno y proteoglicanos. Esta capa ayuda a que la córnea mantenga su forma y cuando se lesiona, no se regenera pudiendo formar una cicatriz. No posee capacidad regenerativa y no se le considera una verdadera membrana. En realidad, es un condensado acelular de la porción más anterior del estroma, la siguiente capa más profunda [2].

El estroma constituye la mayor parte del tejido (80-85% del grosor corneal). Está constituido por tres componentes no acuosos: colágeno, proteoglicanos y células, así como de glicoproteínas especializadas [3] e iones que juegan un papel crucial en la organización de las fibrillas de colágeno para mantener la transparencia [4]. La característica principal es su transparencia, resultado de la organización precisa de las fibras estromales y la matriz extracelular. Estas fibras están empaquetadas en capas

dentro de las cuales se encuentra el colágeno. En el ojo humano hay entre 200 y 250 capas distintas, cada una dispuesta en un ángulo recto con respecto a las fibras de las capas adyacentes. La densidad de empaquetamiento es mayor en las capas anteriores que en el estroma posterior puesto que están muy entrelazadas y la mayoría parece insertarse en la capa de Bowman. Por el contrario, las capas posteriores de la córnea central están más hidratadas [2].

La membrana de Descemet es una estructura elástica de 7 µm. Está compuesta de colágeno tipo IV y laminina. Las células endoteliales secretan continuamente la membrana de Descemet [2].

El endotelio está formado por células endoteliales de una sola capa que recubren la parte posterior, la más interna de las cinco y de tan solo 5 µm de espesor [2,5]. Su transparencia es el resultado de la homogeneidad del índice de refracción de todas sus células constituyentes [6]. Su curvatura ayuda a enfocar la luz en la retina y una correcta percepción visual depende de su estado saludable. Es un tejido avascular ricamente inervado [7]. Los dos sistemas de transporte de iones más importantes son el Na^+ K^+ unido a la membrana ATP_{asa} y la vía de la anhidrasa carbónica intracelular. La actividad en ambas vías produce el flujo neto de iones del estroma al humor acuoso [2].

Esclera

La esclera se fusiona con el perímetro corneal en el limbo y se extiende hacia atrás terminando de formar la esfera ocular. En la parte posterior del ojo, el tejido conjuntivo escleral se fusiona con la vaina dural del nervio óptico, cuya entrada perfora la esclera. La composición aproximada de la esclera humana es en su mayoría agua (68%), seguido de colágeno (28%), otras proteínas (<3%), proteoglicanos (<1%) y elastina (0,6%) [8]. Dicha estructura está formada por:

Cápsula de Tenon: paralelamente a la superficie escleral se encuentran haces de colágeno y fibras de elastina formando una capa compacta. Es importante su capacidad funcional de actuar como un sistema de poleas para transferir fuerzas de los músculos oculares a la esclerótica durante los movimientos oculares [8].

Episclera: se encuentra a continuación de la cápsula de Tenon en su parte inferior. Se trata de una capa fina pero bien vascularizada. Contiene una alta densidad en tejido conectivo compuesta de haces de colágeno dispuestos circunferencialmente además de una pequeña cantidad de tejido elástico junto con melanocitos y macrófagos [9].

Limbo: las fibras superficiales de la esclera se mezclan con las fibras epiesclerales en el limbo. Sobre la zona trabecular, a través del limbo, se produce la transición de la esclera a la córnea en una región de 1 a 2 mm de ancho [9].

Estroma: capa principal del tejido escleral que domina el funcionamiento biomecánico de la esclera con su propiedad viscoelástica no lineal. Los haces de fibrillas de colágeno individuales se encuentran alineadas en paralelo, intercaladas en lugares con microfibrillas y fibras elásticas, formando laminillas de 0,5 a 6 μm de grosor que se encuentran aproximadamente en el plano de la superficie del globo ocular. Las laminillas esclerales en general muestran muchas más ramificaciones y entrelazamientos que las del estroma corneal vecino, y la extensión de esto varía con la profundidad del tejido y la ubicación anatómica [10].

Espolón escleral: las fibras profundas de la esclera se condensan en un anillo para formar el espolón escleral. Esta estructura de anillo rígido, junto con el anillo corneal, proporciona estabilidad al contorno corneal [9].

La esclera, a diferencia de la córnea, tiene fibrillas de colágeno que son más desordenadas y aleatorias. La mayor parte del estroma es principalmente acelular, excepto por la capa epiescleral externa [2].

Humor acuoso

El humor acuoso es el líquido que hay en las cámaras anterior y posterior (Figura 1). Proporciona un medio transparente e incoloro entre la córnea y el cristalino y constituye un componente importante del sistema óptico del ojo. El humor acuoso se forma en el epitelio ciliar y entra en la cámara posterior. Su principal función es la de nutrir a las estructuras carentes de vascularización, la córnea y el cristalino. Para llegar a la cámara posterior, los diversos componentes del humor acuoso deben atravesar los tres componentes tisulares de los procesos ciliares: la pared capilar, el estroma y la bicapa epitelial [11].

Iris

Haro contráctil posterior al cristalino que controla la cantidad de luz que penetra a través de la apertura o cierre de la pupila, su apertura central. La parte más externa de la capa vascular. El iris se estrecha (miosis) o dilata (midriasis), lo que hace que aumente o disminuya la cantidad de luz que llega al cristalino [12].

Cristalino

Estructura transparente, intraocular que tiene forma de lente biconvexa. No está vascularizada ni posee terminaciones nerviosas. Se encuentra entre el iris y el humor

vitreo (Figura 1). Su composición está formada por fibras de colágeno que llegan hasta el cuerpo ciliar y gracias a lo cual posee una consistencia elástica. El cristalino posee poder dióptrico de entre 19 y 20 dioptrías positivas. Su función es modificar la potencia dióptrica mediante el musculo ciliar permitiendo la visión nítida de cerca [13].

Humor vítreo

Se encuentra en la cámara vítrea, o cámara posterior (Figura 1). Situada directamente detrás de la lente. Ocupa 2/3 del globo ocular. Es una masa gelatinosa que reúne fibras delgadas y refringentes. La sustancia fundamental es transparente y gelatiniforme. Actúa como barrera estática y como barrera dinámica. La barrera estática es la estructura vítrea en sí misma, aunque no es una barrera muy restrictiva. La barrera dinámica consiste en los procesos de flujo y limpieza [12,14]. Es en esta estructura donde adquiere importancia la carga positiva de las moléculas, debido a la interacción con los componentes de la red vítrea, como el ácido hialurónico (cargado negativamente), donde difundirán con dificultad, contrariamente a las moléculas cargadas negativamente que lo difundirán sin problemas [15].

Retina

Situada en la capa interna del globo ocular (Figura 1), ubicándose a lo largo de la cara profunda de la capa vascular [12]. En ella nacen las fibras del nervio óptico y constituye el órgano receptor de los estímulos luminosos gracias a los fotorreceptores: conos y bastones. Los conos son las células encargadas de la visión en luminosidad, de lo colores y del espacio, hay tres tipos en humanos, las sensibles a longitudes de onda larga (luz roja), a longitud de onda media (luz verde) y a longitud de onda corta (luz azul). Los bastones, por el contrario, son células más sensibles, y se encargan de la visión cuando la luminosidad es baja (visión nocturna), longitudes de onda corta (uz-verde-azul) [16]. Se extiende desde el nervio óptico, desde donde nacen sus fibras, hasta la cara posterior del iris con dos partes diferenciadas, la porción óptica y la porción ciega [12].

Coroides

Capa media vascular que se encuentra a continuación de la esclera, en su parte interna (Figura 1), unidas por los vasos y nervios que las atraviesan, así como de una capa de tejido conjuntivo. Es una membrana con mayor espesor en la parte trasera del ojo (4 mm) que delante (3 mm). Además, es bastante frágil [12]. Se divide en cinco capas: la membrana de Bruch, la capa coriocapilar, dos capas vasculares y la capa supracoroidea. La membrana de Brunch (2-4 µm) está compuesta por fibras de colágeno y elastina. La capa coriocapilar está formada por capilares muy fenestrados con un

tamaño de poro de 6 a 12 nm, lo que permite el paso de moléculas grandes. El supracoroide se encuentra entre la esclerótica y la coroides y está compuesto por fibras de colágeno, melanocitos y fibroblastos [14].

En la administración ocular de medicamentos, se debe tener en cuenta que el coroides actúa como una barrera estática debido a la estructura supracoroidea y además, proporciona una barrera dinámica como consecuencia de una capa coriocapilar alta el flujo de sangre. Ambas acciones evitan el paso de compuestos hidrófilos, mientras que los fármacos lipófilos cargados positivamente pueden estabilizar las uniones con el tejido, lo que conduce a depósitos de liberación lenta [15].

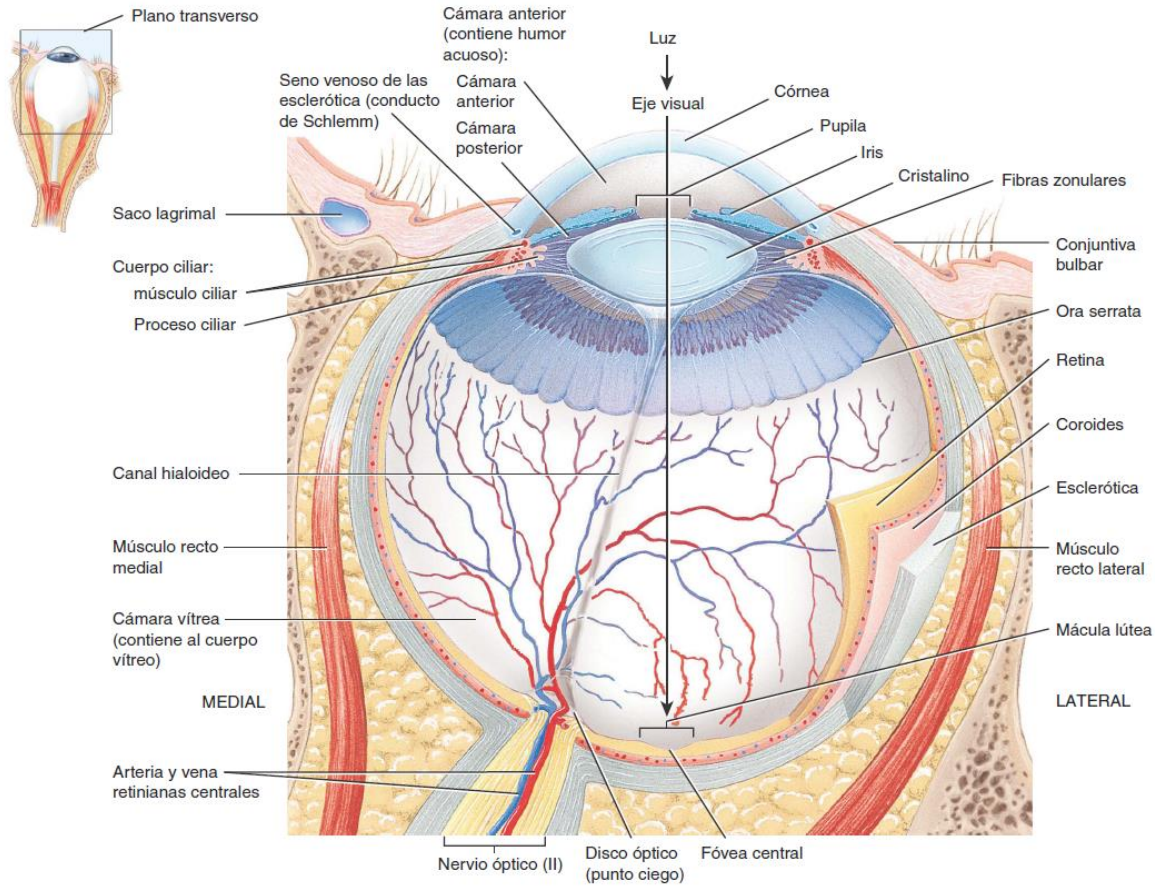


Figura 1. Corte transversal del globo ocular derecho. Imagen reproducida de "Principios de Anatomía y Fisiología" 11ª edición [17]

Párpados

Los parpados (Figura 1) son los encargados de la protección mecánica de la superficie ocular. El parpadeo constante que se produce de manera inconsciente en los párpados renueva la película lagrimal sobre la superficie del ojo, eliminando mecánicamente los cuerpos extraños y las bacterias adheridos superficialmente. Dentro

del tarso hay numerosas glándulas sebáceas especializadas llamadas glándulas de Meibomio que se abren en el borde del párpado. Sus secreciones son la principal fuente de la capa lipídica superficial de la película lagrimal. Cada folículo de las pestañas también está asociado con glándulas sebáceas llamadas glándulas de Moll. Además, hay glándulas apocrinas asociadas llamadas glándulas de Zeis [2].

2. La retinosis pigmentaria

La visión es uno de los sentidos mediante el cual, tanto el ser humano como cualquier ser vivo vertebrado y en la gran mayoría de invertebrados, obtienen información del mundo que les rodea. Es, quizás, el sentido más apreciado y del que más dependemos en nuestro día a día. El ojo es el órgano encargado de procesar la luz recibida en señales nerviosas para que nuestro cerebro las interprete. Esta función se produce en la retina, lamina translúcida de tejido nervioso que tapiza la parte posterior del globo ocular, más concretamente en las células fotorreceptoras, los conos y los bastones.

Las distrofias de retina son un grupo de enfermedades hereditarias, para las cuales no existe una terapia definitiva en la actualidad [18]. Entre estas enfermedades que pueden causar ceguera podemos encontrar principalmente el glaucoma, la degeneración macular asociada a la edad (DMAE), el edema macular secundario a la oclusión venosa de la retina (OVR), la retinitis por citomegalovirus (CMV), la uveítis posterior, la retinopatía diabética (RD) y la retinosis pigmentaria (RP) [14,19,20]. La anatomía del ojo y su fisiología hacen que el desarrollo de fármacos eficaces para el tratamiento de estas enfermedades sea un reto. Además, las diferentes partes del ojo pueden verse afectadas en distinto grado, lo que repercute en la administración de fármacos.

La retinosis pigmentaria (RP), también denominada retinitis pigmentosa, hace referencia a un conjunto de enfermedades degenerativas, todas ellas retino afectivas y hereditarias. En el transcurso de la enfermedad se produce la degeneración de las células fotorreceptoras de la retina [21,22].

Atendiendo a los patrones hereditarios, la RP puede clasificarse como RP autosómica dominante, RP autosómica recesiva, RP ligada al cromosoma X, RP digénica y RP mitocondrial. Tan solo en la RP no sindrómica, aquella en la que la RP se presenta sola (sin otras enfermedades), hay más de 80 genes asociados [23]. No obstante, dicha distrofia también puede aparecer junto a otros síndromes como el

síndrome de Usher, la forma más común de sordo-ceguera asociada con ataxia vestibular [21,24]. Asimismo, este puede ir asociado al síndrome de Bardet-Biedl, trastorno multisistémico tipificado por defectos degenerativos en el desarrollo vinculado con la disfunción cilial, que se asocia de forma variable con obesidad, deterioro cognitivo, polidactilia, hipogenitalismo y enfermedad renal [21,25] y, también a otras formas sindrómicas raras como el síndrome de Bassen-Kornzweig, la enfermedad de Refsum y la deficiencia de la proteína transportadora de α-tocoferol [21]. El principal inconveniente de la RP es que una sola mutación puede ser la responsable de una gran variedad de fenotipos clínicamente distintos, por lo que existe una gran heterogeneidad tanto clínica como genética, además de que distintas mutaciones pueden causar el mismo síndrome [26].

La RP es la degeneración retiniana hereditaria más común causante de ceguera [22], y tiene una prevalencia de 1:4000 en todo el mundo, afectando a más de 1,5 millones de personas [21,23]. Cursa principalmente con pérdida de visión nocturna, periférica después y por último la visión central. Esta pérdida progresiva se debe a la paulatina muerte de los fotorreceptores. En las primeras etapas de la enfermedad, los fotorreceptores tipo bastón son los que se ven afectados, apareciendo ceguera nocturna y pérdida del campo visual periférico medio. Los pacientes pueden perder el 90% de los conos en la fóvea antes de tener una reducción en la agudeza visual [21].

Los bastones son el tipo de célula más numeroso en la retina y también los más metabólicamente activos y por tanto los mayores consumidores de oxígeno [27]. La muerte celular de los bastones provoca un incremento en los niveles de oxígeno en la retina, los fotorreceptores son muy vulnerables a los altos niveles de oxígeno en los tejidos y una consecuencia del exceso de oxígeno en los tejidos es el estrés oxidativo (EO) [28]. Es esta sensibilidad de los fotorreceptores a los altos niveles de oxígeno lo que provoca la posterior muerte de los conos [27–29].

Para su diagnóstico es necesario realizar un fondo de ojo, en el que, por lo general, se apreciaran espículas óseas, vasos atenuados y palidez cerosa del nervio óptico en los tipos de RP más comunes [23,30].

El desarrollo de la enfermedad es variable según la edad del paciente. Algunas personas desarrollan pérdida visual sintomática en la infancia, mientras que otros permanecen asintomáticos hasta la edad adulta [21]. Los primeros síntomas en la adolescencia suelen referirse a la dificultad en la adaptación en visión nocturna, a la oscuridad y ceguera nocturna. Sin embargo, en la edad adulta, la persona pierde progresivamente el campo visual periférico. A medida que avanza la enfermedad, se

pierde la visión periférica distante y eventualmente se desarrolla una visión tipo túnel (Figura 2). Finalmente, generalmente a los 60 años, la visión central se anula. No obstante, según las autoridades sanitarias, la mayoría de los pacientes son técnicamente ciegos a los 40 años cuando el campo visual se encuentra severamente dañado [21].

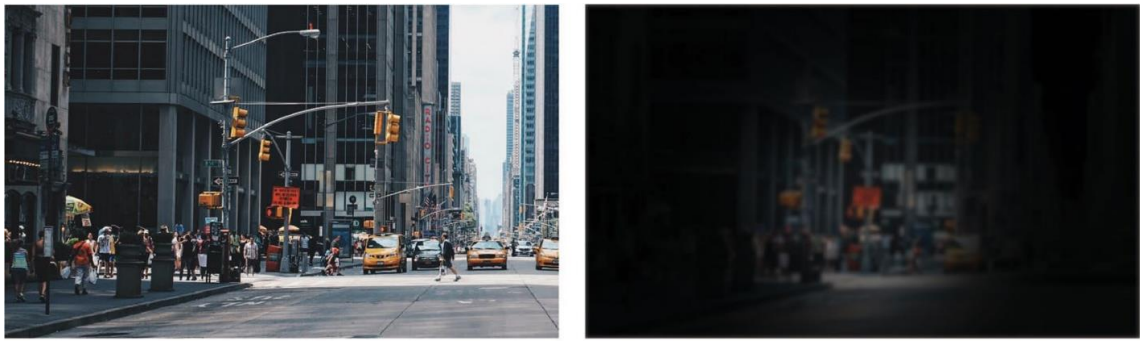


Figura 2. Simulación de vista de paciente con Retinosis Pigmentaria en fase avanzada [31].

Se han adoptado diferentes enfoques terapéuticos según la etapa de la enfermedad. En las primeras etapas, el objetivo es detener la degeneración mediante terapia génica o tratamientos farmacológicos (factores de crecimiento neurotróficos o factores antiapoptóticos), reduciendo la producción de moléculas retinotóxicas y limitando el daño oxidativo. En estadios avanzados, cuando apenas quedan fotorreceptores funcionales, puede ser necesario un trasplante de retina o implantes electrónicos de retina [32].

Se ha demostrado que el EO juega un papel relevante en la patogénesis de la RP y la progresión de esta enfermedad, ya que la muerte de los bastones hace que niveles más altos de oxígeno penetren en el tejido, aumentando así la producción de especies reactivas de oxígeno (ROS, por sus siglas en inglés) e induciendo daño oxidativo celular en los conos [28].

La supervivencia de las células retinianas requiere un equilibrio entre el oxígeno, las ROS y las moléculas antioxidantes que contrarrestan el daño por EO. El EO altera la homeostasis celular y provoca una respuesta protectora por parte de las células, que es más relevante en los fotorreceptores y las células ganglionares de la retina, neuronas con una tasa metabólica alta por lo que están continuamente sujetas a agresiones por estrés oxidativo. El EO provoca una respuesta celular que depende de la gravedad y parece evidenciarse que hay un umbral de estrés. Por debajo de ese umbral, las células provocan mecanismos de protección diseñados para asegurar la supervivencia, mientras que si el estrés supera al umbral o la activación de mecanismos de protección

fallan, las células activan vías de señalización alternativas que eventualmente conducen a la apoptosis, necrosis, piroptosis o muerte celular mediada por autofagia [33].

Se ha establecido que el daño celular por EO aumenta notablemente en la retina externa de los modelos animales con RP. La retina es extremadamente rica en membranas con lípidos poliinsaturados lo que la hace especialmente sensible al estrés oxidativo, ya que pueden sufrir peroxidación. Además, los bastones son células muy activas por lo que consumen grandes cantidades de oxígeno. Durante la progresión de la RP, cuando los bastones mueren, el consumo de oxígeno en la retina disminuye y la concentración de oxígeno en la retina aumenta. Por lo tanto, se produce una situación de hiperoxia que podría inducir el daño oxidativo. Es por ello que la terapia con antioxidantes se considera como una posible forma de frenar el proceso degenerativo en la RP, ya que puede disminuir el estrés oxidativo [34]. El tratamiento con antioxidantes farmacológicos en modelos animales de RP (rd1, rd10 y rds) retrasan sustancialmente la muerte de las células fotorreceptoras, lo que sugiere que el estrés oxidativo es un factor clave que contribuye a la degeneración de la retina en la RP [35–38].

3. Progesterona como tratamiento para la RP

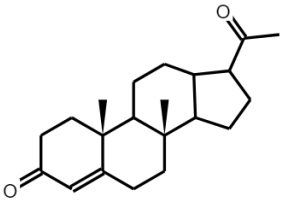
Recientemente, se ha demostrado el papel de las hormonas esteroideas sexuales y sus metabolitos como supresores de la muerte celular frente a EO [39]. Se utiliza el termino neuroesteroide para aquellas sustancias que podrían sintetizarse en el sistema nervioso central (SNC) y afectan a la neurotransmisión interaccionando con los receptores neuronales [40]. Se cree que estos esteroides juegan un importante papel en enfermedades oculares y en la prevención del daño en retina por isquemia [41,42], sobre todo considerándose a la retina como una estructura esteroidogénica del SNC [38].

En los últimos años, la influencia de los antioxidantes en los procesos oculares ha tomado una relevancia importante por sus efectos en la salud visual. Existen numerosos estudios publicados de su uso en el tratamiento de las patologías oculares como cataratas, glaucoma, degeneración macular asociada a la edad y RP [43–46].

La progesterona (PG), pregn-4-eno-3, 20-diona (véase Tabla 1), es una hormona esteroidea C-21 que ha demostrado su capacidad en la inhibición de apoptosis e inflamación [47,48]. Es por ello que, tanto la PG como sus derivados, pueden promover la formación de nueva mielina, reduciendo el grado de pérdida de la vaina de mielina,

mostrando potencial terapéutico contra la degeneración de la retina [38,49–52]. Debido a ello, la PG puede tener potencial terapéutico para el tratamiento de diversas enfermedades neurodegenerativas. Su seguridad y viabilidad se han demostrado en varios modelos de lesión cerebral traumática [48] y se ha demostrado que la PG puede reducir el daño de los radicales libres a altos niveles después de una lesión cerebral traumática en ratas [37,38,53].

Tabla 1. Características químicas de la progesterona

Estructura química 	Fórmula química	$C_{21}H_{30}O_2$
	Peso molecular	314,5 g/mol
	Solubilidad en agua	7 µg/mL
	Lipofilia (log P)	3,58

El estrecho intervalo de dosificación es uno de los principales inconvenientes de la administración de fármacos en la parte posterior del ojo, debido principalmente a la baja eficacia y biodisponibilidad de los fármacos administrados. Las vías intravítrea y periocular son altamente invasivas, y están estrechamente relacionadas con el incumplimiento del paciente y con malestar. Por tanto se busca una administración intraocular de liberación prolongada, lo menos invasiva posible, que conlleve a la aceptabilidad del paciente y al aumento de su calidad de vida, algo que paralelamente se traduciría en una notable reducción de los costes económicos asociados a las estancias hospitalarias por las frecuentes inyecciones repetidas que son necesarias para completar el tratamiento [14,54].

La PG administrada a altas concentraciones por vía intraperitoneal (8 mg/kg) promueve la supervivencia y la proliferación celular en tejidos no neuronales, así como administrada vía oral (100 mg/kg) [52,55], proporciona protección a la retina durante la distrofia objeto de estudio. Hernandez-Rabaza *et al.* [38] estudiaron los efectos de la PG sobre la RP destacando beneficios como la reducción de la gliosis típica, disminución de la concentración de glutamato en la retina y aumento de la concentración de glutatión. Se muestran resultados similares cuando se administra PG en el modelo de ratón RP rd10. Sin embargo, la administración oral o intradérmica de altas concentraciones de PG acarrearía problemas hormonales. Por ello, el presente trabajo busca alternativas terapéuticas para vehiculizar la PG con el fin de administrar la topicamente y a bajas concentraciones. No obstante, la administración ocular tópica de la PG tiene sus inconvenientes. La PG es una hormona esteroide sexual altamente hidrófoba (log P =

3,9) con muy baja solubilidad en agua a temperatura ambiente (7-10 µg/mL) [56]. La PG se disuelve de forma lenta e incompleta en los fluidos gastrointestinales, lo que, junto con su baja solubilidad y su rápido metabolismo hepático, limita su biodisponibilidad oral, por lo que son necesarias concentraciones muy elevadas para que una pequeña cantidad llegue al ojo [57].

El desarrollo de formulaciones oculares tópicas seguras y eficaces sigue siendo una tarea desafiante debido a las barreras anatómicas y fisiológicas inherentes al ojo y a las características celulares [58–61]. La administración tópica es un método de administración de medicamentos simple, indoloro y no invasivo, pero el tejido corneal es una barrera para la penetración del fármaco [27,49]. El epitelio corneal (lipofílico) limita la permeación de moléculas hidrófilas, mientras que el estroma, es decir, la capa inferior, está compuesto por una matriz extracelular altamente hidratada que limita la difusión de sustancias lipofílicas [22]. Además, la aplicación de formulaciones para los ojos activa los mecanismos de defensa, como el lagrimeo, que, junto con el recambio de lágrimas, diluyen el fármaco y lo eliminan de la superficie ocular. En general, la biodisponibilidad ocular del fármaco es inferior al 5% [54,62–64].

La PG se disuelve lenta e incompletamente en los fluidos gastrointestinales, lo que, junto con su baja solubilidad y su rápido metabolismo hepático, limita su biodisponibilidad oral. Todo esto, en conjunto, plantea un problema importante para el desarrollo de formulaciones orales del fármaco [57]. La baja solubilidad de la PG también dificulta la aplicación terapéutica de PG en patologías oculares. La difusión de moléculas aplicadas tópicamente a nivel ocular es muy baja, debido a las barreras anatómicas y fisiológicas inherentes presentes en el ojo [65]. La administración tópica de fármacos sigue siendo la vía de administración preferida frente a las inyecciones para el tratamiento de enfermedades oculares. Por ello, es necesario aumentar su solubilidad acuosa sin cambiar su capacidad intrínseca de permeación a través de membranas biológicas lipófilas, para hacer posible su aplicación ocular, y con este fin se pueden utilizar las ciclodextrinas [66].

Químicamente, las ciclodextrinas (CD) son oligosacáridos cíclicos producidos por degradación enzimática del almidón. Presentan una estructura de cono truncado, en la que el diámetro de la cavidad interna aumenta en función del número de unidades de glucopiranosa. Las caras externas de las CD son hidrófilas con los grupos hidroxilo y la cavidad interna es relativamente apolar, pero contiene moléculas de agua, cuya cantidad varía con la humedad relativa. Esta conformación permite la inclusión de moléculas apolares o partes de moléculas en el interior de la cavidad de las CD, incrementando la aparente solubilidad en agua de la molécula huésped y muy a

menudo, como consecuencia, su biodisponibilidad. La solubilidad en agua de las CD naturales es 145, 18,5 y 232 g/L para las CD α, β y γ, respectivamente. La β-CD es la CD que presenta con mayor frecuencia la mejor aptitud para la inclusión de moléculas de fármacos [67,68]. Además, las propiedades de las ciclodextrinas también pueden mejorar las características mucoadhesivas de las formulaciones, lo que lleva a un aumento del tiempo de retención en la superficie ocular [68].

La mayoría de los fármacos de aplicación ocular están destinados al tratamiento de enfermedades que afectan a diferentes capas de la córnea, la conjuntiva, el iris o el cuerpo ciliar [69]. La administración tópica oftálmica mediante **gotas** se utiliza habitualmente para el tratamiento de enfermedades que afectan al segmento anterior [70]. Sin embargo, el tratamiento de enfermedades oculares posteriores mediante la administración tópica se considera una estrategia terapéutica compleja ya que es difícil alcanzar concentraciones terapéuticas del fármaco en el segmento posterior del ojo debido a la baja penetración del fármaco y al elevado número de capas que ha de atravesar. Pese a ello, hay estudios que avalan la distribución eficiente de moléculas como el clorhidrato de memantina en la coroides y la retina tras la aplicación tópica de un anillo que funcionaba como depósito en la superficie de clorhidrato de memantina en un ensayo *ex vivo* corneal con ojos de vaca [71]. La memantina se encuentra en la actualidad en estudios de fase III en humanos enfermedades oftálmicas como neuroprotector y neuroreparador.

También se ha investigado la aplicación oftálmica de brimonidina en solución en monos, ratas y conejos. Los resultados han demostrado que su administración repetida permite una concentración de fármaco suficiente para proporcionar neuroprotección en la retina [72]. Asimismo, en el tratamiento de la degeneración macular asociada a la edad (DMAE), la aplicación tópica de complejos de dexametasona-ciclodextrina ha dado resultados prometedores en retina de conejo [73,74].

Las **micelas poliméricas** constituyen otra opción atractiva para la administración ocular debido a su capacidad para encapsular fármacos hidrófobos, facilitando el desarrollo de soluciones acuosas transparentes que evitan la sensación pegajosa y la visión borrosa de las formulaciones semisólidas. Los excipientes anfifílicos utilizados en las micelas pueden facilitar la permanencia de la formulación en la superficie del ojo e inhibir las bombas de eflujo en el tejido corneal [75]. Además, las micelas pueden facilitar la penetración del fármaco a través de la córnea y la esclera, proporcionando niveles terapéuticos del fármaco en la parte posterior del ojo, diana terapéutica en el tratamiento de la RP [76,77]. A continuación, se detallan diversos polímeros empleados en el desarrollo de las micelas poliméricas.

El Soluplus es un copolímero de injerto de polivinil caprolactama-acetato de polivinilo-polietilenglicol (PCL-PVAc-PEG) (Figura 3). El Soluplus tiene un valor de concentración micelar crítica (CMC) muy bajo ($6{,}6 \cdot 10^{-5}$ mM). Las micelas formadas con dicho co-polímero son muy estables frente a la dilución y tienen la capacidad de sufrir una gelificación *in situ* en la superficie ocular, lo que mejora la penetración en las estructuras oculares [76,78]. El núcleo hidrófobo de las micelas de Soluplus permite la encapsulación de fármacos poco solubles, mejorando notablemente su solubilidad aparente [46].

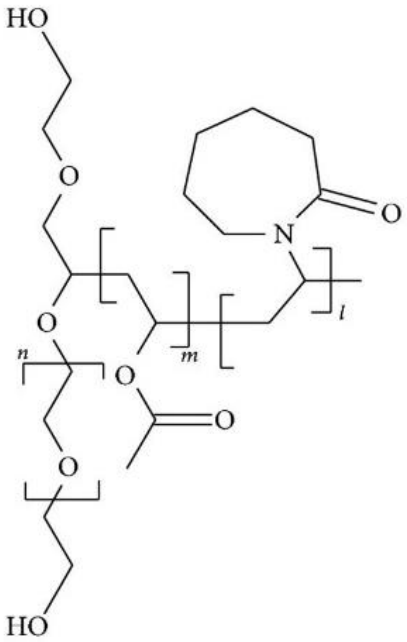


Figura 3. Estructura química del Soluplus®

Pluronic F68 es un copolímero tribloque lineal de poli óxido de etileno - poli óxido de propileno - poli óxido de etileno (PEO-PPO-PEO) (Figura 4) que, además de las propiedades de autoensamblaje, exhibe la capacidad para inhibir las bombas de eflujo de glicoproteína P en la superficie del ojo y para experimentar transiciones sol-gel al calentarse [78].

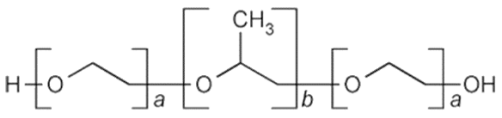


Figura 4. Estructura química del Pluronic® F68

La biodisponibilidad general de los fármacos administrados en gotas como la solución o las micelas es baja debido a la dificultad para su administración, a la perdida de formulación tras el pestañeo y la consiguiente visión borrosa [62]. Para superar estas limitaciones se emplea el uso de insertos oculares, que han dado resultados prometedores para el tratamiento de diversas patologías oculares [79–82].

Los **insertos oculares** se colocan en el fondo de saco superior o inferior y, en algunos casos, directamente en la córnea; su propósito es actuar como un depósito de fármaco de liberación controlada [79]. Son preparaciones estériles sólidas o semisólidas, generalmente hechas de materiales poliméricos [metilcelulosa (MC), hidroxipropilmetilcelulosa (HPMC), etilcelulosa (EC), polivinilpirrolidona K30 (PVP-K30), alcohol polivinílico (PVA), quitosano (QS), alginato de sodio (AS), gelatina y otros polímeros] [83]. Estos sistemas terapéuticos pueden ser dispositivos insolubles que se retirarían posteriormente o pueden diseñarse para disolverse, erosionarse o biodegradarse en la superficie ocular, por lo que no sería necesario retirarlos. Una de las propiedades más importantes que deben tener los insertos oculares es su discreción y comodidad para con el paciente, para que sean aceptados. El objetivo principal de esta formulación es extender el tiempo de contacto ocular, mejorando así la biodisponibilidad [79]. Existen insertos oculares comercializados, como los que se mencionan a continuación:

Ocusert® (Alza Corporation, Palo Alto) es un inserto para la administración de pilocarpina para el tratamiento de la hipertensión ocular, comercializado en 1974. Formado por depósitos de pilocarpina y alginato intercalados entre películas de acetato de etileno-vinilo; diseñado para administrar pilocarpina a 20 µg/h o 40 µg/h [84,85].

Ocufit SR® (Escalon® Medical Corp) son dispositivos de liberación sostenida de forma cilíndrica hechos de elastómero de silicona, diseñados para administrarse en el fondo de saco conjuntival [79].

Mydriasert® son dispositivos insolubles comercializados por IOLTech (Alemania) para la administración de fenilefrina y tropicamida para inducir la midriasis preoperatoria [86].

Minidisc (OTS) de Bausch & Lomb (Reino Unido) fue diseñado para administrar gentamicina o sulfisoxazol entre 3 y 14 días. Son discos de polímero cargados con fármaco con una forma similar a las lentes de contacto, pero más pequeños (4-5 mm). Los insertos son cómodos y fáciles de usar para la mayoría de los pacientes [79]. Esta forma farmacéutica puede ser tanto hidrofílica como hidrofóbica, lo que permite prolongar el periodo de liberación de fármacos hidrosolubles y poco hidrosolubles [83].

NODS® de Smith & Nephew Pharmaceutical Ltd (Reino Unido), ofrece administración precisa de pilocarpina para pacientes con glaucoma a partir de películas de alcohol polivinílico (PVA). Estos dispositivos se adhieren a la superficie ocular del saco conjuntival y son capaces de absorber el líquido lacrimal, se hinchan y administran el fármaco a una velocidad predeterminada a medida que se disuelve lentamente [84].

La principal ventaja de los insertos es que pueden ayudar a aumentar la adherencia del paciente al tratamiento. Sentir la presencia de un cuerpo extraño en el ojo es la razón principal que lleva a los pacientes a rechazar este tipo de formulación y, por lo tanto, es importante desarrollar insertos que sean lo más finos posible [79,87].

4. Modelos de experimentación oftálmica

Existen diferentes diseños experimentales para estudiar la difusión, la distribución y la toxicidad de fármacos a través de los tejidos que conforman en globo ocular. Estos son a menudo modificados según las características del experimento y de las necesidades experimentales.

Modelos de experimentación in vitro:

Los modelos celulares *in vitro* favorecen la reducción del número de animales de laboratorio utilizados para los estudios de penetración de fármacos, además de reducir los costes asociados. Los cultivos de córnea de conejo se utilizan con mayor frecuencia puesto que son más fáciles de obtener, contrariamente a las células humanas, que son difíciles de obtener y proliferan a un ritmo muy lento pudiendo tardar meses en generar datos significativos [88].

Por normal general, los modelos oculares basados en células para el estudio de la absorción y la penetración están enfocados en evaluar la citotoxicidad, más que para la penetración y la absorción del fármaco, puesto que no presentan todo el tejido ocular ni la función de barrera natural. Además, podrían faltar enzimas que metabolicen el fármaco o los transportadores activos en los cultivos *in vitro* [89].

Existen tres grupos distintos de cultivos celulares.

- Cultivos celulares primarios, obtenidos a partir de células epiteliales corneales de la córnea de ratas o conejos o incluso del humano, a partir de células epiteliales de córnea humana, opción que suele ser más limitada en su disponibilidad [90].

- Líneas celulares inmortalizadas, células que se modifican por mutación para evadir la senescencia celular normal, acortamiento de telómeros tras someterse a la división celular un gran número de veces y que provoca la muerte celular. Estas líneas celulares provienen de tumores o se producen mediante transformación con oncogenes virales [91,92].
- Cultivos de tejidos reconstruidos, se reconstruye un equivalente corneal humano. Consta de los tres tipos de células (epiteliales, estromales y endoteliales). El cultivo conjunto de estos tipos celulares proporciona resultados más precisos que los cultivos epiteliales tradicionales [92].

Por lo general, se estima que la permeabilidad del fármaco a través de cultivos celulares corneales sea mayor que a través del tejido corneal animal o humano.

Para estudiar la compatibilidad ocular se utiliza la membrana corioalantoidea del huevo de gallina (HET-CAM), a la cual, se le pone en contacto con la formulación a estudiar. Esta prueba es una alternativa a la prueba de *in vivo* de Draize que también evalúa la irritabilidad que pueden causar las formulaciones oculares [46,93,94]. La CAM es una membrana fetal que no está inervada pero sí muy vascularizada y responde a las lesiones de forma similar a la conjuntiva del conejo, formada por corion y alantoides fusionados [95]. Los efectos inducidos por la sustancia a ensayar en los pequeños vasos sanguíneos y proteínas de esta membrana de tejido blando se utilizan como indicador de los efectos inducidos por la misma sustancia de ensayo en el ojo de un conejo tratado. Los huevos se monitorizan para comprobar el sangrado, la lisis vascular y la coagulación de los vasos de la CAM durante 300s. La puntuación de irritación (IS) se calcula como:

$$\mathrm{IS} = \frac{(301 - \mathrm{tH}) * 5}{300} + \frac{(301 - \mathrm{tL}) * 7}{300} + \frac{(301 - \mathrm{tC}) * 9}{300}$$

En esta ecuación, tH representa el tiempo de hemorragia (s), tL, el tiempo de lisis (s) y tC, el tiempo de coagulación (s) [78]. Según los valores del IS, las sustancias se clasifican como no irritantes (IS <1), ligeramente irritantes (1 ≤ IS <5), moderadamente irritantes (5 ≤ IS <10) o severamente irritantes (IS> 10) [94]. Se utiliza un control negativo (0,9% NaCl) que no induce hemorragia, lisis o coagulación en el momento del estudio (IS = 0). El IS para el control positivo (0,1 N NaOH) se calcula en función de tH (s), tL (s) y tC (s).

Modelos de experimentación ex vivo:

Los modelos *ex vivo* se han desarrollado para evaluar y predecir la biodisponibilidad del fármaco que penetra o se absorbe al aplicarse tópicamente a través de celdas de difusión, utilizando córneas o escleras extirpadas del animal.

Estudios de difusión transcorneal/transescleral: se utiliza un sistema de dos cámaras que consta de un compartimento donante y otro compartimento receptor. La formulación objeto de estudio se coloca en un compartimento donante (compartimento superior, Figura 5. Celda de difusión. Imagen de elaboración propia.) mientras que el receptor se rellena de un medio de disolución, que se mantiene a temperatura corporal 37 °C ± 0,5 °C parcialmente sumergido en un maño y en agitación continua utilizando un agitador magnético (50 rpm). Ambos compartimentos se encuentran separados por la membrana a estudiar, que puede ser sintética o córnea/esclera de un modelo animal previamente establecido. Durante el estudio, en intervalos de tiempo específicos, se toman muestras del medio del compartimento receptor y se analiza el fármaco mediante una técnica analítica adecuada [83].

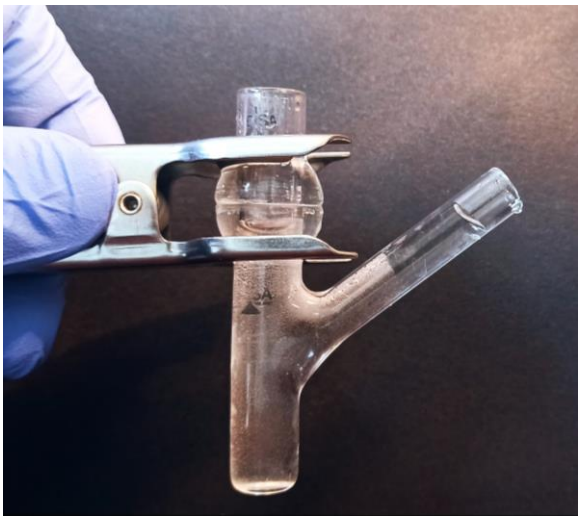


Figura 5. Celda de difusión. Imagen de elaboración propia.

La evaluación preclínica de los sistemas de administración de fármacos oculares normalmente implica estudios de permeabilidad *ex vivo* utilizando córnea o esclera no humana. La disponibilidad de cada especie animal es diferente según la región [92], por lo que en la literatura se han descrito ensayos *ex vivo* de formulaciones para una variedad de tejidos animales, principalmente conejo, porcino y bovino [96]. Estas pruebas en tejidos animales se utilizan principalmente para predecir la permeabilidad humana a los fármacos, asumiendo que la prueba proporciona resultados similares sin tener en cuenta la especie animal utilizada.

La córnea y la esclera de conejo se han preferido en la mayoría de los estudios de permeación *ex vivo* para correlacionarse con las pruebas preclínicas *in vivo* de seguridad y eficacia realizadas también en conejos, aunque ahora estas pruebas *in vivo* están siendo restringidas por la mayoría de los comités de ética animal en el mundo [32]. El ojo de conejo se considera generalmente el animal de referencia para experimentos oculares debido a sus similitudes entre la anatomía del conejo y el ojo humano, a pesar de tener una membrana nictitante (tercer parpado) y una frecuencia de parpadeo muy baja [92]. Este modelo es relativamente más fácil de trabajar en comparación con los modelos porcinos y bovinos. Las principales diferencias que presenta frente al humano es que la córnea es más larga y delgada, carece de la membrana de Bowman y moléculas de bajo peso molecular, como el metronidazol, tienen tasas de flujo más altas que a través del humano.

Los tejidos oculares porcinos parecen ser los más similares a los humanos: los globos oculares tienen un peso y tamaño similares, y también los volúmenes de humor acuoso y el cuerpo vítreo son comparables [97]. Se estima que la permeabilidad de ciertas sustancias activas a través de la córnea porcina es similar o ligeramente menor que a través de la córnea humana, ya que la capa epitelial de la córnea porcina es un poco más gruesa.

Paralelamente, la permeabilidad de las córneas bovinas puede ser menor porque el epitelio bovino tiene casi el doble de capas de células que el epitelio corneal humano [97]. A pesar de las diferencias entre las córneas humanas y bovinas, la prueba de opacidad/permeabilidad de la córnea bovina es una de las alternativas aprobadas actualmente al ensayo de irritación ocular *in vivo* en conejos [98].

Existen pocos estudios que informen sobre las diferencias entre especies (porcino, conejo y bovino) en el coeficiente de permeación aparente, P_{app}. Moiseev y col. [97], informaron de las diferencias en P_{app} que presentan el clorhidrato de ciprofloxacino, el clorhidrato de lidocaína, el maleato de timolol y la dexametasona disueltos en una solución tampón. Sin tener en cuenta el fármaco y el animal, el P_{app} fue más alto para la esclera que para la córnea. Los valores registrados para conejos fueron en general más elevados y también mostraron mayor variabilidad que los registrados para tejidos porcinos. Además, se demostró que las diferencias de tejido no eran la única causa de las diferencias de P_{app}. La córnea tiene un corte de peso molecular de 400 a 600 Da, mientras que la esclerótica permite el paso de moléculas de 70 kDa [99]. También Loch y col. [96] estudiaron y compararon el coeficiente de permeabilidad aparente de las córneas de conejo, porcino y bovino y encontraron que las córneas bovinas eran las

menos permeables de las tres especies. La lipofília de la membrana (log P entre 1 y 3) parece facilitar la penetración en todos los tejidos investigados [64,96].

Existen diferencias morfológicas entre córneas de especies distintas. El límite entre la capa de Bowman y el estroma no está claramente definido en el ratón, pero sí en conejo, vaca y humano. También se sabe que la capa de Bowman es más delgada en los mamíferos inferiores y más gruesa en los mamíferos superiores. Por otro lado, los diámetros de las fibras de colágeno en la capa de Bowman y el estroma son casi iguales en todas las especies. La capa de Bowman es plana en mamíferos "inferiores", como conejos. Por el contrario, está ondulada en los mamíferos superiores, como el ganado y el ser humano [100]. Los tres modelos animales han sido estudiados y han demostrado ser útiles para estudiar el coeficiente de permeabilidad de fármacos oftálmicas [96].

Estudios de biodistribución oftálmica:

Estos estudios de biodistribución tienen como objetivo determinar en qué cantidades se distribuyen los fármacos a través de cada uno de los tejidos y cámaras que conforman el globo ocular. Los globos oculares enteros, generalmente porcinos, se colocan en células de Franz, donde se les aplica tópicamente la formulación a evaluar durante un tiempo determinado. Tras su almacenamiento durante 24 h a -80 °C y en estado de congelación, los ojos se diseccionan, separando las diferentes partes a estudiar (cornea, esclera, humor acuoso, humor vitreo, iris, coroides y neurorretina. Este procedimiento se ha descrito para permitir la separación de las partes oculares limitando el riesgo de contaminación cruzada [101]. Posteriormente se pesan los tejidos aislados y se extrae en una mezcla de disolventes durante 12 horas en agitación constante. Al día siguiente, las muestras se centrifugan y se filtran. Por último, se analizan mediante una técnica analítica adecuada [102].

Modelos de experimentación *in vivo*:

En el estudio de las patologías oculares se utilizan diversos modelos animales *in vivo*. A modo de ejemplo, se utilizan las ratas para el estudio del glaucoma crónico [103], o ratas diabéticas para estudiar las cataratas [104]. Los ratones se han utilizado como modelo para el estudio de la DMAE [105], la retinopatía diabética [106] o la retinosis pigmentaria [52] y los pollos han sido utilizados como modelos de experimentación para el estudio de la miopía [107].

En concreto, para el estudio de la RP, Benlloch-Navarro *et al.* utilizaron ratones rd10 a los que se le administró oralmente PG (150 mg/kg) desde el día 15 hasta el 21

postnatal. Posteriormente se eutanasiaron y se enuclearon los ojos, en los que se evidenció que la PG disminuye la muerte celular de los fotorreceptores. Esto se demostró gracias a la tinción inmunohistoquímica de la desoxinucleotidil transferasa terminal (TUNEL) y mediante una tinción con Iba1 se obtuvo que los ratones tratados mostraron un menor número de células microgliales [108]. Del mismo modo en ratones rd10 se estudió el norgestrel, análogo de la PG, obteniendo resultados similares, preservación de la morfología de los fotorreceptores cónicos en los ratones tratados con norgestrel durante al menos 50 días, hasta P80 [109].

Otro proyecto desarrollado en el modelo de ratón RD1 de RP tratado con PG mostró resultados similares. En este caso, los ratones fueron tratados con una dosis menor de PG (100 mg/kg), administrada por vía oral, a partir del día postnatal 7 en días alternos. Posteriormente, los ratones fueron eutanasiados en los días postnatales 11, 13, 15 y 17. Los resultados mostraron un efecto conservador del número en las filas nucleares en la ONL en los días postnatales 13 y 15. TUNEL mostró una disminución de la muerte celular estadísticamente significativa en los ratones RD1 a los que se les administro PG en comparación con los ratones RD1 control. Además, se comprobó que existe una preservación significativa de la función en los fotorreceptores en el día postnatal 17 [52]. Los mismos resultados se obtuvieron si la PG seadministraba a los ratones en combinación con otras sustancias como el ácido lipoico [110].

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Hipótesis y objetivos

El trabajo que se describe en la presente memoria forma parte de un proyecto de investigación cuyo objetivo es desarrollar sistemas terapéuticos de liberación controlada, de administración ocular, que puedan ser empleados en el tratamiento de la retinosis pigmentaria.

Por ello, como hipótesis de partida se planteó la posibilidad de utilizar la vía tópica ocular para el tratamiento de dicha degeneración retiniana. El objetivo general de este trabajo es el desarrollo de formas farmacéuticas de PG que permitan utilizar la vía de administración ocular para el tratamiento de la retinosis pigmentaria, patología que se destaca por ejercer un impacto negativo sobre la calidad de vida de la persona que la padece.

Por lo tanto, el trabajo se ha planteado con los siguientes objetivos específicos:

1. Validar un método cromatográfico adaptado para determinar PG en estudios oculares *ex vivo.*
2. Determinar la difusión *ex vivo* de PG en solución a través de córnea y esclera de conejo, así como la permeabilidad de estas membranas.
3. Diseñar formulaciones de PG a base de micelas que puedan superar las limitaciones de solubilidad y permeabilidad del fármaco cuando se destinan al tratamiento óptico-local de la RP, comparar la difusión del fármaco a través de la córnea y la esclerótica de tres especies animales (conejo, cerdo y vaca) y estudiar su irritabilidad ocular.
4. Diseñar y caracterizar físicamente un inserto ocular para administrar PG, estudiar su irritabilidad ocular, estudiar la liberación de PG del inserto y analizar su difusión *ex vivo* de PG a través de córnea y esclera de conejo.
5. Determinar la biodistribución ocular de la PG administrada en solución e insertos en ojos de porcino.
6. Determinar el posible efecto terapéutico de la administración ocular en forma de solución de PG *in vivo* en un modelo experimental de RP: ratones rds.

Capítulo 1

HPLC-UV analytical validation of a method for quantification of progesterone in *ex vivo* trans-corneal and trans-scleral diffusion studies.

Abstract

Progesterone (PG) diminishes free radical damage and thus can afford protection against oxidative stress affecting the retina. The therapeutic use of PG is limited because it is a highly hydrophobic steroid hormone with very low solubility in water. This is the main drawback for the therapeutic application of PG at ocular level. The aims of this study were: (i) to analyze if PG causes ocular irritation (ii) to validate a HPLC method to determine PG in *ex vivo* studies and (iii) to evaluate PG permeation through cornea and sclera. A high-performance liquid chromatographic method was developed and validated to detect PG incorporated to β-cyclodextrin using a Waters Sunfire C18 (150 × 4.6 mm) reverse-phase column packed with 5 µm silica particles using a mobile phase consisted of a mixture of acetonitrile (ACN) and pure water 80:20 (v/v), pH 7.4. The limit of detection and the limit of quantification for 50 µL injection of PG were found to be 0.42 and 1.26 µg/mL, respectively. The calibration curve showed excellent linearity over the concentration range (0.5 µg/mL to 100 µg/mL). As proof of concept, *ex-vivo* experiments to investigate PG permeation through cornea and sclera with vertical diffusion cells were carried out to quantify PG diffusion. *Ex vivo* experiments demonstrate its applicability to investigate permeation levels of PG from 6.57 ± 0.37 µg/cm^2 at cornea and 8.13 ± 0.85 µg/cm^2 sclera. In addition, at the end of diffusion studies the amount of PG retained in each tissue was also quantified, and it was 40.87 ± 9.84 µg/cm^2 (mean ± SD; n = 6) in cornea and 56.11 ± 16.67 µg/cm^2 (mean ± SD; n = 6) in sclera.

1. Introduction

Progesterone (PG), pregn-4-ene-3, 20-dione (Figure 1.1), is a C-21 steroid hormone which has been shown to inhibit apoptosis and inflammation [1,2] and hence it may have therapeutic potential for the treatment of various neurodegenerative diseases. Its safety and viability have been demonstrated in several models of traumatic brain injury [2]. Although it has been shown that PG can reduce free radical damage after traumatic brain injury in rats, its chemical structure does not resemble that of typical antioxidants [3].

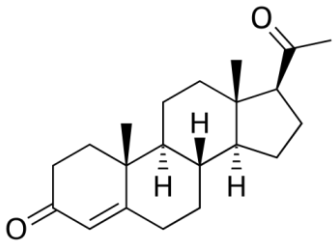

Figure 1.1. Structure of progesterone (PG).

Retinitis pigmentosa (RP) is a heterogeneous group of genetic retina degenerative diseases affecting photoreceptor cells. RP is the most frequent cause of hereditary blindness [4]. RP causes the death of the photoreceptor cells and begins affecting rod cells [5]. As the number rod photoreceptors decrease, tunnel vision and nyctalopia (night blindness) ensue. As the disease progresses, cone cells suffer significant reduction in acuity of the central vision and even full blindness, when the disease is advanced. It has been shown that PG has a positive effect in the treatment of RP after being administered orally, either alone or with oil as a carrier [3].

Chemically PG is a highly hydrophobic sex steroid hormone (log P = 3.9) with very low solubility in water at room temperature (7-10 µg/mL) [6]. PG dissolves slowly and incompletely in gastrointestinal fluids, which together with its low solubility and rapid liver metabolism, limits its oral bioavailability. All this, taken together pose a significant problem for the developing of oral formulations of the drug [7]. PG's low solubility also hinders the therapeutic application of PG in ocular pathologies.

Diffusion of topically applied molecules at the ocular level is very low, due to the inherent anatomical and physiological barriers present in the eye [8]. Ocular bioavailability of drug diffusion after ocular application represents less than 5% and in some cases can be as low as 1% [9–11]. Topical drug administration remains the preferred route of administration over intracameral or intravitreal injections for the treatment of eye diseases such as RP. Ease of application and high patient compliance justify this preference. Analytical methods for the determination of PG by HPLC with UV detection have been previously described [12–15] but the PG concentrations detected are relatively high. In *ex vivo* ocular diffusion studies, some contamination from biological material is unavoidable and may interfere with chromatographic detection of PG. Additionally, the methods previously described identify several molecules in addition to PG which resulted in lengthy procedures. We therefore needed a method free from interferences, with lower limits of detection and quantification and preferably shorter retention times which would result in an optimized method for HPLC determination of PG.

The aims of this study were to validate a method to determine PG in ocular *ex vivo* studies. but it becomes necessary to adapt these methods to our specific situation, and to assess the feasibility of ocular administration of PG by determination of its diffusion through cornea and sclera. For this, *ex vivo* trans-corneal and trans-scleral permeation of PG was determined.

2. Materials and methods

2.1. Materials

Progesterone (PubChem: 5994):methyl-β-cyclodextrin complex (85.2 mg of progesterone per gram), was obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile, ultrapure water, NaOH (sodium hydroxide) and NaCl (sodium chloride) were obtained from Análisis Vínicos, S.L. (Tomelloso, Spain).

2.2. Preparation of Standard Solutions

Stock solutions of PG (1 mg/mL) were prepared in Phosphate Buffer Solution (PBS) (pH 7.4; 150 mM). Six standard solutions for PG (0.5, 1, 5, 10, 50 and 100 µg/mL) were prepared by further dilution of the stock solution in isotonic buffer. These standard fresh solutions were used for the calibration curves.

2.3. Instrumentation and chromatographic conditions

HPLC was performed using a Waters system equipped with a quaternary pump (Waters 1525), a UV/VIS diode-array detector (Waters 2707) and an automatic injector fitted with 50 µL sample loop (Waters 2998 Plus). Computerized data acquisition and treatment were performed with the Breeze2® software. The chromatographic separation of the analytes was performed at room temperature (25 ± 2 ºC) using a Waters Sunfire C18 (150 x 4.6 mm) reverse-phase column packed with 5 µm silica particles. The mobile phase consisted of a mixture of acetonitrile (ACN) and pure water 80:20 (v/v), pH 7.4. The mobile phase was filtered through a 0.45 µm ester cellulose membrane filter DURAPORE® (Millipore Corporate, Billerica, MA, USA). Samples were eluted at a flow rate of 1.0 mL/min and absorbance was measured at 240 nm.

2.4. Validation

The method was evaluated in terms of specificity, linearity, precision, accuracy, limit of detection, limit of quantification and robustness. The analytical method was validated with six different concentrations of progesterone (0.5, 1, 5, 10, 50, 100 µg/mL).

Four aliquots were assayed to determine within-day reproducibility. Analysis was repeated on three different days to determine between-day validation.

Calibration curves were obtained by the least squares linear regression of the peak area as a function of PG concentration. The linearity of the calibration curves was tested by statistical comparison of the slopes with zero and the correlation coefficient with 1. Accuracy expresses the closeness of agreement between a calculated value and the accepted reference value (true value) and it is calculated as the relative error of known concentration solutions. To be considered acceptable, measures should fall within ± 10% for all concentrations [16]. The precision of the analytical method provides information about the random error and corresponds with the closeness of agreement between a series of measurements obtained from multiple analyses of the same homogeneous sample under prescribed conditions. It is measured as the relative standard deviation [RSD (%)] of the areas analyzed for each concentration. The RSD is considered acceptable when it is lower than 10% at all analyzed concentrations [17,18]. In order to determine the specificity of the method, absence of interference was evaluated by analyzing 10 blank samples from the same isotonic buffer used to prepare the standard solutions for calibration [PBS, pH 7.4; 150 mM].

The limit of detection (LOD), the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified, was expressed as the concentration that yielded a peak area equal to three times that of baseline noise at the retention time of the drug. The limit of quantification (LOQ) was the smallest quantity of a substance in a sample that could be quantitatively determined with well-defined accuracy under the experimental conditions. Both limits were determined chromatographically for the drug by examining of the signal to noise at rate the lowest concentration [19,20].

Assuming a normal distribution of measured concentration values, LOD and LOQ were calculated from the residual standard deviation of the regression data according to the criteria LOD=3.3x(S.D./b) and LOQ=10x(S.D./b) where S.D. is the residual standard deviation and b is the slope of the linear regression equation. [19,20]

The robustness of a method is the ability to remain unaffected by small changes in operating conditions. To assess robustness (i.e. the ability of a method to remain unaffected by small changes in operating conditions), experimental conditions were purposely altered at three different levels and chromatographic response and retention times were evaluated as described elsewhere [19]. Briefly, in order to study their effect, factors were altered one at a time as follows: a) the detector wavelength was varied by 2 nm (238 nm and 242 nm); b) the composition of the mobile phase at buffer solution

and ACN ratio were varied to 82:18 (v/v) and 78:22 (v/v); c) the pH of the mobile phase was modified by 0.2 units (7.2 and 7.6 buffer pH) and d) the flow rate of mobile phase by 0.1 units (0.9 and 1.1 mL/min).

2.5. Stability of Drugs in Solution

Seven groups of a concentration sample (50 µg/mL) of PG in cyclodextrins were stored in different conditions to determine the stability of the compound in aqueous solution. Firstly, these solutions were injected, and quantified while being protected from light. They were then divided into aliquots and stored in darkness under different conditions: in a freezer (-80 ºC), refrigerator (4 ºC), laboratory incubator (37 ºC) and at room temperature (25 ± 2 ºC). Other samples of the solution were stored at room temperature but exposed to light. All samples were assayed 3, 7 and 17 days after preparation to determine the residual PG concentration.

2.6. *Ex vivo* Ocular Diffusion of Progesterone.

The validated HPLC method was used to quantify progesterone in the samples obtained from *ex vivo* ocular diffusion studies. These experiments were carried using all-glass Franz diffusion cells purchased from DISA (Milan, Italy) with a diffusion area of 0.567 cm^2. Corneas and scleras from rabbits' eyes where used as membranes to separate the donor and receptor compartments (Figure 1.2). Whole eyes from two-month-old hybrid albino rabbits of either sex were obtained immediately after sacrifice. Eyeballs were rinsed in saline solution to remove any trace of blood, after which the adherent muscle was removed with scissors. Fresh excised corneas and scleras were obtained by cutting along the sclera-limbo junction and the individual excised tissue was used for diffusion studies [21,22]. The experimental protocol was approved by the Ethical Committee of University CEU Cardenal Herrera (Ref. 2011/010) and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (Ref. No. 2017/VSC /PEA/00192). Prior to sacrifice, animals were housed, fed, and handled according to current animal welfare principles (Spanish Royal Decree 1201/2005, (BOE 2005)).

A solution of PG in β-cyclodextrins (102 µg/mL) was prepared in an isotonic buffer [PBS, pH 7.4] and 0.5 mL of this solution was placed in the donor compartment. The receptor chamber was filled with propylenglycol:water (40:60 v/v) pH 7.4 (4.2 mL) [23], the receptor chambers were submerged in a water bath at 37 ºC and stirred by a rotating magnet placed inside the cell to prevent boundary layer effects. Samples (200 µL) were manually obtained from the receptor chamber at 30, 60, 90, 120, 180 min, and an identical volume of pre-warmed fresh medium was added. The amount of PG in each sample was quantified by HPLC. At the end of the *ex vivo* ocular diffusion studies, the amount of drug retained in each cornea and sclera was extracted by placing them in a solution of acetonitrile: water (80:20) for 12 hours. PG concentration was measured by the HPLC method described previously.

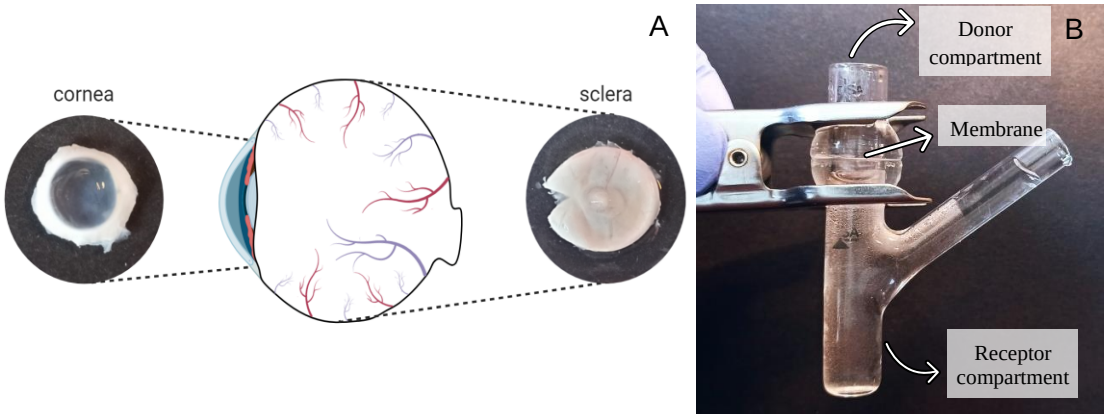

Figure 1.2. Diagram of an eye with photographs of the dissected cornea and sclera from the eye of a rabbit (A). Franz cell used in ocular ex vivo diffusion experiments (B).

2.7. Statistical analysis

Statistical analysis was performed using the Mann-Whitney test between the values obtained for both membranes at each of the ocular diffusion study times. The confidence level was 95%. The same test was also used to evaluate differences in accumulated amount between the different membranes. ANOVA was used to detect statistical comparison of the slopes with zero and the correlation coefficient with 1 in regression curves. In order to assess the robustness of the assay a one-way ANOVA, which would be followed if appropriate by the Scheffé post hoc test, was used to compare the effect of these variations on the detector response or on the retention time (depending on the change applied).

3. Results and discussion

3.1. Validation method

The solubility of PG in isotonic buffer solution [pH 7.6, 25 °C] is 7 µg /mL [24]. Given its log P value, it can be catalogued as practically insoluble in water, thus PG enclosed in cyclodextrins was used to ensure adequate solubility. An analytical method for quantifying PG in samples from *ex vivo* corneal or scleral permeation experiments needs to be highly specific; as such, samples usually contain endogenous compounds released from the eye. Furthermore, the method must be rather sensitive because the frequently low concentrations of PG in the collected samples.

Various HPLC methods that rely on UV-vis detection for PG determination have been described [14,15]. In order to identify which method is more sensitive and specific and to adapt it to the detection of PG in samples from *ex vivo* experiments, we have made modifications to avoid having overlapping peaks from biological material present in the samples.

The chromatographic method devised by Maliwal *et al.* (2009) consists of a Linchrocart C18 column (4.0 x 250 mm) with a 5 µm pore, a mobile phase of methanol and water 80:20 (v/v), a flow-rate of 1 mL/min, a wavelength of 254 nm, and an injection volume of 20 µL. These researchers reported a retention time of 6.39 min for PG. The chromatographic method reported by Wilson (2009) uses a Waters µBondapak C18 column (3.9 x 300 mm) with a 10 µm particle size, a mobile phase of ACN and water 50:50 (v/v), a flow-rate of 1 mL/min monitored at 270 nm, and an injection volume of 40 µL. Using this approach, the authors reported a retention time of 20.7 min. Our method consists of a Waters Sunfire C18 (150 x 4.6 mm) with a 5 µm pore, a mobile phase of ACN and water, 80:20 (v/v), at pH 7.4, an injection volume of 50 µL a flow-rate of 1 mL/min. and monitoring the signal at 240 nm. In our method, PG spectra displayed a maximum peak of absorbance at 240 nm. We selected this for detection, as there were not interferences from the biological samples at the molecule retention time at this wavelength, which proved to be sensitive and specific enough to analyze PG at all the concentrations found in our samples.

Table 1.1. Summary of validated progesterone detection methods between Maliwal *et al.* (2009), Wilson (2009) and our method. The flow rate is 1mL/min in all of them.

Method	Column	Mobile phase	Wavelength	Injection volume	Retention time	Range µg/mL	LOD µg/mL	LOQ µg/mL
Maliwal *et al.* (2009)	Linchrocart C18, (250 X 4.0 mm), 5 µm pore	Methanol : water 80:20 (v/v)	254 nm	20 µL	6.39 min	Not reported	Not reported	Not reported
Wilson (2009)	Waters µBondapak C18, (300 X 3.9 mm), 10 µm pore	ACN : water 50:50 (v/v)	270 nm	40 µL	20.7 min	32.2–161	0.8	1.6
Our method	Waters Sunfire C18, (150 x 4.6 mm), 5 µm pore	ACN : water 80:20 (v/v)	240 nm	50 µL	3.42 min	0.5-100	0.42	1.26

Using the method described here retention time for PG was found to be 3.42 min (Figure 1.3) in contrast to retention times of 20.7 min and 6.39 min reported by Wilson and Maliwal *et al.* respectively [14,15], as shown in table 1.1.

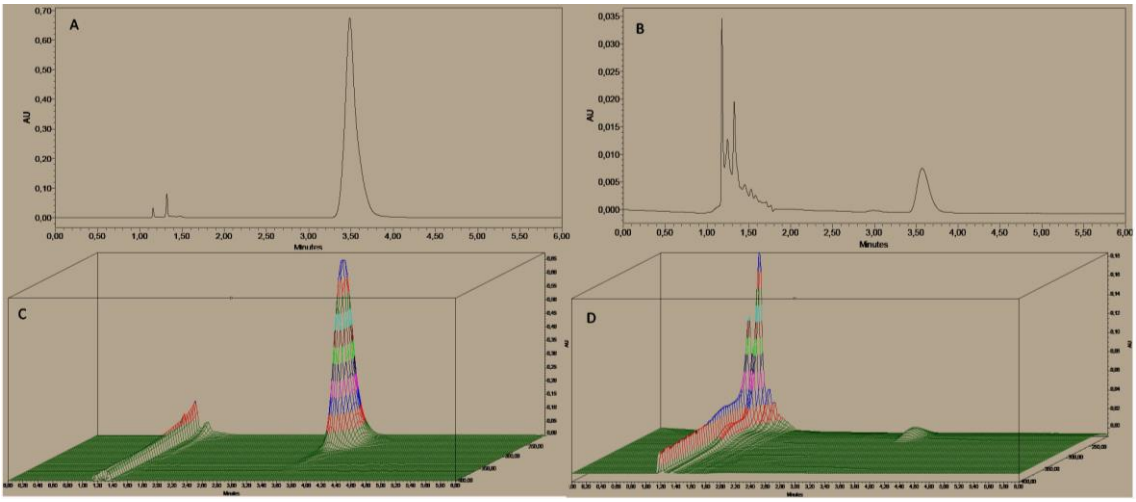

Figure 1.3. 2D chromatograms of 50 µg/mL PG standard solution (A) and sample obtained from ex vivo ocular diffusion experiments (B) at 240 nm. 3D chromatograms of 50 µg/mL PG standard solution (C) and sample obtained from ex vivo ocular diffusion experiments (D) for all wavelengths assayed (z-axis).

The LOD and LOQ for PG were 0.42 and 1.26 µg/mL respectively in contrast to 0.8 and 1.6 µg/mL reported by Wilson in 2009 (Table 1.1) [15]. Linearity was obtained in a concentration range of 0.5–100 µg/mL, in contrast to values reported by other authors (32.2-161.0 µg/mL) [15] (Table 1.1). These modifications of the analytical method allow a larger number of samples from ocular diffusion experiments to be processed faster and more efficiently and also allows PG to be quantified at lower concentrations than those reported by other groups [14,15] without the interferences produced when other detectable molecules from the ocular membranes were present in the samples.

Under these chromatographic conditions, we were able to separate and identify PG from other endogenous compounds present in the eye when our samples were analyzed. Representative chromatograms for standards and samples obtained from our

ocular diffusion studies are shown in Figure 1.3. Ten blank samples were analyzed to investigate the specificity of the method. No interference was detected at the retention time of PG. The method exhibited linearity between the response (y) and the corresponding concentration of PG (x) over the range of concentrations assayed. An average calibration curve was constructed from the results obtained: y = 232554 (±315) x-19079 (±13420). The calibration regression curve together with the 95% confidence interval (CI) is shown in figure 1.4. The results of the least square linear regression analysis showed a correlation coefficient of $r^2 \geq 0.99999$. The slope of the calibration curve was statistically different from zero, and the intercept was not statistically different from zero. The results of between-day and within-day precision and accuracy are shown in Table 1.2. It can be seen that calculated values were below 10% in all cases. The highest RSD value (4.98%) was obtained by the 1 µg/mL concentration studied in the within-day condition, hence, being within the percentage limits.

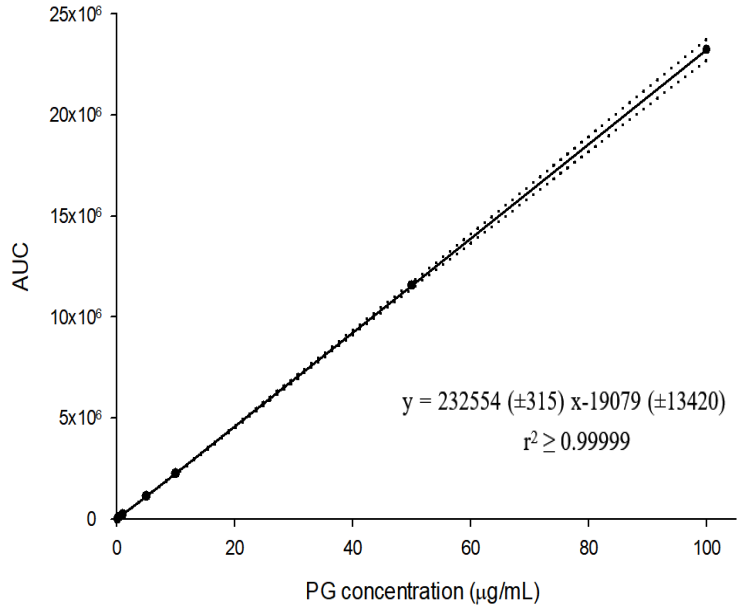

Figure 1.4. Linear regression of the average calibration curve of PG represented with the 95% confidence intervals (CI).

Table 1.2. Between- and within-day variability, accuracy and precision of the HPLC method for determining progesterone concentrations in saline-buffered samples.

Real concentration (µg/mL)	Between-day Variability (n=5)			Within-day Variability (n=5)		
	Concentration Found (mean ±SD) (µg/mL)	Accuracy (%)	RSD (%)	Concentration Found (mean ± SD) (µg/mL)	Accuracy (%)	RSD (%)
0.5	0.46 ± 0.01	-7.0	2.71	0.47 ± 0.01	-5.0	1.20
1	0.90 ± 0.01	-9.4	1.14	1.00 ± 0.05	-0.1	4.98
5	4.79 ± 0.12	-4.0	2.55	4.70 ± 0.09	-5.9	1.95
10	9.67 ± 0.13	-3.4	1.39	10.43 ± 0.37	4.4	3.54
50	49.70 ± 0.65	-0.8	1.31	49.42 ± 0.58	-1.2	0.58
100	99.92 ± 2.22	0.1	2.22	100.18 ± 4.30	0.5	4.30

To determine the robustness of the method, experimental conditions were deliberately altered at different levels. Variation of the detector wavelength, composition, pH and flow rate of the mobile phase had no statistically significant effect (ANOVA; $p>0.05$) on the retention time and chromatographic response of the method. The results for the robustness of the PG method are shown in Table 1.3 and confirm that the modifications carried out did not have an important effect on parameters. Thus, we can confirm that our method is robust.

Table 1.3. Robustness values of detector and mobile phases. Composition is expressed as percentage of ACN:water (v/v).

Condition		Modification	Area (mean ± SD)	RSD (%)	Retention Time (min) (mean ± SD)
Detector wavelength (nm)		238	6654444 ± 41232	0.62	3.42 ± 0.012
		240	6672351 ± 4262	0.06	3.42 ± 0.010
		242	6642486 ± 43059	0.65	3.41 ± 0.015
Mobile phase	pH	7.2	6654711 ± 27983	0.42	3.41 ± 0.014
		7.4	6649987 ± 3102	0.05	3.41 ± 0.011
		7.6	6661878 ± 19235	0.29	3.41 ± 0.016
	Composition (v/v)	78:22	6620115 ± 19847	0.30	3.42 ± 0.003
		80:20	6646364 ± 36694	0.55	3.41 ± 0.003
		82:18	6679568 ± 21338	0.32	3.42 ± 0.004
	Flow rate ($mL \cdot min^{-1}$)	0.9	6655213 ± 3598	0.05	3.42 ± 0.009
		1.0	6661676 ± 30616	0.46	3.41 ± 0.001
		1.1	6689396 ± 52098	0.78	3.41 ± 0.002

3.2. Stability of Drugs in Solution

The results obtained in the stability test are shown in Figure 1.5 as concentration percentage ± SD vs. time (days) for each condition studied. On day 3, none of the samples showed a concentration below 90% of the initial concentration, and on day 7 only samples stored in the dark at room temperature and those stored at room temperature under light exposure showed a concentration below 10% of the initial value. The stability results obtained show a significant decrease in PG values after 17 days

storage regardless of temperature and light conditions. In all cases the level of PG quantified was between 60 and 80% of initial PG values. Complexing PG with cyclodextrins to make it more water soluble does not seem to increase the stability of the molecule. Although it has been described that PG stock solutions in ethanol are stable for up to 6 months, we dissolved PG in PBS solution. Similar results have been also described showing degradation of PG in bovine milk [25].

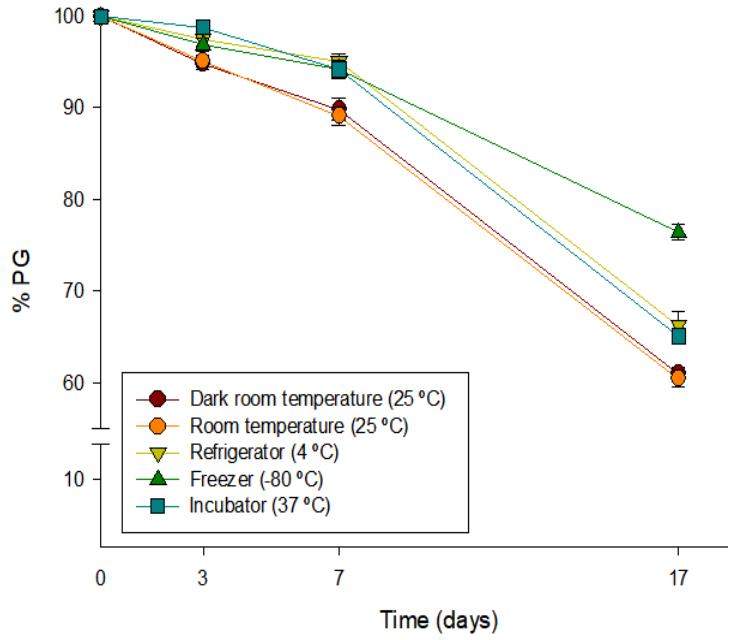

Figure 1.5. Stability results obtained for PG in standard solutions over 17 days. Recovery (%) ± SD (%), n=3.

3.3. Ex vivo Ocular Diffusion of Progesterone.

The amounts of PG accumulated in the receptor compartment of a PG solution (initial solution concentration 102 µg/mL) were plotted against time (Figure 1.6). PG was first detected after 60 minutes in the diffusion experiments using corneas as the membrane whereas in experiments with sclera PG was detected after 15 minutes. Statistical differences ($p<0.05$) were detected between corneal and scleral concentrations for all sample times after PG was detected (Figure 1.6).

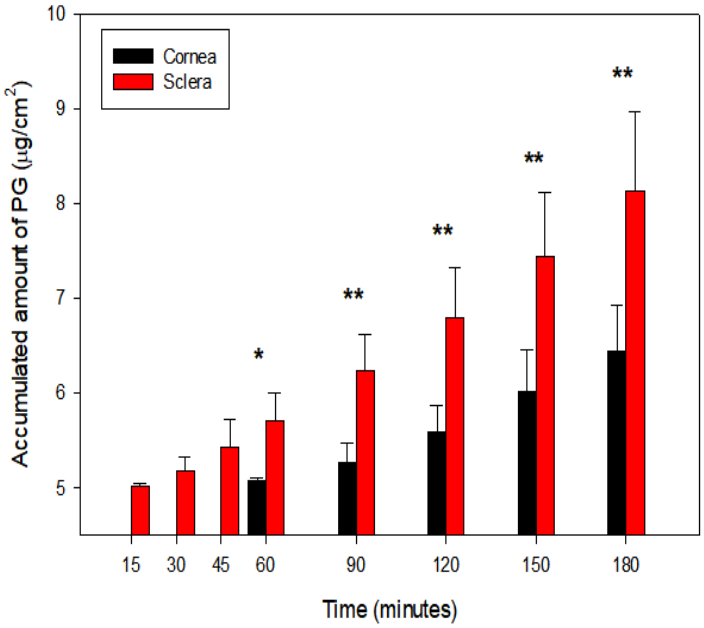

Figure 1.6. Amounts of PG accumulated in receptor chamber (µg/cm^2) vs. time (minutes) obtained in the ocular ex vivo experiments developed with cornea and sclera. Error bars show standard deviation of the observed values (n = 6). Significant differences were found between amounts of PG diffused through cornea and sclera at different times, *($p<0.05$) and **($p<0.01$).

After 3 hours of experiment, PG retained in the cornea and sclera were 40.87 ± 9.84 µg/cm^2 and 56.11 ± 16.67 µg/cm^2 (mean ± SD; n = 6) respectively. Statistical differences were found between the amount of PG retained in cornea and sclera (p = 0.009).

The accumulated amount of PG in receptor compartments was 6.57 ± 0.37 µg/cm^2 (n = 6) for trans-corneal and 8.13 ± 0.85 µg/cm^2 (n = 6) for trans-scleral diffusion. Thus, trans-scleral diffusion of PG was statistically higher than trans-corneal diffusion (p= 0.002). PG has two predicted pKa values, one in acid media (pKa = 18.92) and one in basic media (pKa = -4.8) so the protonation state of the drug is dependent on the pH of the solution; at physiological pH the molecule would not be charged. PG is a lipophilic and small molecule (log Poct = 3.9; MW = 314.5 g/mol) [26], therefore, as it is expected, PG has a reasonable trans-ocular permeation [27]. The accumulated results showed greater amounts of PG permeating through sclera than through cornea. T he may be due to different histological characteristics of the two tissues. The cornea is a complex tissue with a minimum of five different layers [28] with an outer epithelial layer (stratified squamous non keratinized) Bowman and Descemet membrane, which would interfere differently with hydrophilic and lipophilic molecules [28]. Diffusion to the internal part of the eye through the cornea may be rather difficult. On the other hand, the sclera is made up of collagen fibers arranged to create a dense connective tissue which may make trans-scleral diffusion of PG easy [29].

After 3 hours of experiment, the amount of PG retained in corneal and scleral tissue was quantified and found to be 40.87 ± 9.84 µg/cm^2 and 56.11 ± 16.67 µg/cm^2 (mean ± SD; n = 6) respectively. Statistical differences were found between the amount of PG retained in cornea and sclera ($p = 0.009$).

The results obtained show that PG is quantifiable and passes through cornea and sclera accumulating moderately in these structures, probably due to its lipophilicity. Furthermore, a modification of this HPLC-UV analytical method has been used for the quantification of PG (without cyclodextrins) in encapsulated micelles of Soluplus and Pluronic F68 [30]. Although the target concentration of PG in the retina needed to produce a significant therapeutic effect is unknown, the results obtained in this study provide interesting data for the development of eye formulations of PG as a possible treatment for RP. Future studies will complement these results with new formulations with greater capacity to control release and with a longer ocular residence time than the aqueous or micellar drops previously studied.

4. Conclusions

We report the validation of a simple chromatographic method for the rapid and precise determination of PG after ocular *ex vivo* diffusion studies. The specificity, limits of detection and quantification, accuracy, precision and robustness of the HPLC method here reported allow quantitative determination of PG contained in samples obtained from *ex vivo* ocular permeation experiments adequately. The stability results obtained, showed that PG can be stored refrigerated (4 ºC) or frozen (-80 ºC) for at least 7 days without its concentration dropping to less than 10%. Trans-corneal and trans-scleral diffusion of PG has been characterized under passive diffusion conditions. Therefore, we can confirm that the described method is adequate for the quantification of PG *ex vivo.* The present work will lay the foundations for future research studies of new ocular formulations for the release of PG after its topical application to the eye.

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Capítulo 2

Micelles of progesterone for topical eye administration: Interspecies and intertissues dierences in *ex vivo* ocular permeability.

Abstract

Progesterone (PG) may provide protection to the retina during retinitis pigmentosa, but its topical ocular supply is hampered by PG poor aqueous solubility and low ocular bioavailability. Development of efficient topical ocular forms must face up two relevant challenges: protective barriers of the eyes and lack of validated *ex vivo* tests to predict drug permeability. The aims of this study were: (i) to design micelles using Pluronic F68 and Soluplus copolymers to overcome PG solubility and permeability; and (ii) to compare drug diffusion through cornea and sclera of three animal species (rabbit, porcine and bovine) to investigate interspecies differences. Micelles of Pluronic F68 (3-4 nm) and Soluplus (52-59 nm) increased PG solubility by one and two orders of magnitude, respectively and exhibited nearly 100% encapsulation efficiency. Soluplus systems showed *in situ* gelling capability in contrast to the low viscosity Pluronic F68 micelles. The formulations successfully passed the HET-CAM test. PG penetration through rabbit cornea and sclera was faster than through porcine or bovine cornea, although the differences were also formulation-dependent. Porcine tissues showed intermediate permeability between rabbit and bovine. Soluplus micelles allowed greater PG accumulation in cornea and sclera whereas Pluronic F68 promoted a faster penetration of lower PG doses.

1. Introduction

Retinitis pigmentosa (RP) is a degenerative disease that involves heterogeneous inherited photoreceptors apoptosis affecting the peripheral retina. RP is the most common hereditary retinal degeneration, causing blindness [1]. One of the concerns in RP is that a single mutation can cause a variety of different clinical phenotypes, so there is a large clinical and genetic heterogeneity; also, different mutations can cause the same syndrome [2]. In the early stages of the disease, rod photoreceptors are affected causing difficulties with dark adaptation, night blindness and loss of mid-peripheral visual field in adolescence or young adulthood. As the RP advances and cones die, central vision is lost. Nevertheless, RP is a highly variable disorder; some patients develop symptomatic visual loss in childhood whereas others remain asymptomatic until mid-adulthood [1,3,4].

Different therapeutic approaches have been adopted depending on the stage of the disease. In the early stages, the aim is to halt the degeneration using gene therapy or pharmacological treatments (neurotrophic growth factors or anti-apoptotic factors), reducing the production of retino-toxic molecules, and limiting oxidative damage. In advanced stages, when there are barely any functional photoreceptors left, retinal transplantation or electronic retinal implants may be needed [4]. In the early stages when rods die and oxygen consumption in the outer retina is reduced, the supply of oxygen is not decreased because there is no self-regulation of choroidal blood vessels. This leads to increased levels of oxygen in the external retina. It has been suggested that long-term protection against oxidative damage can delay the death of cones in patients with RP regardless of the causative mutation [5].

Estrogens exert neuroprotection on the retina and optic nerve. Progesterone (PG; a C-21 steroid hormone, Figure 2.1a-A) and its derivatives may promote the formation of new myelin and reduce the extent of myelin sheath loss, showing therapeutic potential against retinal degeneration [6–10]. The retina is considered a true steroidogenic CNS structure [9]. PG has been reported to promote cell survival and proliferation in non-neuronal tissues (10) and to provide protection to the retina during retinitis pigmentosa [11,12]. Recently, Hernandez-Rabaza *et al.* [13] studied the effects of PG on RP highlighting benefits such as reduction of the typical gliosis, decrease in the concentration of glutamate in the retina and increase of the concentration of glutathione. Similar results were found when PG administration was performed in RP mouse model rd10. However, topical ocular delivery of PG is hindered by its poor aqueous solubility and low ocular bioavailability [13]. To the best of our knowledge PG dosage forms for eye administration have not been investigated yet.

Development of safe and effective topical ocular formulations remains a challenging task due to the inherent anatomical and physiological barriers of the eye [14–17]. Topical administration is a painless, non-invasive and simple practice method, but corneal tissue is a formidable barrier for drug penetration [5,6]. Corneal epithelium (lipophilic) limits the permeation of hydrophilic molecules, while the stroma, i.e. the layer below, is composed of a highly hydrated extracellular matrix that limits the permeation of lipophilic substances [1]. Moreover, instillation of eye drops activates defense mechanisms, such as lacrimation, which together with tear turnover, dilute the drug and clear it from the ocular surface. The excess volume is drained via the nasolacrimal duct into systemic circulation [3,5,6]. Overall, ocular drug bioavailability is less than 5% [18–20]. Polymeric micelles are an attractive option for ocular delivery due to their capability to encapsulate hydrophobic drugs, facilitating the development of clear aqueous solutions that avoid the sticky sensation and blurred vision of semisolid formulations. The

amphiphilic excipients used in micelles can facilitate the permanence of the formulation on the eye surface and inhibit efflux pumps on corneal tissue [21]. Moreover, micelles can facilitate drug penetration through both cornea and sclera, providing therapeutic drug levels to the back of the eye [22,23].

Preclinical assessment of ocular drug delivery systems commonly involves *ex vivo* permeability studies using non-human cornea or sclera. The availability of each animal species is different according to the region [24] and thus *ex vivo* tests of human formulations have been reported in literature for a variety of animal tissues, mainly rabbit, porcine and bovine [25]. These tests in animal tissues are mainly intended to predict human drug permeability assuming that the test renders similar results disregarding the species. Rabbit cornea and sclera have been preferred in the majority of *ex vivo* permeation studies in order to correlate with *in vivo* preclinical tests of safety and efficacy carried out also in rabbits, although now these *in vivo* tests are being restricted by most Animal Ethical Committees over the world [4]. Porcine eye tissues seem to be the most similar to the human ones: the eyeballs have similar weight and size, and also the volumes of aqueous humor and the vitreous body are comparable [26]. The permeability of certain active substances through the porcine cornea is estimated to be similar or slightly lower than through human cornea since the porcine corneal epithelial layer is a little thicker. In parallel, the permeability of bovine corneas may be lower because bovine epithelium has almost twice as many cell layers as the human corneal epithelium [26]. Despite differences between human and bovine corneas, the Bovine Cornea Opacity/Permeability test (BCOP) is one of the currently approved alternatives to *in vivo* ocular irritation assay in rabbits [27]. There is only one study that reports on the interspecies (porcine, rabbit and bovine) differences in the apparent permeation coefficient, P_{app}, of ciprofloxacin hydrochloride, lidocaine hydrochloride, timolol maleate, and dexamethasone dissolved in buffer solution. P_{app} values were recorded for each drug through sclera, cornea, conjunctiva, choroid and retina, and the combination of the different tissue layers. As expected, disregarding the drug and the animal, P_{app} was higher for conjunctiva and sclera and lower for cornea. The values recorded for rabbits were in general higher and also showed greater variability than those recorded for porcine tissues. Moreover, tissue dissimilarities were proved not to be the only cause of the P_{app} differences. Drug lipophilia (log P between 1 and 3) seems to facilitate the penetration in all tissues investigated [20, 25]. The cornea has a molecular weight cut-off of 400–600 Da while sclera allows the pass of 70 kDa molecules [28].

The aim of the present study was two-fold: (i) to design micelle-based formulations of PG that can overcome the drug solubility and permeability limitations when intended for topical treatment of RP; and (ii) to compare drug diffusion through

cornea and sclera of three animal species, namely, rabbit, porcine and bovine, in order to investigate interspecies differences. Specifically, two polymers, Soluplus and Pluronic F68, were selected to prepare the micelles. Soluplus is a polyvinyl caprolactam–polyvinyl acetate–polyethylene glycol graft copolymer (PCL-PVAc-PEG) (Figure 2.1a-B). Micelles prepared with Soluplus are highly stable against dilution due to its low critical micelle concentration (CMC) value and may also undergo *in situ* gelling on the ocular surface, enhancing penetration into ocular structures [22,29]. The hydrophobic core of Soluplus micelles allows the encapsulation of poorly soluble drugs, notably enhancing their apparent solubility [30]. Pluronic F68 is a linear triblock poly(ethylene oxide) (PEO)–poly(propylene oxide) (PPO)–poly(ethylene oxide) (PEO) copolymer (Figure 2.1a-C) that, in addition to the self-assembling properties, exhibits capability to inhibit P-glycoprotein efflux pumps at the eye surface and to undergo sol-gel transitions upon heating [29]. To carry out the study, first the capability of micelles prepared covering a wide range of copolymers concentrations to solubilize PG was investigated. After a screening of ocular compatibility using HET-CAM test, *ex vivo* drug permeability through the cornea and sclera from the three selected animal species was investigated.

2. Materials and Methods

2.1. Materials

Progesterone (PG, 314.46 g/mol), Pluronic® F68 (8,350 g/mol) and PG:methyl-β-cyclodextrin complex (85.2 mg PG/g; Progresterone-water soluble) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Soluplus® (115,000 g/mol) from BASF (Ludwigshafen, Germany), acetonitrile was provided from Scharlab SL (Barcelona, Spain), ethanol absolute from VWR (Fontenay-sous-Bois, France), propylene glycol from Guinama (La Pobla de Vallbona, Spain). Ultrapure water was obtained by reverse osmosis (MilliQ, Millipore Ibérica, Madrid, Spain). All other chemicals used were of analytical reagent grade and used as received.

2.2. Micelles Preparation and Characterization

Micelles were prepared by dispersing Soluplus or Pluronic F68 at different concentrations (4%, 8%, 12%, 16% and 20% w/w) in triplicate in PBS pH 7.4. The dispersions were kept under magnetic stirring for 24 h at room temperature. Size, zeta potential, and polydispersion index (PDI) of the micelles were measured in triplicate in a Zetasizer® 3000HS (Malvern Instruments, UK), previous filtration of each sample through 0.22 µm membranes (Acrodisc® Syringe Filter, GHP Minispike, Waters). The pH was

measured using a calibrated GLP22 pH meter (Crison Instruments, L'Hospitalet de Llobregat, Spain) [23].

2.3. Solubility of PG in Micelle Dispersions

Aliquots (10 mL) of Soluplus and Pluronic F68 dispersions prepared as above were placed in tubes containing PG (~ 3 mg). PBS pH 7.4 medium without copolymer was used as control. The resulting dispersions were prepared in triplicate and kept under constant magnetic agitation (Unitronic, JP Selecta, Barcelona, Spain) at 300 rpm for 96 h and room temperature [23,29]. Then, they were centrifuged (centrifuge model 5804R, Eppendorf AG, Germany) at 5000 rpm for 30 min to separate the non-solubilized PG. The supernatants were collected gently and diluted with ethanol:water (30:70 v/v) mixture to determine the apparent solubility of PG.

PG concentration was measured in a HPLC (AS-4140 Autosampler, PU-4180 Pump, LC-NetII/ADC Interface Box, CO-4060 Column Oven, MD-4010 Photodiode Array Detector) from Jasco (Tokyo, Japan) fitted with a C18 column (LiquidPurple ODS C18, 5 µm, 4.6 × 150 mm) and operated using ChromNAV software (ver. 2, Jasco, Tokyo, Japan). The mobile phase was acetonitrile:water (20:80 v/v) at 1 mL/min and 25 ºC. The injection volume was 50 µL. PG was quantified at 245 nm (retention time 5.1 min). Standard solutions of PG (0.01–5 µg/mL) in ethanol/water (30:70 v/v) were prepared.

The apparent solubility data were used to calculate the following parameters [23,29]. Molar solubilization capacity (moles of drug solubilized per mol of copolymer forming micelles):

$$X = \frac{Stot - Sw}{Ccopol - CMC} \quad (1)$$

Micelle–water partition coefficient (ratio between the drug concentration in the micelle and the aqueous phase):

$$P = \frac{Stot - Sw}{Sw} \quad (2)$$

Molar micelle–water partition coefficient, assigning a default copolymer concentration of 1 M:

$$PM = \frac{X*(1-CMC)}{Sw} \quad (3)$$

Gibbs standard-free energy of solubilization was estimated from the molar micelle/water partition coefficient (PM) and the micelle-water partition coefficient (P), as:

$$\Delta Gs = -RT * \ln(PM) \quad (4)$$

$$\Delta Gs = -RT * \ln(\mathrm{P}) \quad (5)$$

The proportion of drug molecules encapsulated in the micelles:

$$mf = \frac{Stot - Sw}{\mathrm{Stot}} \quad (6)$$

In these equations, Stot represents the apparent solubility of PG in the micellar solution, Sw is the PG solubility in water, Ccopol is the copolymer concentration in each micelle solution, CMC is the critical micelle concentration, and R is the universal constant of gases.

2.4. Rheological Analysis

The dependence on temperature of storage (G') and loss (G'') moduli of Soluplus and Pluronic F68 dispersions was evaluated in a Rheolyst AR-1000N rheometer (TA Instruments, Newcastle, UK) equipped with an AR-1000N data analyzer, a Peltier plate, and cone geometry (59 mm diameter, 2.1°). The angular frequency was fixed at 5 rad/s and the temperature increased from 15 °C to 40 °C with ramp rate of 2 °C/min, while the oscillation stress was kept at 0.1 Pa.

2.5. Hen's Egg Test Chorioallantoic Membrane (HET-CAM)

Fertilized hen´s eggs were donated by the Coren Technological Incubation Center (San Cibrao das Viñas, Spain) and incubated at 37 °C and 60% RH (Ineltec, model CC SR 0150, Barcelona). Each of the eggs was manually rotated 180° every 8 h to ensure the correct embryo development. After 9 days of incubation, a circular cut (1 cm) was made in the air chamber of the eggshell. The inner membrane was moistened with 0.9% NaCl for 30 min and then carefully removed to expose the chorioallantoid membrane (CAM). Dispersions of Soluplus and Pluronic F68 micelles loaded with PG (200 µL) were deposited in the CAM of different eggs. Solutions of 0.1 N NaOH and 0.9% NaCl were used as positive and negative controls respectively (300 µL). The eggs were monitored for bleeding, vascular lysis and coagulation of the CAM vessels for 300s. The irritation score (IS) was calculated as

$$\mathrm{IS} = \frac{(301-\mathrm{tH})*5}{300} + \frac{(301-\mathrm{tL})*7}{300} + \frac{(301-\mathrm{tC})*9}{300} \quad (7)$$

In this equation, tH represent haemorrhage time (s), tL, lysis time (s) and tC, coagulation time (s) [29]. According to the IS values, the substances are classified as non-irritant (IS <1), mild irritant (1 ≤ IS <5), moderately irritating (5 ≤ IS <10) or severe irritant (IS> 10) [31].

2.6. Ex-Vivo Corneal and Sclera Permeability Assay

Bovine, porcine and rabbit eyes were obtained just after sacrifice from a local slaughterhouse and transported immersed in PBS medium without antibiotics in an ice bath. After cleaning of muscle and connective tissues around the eye bulb, the anterior segment of the eye was circumferentially cut behind the limbus and separated. Corneas and scleras were isolated, immersed in PBS, and immediately placed on vertical diffusion Franz cells between the donor and receptor compartments [32–36].

The receptor chamber was filled with propyleneglycol:water (40:60% w/w) pH 7.4 (6 mL). Donor chamber (2 mL) was filled with the same solution and the tissue allowed to balance for 1 h. The solubility of PG in the receptor medium has already been established [37] and ensured sink conditions. The receptors were kept immersed inside a bath at 35 °C, and gentle magnetic stirring was applied for 1 h. After 1 h, the solution at the donor chamber was completely removed using a Pasteur pipette and replaced by the micelles formulations (1 mL) prepared as described above in PBS pH 7.4. The donor compartments (0.785 cm^2 permeation available area) were covered with parafilm. Samples (1 mL) were taken manually from the receptor chamber at 30, 60, 90, 120, 180 and 240 min, and the same volume was replaced with fresh medium, making sure to remove all bubbles from the diffusion cells. All the experiments were carried out in quadruplicate. As a control a solution of PG (343.04 µg/mL) was prepared by dispersion of the commercially available PG:methyl-β-cyclodextrin complex powder (85.2 mg PG/g) (Sigma Aldrich Co.; St. Louis, MO, USA) in buffer pH 7.4 and evaluated in parallel.

PG permeated was analyzed by HPLC and the cumulative amount of the drug (µg/cm^2) measured in the receptor solution was plotted against time to estimate the apparent permeability coefficients (P_{app}, cm/s), as follows

$$\mathrm{P_{app}} = \frac{\Delta \mathrm{Q}}{\Delta t} * (A * Co * 60)^{-1} \quad (8)$$

In this equation ΔQ/Δt (µg/min) is the steady state flow (J) across corneal or scleral tissue, A is the exposed surface area of ocular tissue (0.785 cm^2 in bovine and porcine

tissues; 0.567 cm^2 in rabbit), C_0 is the initial PG concentration (µg/mL) in the donor compartment, and 60 is taken as the factor to convert minute into second [29,38].

Each experiment was performed in triplicate, and the results were reported as mean values ± standard deviation. The steady state flow (J) was calculated from the slope as well as the lag time (T_0) by intersecting with the x-axis of the linear regression [30]. Drug concentration in the donor chamber was also quantified at the end of the test (4 h). All corneas and scleras were visually inspected after test to verify that all of them were in good conditions, without cracks or changes in their appearance. Corneas and scleras were immersed in acetonitrile (2 mL) medium at 37 °C for 24 h in Falcon tubes. Then, they were sonicated during 99 min at 37 °C, centrifuged (1000 rpm, 5 min, 25 °C), and the supernatants were filtered (Acrodisc® Syringe Filter, 0.22 µm GHP Minispike, Waters), centrifuged again (14,000 rpm, 20 min, 25 °C), and filtered to be analyzed in HPLC.

2.7. Statistical Analysis

Data analysis was performed using the Mann-Whitney or the Kruskal-Wallis tests as appropriate. Post-hoc multiple comparisons were carried out using the Man-Whitney test applying Bonferroni's correction for significance. Statistical analysis was carried out using SPSS 24.0.

3. Results and Discussion

3.1. Micelles Preparation and PG Solubilization

Both Pluronic and Soluplus copolymers (Figure 2.1a-B and C) are attractive excipients for ocular drug delivery owing their self-assembling properties, which enable forming micelles and also *in situ* gelling systems at moderate concentrations. Compared to Pluronic, literature on the applications of Soluplus to formulate eye drops is more limited. Soluplus has the advantage of its extremely low CMC (6.6×10^{-5} mM vs. 4.0×10^{-2} mM for Pluronic F68) [39] because of the higher hydrophobicity of the caprolactam moieties, and thus Soluplus micelles are more stable against dilution [40]. Also, differently, Soluplus solutions in water are slightly acid (pH 5.6) while Pluronic F68 solutions are neutral (pH 7.4). Although these pH values are well tolerated by the eye surface, for comparison purposes all micelle dispersions were prepared in a buffered medium. Accordingly, Soluplus and Pluronic F68 micelles were prepared in PBS pH 7.4 covering a wide range of copolymer concentrations (4, 8, 12, 16 and 20% w/w) (Table 2.1). The concentrations of each copolymer chosen for the study were well above the

CMC values, and ranged between 0.35 and 1.74 mM for Soluplus, and 4.79–23.95 mM for Pluronic F68 (corresponding to 4–20% concentration for both copolymers).

Unimodal number-based size distributions were observed for all micelle dispersions. Soluplus micelles had an average size of 52.32 ± 10.13 nm and a polydispersity index of 0.24 ± 0.01, while Pluronic F68 micelles were much smaller showing an average size of 3.74 ± 1.13 and a polydispersity index of 0.53 ± 0.08 (Table 2.2). The PG load did not cause a relevant change in the size of the micelles (Table 2.1). The surface charge of all polymeric micelles was slightly negative or near zero according to the composition of the two copolymers.

Table 2.1. pH, particle size, polydispersion index (PDI) and Z-potential (mV) of Soluplus and Pluronic (12%) micelles in PBS pH 7.4 at 25 ºC before and after being loaded with progesterone (PG). Mean values ± standard deviations; n=3.

Formulation	pH	Particle size (nm)	PDI	Z-potential (mV)
Soluplus 12%	6.46	52.32 ± 10.13	0.24 ± 0.01	-1.17 ± 0.38
Soluplus 12% + PG	6.38	59.19 ± 0.41	0.24 ± 0.02	-1.67 ± 0.62
Pluronic F68 12%	7.39	3.70 ± 1.01	0.74 ± 0.08	-1.76 ± 1.50
Pluronic F68 12% + PG	7.30	3.52 ± 0.15	0.73 ± 0.07	-0.46 ± 0.50

Table 2.2. Particle size of micelles of Soluplus and Pluronic micelles in PBS pH 7.4 at 25 ºC before and after being loaded with progesterone (PG). Mean values ± standard deviations; n=3.

Copolymer (% w/w)	SP	SP+PG	PL	PL+PG
4	51.23 ± 0.62	53.45 ± 2.68	5.49 ± 0.88	4.35 ± 0.17
8	53.33 ± 1.07	48.72 ± 0.48	2.94 ± 1.07	4.36 ± 0.56
12	52.32 ± 10.13	59.19 ± 0.41	3.70 ± 1.01	3.52 ± 0.15
16	60.89 ± 12.94	72.09 ± 3.67	3.22 ± 0.49	3.36 ± 0.02
20	53.58 ± 5.05	66.18 ± 21.32	3.32 ± 0.13	2.20 ± 0.15

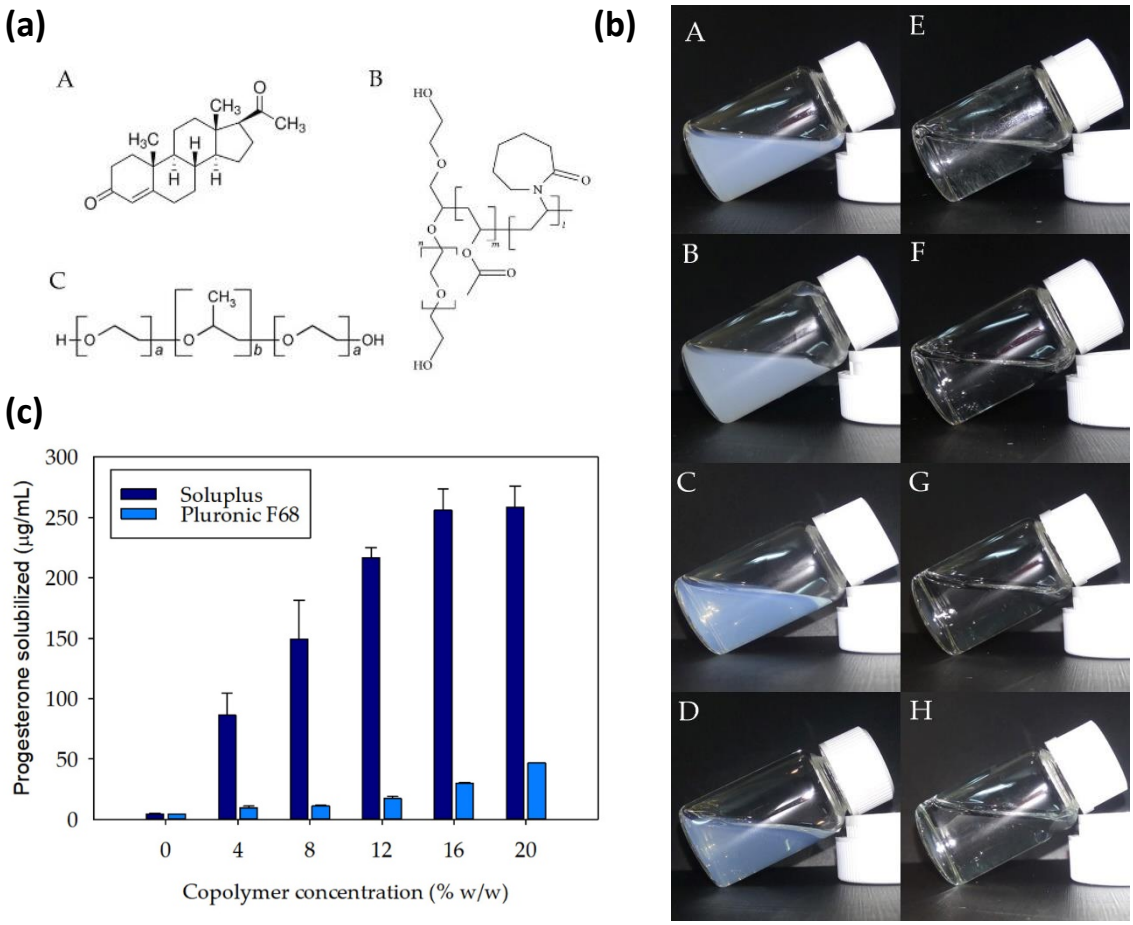

Figure 2.1. (a) Structure of PG (A), Soluplus® (B), and Pluronic® (C); (b) Appearance of micelle dispersions (A) 16% Soluplus + PG, (B) 20% Soluplus + PG, (C) 16% Soluplus, (D) 20% Soluplus, (E) 16% Pluronic + PG, (F) 20% Pluronic + PG, (G) 16% Pluronic, and (H) 20% Pluronic; (c) PG solubility in Soluplus and Pluronic F68 micelle dispersions formulated in PBS pH 7.4. Error bars represent the standard deviations (n = 3).

The initial transparent (Pluronic F68) or opalescent (Soluplus) dispersions kept the same appearance when adding PG (Figure 2.1b). Pluronic produced clearer formulations due to its high HLB (HLB = 29), while Soluplus dispersions opalescence is related to that the aggregates are larger and less hydrophilic (HLB = 16). The transmittance of 20% Soluplus dispersion in PBS pH 7.4 measured using a 10-mm light path cuvette is shown in Figure 2.2. Dispersions including PG behaved similarly. The transmittance of a liquid layer of Soluplus/PG system of about 10 microns, which corresponds to the thickness of the human tear film [41], can be estimated to be close to 100% in the visible light range. Therefore, no change in visual acuity is expected after instillation.

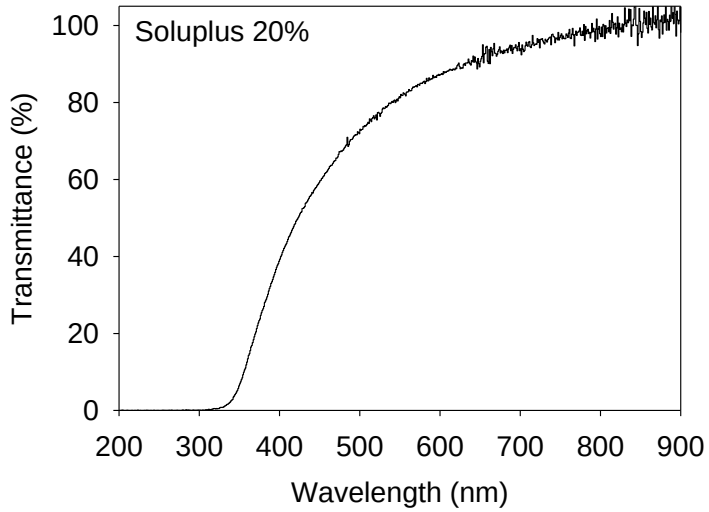

Figure 2. 2 Transmittance at room temperature of 20 %w/w Soluplus dispersion prepared in PBS pH 7.4 and measured using 10-mm light path cuvette

Once the micelles were prepared, their capability to encapsulate PG was investigated. PG solubility in PBS at 25 ºC was 4.4 µg/mL, which was lower than the solubility in water reported in literature, i.e. 7.0 µg/mL [42]. In the presence of the copolymers, PG solubility experienced a remarkably increase (Figure 2.1c). Pluronic F68 micelles (20% in PBS) increased the apparent solubility up to 46.8 ± 0.2 µg/mL. In the case of Soluplus (20% in PBS) the increase was even more remarkable, as an apparent solubility of 258.2 ±17.6 µg/mL was recorded. Parameters used to quantify the solubilization efficiency are summarized in Tables 2.3 and 2.4. Micelle-water partition coefficients, expressed as log P values, ranged between 3 and 4 for Pluronic micelles and between 4 and 5 for Soluplus micelles. These values clearly indicated that the proportion of PG encapsulated inside the micelles is to 3-to-5 orders of magnitude higher that the free drug remaining in the aqueous medium. Indeed, an increase in the copolymer concentration favored the encapsulation of more PG molecules, and according to the mf values (Table 2.3), more than 99.9% of drug was hosted inside the micelles for the higher Soluplus concentrations tested. Therefore, the outstanding encapsulation efficiency of ca. 100% means that almost no free drug is outside the micelles (when solubilization equilibrium was reached and excess of solid drug was removed). Moreover, solubilization data suggested that Soluplus 20% micelles dispersions could even encapsulate larger amounts of PG. The huge range of PG amounts that can be solubilized using Pluronic and Soluplus micelles may offer an enormous versatility to formulate the dose required for each patient.

Table 2.3. PG solubilization capacity of Soluplus dispersions in PBS pH 7.4, calculated using Equations 1-6. SP: Soluplus; PG: progesterone; χ: molar solubilization capacity; log P: logarithm of the partition coefficient; PM: molar partition coefficient; ΔG: Gibbs free standard solubilization energy; mf: molar fraction of the encapsulated drug within the micelle.

Copolymer (% w/w)	SP (M)	PG (M)	PG (μg/mL)	χ	log P	PM	ΔG for PM (KJ/mol)	mf
4	3.47×10^{-4}	2.74×10^{-4}	86.25	7.89×10^{-1}	4.29	56163941.1	-44211.8	0.99995
8	6.95×10^{-4}	4.74×10^{-4}	149.12	6.82×10^{-1}	4.53	48546060.2	-43850.6	0.99997
12	1.04×10^{-3}	6.88×10^{-4}	216.50	6.60×10^{-1}	4.69	46987265.0	-43769.7	0.99998
16	1.39×10^{-3}	8.13×10^{-4}	255.77	5.85×10^{-1}	4.76	41631784.1	-43469.9	0.99998
20	1.73×10^{-3}	8.21×10^{-4}	258.18	4.72×10^{-1}	4.77	33619107.1	-42940.3	0.99998

Table 2.4. PG solubilization capacity of Pluronic F68 dispersions in PBS pH 7.4, calculated using Equations 1-6. PL F68: Pluronic F68; PG: progesterone; χ: molar solubilization capacity; log P: respect of the partition coefficient; PM: molar partition coefficient; ΔG: Gibbs free standard solubilization energy; mf: molar fraction of the encapsulated drug within the micelle

Copolymer (% w/w)	PL F68 (M)	PG(M)	PG (μg/mL)	χ	log P	PM	ΔG for PM (KJ/mol)	mf
4	4.79×10^{-3}	3.07×10^{-5}	9.67	6.47×10^{-3}	3.34	460565.1	-32309.9	0.99954
8	9.58×10^{-3}	3.52×10^{-5}	11.08	3.69×10^{-3}	3.40	262942.2	-30921.1	0.99960
12	1.44×10^{-2}	5.47×10^{-5}	17.21	3.82×10^{-3}	3.59	271837.0	-31003.5	0.99974
16	1.92×10^{-2}	9.60×10^{-5}	30.18	5.02×10^{-3}	3.83	357388.1	-31681.5	0.99985
20	2.39×10^{-2}	1.49×10^{-5}	46.81	6.23×10^{-3}	4.02	443288.4	-32215.2	0.99991

The free energy of solubilization was negative in all cases; probably the encapsulation process was thermodynamically driven by hydrophobic interactions with the micelle cores. So far the largest increases in PG solubility have been reported for cyclodextrins in PEG:water medium, attaining values close to 1 mg/mL [42]. The remarkable advantages of PG encapsulation in the prepared polymeric micelles compared to inclusion complex formation with cyclodextrins rely on: (i) the outstanding 100% encapsulation efficiency in micelles, which is never reached with cyclodextrins; (ii) the higher thermodynamic and kinetic stability of the micelles, which are less prone to release the encapsulated drug than the cyclodextrins [43,44], and (iii) that organic cosolvents are not needed.

3.2. Rheological Properties of the Formulations

The rheological properties of a topical ocular formulation notably determine the time of permanence on the ocular surface and, in turn, the drug ocular bioavailability [45]. The use of thickening agents is frequent, but their concentration requires a fine balance between the required flowability of the liquid formulation during dosing (viscosity under shearing conditions below $25 \cdot 10^{-3}$ Pa·s) and the consistency demanded to remain on the eye (shear viscosity ≥ $10 \cdot 10^{-3}$ Pa·s) [46]. The upper limit of viscosity could be expanded

by means of polymers that undergo *in situ* gelling phenomena [47]. Thus, the next step was to investigate whether the presence of the micelles can increase by themselves the viscosity of the formulation and to determine the effect of temperature on the rheological properties.

Viscoelastic behavior of copolymer dispersions with and without PG is depicted in Figure 2.3. Pluronic F68 dispersions (4-20% w/w) with and without PG showed a viscous behavior with negligible storage modulus (G´) in the 15-40 °C range. The G" values were constant in the temperature interval evaluated and no sol-gel transition was observed. The presence of PG caused minor changes in the loss modulus of Pluronic F68. Differently, Soluplus blank dispersions exhibited the typical sol-gel transition showing remarkable increase in G' when a certain temperature was reached (Figure 2.4). The transition temperature decreased as the copolymer concentration increased: 4%, 37.5 ºC; 8%, 35.6 ºC; 12%, 35.7 ºC; 16%, 34.6 ºC and 20%, 32.7 ºC. For the PG-loaded micelle dispersions the transition temperature was: 4%, 37.4 ºC; 8%, 34.9 ºC; 12%, 33.5 ºC; 16%, 36.3 ºC and 20%, 35.6 ºC. Nearby and above the transition temperature both G' and G'' showed a progressive increase in their values. This temperature-dependence pattern agreed well with other reports on Soluplus dispersions [25] and was quite different to the typical *in situ* gelling behavior reported for other temperature-responsive copolymers that exhibit brusque transitions. Interestingly, at 35 ºC Soluplus 12-20% micelle dispersions could transform into soft gels on the eye surface prolonging the retention time and the drug release. The complex viscosity values (i.e., the frequency-dependent viscosity) recorded at 35 °C for Soluplus 16 and 20% in PBS with PG were 4.08 and 4.63 Pa·s, respectively, while for Pluronic 16 and 20% in PBS with PG, the values of complex viscosities were $9.87 \cdot 10^{-3}$ and $14.21 \cdot 10^{-3}$ Pa·s, respectively. These findings indicate that Pluronic micelles communicate shear viscosity above the critical level to maintain precorneal residence in man [46].

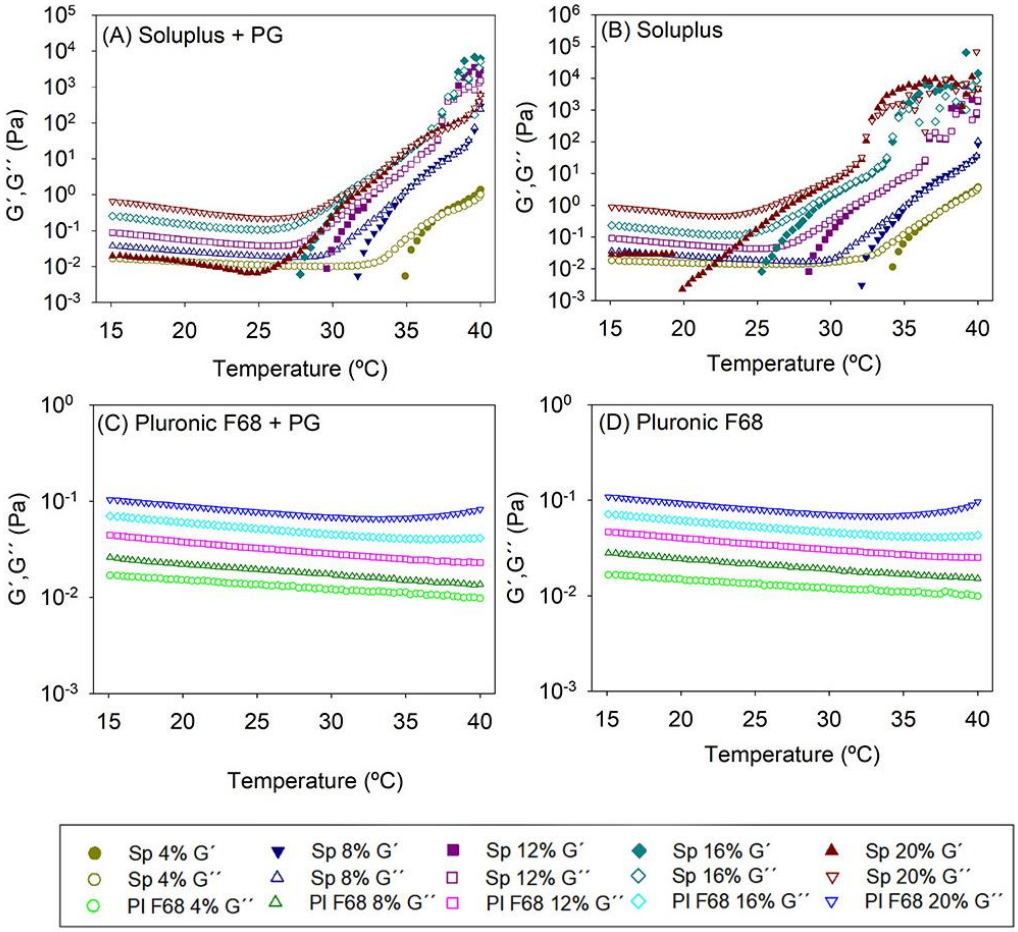

Figure 2.3. Evolution of the storage (G´) and the loss (G") moduli as a function of temperature of (A) PG-loaded Soluplus micelle dispersions, (B) blank Soluplus dispersions (C) PG-loaded Pluronic F68 micelle dispersion (D) blank Pluronic dispersions. Total copolymer concentration was 4, 8, 12, 16 and 20% w/w in PBS pH 7.4 buffer (n=4).

Figure 2.4. Appearance of the viscous 20 %w/w Soluplus dispersion in PBS pH 7.4 at 37 ºC.

3.3. HET-CAM test

A first screening of ocular compatibility was carried out placing the formulation in contact with the hen´s egg chorioallantoic membrane (HET-CAM). This test is an

alternative to the *in vivo* Draize test to evaluate the irritancy that ocular formulations may cause [29,30,48]. CAM is a fetal membrane that is not innervated but highly vascularized and responds to injury similarly to the rabbit conjunctiva made of fused chorion and allantois [49]. The effects induced by the substance to be tested on the small blood vessels and proteins of this soft tissue membrane are used as an indicator of the effects induced by the same test substance on the eye of a treated rabbit [31].

The HET-CAM test revealed that Soluplus and Pluronic F68 micelle dispersions behaved as the negative control (0.9% NaCl) without inducing bleeding, lysis or coagulation at the time of the study (tH, tL and tC > 301s; IS= 0), which means that they can be considered non-irritants (Figure 2.5). The IS for the positive control (0.1N NaOH) was 18.73 (tH =30s, tL = 30s, tC = 35s). The non-irritant behavior of the Soluplus and Pluronic F68 micelle dispersions is in good agreement with previous reports on HET-CAM assay results recorded for 23 %w/v Soluplus dispersions [30] and *in situ* gel formulations prepared with 23 %w/v Pluronic F127 and 15 %w/v Pluronic F68 [50].

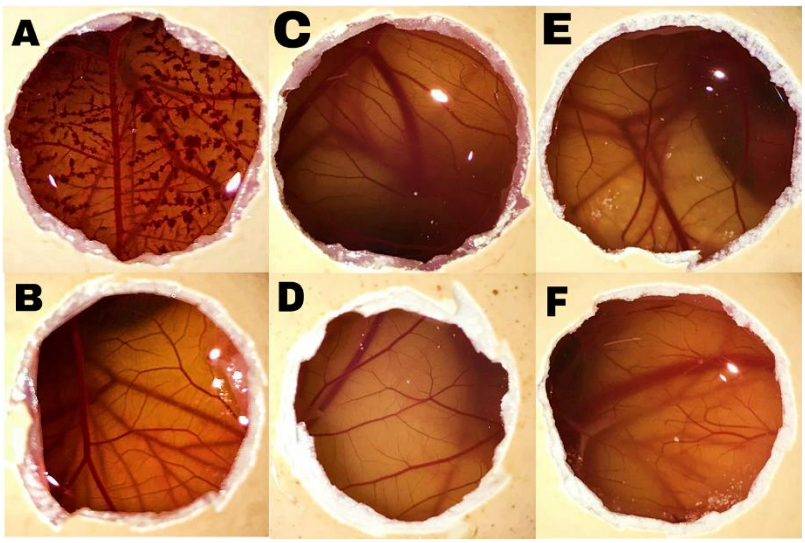

Figure 2.5. Effect of controls and test substances on the surface of the chorioallantoic membrane (CAM) after treatment for 3 min (A) NaOH 0.1N, (B) NaCl 0.9 %w/w, (C) Soluplus (16%), (D) Soluplus (20%), (E) Pluronic F68 (16%), and (F) Pluronic F68 (20%) dispersions in PBS.

3.4. Ex Vivo Permeation Assay

First, pig eyes were selected as they are structurally similar to human eyes in terms of globe size, corneal thickness, ratio of globe diameter to corneal length, presence of Bowman's layer and sclera histology and collagen bundle organization [24,51]. PG permeability studies were first carried out for both 16 and 20% polymer concentrations with the aim of identifying the formulation that promoted the highest drug penetration through cornea and sclera. Formulations had a PG load of 256 and 258 µg/mL for

Soluplus 16% and 20% dispersions, respectively. In parallel, Pluronic F68 16% and 20% dispersions contained 30.1 and 46.8 µg/mL, respectively.

After 4 h in contact with cornea, the amounts of PG accumulated in the receptor chamber were 1.31 ± 0.21 µg/cm^2 and 2.44 ± 0.17 µg/cm^2 when applied as Soluplus micelles at 16% and 20% respectively. In the case of Pluronic 16% and 20% micelle dispersions, the amounts accumulated were 0.43 ± 0.17 µg/cm^2 and 0.77 ± 0.13 µg/cm^2 respectively (Figure 2.6A).

In parallel, the amount of PG that passed through the sclera in 4 h was 0.26 ± 0.14 µg/cm^2 and 1.12 ± 0.36 g/cm^2 for Soluplus micelles at 16% and 20% respectively, and 0.15 ± 0.06 µg/cm^2 and 0.36 ± 0.23 µg/cm^2 for Pluronic micelles at 16% and 20% respectively (Figure 2.6B). Thus, differently to the common trend observed for most ophthalmic drugs in which sclera permeability is higher than cornea permeability [52], in the present study PG permeability through cornea was somewhat favored. It has been previously observed that sclera and cornea permeabilities become similar when the drug is hydrophobic and, therefore, exhibits high affinity for the hydrophobic epithelium of cornea and low affinity for aqueous porous network of sclera [27]. Indeed, it should be noticed that PG is a BCS Class 2 drug, i.e. it exhibits low solubility (nearly insoluble in buffered medium) and high permeability. PG is a small molecule (MW 314.46 g/mol) with a very low polar surface area (log P= 3.58) [53]. Interestingly, although the BCS classification refers to intestinal permeability, hydrophobic drugs appear as the most suitable to cross most biological membranes and therefore the BCS class may provide preliminary information about the feasibility of that a drug could pass through the cornea [27]. In the particular case of a hydrophobic drug, such as PG, encapsulated in micelles, additional factors should be considered. Micelles in contact with the cornea provide an enhancement in drug gradient concentration and may facilitate the partition of the drug towards the epithelium, but also the drug-loaded micelles may easily cross the stroma [54]. In the case of sclera and for a drug so hydrophobic as PG, only the drug encapsulated inside the micelles is expected to pass through the tissue, in which the arrangement of the collagen bundles allows the formation of a pore network (20-200 nm) fill of water that screens the crossing of the substances as a function of their molecular size [55]. As expected, formulations that provided higher PG apparent solubility led to higher amounts of drug permeated.

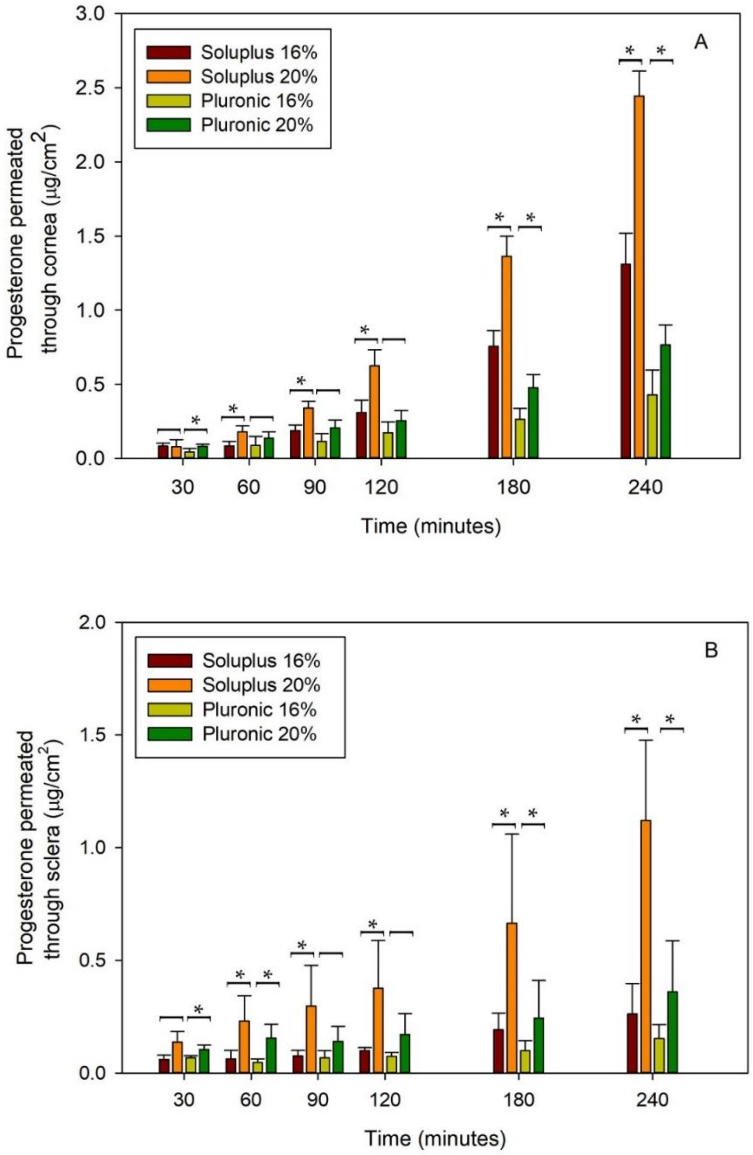

Figure 2. 6. Trans-corneal (A) and trans-scleral (B) permeation of PG as a function of time when formulated in Soluplus or Pluronic F68 micelles. (n=4). *Significant differences were found between 16% and 20% of either Soluplus or Pluronic.

The steady state flux (J) of PG was estimated to be 0.50 ± 0.07, 0.91 ± 0.04, 0.13 ± 0.07 and 0.26 ± 0.04 µg/($cm^2 \cdot h^1$) for Soluplus 16% and 20% and Pluronic 16% and 20% respectively through cornea; and 0.08 ± 0.06, 0.37 ± 0.08, 0.04 ± 0.03 and 0.09 ± 0.07 µg/($cm^2 \cdot h^1$) for Soluplus 16% and 20% and Pluronic 16% and 20% respectively through sclera. Statistical differences ($p<0.05$) were detected at various times between different concentrations of each polymer used as illustrated in Figures 2.6A and 2.6B. As permeability values recorded through cornea and sclera were larger for Soluplus and Pluronic 20% micelle dispersions, these formulations were selected for the studies in different species.

Bovine eyes are significantly larger than human eyes; but they are widely used in diffusion studies too [23,30,52,56–57]. Bovine corneal epithelium has been reported to be at least two times thicker than the human one, which may lead to lower drug permeability [26]. Rabbit eyes have been widely used for *ex vivo* models [32,58–60], although they are smaller than human eyes and lack from Bowman's layer, which may facilitate drug penetration [26]. To elucidate the potential impact of these differences on PG permeability results, the thicknesses of the cornea and sclera tissues of the as-supplied eyes were measured using a caliper. Cornea thickness was 91.2 ± 0.8 μm, 87.0 ± 2.1 μm and 51.7 ± 7.1 μm for bovine, porcine and rabbit, respectively. Sclera thickness was 129.8 ± 14.7 μm, 73.2 ± 2.7 μm and 24.3 ± 4.9 μm bovine, porcine and rabbit, respectively.

In the case of PG-loaded Soluplus micelles, the amounts accumulated in the receptor compartment of cornea and sclera ranked as follows: rabbit > porcine > bovine (Table 2.5). Differently, Pluronic micelles did not follow such a clear trend; the amount of drug that passed through cornea ranked rabbit > porcine ~ bovine, while in the case of sclera ranked rabbit ~ bovine > porcine (Figure 2.7). In the three species, the amount of PG permeated was higher through cornea than through sclera ($p<0.05$).

Table 2.5. Accumulated amounts of PG in receptor compartments (μg/cm^2) at 240 minutes in diffusion tests from 20% copolymer micelle dispersion using rabbit, porcine and bovine cornea and sclera.

Micelles	**Cornea**			**Sclera**		
	Rabbit	Porcine	Bovine	Rabbit	Porcine	Bovine
Soluplus	3.65 ± 0.50	2.44 ± 0.17	1.13 ± 0.09	2.30 ± 0.64	1.12 ± 0.36	0.83 ± 0.02
Pluronic	1.75 ± 0.48	0.77 ± 0.13	0.92 ± 0.06	0.71 ± 0.13	0.36 ± 0.23	0.82 ± 0.03

Statistical differences were also detected between the different species under investigation and in both corneas and scleras (Figure 2.7). The amounts accumulated were higher for Soluplus than Pluronic micelle dispersions in good agreement with the higher amount of PG supplied.

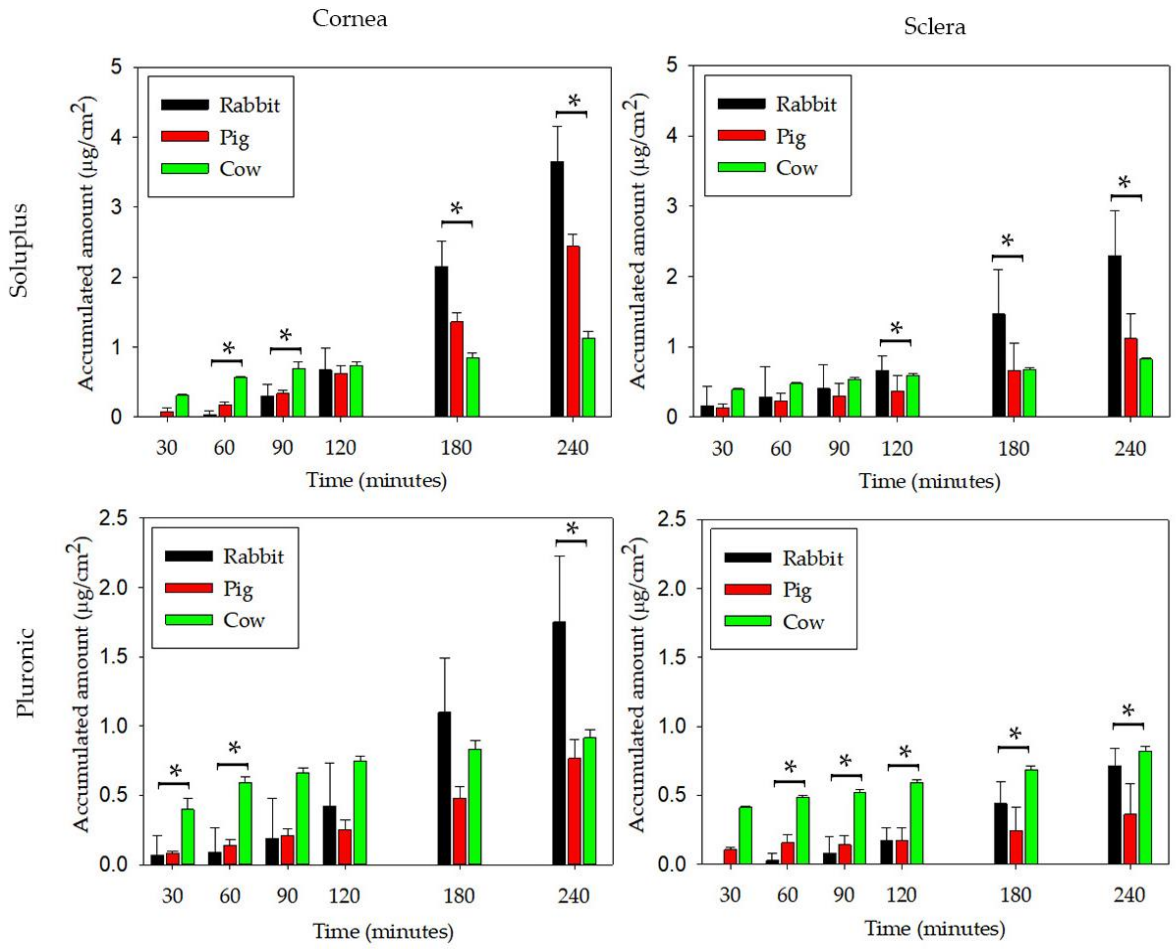

Figure 2.7. Accumulated amount of PG permeated through rabbit, porcine and bovine cornea and sclera and measured in the receptor chamber as a function of time. PG was formulated in Soluplus and Pluronic micelles, Total copolymer concentration was 20 %w/w in all cases (n=4). *Significant differences were found between the three species ($p < 0.05$).

After the 4 h assay, corneas and scleras were visually inspected and all were in good condition, without holes or cracks. The mass balance was determined from the concentrations in the donor and receptor compartment and in the membrane after the 24-hour extraction (Figure 2.8).

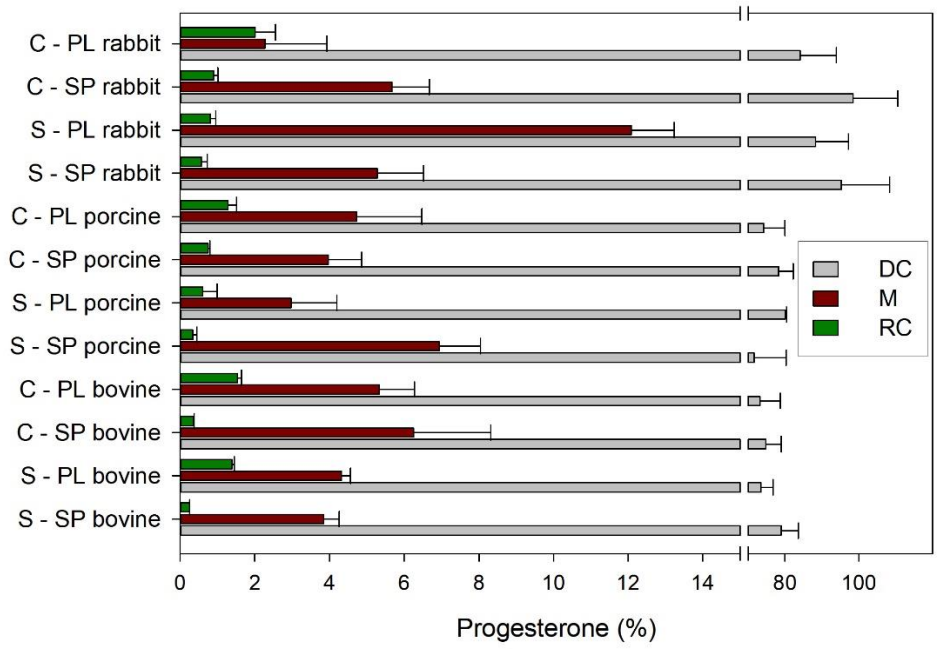

Figure 2.8. Mass balance, percentage (%) of PG accumulated in the donor compartment (DC); in the membrane (M) of bovine, porcine and rabbit and in the receiving compartment (RC) after 4 h of experiment of permeation of micelles Soluplus (SP) and Pluronic (PL) in corneas (C) and scleras (S). The total polymer concentration was 20% w / w in all cases. (n=4).

The coefficient of apparent permeability (P_{app}) of PG through the cornea and sclera of the three animal species was calculated from the flow ratio (J) and the concentration of PG in the donor phase (Table 2.6). The smallest permeability coefficient was for Soluplus micelles in sclera bovine, while the highest P_{app} corresponded to Pluronic in rabbit cornea. P_{app} values recorded for corneas were greater than those estimated for scleras. Among the species, the P_{app} values recorded in rabbits were greater than those obtained for bovine and porcine in both cornea and sclera. Except for sclera data recorded for Pluronic formulations, P_{app} values were higher when tested in porcine compared to bovine tissue (Table 2.6). Since there are no commercially available ocular formulations of PG, as a control we prepared a PG solution (343.04 µg/mL) from a commercially available PG:methyl-β-cyclodextrin complex powder. Permeability tests carried out in rabbit cornea and sclera led to J values of 2.02 ± 0.92 µg/($cm^2 \cdot h^1$) and 4.86 ± 2.37 µg/($cm^2 \cdot h^1$), respectively. The permeability coefficient, P_{app}, values were 16.2 ± $7.5 \cdot 10^{-7}$ and $39.3 \cdot \pm 19 \cdot 10^{-7}$ cm/s for cornea and sclera, respectively. P_{app} values recorded in cornea were similar to those recorded for the polymeric micelle dispersions, but P_{app} values recorded in sclera were larger for the PG:methyl-β-cyclodextrin complex. This

finding may be related to the fact that the complex is smaller than a PG-loaded micelle, and thus it can cross the sclera porous network more easily [55].

Table 2.6. Flux (J, μg/(cm^2·h1)) and permeability coefficient (Papp, cm/s) in rabbit, pig and cow cornea and sclera for PG formulated in Soluplus and Pluronic micelles (n=4). The total polymer concentration was 20% w / w in all cases. (n=4).

Animal	Soluplus/Cornea		Soluplus/Sclera		Pluronic/Cornea		Pluronic/Sclera	
	J	P_{app} (x10^7)	J	P_{app} (x10^7)	J	P_{app} (x10^7)	J	P_{app} (x10^7)
Rabbit	1.38 ± 0.15	16.5 ± 1.8	0.77 ± 0.17	9.2 ± 2.0	0.66 ± 0.19	37.3 ± 10.5	0.26 ± 0.03	14.5 ± 1.5
Pig	0.91 ± 0.04	9.8 ± 0.5	0.37 ± 0.08	4.0 ± 0.9	0.26 ± 0.04	15.2 ± 2.3	0.09 ± 0.07	5.6 ± 3.9
Cow	0.31 ± 0.07	3.4·± 0.8	0.11 ± 0.01	1.2 ± 0.1	0.10 ± 0.03	6.1 ± 1.5	0.11 ± 0.01	6.8 ± 0.8

Finally, the dependence of the permeability coefficient values on the thickness of cornea and sclera was analyzed (Figure S3 in Supplementary Material). Except for the small PG-loaded Pluronic micelles that did not show significant dependence of P_{app} on sclera thickness, all other formulations showed a negative correlation between P_{app} and the tissue thickness. In the absence of other mechanisms, drug penetration in ocular tissues may be driven by diffusion. Diffusion coefficient is well known to be inversely proportional to the thickness of the membrane and, although cornea and sclera have a non-homogenous structure and several mechanisms may interplay, the thickness of the tissue (particularly of the complex cornea) is an additional barrier to the pass of the drug. Our results are in good agreement with previous findings on that the lowest permeability coefficients corresponded to the thicker bovine cornea [25].

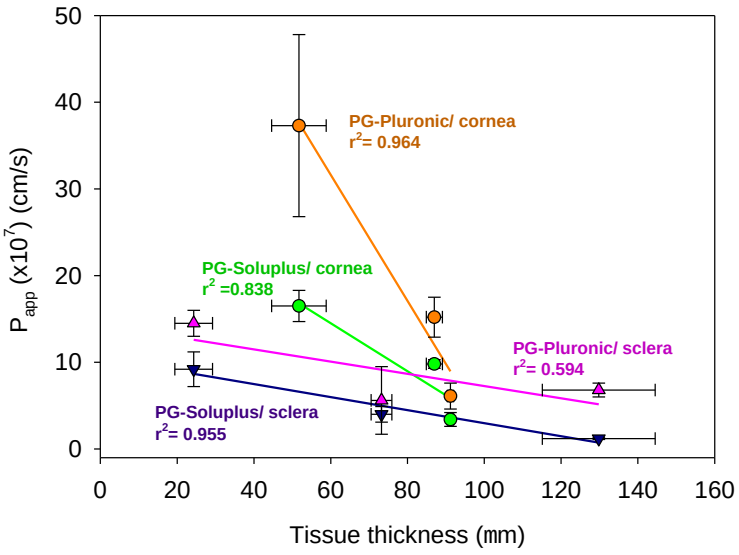

Figure 2.9. Dependence of P_{app} of PG on the thickness of cornea (orange and green lines) and sclera (pink and purple lines) when administered formulated in Pluronic F68 or Soluplus micelles. Thickness increased in the order rabbit < porcine < bovine tissue.

4. Conclusions

Soluplus and Pluronic F68 exhibited different abilities to encapsulate PG, which may be related to their different HLB. Pluronic micelles increased PG solubility one order of magnitude in buffered pH 7.4 medium, while Soluplus formulations enhanced the apparent solubility by two orders of magnitude. Relevantly, the tested micelle dispersions, particularly those prepared with Soluplus 20% exhibited an encapsulation efficiency of approx. 100%, which indicates that PG has strong affinity for the micelle core. The wide range of PG that can be encapsulated in Pluronic and Soluplus micelles may offer an enormous versatility to formulate the dose required for each patient affected by retinitis pigmentosa. Importantly, all copolymer concentrations tested successfully passed the HET-CAM assay.

Relevant differences in PG permeability through cornea and sclera were observed depending on the copolymer used to prepare the formulations and the animal chosen as the source of the tissues. Regarding the copolymer, Pluronic micelles provided higher permeability coefficients (P_{app}) which may be related to their few nanometers size and less stable micelle structure. Thus, depending on the clinical demands, Soluplus and Pluronic micelles may offer different performances; namely, Soluplus micelles can provide overall greater amounts of PG accumulated in cornea and sclera and transferred to the eye tissues, while Pluronic F68 promote a faster penetration of lower PG doses.

Regarding the interspecies differences, PG penetration through rabbit´s cornea occurred faster than through porcine or bovine cornea. In the case of sclera, although faster for rabbit, interspecies differences were more relevant for Soluplus micelles than for Pluronic micelles. This latter finding maybe related again to the greater size of Soluplus micelles and suggests that sclera tissues from different species may have different pore network architecture in addition to different thickness. Also, the *in situ* gelling capability of Soluplus-based formulations may enhance the retention time on the eye surface. Overall, the developed micelle formulations can be pointed out as interesting tools for the ocular delivery of PG, and porcine tissues may provide a more balanced view of the performance of the formulations compared to the highly permeable rabbit tissues and the poorly permeable bovine tissues.

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Capítulo 3

Development, characterization, and ex vivo evaluation of an insert for the ocular administration of progesterone.

Abstract

Progesterone (PG) affords neuroprotection in degenerative diseases associated to oxidative stress, such as cataracts, age-related macular degeneration, glaucoma, diabetic retinopathy and retinitis pigmentosa. The aim of this project was to develop ocular inserts for delivery of PG to the eye. Different inserts with PG in its composition were formulated and the insert with the best characteristics (59% polyvinyl alcohol, 39% polyvinylpyrrolidone K30 and 2% propylene glycol) was selected for ex vivo studies. Physical characteristics and drug release patterns of the insert were analysed. *In vitro* diffusion studies revealed a controlled diffusion of progesterone. *Ex vivo* experiments demonstrated similar trans-corneal and trans-scleral PG diffusion (corneal apparent permeability coefficient 6.46 ± 0.38 × 10^{-7} cm/s and scleral apparent permeability coefficient 5.87 ± 1.18 × 10^{-7} cm/s; mean ± SD; n = 5). However, the amount of PG accumulated in scleras was statistically higher than in corneas (30.07 ± 9.09 μg/cm^2 and 15.56 ± 4.36 μg/cm^2 respectively). The PG-loaded inserts (55.6 μg/cm^2) were thin, translucent, showed no irritancy (HET-CAM test) and were elastic and robust, all suitable properties for its potential use in the treatment of several ocular diseases.

1. Introduction

Progesterone (PG) is a sexual hormone with demonstrated neurosteroidal properties. PG affords neuroprotection in multiple animal models of stroke [1] as well as in various animal models of neuronal injury (central nervous system, traumatic brain and spinal cord) [2]. It has also been shown that PG reduces infarct volume and improves functional recovery by acting upon mechanisms involved in ischemic brain injury. Researchers investigating the impact of treatment with progesterone after cerebral ischemia have concluded that it reduces glial activation and diminishes brain and systemic inflammation [2]. Endogenous PG synthesis may be involved in regulation of microglial activity, acting therefore as a mediator in neuroprotection [3]. Administration of high PG dose seem to be able to reduce cell death produced by free radicals. PG increases expression of antioxidant enzymes and reduces lipid peroxidation and oxidative stress, probably as a consequence of lowering free radical concentration [4]. It has been proven that PG has a protective effect on degenerative eye diseases related

to oxidative stress, such as cataracts, age-related macular degeneration and glaucoma and other retinopathies such as diabetic retinopathies or retinitis pigmentosa [5,6].

Retinitis pigmentosa (RP) is a group of genetically degenerative and clinically heterogeneous retinopathies, in which there is a progressive loss of rods followed by the death of cones [7]. RP is the most common cause of inherited blindness [8]. This disorder causes the death of photoreceptor cells, affecting the rod cells at the beginning of the disease [9] and later progressing to affect the cones. Rod photoreceptors are responsible for peripheral vision and as their number decreases, patients start to suffer tunnel vision and nyctalopia (night blindness). Cone cells also become affected as the disease progresses, causing a significant visual acuity reduction including loss of central vision which eventually results in blindness in advanced stages of the disease [4]. The symptoms of RP typically appear in childhood and progress generally until the affected individual reaches 40-50 years of age, at which point most of his or her sight has been lost [10].

Currently there is no satisfactory treatment for RP, but different therapeutical strategies are under investigation [10–12]. Promising results about the administration of progesterone (PG) or its analogue, norgestrel, have been reported showing that these drugs may be helpful in delaying photoreceptor cell death in cases of RP [4,13,14].

Topical administration of ophthalmic gels or eye drops are the common preparations for the treatment of ocular pathologies. With these conventional pharmaceutical forms, bioavailability of the administered drugs is low and together with the difficulty for administration and ensuing blurred vision, often results in poor therapeutical compliance [15,16]. To overcome these limitations the use of ocular inserts, which are giving promising results for the treatment of various eye pathologies, is on the rise [16–19]. Ocular inserts are solid or semisolid sterile preparations, usually made of polymeric materials (methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), ethylcellulose (EC), polyvinylpyrrolidone K30 (PVP-K30), polyvinyl alcohol (PVA), chitosan (CS), sodium alginate (SA), gelatine and other polymers) [20]. Drug formulations using these polymers are meant to be placed in the eye to deliver drugs to the ocular surface [17]. There are commercialised ocular inserts, such as Minidisc® and Ocusert® that have yielded satisfactory results [16,21]. The main advantage of the inserts is that they may help to increase the patient's adherence to treatment. Feeling the presence of a foreign body in the eye is the principal reason prompting patients to refuse this type of formulation and therefore it is important to develop inserts which are as thin as they can possibly be. One inconvenience for the development of an ocular pharmaceutical form of PG is the low aqueous solubility of the molecule. This can be

solved by incorporating PG in β-cyclodextrins (β-CD), which has been demonstrated to enhance transdermal PG permeability [22].

The aims of this work were (1) to design and characterize physically an ocular insert to administrate PG, (2) to perform HET-CAM studies to examine ocular irritancy of the PG formulation, (3) to study PG release from the insert and (4) to analyse *ex vivo* trans-corneal and trans-scleral PG diffusion using rabbit's eyes.

2. Material and methods

2.1. Compounds

Progesterone (PG, C21H30O2, MW 314.5 g/mol) incorporated to β-CD (85.2 mg/g), was purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). MC, HPMC, PVP-K30, SA and PVA were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Plasticizers, propylene glycol (PGL) and glycerine (GL) were acquired from Guinama (Valencia, Spain). High-performance liquid chromatography (HPLC) grade acetonitrile and water were obtained from Honeywell, Riedel-de Haën (Seelze, Germany).

2.2. Preparation and physical evaluation of the inserts without PG

As a first step, inserts without PG were formulated and their properties were evaluated. Table 3.1 shows the composition of the 11 inserts prepared using MC, HPMC, SA, PVA and PVP-K30 as polymers and PGL and GL as plasticizer.

Polymers were dissolved in 5 mL of water using a magnetic stirrer. Then, the required amount of plasticizer was added and stirred for 12 h. Samples were sonicated at 80 MHz for 20 min in an ultrasonic water bath (Model 275T, Crest Ultrasonics Corp., Trenton, NJ, USA) to remove air bubbles [23]. A volume of 5 mL of the formulations F03, F05-F08, F10 and F11 (table 3.1) were poured onto Petri dishes (50 mm diameter) because of their liquid consistency in which they were allowed to dry [23–26]. The rest of the formulations were laminated using a laminator device to a thickness of 0.6 mm (utility model patent registration number U200502256) on a film support (3M-ScotchpackTM 9733 Backing Polyester Film Laminate). Inserts were left to dry in darkness at room temperature for 24-48 h.

After an extensive review of polymers used for topical application, those showing better results in previous studies with other molecules were chosen (tizanidine hydrochloride, moxifloxacin hydrochloride, fluconazole and ofloxacin) [23–25,27]. The

percentages of the polymers were adjusted to make a selection based on preliminary results.

Table 3.1. Polymers: methylcellulose (MC), polyvinyl alcohol (PVA), polyvinylpyrrolidone K30 (PVP-K30), hydroxypropyl methylcellulose (HPMC) and sodium alginate (SA) (%, w/w), and plasticizer: propylene glycol (PGL) and glycerine (GL) (%, w/w) of each formulation developed.

Formulation code	Polymers (%, w/w)	Plasticizer (%, w/w)
F01	MC:PVA (39:59)	PGL (2)
F02	MC:PVA (59:39)	PGL (2)
F03	PVP-K30:PVA (37: 61)	PGL (2)
F04	MC (93)	PGL:GL (2:5)
F05	HPMC:PVP-K30 (38:55)	PGL:GL (2:5)
F06	MC:PVP-K30 (38:55)	PGL:GL (2:5)
F07	PVP-K30:PVA (39:59)	PGL (2)
F08	PVP-K30:PVA (20:78)	PGL (2)
F09	PVA:SA (49:49)	GL (2)
F10	PVA:SA (83:15)	PGL (2)
F11	PVA:SA (83:15)	GL (2)

Thickness and translucency of the formulated ocular inserts were evaluated after detaching them from the backing film. Insert thickness was measured at three different points using an electronic digital calliper (Ratio, 6369 H 15; Barcelona, Spain) and mean film thickness was noted (n=3). Insert translucency was evaluated with a digital luxmeter (iClever® LX1330B). It was measured as the fraction of incident light detected by the sensor with and without the insert (eq. 1).

$$Translucency\ (\%) = \frac{illuminance\ through\ insert}{Illuminance} \cdot 100\ (eq.1)$$

The selected inserts (F03, F04, F07 and F08; n=3) were cut (1 cm^2), weighed individually and kept in a desiccator containing solid anhydrous calcium chloride. After three days, the inserts were weighed again. A high precision electronic weighing balance was used to weigh the individual inserts (Radwag AS 220.R2). The percentage of moisture loss was calculated using eq. 2 [28].

$$Moisture\ loss\ (\%) = \left[\frac{Initial\ weight - final\ weight}{Initial\ weight}\right] \cdot 100\ (eq.2)$$

To assess moisture absorption, 1 cm^2 of each insert were weighed and placed in a desiccator containing a saturated solution of NaCl to maintain high relative humidity. The inserts were weighted daily. After three days, when the weight became constant, the inserts were removed and the percentage of water uptake was calculated using eq. 3 [28].

$$Moisture\ uptake\ (\%) = \left[\frac{Final\ constant\ weight - Inital\ weight}{Initial\ weight}\right] \cdot 100\ (eq.3)$$

To measure the mechanical properties of the formulation, a strip of 10 x 70 mm was cut from each insert. The selected strips did not show any physical imperfections. Breaking force and stretching were assessed at breaking point using an electronic dynamometer (Instruments J. Bot; Barcelona, Spain). The load cell weight was 5 Kg and the break point was established at 5%. These parameters allowed to determine approximately the resistance and elongation of the different inserts under evaluation [29]. Each strip was held between the two clamps of the dynamometer for analysis: the upper clamp is mobile while the lower one is static. The strip was pulled at a rate of 100 mm/s. The tensile strength and elongation at breaking point were calculated following eq. 4 and 5 [29].

$$\text{Tensile strenght } \left(\frac{\text{N}}{\text{mm}^2}\right) = \frac{\text{Break force (N)}}{\text{Cross sectional area } (mm^2)} \ (eq.4)$$

$$\text{Elongation } (\%) = \frac{Increase\ in\ lenght\ at\ break\ point\ (mm)}{Original\ length\ (mm)} \cdot 100 \ (eq.5)$$

2.3. Preparation and characterization of inserts with PG

The inserts selected to continue the studies by addition PG were: F03, F04, F07 and F08. The procedure described above (2.2) was followed, but as PG is highly insoluble in water, PG enclosed in β-CD (85.2 mg PG per gram) was used. The manufactured inserts contained 55.6 μg/cm^2 of PG.

After drying the inserts, the external morphology was evaluated using optical microscopy and polarized light microscopy (Leica DM 2000) to check for absence of crystallization. Photographs were obtained using a digital camera (Shift Ds-H2, Nikon). To determine the uniformity of drug content, three 1 cm^2 samples of each insert (n=3) were dissolved in 1 mL PBS. The amount of PG was determined from a 200 μL aliquot using a HPLC validated method [30] and the results were expressed as the average of the three measurements.

Scanning Electron Microscopy (SEM) was used to check the surface and internal morphology of the insert. The SEM characterization of the selected insert was performed using a HITACHI S-4800 Scanning Electron Microscope with Field Emission Gun (FEG) with a resolution of 1.4 nm at 1kV RX Bruke detector (accelerative voltage 5 kV). Samples of the inserts were peeled out and then placed on a SEM sample holder using graphite-impregnated adhesive conductive black carbon tape. The sample was then coated with platinum and visualized under SEM at various magnifications.

2.4. Ocular Tolerance Test

Fertilized eggs form White Leghorn hens (50-60 g) were purchased from Granja Santa Isabel, Córdoba, Spain. An incubator (Covatutto 24 digitale) and an egg turner Turner (Girauova automatic) were purchased from Novital, Varese, Italy. The fertilized hen eggs were placed in the incubator at 37 °C with 60% environmental humidity. They were maintained in the incubator for 8 days, being turned automatically to prevent the attachment of the embryo to one side of the egg. At the end of the 8th day, they were left to rest with the large end of the eggs facing up for 24 h to ensure the embryo moved to the bottom of the egg. Eggs were placed on a support outside the incubator and the shells were carefully cut with a rotatory blade without damaging the membrane. With a scalpel, the shell that had been cut was dislodged and the internal membrane was moistened for 30 min with 2 mL of 0.9% NaCl solution, before removing it.

The Hen's Egg Test on the Chorio-Allantoic Membrane (HET-CAM) with PG solution and PG insert was carried out to assess the potential ocular irritancy. To carry out the test, 500 µL of the PG solution (500 µg of PG/mL in PBS) and a 0.567 cm^2 insert containing 55.6 µg of PG/cm^2 as well as an identical volume of a positive and a negative control solution were placed on the CAM of different eggs with a pipette. The eggs were observed for 5 min to see whether any haemorrhages, vascular lysis or coagulation developed. The egg containing the PG solution and the PG-loaded insert were compared with eggs serving as positive and negative controls. The irritation index (IS) was calculated using the formula shown [31] (eq. 6):

$$IS = \frac{(301 - tH) * 5}{300} + \frac{(301 - tL) * 7}{300} + \frac{(301 - tC) * 9}{300} \quad (eq. 6)$$

where tH represents haemorrhage time, tL is lysis time and tC is coagulation time in seconds.

The CAM responds to an ocular irritant by developing an inflammatory reaction in terms of coagulation, lysis or haemorrhage. Depending on its IS a substance can be classified as not irritant (IS < 1), weak or slight irritant(1 ≤ IS < 5), moderate irritant (5 ≤ IS < 10) or strong or severe irritant (IS > 10) [31]. Tests for each concentration were performed at least in triplicate.

2.5. In vitro PG release studies

Drug-loaded inserts (0.567 cm^2, n=3) were placed in vials containing 5 mL of propylene glycol:water (40:60%, v/v) at 37 °C under magnetic stirring. A sample of 0.2 mL was taken from each vial at 1, 5, 15, 30, 60, 180, 360 minutes and 24 hours to

determine the amount of drug released from the insert. After taking each sample, the same volume of fresh propylene glycol:water (40:60%, v/v) was immediately replaced in each vial. Collected samples were analysed by HPLC-UV using a previously validated method [30].

2.6. Trans-corneal and trans-scleral ex vivo diffusion of PG from the insert

Ex vivo diffusion studies were performed using eyes obtained post-mortem from 2-month old hybrid albino rabbits weighing 2.0-2.5 kg housed at the "Granja Docente y de Investigación Veterinaria", University CEU Cardenal Herrera. The experimental protocol was approved by the Ethical Committee of University CEU Cardenal Herrera (Ref. 2011/010) and by the Conselleria d'Agricultura, Pesca i Alimentació, Generalitat Valenciana (Ref. No. 2017/VSC/PEA/00,192). The eyeballs were rinsed in saline solution to remove blood and adhered muscles were scissored away. Corneas and scleras were obtained by cutting along the sclera-limbo junction. The average thickness for cornea and sclera were 51.7 ± 7.1 µm and 24.3 ± 4.9 µm, respectively.

Trans-corneal and trans-sclera diffusion of PG were determined using vertical standard Franz type diffusion cells (DISA, Milan, Italy) with an available permeation area of 0.567 ± 0.008 cm^2. Corneas and scleras were placed between the two compartments with the corneal epithelium or the outermost layer of the sclera, facing the donor compartment of the diffusion cell.

To simulate tears, 7 µl of phosphate buffer pH 7.4 were added to the donor compartment of each cell. After a PG-loaded insert was placed on the upper surface of the corresponding membrane, the donor compartment was sealed with Parafilm® to avoid evaporation.

As an aqueous receptor medium, such as phosphate buffer, is not suitable for drugs with low hydrosolubility, the receptor chamber was filled with propylene glycol:water, pH 7.4 (40:60%, v/v) (4.2 ± 0.1 mL) at 37.0 ± 0.1 ºC and was stirred using a rotating teflon-coated magnet. This receptor medium had been found to be suitable for *in vitro* skin diffusion studies involving PG [22,32,33]. Propylene glycol 20% (w/w) as a solubilizer has been used in studies involving drugs with low water solubility without affecting neither cell viability nor permeability [34]. Furthermore, propylene glycol has been proposed as a vehicle for ophthalmic use up to 15% (w/w), and has been proven to be non-toxic [35,36]. The Franz cell receptor was sealed with paraffin to avoid evaporation of the medium. At set time intervals (15, 30, 45, 60, 90, 120, 150 and 180 min), 0.2 mL of samples were withdrawn from the receiving compartments to measure PG amounts by HPLC [30]. An equal amount of propylene glycol:water was then added to maintain the original volume.

The concentrations of PG in the receptor compartment ($C_{receiver}$) were plotted against time to estimate the apparent permeability coefficients (P_{eff}, cm/s). Permeability coefficients through the cornea and sclera were estimated using equation 7 [37]. This equation considers a continuous change in donor and recipient concentrations and is valid under sink or non-sink conditions.

$$C_{receiver,\,t} = \frac{Q_{total}}{V_{receiver}+V_{donor}} + \left[\left(C_{receiver,t-1}\cdot f\right) - \left(\frac{Q_{total}}{V_{receiver}+V_{donor}}\right)\right]\cdot e^{P_{eff}\cdot S\cdot\left(\frac{1}{V_{receiver}}+\frac{1}{V_{donor}}\right)\cdot\Delta t} \quad (eq.\,7)$$

where $C_{receiver}$, t is the PG concentration (µg/mL) in the receptor compartment at time t, Q_{total} is the total amount of PG in the insert, $V_{receiver}$ is the volume in the receptor compartment, V_{donor} is the volume in the donor compartment, $C_{receiver,\,t-1}$ is the amount of PG in the receptor compartment at previous time, f is the replacement dilution factor of the sample, S is the surface area of the membrane and Δt is the time interval. The curve fittings were performed by non-linear regression, minimizing the sum of the squared residuals.

At the end of the diffusion study, the PG in the membranes was extracted by cutting the membranes in small portions and placing them in 5 mL of extraction solution (Acetonitrile:Water, 80:20 v/v) at 25 °C for 12 h at 300 rpm, after which the solutions were filtered (Acrodisc® Syringe Filter, 0.22 µm GHP Minispike, Waters) to determine the amount of PG by HPLC.

2.7. Statistical analysis

Values were expressed as mean ± standard deviation. To determine statistically significant differences among the experimental groups, depending on normality and homoscedasticity, parametric tests (ANOVA followed by Tukey´s test for multiple comparisons and Student t-test) or non-parametric testing (Mann-Whitney U-test) were used as deemed appropriate. The confidence level was 95%. Statistical analysis was carried out using SPSS 24.0.

3. Results and discussion

3.1. Development and characterization of the inserts

Several inserts were prepared with various combinations of the different polymers: HPMC, MC, PVP, PVA and SA (Table 3.1) but without incorporating PG. All systems contained PGL or GL as plasticizers. All materials are biocompatible and may possibly be suitable to be used on the eye surface [38–40].

After lamination, insert F01 was found to have a rough surface and inserts F02, F05 and F06 were too brittle and could not be separated without breaking from the lamination support. These inserts were discarded for further studies. Table 3.2 shows the thickness, weight, and translucency values of the inserts. The inserts F09 and F11 were discarded due to their high translucency. Furthermore, F10 was so thin that it wrinkled easily, so was also eliminated. Inserts mentioned above were discarded for further studies because they did not fulfil expected properties.

Best result were obtained for inserts manufactured using a combination of PVA and PVP-K30. The properties of each insert can be explained by analyzing their composition.

PVA has the ability to retain a large amount of liquid, which gives the insert elasticity and structural integrity [41]. It also has other properties such as ease of preparation, good adhesiveness, good mechanical properties, and excellent chemical resistance and can also be an oxygen barrier. Blends of PVA with other polymers have been shown to change the properties of PVA-based materials. Mixing it with another polymer with strong proton receptor sites improves heteropolymer interactions [41]. The ocular inserts could form hydrogen bonds with the mucosa generating mucoadhesion, which appears as result of the presence of hydroxyl groups in the inserts provided by the PVA. PVA has excellent film-forming and adhesive properties [42].

PVP films have a shiny appearance and when dry, become translucent and resistant (Teodorescu and Bercea 2015). PVP is a commonly used polymer because of relevant properties, such as good stability and biocompatibility [43], thermal and chemical resistance, ability to form complexes with hydrophilic and hydrophobic molecules and solubility both in water and organic solvents [41].

Previous studies have shown that the percentage between PVA and PVP in the formulations results in changes the polymer behaviour [44]. Most of the formulations containing the highest concentration of PVP showed the presence of pores and a high swelling index [44]. It is the relationship between PVA and PVP that gives the insert its properties; in fact, in our study, inserts manufactured with PVA and PVP with different percentages (F03, F07 and F08, Table 3.1), did not show the same characteristics.

Table 3.2. Thickness, weight and translucency of the prepared inserts. Mean ± SD (n = 3).

Insert	Thickness (μm)	Weight (mg)	Translucency (%)
F03	53 ± 11	1.53 ± 0.31	93.07 ± 0.49
F04	< 10	0.60 ± 0.17	91.00 ± 0.31
F07	< 10	0.33 ± 0.05	93.07 ± 0.39
F08	17 ± 5	0.97 ± 0.51	92.88 ± 0.40
F09	< 10	0.37 ± 0.06	88.09 ± 0.62
F10	< 10	< 0.1	91.59 ± 0.30
F11	< 10	0.33 ± 0.05	88.87 ± 0.49

Inserts F03, F04, F07 and F08 maintained thickness, flexibility, and adaptability to the ocular surface after formulation. Furthermore, the consistency and translucency of these inserts was deemed to be optimal for our studies and hygroscopicity studies were carried out on all four. The percentage of moisture loss and absorption of water for each of the selected inserts was calculated and the results are shown in Figure 3.1.

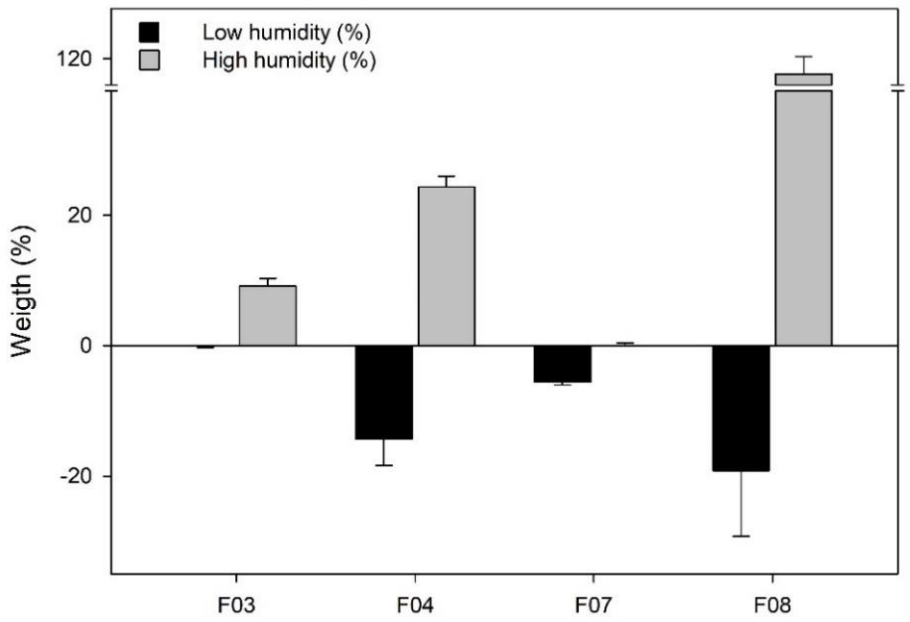

Figure 3.1. Percentage of weight lost and gained by the inserts after exposure to low and high humidity environments. Data are mean ± SD (n = 3).

The data obtained from the evaluation of the mechanical properties of the inserts, namely resistance and elongation, are shown in Figure 3.2A and 3.2B respectively.

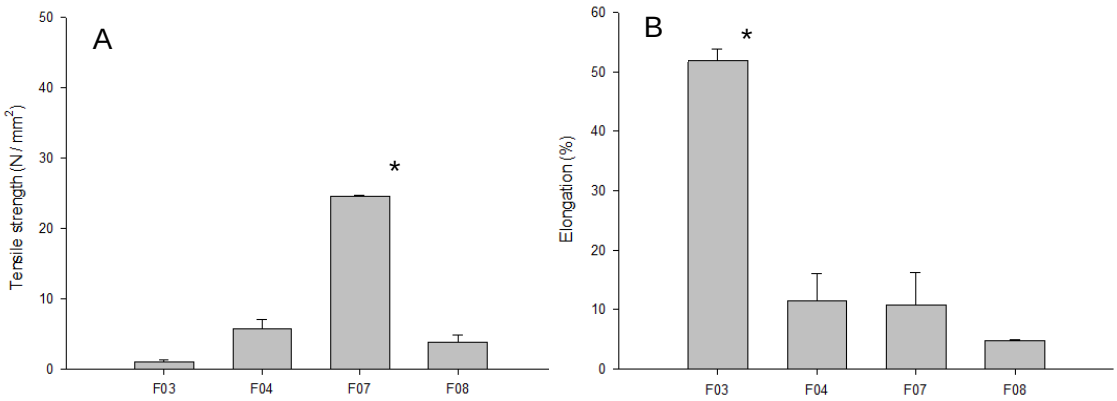

Figure 3.2. (A) Tensile strength values (N/mm2) obtained for each ocular insert. (B) Maximal elongation at break-point values (%) obtained for each ocular insert (* $p < 0.05$).

In Figure 3.2A tensile strength values can be observed, the highest value was 24.5 ± 0.01 N/mm2 belonging to the F07 insert. Figure 3.2B shows the elongation values (Maximal elongation at breaking point) of the inserts studied. The insert F03 showed greater elasticity ($p < 0.05$). It stretched up to 51.88% of its original size.

Since the inserts F03, F04, F07 and F08 showed good characteristics, they were re-formulated with PG in their composition (55.6 µg/cm^2) following the methodology previously described and observed under microscope. Inserts were observed under optical and polarized light microscopy in search of imperfections. Microscopic images of the inserts are shown in Figure 3.3 either viewed under polarized light (Figures 3.3A-D) or under conventional illumination (Figures 3.3E-H).

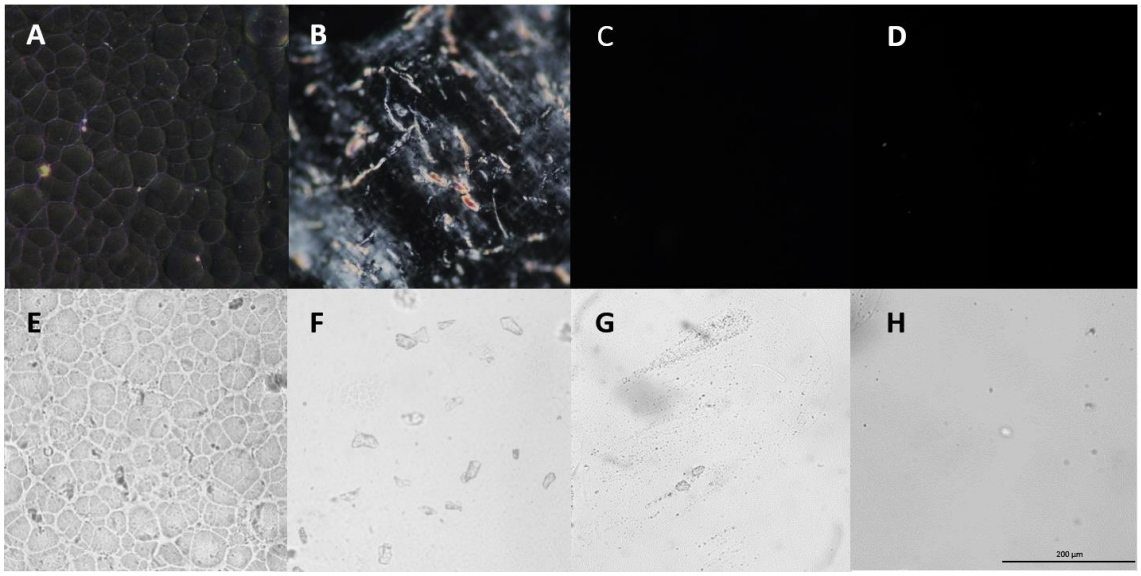

Figure 3.3. Photographs (Leica DM 2000) taken under a polarized light microscope (20 × magnification) and photographs taken under a normal light microscope (A & E= F03; B & F, F04; C & G, F07 and D & H, F08).

The presence of imperfections was observed in F03 and F04 inserts. In Figures 3.3A and 3.3B cracks can be observed, while in Figure 3.3E and 3.3F small crystals and the presence of imperfections can be seen. On the other hand, F07 and F08 inserts showed no imperfections or cracks. The insert F07 was selected because it had the best characteristics: besides having no imperfections it has a higher resistance to breakage and a lower moisture uptake than F08.

The tensile strength and elongation studies were repeated with the selected insert F07 to assess the effect of addition of PG on the properties of the insert. Addition of PG to F07 with PG increased breaking strenght to 49.0 ± 0.2 N/mm^2, compared to 24.5 ± 0.2 N/mm^2 shown by the same insert without PG. However, PG addition to the insert formulation did not represent a significant modification on the elongation (15.5 ± 0.7% with PG compared to 10.8 ± 5.5% without PG, $p > 0.05$).

To compare the insert (Figure 3.4A-C) with commercial contact lenses (Acuvue®, Johnson & Johnson vision care Inc., Jacksonvill, FL, USA) (Figure 3.4D-F) both were observed under SEM. As can be seen, the surface of insert F07 (Figure 3.4A) and its transversal section (Figure 3.4C) show a homogeneous structure, free of indentations and bumps, and it is very thin (500 µm thickness compared to 1500 µm of commercial contact lenses). The porosity of the insert can also be observed (Figure 3.4B).

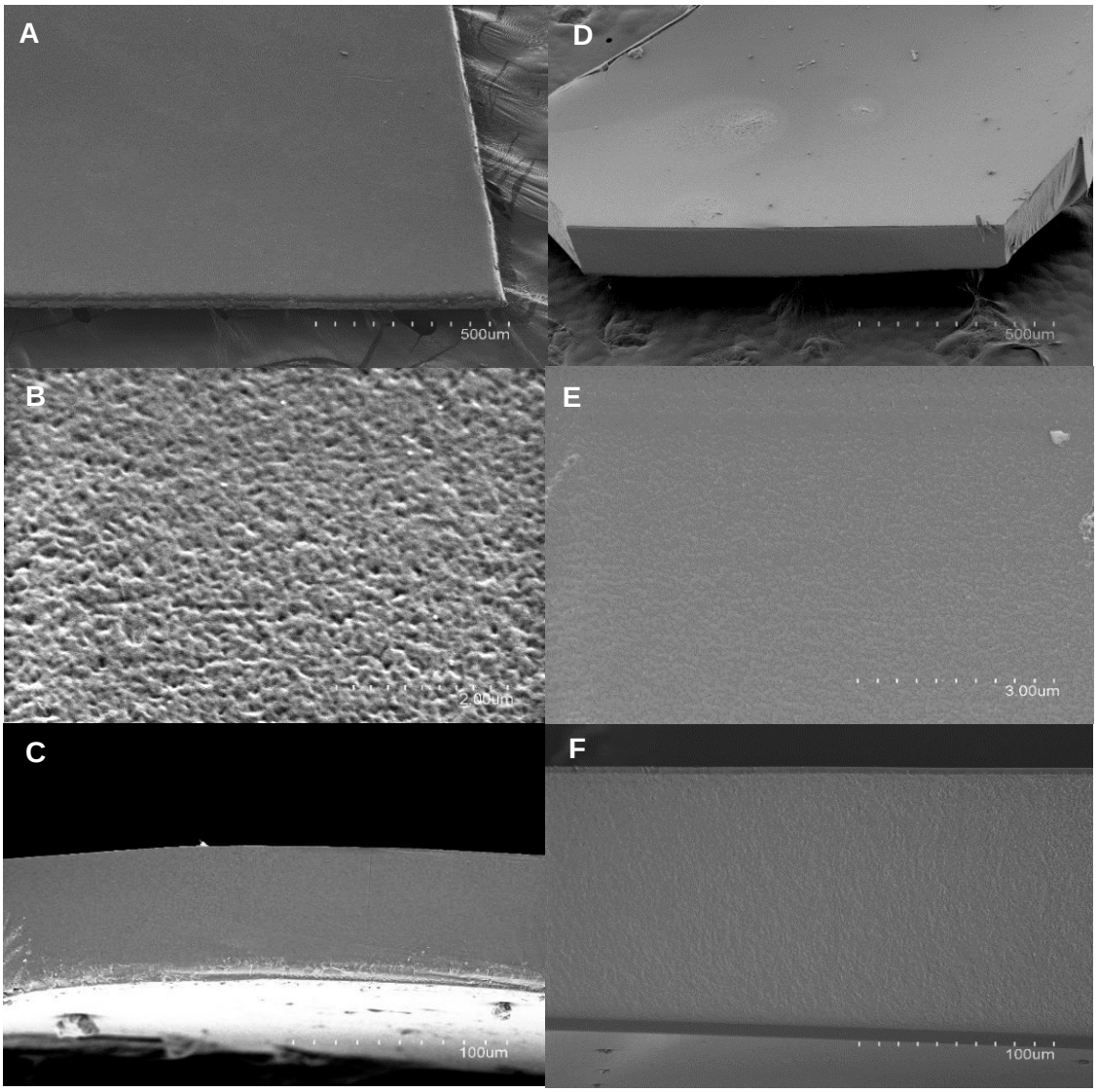

Figure 3.4. SEM images of the surface (A), at higher magnification (B) and cross-sectional view (C) of the ocular insert F07 and of commercial contact lens: surface view (D), higher magnification (E) and cross-sectional view (F).

Our insert (F07) was thinner and lighter than those formulated by other authors [23,28,45,46]. In figure 3.5 it can be observed how the insert fits into the ocular surface of a rabbit's eye; it is thin, translucent and therefore it should be comfortable for the patient.

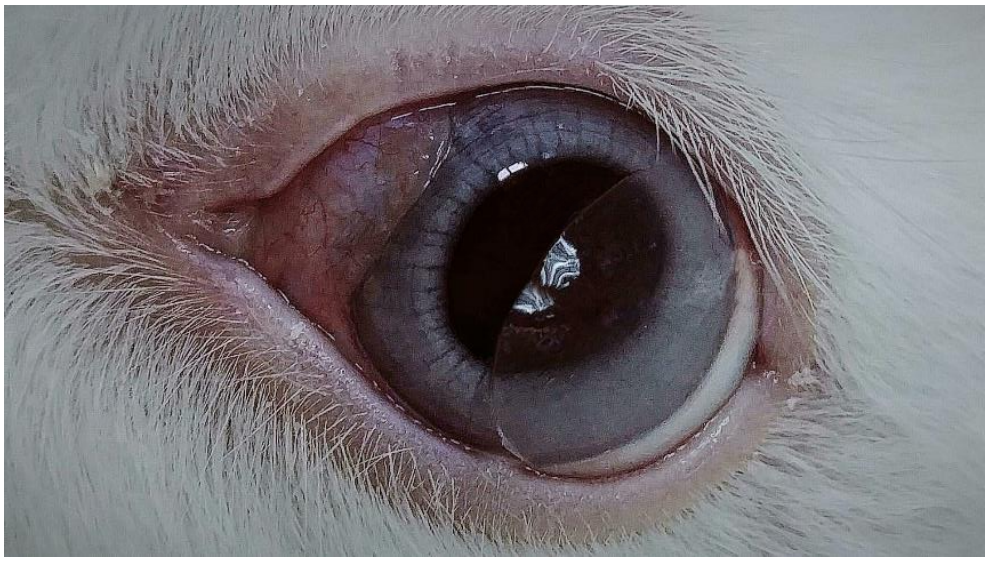

Figure 3.5. Photograph of insert F07 containing PG on a rabbit's eye. The insert was placed in the rabbit´s eye lopsided to allow visualization of the insert.

3.2. HET-CAM test

The HET-CAM, alternative to the Draize eye irritation test, is a test based in the response to injury of the highly vascularized but not innervated foetal membrane which is similar to that elicited by the rabbit conjunctiva.

HET-CAM test is used to assess the irritation that can be caused by ocular drug solutions and formulations [31]. To assess that PG and the formulation insert does not cause irritation, the HET CAM test was performed. Lysis, haemorrhage and coagulation time for positive controls were 32, 32 and 36 seconds respectively (Figure 3.6A). This gave an IS for the positive control of 18.71 (strong irritant), while the negative control did not produce any effect on blood vessels (Figure 3.6B). PG in β-CD in aqueous solution at 500 µg/mL (Figure 3.6C) and PG insert F07 (Figure 3.6D), did not produce any observable effects on the blood vessels during the 3 min of observation (IS = 0).

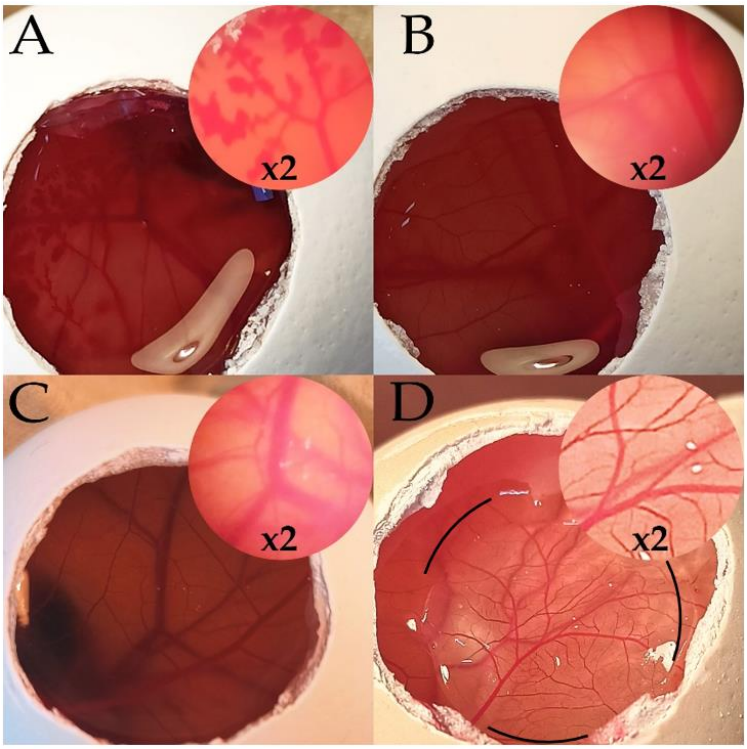

Figure 3.6. HET-CAM test. Effect of positive (A) and negative (B) controls, PG in β-CD in aqueous solution (500 µg/mL) (C) and PG-loaded insert (55.6 µg/cm^2) (D) on the surface of the chorioallantois membrane (CAM) after treatment for 3 min. Positive and negative controls were: NaOH 0.1N solution and NaCl 0.9% w/w respectively. The black segments in D serve to highlight the edges of the insert. A magnified (x2) image is shown in the small circle

The results obtained demonstrate that PG in β-CD in aqueous solution and formulated as an insert with PVP-K30 and PVA as polymer and PGL as plasticizer (Insert F07) did not cause ocular irritation and could be administered at the ocular surface. Our results are in agreement with other studies that have shown that the PG formulated in Soluplus® and Pluronic F68® micelles are not irritating [47].

3.3. In vitro release of PG from the insert

In vitro release studies of the selected insert were performed to confirm that the insert was able to release its load when placed in contact with an aqueous media. The release profile of PG from the insert is shown in figure 3.7. PG flux into the receptor chamber reduced with time as the concentration of PG in the insert became lower. As can be seen in Figure 3.7, in the first 3 h the percentage of PG released from the insert was 80%, while the remaining 20% was released in a 21 h period. The conditions of this assay are not realistic as there is an excess of water and stirring, but the results obtained show that PG is released from the insert. Furthermore, it seems that the insert has the necessary porosity to allow an almost complete emptying of its load.

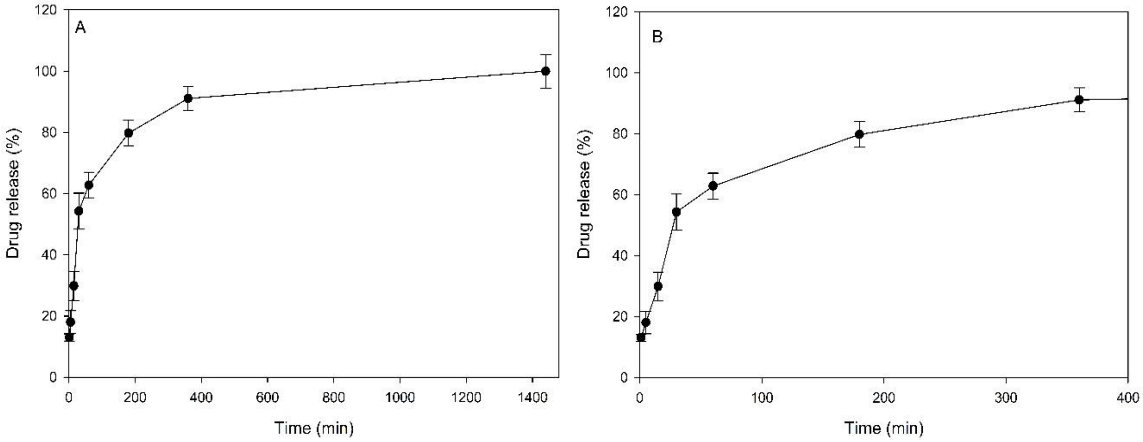

Figure 3.7. Percentage of PG released from the insert F07 during 24 h (A) and the PG release from the same insert during the first 200 min (B).

3.4. Trans-corneal and trans-scleral ex vivo diffusion studies of PG

The diffusion of PG through rabbit cornea and sclera was analysed (Figure 3.8), as well as the retention of PG by both membranes.

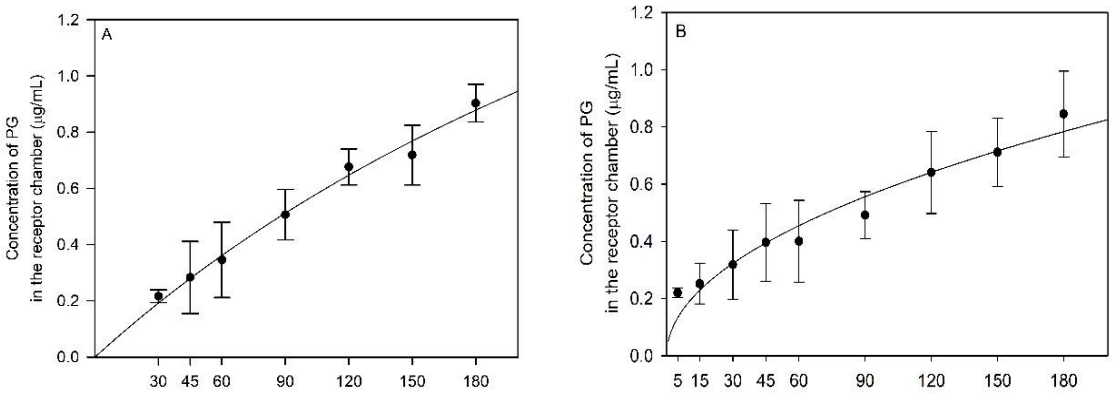

Figure 3.8. Concentration of PG in the receptor chamber (µg/mL) vs time in trans-corneal (A) and trans-scleral (B) diffusion studies. The error bars show the standard deviation of the observed values (n = 5).

Once diffusion studies were completed, corneas and scleras were visually inspected to check for holes or cracks in the membranes. All were found to be in good condition. The percentage of PG diffused to the receptor compartment is shown in Figure 3.9, as well as the amount extracted from both membranes, cornea and sclera and the PG in the insert.

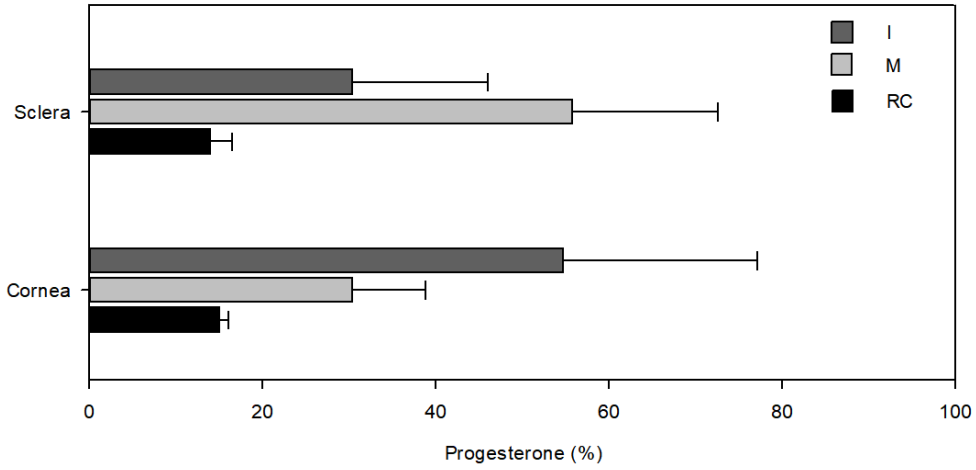

Figure 3.9. Mass balance of PG: percentage (%) of PG in the insert (I); in the membrane (M) and accumulated in the receptor compartment (RC) after 3 h of trans corneal and trans scleras diffusion studies with the insert (n = 5).

When the insert comes into contact with the ocular membrane, PG begins to diffuse from the insert to the tear fluid, then from the tear to either membrane, cornea or sclera, to finally diffuse further into the eye. Although a quick release from the insert (more than 80% of the dose incorporated was released in about 3 h) (Figure 3.7) was shown, its needs to be taken into account that the assay was performed in an excess of water. Consequently, it could be possible that trans-corneal and trans-scleral diffusion of PG would be limited either by its interaction with the membranes (cornea and sclera) or by the release from the insert.

The ocular apparent permeability coefficients, P_{eff} (cm/s), calculated for rabbit corneas and scleras were 6.46 ± 0.38 × 10^{-7} cm/s and 5.87 ± 1.18 × 10^{-7} cm/s, respectively. No statistically significant differences between apparent permeability coefficients across both membranes were observed. Nevertheless, the accumulated amount of PG in cornea (15.56 ± 4.36 µg/cm^2) was lower than in sclera (30.07 ± 9.09 µg/cm^2). The amount of PG that remained in the insert when it was placed on top of the cornea was 54% of its initial concentration, whereas when sclera was the membrane the amount of PG remaining in the insert was 30.35%, which indicates that an important fraction of PG remains in the insert pending its release.

It is well known that passive permeability coefficient is inversely proportional to the thickness of the membrane. The dependence of the permeability coefficient values on the thickness of the cornea and sclera were analysed. Although cornea was much thicker than sclera (51.7 ± 7.1 µm *vs.* 24.3 ± 4.9 µm), there were no significant differences in the amount of PG that diffused through both membranes (Figure 3.9). However, sclera's higher lipophilicity allowed greater retention of PG and its greater release from the insert.

Previous *ex vivo* diffusion studies with PG in eye drops (PG incorporated in β-CD in aqueous solutions 343.04 µg/mL) showed apparent permeability coefficients of 22.6 ± 5.52 × 10^{-7} and 42.9 ± 7.38 × 10^{-7} cm/s for cornea and sclera, respectively (n = 10). In contrast, pure PG micelles formulated in the polymeric solubilizer Soluplus® showed apparent permeability coefficients of 16.5 ± 1.8 × 10^{-7} and 9.2 ± 2.0 × 10^{-7} cm/s for cornea and sclera respectively, whereas using PG micelles in Pluronic® F68 apparent permeability coefficients were 37.3 ± 10.5 × 10^{-7} and 14.5 ± 1.5 × 10^{-7} cm/s for cornea and sclera, respectively. These results show that PG permeability from the insert was 3-7 times lower than permeability coefficients reported with eye drops of PG in β-CD. Similarly, apparent permeability coefficients from the insert were 2-6 times lower than those found when using PG drops in micelles [47]. This lower permeability could be attributable to the fact that the insert controls PG release to cornea and sclera. Although the permeability of PG from the insert presented here is lower than that found in formulations previously described [30,47], it is important to consider that this insert could control the release of the drug over time better, due to longer contact-time with the eye membranes. Furthermore, ocular inserts have some additional advantages compared to liquid formulations such as higher availability of the drug in ocular compartments. Additionally, there are also lower losses of drug and minimal systemic absorption because there is no involuntary lacrimation. Finally, higher precision dosing with controlled release allows to reduce the frequency of administration [16]. Thus, the formulated insert we have designed and evaluated may provide a suitable promising alternative for the treatment of eye diseases requiring PG administration.

4. Conclusions

In the present study, several PG inserts were formulated and evaluated leading to the selection of a PG insert manufactured with 59% polyvinyl alcohol, 39% polyvinylpyrrolidone K30 and 2% propylene glycol with progesterone in its composition (55.6 µg/cm^2). The formulated insert shows good biocompatibility, it is flexible, transparent and has the required mechanical properties for its ocular application. *In vitro* PG release experiments show that the release of PG occurs in a controlled manner. *Ex vivo* diffusion studies performed with the insert showed that PG diffuses similarly through scleral and corneal tissues, but PG accumulates in greater amounts in the sclera than in the cornea. *In vivo* experiments will need to be carried out to demonstrate the efficacy of PG in the treatment of certain ocular diseases, particularly those caused by oxidative stress. Furthermore, the formulated insert with PG would need to be tested in the human eye to assess its suitability for such administration.

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Capítulo 4

Ex vivo ocular biodistribution of progesterone after topical administration of drops and inserts

Abstract

Progesterone (PG) has been shown to have a slowing effect on photoreceptor cell death in mouse models of retinitis pigmentosa when administered orally. The aim of this study was to investigate whether topically administered PG was able to overcome ocular barriers and reach the neuroretina and the PG biodistribution in different ocular tissues was also determined using the porcine eye globe model. Different PG-containing formulations were prepared: drops (1mg/mL), corneal/scleral-insert (325.7 μg/cm^2) and 3 scleral-inserts (32.57, 325.7 and 3257 μg/cm^2) to investigate the effect of drug loading. The effect of tear clearance was also investigated. Upon completion of the experiments, the eye globes were stored at -80 °C for 12h before dissection and extraction of PG from each tissue fraction using acetonitrile (12h). Quantification of PG in each tissue was performed by UHPLC-MS/MS.

The results showed that after topical administration, PG diffused from the ocular surface and distributed throughout the eye including the neuroretina. Lower levels of PG were found in sclera, choroid and neuroretinal tissues when PG was applied in drops than in inserts. Only the corneal/scleral-insert showed statistical differences in PG amounts in cornea when compared to drops, probably due to the longer contact time of the drug with the ocular surface when applied in the insert. An increase in the initial PG concentrations applied resulted in a statistically significant increase in the amounts of PG in aqueous humour, sclera, choroid and neuroretina. There were no statistical significant differences between performing the experiment using the ocular insert with or without a constant drip of saline solution over the cornea (1.2 μl/min) for 24 h to simulate tear clearance and lacrimal function.

1. Introduction

The eye is divided into several clearly differentiated anatomical structures which include the ocular surface (cornea and sclera), the iris, the lens, the ciliary body, aqueous and vitreous humours, choroid and retina. When light enters the eye, it passes through the cornea, pupil and lens to reach the retina, where the electromagnetic energy of the light is converted into nerve impulses by two types of specialized photoreceptor cells, the cones and rods, which send the nerve impulses via the optic nerve to the brain where they are processed in the visual cortex.

The survival of retinal cells requires a balance between oxygen, reactive oxygen species (ROS) and antioxidant molecules that counteract oxidative stress damage. Oxidative stress disrupts cellular homeostasis and elicits a protective response from cells, which is most relevant in photoreceptors, as they are continuously subjected to oxidative stress aggressions. It elicits a severity-dependent cellular response and there seems to be evidence of a stress threshold. Below that threshold, cells trigger protective mechanisms designed to ensure survival, whereas if stress exceeds the threshold or activation of the protective mechanisms fail, the cells activate alternative signalling pathways that eventually lead to apoptosis, necrosis, pyroptosis or autophagy-mediated cell death [1].

Among eye disorders associated with ROS which can cause blindness we can find mainly glaucoma, age-related macular degeneration (AMD), macular edema secondary to retinal vein occlusion (RVO), cytomegalovirus retinitis (CMV), posterior uveitis, diabetic retinopathy (DR) and retinitis pigmentosa (RP) [2–4]. Furthermore, various eye disorders can affect different parts of the eye to different degrees, which would have implications for drug delivery and therapeutic effectiveness of pharmacological treatments. The need to deliver drugs to the posterior segment of the eye is currently a pharmacological challenge given its unique anatomy and physiology which pose a serious difficulty for the development of effective drug formulations.

When a drug is administered to the eye via drops, a high percentage is eliminated by blinking and diluted by tears. In addition, the substance in solution is in contact with the eye surface for a short period of time. The difficulty in administering the drops and their high percentage of elimination, in addition to their short contact time, before the drug is washed away are the main disadvantage of this formulation. When the therapeutic target is the posterior segment of the eye, a greater amount of the drug needs to permeate through the different structural layers of the eye, making it necessary to study possible alternatives for drug delivery. Eye inserts fulfil the requirements to act as a controlled-release drug reservoir [5] and they can be placed in the cornea or, in some cases, directly on the upper or lower sclera. Inserts are solid or semi-solid sterile preparations usually made of polymeric materials [6]. One of the most important properties of ocular inserts is their ability to increase the contact time between the preparation and the ocular membranes, thus improving drug bioavailability [5]. Feeling the presence of a foreign body in the eye is the main reason that leads patients to reject this type of formulation and therefore it is important to develop inserts that are as thin as possible [5,7].

Retinitis pigmentosa (RP) is a group of inherited diseases that cause progressive degeneration of photoreceptor cells and ultimately blindness and for which there is currently no effective treatment. Research has focused on trying to find drugs that can slow the degeneration of photoreceptors in patients affected by RP. One such drug is progesterone (PG): when administered orally, it has been shown to have a beneficial effect in rd10 and rd1 mouse models (RP mouse models) by slowing death cell of photoreceptors, decreasing the amount of oxidative stress, increasing the amount of antioxidant molecules and decreasing the number of inflammatory cytokines [8–10] but it is subject to hepatic first pass metabolism and has very poor oral bioavailability in humans.

The low hydrosolubility of PG is a disadvantage for the development of topical formulations which can be solved by combining them with cyclodextrins, oligosaccharides with a hydrophilic outer surface and a hydrophobic core capable of forming inclusion complexes with drug molecules [11]. The cyclodextrin conformation allows inclusion of apolar molecules such as PG in its inner cavity, whereas the outer part of the molecule is hydrophilic, thus conferring new physicochemical characteristics to the drug-cyclodextrin complex: there is an apparent increased water solubility of the drug and often, as a consequence, better bioavailability. There is a dynamic balance between the cyclodextrin-drug complex and the dissociated drug, therefore making possible the release of the drug [12]

Our group has recently published results documenting the diffusion of PG through the cornea and sclera from different animal models using different pharmaceutical formulations. However, it is still unknown whether topically administered PG would be able to reach the neuroretina and how it is distributed within the eye. The aim of the present study was three-fold: (i) to determine whether PG was able to reach the neuroretina, as well as to know the amount of PG that was distributed after its administration through drops or inserts in the different parts of the eye, (ii) to study the variations upon application at different initial concentrations and (iii) to study the effect of tear clearance on the ocular distribution of PG administered through inserts.

2. Material and methods

2.1. Compounds

Progesterone (PG, $C_21H_{30}O_2$, PubChem: 5994) incorporated to Methyl-β-cyclodextrin (βCD) complex powder (PG-βCD, 86.8 mg of PG per gram), polyvinylpyrrolidone K30 (PVP-K30) and polyvinyl alcohol (PVA) were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). UPLC grade solvents (HiPerSolv Chromatonorm; Darmstadt, Germany) and deionized water (Milli-Q Gard 1 Purification Pack resistivity > 18MΩcm) were used to prepare all the solutions and were purchased from Millipore, Zug, Switzerland. UPLC/MS grade formic acid (FA) was purchased from Brunschwig (Basel, Switzerland) and Propylenglycol (PGL) was obtained from Acros Organics (Geel, Belgium).

2.2. Analytical method

A UHPLC-MS/MS system comprising a Waters Acquity® UPLC®/Xevo® TQ-MS detector; ESI positive mode and multiple reaction monitoring (MRM) were employed to ionize and to quantify PG. Gradient separation was achieved using a Waters XBridge® BEH C18 (100×2.1 mm, 2.5 µm) reverse phase column at room temperature. The mobile phase consisted of acetonitrile and water (80:20) to which 0.1% FA (v/v) was added. The flow rate was 0.4 mL/min and the injection volume was 5 µL. The run time was 1.1 min and the limits of detection and quantification were 1 ng/mL and 5 ng/mL, respectively.

2.3. PG Formulations

2.3.1. Drops of PG

Due to the low solubility of PG in water (7 µg/mL) [13] and in PBS (4.4 µg/mL) [14], a methyl-β-cyclodextrin (βCD) complex was used which increased the solubility of PG to 26.04 mg/mL in PBS. A solution of PG-βCD (1 mg/mL) was prepared and stirred for 1 h at room temperature with a magnetic stirrer prior to use in the distribution experiments.

2.3.2. PG Inserts

PG inserts previously developed and studied in trans-corneal and trans-scleral diffusion experiments were formulated as previously described [15]. Briefly, PVA (59%)

was dissolved in 5 mL of water in a magnetic stirrer at high temperature (approx. 100 °C) during ~10 min to ensure it was fully dissolved [16]. After complete PVA solution, PVP-K30 (39%) was incorporated and allowed to cool with constant stirring. Once the polymers were dissolved, PG-βCD and propylene glycol (PGL) were incorporated with a further 2 h stirring. The polymer was then poured into Petri dishes and allowed to dry for 24-48 h. Using a mould, inserts were cut to obtain scleral inserts (S-I) of 3.07 cm^2 (figure 4.1). Inserts containing increasing amounts of PG (0.1, 1 and 10 mg) were prepared, thus rendering PG concentrations in the inserts of 32.57, 325.7 and 3257 $\mu g/cm^2$, respectively.

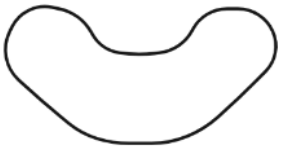

Figure 4.1. Design of the S-I. Surface of 3.07 cm^2.

A corneal and scleral insert (C/S-I) was formulated with the same components and methodology as described above but increasing its size and adapting its shape so that it was in contact with the visible part of the eye (both cornea and sclera). The insert loading was maintained (1 mg of PG per insert) so that circular inserts of 7.64 cm^2 (130.9 $\mu g/cm^2$) were obtained.

2.4. Ex vivo biodistribution studies of PG

2.4.1. Ocular tissue source

Adult porcine eyes (from animals weighing 80-100 kg) were supplied by a local slaughterhouse (Abattoir de Loëx Sarl; Loëx, Switzerland) and transported to the laboratory promptly after sacrifice. There, the surrounding tissues were carefully removed with a scalpel, so as not to damage the eye and were used immediately.

2.4.2. PG Ocular ex vivo biodistribution studies

The entire eyeball was placed in a vertical Franz diffusion cell, with the cornea facing upwards. The diffusion cells were designed to embed 1/3 of the eyeball in a hemispherical glass cup attached to a water-filled receptor compartment, without being in contact with the eye. S-I were placed over the sclera, just next to the cornea. Once

the insert was placed, a water-soaked absorbent disposable paper towel was placed around the Franz's cell and wrapped in a plastic bag to keep moisture in and prevent dryness of the eye. Large inserts were placed directly in the centre of the cornea, being in contact with the entire cornea (Corneal mean surface area: 1.90 ± 0.26 cm^2) and with part of the sclera (Sclera mean surface area: 5.74 ± 0.26 cm^2).

To administer the drops, a donor compartment with a total permeation area of 2.835 cm^2 was placed over the eye and fixed with clamps. The drops (250 µL) were administered every 2 h and left in contact with the ocular surface for 5 min inside the donor chamber. After the exposure time had elapsed, the donor compartment was disassembled and the surface in contact with the drops was washed twice with distilled water (approximately 5 min) and covered with the bag. Then it was left to stand for 1 h and 50 min and the process was repeated. Four instillations of the drops were made to administer a total of 1 ml of formulation in 8 hours. A diagram of the assembly can be seen in figure 4.2.

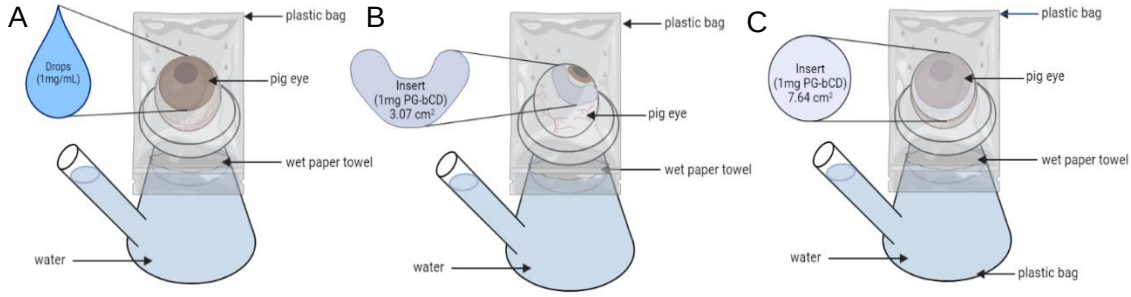

Figure 4.2. Design of the set-up of *ex vivo* distribution studies. With drops (A), the S-I (B) and the C/S-I (C). For clarity, the donor compartment in A has not been drawn.

To study the influence of PG concentration on its distribution through the different anatomical structures of the eye, two scleral inserts preloaded with either 10 times less PG (0.1 mg/insert) or ten-fold higher PG concentration (10 mg/insert) were investigated. *Ex vivo* biodistribution studies were carried out under the same conditions as described above for the scleral insert.

Finally, to study the effect of the elimination of PG by tears, the experiment using the scleral insert was repeated with a constant drip of saline solution (NaCl, 0.9%) simulating human lacrimation (1.2 µL/min) by means of a syringe pump (KD Scientific 789220F) [17]. After 8 h, the insert was removed while maintaining the artificial tears dripping onto the eye for a total of 24 hours (Figure 4.3).

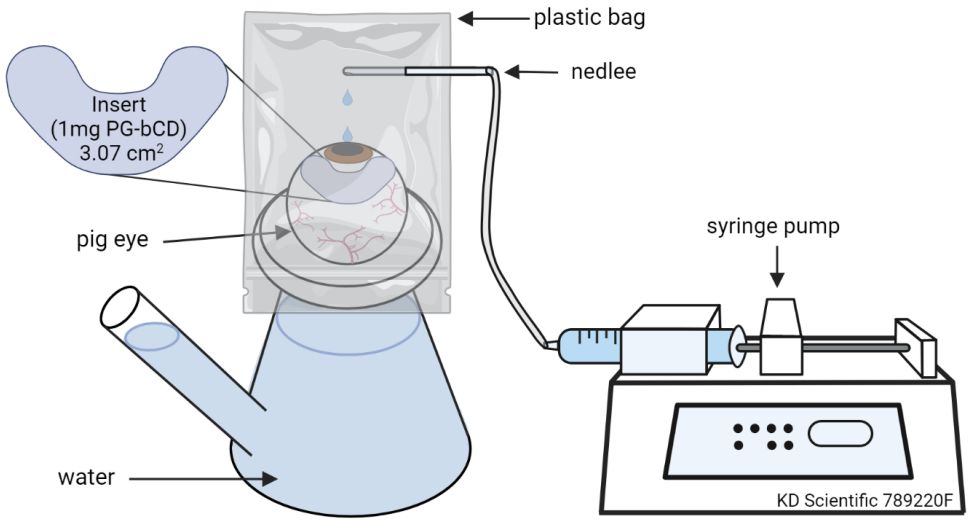

Figure 4.3. Design of the set-up of *ex vivo* distribution studies of 24 hours with tear drip

After the exposure time to the formulation had elapsed, the eyes were washed with water to remove any remaining formulation from the eye surface. The eyes were wrapped in Parafilm® and frozen at -80 °C for 12 h. The eyes were taken out of the freezer and dissected before they fully thawed, separating the different tissues and humours (cornea, sclera, iris, aqueous humour, vitreous humour, choroid and retina) which were individually weighed, extracted in acetonitrile for 12 h with constant stirring. The samples were then centrifuged at 3000 rpm, during 5 min and the supernatant was filtered (Chromafil® Xtra PTFE-20/13, pore size 0.20 µm) prior to their analysis by UHPLC-MS/MS for PG quantification.

2.5. Statistical analysis

Values were expressed as mean and standard deviation. To determine statistically significant differences among the experimental groups, data analysis was performed using the Kruskal-Wallis or the Mann-Whitney tests as appropriate. Post-hoc multiple comparisons were carried out using the Man-Whitney test applying Bonferroni's correction for significance. The confidence level was 95%. Statistical analysis was carried out using SPSS 27.0.

3. Results and discussion

3.1. Selection of the animal model for biodistribution ocular studies

Several animal models are being used to investigate ocular drug administration but all have differences. The main characteristics of human eyes and animal models most used in ocular studies, in terms of scleral and corneal thickness and size, are highlighted in Table 4.1. Human and porcine eyeballs are very similar not only in size, but also in weight and volume of aqueous humour and the vitreous body. The permeability of certain drugs through the porcine cornea is three to nine times less than that of the rabbit cornea, because the greater thickness of the epithelium. In turn, penetration through the porcine cornea is expected to be slightly less than the penetration through human cornea, since the epithelial layer of the porcine cornea is thicker. On the other hand, diffusion through the bovine cornea or sclera is less than through human cornea or sclera due to its greater thickness [18].

Table 4.1. Key differences between human, bovine, porcine and rabbit eyes [18–26].

Parameter	Human	Bovine	Porcine	Rabbit
Corneal thickness	0.5-0.7 mm	0.9-1.1 mm	0.5-0.7 mm	0.3-0.4 mm
Scleral thickness	0.49-0.97 mm	1.46-1.70 mm	0.31-1.12 mm	0.29-0.33 mm
Diameter of ocular globe	23.5 mm	28.5-30.5 mm	24.5 mm	18-20 mm
Bowman´s layer	Present	Present	Present	Absent

Trans-corneal and trans-scleral diffusion of PG has been studied in previous publications [14,15,27] using different animal models (bovine, porcine and rabbit) in order to study the influence of the model in the permeation of PG. Those reports indicated that there were significant differences in the penetration of PG. Higher amounts of PG diffused through cornea and sclera from rabbit followed by porcine and bovine tissue, which was attributed to differences in corneal thickness. In fact, porcine corneal tissue is often used as an alternative to rabbit tissue in experimental eye studies. It is easily accessible, large and sturdy enough, making it easy to handle. For all of these and since the characteristics of the porcine pig eye model most closely resemble those of the human, in this paper PG ocular distribution was studied in the porcine eye.

3.2. Biodistribution profile of PG in eye tissues

3.2.1. Comparison of PG amounts in tissues from different formulations

In the present study to investigate PG distribution in the eye, three different formulations were investigated: drops, an insert placed over the sclera (S-I) and a larger insert which covered the cornea and part of the sclera (C/S-I). The application time of the inserts was 8 hours, whereas the drops had a shorter actual contact time, as they were administered four times being in contact with the eye surface for 5 minutes on each administration.

The amounts of PG in each tissue and humour analysed revealed that there were higher PG concentrations in each of the anatomical structures when PG was applied via either of the inserts when compared to drops (Fig 4.4). Although PG concentrations were always lower when PG was administered as drops, statistically significant differences were found in concentrations achieved between S-I and drops in sclera, choroid, and neuroretina. On the other hand, the drops compared to the C/S-I showed a lower concentration per tissue with statistically significant differences in sclera, choroid, neuroretina and also cornea. We believe the differences between drops and both inserts might be mainly due to PG contact time, since inserts allow a greater distribution of the PG by remaining in direct contact with the ocular tissues for longer than the drops. The polymer characteristics used in the manufacture of the inserts may explain those differences in terms of bioadhesion, permeation and contact time. PVA has the ability to retain a large amount of liquid, which gives the insert elasticity and structural integrity [28] and additionally it has excellent mucoadhesion properties as it forms hydrogen bonds with the mucosa and becomes a film [29]. PVP is a polymer with the ability to form complexes with hydrophilic and hydrophobic molecules [28] and has a high swelling index [30]. All of these will be responsible for the better transfer of PG from the insert to tissues in the eye surface and from there, to all other ocular structures.

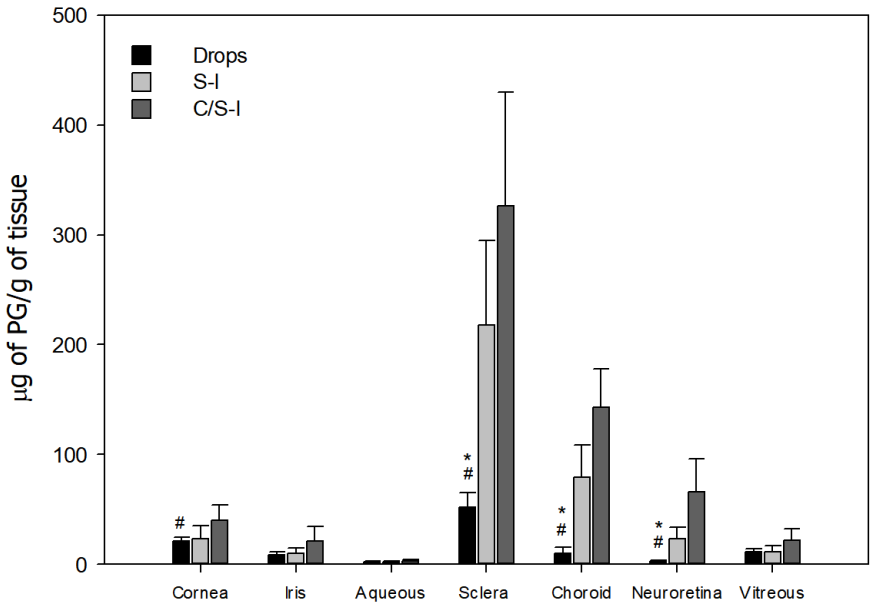

Figure 4.4. Amount of PG distributed from the different formulations per gram of each of the ocular tissues. *represents that there are significant differences between S-I and drops ($p < 0.05$). # represents significant differences between drops and C/S-I ($p < 0.05$). No tissue showed significant differences between the S-I and the C/S-I ($p > 0.05$). Data are mean ± SD (C/S-I, n = 5; Drops and S-I, n = 6).

Regarding the inserts, both S-I and C/S-I, had the same amount of PG (1 mg/insert), but their dimensions were different (3.07 cm^2 vs. 7.64 cm^2) and therefore the PG concentrations in them varied accordingly (325.7 $\mu g/cm^2$ vs. 130.9 $\mu g/cm^2$). However, although greater PG amounts reached almost all structures analysed when the C/S-I was used, the differences with S-I were not found to be statistically significant. However, the power of the test calculated for the Kruskal-Wallis test performed for sclera, choroid and neuroretina was greater than 95%. Interestingly, no statistical differences were found even in cornea for both inserts, a tissue for which the S-I was not in contact. However, for cornea the power of the Kruskal–Wallis revealed that the probability of having made at least one type-II error is approximately 50%. It would seem that PG is distributed to all ocular tissues, even from sclera to cornea. It is clear from these results that PG administered through the sclera (S-I) can reach all ocular structures analysed (Fig 4.4). Therefore, the S-I was selected as the best formulation for further distribution studies. In addition, this insert can probably be more comfortable to wear, easier to apply due to its shape and because its smaller surface area, wrinkles will not appear too easily.

An increase in the size of the inserts from their original size was observed after the exposure time. The size was measured before and after application of each insert. The increase with respect to the original size (3.07 cm^2) was 80.13 ± 8.47% (5.53 ± 0.26

cm^2). Natural and synthetic polymers have properties that improve viscosity in ocular drug formulations by improving drug residence time. This is due to the fact that these materials absorb water, forming viscoelastic gels, which then become suitable vehicles for the administration of drugs [5]. The release of the drug incorporated into the polymer depends on its chemical structure, network arrangement, and swelling properties [31]. One of the advantages of polymer-incorporating eye formulations is that they improve viscosity and resist tear drainage when in the lower conjunctival cul-de-sac. However, they have focusing drawbacks, as they casue initial blurring due to changes in the refractive index at the corneal surface [32,33], although this does not happen in the case of the scleral insert.

3.2.2. Comparison of the amounts of PG reaching ocular structures when administered via S-I with different PG concentrations.

To study how the difference in the amount of PG in the insert affected the time for distribution and especially the time to reach the neuroretina, another two S-I were formulated with the same characteristics except for the amount of PG in the insert. One of the inserts contained 0.1 mg of PG whereas the other was loaded with 10 mg PG (32.57 µg/cm^2 and 3257 µg/cm^2, respectively). Figure 4.5 shows how the amounts of PG per gram of tissue increased as a function of the PG loaded in the insert.

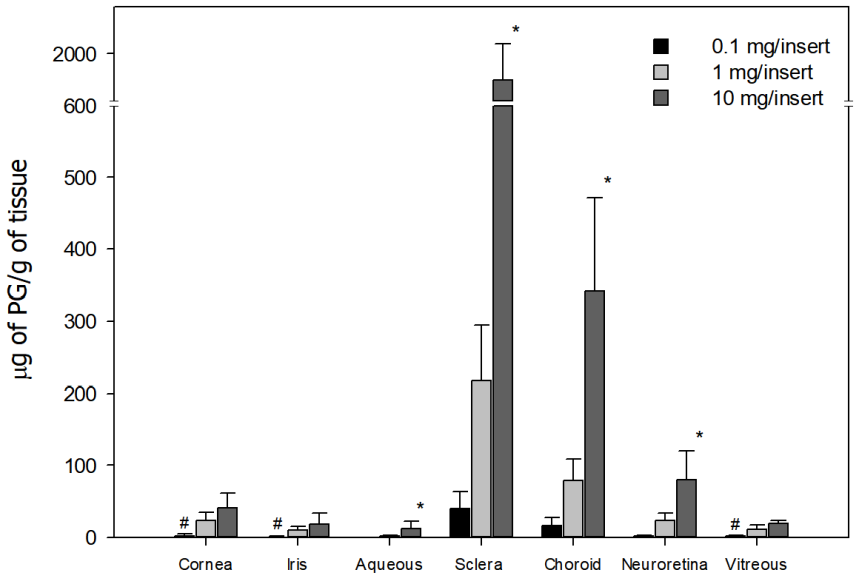

Figure 4.5. Amount of PG distributed from inserts (S-I) with different PG amounts (0.1, 1 and 10 mg/insert) to each of the ocular structures. Significant differences between all concentrations in that tissue ($p < 0.05$) are marked with *. Significant differences between 0.1-1 and 0.1-10 mg/insert are represents with # ($p < 0.05$). No tissue showed significant differences between the S-I and the C/S-I ($p > 0.05$). Data are mean ± SD (n = 6).

As the PG concentration in the insert increased, so did the amount of PG reaching the different tissues (Fig 4.5). Statistically significant differences in PG concentration between the different inserts were found in aqueous humour, sclera, choroid and neuroretina. However, in cornea, iris and vitreous body the differences seen in the amount of PG between the three inserts used were not found to be significant, only between 0.1-1 and 0.1-10 mg/insert statistically significant differences were found.

The amount of PG that can reach the neuroretina can be modulated by varying the PG concentration in the inserts (Figure 4.5). Furthermore, similar amounts of PG reach the neuroretina using a S-I loaded with 10 mg PG (Figure 4.5) than with the C/S-I loaded with 1mg PG (Figure 4.4).

3.1.3. Effect of tear clearance on PG ocular biodistribution

In order to study the distribution of PG in the eye under more realistic conditions, the study of PG distribution following application of the S-I 1 mg/insert was repeated. It was placed onto the sclera for 8 h as previously described, but additionally drops of saline solution (1.2 µl/min) were constantly dripping onto the eye surface while the insert was in place and after its removal for an additional 16 h, for a total of 24 h. Analysis of PG distribution through the eye's anatomical structures did not show any statistically significant differences in any of them (figure 4.6). Therefore, we can conclude that the amount of PG reaching the neuroretina were not affected by tear clearance.

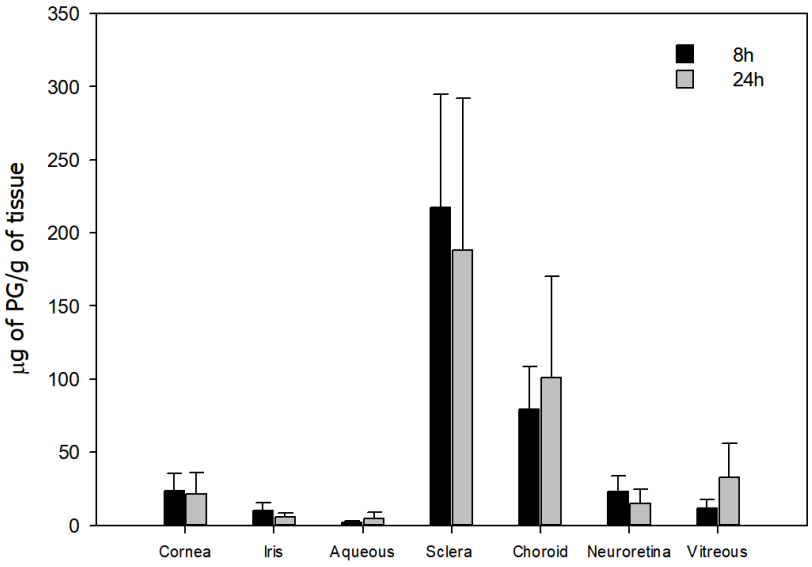

Figure 4.6. Amount of PG distributed from inserts with the same concentration administered for 8 hours without clearance (black), for 8 hours and without insert for an additional 16 hours with tear clearance for 24 hours (grey). No statistically significant differences were found in any tissue. Data are the mean ± SD (n = 6).

The amount of PG that must reach the neuroretina to show a pharmacological effect is still unknown, but our research group has evidence showing the protective effect of PG administered in drops (1 mg/mL, one drop every 12 h), to an animal model of retinitis pigmentosa (RDS mice. Statistically significant differences have been found between the treated group and the control group (unpublished results).

4. Conclusions

PG can diffuse from the ocular surface and can be distributed throughout the eye tissues to the neuroretina after topical administration via eye drops and two types of inserts (S-I, C/S-I). PG administered with both inserts developed was able to reach the neuroretina in greater quantities than when administered using drops. S-I was selected because it showed better physical characteristics (swelling, wrinkling, etc.) and as it is small and placed over the sclera rather that the cornea, it can probably be more comfortable to wear and easier to apply. Furthermore, there is a relationship between the concentration of PG in the neuroretina and the amount of PG loaded to the S-I, which should provide an easy mechanism to control of the amount of PG to be administered. We have also demonstrated that tear clearance does not seem to have an effect on the *ex vivo* PG distribution through the eye following administration using th S-I.

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Capítulo 5

Effect of progesterone on the survival of photoreceptors in the retina of RDS mice after ocular administration

Abstract

Retinitis pigmentosa (RP) is an inherited eye disorder with no effective treatment so far. RP triggers a cascade of retinal disorders leading to photoreceptor death. The purpose of this study was to study whether topical ocular administration of progesterone (PG) could decrease photoreceptor death in an animal model of RP (rds mouse) and to evaluate possible topical formulations for its administration. A group of rds mice received one drop of a solution of PG in β-cyclodextrins (CD) every 12 h for 10 days in one eye, the left eye, while the right eye was left untreated. Another control group of rds mice was treated with saline in the left eye, and again the right eye was left untreated. After treatment, the animals were euthanized on postnatal day 21. TUNEL, GFAP, and DAPI staining were performed. PG in CD (CD-PG) drops in solution administration significantly decreased cell death and inflammation in the retinas of PG-treated rds mice. Significant differences were only found in the treated eye, demonstrating no systemic effect. In conclusion, CD-PG applied topically in drops to the eye decreases photoreceptor death in the early stages of RP, delaying vision loss and decreasing gliosis. Additionally, several PG formulations (solution, micelles, and inserts). for the treatment of RP by topical administration have been compared using mathematical analysis of previously studied apparent permeation coefficients.

1. Introduction

Retinitis pigmentosa (RP) is a group of inherited diseases that produce progressive degeneration of photoreceptor cells evolving towards complete blindness. Rods are the first photoreceptor type affected by RP, producing night blindness and the reduction of the visual field. Rod cell death leads to a pathological environment in the retina that affects cone photoreceptors and induces the death of the latter cells. In the final stages of this disease electroretinogram changes and accumulation of pigment deposits (clusters) can be seen in the peripheral retina [1].

One in 4000 people is affected by RP, which means there are almost two million people all over the world affected by this disease. This means that even though this is a rare disease, RP is the most common cause of inherited blindness. Nowadays no

effective treatment for RP exists. Because of the heterogenic group of over 100 genes involved in RP, it is hugely difficult to find an efficient genetic treatment for this disease [1,2]. Among autosomal dominant forms of RP, which constitutes 20% to 25% of the non-syndromic form of RP, there are two forms of mutation found in humans: mutations within rhodopsin and mutations in peripherin (PRPH2/RDS) genes [3]. The human PRPH2/RDS gene has been associated with 151 individual disease-causing mutations [4]. In the present study, we have used the rds mice model, that have a mutation in PRPH2, because the rate of cell death in the mice closely resembles the rate observed in human RP and other forms of progressive neuronal degeneration [5].

Different therapeutic approaches have been adopted to halt photoreceptor degeneration by pharmacological treatments. It has been suggested that long-term protection against oxidative damage may delay cone death in RP patients, regardless of the causative mutation [6] . It has been reported that PG alone or in combination with other substances, administered orally, have a beneficial effect in the rd10 and rd1 mice models (RP mice models) by slowing down photoreceptor cell death, diminishing oxidative stress, inflammatory cytokines and increasing antioxidant molecules [7–12].

Natural PG can be rapidly inactivated when administered orally. It has extensive hepatic and intestinal metabolism, and the plasma half-life is 25 min. PG administration has drawbacks due to its solubility. PG (log P = 3.9) is considered practically insoluble in isotonic buffer solution (7 µg/ml) [13]. PG dissolves slowly and incompletely in gastrointestinal fluids and has rapid hepatic metabolism, so its oral bioavailability is limited. High concentrations of PG administered orally are needed for a small amount to reach the eye [14]. Therefore, topical administration of PG to the eye may be an interesting alternative to oral administration to prevent this therapeutic agent from reaching the systemic circulation and to reduce the concentration needed to be administered. However, although it is known that oral PG administration at high doses can reach photoreceptors, it is not known whether it would affect neuroretinal cell death [7,15].

Our group has investigated the incorporation of PG into different topical ocular formulations: solution, micelles and inserts. These formulations were for corneal and/or scleral administration. The diffusion of PG through the cornea and sclera into the eye has been studied in *ex vivo* experiments using rabbit eyes. The drops formulated and used for the trans-corneal and trans-scleral diffusion studies consisted of a solution of PG incorporated in β-cyclodextrins (CD-PG) [16]. It is interesting to note the use of cyclodextrins in drops can increase their solubility and increase drug permeability [17–19] . Other PG ocular formulations developed by our group were polymeric micelles of

Soluplus (20%) and Pluronic F68 (20%) micelles, both of them loaded with pure PG [20]. Polymeric micelles were used for their ability to encapsulate hydrophobic drugs, facilitating the development of formulations with poorly soluble active ingredients such as PG. Our previous research results showed that encapsulation in polymeric micelles used in Soluplus and Pluronic micelles facilitate the development of aqueous formulations by avoiding the feeling of stickiness or blurred vision, as well as increasing the permanence on the ocular surface and increasing molecule penetration into deeper ocular structures as indicated by other authors [18,20–22]. The other formulation developed by our group was a polymeric eye insert, composed of 59% polyvinyl alcohol, 39% polyvinylpyrrolidone K30 and 2% propylene glycol, loaded with CD-PG [16]. The advantages of eye inserts compared to liquid formulations are numerous. Ocular inserts increase drug contact time, increasing drug availability [23]. The drug-loaded insert is designed to adhere to the conjunctiva or directly to the cornea, allowing longer residence time and bioavailability; it allows precise dosing with controlled release, has minimal systemic absorption and also the lower frequency of administration [24].

The experiments carried out and presented in this paper try to solve the next objectives: 1) to study the effect of CD-PG solution in delaying photoreceptor cell death; 2) to study the effect of CD-PG solution in the gliosis process of the rds (slow retinal degeneration) mice model; 3) to study the possible systemic effect of CD-PG solution applied topically to the eye and additionally, 4) to discuss and evaluate the diffusion of the drug from possible topical formulations and their suitability for PG ocular administration.

2. Materials and Methods

2.1. Experimental design

Rds mice were housed in the facilities of the Research Unit at CEU Cardenal Herrera University. Animals were kept in cages at 25 °C, 60% humidity and standard conditions of dark and light cycles (12 hours) with free access to food and water (Harlan Ibérica S.L. (Barcelona, Spain)). Handling and care of the animals were approved by the CEU Cardenal Herrera Universities Committee for Animal Experiments (reference 2017/VSC/PEA/00243) and were also performed following the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in Ophthalmic and Vision Research.

In the present work, *in vivo* studies were performed on rds mice (n = 8-10 per group) by administering CD-PG solution as eye drops. The eye drops were prepared

with a PG concentration of 1mg/mL and were administered twice daily (every 12/h). The day mice were born was marked as postnatal day 0 (PN0). The mice were separated into two groups to receive PG treatment (PG-treated mice) or to serve as controls (Control Mice) (Figure 5.1). From PN11 to PN20 (both inclusive) (Figure 5.1C), the left eyes of PG-treated mice or Control mice received either a drop of CD-PG solution or a drop of 0.1M phosphate buffer saline (PBS) pH 7.2 respectively (Figure 5.1) every 12 hours. The right eyes of each mouse were not treated with anything, leaving them as control eyes. PG-Blank (Figure 5.1B) would serve to evaluate PG possible systemic effects derived from the drug administration to the contralateral eye (PG-T) (Figure 5.1B).

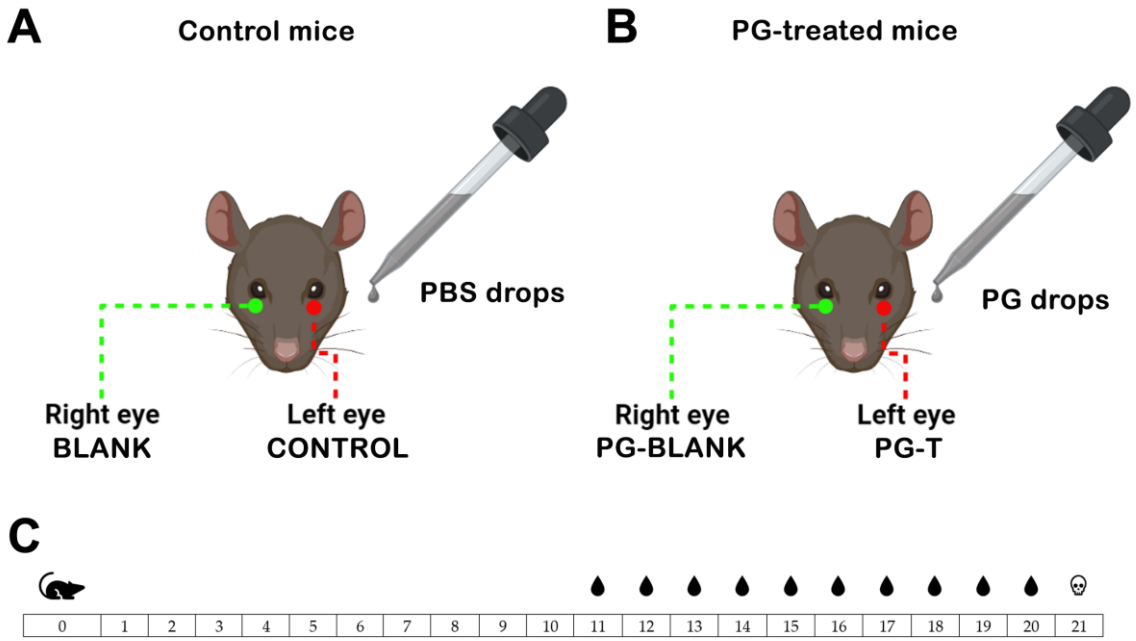

Figure 5.1. Scheme of the experimental design. (A) Treatment applied in the PBS 0.1% treated mice. (B) Treatment applied in the PG treated mice. (C) Scheme of the treatment days and the euthanasia day of the mice.

2.2. Histological and Immunofluorescence studies

At PN21 mice were euthanized, the eyes were enucleated and after puncturing the corneas with a needle, the eyes were submerged in 4% paraformaldehyde for 2 hours. The eyes were then washed three times in PBS solution for 10 minutes and submerged overnight at 4 °C in a PBS solution containing a final sucrose concentration of 0.3M. The eyes were transferred to a PBS solution with 0.9M sucrose 24h later. The eyes were embedded in Tissue Tek (Sakura Europe, Spain) and 8µm sections were obtained using a Leica CM 1850 UV Ag protect cryostat, (Leica Microsistemas SLU, Barcelona, Spain). The sections were transferred to superfrost slides (Thermo Fisher Scientific, Braunschweig, Germany) and kept at −20 °C.

A double immunostaining process was carried out to detect the dying cells

Terminal deoxynucleotidyl transferase nick end labelling assay (TUNEL) and glial fibrillar acid protein expression (GFAP). Tissue sections were washed three times with PBS 1% and triton 0.1% solution for 10 minutes. After unspecific epitopes were blocked by incubation, the tissue sections were washed in a 1% PBS solution and a 5% normal goat serum solution for 1 hour at 4 °C. The sections were then washed with 1% PBS and the TUNEL in situ cell death kit (Roche Diagnostics, Mannheim, Germany) was performed, as previously reported [7]. The sections were incubated at 4 °C overnight in a solution of GFAP antibody in PBS 1% (1:500, Dako cytomation, Denmark). The primary antibody was detected with the fluorescence-conjugated secondary antibody Alexa Fluor 568 (Invitrogen, Life Technologies, Madrid, Spain). Finally, the sections were mounted using Vectashield® mounting medium with DAPI (Vector, Burlingame, United States).

Images of the retina were taken at 20X magnification with a Nikon DS-Fi1 camera attached to a Leica DM 2000 microscope. The software used for the image capture was the application Suite version 2.7.0 R1 (Leica Microsystems SLU, Barcelona, Spain). To ensure that the area of the retina pictured was the same in all sections, photos were taken using the optic nerve as a positioning reference. The dying cells were counted concerning the retina area and multiplied by 106 to obtain integer numbers. GFAP was quantified as the percentage of the labelled space compared to the whole retina. Finally, the number of rows in the outer nuclear layer (ONL), which would correspond to the nucleus of photoreceptors, were counted. These quantifications were carried out with the help of the software ImageJ Fiji.

2.3. PG permeability coefficients

To compare the topical formulations for ocular administration of PG we have analyzed the trans-corneal and trans-scleral permeability of PG from different ocular formulations studied by our research group [16,20]. The apparent permeability coefficients (P_{eff}) were recalculated using equation (1) [25] using the data obtained from *ex vivo* experiments with rabbit corneas and scleras from solution, micelles and polymeric PG-embedded insert. This equation considers a continuous change in donor and acceptor concentrations and is valid under both sinking and non-sinking conditions.

$$C_{receiver,\,t} = \frac{Q_{total}}{V_{receiver}+V_{donor}} + \left[\left(C_{receiver,t-1}\cdot f\right) - \left(\frac{Q_{total}}{V_{receiver}+V_{donor}}\right)\right]\cdot e^{P_{eff}\cdot S\cdot\left(\frac{1}{V_{receiver}}+\frac{1}{V_{donor}}\right)\cdot\Delta t}$$

$C_{receiver,\,t}$ is the PG concentration (µg/mL) in the receptor compartment at time t, Q_{total} is the total amount of PG in the insert, $V_{receiver}$ is the volume in the receptor compartment,

V_{donor} is the volume in the donor compartment, $C_{receiver,\ t-1}$ is the amount of PG in the receptor compartment at a previous time, f is the replacement dilution factor of the sample, S is the surface area of the membrane and Δt is the time interval. The curve fittings were performed by non-linear regression, minimizing the sum of the squared residuals.

2.4. Statistical analysis

The Shapiro-Wilk test was used to assess for normality of the histological and immunofluorescence data and the Levene test was used to check for homoscedasticity. The analysis of variance (ANOVA) test and mean comparisons using the Bonferroni test was used to compare the groups. The student t-test was used to analyze the permeability coefficient obtained with the mathematical treatment from the value obtained by assuming that the concentration in the donor compartment remained constant. Statistical analysis for permeability coefficients was performed using the Mann-Whitney test between the values obtained for both membranes (cornea and sclera) and each formulation. Analyses were carried out using the software IBM SPSS v27 with $\alpha = 0.05$. The results are presented as mean ± SD.

3. Results

Topical PG solution treatment of the eye shows a statistically significant difference in the amount of cell death of photoreceptor cells. Figure 5.2 shows how PG treatment diminishes the amount of TUNEL labelled cells per area in the PG-T eye compared with the Control which received PBS. Furthermore, the study showed a statistically ($p < 0.05$) significant decrease of TUNEL positive cells in PG-T in contrast to PG-Blank in the same mice but not between Control and Blank eyes. In addition, there are no statistical differences between the number of TUNEL cells in the retina of PG-Blank, Control and Blank eyes. These results show a decrease in the photoreceptor cell death in the PG-T, and also prove that PG does have not any effect on the PG-Blank eye. This result also proves the capability of PG topically applied to protect photoreceptors and delay cell death. In addition, we have demonstrated that PG applied in on eye do not affect the non-treated eye, suggesting that there is not any systemic effect.

The results related to GFAP expression in the retina are similar to that obtained in the TUNEL staining, showing an important decrease in the PG-T GFAP staining and statistically significant difference in GFAP expression between PG-T and the other

groups (figure 5.3). When GFAP expression between Control, Blank and PG-Blank eyes is compared, no statistical differences were found. In addition, there is a decrease in the amount of GFAP expression in the PG-T but not in the PG-Blank as was reported for TUNEL staining. As before, these results also show that there is no systemic effect from PG treatment in the contralateral eye (PG-Blank) to that receiving PG (PG-T).

Finally, the results obtained by counting the number of cell rows in the ONL, as a means to evaluate photoreceptor cell death and retinal degeneration, do not show any statistical differences between PG-T, PG-Blank, Control and Blank (Figure 5.4). Although the maximal amount of cell death in the rds mouse model occurs between PD17 and PD21 [26], a significant decrease in the amount of ONL cell rows would have occurred later in time [27,28] and therefore has not been detected in our experimental model.

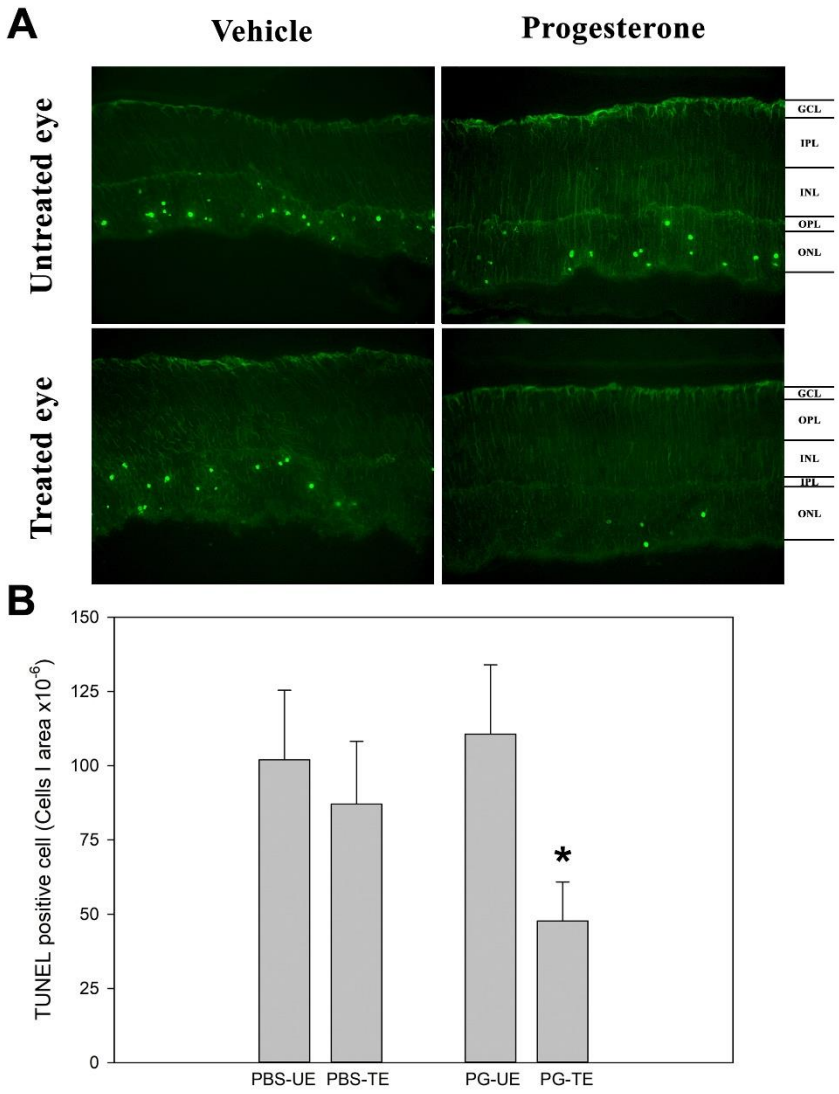

Figure 5.2. TUNEL immunohistochemistry. (A) An example of TUNEL staining of each different group in the experiment. (B) Results of TUNEL quantification. *: Statistically significant difference (p-value < 0.5) of PG-TE vs PG-UE, PBS-TE and PBS-UE.

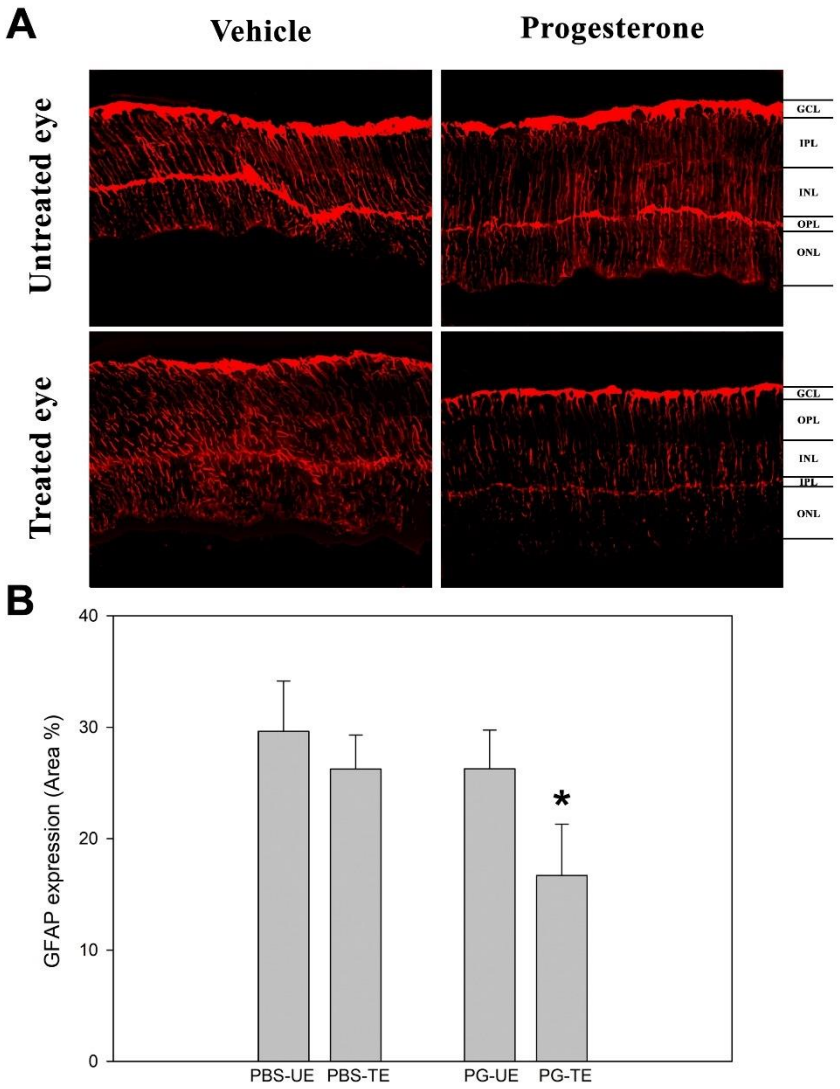

Figure 5.3. GFAP immunohistochemistry. (A) An example of GFAP staining of each different group in the experiment. (B) Results of GFAP quantification. *: Statistically significant difference (p-value < 0.5) of PG-TE vs PG-UE, PBS-TE and PBS-UE.

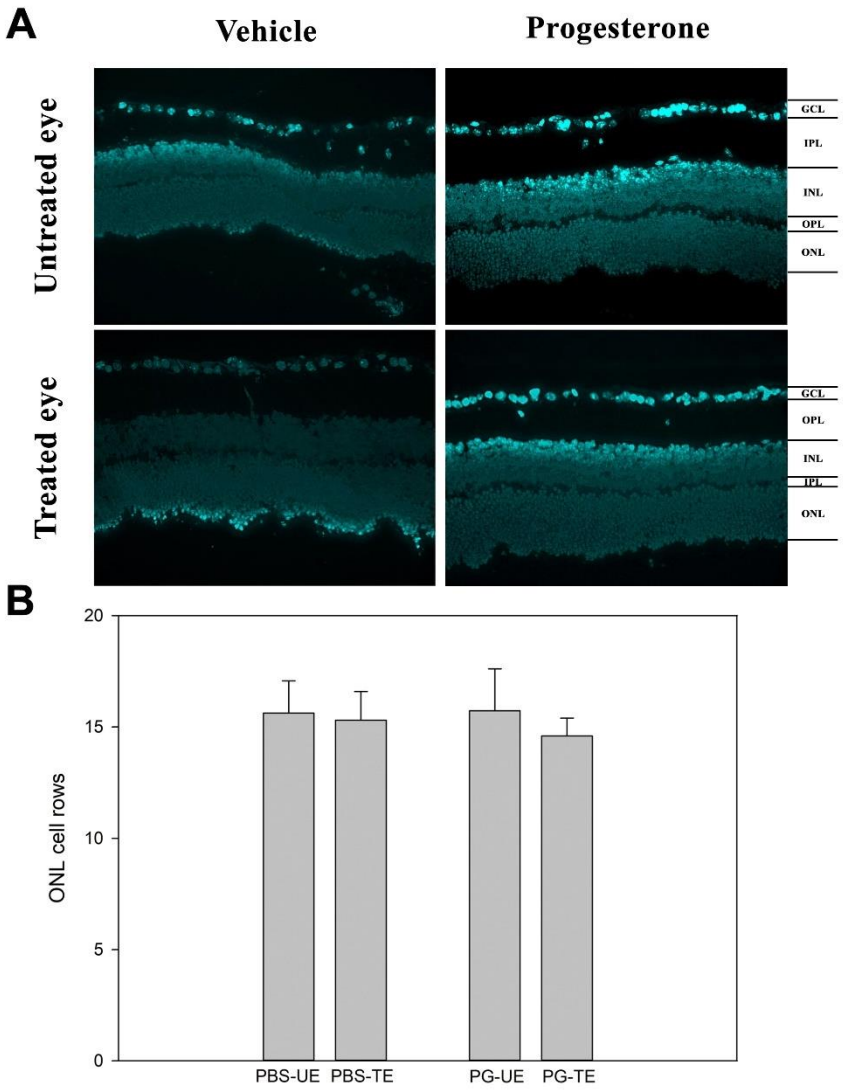

Figure 5.4. DAPI staining. (A) An example of DAPI staining of each different group in the experiment. (B) Results of DAPI quantification.

4. Discussion

The main objective of RP treatments is to preserve photoreceptors and avoid, or at least decrease the photoreceptor cell death rate . In this sense, TUNEL staining, which labels cells that have started a procedure of death, confirm the capability of PG topical treatment to reverse partially photoreceptor cell death (Figure 5.2B) (p-value < 0.05). This result means that a higher amount of photoreceptor cells remains in the neuroretina and the photoreceptor cell death rate decrease with PG topical treatment.

Gliosis is a repose to any kind of damage of any tissue, including the retina. Recruitment of glial cells to the damaged area was proved in several retinal degenerative

diseases as RP, diabetic retinopathy, glaucoma, retina detachment and in several mice models. In the retina, the most well-known gliosis marker is the upregulation of GFAP expression by Müller cells [29,30]. Our results show that topical treatment with PG partially inhibits GFAP expression (Figure 5.3B) (p-value < 0.05).

These results point to the conclusion that PG local treatment can decrease the rate of photoreceptor cells in the rds mice model and, therefore, delay RP progression and preserve visual function longer in time.

As there are no statistical differences between data obtained between the eye contralateral to the one receiving PG treatment (Blank PG) and those receiving the vehicle (Control) or no treatment (Blank) it can be concluded that PG local treatment in one eye does not have an effect in the contralateral eye, suggesting that there is not a cross of the blood-retinal barrier or distribution of sufficient PG amounts through the systemic circulation to to trigger a response.

Despite there is not any previous research project in which rds mice have been treated with PG, oral treatment in the rd1 and rd10 RP mice model has proved the capability of RP in delaying photoreceptor cell death. In the case of rd10 oral PG treatment induced a decrease in TUNEL positive cells of 50% in three studied areas of the retina (far periphery, mid periphery and central retina), but only in the far periphery there was a statistically significant difference [7]. In this study we achieve a similar TUNEL expression reduction (45.21%) proving the capability of PG to decrease cellular death in a similar amount as the other experiments done before. Regarding GFAP expression there is not any difference in rd10 PG oral treatment compared with the rd10 untreated group [7]. Nevertheless, results of oral PG treatment in the rd1 mice model in reports done by Ramirez-Lamelas [8] and Sanchez-Vallejo [15] prove that the treatment delays the reactive gliosis in RP. In our case, GFAP expression decrease as it is shown in Figure 5.3, verifying the previous results of PG oral treatment.

Concerning the number of ONL nuclear rows, results of oral PG treatment in rd1 mice show that the treatment has not any effect at the first studied age (PN11) but preserve ONL nuclear rows at longer ages (PN13 and PN15). [15]. Despite the fact, that our result does not show any effect at this level, it would be interesting to explore in future projects the effect of PG eye topical treatment in the rds mice model at longer ages.

Recently, our group has studied different formulations of PG for topical delivery to the eye, including eye solution and inserts with cyclodextrins and micelles of soluplus and pluronic with pure PG [20]. To select the best ocular formulation for topical

administration of PG all the results obtained in *ex vivo* diffusion PG studies carried out in rabbit eyes have been re-analyzed. In the diffusion experiments previously performed with the inserts, the concentration of PG in the donor compartment decreased with time [16]. The PG in the inserts was less than 90%, exactly 55% and 30% when the membranes were cornea and sclera, respectively. We considered less accurate to calculate permeability coefficients assuming constant concentrations in the donor compartment and for this reason equation 1 (see Material and methods) was used. This equation considers that there is a continuous change in the concentrations in the donor compartment and is valid under sink or non-sink conditions and therefore it allowed us to compare all the data obtained from previous studies. We re-calculated the P_{eff} from all data obtained in previous studies performed with drops and micelles [13,14]. Figure 5.5 shows the P_{eff} of PG calculated with the different PG formulations through the corneas and sclera of rabbits. These formulations were prepared with CD (solution and inserts) and micelles of Soluplus and Pluronic with pure PG.

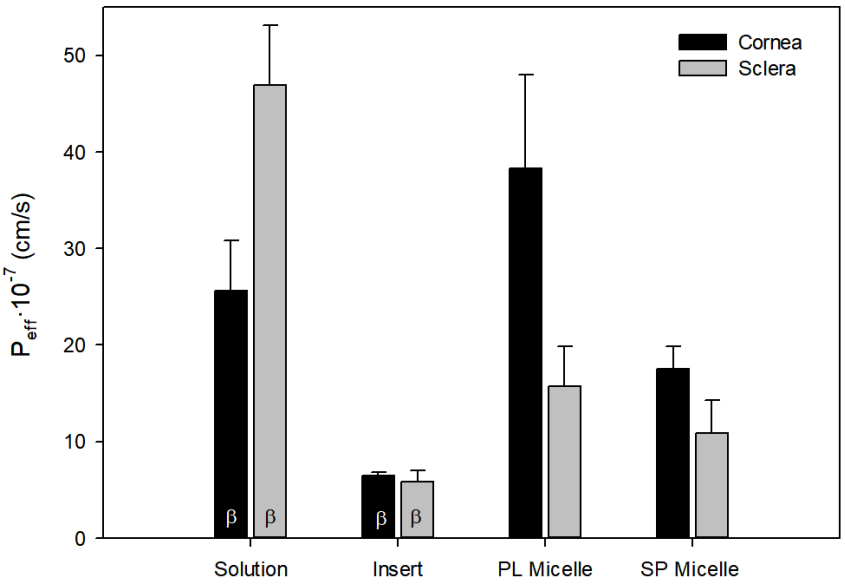

Figure 5.5. Apparent permeation coefficients (P_{eff}, cm/s) of PG, in the cornea and sclera of rabbits, were obtained with different PG ocular formulations. β means that formulations were prepared with CD (solution and inserts) and micelles of Soluplus and Pluronic with pure PG.

Ocular formulations of PG showed significant differences in P_{eff} values (Figure 5.5). On the one hand, comparing liquid formulations solution and micelles, solution showed higher permeability compared to micelles in the sclera ($p<0.05$). Polymeric micelles are capable of encapsulating hydrophobic drugs such as PG. The amphiphilic excipients used in micelles can facilitate the permanence of the formulation on the ocular

surface compared to solution and can inhibit efflux pumps in the corneal tissue [31]. Micelles can facilitate drug penetration through the cornea and sclera, providing therapeutic drug levels at the back of the eye [22,32]. Pluronic micelles provided higher permeability coefficients than Soluplus micelles, which may be related to their size of a few nanometres [20]. However, the polymeric micelles studied do not provide higher PG permeability than solution in the sclera and only Pluronic micelles provided higher permeability coefficients than solution in the cornea ($p<0.05$). This may be explained by the presence of cyclodextrins, which have been shown to increase drug permeability [19]. Both solutions and micelles have the disadvantage that they remain in contact with the ocular membrane for a short time before they are completely removed by blinking and tearing. Approximately 90% of drug formulations for ophthalmic administration are eye drops, mainly due to their ease of administration and excellent acceptance by patients [33]. In contrast, the bioavailability of drugs administered topically in drops is less than 5% [34–36], as defence mechanisms such as lacrimation or tear turnover are activated, which dilute the drug and remove it from the ocular surface. Excess volume is drained through the nasolacrimal duct into the systemic circulation [1,6,37]. Additionally, ocular solutions are primarily intended for the treatment of affections to the anterior segment of the eye.

Finally, PG inserts showed lower permeability compared to solution and micelles (figure 5.5), but the advantages of eye inserts compared to liquid formulations are numerous. The main advantage of inserts is that they increase the contact time of the formulation, increasing drug availability and allowing controlled release. Thus, effective drug concentration in the eye can be ensured for a longer period than with drops. Drug dosing is also more precise, and the risk of systemic side effects is reduced. In addition, the inserts have a longer shelf life and the presence of additives such as preservatives is not necessary [38]. The constant contact of the insert with the ocular membranes suggests that it would be capable of transporting PG in greater amounts to neuroretina, albeit more slowly than solution or micelles. This is demonstrated by biodistribution studies in pig eyes performed by our research group, in which, at the same initial concentration, the solution delivered 2.63 ± 1.08 µg of PG to neuroretina compared with 66.32 ± 29.52 µg delivered by the insert [data pending publication]. Furthermore, in that study, the relationship between increased PG concentration in the formulation and increased amounts of PG achieved in neuroretina has been demonstrated, so there is a correlation between the concentration in the insert and the amount of PG reaching the neuroretina. Therefore, although in the *in vivo* studies described in this paper we have resorted to PG solution because of the small size of eyes in the newborn mice used as

a model, PG inserts are nevertheless a very interesting ocular formulation for topical application of PG.

5. Conclusions

The results shown demonstrate that CD-PG solution applied topically in drops to the eye has a beneficial effect on delaying photoreceptors death and gliosis in the rds mice model. Furthermore, the lack of any effect on the PG-Blank shows that there is no systemic effect from the topically administered PG to one eye on the contralateral one. These results that the feasibility of using topically administered PG to the eye as a possible treatment for retinal degeneration. Although the PG solution provided greater permeability through the sclera and the Pluronic F68 micelles through the cornea, the insert is considered to be the best ocular formulation tested as it releases PG in a controlled manner.

6. References

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Resumen

La retinosis pigmentaria (RP) representa a un conjunto de enfermedades degenerativas, que cursan con la degeneración de las células fotorreceptoras de la retina, conos y bastones. Es una enfermedad de origen genético, heredable y que padece una de cada 4000 personas, afectando aproximadamente a 1,5 millones de personas en todo el mundo. La RP cursa, principalmente, con una pérdida inicial de la visión nocturna, para posteriormente verse reducida la visión periférica y por último la visión central. Esta pérdida progresiva de visión se debe a la paulatina muerte de los fotorreceptores. En la progresión de la RP, cuando los bastones mueren, el consumo de oxígeno en la retina disminuye y, por ello, la concentración de oxígeno en la retina aumenta. La hiperoxia que se produce induce el daño oxidativo. Es por ello que, el tratamiento con antioxidantes, que disminuyen el estrés oxidativo, se considera como un posible modo de frenar el proceso degenerativo en la RP. De hecho, se ha demostrado que el tratamiento con antioxidantes en modelos animales de RP (rd1, rd10 y rds) retrasa la muerte de fotorreceptores.

Recientemente se ha estudiado el papel de las hormonas esteroideas sexuales y sus metabolitos como supresores de la muerte celular frente al estrés oxidativo. La progesterona (PG) es una hormona esteroidea que, en estudios previos, ha demostrado su capacidad inhibitoria de apoptosis e inflamación. Asimismo, es capaz de reducir el daño causado por los radicales libres en concentraciones altas tras una lesión cerebral traumática en ratas. Por ello, podría tener un potencial terapéutico para el tratamiento de diversas enfermedades degenerativas como la RP. De hecho, en estudios realizados en el modelo de ratones rd10, se ha demostrado que la administración oral de PG, 150 mg/kg, desde el día 15 hasta el 21 postnatal, disminuye la muerte celular de los fotorreceptores. El mecanismo de acción del tratamiento con PG sigue sin estar totalmente elucidado, pero se sugiere que una de las vías de acción de la PG podría estar relacionada con sus efectos antioxidantes en la retina. En este sentido, se demostró que el tratamiento con PG disminuye la cantidad de peroxidación lipídica. Otros estudios mostraron resultados similares en el modelo de ratón rd1 tratado con PG administrada por vía oral, a dosis menor (100 mg/kg), a partir del día postnatal 7 en días alternos. El efecto antioxidante fue el mecanismo de acción propuesto para la PG, tanto en la administración oral en el estudio realizado en el modelo de ratón rd10 como en rd1.

Sin embargo, la administración oral de PG presenta inconvenientes debido a las características de la molécula. Es una molécula muy lipófila (log P = 3,9) que se considera prácticamente insoluble en agua y se disuelve lenta e incompletamente en los fluidos gastrointestinales. La PG se inactiva rápidamente cuando se administra por vía

oral. Tiene un amplio metabolismo hepático e intestinal y su semivida plasmática es de 25 minutos. Su biodisponibilidad oral es limitada, se necesitan administrar altas concentraciones de PG para que una pequeña cantidad llegue hasta el ojo. Por ello, es importante estudiar si es posible administrar la PG por vía tópica, localmente en el ojo y minimizar la concentración a administrar.

En el trabajo de Tesis Doctoral que se presenta se han analizado diferentes estrategias para la posible administración ocular tópica administrar la PG oftálmicamente a nivel local. Se investigó el uso de ciclodextrinas, capaces de incrementar su solubilidad y aumentar la permeabilidad de PG, así como la encapsulación en micelas poliméricas, que facilitan el desarrollo de formulaciones acuosas aumentando la permanencia y la difusión de las moléculas en la superficie ocular. Por último, también se han estudiado insertos oculares, que aumentan el tiempo de contacto del fármaco con la superficie ocular, incrementan su biodisponibilidad y permiten una liberación controlada, dosificación precisa y una menor frecuencia de administración.

Para ello, se plantearon en el trabajo de Tesis Doctoral los siguientes objetivos específicos: (1) validar un método cromatográfico para poder cuantificar la PG; (2) determinar la permeabilidad *ex vivo* de la PG en solución a través de córnea y esclera de conejo; (3) elaborar micelas de PG que puedan superar las limitaciones de solubilidad y permeabilidad del fármaco cuando se destinan al tratamiento óptico-local de la RP y comparar la difusión del fármaco a través de la córnea y la esclera de tres especies animales (conejo, porcino y vacuno), además de estudiar su irritabilidad ocular; (4) elaborar y caracterizar un inserto ocular para administrar PG, estudiar su posible irritabilidad ocular, estudiar la liberación de PG del inserto y analizar la difusión *ex vivo* de PG a través de córnea y esclera de conejo; (5) determinar la biodistribución ocular de la PG, utilizando gotas e insertos en ojos de porcino y, (6) determinar el posible efecto terapéutico de la administración ocular de PG *in vivo* en ratones rds, un modelo experimental de RP.

En primer lugar, se puso a punto y se validó un método de cromatografía líquida HPLC, para detectar PG incorporada a β-ciclodextrina, utilizando una columna de fase inversa Waters Sunfire C18 (150 × 4,6 mm), empaquetada con partículas de sílice de 5 µm, y como fase móvil una mezcla de acetonitrilo (ACN) y agua de calidad HPLC (80:20, v/v), a pH 7,4. El tiempo de retención de la PG fue de 3,42 minutos el límite de detección y el límite de cuantificación fue de 0,42 y 1,26 µg/mL, respectivamente. La curva de calibración mostró una linealidad excelente en el rango de concentraciones de 0,5 µg/mL

a 100 µg/mL. Con el fin de analizar la aplicabilidad del método, se llevaron a cabo experimentos *ex vivo*, en celdas de difusión, para cuantificar la permeabilidad de la PG a través de la córnea y la esclera de conejo. Los experimentos *ex vivo* demostraron que se pueden cuantificar las cantidades acumuladas de PG que difunden por cm^2 de membrana (Q = 6,57 ± 0,37 µg/cm^2 a través de la córnea y Q = 8,13 ± 0,85 µg/cm^2 en esclera; media ± DE, n=6), así como las cantidades de PG retenida en cada tejido, (Q = 40,87 ± 9,84 µg/cm^2 en córnea y Q = 56,11 ± 16,67 µg/cm^2 en esclera; media ± DE, n=6).

Las micelas de Pluronic F68 (3-4 nm) y Soluplus (52-59 nm) elaboradas aumentaron la solubilidad de la PG en uno y dos órdenes de magnitud respectivamente, y exhibieron una eficiencia de encapsulación cercana al 100%. Los sistemas Soluplus mostraron capacidad de gelificación *in situ* en contraste con las micelas Pluronic F68 de baja viscosidad. Las formulaciones mostraron ausencia de irritación ocular en la prueba en membrana corioalantoidea de huevo de gallina (HET-CAM). Se realizaron estudios de permeabilidad *ex vivo* en celdas de difusión, utilizando diferentes especies animales; bovino, porcino y conejos, y se cuantificó la PG en las muestras obtenidas mediante HPLC. La difusión de PG a través de la córnea y la esclera del conejo fue más rápida que a través de la córnea porcina o bovina, aunque las diferencias también dependieron de la formulación. La permeabilidad de la PG en los ojos del cerdo fue intermedia entre las permeabilidades encontradas para conejo y bovino. Las micelas de Soluplus permitieron una mayor acumulación de PG en la córnea y la esclera, mientras que las micelas de Pluronic F68 proporcionaron una difusión más rápida con dosis más bajas de PG.

Se formularon diferentes insertos con PG en su composición y se analizaron sus características: grosor, peso, translucidez, pérdida y ganancia de humedad, resistencia a la rotura, elongación, uniformidad de contenido, presencia de imperfecciones microscópicas y liberación de fármaco desde el inserto. Se seleccionó el inserto con las mejores características que tenía la siguiente composición: 59% alcohol polivinílico, 39% polivinilpirrolidona K30 y 2% propilenglicol. Se realizaron estudios *ex vivo* de difusión con el inserto, en celdas de difusión, utilizando córnea y esclera de ojos de conejos. La cuantificación de PG en las muestras obtenidas en los estudios de difusión se realizó por HPLC. El estudio realizado puso de manifiesto que se producía una difusión controlada de la PG. La difusión de la PG a través de la córnea y de la esclera se calculó utilizando un tratamiento matemático (capítulo 4, ecuación 7) que considera cambios continuos en las concentraciones del compartimento dador. La difusión obtenida es similar (P_{app} corneal = 6,46 ± 0,38 × 10^{-7} cm/s y P_{app} escleral = 5,87 ± 1,18

× 10^{-7} cm/s, media ± DE, n=5). Sin embargo, la cantidad de PG acumulada en las escleras fue significativamente superior a la acumulada en las córneas (Q = 30,07 ± 9,09 µg/cm^2 y Q = 15,56 ± 4,36 µg/cm^{2,} respectivamente). Los insertos elaborados con PG (55,6 µg/cm^2) eran delgados, translúcidos, no producían irritación ocular (prueba HET-CAM) y eran elásticos y resistentes, propiedades que hacen que esta formulación sea adecuada para su uso potencial en el tratamiento de enfermedades oculares.

Una vez estudiadas las distintas formulaciones oftálmicas para la administración tópica de PG (solución e insertos con ciclodextrinas y micelas de Soluplus y Pluronic F68 con PG pura), se analizaron en conjunto los resultados obtenidos en los estudios de difusión *ex vivo* de PG en los ojos de conejo, con el fin de seleccionar la mejor formulación ocular para administrar PG tópicamente. Para comparar todas las formulaciones ensayadas, se utilizó el mismo tratamiento matemático utilizado con los insertos de los datos obtenidos en los estudios de difusión previos (solución y micelas), ya que aquella ecuación es válida tanto para las condiciones “sink” (utilizadas en los experimentos realizados con soluciones y micelas) como “no sink” (condiciones de los experimentos realizados con los insertos). De este modo se calcularon los coeficientes de permeabilidad aparentes de la PG (P_{eff}) con el fin de comparar todos los resultados obtenidos (Tabla Resumen 1).

Tabla Resumen 1. Coeficientes de permeabilidad aparentes (P_{eff}, cm/s) de PG en córnea y esclera de conejos, obtenidos a partir del tratamiento matemático.

Formulación	Córnea (P_{eff}·10^{-7} cm/s)	Esclera (P_{eff}·10^{-7} cm/s)
Solución	25,6 ± 5,26	46,9 ± 6,16
Micelas Soluplus	15,3 ± 2,52	8,66 ± 2,86
Micelas Pluronic F68	34,7 ± 9,49	13,7 ± 3,97
Inserto	6,46 ± 0,38	5,87 ± 1,18

Como se observa en la Tabla Resumen 1, la solución de PG con ciclodextrinas proporcionó una penetración de la PG significativamente superior a través de la esclera en comparación con el resto de las formulaciones. Sin embargo, fueron las micelas poliméricas de Pluronic F68 las que proporcionaron coeficientes de permeabilidad de la PG más elevados a través de la córnea. Si se analizan en conjunto los resultados obtenidos se observa que cada formulación tiene sus ventajas: las ciclodextrinas son capaces de incrementar la solubilidad y la permeabilidad de la PG en la solución oftálmica, mientras que las micelas poliméricas son capaces de encapsular fármacos hidrófobos, como la PG, y facilitan la permanencia de la formulación en la superficie ocular en comparación con la solución. Las micelas de Pluronic proporcionaron coeficientes de permeabilidad de PG significativamente superiores a los de las micelas Soluplus, lo que puede ser debido a su pequeño tamaño (Pluronic, 3 - 4 nm frente a

Soluplus, 52 - 59 nm). Tanto la administración de la PG en solución como en micelas tiene la desventaja de que permanece en contacto con la membrana ocular durante un corto espacio de tiempo antes de que se elimine por completo mediante el parpadeo. Finalmente, se demostró que la permeabilidad de la PG administrada en forma de inserto era menor en comparación con las gotas y micelas, pero las ventajas de los insertos oculares en comparación con las formulaciones líquidas son numerosas. El inserto puede incorporar gran cantidad de PG y es capaz de liberarla de manera controlada y sostenida en el tiempo, permitiendo que se pueda garantizar durante un período de tiempo más largo la concentración eficaz de fármaco en comparación con la solución y las micelas. La dosificación del fármaco en los insertos también es más precisa y se reduce el riesgo de efectos secundarios. Además, los insertos tienen una vida útil más larga y no es necesaria la adición de conservantes a la formulación. Sin embargo, la ventaja principal de los insertos es que aumentan el tiempo de contacto de la formulación, aumentando la biodisponibilidad del medicamento. El contacto constante y prolongado del inserto con las membranas oculares sugiere que sería capaz de difundir mayor cantidad de PG a la neurorretina, aunque más lentamente que las gotas o las micelas.

Para investigar si la PG administrada tópicamente por vía oftálmica es capaz de llegar a la neurorretina, se estudió su distribución a través de los tejidos oculares. Asimismo, se analizó el efecto de la concentración inicial de PG en la formulación, así como el efecto del aclaramiento lagrimal. Se prepararon diferentes formulaciones de PG: gotas (solución de PG de concentración 1 mg/mL), inserto córneo-escleral (de concentración de PG 325,7 $\mu g/cm^2$) y 3 insertos esclerales (de concentración de PG 32,57, 325,7 y 3257 $\mu g/cm^2$, respectivamente). Las diferentes formulaciones se administraron tópicamente en la superficie ocular utilizando ojos de porcino enteros. Tras congelar el ojo durante 12 horas a -80 °C, se diseccionaron las diferentes estructuras anatómicas del ojo y se extrajo la PG de cada fracción en acetonitrilo durante 12 horas. La cuantificación de PG en cada tejido se realizó mediante UHPLC-MS/MS. Se demostró que, tras la administración tópica, la PG se difunde desde la superficie ocular y se distribuye por todas las estructuras oculares, incluida la neurorretina. Se encontraron niveles más bajos de PG en la esclera, la coroides y los tejidos neurorretinianos cuando se administró la PG en gotas que cuando la administración se realizó mediante insertos. Solo el inserto corneo-escleral mostró diferencias estadísticamente significativas en las cantidades de PG en la córnea en comparación con las gotas, probablemente debido al mayor tiempo de contacto del fármaco con la superficie ocular cuando se aplica en el inserto. Un aumento de las

concentraciones iniciales de PG aplicadas proporcionó un incremento estadísticamente significativo de las cantidades de PG en humor acuoso, esclera, coroides y neurorretina. Además, los resultados obtenidos en la simulación de la eliminación mediante lagrimeo, utilizando el inserto ocular con o sin un goteo constante de solución salina sobre la córnea, 1,2 µL/min durante 24 h, no revelaron diferencias estadísticamente significativas, por lo que las lágrimas parecen no afectar a la cantidad de PG en neurorretina.

Como se ha descrito anteriormente la RP desencadena una cascada de trastornos retinianos que conducen a la muerte de los fotorreceptores. Para estudiar el efecto de la PG sobre la muerte de estos fotorreceptores, se ha estudiado en un modelo animal de RP (ratón rds) si la administración tópica ocular de PG incorporada en ciclodextrinas es capaz de disminuir la muerte de los fotorreceptores. En el estudio realizado, un grupo de ratones rds recibió una gota de PG-βCD, cada 12 h, durante 10 días en un ojo, el izquierdo, mientras que el ojo derecho se dejó sin tratar. Otro grupo control de ratones rds fue tratado con solución salina en el ojo izquierdo, y de nuevo el ojo derecho se dejó sin tratar. Tras el tratamiento, los animales fueron sacrificados en el día 21 postnatal. Se realizaron tinciones de TUNEL, GFAP y DAPI. La administración de PG-βCD disminuyó significativamente la muerte celular en las retinas de los ratones rd1 tratados con PG, así como la inflamación. Solo se encontraron diferencias estadísticamente significativas en el ojo tratado, lo que demuestra que no hay un efecto sistémico de la PG. Se ha demostrado que el tratamiento con PG-βCD disminuye la muerte de fotorreceptores en las primeras etapas de la RP, pudiendo retrasar la pérdida de visión y disminuir la inflamación.

Los resultados obtenidos en este trabajo demuestran que la PG puede incorporarse en diversas formulaciones oculares como soluciones, micelas e insertos. Además, la PG puede administrarse tópicamente en el ojo, difunde, permea y se distribuye en el ojo en cantidades suficientes para llegar a la neurorretina, reducir la muerte de los fotorreceptores en el modelo animal rds y, por tanto, retrasar la perdida visual. Además, la PG mostró un perfil citotóxico adecuado, al no producir irritación ocular en estudios HET-CAM. Los resultados aquí recopilados demuestran, por primera vez, la viabilidad de la administración ocular tópica de la PG y abren la posibilidad de seguir investigando estas formulaciones como una nueva estrategia terapéutica para los pacientes con RP, y por extensión, para pacientes con otras enfermedades oculares en las que el estrés oxidativo sea un factor de riesgo, como glaucoma, degeneración macular asociada a la edad, edema macular secundario a la oclusión venosa de la retina, retinitis por citomegalovirus, uveítis posterior y retinopatía diabética.

Abstract

The term retinitis pigmentosa (RP) is used to describe a group of degenerative diseases, which involve degeneration of the photoreceptor cells of the retina, cones and rods. It is an inheritable, genetic disease which is estimated to affect 1:4,000 people worldwide, approximately 1.5 million people. Initially RP manifests itself by a loss of night vision which is followed by a reduction in peripheral vision and finally central vision. This progressive loss of vision is due to the gradual death of photoreceptor cells. When the rods die as a result of RP progression, oxygen consumption in the retina decreases and therefore, the oxygen concentration in the retina increases. The resulting hyperoxia causes oxidative damage. Treatment with antioxidants to decrease oxidative stress, is considered to be a possible intervention to slow down the degenerative process in RP. In fact, antioxidant treatment in animal models of RP (rd1, rd10 and rds) has been shown to delay photoreceptor cell death.

The role of sex steroid hormones and their metabolites, as suppressors of cell death has recently been studied. Progesterone (PG) is a steroid hormone that has been shown in previous studies to inhibit apoptosis and inflammation. It is also able to reduce free radical damage at high concentrations of PG following traumatic brain injury in rats. It may therefore have therapeutic potential for the treatment of various neurodegenerative diseases such as RP. In fact, studies in the rd10 mouse model, have shown that oral administration of PG, 150 mg/kg, from postnatal day 15 to 21 decreases photoreceptor cell death. The mechanism of action of PG treatment remains unclear, but it has been suggested that it could be related to its antioxidant effects in the retina. PG treatment was shown to decrease the amount of lipid peroxidation. Other studies showed similar results in the rd1 mouse model treated with orally administered PG at a lower dose (100 mg/kg) from postnatal day 7 on alternate days. The proposed mechanism for oral administration of PG was the same as in the study conducted in the rd10 mouse model, its antioxidant effects.

Oral administration of PG has drawbacks due to the characteristics of the molecule. It is very lipophilic (log P = 3.9), practically insoluble in water and dissolves slowly and incompletely in gastrointestinal fluids. PG is rapidly inactivated when administered orally. It undergoes extensive hepatic and intestinal metabolism and has a plasma half-life of 25 minutes. Its oral bioavailability is limited, which implies that very high concentrations of PG needs to be administered for a small amount of the drug to be able to reach the eye. It is therefore important to study whether it is possible to administer PG topically, locally to the eye, and minimizing the concentration to be administered.

In our research, we have studied the incorporation of PG to various formulations

and different strategies have been analyzed to assess their possible administration topically to the eye. We have evaluated the use of cyclodextrins, capable of increasing PG solubility and increasing its permeability; as well as encapsulation in polymeric micelles, which facilitate the development of aqueous formulations and increase the permanence of molecules on the ocular surface and their diffusion into the eye.; We have also used ocular inserts, which increase the contact time of the drug with the eye, increasing its bioavailability, and allowing controlled release, precise dosage and less frequent administration.

The following specific objectives were set out: (1) to validate a chromatographic method to be able to quantify PG (2) to determine the *ex vivo* permeability of PG in solution through rabbit cornea and sclera, (3) to elaborate PG micelles that can overcome the solubility and permeability limitations of the drug when intended for the ocular local treatment of RP by assessing the diffusion of the PG-loaded micelles through the cornea and sclera of three animal species (rabbit, pig and bovine) and to study their ocular irritation potential, (4) to develop and characterize an ocular insert to administer PG, by evaluating its ocular irritation potential and to analyze the *ex vivo* the release of PG from the insert and its diffusion through rabbit cornea and sclera, (5) to determine the ocular biodistribution of PG, using solution and inserts, in pig eyes and (6) to determine the possible therapeutic effect of ocular administration of PG *in vivo* in rds mice, an experimental model of RP.

A high-performance liquid chromatography method to detect PG incorporated into β-cyclodextrin was developed and validated using a Waters Sunfire C18 reversed-phase column (150 × 4.6 mm) packed with 5 µm silica particles and a mixture of acetonitrile (ACN) and pure water 80:20 (v/v) at pH 7.4 as mobile phase. The limit of detection and limit of quantification of PG were 0.42 and 1.26 µg/mL, respectively. The calibration curve showed excellent linearity in the concentration range of 0.5 µg/mL to 100 µg/mL, with a retention time of 3.42 minutes. To analyze the applicability of the method, *ex vivo* experiments were carried out in diffusion cells to quantify the permeability of PG across rabbit cornea and sclera. The *ex vivo* experiments demonstrate their applicability to quantify the cumulative amounts of diffused PG per cm^2 of membrane ($Q = 6.57 \pm 0.37$ µg/cm^2 across the cornea and $Q = 8.13 \pm 0.85$ µg/cm^2 in sclera; mean ± SD, n=6), as well as the amounts of PG retained in each tissue, ($Q = 40.87 \pm 9.84$ µg/cm^2 in cornea and $Q = 56.11 \pm 16.67$ µg/cm^2 in sclera; mean ± SD, n=6).

Pluronic F68 (3-4 nm) and Soluplus (52-59 nm) micelles increased PG solubility by one and two orders of magnitude, respectively and exhibited close to 100%

encapsulation efficiency. The Soluplus systems showed *in situ* gelling ability in contrast to the low-viscosity Pluronic F68 micelles. The formulations showed no irritation in the hen's egg test chorioallantoic membrane (HET-CAM) test method. *Ex vivo* permeability studies were performed in diffusion cells, using eyes from different animal species; cow, pig and rabbit, PG was quantified by HPLC from the samples obtained. PG penetration through rabbit cornea and sclera was faster than through the porcine or bovine cornea, although the differences also depended on the formulation used. PG permeability in pig's eyes was intermediate between the permeability found in rabbit and bovine. Soluplus micelles allowed a higher accumulation of PG in cornea and sclera, while Pluronic F68 micelles provided faster penetration of lower doses of PG.

Different inserts were formulated with PG in their composition and their characteristics were analyzed: thickness, weight, translucency, moisture loss and gain, tear resistance, elongation, PG content uniformity, presence of microscopic imperfections and drug release from the insert. The insert with the best characteristics was selected, which was a polymer made up with 59% polyvinyl alcohol, 39% polyvinylpyrrolidone K30 and 2% propylene glycol. *Ex vivo* diffusion studies were performed with the insert in Franz cells using rabbit cornea and sclera. The quantification of PG in the samples obtained in diffusion studies was performed by HPLC. The study showed sustained diffusion of PG. The diffusion of PG through the cornea and sclera was calculated using a mathematical treatment (chapter 4, equation 7) that considers continuous changes in donor compartment concentrations. The diffusion obtained was similar (corneal P_{app} = 6.46 ± 0.38 × 10^{-7} cm/s and scleral P_{app} = 5.87 ± 1.18 × 10^{-7} cm/s, mean ± SD, n=5). However, the amount of PG accumulated in the scleras was significantly higher than in the corneas (Q = 30.07 ± 9.09 μg/cm^2 and Q = 15.56 ± 4.36 μg/cm^2, respectively). The inserts made with PG (55.6 μg/cm^2) were thin, translucent, did not cause ocular irritation (HET-CAM test) and were elastic and resilient, properties that make this formulation suitable for potential use in the treatment of ocular diseases. Once the different ophthalmic formulations for topical administration of PG (eye solution and inserts with cyclodextrins and micelles of Soluplus and Pluronic F68 with pure PG) had been studied, the results obtained in *ex vivo* diffusion studies of PG in rabbit eyes were analyzed together in order to select the best ocular formulation for topical PG administration.

To compare all the formulations tested, the data obtained in the previous diffusion studies (solution and micelles) have been used. And re-analysed using the same mathematical treatment used for the sinserts. As the equation is valid for both "sink" (solution and micelles) and "non-sink" (inserts) conditions. The apparent

permeability coefficients of the PG were thus calculated (table 1) and all data obtained were compared.

Summary Table 1. Apparent permeability coefficients (P_{eff}, cm/s) of PG in rabbit cornea and sclera, obtained from the mathematical treatment using equation 7 from chapter 4.

Formulation	Cornea ($\cdot 10^{-7}$ cm/s)	Sclera ($\cdot 10^{-7}$ cm/s)
Solution	25.6 ± 5.26	46.9 ± 6.16
Soluplus micelles	15.3 ± 2.52	8.66 ± 2.86
Pluronic F68 micelles	34.7 ± 9.49	13.7 ± 3.97
Insert	6.46 ± 0.38	5.87 ± 1.18

The eye solution showed a higher scleral permeability compared to the other formulations. However, higher permeability coefficients across the cornea were obtained with Pluronic F68 polymeric micelles. Each formulation has its advantages, cyclodextrins can increase the solubility and permeability of PG in eye solution, while polymeric micelles are able to encapsulate hydrophobic drugs such as PG and facilitate the permanence of the formulation on the ocular surface compared to solution because of their higher viscosity. The Pluronic micelles provided higher permeability coefficients than the Soluplus micelles, which may be due to their smaller size (Pluronic, 3 - 4 nm vs. Soluplus, 52 - 59 nm). However, both solution and micelles have the disadvantage that they remain in contact with the ocular membrane for a short time before they are completely removed by blinking. Finally, the PG insert showed lower permeability compared to solution and micelles, but the advantages of eye inserts compared to liquid formulations are numerous. Inserts have higher PG-loading capacity of the insert and is able to provide a sustained release, allowing the effective drug concentration to be guaranteed for a longer period of time, compared to solution and micelles. Drug dosage in the inserts is also more accurate and the risk of side effects might be reduced. In addition, the inserts have a longer shelf life and the addition of preservatives is not necessary. But the main advantage of inserts is that they increase the contact time of the formulation, increasing the availability of the drug. The constant contact of the insert with the ocular membranes suggests that PG would be released and transported in greater amounts to the neuroretina, albeit more slowly than solution or micelles.

To investigate whether topically administered PG via the ophthalmic route can reach the neuroretina, its distribution through ocular tissues was studied. The effect of the initial concentration of PG in the formulation was also analysed, as well as the effect of tear clearance. Different formulations of PG were prepared: solution (1 mg/mL), corneo-scleral insert (325.7 µg/cm^2) and 3 scleral inserts (32.57, 325.7 and 3257

μg/cm^2). The different formulations were administered topically to the ocular surface using whole porcine eyes. After freezing the eye for 12 hours at -80 °C, the different anatomical structures of the eye were dissected, and PG was extracted from each tissue fraction in acetonitrile for 12 hours. Quantification of PG in each tissue was performed by UHPLC-MS/MS. The results revealed that, after topical administration, PG diffuses from the ocular surface and it is distributed throughout all ocular structures, including the neuroretina. Lower levels of PG were found in the sclera, choroid and neuroretinal tissues when PG was applied as solution compared to inserts. Only the corneo-scleral insert showed statistically significant differences in the amounts of PG in the cornea compared to solution, probably due to the longer contact time of the drug with the ocular surface when applied by means of the insert. An increase in the initial concentration of PG applied, provided a statistically significant increase in the amounts of PG in the aqueous humour, sclera, choroid and neuroretina. In addition, the results obtained in the simulation of PG elimination by tears, using the ocular insert with or without a constant drip of saline over the cornea, 1.2 μL/min for 24 h, showed there were no statistically significant differences, therefore we can conclude that tears do not affect the amount of PG reaching the neuroretina.

As described above, RP triggers photoreceptor death. To study the effect of PG on the death of these photoreceptors, we have studied in an animal model of RP (rds mice) whether topical ocular administration of PG incorporated to cyclodextrins is able to decrease photoreceptor death. In the study, one group of rds mice received one drop of PG-βCD every 12 h for 10 days in one eye, the left eye, while the right eye was left untreated. Another control group of rds mice was treated with saline in the left eye, and again the right eye was left untreated. After treatment, the animals were euthanized on postnatal day 21. TUNEL, GFAP and DAPI staining were performed. Administration of PG-βCD significantly decreased cell death in the retinas of PG-treated rds mice, as well as inflammation. Statistically significant differences were only found in the treated eye, thus revealing that there is no systemic effect from topically administered PG. There seems to be evidence that PG-βCD treatment decreases photoreceptor death in the early stages of RP, which may delay vision loss and decrease inflammation.

The data obtained in this work demonstrate that PG can be incorporated into various eye formulations such as eye solution, micelles and inserts. In addition, PG administered topically to the eye, diffuses, permeates and is distributed within the eye in sufficient quantities to reach the neuroretina. It has also been shown that topical ocular administration of PG, reduces photoreceptor death in the rds animal model and thereby delays visual loss. In addition, PG exhibits no toxicity or irritation in HET-CAM studies.

Therefore, the data collected here demonstrate for the first time that PG can be administered topically and open the possibility to further investigate these formulations as a new therapeutic strategy for patients with RP, and by extension for patients with other eye diseases associated with oxidative stress such as glaucoma, age-related macular degeneration, macular edema secondary to retinal venous occlusion, cytomegalovirus, retinitis, posterior uveitis and diabetic retinopathy.

Conclusiones

A partir de los resultados obtenidos se pueden extraer las siguientes conclusiones:

1. Se ha validado de un método analítico por HPLC, rápido y sencillo, para la determinación de PG cuya especificidad, exactitud, precisión, robustez, límite de detección y límite de cuantificación, permiten la cuantificación de PG en muestras obtenidas de experimentos de permeación ocular *ex vivo*.

2. Se formularon micelas de PG con Soluplus y Pluronic que aumentaron la solubilidad de la PG y su dispersión, y además, mostraron una eficiencia de encapsulación de aproximadamente el 100%, que indica que la PG tiene una fuerte afinidad por el núcleo de la micela.

3. Las micelas de Pluronic proporcionaron valores de permeación de PG significativamente superiores a las micelas de Soluplus en conejo, porcino y bovino. Se observaron marcadas diferencias entre especies: la penetración de la PG a través de la córnea y de la esclera se produjo con mayor rapidez en los ojos de conejo que en los de porcino o bovino.

4. Se han formulado distintos insertos de PG de los cuales se seleccionó el inserto elaborado con alcohol polivinílico (59%), polivinilpirrolidona K30 (39%) y propilenglicol (2%). Este inserto es biocompatible, flexible, transparente y posee las propiedades mecánicas requeridas para su aplicación ocular.

5. El inserto libera la PG de forma controlada. Los estudios de difusión *ex vivo* realizados con el inserto mostraron que la permeabilidad de la PG a través de los tejidos esclerales y corneales es similar, pero la PG se acumula en mayor cantidad en la esclera que en la córnea.

6. Los resultados obtenidos en los ensayos HET-CAM evidenciaron que ninguna de las formulaciones estudiadas producía irritación ocular.

7. Mediante los estudios de biodistribución realizados se ha puesto de manifiesto que la PG es capaz de difundir desde la superficie ocular hasta la neurorretina tras su administración tópica mediante soluciones oculares o insertos corneo-esclerales o esclerales, siendo la difusión de la PG significativamente superior cuando la administración se realiza mediante insertos.

8. Se ha demostrado que a mayor concentración de PG en el inserto, mayor cantidad de PG llega a la neurorretina y que el aclaramiento de las lágrimas sobre el inserto no tiene un efecto limitante en la distribución de la PG a través del ojo.

9. Aunque la solución de PG con ciclodextrinas proporcionó mayor permeabilidad de la PG a través de la esclera, y las micelas de Pluronic F68 a través de la córnea, se considera que el inserto es la mejor formulación ocular de las ensayadas, ya que libera de forma sostenida la PG y proporciona cantidades superiores de fármaco en neurorretina.

10. La administración ocular tópica de solución de PG incorporada en ciclodextrinas retrasa la muerte de los fotorreceptores y disminuye el proceso inflamatorio en un modelo de ratón rds. Por ello, la administración ocular de PG se vislumbra como posible tratamiento para la fotodegeneración de enfermedades que cursan con estrés oxidativo, como la retinosis pigmentaria.

Conclusions

The following conclusions can be drawn from the results obtained:

1. A rapid and simple HPLC analytical method has been validated for the determination of PG. The specificity, accuracy, precision, robustness, limit of detection and limit of quantification of the method allow the quantification of PG in samples obtained from *ex vivo* ocular permeation experiments.

2. Soluplus and Pluronic micelles increased PG solubility and dispersion and additionally showed an encapsulation efficiency of approximately 100%, indicating that PG has a strong affinity for the micelle core.

3. Pluronic micelles provided superior PG permeability values than Soluplus in eyes of rabbit, pig and bovine. Marked diffusion differences were observed between species and in Soluplus and Pluronic micelles: diffusion through both cornea and sclera occurred more rapidly in rabbit eyes than in porcine or bovine eyes.

4. Different PG inserts have been formulated to select one [polyvinyl alcohol (59%), polyvinylpyrrolidone K30 (39%) and propylene glycol (2%)] that was biocompatible, flexible, transparent and had the required mechanical properties needed for its ocular application.

5. PG is released from the insert in a suitable manner. *Ex vivo* diffusion studies performed with the insert showed that PG diffuses similarly through scleral and corneal tissues, but PG accumulates in the sclera in higher quantities than in the cornea.

6. The formulations developed do not cause ocular irritation as the results from the HET-CAM test showed.

7. The biodistribution studies carried out show that PG is capable of diffusing from the ocular surface to the neuroretina after topical administration by means of eye solution or the corneo-scleral or scleral inserts. Greater diffusion was attained with the inserts.

8. It has been shown that the higher the concentration of PG in the insert, the more PG reaches the neuroretina and that the tear clearance over the insert does not have a limiting effect on the distribution of PG throughout the eye.

9. Although the cyclodextrin PG solution provided greater permeability of PG through the sclera and the Pluronic F68 micelles through the cornea, the insert is considered to be the best ocular formulation of those tested as it releases PG in a sustained manner and greater amounts of the drug can reach the neuroretina.

10. Topical ocular administration of solution with PG incorporated in cyclodextrins delays photoreceptor cell death and decreases the inflammatory process in the rds mouse model. Thus, ocular administration of PG is envisioned as a potential treatment for photodegeneration in diseases involving oxidative stress, such as retinitis pigmentosa.