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Research paper

Prospective insulin-based ophthalmic delivery systems for the treatment of dry eye syndrome and corneal injuries

Estael L.C. Cruz-Cazarim[a], Maurílio S. Cazarim[a], Abayomi T. Ogunjimi[a], Raquel Petrilli[a], Eduardo M. Rocha[b], Renata F.V. Lopez[a,*]

[a] School of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Av. Cafe s/n, 14040-903 Ribeirao Preto, SP, Brazil
[b] School of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirao Preto, SP, Brazil

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ABSTRACT

The presence of insulin (INS) receptors on the ocular surface (OS) and lacrimal gland (LG), and the high prevalence of dry eye syndrome (DES) and corneal lesions in diabetic patients suggest that INS is relevant for OS homeostasis and wound healing. The study aims at developing delivery systems for the topical administration of INS to the OS in order to improve INS local bioavailability and evaluate the influence of the delivery systems on DES in diabetic rats (DM) (n = 05/group). Chitosan microparticles (MP), chitosan/poloxamer gel (GEL) and MP-loaded GEL (GELMP), with or without INS were developed. Formulations were instilled into the eyes of diabetic rats (DM) for 15 days and the tear fluid volume, corneal cells morphology and cornea thickness were assessed and compared with an aqueous dispersion of INS (DISP-INS). All delivery systems had pH of about 6, osmolality suitable for topical application and positive zeta potential. The MPs with or without INS had sizes close to 4 μm, spherical morphology and INS encapsulation efficiency of 77 ± 6%. DISP-INS and GELMP-INS formulations produced tear secretion amounts significantly higher than those receiving formulations containing no INS and similar to healthy animals. Cornea surface impression cytology showed that treatment with INS-delivery systems and not DISP-INS almost normalized cells morphology. Treatment with GELMP-INS increased INS by 2.5 in the LG and eyeball as compared to the groups treated with GEL-INS and MP-INS, while treatment with DISP-INS left no traces of drug in the eye after treatment termination. GEL and GELMP containing INS were also able to normalize the thickness of the corneal epithelia. In conclusion, GELMP-INS normalized tear fluid volume, corneal thickness, protected corneal cells morphology and increased ocular bioavailability of INS, making it a promising treatment strategy for DES and corneal lesions.

1. Introduction

Diabetes mellitus (DM) is one of the major risk factors responsible for diseases in the eyeball [1,2]. In addition to the well-known DM complications, such as cataract and diabetic retinopathy, its relation to alterations in the ocular surface (OS) and disruption in the functioning of the lacrimal glands (LG) has been much discussed in recent years [3–5]. Chronic hyperglycemia induce corneal epitheliopathy, neural damage and tears reduction [6–8]. Oxidative stress and inflammatory mediator play key roles in the development of these alterations, disrupting the signaling pathways and the secretory apparatus of the LG [9–11]. All these events generally contribute to tissue damage, promotes an inflammatory micro-environment that slows down or prevents healing of lesions on the surface of the cornea and lacrimal gland, resulting in dry eye syndrome (DES) [3], which is generally associated with symptoms such as discomfort, visual disturbance, tear instability and potential damage to the ocular surface [12].

Although corneal lesions and DES are certainly not solely related to DM, problems associated with corneal wound healing are considered to be one of the most frequent global eye problems without efficient therapeutic strategies [13,14]. DES is an underestimated risk factor for corneal lesions and its prevalence is as high as of DM in the general population [15–17]. Also, for DES the treatment strategies are predominantly symptomatic [18–20].

The discovery of insulin (INS) receptors exists on the OS and LG indicates the relationship between INS and the pathophysiology of those organs [21,22]. In fact, for years, INS has been a constant target of researches involving the promotion of corneal healing [3,21–28] and the prevention of lesions in the lacrimal gland [8].

While topical administration of INS appears to be a promising

* Corresponding author.
E-mail address: rvianna@fcfrp.usp.br (R.F.V. Lopez).

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strategy for treating these disorders, most ophthalmic solutions applied topically drain from the eye through various mechanisms including tearing, drug dilution and tear renewal, resulting to low local bioavailability of topically applied drugs [29]. Thus, the development of formulations, which can increase the residence time of INS in the ocular micro-environment and facilitate delivery to the lacrimal glands and corneal cells will be highly beneficial.

Till date, ocular administration of INS has been from invasive release systems, such as implants [23], or simple aqueous solutions [25]. The latter requires frequent instillation [30] and high INS concentration, both having the propensity of triggering undesirable toxic effects and damage to the ocular surface cells [31].

Microparticulate delivery systems have been shown to be a promising strategy that can overcome some drawbacks of ocular formulations [29,32]. Microparticles allow delivery of higher quantity of drugs than nanoparticles, release drugs slowly while avoiding their immediate metabolism [32–34]. To further increase the residence time of the microparticles on the ocular surface, their dispersion in thermoreversible gels *in situ* has been shown to be a good strategy [35,36]. These gels are prepared from polymers that usually exist in a liquid phase at room temperature, having the viscosity of conventional eye drops, making it easy to administer but thereafter forms a gel when in contact with the eye [37], thereby increasing its mechanical strength and reducing drug leakage due to lacrimal drainage [36].

Thus, in this study, microparticles and *in situ* thermo-reversible gels containing INS were developed and administered topically alone or in combination to the eye of diabetic rats. The influence of these release systems with or without INS on lacrimal gland disorders and corneal regeneration were thereafter evaluated.

2. Material and methods

2.1. Chemicals

Recombinant human INS was kindly supplied by Novo Nordisk Brazil (São Paulo, Brazil). Chitosan (MMW 190,000–310,000 Da, 75–85% deacetylated – information provided by the manufacturer) was purchased from Sigma Aldrich (St. Louis, MO, USA). Poloxamer 407 was purchased from Embrafarma (São Paulo, Brazil). Streptozotocin was purchased Sigma-Aldrich (St. Louis, MO, EUA) and Ketamine hydrochloride (Dopalen) was purchased from Vetbrands (Jacareí, Brazil) and Xylazine (Dopaser) from Hertape Calier (Juatuba, Brazil). All other reagents were analytical or HPLC grade. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, MA, USA) was used to prepare all solutions.

2.2. Quantification of INS

A commercial ELISA kit (Invitrogen™, USA) specific for recombinant human INS was used to quantify INS in the formulations, lacrimal gland and eyeball at 450 nm on an ELISA plate reader (Multiskan™ FC). The method was evaluated for specificity, linearity, accuracy and precision according to the ICH guideline [38] with keen attention to eliminating possible interferences from the formulation and tissues.

2.3. Preparation of formulations

2.3.1. Chitosan microparticles

Chitosan microparticles with INS (MP-INS) or without INS (MP) were prepared by simple spray drying technique according to established method [39]. Briefly, 1 g of chitosan was dispersed in 100 mL of 0.5% acetic acid solution to obtain the MP. About 3.5 mg of INS was solubilized in this dispersion to obtain the MP-INS containing, theoretically, INS at 3.5 mg/g of chitosan. The dispersions were sprayed in a Spray Dryer (MSD 0.5, Labmaq, Brazil) at a flow rate of 6.0–6.5 mL/min using a 1 mm diameter atomizer spray nozzle, atomizer air rate of 6 m^3/min, with inlet and outlet temperature at 100 °C and 75–85 °C respectively. Recovered microparticles were characterized by size, morphology, zeta potential and INS content in the chitosan microparticles where applicable.

Particle size distribution was evaluated by laser diffraction technique using a diffractometer (LS13320, Beckman Coulter, USA). Simply, 1 mg of chitosan microparticles (n = 3 from different batches) with or without INS was dispersed in 2 mL methanol and immediately analyzed [39]. Particle morphology was evaluated by scanning electron microscopy (SEM, Phillips XL 30 FEG Microscope) in triplicate at an acceleration voltage of 20.0 kV and magnifications of 3000 to 20,000 times.

To quantify INS content in the microparticles, a known amount of the MP-INS powder (n = 3 from different batches) was dispersed in a 1% acetic acid solution under continuous stirring for 24 h to completely extract INS. The dispersion was then filtered, and the resulting solution was analyzed by ELISA to determine INS amount in the MP ($Q_{Experimental}$). The %INS in MP-INS was calculated according to Eq. (1);

$$INS\% = \left(\frac{Q_{Experimental}}{Q_{Theoretical}} \times 100 \right) \quad (1)$$

where $Q_{Experimental}$ is the INS amount extracted from a known amount of MP-INS and $Q_{Theoretical}$ is the theoretical INS amount expected to be detected in an equivalent amount of MP-INS considering the initial amount of INS used to prepare the MP (3.5 mg of INS/g of chitosan).

Zeta potential of the microparticles (n = 3 from different batches) with or without INS was determined using Zetasizer Nano ZS 4.0 (Malvern®, UK) by dispersing about 1.3 g of the MP-INS in 100 mL of sterile 0.9% NaCl solution. These dispersions produced microparticle formulations without INS (MP) and with 35 ug/mL of INS (corresponding to 1 IU/mL INS) (MP-INS) used in the *in vivo* experiments.

2.3.2. In situ thermoreversible gels

In situ thermoreversible gel was prepared from a dispersion of chitosan and poloxamer 407 (P407) according to a previously described method [36]. Briefly, 1% w/v chitosan was dispersed in acetic acid (0.5%), the pH was adjusted to 6.0 ± 0.2 and kept under refrigeration overnight prior to the addition of sufficient amounts of INS to obtain a final INS concentration of 1 IU/mL (35 µg/mL INS). Thereafter, 16 g of P407 was added slowly to each 100 mL of the INS-chitosan dispersion on an ice bath. The resulting INS-chitosan-P407 dispersion was kept under refrigeration protected from light and named GEL-INS. A blank gel was produced following the same procedure, however without INS and was named GEL.

The *in situ* thermosetting gels (n = 3 from different batches) were characterized by osmolality, final pH and zeta potential. The osmolality of the gels was determined by a direct freeze point method without dilution using an Osmometer (Semi-Micro K-7400, KNAUER, Germany) while the final gel pH was determined using a digital pH meter (DM20, Digmed®, Brazil) after 1:10 dilution with freshly prepared deionized water to prevent interference due to the viscosity of the gels.

2.3.3. In situ thermoreversible gel containing microparticles

The MP were incorporated into the *in situ* thermoreversible gel by adding enough amounts of the MP to produce formulations with equivalent amounts of INS. Briefly, *in situ* thermoreversible gels were prepared as previously described; however, the MP-INS instead of free INS crystals were incorporated into the chitosan dispersion. The amount added was such that the final INS concentration in the formulation was 1 IU/mL. Then, P407 was added to this dispersion under constant stirring in an ice bath, the final formulation was kept under refrigeration protected from light and named GELMP-INS. Osmolality, pH and zeta potential of the GELMP-INSs (n = 3 from different batches) were evaluated the same way as described for the GEL and GEL-INS.

2.3.4. Simple INS dispersion

A simple INS dispersion (n = 3 from different batches) was prepared by adding 3.5 mg INS crystals to 100 mL of a 0.9% sterile NaCl solution to obtain 1 IU/mL INS dispersion. This dispersion was named DISP-INS.

2.4. In vivo studies: treatments with INS-based formulations and their influence on corneal lesions and DES

2.4.1. Experimental model

Forty (40) male Wistar rats (*Rattus norvegicus*), 6–8 weeks old (180–220 g body weight) were used. The protocol employed in this study was approved by the Ethics Committee of the University of Sao Paulo (Protocol No. 12.1.915.53.3) and it was in accordance with the National Institutes of Health (NIH) and ARVO Guidelines for the Care and Use of Laboratory Animals.

To induce DES and cornea surface changes, DM was induced by administering a single dose of streptozotocin (STZ, solubilized in 0.01 mol/L citrate buffer) at 60 mg/kg body weight to 35 animals (n = 5 animals/group) through the caudal vein [40]. Serum glucose levels were monitored two days after streptozotocin induced DM, on the first day of treatment with the formulations and at the end of the treatment on the last day of experiments (day 15). Serum glucose levels greater than 200 mg/dL (556 ± 60 mg/dL) under fasting after 2 days of STZ administration confirms the induction of DM. Non-diabetic positive control group (fasting glycemia of 117 ± 8 mg/dL) received only the vehicle through the caudal vein. Treatment with the ophthalmic formulations was initiated only after 8 weeks of DM induction, a period that guarantees necessary cornea surface changes and reduction in tear secretion [8].

2.4.2. Animal treatment

Drug treatment regimen which was a daily instillation of 50 µL in each eye for 15 days was the same for all animal groups regardless of the formulation employed. Diabetic animals were divided into 7 groups with each group having 5 animals. One group received no treatment (negative control), two groups received formulations without INS (MP and GEL) while four groups were treated with formulations containing INS (DISP-INS, MP-INS, GEL-INS and GELMP-INS). In addition, a separate group of 5 animals was maintained healthy, without DM induction (positive control). The animal treatment protocol is as shown in Fig. 1.

2.4.3. Quantification of tear secretion

Phenol Red Test (PRT) was deployed in quantifying the amount of tear secretion produced on days 0, 5, 10 and 15 of the treatment regimen every morning before the daily instillation of the formulations to both eyes of the animals (n = 5/group). A PRT cord (Zone-Quick®, Showa YakuhinKako Co. Ltd., Japan) approximately 20 mm long was placed on the lower portion of the eyelid without anesthesia and kept in contact with each eye for 30 s. Through the color changes observed, the moistened length of the cord was measured in millimeters using a caliper.

2.4.4. Corneal impression cytology

After 15 days of treatment, prior to sacrifice, the animals (n = 5 animals/group) were anesthetized using ketamine (120 mg/kg body weight) and xylazine (12 mg/kg body weight) before the print cytology was performed. Corneal epithelial cells were collected with a 45 µm porosity filter paper (Millipore, USA) previously cut into 1 cm^2 and kept in contact with each eye for 30 s. The filter paper was thereafter stained with Periodic acid-Schiff (PAS) and hematoxylin [8]. The samples were analyzed under a Primo Vert microscope (Carl Zeiss GmbH, Germany) at a 40x magnification and graded from 0 to 3 according to the Nelson scale with healthier epithelium having lower scores. Briefly, in the Nelson scale [41] the presence of polymorphonuclear cells, intercellular junctions, mucin, amount of caliciform cells and nucleus/cytoplasm ratio are considered for the classification of the cells in four levels of viability, from 0 (healthy) to 3 (abnormal) [42], as described below:

Grade 0: Epithelial cells are small and round with eosinophilic cytoplasm. The nucleus is large and basophilic, with nucleus/cytoplasm ratio of 1:1 or 1:2. There are large numbers of cells per field.

Grade 1: Epithelial cells are slightly larger, more polygonal and with eosinophil cytoplasm. The nucleus is smaller, with nucleus/cytoplasm ratio of 1:3. There is a good number of cells per field.

Grade 2: Epithelial cells are large and polygonal. Occasionally they are multinucleated and the cytoplasm of variable staining. The nucleus is lower with nucleus/cytoplasm ratio of 1:4 or 1:5. There are few cells per field.

Grade 3: Epithelial cells are large, polygonal and with basophilic cytoplasm. The nucleus is small, pyknotic or absent in some cells. The nucleus/cytoplasm ratio is greater than 1:6. Rare cells per field.

The scoring evaluation using the Nelson scale was performed by two different researchers who randomly selected and evaluated 6 fields from each slide (n = 5 slides/group) in a double-blind analysis. In this way, 30 distinct fields of the printed cornea were analyzed for each treated group by two researchers.

2.4.5. Recovery of LG and eyeball INS

After animal sacrifice, the LG and left eyeballs (n = 5 animals/group) were harvested for processing and quantification of their recombinant INS content. The organs were weighed, immersed in 10 mL HCl solution (0.01 M v/v), homogenized in an Ultra Turrax (T25 Basic, IKA®, Germany) at 24,000 rpm for 1 min, filtered and quantified for INS using ELISA kit.

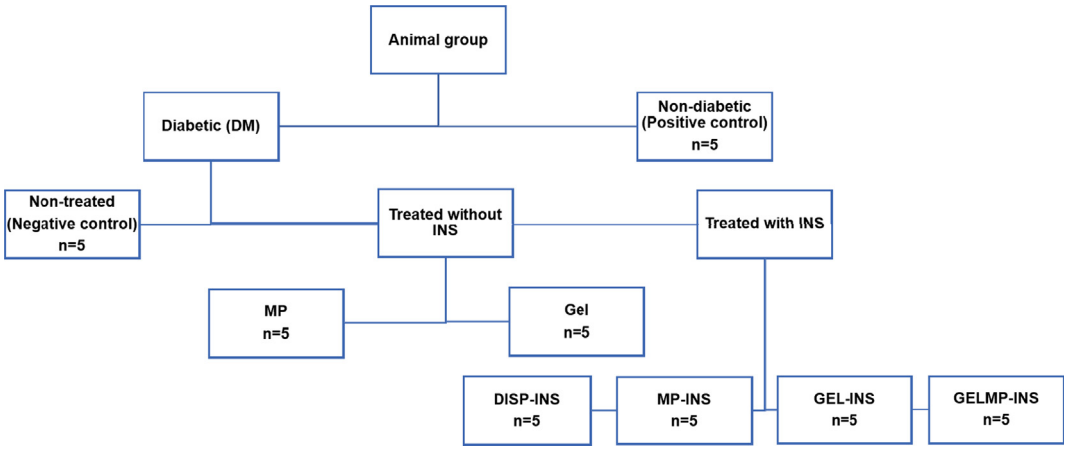

Fig. 1. Schematic representation of *in vivo* treatment groups.

2.4.6. Evaluation of corneal epithelial thickness

The right eyeballs of the animals were removed (n = 5 animals/group) after sacrifice and subjected to histological analysis in order to measure the corneal epithelium thickness. Before the measurement procedure, a small incision was made in the eyeballs to facilitate easy entry of the fixing solution (4% paraformaldehyde in PBS). The samples were maintained in the fixing solution for 24 h, mounted in Tissue-Tek (Tissue-Tek O.C.T. compound, Sakura®, USA) and frozen in histological block format with the aid of dry ice and acetone. The blocks were kept in freezer at −80 °C before cutting into 6 μm sections with a cryostat (CM1850, Leica®, Germany). The sections were fixed on a previously gelatinized glass slide and then stained with Harris Hematoxylin and Eosin (HE). The stained sections were visualized and captured under a Primo Vert microscope (Carl Zeiss, GmbH, Germany) at 40x magnification and the corneal epithelium thickness was measured using Axion Vison software (Release 4.8.2.0; Carl Zeiss, GmbH, Germany).

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Prism, USA) at significance level of 5%. A *Two-Way ANOVA* was used to determine statistical differences for tear secretion, animal weight and capillary glycemia. A *One-Way ANOVA* was employed in evaluating differences in the concentration of INS recovered from the lacrimal gland and eyeball and the corneal epithelium thickness. A student *t-test* was deployed in analyzing differences in capillary glycemia before and after DM induction, weight of lacrimal gland of diabetic animals treated with different formulations, tear secretion before the first day of treatment as a function of diabetic and non-diabetic animals. The student *t-test* was also used in evaluating the differences of the above parameters with considerations to MPs with or without INS. The impression cytology scores were classified in the categories mentioned above and the level of agreement between the two researchers was determined by calculating kappa which varies between −1 to +1, being smaller and greater agreement respectively [43]. The kappa value obtained was thereafter classified according to Nigel Paneth's scale, where kappa values greater than 0.8, between 0.60 and 0.80, between 0.40 and 0.60 and lower than 0.40 indicate excellent, good, regular and poor agreements respectively [44].

3. Results

3.1. INS quantification

The ELISA method used for the quantification of recombinant human INS from the formulation was linear ($R^2 \geq 0.9994$) within the concentration range of 11.7–337 μIU/mL, accuracy was between 96.3 ± 1.34 to 104.88 ± 2.35% and coefficient of variation was lower than 4.16% while the limit of quantification was 11.7 μIU/mL. The method was specific for human INS as materials from the formulations (MP and GEL), eyeball and lacrimal gland did not interfere with quantification of INS, confirming no cross interaction between the natural insulin present and the animal tissue extracts used for the *in vivo* studies.

3.2. Formulation characterization

The MPs produced with or without INS had an average diameter of 3.7 ± 0.6 and 4.2 ± 0.3 μm respectively, with no significant [*t-test*, $p = 0.099$] difference between them. Only one microparticulate population was observed suggesting a monomodal distribution with approximately 25% and 75% of the particles in the size range of 2 and 7 μm respectively. Typically, about 90% of the microparticles were smaller than 11 μm.

Fig. 2 shows the representative photomicrographs of INS crystals (Fig. 2A and B), MPs (Fig. 2C and D) and MP-INS (Fig. 2E and F) obtained by SEM. The average diameter and polydispersity of the microparticles were compatible with that obtained using laser diffraction analysis. Regardless of the presence of INS, the microparticles showed relatively spherical morphologies, with surface full of indentations with appearance of 'deflated balls' (Fig. 2). There was no visible characteristic INS crystal (Fig. 2A and B) observed in the SEM of the MPs containing INS (Fig. 2E and F) while INS% present in the microparticles was 77 ± 6%, representing 2.7 ± 0.2 mg INS/g of MP.

The osmolality varied approximately from 280 to 400 mOsm/Kg. The presence of INS not significantly altering the formulation's osmolality when compared to the blank formulations [*t-test*, $p > 0.05$]; however, different formulations exhibited distinct osmolarities. Comparing the GEL to the other formulations there was difference in the osmolarity [*t-test*, $p < 0.001$]. MP formulations showed osmolality similar to that of DISP-INS [*t-test*, $p = 0.772$], while the GEL had higher osmolarity than DISP-INS [*t-test*, $p = 0.001$] (Table 1).

The DISP-INS showed a slightly negative zeta potential, MP with or without INS was slightly positive while the gels showed a highly positive (> 30 mV) zeta potential. All ophthalmic formulations developed and employed during *in vivo* studies had a final pH of 6.0 ± 0.2.

3.3. In vivo studies

Fig. 3 shows the tear secretion after treatment with formulations as a function of the treatment time. Differences in tear secretion were compared between all animal groups across all experimental days using a Two-Way ANOVA followed with a Tukey's multiple comparison test. The result shows significant differences in tear secretion between animal groups treated with different formulations [$F(7,288) = 65.41$, $p < 0.0001$] and across treatment days [$F(3,288) = 98.49$, $p < 0.0001$]. On day 0, all DM animals had significantly lower tear secretion [Tukey's multiple comparison test, $p < 0.0001$] than that presented by healthy animals, being 5.3 ± 0.7 and 10.7 ± 1.7 mm respectively.

Regardless of treatment, all formulations containing INS increased tear secretion from the 5th day, which demonstrates that the amount of INS released from the formulations is sufficient to improve tear secretion. After day 15 of treatment, only animals treated with DISP-INS and GELMP-INS showed similar tear secretion to the positive control [Tukey's multiple comparison test, $p > 0.05$]. It should however be noted that animals treated with GELMP-INS produced tear secretion statistically similar [Tukey's multiple comparison test, $p > 0.05$] to the positive control as early as the 5th day, while animals treated with DISP-INS produced comparable tear secretion to the positive control only slightly later on the 10th day of treatment [Tukey's multiple comparison test, $p = 0.9838$]. Animals treated with formulations containing no INS (MP and GEL) had no difference in tear secretion as a function of time, while the tear secretion was similar to that produced by the negative control [Tukey's multiple comparison test, $p > 0.05$].

The results obtained from the analysis of the cytology of ocular surface impression of animals after 15 days of treatment are shown in Table 2.

Fig. 4 shows representative images of the cytology of ocular surface impression of animals after 15 days of treatment.

Corneal impression cytology analysis of animals treated using the Nelson scale showed that healthy animals (positive control) were scored as Grade 0 while untreated DM animals (negative control) were scored as Grade 3. Animals treated with MP-INS, GEL-INS and GELMP-INS were scored as Grade 1, while animals treated with DISP-INS, INS-free GEL and MP were scored as Grade 2. The concordance index obtained between the two researchers who performed the qualitative analysis (kappa) was 0.72; this index was considered "good" according to the classification of Nigel Paneth [44].

At the end of the 15 days treatment, *Two-Way ANOVA* with Bonferroni post-test analysis showed that DM animals (224 ± 31 g) presented significantly different body weights from those of healthy

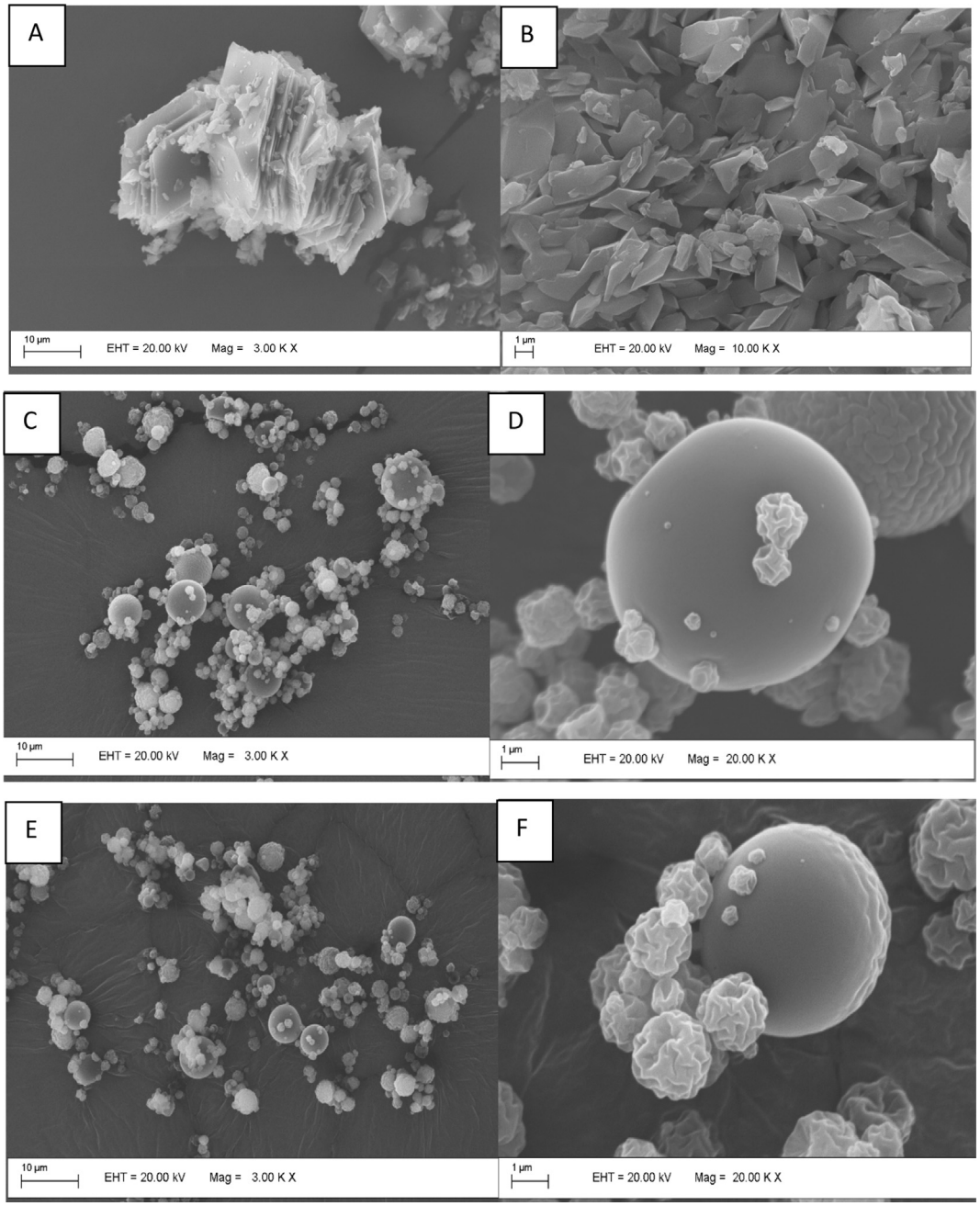

Fig. 2. Scanning Electron Photomicrograph at two magnifications (3Kx and 10Kx). A and B: crystals of recombinant human insulin (INS); C and D: microparticles without insulin (MP); E and F: insulin microparticles (MP-INS).

animals (435 ± 40 g) [F(2,12) = 23.9; $p < 0.05$]. The treated DM animals presented no significant body weight differences with (209 ± 33 g) or without (218 ± 24 g) INS in their treatment formulations. However, treatment resulted in significant weight reduction when compared to untreated DM animals (negative control) with the animals presenting body weights of 214 ± 29 and 245 ± 35 g respectively [*Two-Way ANOVA*, F(2,12) = 23.9; $p < 0.05$]. Student *t-test* showed significantly different lacrimal gland weights of healthy animals (0.12 ± 0.02 g) as compared to DM animals (0.07 ± 0.01 g), [$p < 0.05$]; however, there was no statistical difference between the lacrimal gland weights of DM animals that received or not any form of treatment [*Two-Way ANOVA*, F(1,9) = 0.085; $p > 0.05$].

After 15 days of treatment, no INS was detected in the lacrimal glands and eyeball of animals in the positive and negative control groups, and animal groups treated with GEL, MP and DISP-INS. In this last case, the amount of INS was below the quantification limit of the analytical method. In contrast, recombinant human INS was quantified in the lacrimal gland and eyeball of animals treated with MP-INS, GEL-INS and GELMP-INS formulations, with GELMP-INS treatment resulting in significantly [*One-Way ANOVA*, F(7,33) = 16.24; $p < 0.05$] higher INS amounts in the lacrimal gland and eyeball than the other treatments (Fig. 5).

Corneal epithelium thickness values obtained after histological analysis at the end of 15-day treatment are as shown in Table 3.

A One-Way ANOVA followed with Tukey's comparison test was performed to compare epithelium thickness between all animal groups after the 15th day of treatment. The average corneal epithelium thickness of untreated DM animals (negative control) was significantly lower than that of healthy animals (positive control) [*One-Way ANOVA,* F(7;56) = 3.092; p = 0.0439]. Animals that received GEL-INS and

Table 1
The osmolarity and zeta potential of the formulations used in the *in vivo* studies.

Formulations	Osmolarity (mOsmol/Kg)	Zeta potential (mV)
MP	284(± 5)	+14(± 0)
GEL	387(± 3)	+35(± 1)
DISP-INS	284(± 3)*	−4(± 1)
MP-INS	282(± 14)*	+11(± 1)
GEL-INS	382(± 11)†	+34(± 2)
GELMP-INS	397(± 3)†	+33(± 3)

Values reported are Mean ± SD of 5 determinations for 3 different formulations. MP = microparticles; DISP-INS = insulin dispersion; MP-INS = Insulin microparticles; GEL-INS = insulin gel; GELMP-INS = insulin gel microparticles. The statistics was made for the significance level of 5% [$p < 0.005$] and power test of 80% with the aim to test the hypothesis regarding osmolality is not shifted in INS presence, for this, the student's *t*-test was run for paired samples. † = there was statistical difference compared to MP formulation; * = there was statistical difference compared to GEL formulation.

GELMP-INS presented average epithelial cornea thickness statistically similar to that of the positive control [$p > 0.98$] and significantly higher than that of negative control animals (GEL-INS, $p = 0.0040$; GELMP-INS, $p = 0.0269$).

4. Discussion

Chitosan was the choice polymer for microparticles preparation because of its known properties of biodegradability and biocompatibility [45], mucoadhesion [46] and penetration enhancer [47], which could favor the bioavailability of INS. The presence of opposing charges between INS (negative) and chitosan (positive) [48–50], seems to have led to high incorporation of INS into the microparticles, with approximately 80% of the theoretical INS amount incorporated after spray drying. Chitosan-based microspheres prepared by the polyelectrolyte complexation method [51] and chitosan nanoparticles [48] have also shown capacity to encapsulate high percentages of INS.

The micrometric size presented by MP and MP-INS will not cause vision blurring [29,52,53] and may be deposited in the lacrimal sac [53,54], further allowing the continuous release of the drug at the ocular surface without causing any discomfort to the patient. The incorporation of INS into MP did not also significantly alter the size and morphology (Fig. 2) of the microparticles; microparticles generally were fairly spherical with non-porous surfaces and absence of grooves or fissures, making them compatible and adequate for topical ocular administration [53]. They however possess a scaly appearance, similar to previously developed chitosan microparticles [39].

Table 2
Results from the evaluation of the cytology of ocular surface im pression using the Nelson scale. For each treatment (n = 5), 5 slides were evaluated in 6 different fields each by two researchers who randomly selected the slides in a double-blind analysis.

Treatment	Grade on the Nelson scale	Kappa value	Level of agreement*
Positive control	0	0.72	GOOD
Negative control	3		
MP	2		
GEL	2		
DISP-INS	2		
MP-INS	1		
GEL-INS	1		
GELMP-INS	1		

* Classified according to Nigel Paneth's scale.

Poloxamer 407, a synthetic surfactant, nontoxic, amphiphilic and non-ionic polymer was applied for the development of the thermoreversible gel. Chitosan was added to the polymer dispersion since it was previously demonstrated that this mixture improves the mechanical and rheological characteristics of the Poloxamer gel, as well as prolonged residence time on the ocular surface [36]. In this work, binary chitosan/polaxamer (GEL) mixture, without and with INS (GEL-INS), was characterized for osmolality and pH. It is noteworthy, however, that the addition of INS or MP-INS to GEL did not change the macroscopic characteristics of the sol/gel transition, i.e. GEL-INS and GELMP-INS continued to exist in a solution form at 25 °C and had a consistency, such as that of a gel, at temperatures above 32 °C.

Addition of INS or MP-INS to GEL did not significantly alter GEL osmolality (Table 1). Nevertheless, it was slightly above the recommended limit of 340 mOsmol/Kg [55,56]; however, it has been shown that after the application of a formulation on the ocular surface, it mixes rapidly with the tear fluid, making it recover its tonicity within about one to two minutes of application [55,56]. Moreover, patients who suffer from DES generally presents lacrimal osmolality higher than

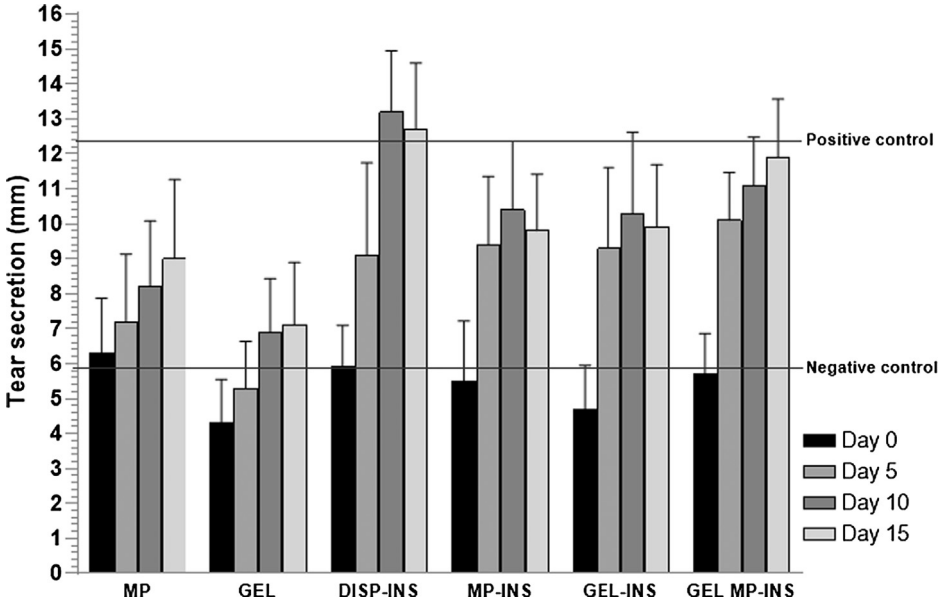

Fig. 3. Tear secretion (mean ± SD, n = 5) from the eyes of treated animals as a function of time. Lower and upper horizontal black lines correspond to mean tear secretion from untreated DM animals and healthy animals respectively in the 15 days of treatment.

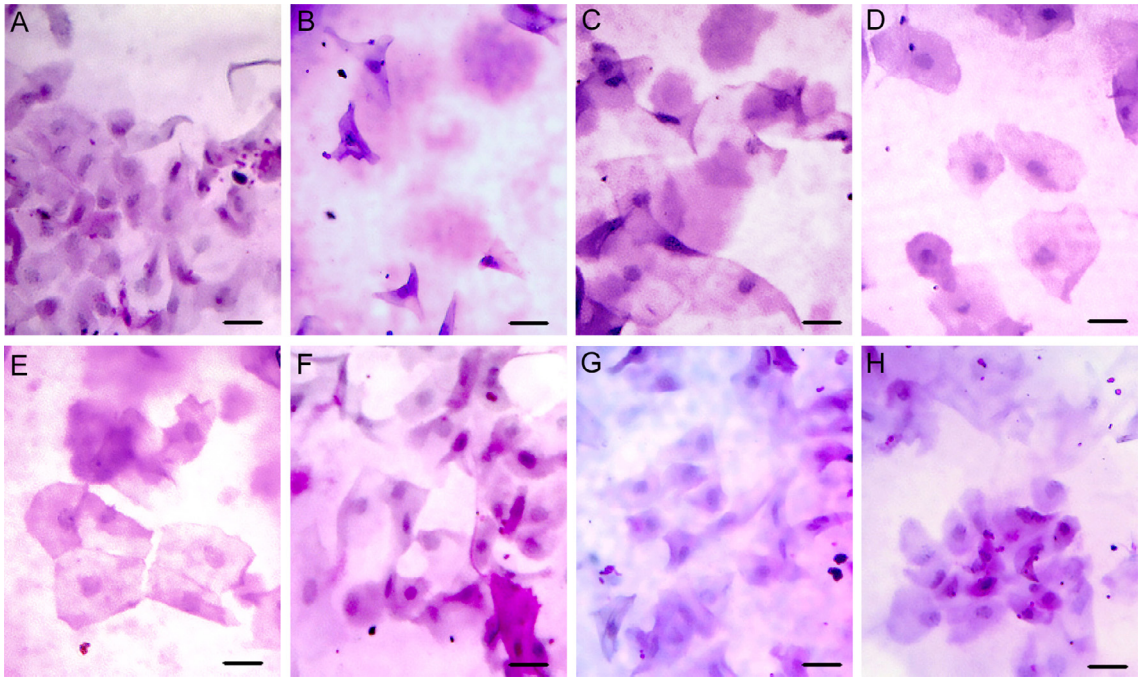

Fig. 4. Representative photomicrographs of the cytology of the ocular surface impression of animals at the end of treatment, Day 15. 40x. (A) positive control, many cells per field, 1:2 nucleus/cytoplasm ratio, Grade 0 (B) negative control, rare cells per field, spiculated cells, Grade 3, (C) MP, Grade 2, (D) GEL, Grade 2, (E) DISP-INS, Grade 2, (F) MP-INS, Grade 1, (G) GEL-INS, Grade 1 and (H) GELMP-INS, Grade 1. Grades are based on the Nelson scale and determined by two researchers who randomly analyzed 6 different fields in 5 slides of corneal impressions of each treated group in a double-blind analysis (125 slides for each researcher). The level of agreement between the researchers was determined by the calculation of kappa, which was 0.72, indicating, according to the scale of Nigel Paneth, a good agreement.

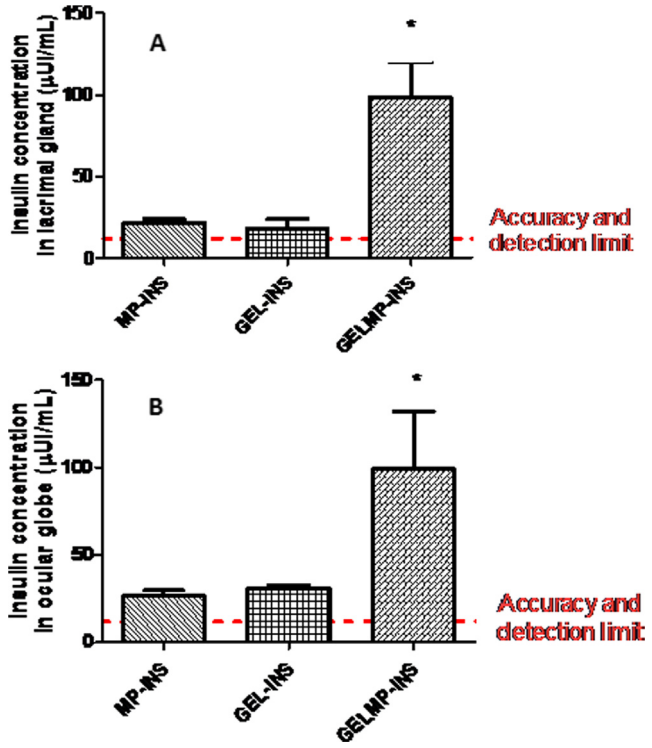

Fig. 5. INS concentration in (A) lacrimal gland; (B) eyeball of animals after treatment completion. The results were expressed as the mean ± standard deviation (n = 5). *[*One-Way ANOVA*, F(7;33) = 16.24; p < 0.05]. GEL, MP, and DISP-INS treatments did not produce quantifiable amounts of INS (below 4.0 IU/mL) measured by ELISA.

Table 3
Corneal epithelium thickness after 15 days of treatment.

Treatment groups	Thickness (µm)
Positive control	16 ± 3
Negative control	12 ± 3
MP	15 ± 2
GEL	14 ± 1
DISP-INS	15 ± 3
MP-INS	15 ± 3
GEL-INS	17 ± 4*
GELMP-INS	16 ± 2*

Results are expressed as mean ± SD (n = 5). MP = microparticles; DISP-INS = insulin dispersion; MP-INS = insulin microparticles; GEL-INS = insulin gel; GELMP-INS = insulin microparticles gel. *Value statistically similar to the positive control (One-Way ANOVA followed with Tukey's comparison test, p > 0.05).

316 mOsmo/Kg [57]; the greater osmolality of the gels should therefore not be a problem for *in vivo* administration

The lower pH of the GEL and other formulations (pH about 6) as compared to physiological pH is due to the presence of chitosan, which normally does not disperse completely in aqueous medium at pH above 6.3. Also, it is known that only formulations with pH < 5 stimulate tear production and increase drainage. Thus, it is observed that, although slightly lower than physiological, the pH of the formulations developed is not a limiting condition for their topical ocular application.

The zeta potential of the formulations varied according to the components of the formulations and their organizations (Table 1). DISP-INS, for example, had a slightly negative zeta potential which is characteristic of the INS molecule [58]. However, the zeta potential of microparticles and gels, regardless of the presence of INS, was positive. This is because chitosan, which has pKa ~ 6.5 [59], is ionized at lower

pHs. These positive charges may favor the interaction of the formulations with the ocular surface, which has residual of negative charges, thereby increasing the residence time of the gel and consequently INS in the eye [36,46].

Characteristics of the formulation may alter the way INS molecules interact in the medium. Depending on the formulation and INS concentration, INS molecules can arrange themselves in the form of dimers, hexamers and other aggregates [60], which may influence the release rate of INS from the formulation and its bioavailability. To verify the impact of the formulations on the bioavailability of INS and its expected action to stimulate tear secretion, *in vivo* experiments were performed on diabetic animals.

The induction of the experimental DM with a single STZ treatment through the caudal vein was, as predicted [3,8,61], capable of reducing tear secretion and alter corneal epithelium. At day 0, prior to the first treatment with the formulations, the tear secretion produced by healthy animals was at least 2 times higher than that of DM animals [*t-test,* $p < 0.05$]. Treatment with blank formulations (without INS), did not alter the tear secretion when compared to those animals with untreated DM (negative control). In contrast, treatment with the formulations containing free or encapsulated INS was able to increase, after 5 days of treatment, the production of tear secretion. At the end of the 15-day treatment, animals receiving DISP-INS and GELMP-INS presented similar tear fluid volume which was not significantly different from that of healthy animals (positive control) (Fig. 3). These results suggest that treatment with these formulations was able to regularize the amount of tears secretion, a particularly important response in the treatment of DES and, more importantly, it means that the INS released from the formulations is active. Although, DISP-INS and GELMP-INS tear secretion was similar to that of positive control animals, GELMP-INS increased tear secretion by 77% in treated animals by the 5th day of treatment as compared to 54% tear secretion in DISP-INS treated animals within the same period; this suggests a more rapid tear secretion regularization when GELMP-INS was employed.

Impression cytology results showed that only healthy animals (positive control) had a large population of morphologically small and rounded cells in several evaluated fields, a large basophilic nucleus and a nucleus:cytoplasm ratio of 1:2 (Fig. 4) and were classified as Grade 0 by the Nelson scale [41]. The DM animals presented metaplasic epithelia and the following formulations protected the epithelia because of the gel formulation and the presence of INS. Received no treatment animals (negative control) presented small number of cells per field with deformed morphology, no nucleus in most cells and a nucleus:cytoplasm ratio of 1: 6 and were classified as Grade 3 [42,62,63]. In addition, the animals who received blank formulations showed some improvement in their condition and were classified as Grade 2. This improvement was similar to that presented by treatment with DISP-INS while other formulations containing INS (MP-INS, GEL-INS and GELMP-INS) presented cells with morphology close to the positive control and were classified as Grade 1 (Table 2, Fig. 4). Thus, MP, GEL and GELMP formulations appear to have somehow protected corneal cells against DM-induced changes, even in the absence of INS. Addition of INS to the delivery systems resulted in cells with characteristics closer to normal cells than treatment with DISP-INS probably because of the increased local bioavailability of INS conferred by such systems.

To substantiate the evidence of increased local bioavailability, INS was quantified in the LG and other eye ball compartments in animal groups treated with GEL-INS, MP-INS and GELMPINS, as shown in Fig. 5, after 18 h of last instillation. In the case of GEL-INS, the presence of the INS in the compartments can be explained by the increased residence time of INS in the eye as conferred by the GEL [36]. The mucoadhesive [59] and penetration enhancer characteristics [47] of chitosan associated with the positive zeta potential of the formulation seems to have favored the interactions of the release systems with the ocular surface, leading to a decrease in INS drainage and increased INS availability in the cornea and lacrimal gland, without, however, compromising cell viability (Fig. 4). Considering MP-INS, in addition to the possible sustained release of INS by the microparticles, their retention in the lacrimal sac [53] and positive zeta potential may have generated a possible cumulative effect leading to a reduction in INS loss due to lacrimation. Finally, GELMP-INS, which delivered the highest amount of INS in the eye, might have benefited from the synergistic action of the GEL and MP. This is because the amount of INS recovered in the LG was higher than the sum of INS released individually by GEL-INS and MP-INS. This may allow us to infer that GELMP-INS seems to be the most appropriate formulation for the treatment of DES based on the tear secretion and INS concentrations recovered when the formulation was employed.

In addition, with reference to the treatment of cornea wounds, the knowledge of the presence of INS receptors on an ocular surface and the known metabolic and mitogenic effects of INS [3,21,24], it is fundamental to develop and deploy strategic formulations capable of providing INS to its receptors over a longer duration. This could favor INS local action and be promising for the treatment of corneal lesions. In this study, GELMP-INS has increased the amount of INS in the eyeball (Fig. 5) as compared to other formulations.

Another important parameter in the clinical evaluation of patients presenting with various types of ocular changes such as DES, DM [64] and corneal lesions [23,25] is the cornea thickness, a measure that reflects tissue health as a function of the correct functioning of the endothelial pump. Our study showed statistical difference [*One-Way ANOVA,* $F(7;56) = 3.092$; $p = 0.0439$] between the epithelium thickness of healthy animals as compared to untreated DM animals (Table 3), a result similar to this study by Cui et. al. [65] reported a smaller thickness of the corneal epithelium in patients with DES. Tukey multiple comparisons test revealed that corneal thickness of the animals treated with GEL-INS ($p = 0.0040$) and GELMP-INS ($p = 0.0269$) was statistically superior to that presented by the untreated DM animals (negative control) but not significantly different from that of healthy animals (positive control) [$p > 0.98$] (Table 3). This allows us to infer a conclusion in conjunction with results of the impression cytology that, treatment with these two formulations may have allowed a better restructuring of the corneal epithelium. Only slight increase in cornea thickness after topical and subcutaneous administration of DISP-INS in other studies were observed [23,25], further reinforcing the importance and superiority of the delivery system developed in the current study.

It is also worth of note to mention that none of the animals that received INS treatment from any of the formulations presented with lens opacity, characteristic of cataract. It is common after five weeks of the induction of DM by STZ that animals show first signs of cataract manifestation, with a greater light-mirroring and some visual impairment, characteristic of normoglycemic imbalance [66,67]. Only two animals, each belonging to the untreated DM (negative control) and DM group treated with GEL (without INS), presented macroscopically these alterations, on the 12th and 14th day of treatment. In this sense, we believe that topical treatment with localized INS may have delayed the onset and/or progression of lens opacity. However, more specific studies must be carried out in order to prove this hypothesis.

5. Conclusion

We successfully developed microparticulate release systems for topical ocular administration of INS with high INS loading and particle size diameter and other physicochemical characteristics that make them adequate for ocular administration. These systems provided favorable pre-clinical results for the treatment of DES and corneal lesions. GELMP-INS administration provided adequate tear fluid volume, cytological and histological analyzes closer to healthy animal in addition to higher INS recovery from target tissues. Thus, the developed GELMP-INS may be a plausible delivery platform for INS in the treatment of DES and corneal lesion.

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