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

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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.

KEY WORDS:

Progesterone, ocular insert, oxidative stress, retinitis pigmentosa, *ex vivo* diffusion studies, trans-corneal and trans-scleral drug delivery, HET-CAM assay.

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, $C_{21}H_{30}O_2$, 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 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.

102 Polymers were dissolved in 5 mL of water using a magnetic stirrer. Then, the
103 required amount of plasticizer was added and stirred for 12 h. Samples were sonicated at
104 80 MHz for 20 min in an ultrasonic water bath (Model 275T, Crest Ultrasonics Corp.,
105 Trenton, NJ, USA) to remove air bubbles [23]. A volume of 5 mL of the formulations
106 F03, F05-F08, F10 and F11 (table 1) were poured onto Petri dishes (50 mm diameter)
107 because of their liquid consistency in which they were allowed to dry [23–26]. The rest
108 of the formulations were laminated using a laminator device to a thickness of 0.6 mm
109 (utility model patent registration number U200502256) on a film support (3M-
110 Scotchpack^{TM} 9733 Backing Polyester Film Laminate). Inserts were left to dry in
111 darkness at room temperature for 24-48 h.

112 After an extensive review of polymers used for topical application, those showing
113 better results in previous studies with other molecules were chosen (tizanidine
114 hydrochloride, moxifloxacin hydrochloride, fluconazole and ofloxacin) [23–25,27]. The
115 percentages of the polymers were adjusted to make a selection based on preliminary
116 results.

117

118 **Table 1.** Polymers: methylcellulose (MC), polyvinyl alcohol (PVA), polyvinylpyrrolidone K30 (PVP-
119 K30), hydroxypropyl methylcellulose (HPMC) and sodium alginate (SA) (%, w/w), and plasticizer:
120 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

144 the break point was established at 5%. These parameters allowed to determine
145 approximately the resistance and elongation of the different inserts under evaluation [29].
146 Each strip was held between the two clamps of the dynamometer for analysis: the upper
147 clamp is mobile while the lower one is static. The strip was pulled at a rate of 100 mm/s.
148 The tensile strength and elongation at breaking point were calculated following *eq. 4 and*
149 *5* [29].

150 $$\text{Tensile strenght}\left(\frac{\text{N}}{\text{mm}^2}\right) = \frac{\text{Break force (N)}}{\text{Cross sectional area } (mm^2)} \ (eq.4)$$

151

152 $$\text{Elongation (\%)} = \frac{Increase\ in\ lenght\ at\ break\ point\ (mm)}{Original\ length\ (mm)} \cdot 100 \ (eq.5)$$

153

154 ***2.3.*** *Preparation and characterization of inserts with PG*

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

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

166 Scanning Electron Microscopy (SEM) was used to check the surface and internal
167 morphology of the insert. The SEM characterization of the selected insert was performed
168 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 ± 0.1ºC 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.

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

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

266 homoscedasticity, parametric tests (ANOVA followed by Tukey´s test for multiple
267 comparisons and Student *t*-test) or non-parametric testing (Mann-Whitney U-test) were
268 used as deemed appropriate. The confidence level was 95%. Statistical analysis was
269 carried out using SPSS 24.0.

270

271 ## 3. Results and discussion

272 ### ***3.1.*** *Development and characterization of the inserts*

273 Several inserts were prepared with various combinations of the different
274 polymers: HPMC, MC, PVP, PVA and SA (Table 1) but without incorporating PG. All
275 systems contained PGL or GL as plasticizers. All materials are biocompatible and may
276 possibly be suitable to be used on the eye surface [38–40].

277 After lamination, insert F01 was found to have a rough surface and inserts F02,
278 F05 and F06 were too brittle and could not be separated without breaking from the
279 lamination support. These inserts were discarded for further studies. Table 2 shows the
280 thickness, weight, and translucency values of the inserts. The inserts F09 and F11 were
281 discarded due to their high translucency. Furthermore, F10 was so thin that it wrinkled
282 easily, so was also eliminated. Inserts mentioned above were discarded for further studies
283 because they did not fulfil expected properties.

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

287 PVA has the ability to retain a large amount of liquid, which gives the insert
288 elasticity and structural integrity [41]. It also has other properties such as ease of
289 preparation, good adhesiveness, good mechanical properties, and excellent chemical
290 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 1), did not show the same characteristics.

Table 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	0.6 ± 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 1.

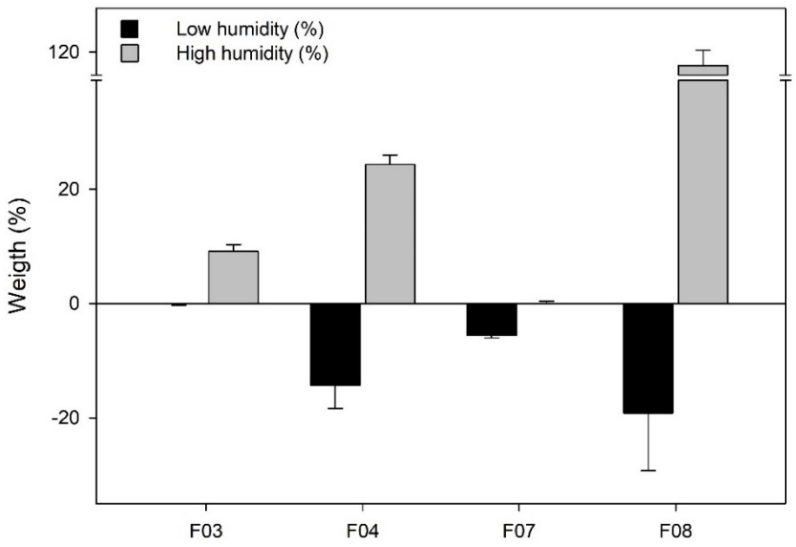


Figure 1. Percentage of weight lost and gained by the inserts after exposure to low and high humidity environments. Data are mean ± SD (n = 3).

The data obtained from the evaluation of the mechanical properties of the inserts, namely resistance and elongation, are shown in Figure 2A and 2B respectively.

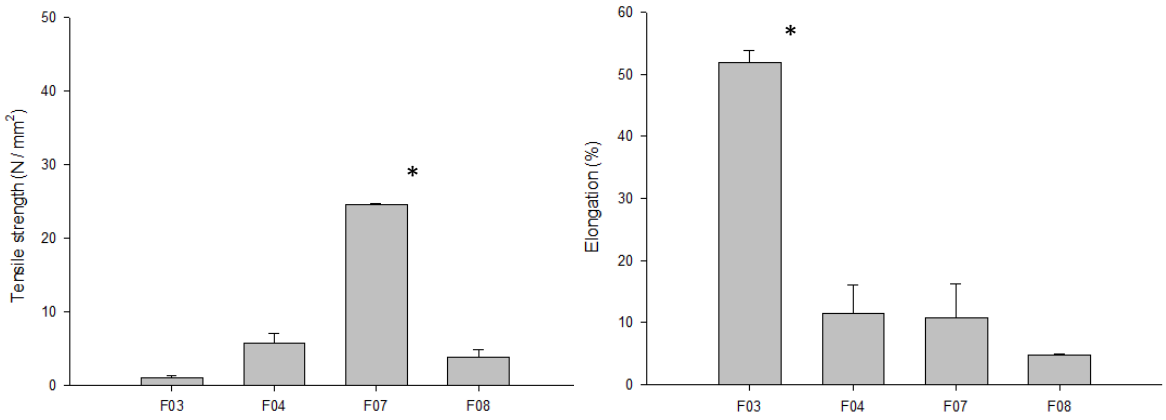
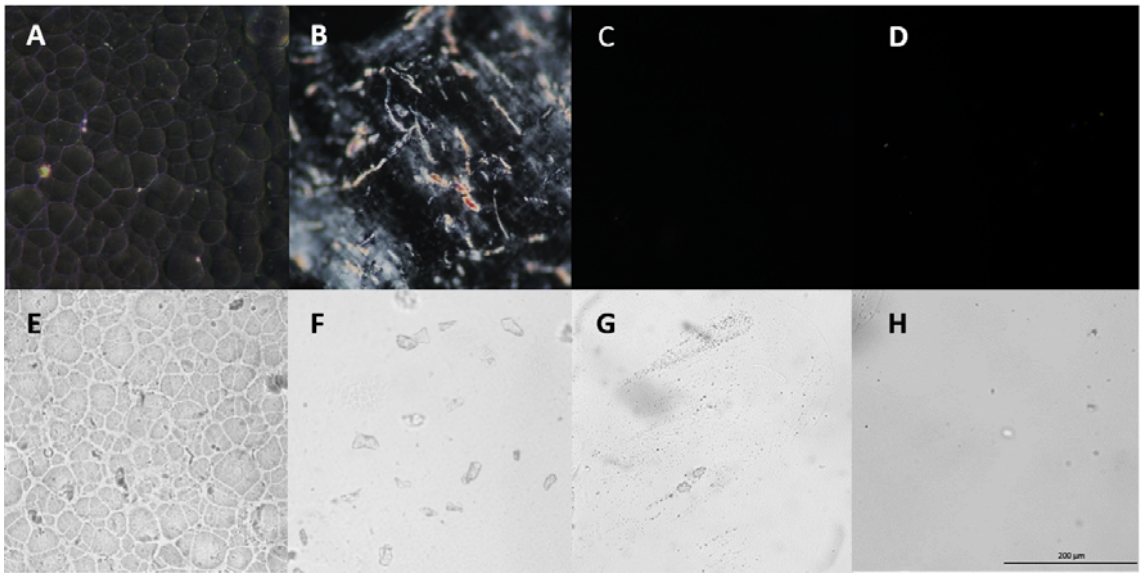


Figure 2. (A) Tensile strength values (N/mm^2) obtained for each ocular insert. (B) Maximal elongation at break-point values (%) obtained for each ocular insert (* $p < 0.05$).

In Figure 2A tensile strength values can be observed, the highest value was 24.5 ± 0.01 N/mm^2 belonging to the F07 insert. Figure 2B shows the elongation values

328 (Maximal elongation at breaking point) of the inserts studied. The insert F03 showed
329 greater elasticity ($p < 0.05$). It stretched up to 51.88% of its original size.

330 Since the inserts F03, F04, F07 and F08 showed good characteristics, they were
331 re-formulated with PG in their composition (55.6 µg/cm^2) following the methodology
332 previously described and observed under microscope. Inserts were observed under optical
333 and polarized light microscopy in search of imperfections. Microscopic images of the
334 inserts are shown in Figure 3 either viewed under polarized light (Figures 3A-D) or under
335 conventional illumination (Figures 3E-H).



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337 **Figure 3.** Photographs (Leica DM 2000) taken under a polarized light microscope (20 × magnification)
338 and photographs taken under a normal light microscope (A & E= F03; B & F, F04; C & G, F07 and D &
339 H, F08).

340 The presence of imperfections was observed in F03 and F04 inserts. In Figures
341 3A and 3B cracks can be observed, while in Figure 3E and 3F small crystals and the
342 presence of imperfections can be seen. On the other hand, F07 and F08 inserts showed no
343 imperfections or cracks. The insert F07 was selected because it had the best
344 characteristics: besides having no imperfections it has a higher resistance to breakage and
345 a lower moisture uptake than F08.

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

352 To compare the insert (Figure 4A-C) with commercial contact lenses (Acuvue®,
353 Johnson & Johnson vision care Inc., Jacksonvill, FL, USA) (Figure 4D-F) both were
354 observed under SEM. As can be seen, the surface of insert F07 (Figure 4A) and its
355 transversal section (Figure 4C) show a homogeneous structure, free of indentations and
356 bumps, and it is very thin (500 µm thickness compared to 1500 µm of commercial contact
357 lenses). The porosity of the insert can also be observed (Figure 4B).

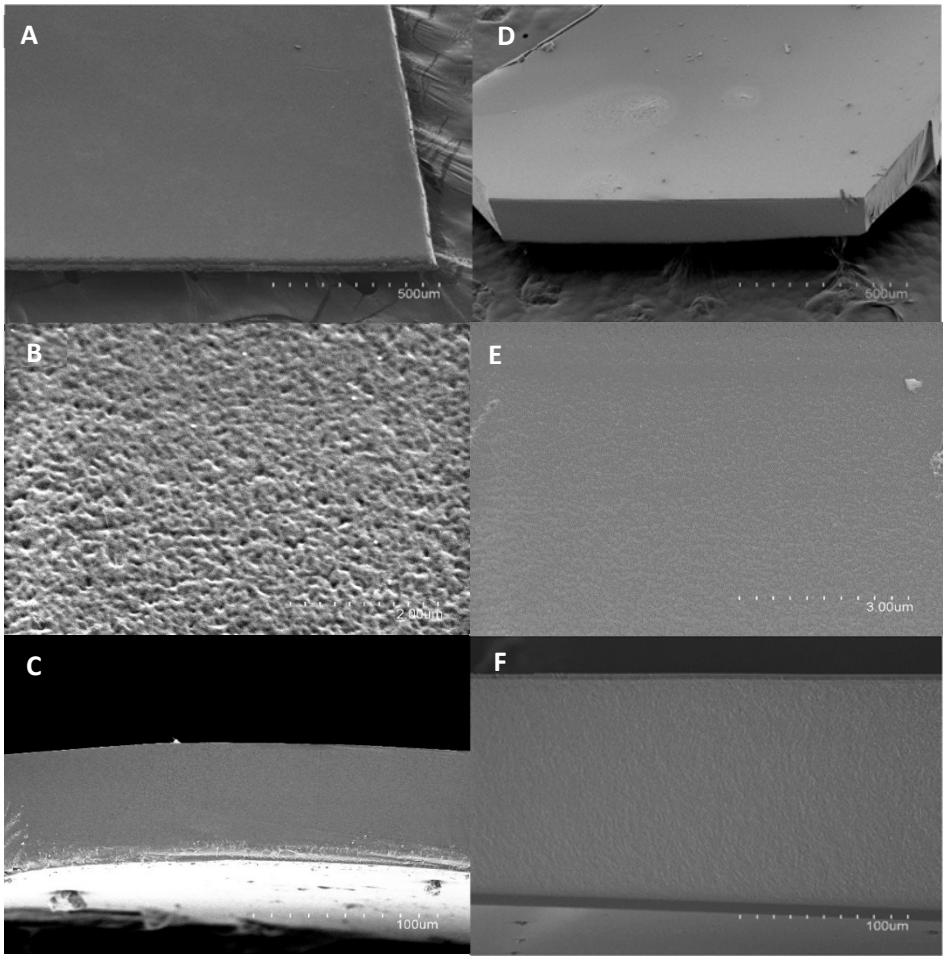

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Figure 4. SEM images of the surface (A), at higher magnification (B) and cross-sectional view (C) of the ocular insert F07 and of commercial contact lens: surface view (D), higher magnification (E) and cross-sectional view (F).

Our insert (F07) was thinner and lighter than those formulated by other authors [23,28,45,46]. In figure 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.

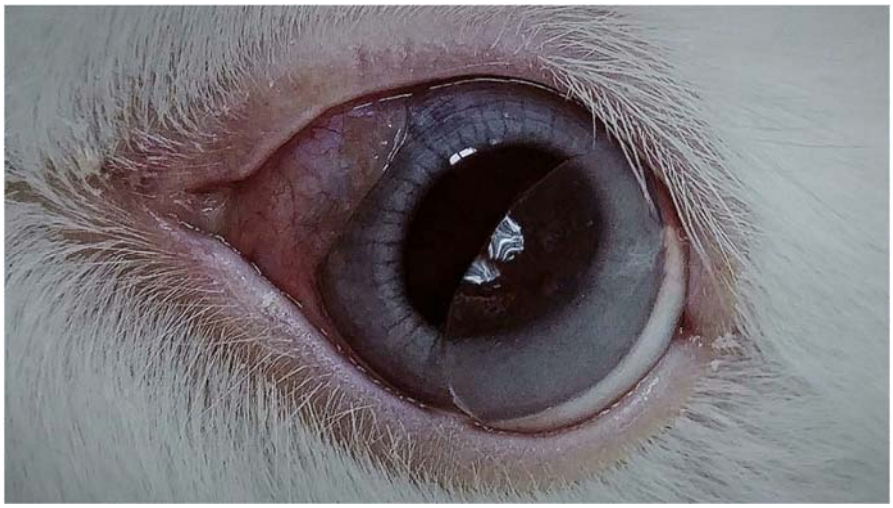

Figure 5. Photograph of insert F07 containing PG on a rabbit's eye. The insert was placed in the rabbit´s eye lopsided to allow visualization of the insert.

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 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 6B). PG in β-CD in aqueous solution at 500 µg/mL (Figure 6C) and PG insert F07 (Figure 6D), did not produce any observable effects on the blood vessels during the 3 min of observation (IS = 0).

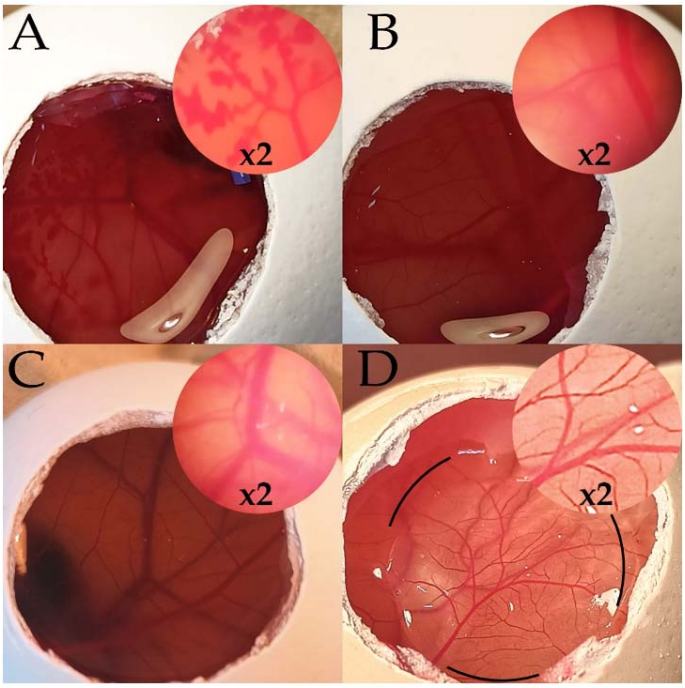

Figure 6. HET-CAM test. Effect of positive (A) and negative (B) controls, PG in β-CD in aqueous solution (500 µg/mL) (C) and PG-loaded insert (55.6 µg/cm^2) (D) on the surface of the chorioallantois membrane (CAM) after treatment for 3 min. Positive and negative controls were: NaOH 0.1N solution and 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 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 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.

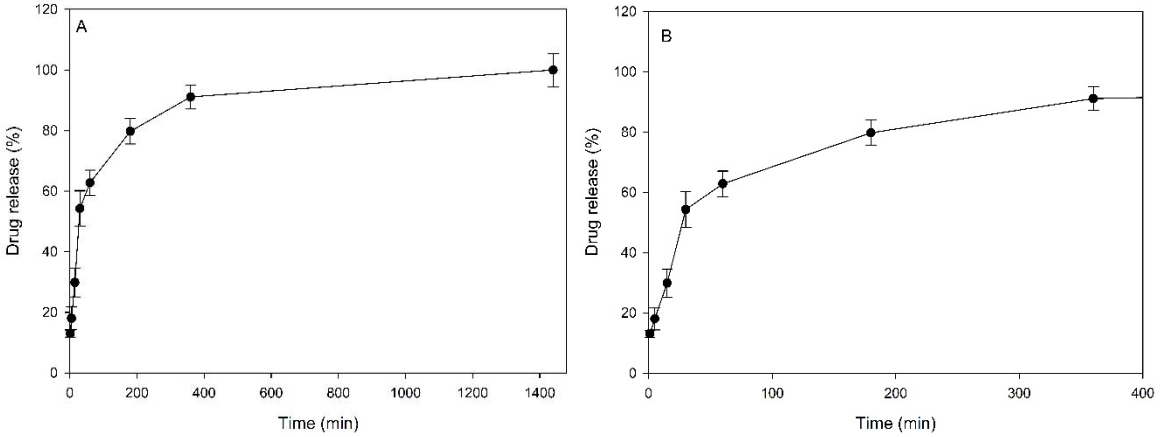

Figure 7. Percentage of PG released from the insert F07 during 24 h (A) and the PG release from the same insert during the first 200 min (B).

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 8), as well as the retention of PG by both membranes.

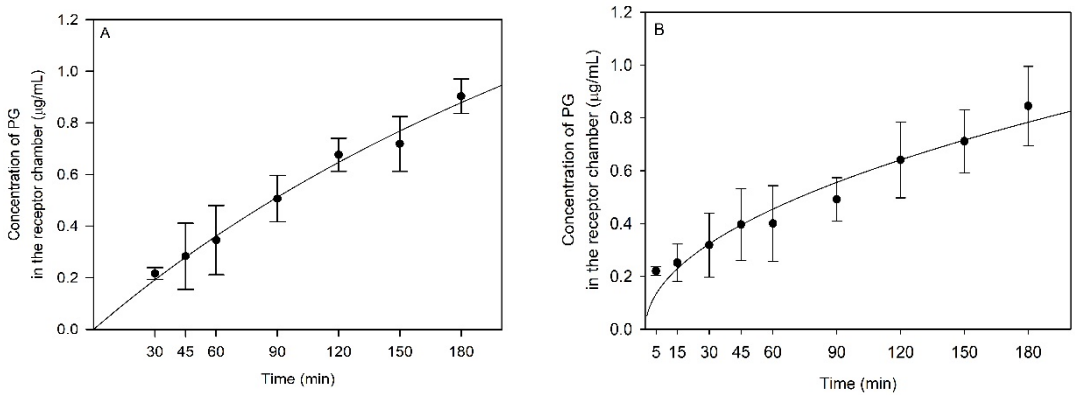

Figure 8. Concentration of PG in the receptor chamber (µg/mL) *vs* time in trans-corneal (A) and trans-scleral (B) diffusion studies. The error bars show the standard deviation of the observed values (n = 5).

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 9, as well as the amount extracted from both membranes, cornea and sclera and the PG in the insert.

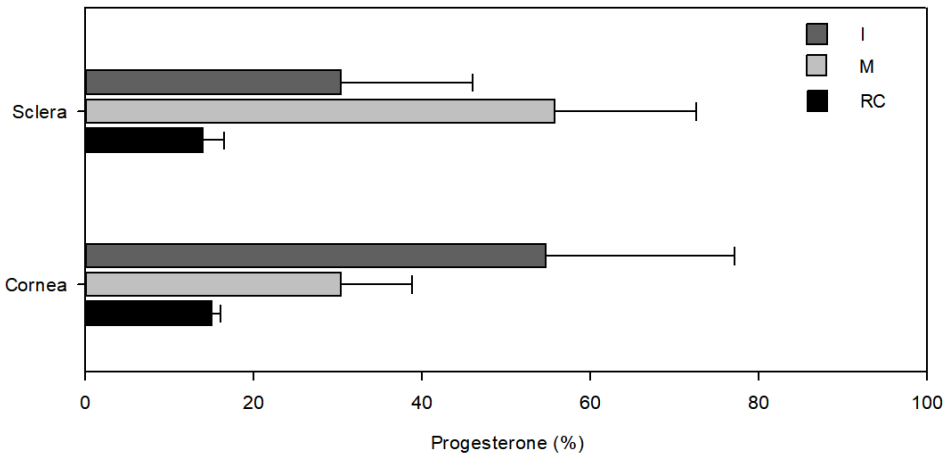

Figure 9. Mass balance of PG: percentage (%) of PG in the insert (I); in the membrane (M) and accumulated in the receptor compartment (RC) after 3 h of trans corneal and trans scleras diffusion studies with the insert (n = 5).

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 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 $6.46 \pm 0.38 \times 10^{-7}$ cm/s and $5.87 \pm 1.18 \times 10^{-7}$ 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 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 ±

452 5.52×10^{-7} and $42.9 \pm 7.38 \times 10^{-7}$ cm/s for cornea and sclera, respectively (n = 10). In
453 contrast, pure PG micelles formulated in the polymeric solubilizer Soluplus® showed
454 apparent permeability coefficients of $16.5 \pm 1.8 \times 10^{-7}$ and $9.2 \pm 2.0 \times 10^{-7}$ cm/s for
455 cornea and sclera respectively, whereas using PG micelles in Pluronic® F68 apparent
456 permeability coefficients were $37.3 \pm 10.5 \times 10^{-7}$ and $14.5 \pm 1.5 \times 10^{-7}$ cm/s for cornea
457 and sclera, respectively. These results show that PG permeability from the insert was 3-
458 7 times lower than permeability coefficients reported with eye drops of PG in β-CD.
459 Similarly, apparent permeability coefficients from the insert were 2-6 times lower than
460 those found when using PG drops in micelles [47]. This lower permeability could be
461 attributable to the fact that the insert controls PG release to cornea and sclera. Although
462 the permeability of PG from the insert presented here is lower than that found in
463 formulations previously described [30,47], it is important to consider that this insert could
464 control the release of the drug over time better, due to longer contact-time with the eye
465 membranes. Furthermore, ocular inserts have some additional advantages compared to
466 liquid formulations such as higher availability of the drug in ocular compartments.
467 Additionally, there are also lower losses of drug and minimal systemic absorption because
468 there is no involuntary lacrimation. Finally, higher precision dosing with controlled
469 release allows to reduce the frequency of administration [16]. Thus, the formulated insert
470 we have designed and evaluated may provide a suitable promising alternative for the
471 treatment of eye diseases requiring PG administration.

472

473 **4. Conclusions**

474 In the present study, several PG inserts were formulated and evaluated leading to
475 the selection of a PG insert manufactured with 59% polyvinyl alcohol, 39%
476 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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CRediT authorship contribution statement

Adrián M. Alambiaga-Caravaca: Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Iris M. Domenech-Monsell:** Investigation, Writing - review & editing**. María Sebastián-Morelló:** Validation, Writing - review & editing. **M. Aracely Calatayud-Pascual:** Conceptualization, Visualization, Writing - review & editing, Supervision. **Virginia Merino:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original

draft, Writing - review & editing. **Vicent Rodilla:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Alicia López-Castellano:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration

The authors declare no conflict of interest. Only the authors played a role in the design of the study, in the collection, analyses, interpretation of data as well as in the writing of the manuscript.

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