International Journal of Pharmaceutics 509 (2016) 237–243

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Preliminary characterization of dexamethasone-loaded cross-linked hyaluronic acid films for topical ocular therapy

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ARTICLE INFO

Article history:
Received 28 April 2016
Received in revised form 23 May 2016
Accepted 26 May 2016
Available online 27 May 2016

Keywords:
Hyaluronan
Dexamethasone
Model
Ocular
Inflammation

ABSTRACT

The aim of this work was to design and characterize cross-linked hyaluronic acid (HA)–itaconic acid (IT) films loaded with dexamethasone sodium phosphate salt (DEX) for topical therapy of inflammatory ocular surface diseases. Films were chemically cross-linked with polyethylene glycol diglycidyl ether (PEGDE), then physical and mechanical characterization by stress–strain, X-ray diffraction, X-ray fluorescence spectrometry and swelling assays was conducted. A sequential *in vitro* therapeutic efficacy model was designed to assess changes in interleukin (IL)-6 production in an inflamed human corneal epithelial (HCE) cell line after film exposure. Changes in cell proliferation after film exposure were assessed using the alamarBlue® proliferation assay. Experimental findings showed desirable mechanical properties and *in vitro* efficacy to reduce cell inflammation. A moderately decreased proliferation rate was induced in HCE cells by DEX-loaded films, compared to commercial DEX eye drops. These results suggest that DEX and HA have opposite effects. The sequential *in vitro* therapeutic efficacy model arises as an efficient tool to study drug release from delivery systems by indirect measurement of a biological response.

1. Introduction

The ocular surface is affected by a number of inflammatory disorders. Some can be classified as acute and mild, such as seasonal allergic conjunctivitis and transient infectious conjunctivitis, others as chronic and/or more severe, such as vernal keratoconjunctivitis, atopic keratoconjunctivitis, dry eye syndrome and cicatrizing autoimmune conjunctivitis, involving corneal damage and leading to visual loss (Holland et al., 2013).

The systemic use of corticosteroids to treat ophthalmic inflammatory diseases was widely introduced in the 1950s (Raizman, 1996). However, several systemic and ocular-specific adverse effects, such as cataracts and increased intraocular pressure, were reported after a few years of this clinical practice (Becker and Mills, 1963; Covell, 1958; Urban and Cotlier, 1986). Dexamethasone (DEX) is one of the well-known resources used to treat inflammatory processes. It is a synthetic glucocorticoid with potent anti-inflammatory and immunosuppressive effects, being commonly used to treat inflammation of the anterior structures of the eye.

The high complexity of eye anatomy represents an important challenge in the development of new drug delivery systems. Topical administration is the preferred administration pathway for structures of the front of the eye, such as the cornea and conjunctiva, where the pre-corneal tear film and corneal epithelium represent an important barrier that any drug delivery system has to overcome. Traditional eye drop formulations have important limitations, leading to a reduction of their therapeutic capacity, which is usually affected by blinking and tear drainage and replacement reducing drug bioavailability in the pre-corneal area (Ding, 1998; Kompella et al., 2010; Washington et al., 2001). Research and development in this area of Pharmaceutical Sciences is a strong field of scientific and technological interest.

In recent years, several efforts were focused on optimizing corticoid delivery to ocular structures while minimizing systemic adverse effects, leading to a wide range of topical drops, ointments,

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http://dx.doi.org/10.1016/j.ijpharm.2016.05.054
0378-5173/

delayed-release vehicles and intraocular, periocular and oral corticosteroid preparations (Boddu et al., 2010; Gan et al., 2010; Kassem et al., 2007; Kiernan and Mieler, 2009; Kompella et al., 2003; McGhee, 1992). The chemical form of the drug can be very important for ocular bioavailability. Changing the salt can affect the solubility and lipophilicity of the drug. For example, DEX acetate ester has the preferred solubility and partition coefficient properties for corneal permeation compared to the very water-soluble phosphate salt or very lipophilic freebase. However, the phosphate salt is preferred for eye drop formulations because of its water solubility (Gibson, 2004). Despite the growing number of reported approaches, systemic and local ocular adverse effects remain still high (Bielory et al., 2010; Carnahan and Goldstein, 2000; Chew et al., 2011; Holland et al., 2009; Pavesio et al., 2010).

An additional limitation of traditional eye drops is related to the need to maintain the sterility, and ensuring stability and security of the formulation throughout the treatment period. Benzalkonium chloride (BAK) is the most commonly used ophthalmic preservative; however, it is less and less used because of its reported side effects on patients (Noecker, 2001). Single-dose containers appear as the best alternative; nevertheless, the market cost of these formulations is near fivefold higher than that of multi-dose formulations.

Some of these drawbacks can be overcome by using solid, dry, bioadhesive biopolymer-based systems capable of remaining attached to the conjunctiva while delivering the drug in a preservative-free fashion. Thus, pre-corneal contact time lengthening, an increase in drug biodisponibility into ocular structures, and a reduction in drug elimination rate can be obtained.

The use of biopolymers for drug delivery systems has been the subject of numerous reports in scientific literature (Diebold et al., 2011; Lehr and Haas, 2002). These systems, or the materials used to produce them, should gather some desirable characteristics like zero or minimal biological effects, no toxicity or contamination due to chemical residues, and rapid degradation or excretion. Hyaluronic acid or hyaluronan (HA) is a high molecular mass linear polymer consisting of alternating units of N-acetyl-β-D-glucosamine and β-D-glucuronic acid. It is a naturally occurring biodegradable, non-toxic, non-immunogenic and non-inflammatory biomaterial, widely used in medical practice for many pathological conditions. The well-known biocompatibility of HA makes it a suitable material for different ophthalmic applications, such as enhanced contact lenses wettability (Fonn, 2007), eye drops (Miyauchi et al., 1993), surgery (Polack, 1986), tear film stabilizer (Hamano et al., 1996; Prabhasawat et al., 2007), and in the treatment of dry eye (Johnson and Murphy, 2006; Sand et al., 1989) and other ocular disorders (Aragona, 2004; Stuart and Linn, 1985). We have previously reported the preparation of HA–itaconic acid (IT) cross-linked films with polyethylene glycol diglycidyl ether (PEGDE), which were well tolerated by corneal cells both *in vitro* and *in vivo* (Calles et al., 2013). In this work we characterized HA-based films loaded with DEX to evaluate their potential for ocular delivery, using an *in vitro* model of corneal inflammation.

2. Materials and methods

2.1. Materials

HA sodium salt (Mw: 1,560,000 Da) was purchased from Parafarm® (Buenos Aires, Argentina). Dimethyl sulfoxide (DMSO), PEGDE (average Mn = 500) and DEX were purchased from Sigma–Aldrich Corp. (St. Louis, MO, US). Fetal bovine serum, penicillin, streptomycin, epidermal growth factor, insulin, and Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) were provided by Invitrogen-Gibco (Inchinnan, UK) and alamarBlue® reagent was acquired from AbD Serotec (Oxford, UK). Culture plates were purchased from Nunc (Roskilde, Denmark). The enzyme-linked immunosorbent assay (ELISA) kit to measure interleukin (IL)-6 was purchased from Gen-Probe Incorporated (San Diego, CA, US). Commercially available preservative-free (Dexafree) and BAK-preserved DEX (Colircusí) eye drops were obtained from Laboratoires Théa (Clermont-Ferrand, France) and Alcon-Cusí (Barcelona, Spain), respectively. All other chemicals were of extra pure grade.

2.2. Film synthesis

Films were synthesized from HA/IT/PEGDE solutions by a previously described homogeneous cross-linking method (Calles et al., 2013), where IT and PEGDE were used as chemical cross-linker agents in a twice-distilled water solution. The amount of those reagents was adjusted to produce (1:1:2) molar ratios and the HA solution concentration was 2% (w/w). After a 24 h reaction period under slight stirring at room temperature (RT) (21–23 °C), gels were cast at RT under an extraction hood as circular films of 7.0 cm diameter. DEX was incorporated into the HA films during the cross-linking process to achieve a 0.4% concentration w/w (DEX-loaded film). DEX concentration was chosen according to literature (Calles et al., 2013) to achieve transparent films suitable to be used in cell culture experiments. Obtained films were flexible and clear (Fig. 1). Different size and shape samples were cut for film characterization.

2.3. Physicochemical characterization of films

Films were physically and mechanically characterized in terms of: (a) stress–strain; (b) X-ray diffraction; (c) X-ray fluorescence spectrometry; (d) swelling and (e) oxygen permeability.

Film thickness (mm) was measured using a digimatic caliper MDC-1″SFB (Mitutoyo Corporation, Kanagawa, Japan). Five measurements were made for each different film in central and peripheral areas. The stress–strain properties of the films were studied in 4 × 1 cm rectangular samples using an Instron 3369 tester (Norwood, US) in traction mode at 2 mm/min at RT.

X-ray diffraction and X-ray fluorescence spectrometry analyses were made in unloaded- and DEX-loaded film samples using a Philips PW1710 X-ray diffractometer and a Philips MagiX spectrometer (Amsterdam, The Netherlands), respectively. IT, HA and DEX powders were used as controls. X-ray diffractograms were performed for diffraction angles (°2θ) from 2 to 70 using a copper tube anode. After X-ray fluorescence spectrometry, further semi-quantitative analysis was performed by using IQ + Standardless software from PANalytical (Almelo, The Netherlands).

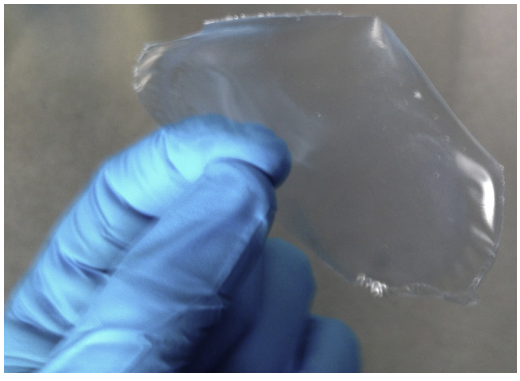

Fig. 1. DEX-loaded films (7.0 cm diameter).

Swelling of DEX-loaded films was performed in distilled water at RT after sample drying to achieve constant weight. Swelling ratio (SR) was calculated as the increase in weight or diameter of immersed films using the following equation: SR = Ws/Wd; where Ws is the weight/diameter of the sample at equilibrium, and Wd is the weight/diameter of the dried sample. Non cross-linked films were not studied because of its immediate dissolution. The experiment was interrupted when a stable swelling was achieved.

Oxygen permeability is an important parameter for topical ocular devices. Materials topically applied onto the ocular surface must have good oxygen permeability to avoid hypoxia-induced complications. The oxygen permeability of DEX-loaded films was measured in an Ox-Tran 2/21 Mocon oxygen transmission rate-testing device (Minneapolis, US) at RT and 50% relative humidity. Masked films with an open area of 2.5 cm^2 were exposed on one side to the carrier gas (nitrogen) and on the other side to the test gas (oxygen). Both flowing gases were automatically controlled to the same temperature. At least three determinations were carried out for each film sample.

2.4. Film sterilization

Two sterilization methods, involving 15 min UV exposure and 5 min immersion in 70% ethanol, were used. After sterilization, films were immersed in DMEM/F-12 culture medium and maintained at 37 °C for 4 days in a Galaxy® CO_2 incubator (New Brunswick Scientific Inc., Enfield, CT, US). Possible bacterial contamination was excluded by visual examination at 24, 48, 72 and 96 h.

Heat sterilization methods were avoided to prevent the degradation of the system. Stress-strain, swelling and anti-inflammatory efficacy studies were performed in sterilized and non-sterilized samples to determine any alteration in DEX-loaded films after the sterilization process used. No significant changes were observed (data not shown).

2.5. Human corneal epithelial cell line

The SV40-immortalized HCE cell line (Araki-Sasaki et al., 1995), derived from human corneal epithelial cells, was used at passages 29–38. Cells were cultured in DMEM/F-12 culture medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml epidermal growth factor, 0.5% DMSO and 5 μg/ml insulin at 37 °C in a 5% CO_2/95% air atmosphere. Media were changed every other day, and daily observation of cultures was made by phase contrast microscopy.

2.6. In vitro therapeutic efficacy

Therapeutic efficacy of DEX-loaded films was studied *in vitro* by measuring their anti-inflammatory and anti-proliferative capacity.

The anti-inflammatory capacity of DEX-loaded films was assessed with a sequential *in vitro* therapeutic efficacy model using a modified version of the model described by Enríquez-de-Salamanca et al. (2008). Briefly, the secretion of the inflammatory cytokine IL-6 by HCE cells in response to 25 ng/ml tumor necrosis factor (TNF)-α exposure ("inflamed cells") was measured after treatment with DEX-loaded films. Four independent sets of inflamed cells were sequentially exposed to the same DEX-film for a 15 min period for each set of cells, and IL-6 secreted into the culture medium was measured by ELISA. Collected supernatants were maintained at −80 °C until use. Non-inflamed, untreated cells, and inflamed untreated cells were used as negative and positive controls respectively. For a better comprehension of the experiment setup, a timeline depicting the procedure followed is shown in Fig. 2.

To measure short-term alterations of cell proliferation rate induced by DEX-loaded film exposure, the alamarBlue® assay was used. This test incorporates a fluorescent non-toxic reagent (resazurin) that is reduced into resorufin and can be quantified. The HCE cells were plated in 24-well plates (40,000 cells/well) and grown for 24 h. Then, commercial DEX eye drops (both preservative-free and including preservative) and sterilized, blank and DEX-

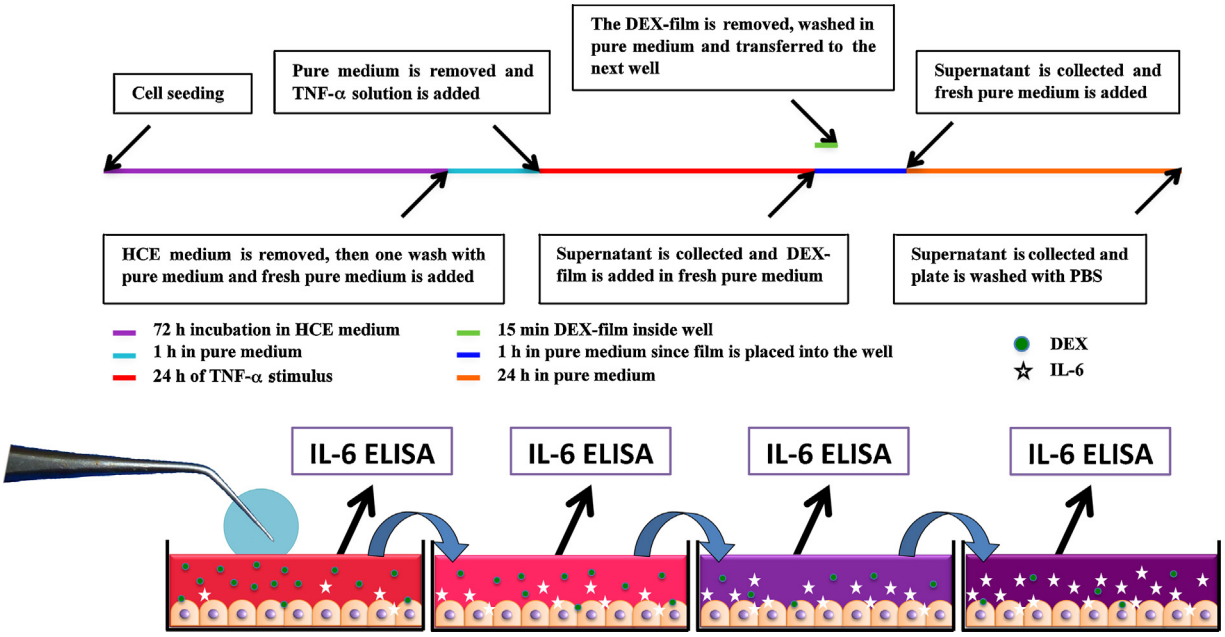

Fig. 2. Schematic showing timeline and procedures followed in the *in vitro* therapeutic efficacy experiment. Pure medium = unsupplemented DMEM/F-12 cell culture medium. Cells were sequentially exposed to the same DEX-film for a 15 min period for each set of cells, and IL-6 secreted to culture medium was measured by ELISA.

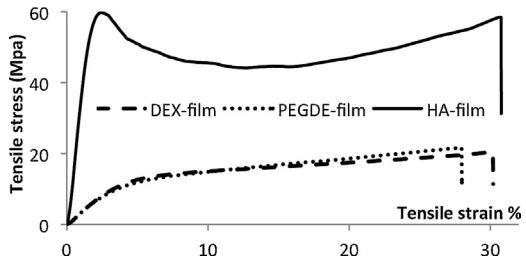

Fig. 3. Stress–strain assay at RT of DEX-film (DEX-loaded cross-linked films), PEGDE-film (unloaded cross-linked films), and HA-film (unloaded and uncross-linked films). Cross-linking changed the tensile stress needed to reach the yield point. All films, modified and unmodified, exhibited plastic deformation.

loaded films were placed in the wells for 24 h. Then, films, eye drops and medium were removed and cells incubated in fresh culture medium with 10% v/v alamarBlue® reagent at 37 °C for 4 h. Afterwards, the medium with the reagent was removed and 100 μl aliquots were transferred to a 96-well plate, where fluorescence was measured at 560 nm (excitation) and 590 nm (emission) wavelengths using a SpectraMax® M5 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, US). Cells were allowed to grow for an additional 72 h, and the procedure detailed above was repeated every 24 h. Cells unexposed to films were used as controls. The proliferation index was then calculated after 24, 48, 72 and 96 h as a percentage by referring to measurements in control cells. Three independent experiments were performed in duplicate. DEX concentration in each well was calculated to be 11 nM for every DEX formulation used (Djalilian et al., 2006; Ebihara et al., 2011; Jones et al., 2010).

Finally, cells exposed to all DEX formulations and blank films were observed and photographed 24, 48, 72 and 96 h after exposure by phase contrast microscopy (Nikon Eclipse TS100, Tokyo, Japan) in order to evaluate possible morphological alterations.

2.7. Statistical analysis

Results were expressed as the mean ± standard error of the mean (n = 3–4 in duplicates). Differences were considered to be significant when $p \leq 0.05$. Significant differences were determined by the Student's *t*-test.

3. Results

3.1. Physicochemical characterization of films

The mean thickness for the stress–strain samples (DEX-loaded, unloaded and non-cross-linked films) was 157 ± 17 μm. Stress–strain assays (Fig. 3) revealed differences in the tensile properties of cross-linked films (DEX-loaded and unloaded), changing the tensile stress needed to reach the yield point. Thus, cross-linked films required lower stress to change their behavior from elastic to plastic. In contrast, both films modified with PEGDE as a cross-linker agent and unmodified films exhibited plastic deformation.

X-ray diffraction assay did not show different crystallographic properties between DEX-loaded and unloaded films (Fig. 4). Also, films were analyzed by elemental analysis using a high sensitive technique such as X-ray fluorescence spectrometry. Spectra analysis of X-ray fluorescence of the samples was performed using the IQ+Standardless software to calculate semi-quantitatively the percentage of elements found (this software does not calculate the statistical analysis). DEX-loaded films showed higher intensity for P atoms (present in DEX molecules) in DEX-loaded films, confirming the presence of DEX in those films. The intensity for P atoms in DEX-loaded films was 0.023% higher than that found for unloaded formulations.

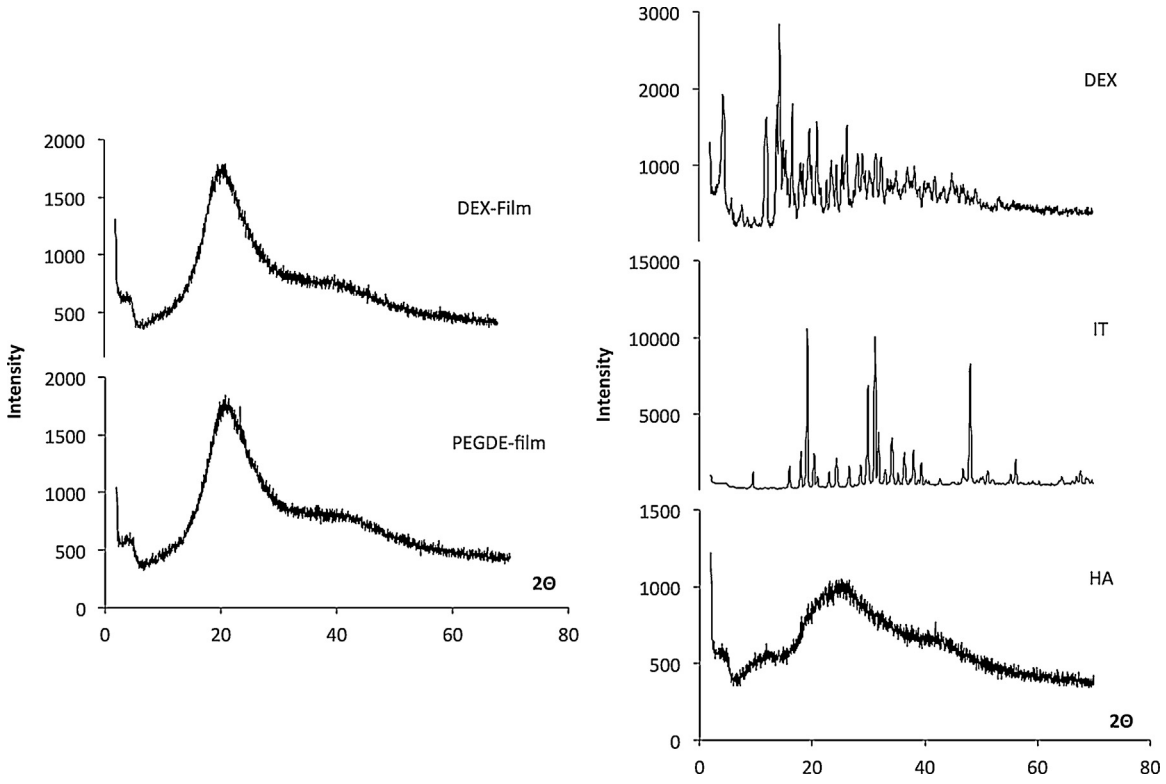

Fig. 4. X-ray diffractogram of DEX-film (DEX-loaded cross-linked films), PEGDE-film (unloaded cross-linked films), HA (HA powder), IT (IT powder), and DEX (DEX powder). Crystallographic properties between DEX-loaded and unloaded films showed no differences.

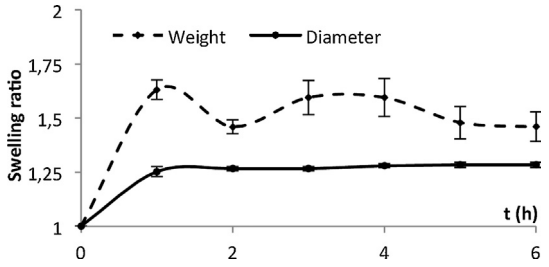

Fig. 5. Swelling behavior of DEX-loaded films calculated in terms of weight or diameter. Swelling was biphasic with the initial size and weight increase in the first hour followed by a sustained phase.

The swelling assay revealed high stability for drug-loaded cross-linked films (Fig. 5). PEGDE was found to be effective as a cross-linking agent, leading to insoluble materials. Films were stable under water immersion up to 6 h.

Oxygen transmissibility (Dk/t) is a parameter well studied in contact lenses (CL). Harvitt and Bonanno (Harvitt and Bonanno, 1999), utilizing the metabolic requirements of the cornea under hypoxic conditions, suggested the necessity of providing the cornea with higher levels of oxygenation to avoid hypoxia through the whole cornea under unfavorable conditions, such as overnight CL wear. In this case, a minimum Dk/t of 125 Barrer/cm would be necessary. Film samples showed good oxygen permeability with a mean value of 329.57 cm^3 mm/m^2 day atm ± 71.39 (equivalent to 323.81 Barrer/cm).

3.2. Film sterilization

Both sterilization methods (70% ethanol immersion and UV radiation) used were equally efficient in preventing bacterial contamination. No bacterial proliferation was observed in the culture medium during the experiments using sterilized materials. This result has special relevance not only from an experimental point of view but also considering that sterility is a key requirement for the production of ophthalmic formulations.

It should be noted that biological indicators are the most accepted means of monitoring the sterilization process because they directly determine whether the most resistant microorganisms (e.g., *Geobacillus* or *Bacillus* species) are present. The techniques used in this work confirmed no contamination for the total length of *in vitro* experiments. However, they cannot confirm actual sterilization instead disinfection. That kind of assessment would be warranted in future studies when optimized formulations for human application are developed.

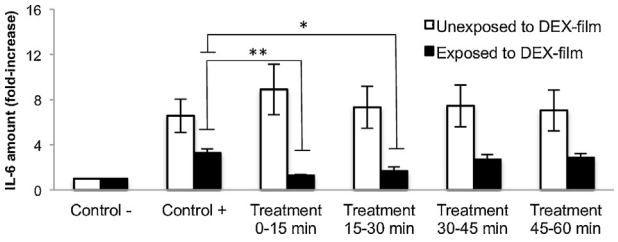

Fig. 6. IL-6 production by TNF-α inflamed HCE cells treated with DEX-films. *p ≤ 0.05; **p ≤ 0.01. Inflamed cells exposed to the DEX-loaded film for the first (0–15 min) or second (15–30 min) time significantly reduced their IL-6 production compared to controls. The third (30–45 min) or the fourth time (45–60 min) showed no significant reduction of IL-6 secreted levels.

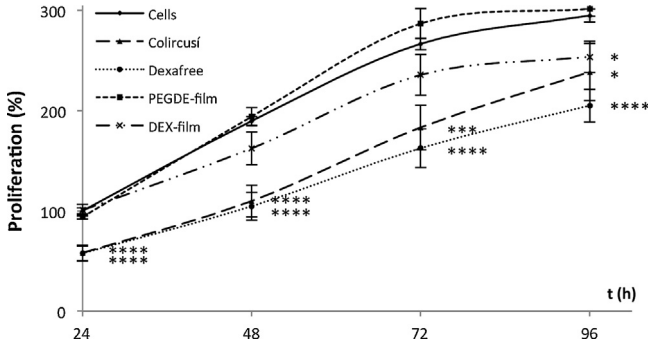

Fig. 7. HCE proliferation rate after 24 h exposure to: Colircusí (eye drops containing BAK), Dexafree (preservative-free commercial eye drops), PEGDE-film (unloaded cross-linked HA film) and DEX-film (DEX-loaded cross-linked HA film). Significant differences regarding control cells: *p ≤ 0.05; ***p ≤ 0.001; ****p ≤ 0.0001. HCE cell proliferation rate was decreased after exposure to all formulations containing DEX. Exposure to unloaded films slightly increased proliferation rate. DEX-loaded film exposure moderately decreased cell proliferation rate compared to commercial eye drops, although the percentage reduction in proliferation was similar to that induced by conventional DEX eye drops after 96 h.

3.3. In vitro therapeutic efficacy

IL-6 levels secreted by TNF-α inflamed cells (Control+) reached an almost sevenfold increase compared to those basally secreted by non-inflamed cells (Control−) (Fig. 6). Inflamed cells exposed to the DEX-loaded film for the first (0–15 min) or second (15–30 min) time significantly reduced their IL-6 production compared to controls. When the DEX-loaded film was used for the third (30–45 min) or the fourth time (45–60 min), levels of secreted IL-6 were still reduced compared to those of Control+, although the reduction was not significant. This would indicate that DEX was quickly released in the first 30 min, possibly due to the sink conditions used (DEX concentration in culture medium was $\sim 8 \times 10^3$ times lower than its water solubility), which are very different from *in vivo* tear volume. Unloaded films had previously shown that IL-6 production is not increased in HCE cells after exposure (Calles et al., 2013).

The corneal epithelial cell proliferation rate (Fig. 7) was decreased after exposure to all formulations containing DEX. A marked decrease ($p \leq 0.001$) was observed 24 and 48 h after exposure to both preservative-free and conventional DEX eye drops. Exposure for 24 h to unloaded films slightly increased proliferation rate. However, DEX-loaded film exposure moderately decreased cell proliferation rate compared to commercial eye drops, although the percentage reduction in proliferation was similar to that induced by conventional DEX eye drops after 96 h.

Morphological details of all exposed and unexposed HCE cells were observed every 24 h up to 4 days. Cells remained intact throughout the proliferation experiment regardless of their exposure to any of the DEX formulations or films (Fig. 8).

4. Discussion

Physicochemical characterization provided valuable information for HA modification, leading to more stable materials. The swelling behavior revealed a slight increase in the size and weight of samples that remained stable throughout the experiment. These findings indicate that cross-linking of HA chains was enough to maintain stability, but not so excessive as to prevent the desired chain mobility needed to allow the bioadhesion process. As previously described in section 3.1, the cross-linking process was effective to bond HA chains covalently; it prevented material solubility and did not affect oxygen permeability or HA plasticity. In fact, PEGDE is a homobifunctional cross-linker composed of two

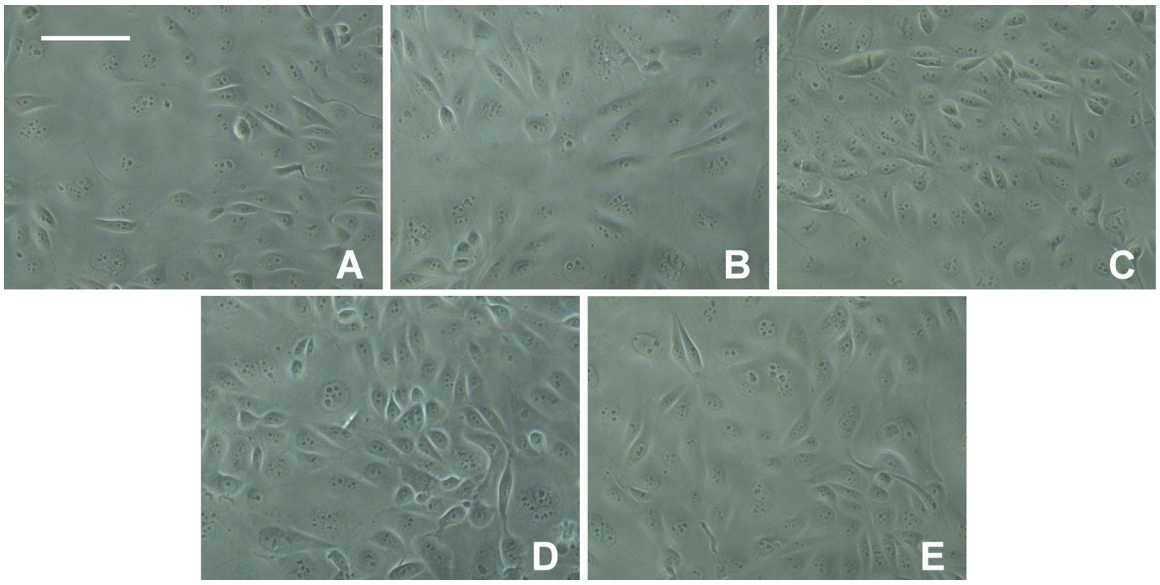

Fig. 8. Morphological details of HCE cells obtained by phase contrast microscopy 72 h after exposure. (A) unexposed cells; (B) Colircusí (DEX eye drops containing BAK); (C) Dexafree (DEX preservative-free commercial eye drops); (D) PEGDE-film (unloaded cross-linked HA film); (E) DEX-film (DEX-loaded cross-linked HA film). Scale bar = 50 μm. Cell morphology remained intact throughout the proliferation experiment after exposure to all formulations.

epoxy terminal groups with a long polyethylene glycol (PEG) chain binding them. Although the cross-linking process usually leads to increased rigidity as a result of the restrained movement of the polymer molecules, the particular structure of PEGDE with long and flexible PEG chains can mitigate these effects. Thus, PEGDE gives the possibility of cross-linking HA without losing polymer ductility as previously reported (Calles et al., 2013). Moreover, the presence of DEX in loaded films did not affect HA flexibility; this is expected considering the low drug amount per film mass.

The high presence of an amorphous material like HA could mask the presence of all other substances in the formulation, leading to no perceptible difference in mechanical behavior as well as in crystallographic properties. Thus, no differences were observed in tensile or X-ray assays. However, much more sensitive techniques such as X-ray fluorescence spectrometry were appropriate to determine the presence of DEX in the formulation by detecting a higher intensity of P atoms.

The sequential *in vitro* therapeutic efficacy model showed a decrease of IL-6 secretion by HCE cells directly related to DEX release from cross-linked films. Films exhibited low drug retention, and the hydrophilic sodium salt of DEX was quickly released into the cell culture medium. Similar findings have been reported for DEX-loaded HA films (Luo et al., 2000), where HA was first converted to the adipic dihydrazide derivative and then cross-linked with the macromolecular homobifunctional reagent poly (ethylene glycol)–propiondialdehyde to give a polymer network. In this work, the DEX base was loaded by using an ethanol solution; and despite the hydrophobic nature of the DEX base, the release profiles reached 80% release in 30 min (Luo et al., 2000).

We proposed a novel sequential *in vitro* study to show cellular response after DEX-loaded film treatment. The advantage of this *in vitro* model is its capacity to study the effect of a drug in a dynamic, sequential, time-dependent fashion. To the best of our knowledge, there are not similar approaches in scientific literature. The closest approach was reported by Ito et al. (2007), where the *in vitro* efficacy of cross-linked HA hydrogels was studied by releasing DEX into the cell culture medium and then exposing peritoneal macrophages to those media. The response to treatment was determined by the quantification of TNF-α secreted from exposed cells. In this work, the authors used longer contact time between cells and DEX-containing media, and did not report whether the determination of TNF-α was in the same DEX-containing media, or the time at which the TNF-α measurement was done. In order to standardize previous variables, we decided to measure the effect of DEX by adding the studied systems directly onto cells seeded in well plates instead of exposing cells to a medium where DEX was released. Moreover, we always measured IL-6 produced by corneal epithelial cells after 24 h (pre- and post-treatment). Thus, we provide a systematic tool for *in vitro* efficacy determination of drug delivery systems. It is important to note that the system reported by Ito et al., showed longer release rate than our DEX-loaded films.

This promising model takes special relevance in the current international context where new and innovative models are demanded to reduce animal experimentation. It could be adapted to different *in vitro* physiological responses to determine the efficacy of solid drug delivery platforms and provide illustrative indirect information about release behaviors when drug quantification is difficult or when drugs are present in a very low concentration as occurs in our system. However, the limitations of the model to mimic physiological conditions, such as ocular tear volumes and turnover rate, should be carefully considered before predicting drug release rates with obtained results. Thus, longer studies should be carried out to assure more accurate predictions in physiological conditions.

The slightly more proliferative behavior of HCE cells after exposition to DEX-loaded films could be attributed to the "protective" effect of HA on the ocular surface. It was previously reported that HA promotes corneal epithelial wound healing by stimulating the proliferation of the corneal epithelium (Inoue and Katakami, 1993). In addition, other authors reported that HA was an effective protective agent with antioxidant properties that could decrease DNA damage and cell apoptosis induced by BAK in HCE cells (Wu et al., 2011). Thus, the anti-proliferative effect of DEX could be masked by the presence of HA in formulations. Nevertheless, this hypothesis was not studied in this work.

5. Conclusions

Cross-linking of HA films leads to insoluble, stable and oxygen permeable materials without loss of plasticity. X-ray fluorescence

spectrometry was the most sensitive method for detecting DEX presence. The *in vitro* efficacy models showed the sustained effect of DEX delivered from DEX-loaded films on IL-6 production by inflamed corneal epithelial cells up to 1 h, and a moderated reduction in proliferation rate.

The sequential *in vitro* efficacy model was shown to be a useful tool as a first approach to study drug delivery performance by indirect measurement of a biological response induced by a vehiculated drug. A reduction in culture media volume and refresh time could be key to overcome the limitations of the model to mimic physiological tear volume and turnover.

Considering all this evidence, cross-linked HA solid platforms are promising materials for ocular drug delivery to be used as preservative-free, topically applied inserts in the eye surface; however, more experiments are warranted to improve drug retention and efficacy.

Acknowledgments

This work was supported by CONICET (Argentinian National Council of Scientific and Technical Research), Universidad Nacional del Sur (Argentina), FEDER-CICYT MAT2013-47501-C02-1-R (Spanish Ministry of Economy and Competitiveness) and 612218/3D-NET (Marie Curie-IAPP Action, 7 EU Program) grants. The authors thank Proof-Reading-Service.com for English review service.

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