International Journal of Pharmaceutics 432 (2012) 1–10

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

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

Conjunctival and corneal tolerability assessment of ocular naltrexone niosomes and their ingredients on the hen's egg chorioallantoic membrane and excised bovine cornea models

Hamdy Abdelkader[a,c,*], Sayed Ismail[b], Amal Hussein[c], Zimei Wu[a], Raida Al-Kassas[a], Raid G. Alany[a,d]

[a] School of Pharmacy, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand
[b] Department of Pharmaceutics, Faculty of Pharmacy, Assiut University, Assiut, Egypt
[c] Department of Pharmaceutics, Faculty of Pharmacy, Minia University, Minia, Egypt
[d] School of Pharmacy and Chemistry, Kingston University London, Kingston upon Thames, UK

ARTICLE INFO

Article history:
Received 24 December 2011
Accepted 12 April 2012
Available online 30 April 2012

Keywords:
Naltrexone hydrochloride
Opioid growth factor
Niosomes
Bovine cornea
Ocular delivery

ABSTRACT

This study aimed at combining the hen's egg test-chorioallantoic membrane (HET-CAM), bovine corneal opacity and permeability (BCOP) test and histological examination of excised corneas to evaluate the conjunctival and corneal toxicity of niosomes and their ingredients. Various surfactant/lipid combinations and concentrations (1–10%, w/v) were investigated for the ocular delivery of an ambitious drug (naltrexone hydrochloride) for treatment of diabetic keratopathy. Four niosomal formulations were investigated and found to be non irritant to the 10 days old HET-CAMs (an acceptable conjunctival model). Only one of the tested ingredients (sodium cholate – CH) showed moderate irritation, however such an effect was diminished when incorporated into niosomes. Corneal opacity and fluorescein permeability scores for the test substances correlated well with the HET-CAM test results. Corneal erosion and stromal thickness were found to be in agreement with the HET-CAM and BCOP results, which discriminated well between moderately and mildly irritant test substances. Corneal histological examination revealed toxicity signs included epithelial erosion, stromal condensation and stromal vacuolisation, which allowed better discrimination between strong and moderate irritants. It is concluded that the prepared niosomes possess good ocular tolerability and minimal ocular tissue irritation. They can be further investigated as ocular delivery systems using appropriate animal models.

1. Introduction

Topical ocular application of naltrexone (NTX) in doses of up to 0.4 mg/ml has been shown to markedly accelerate the wound healing of cornea in humans (Zagon et al., 2000), rats (Zagon et al., 1998b) and rabbits (Zagon et al., 1998a), as well as diabetic rodents (Zagon et al., 2002a). Moreover, topical application of NTX can enhance diabetic corneal epithelial healing without causing morphologic abnormalities in the reassembly of adhesion structures (Zagon et al., 2007). Topical treatment with NTX has been found to normalise tear production and corneal sensitivity in type 1 diabetic rats (Zagon et al., 2009).

NTX accelerates corneal wound healing through the blockade of the opioid growth factor (OGF) interaction with the OGF receptor (Zagon et al., 2002b). Consequently, it can enhance DNA synthesis and corneal epithelialisation. However, its exact mechanism for normalising tear production and restoring corneal sensation in diabetes mellitus is still unclear (Zagon et al., 2009). It is worth mentioning that the corneal safety of topically applied NTX has been studied (Zagon et al., 2006). The results showed naltrexone to be non-toxic at concentrations ranging from 10^{-3} to 10^{-7} M are not toxic when applied topically to the cornea.

Naltrexone can be considered as a promising new ophthalmic pharmaceutical for treatment of diabetic keratopathy (Abdelkader et al., 2011b). Preformulation studies conducted on NTX demonstrated that NTX is a hydrophilic agent with log *P* value of 1.61 at 35 °C (the ocular surface temperature) suggesting that corneal permeation is the likely rate limiting step for its ocular absorption. Additionally, further preformulation studies revealed that NTX is vulnerable to oxidation in aqueous solutions (Abdelkader et al., 2011c). Non-ionic surfactant vesicles (niosomes) were developed for ocular delivery of NTX. The developed niosomes enhanced the precorneal penetration of NTX through excised bovine corneas. Further, preliminary studies conducted using

* Corresponding author at: 93 Grafton Road, School of Pharmacy, The University of Auckland, Auckland 1010, New Zealand. Tel.: +64 21 077 6704; fax: +64 99 367 7192.
E-mail address: h.abdelkader@pharm.miniauniv.edu.eg (H. Abdelkader).

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http://dx.doi.org/10.1016/j.ijpharm.2012.04.063

the hen's egg chorioallantoic membrane (conjunctival models) suggested that these niosomes have minimal ocular irritation (Abdelkader et al., 2011a).

In recent decades, researchers testing ocular dosage forms have recorded toxicological signs of ocular tissues exposed to topically applied drugs. Ocular tissues, such as the cornea and conjunctiva, are susceptible to injuries and adverse ocular effects, either from the administered drug or excipients used in the finished pharmaceutical product (Basu, 1984; Li et al., 2008). For instance, amphotericin B and keroconazole (antifungal agents) can cause corneal oedema and corneal abnormalities when administered topically (Foster et al., 1981). Excessive use of topical anaesthetics can produce corneal lesions and ulcers (Basu, 1984). Anti-inflammatory corticosteroids are found to retard epithelial corneal wound healing and induce glaucoma (Basu, 1984; Basu et al., 1981; Li et al., 2008). Ocular side effects due to pharmaceutical excipients have also been reported. For instance, benzalkonium chloride (BAC), a quaternary ammonium cationic surfactant, is a commonly used preservative in ophthalmic products. BAC has been reported to cause corneal opacity, a decrease in corneal epithelial microvili, conjunctival hyperaemia (red eye) and delayed wound healing (Li et al., 2008; Pfister and Burstein, 1976). Assessment of the toxicity of ophthalmic formulations and the potential for ocular irritation represents an essential step in the development of new ocular delivery systems (Basu, 1984; Lang et al., 2002).

From the regulatory viewpoint, there is relatively little guidance from ICH on the non-clinical toxicity data needed for registration of ocular drugs, including those delivered using novel carriers (Avalos et al., 1997; Short, 2008).

European regulatory authorities recommend ocular tolerance studies (CPMMP/SWP/21/00). These studies consist of a single-dose tested in a small number of rabbits (1–3), with a drop size of 20–30 μl in a single dose administration, along with observation and scoring for any ocular abnormalities (Short, 2008). The *in vivo* ocular test (Draize test) has been highly criticised ethically and scientifically over the past two decades (Anderson and Russell, 1995; Bruner, 1992). Scientifically, the reproducibility of the test is poor, as scoring and interpretation are subjective, depending on visual observation. It tends to over-predict the human response, because it uses a high dose of the test materials and the site of application is at the conjunctival sac of the rabbit eye. Ethically, the use of large numbers of live animals, the application of large doses of painful and stressful test material and the length of recovery time are criticised by animal welfare groups. Hence, the *in vitro* and *ex vivo* tests can offer some advantages over the conventional *in vivo* ones. These include reducing the number of animals involved, and using more quantifiable and objective end-point measurements. These tests are also more convenient and less time-consuming (Avalos et al., 1997).

The HET-CAM test serves as a possible model for conjunctival irritation testing, as it responds to irritant substances with an inflammatory reaction similar to that produced by conjunctival tissue (Alany et al., 2006; Anderson and Russell, 1995; Bruner, 1992). However, good eyesight relies on the cornea as a refractive component. The cornea serves as the gateway to the eye for external images. The transparency and smoothness of the cornea is essential to maintain its protective and refractive functions (Nishida, 2005). Consideration must be given to the safety of the corneal tissue when using the developed ocular formulation. It is not surprising that both corneal and conjunctival damage together constitute 100 out of 110 possible points comprising the Draize test score. Therefore, it has been found more advantageous to develop an *in vitro* alternative model to investigate the safety of the test material on both the cornea and the conjunctiva (Weterings and Vanerp, 1987). Combined isolated enucleated eyes and HET-CAM tests were previously developed. These combined tests were evaluated against the effects observed in the rabbit eye test. A good correlation was obtained with a broad range of chemical substances (Weterings and Vanerp, 1987). Moreover, these methods are easy to perform, inexpensive, reproducible and uses less subjective scores than the *in vivo* rabbit test (Avalos et al., 1997; Budai et al., 2010; Weterings and Vanerp, 1987).

However, predicting ocular irritation using opacity and permeability endpoints is challenging when the test substances produce a delayed reaction by interacting with nucleic acids and mitochondrial proteins, rather than causing an immediate loss of epithelial integrity. Therefore, histological examination of the cornea after treatment with the test substances can provide a more comprehensive assessment of the depth of injury and cellular damage of the three principle layers of the cornea (Curren et al., 2000; Curren and Harbell, 1998).

In this report, the HET-CAM test, BCOP assay and histological examination of excised bovine corneas were jointly used to investigate the ocular irritation potential of niosomes and their ingredients.

2. Experimental

2.1. Materials

NTX was purchased from Mallinckrodt Inc., St. Louis, MO, USA. Span 60, cholesterol and dicetyl phosphate (DCP) were purchased from Sigma–Aldrich, St. Louis, USA. Poly-24-oxyethylene cholesteryl ether (C24) was a generous gift from Lubrizol Inc., Cleveland, USA. Sodium cholate (CH) was a generous gift from New Zealand Pharmaceuticals, Palmerstone North, New Zealand. Fertilised hen's eggs (Brown Shavers) were purchased from Bromley Park Hatcheries Ltd., Tuakau, New Zealand. Freshly excised cow eyes were collected from Auckland Meat Processors, Auckland, New Zealand.

2.2. Methods

2.2.1. Preparation of ocular niosomes

Niosomes encapsulating NTX were prepared using the reverse-phase evaporation (REV) method (Abdelkader et al., 2011a). The prepared niosomes were previously evaluated for size, morphology, gel/liquid transition temperature and entrapment efficiency (EE%) (Abdelkader et al., 2011a).

Table 1 reveals the composition of the prepared niosomes.

2.2.2. Conjunctival (HET-CAM) test

2.2.2.1. Preparing and growing the CAMs. Freshly collected fertilised hen's eggs were incubated at 37.5 ± 0.5 °C and 66 ± 5% relative humidity (RH) for 3 days. On day three, the eggshells were opened by cracking the underside of the egg against the edge of a plastic Petri dish. The content of the shell was then poured into an in-house fabricated growing chamber (Alany et al., 2006). Once in the growing chamber, each egg was examined for the viability of the embryo (intact CAM and yolk sac) (Fig. 1). Only viable embryos with intact CAMs and yolk sacs were further incubated at 37.5 ± 0.5 °C

Table 1
Codes and composition of the prepared niosomal formulations.

Formulation code	Molar ratio				
	Span 60	Cholesterol	DCP	C24	CH
F-S60	7	3	0	0	0
F-DCP	6.75	2.75	0.5	0	0
F-C24	6.9	2.9	0	0.2	0
F-CH	6.75	2.75	0	0	0.5

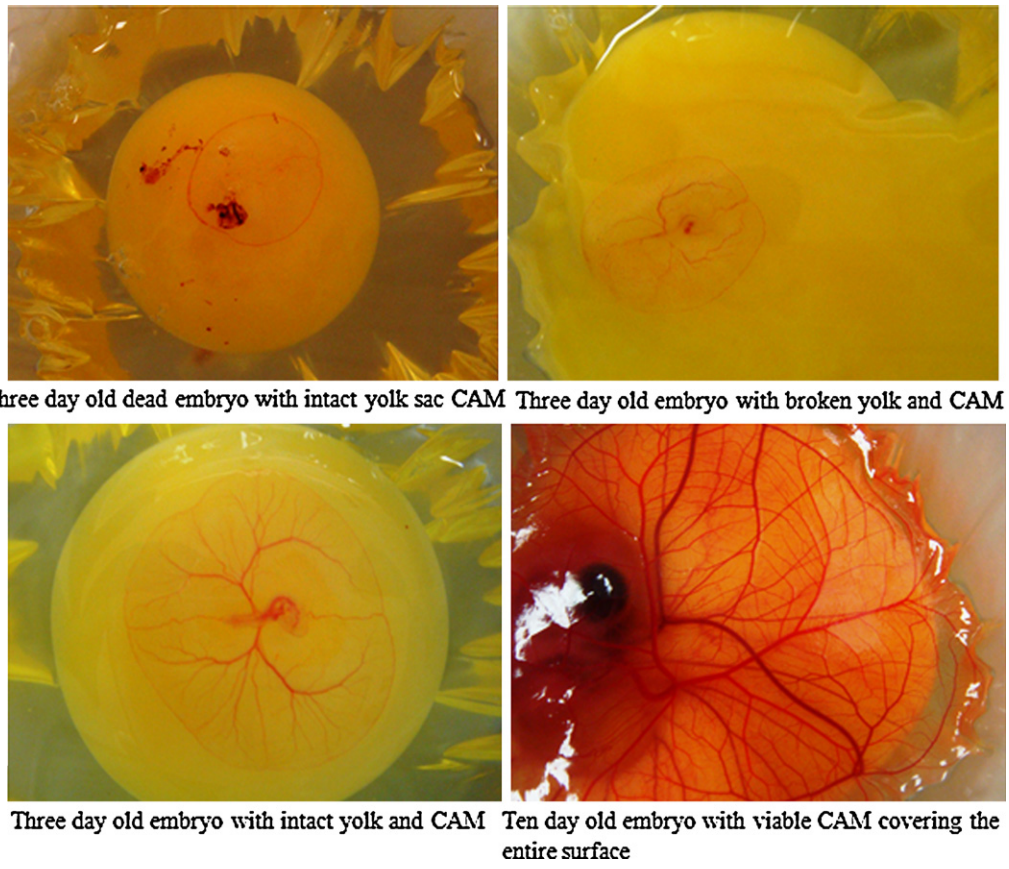

Fig. 1. Development stages of the growing embryos.

and 66 ± 5% RH. The temperature and RH were recorded every hour using RH/Temp data logger, EL-USB-2, UK.

2.2.2.2. Test substances and noisomal formulations. The conjunctival irritation potential of the following test substances was investigated:

- NTX solution (40 mg/ml) in PBS pH 7.4.
- Span 60 and cholesterol, DCP, C24 and CH in their pure (powder) form.
- Surfactant solutions of 0.3% (w/v) CH and 0.4% C24 in PBS representing the actual concentrations of the surfactant additives in the proposed niosomal formulations.
- Drug-free (plain) niosomal formulations (F-S60, F-DCP, F-C24 and F-CH) at three different total surfactant/lipid concentrations (1, 5 and 10%, w/v).
- NTX loaded niosomal formulations F-S60, F-DCP, F-C24 and F-CH (5%, w/v).

2.2.2.3. Irritation testing, scoring and classification. On day 10, 0.2 ml (liquid) or 0.1 g (solid) of the test substances was placed on the membrane. For each test substance three eggs were used. NaOH (0.5 M) was used as a positive control strong irritant, acetone as a moderate irritant, propylene glycol as a slight irritant, and normal saline as a negative control (Alany et al., 2006).

After the application of the test substance, the blood vessels and capillaries were examined for irritant effects. The irritant effects were hyperaemia, haemorrhage and clotting at different time points post-application for 5 min (Abdelkader et al., 2011a). A time-dependent numerical score was allocated to each test substance or formulation (Table 2).

The sum of the time-dependent numerical scores for all three irritant responses gave a single numerical value. This value interpreted the irritation potential of the test substance (Table 2). The mean score value allowed assessment of the irritation potential by a classification system similar to the Draize test (Luepke, 1985).

2.2.3. BCOP test

2.2.3.1. Cow eye's acquisition and examination. Cow eyes were obtained from a local slaughterhouse (Auckland Meat Processors, Auckland, New Zealand). The collected eyes were transported to the laboratory in cold saline (8–10 °C). The eyes were examined for epithelium detachment, corneal opacity and corneal vascularisation. Eyes with corneal damage or abnormalities were discarded.

2.2.3.2. Test substances and niosomal formulations. The same controls, components and formulations described in the HET-CAM test section were also investigated for their corneal irritation potential.

2.2.3.3. Irritation testing, scoring and classification. Small plastic cups were used to hold the eyes (cornea upwards) in the humid atmosphere of a closed water bath at 37 ± 0.5 °C for 10 min (Weterings and Vanerp, 1987). A silicon O-ring (thickness 1.78 mm, an internal diameter 7.6 mm) was carefully placed on the central

Table 2
Irritation scores and interpretations used in HET-CAM test.

	Score			Cumulative score	Irritation assessment
Effect/time (min)	0.5	2	5	0–0.9	None
Hyperemia	5	3	1	1.0–4.9	Slight
Haemorrhage	7	5	3	5.0–8.9	Moderate
Clotting/coagulation	9	7	5	9.0–21.0	Severe

Table 3
Irritation scores and interpretations used in BCOP test.

Opacity	Score	Epithelial integrity	Score	Epithelial detachment	Score	Cumulative score	Irritation assessment
None	0	None	0	No gross abnormalities	0	≤0.5	None
Slight	1	Diffuse and weak	0.5	Wrinkling of corneal surface	2	0.6–1.9	Slight
Marked	2	Confluent and weak	1	Loosening of epithelium	3	2.0–4.0	Moderate
Severe	3	Confluent and intense	1.5	Epithelium absent	4	>4	Severe
Opaque	4						

part of the cornea, to localise the application site and for easy and reproducible test material application. One drop of saline was applied inside the ring and the eyes were equilibrated in a closed water bath for 5 min. The test substance was applied to the cornea inside the ring at a volume of 0.1 ml or 0.1 g. After 30 seconds (s), the eyes were rinsed with saline (approximately 10 ml), followed by further incubation in the closed water bath for another 10 min. The extent of corneal injury was assessed by evaluating the opacity, followed by application of sodium fluorescein solution (2% (w/v) pH 7.4) to examine the integrity of the corneal epithelium, using an examination lamp and cobalt blue filter (Leica, GmbH, Germany). The observations were graded according to individual numerical scores for opacity, epithelial integrity (degree of staining) and epithelial detachment (Weterings and Vanerp, 1987). The sum score was calculated and the mean scores for each of the 3 exposed eyes were used to interpret the corneal irritation potential (Table 3).

2.2.4. Histopathological evaluation of the excised bovine corneas

Bovine corneas previously tested using the BCOP test along with fresh corneas treated with the prepared niosomes for longer periods of time (1, 3 and 8 h) were histologically studied. The samples were dissected and fixed in 10% (v/v) neutral buffered formalin (NBF) for at least 24 h, and then fixed in 70% (v/v) ethanol for another 24 h. The fixed samples were transferred into labelled cassettes and placed in 70% (v/v) ethanol. They were subjected for tissue processing, embedding, sectioning and staining. Each section was paraffin-embedded, bisected into two equal halves and finally mounted in a paraffin block so that a section of each half cut and placed on a single microscopic slide. The slides were stained with haematoxylin and eosin (H&E). The stained corneal sections were imaged using a light microscope (Leica DMRE, GmbH, Germany) and the thickness of stroma was measured using Leica DMRE software.

2.2.5. Statistical analysis

A one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparisons at the 5% significance level was used to test the statistical significance of the stromal thickness values post application of test substances at different time points. These were performed using GraphPad Software Version 3.05, San Diego, CA, USA.

3. Results and discussion

3.1. Conjunctival (HET-CAM) test

Fig. 1 outlines the development stages of the growing embryos. Only embryos having intact yolk and viable CAM were further incubated to day 10. The ten days old CAMs were utilised for application of the test substances and niosomes. The temperature and relative humidity were kept at 37.5 ± 0.5 °C and 67 ± 5% RH. These were found to be the optimum conditions for CAM growing (Auerbach et al., 1974; Luepke, 1985).

According to the original method, the CAMs were fully grown in their eggshells until the test day (Luepke, 1985). On day 10 of incubation (test day), a window was created in the eggshell using a dentist rotary saw-blade and the inner egg membrane was removed to expose the CAM for applying test substances (Luepke, 1985). This method was found to damage the CAM occasionally when eggshell particles fell onto the CAM surface during the drilling procedure. Moreover, the window created in the eggshell is relatively small hence gives limited access to and visibility of the CAM. This impedes the evaluation of vascular responses and minimises the total area of CAM surface available for testing (Lawrence et al., 1986).

A modified method was reported in the literature, where the chick embryo was grown in a Petri dish from day 3 onwards to allow ready access to the entire CAM surface for better visibility and convenience (Auerbach et al., 1974). This method was slightly modified for this study using the in-house made growing chambers to replace the Petri dishes (Alany et al., 2006).

Accordingly, the growing chambers can offer the embryo a more natural environment to grow. The curved, elastic nature of the cellophane membrane mimics the inner membrane of the eggshell compared with the flat, rigid surface of the Petri dish. This could explain the higher survival percentage for embryos (60%) compared with that obtained when using a Petri dish to grow the embryos (50%) (Auerbach et al., 1974). These findings suggest that the cracking procedure and growing conditions are critical to the survival of the embryos and therefore the number of CAMs available for testing.

Fig. 2 shows the cumulative HET-CAM scores for the controls, NTX solution (40 mg/ml) in PBS, the test substances used in fabricating the niosomal formulations and the prepared niosomes.

The average cumulative scores calculated for NTX solution, Span 60 powder, cholesterol powder and DCP powder were found to be <0.9. These results reveal that these test substances are practically non-irritant when applied to the surface of the CAM. Span 60 and cholesterol are regarded as non-irritant and are widely used in the cosmetic and food industry (Buehler, 2003; Lawrence, 2003). In contrast, CH powder was found to be strongly irritant with a cumulative score of 14 ± 1.4. This result is in agreement with the *in vivo* rectal irritation results in rats. A solution of 25 mM (equivalent to 1.1%, w/v) CH was found to be strongly irritant to rectal mucosa of rats. Immediate strong contractions were observed after application into the rectum. Lumen congestion, oedema and haemorrhage were also detected after 20 min (Kinouchi et al., 1996).

All the prepared niosomal formulations (total lipid concentrations up to 10%, w/v) were assessed for the irritation potential and the results did not show any signs of inflammatory reactions at the nominated time points. Similar results have been reported on niosomes assessed using the *in vivo* rabbit eye test (Draize test) (Abdelbary and El-gendy, 2008). Niosome formulations composed of Tween 60:cholesterol:DCP at 1:1:0.1 molar ratio, Tween 80: cholesterol at 1:1 molar ratio and Brij 35:cholesterol: DCP at 1:1:0.1 molar ratio did not show any signs of redness, inflammation or increased tear production when applied onto the eyes of albino rabbits (Abdelbary and El-gendy, 2008).

Application of C24 in a powder form developed minimal irritation potential manifested as hyperaemia after 3 min. This indicates that C24 is slightly irritant when applied on the surface of the CAM. Further, no signs of vascular toxicity were detected after application of niosomes containing sodium cholate (F-CH). The concentrations of CH in the tested F-CH niosomes were 0.13–0.53%

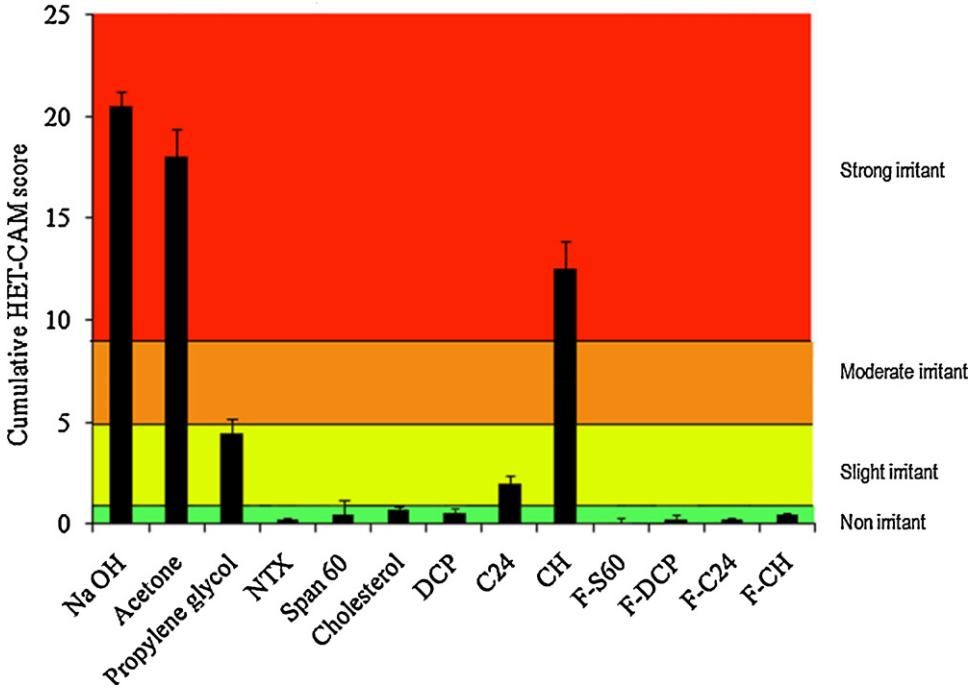

Fig. 2. Cumulative HET-CAM scores of controls, raw materials and niosomal formulations. Results expressed as mean values ± SD, $n = 3$.

(w/v). It is likely that the amount of CH in these formulations was insufficient to produce an irritant effect. The *in vivo* ocular test demonstrated good ocular tolerability and no signs of ocular damage after the application a 0.5% (w/v) CH solution onto rabbit eyes (Furrer et al., 2002). Another possible explanation is that CH molecules were trapped in the bilayer membrane of the prepared niosomes, which is likely to reduce their to interact with the ocular surface.

3.2. BCOP test

The cornea has both refractive and protective functions. The normal cornea is transparent, lustrous and completely impermeable to fluorescein dye, due to the exclusive tight junctions of the corneal epithelium. Fig. 3 shows photographs for bovine corneas before and after staining. Epithelial damage, such as corneal epithelial erosion or corneal oedema, leads to an increase in corneal permeability, loss of its normal lustre and increase in corneal opacity (Fig. 6). The extent of corneal damage was assessed and scored.

Fig. 4 shows the cumulative bovine eye test scores for the controls, NTX solution (40 mg/ml) in PBS, the test substances used in manufacturing the prepared niosomal formulations and the prepared niosomes.

Apart from CH powder, all the test substances and the prepared formulations did not show any signs of corneal injuries [corneal opacity, corneal permeability (flouresceine stained) or epithelial damage], and the cumulative corneal scores were <0.5. Additionally, all the prepared niosomal formulations, in the three concentrations tested, showed no signs of corneal damage. Moreover, the lustre of the niosome-tested corneas was as normal as the negative control. Encouraging results were reported with the *in vivo* rabbit eye test for niosomes (Guinedi et al., 2005). Niosomes composed of Span 60:cholesterol 7:4 mol/mol were applied onto rabbit eye and monitored daily for 40 days. Rabbits' corneas were excised and assessed histologically for corneal irritation. The tested corneas showed slight stromal oedema and no major harmful corneal signs. As such the tested niosomes were regarded as safe for short and long term treatment (Guinedi et al., 2005).

Table 4 shows the interpretation of the cumulative test scores for the HET-CAM and bovine eye tests. The irritation potential for all the prepared niosomes and the excipients used was equally interpreted by the HET-CAM (the conjunctival model) and bovine

Table 4
Summary of HET-CAM and BCOP results.

Test substance	Conjunctiva irritation (HET-CAM)	Corneal irritation (Bovine eye)
Na OH (1M)	Strong	Strong
Acetone	Strong	Moderate
Propylene glycol	Slight	Slight
NTX (40 mg/ml))	None	None
Span 60 (powder)	None	None
Cholesterol (powder)	None	None
DCP (powder)	None	None
C24 (powder)	Slight	Slight
CH (powder)	Strong	Strong
C24 (0.4% w/v)	None	None
CH (0.3% w/v)	None	None
NTX Free F-S60 (1% w/v)	None	None
NTX Free F-DCP (1% w/v)	None	None
NTX Free F-C24 (1% w/v)	None	None
NTX Free F-CH(1% w/v)	None	None
NTX Free F-S60 (5% w/v)	None	None
NTX Free F-DCP (5% w/v)	None	None
NTX Free F-C24 (5% w/v)	None	None
NTX Free F-CH (5% w/v)	None	None
NTX Free F-S60 (10% w/v)	None	None
NTX Free F-DCP (10 % w/v)	None	None
NTX Free F-C24 (10% w/v)	None	None
NTX Free F-CH (10% w/v)	None	None
NTX F-S60 (5% w/v)	None	None
NTX F-DCP (5% w/v)	None	None
NTX F-C24 (5% w/v)	None	None
NTX F-CH (5% w/v)	None	None

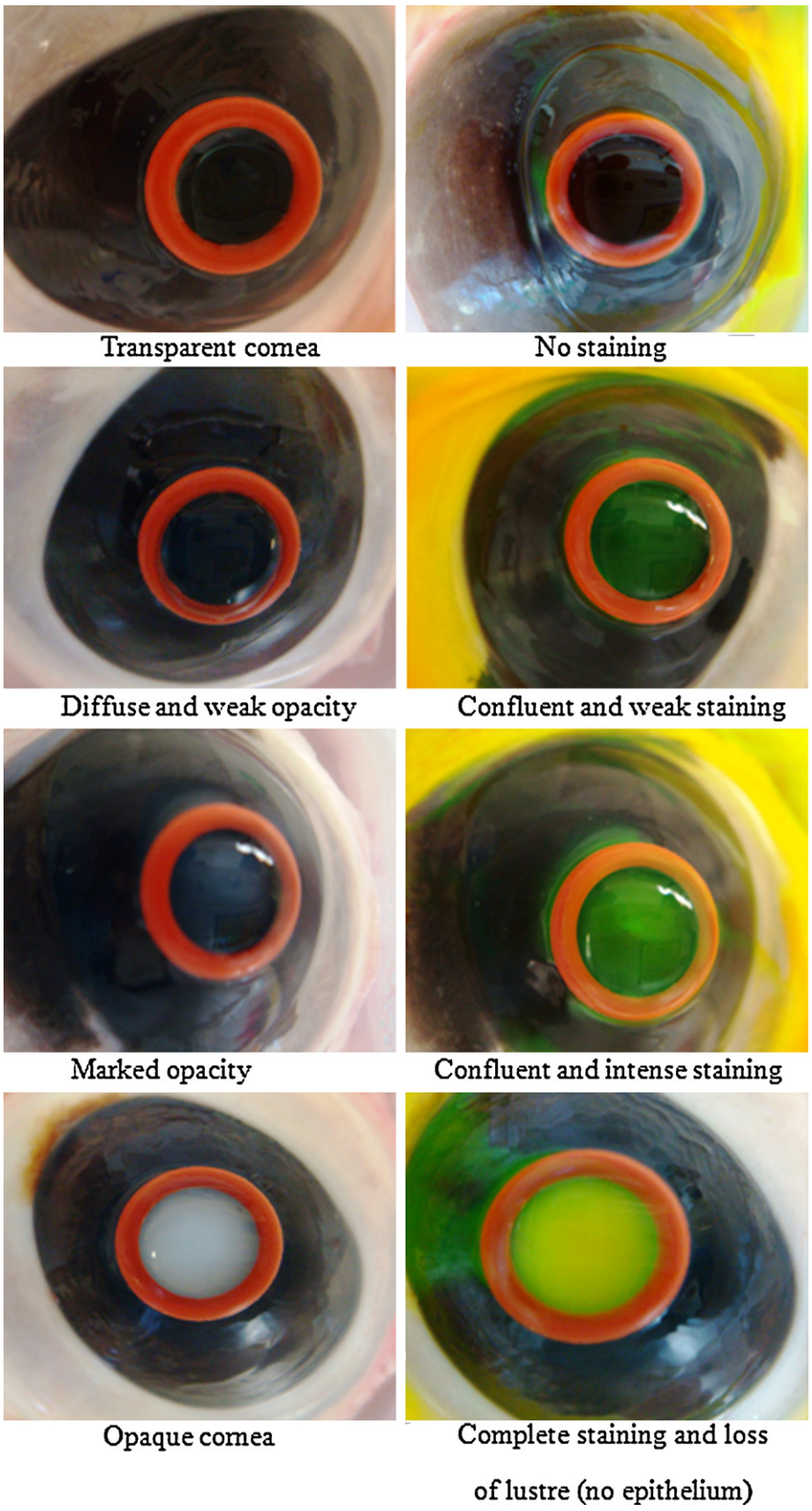

Fig. 3. Corneal injuries used to score the test substances, unstained (left), and fluorescein dye-stained (right).

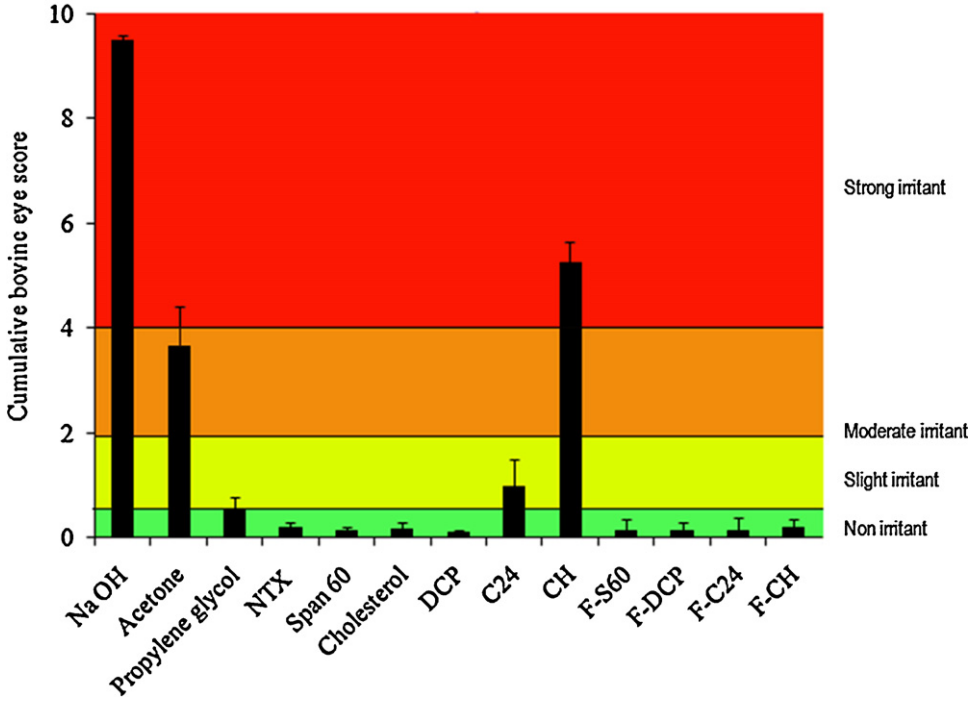

Fig. 4. Cumulative BCOP scores of controls, raw materials and niosomal formulations. Results expressed as mean values ± SD, $n = 3$.

eye assay (the corneal model). These findings indicate that a good correlation exists between the two toxicity models. The only exception was acetone. Based on the HET-CAM test, acetone was graded as a strong irritant, whereas the BCOP results suggested that is a moderate irritant. The scoring system of bovine corneas is based on assessing the disruption of the epithelium barrier and consequently, corneal opacity and permeability induced by the test substances are estimated. Acetone, an organic solvent, could induce damage to the corneal tissues in addition to the effects recorded for the surfactants used. Therefore, histological examination of the corneal layers could be helpful to compliment and further explain the findings from the HET-CAM and BCOP tests.

3.3. Histopathological evaluation of bovine corneas

Histological sectioning, fixing and H&E staining of bovine corneas exposed to different test substance/formulations were carried out to help understand and evaluate the degree of corneal injuries induced.

Negative (Fig. 5) and positive (Fig. 6) controls were included to facilitate the interpretation of the results. The negative controls provide the baseline against which histological changes are compared. Furthermore, the histology of the negative control corneas was used in this study to account for any variability when preparing the sections, to minimise or rule out cattle age effects. Older cattle have thicker corneal sections where Descemet's membrane

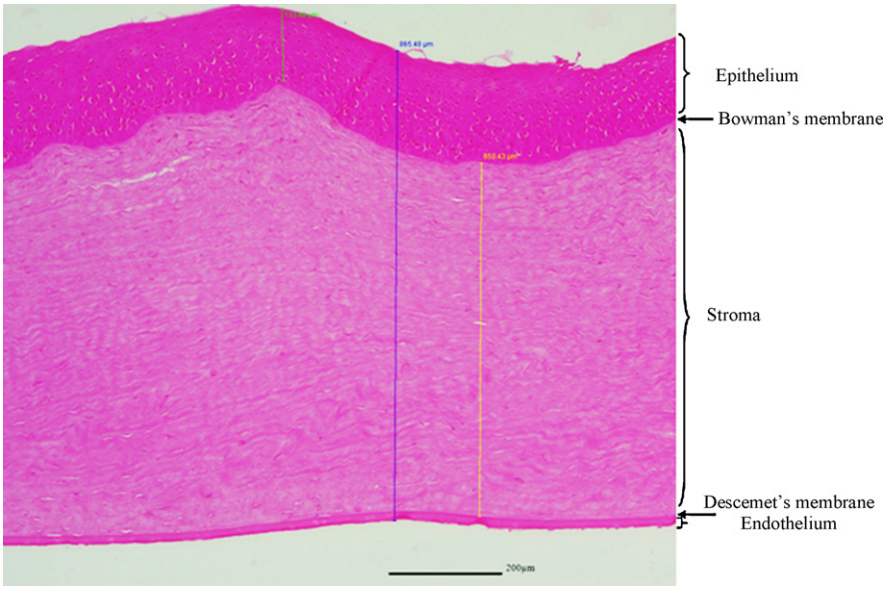

Fig. 5. Photomicrograph of H&E stained corneal section of a negative control treated with normal saline for 30 s (10× magnification).

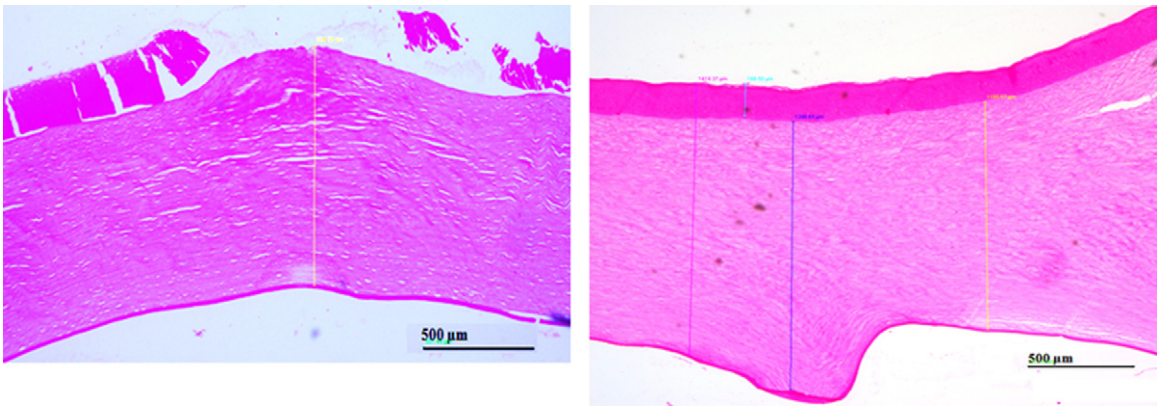

Fig. 6. Photomicrographs of H&E stained corneal sections of sodium hydroxide-treated control (left) and acetone-treated control (right) for 30 s (5× magnification).

increases in thickness with the age of the donor. Also, poorly prepared sections (over-trimmed or poorly embedded) show thicker corneal layers (Cuellar et al., 2002). In this study, a negative control was used as a baseline for each processed batch to evaluate the irritation potential for the test substances (Table 5).

3.3.1. Evaluating the negative control-treated corneas

The cornea is an avascular tissue (no circulatory system), hence no leucocyte infiltration is evident. Since the test materials were applied topically onto a silicon O-ring on the surface of the cornea, the evaluation was conducted top-down, starting with the upper epithelium and proceeding down through the epithelial layers, *via* the stroma and down to the endothelium. The depth of corneal injury basically depends on the penetration of the test substance through the three principles corneal layers; namely, the epithelium, stroma and endothelium.

3.3.2. Evaluating the positive control-treated corneas

Epithelial defects and stromal oedema are two common causes of ocular irritation after topical application of test substances (Harbell et al., 1999, 2006). Cell loss, vacuolisation (presence of vacuoles or holes), pyknosis (nuclei coagulation), saponification (due to the effect of alkali on cellular lipids) and separation of cells from the Bowman's membrane are characteristic lesions observed in the epithelium (Fig. 6).

The lesions of stroma are predominantly presented in form of stromal oedema, keratocytes pyknosis and vacuolisation. The stromal swelling or oedema is associated with the loss of the

Table 5
Histological corneal lesions scores for controls and niosomes at different time points (thickness values are expressed as mean values ± SD, $n = 3$).

Test substance	Epithelium	Stroma		
	Cell loss	Vacuolisation/coagulation	Thickness (µm)	Collagen matrix Vacuolisation
Control (30 s)	-	-/-	657 ± 60	-
NaOH (1M) (30 s)	+++	+++/+++	1180 ± 267	+++
Acetone (30 s)	+	+/+++	1278 ± 110	+
CH powder (30 s)	+++	+++/+++	1280 ± 258	+++
C24 Powder (30 s)	-	+/-	756 ±211	+
F-S60 (30 s)	-	-/-	653 ± 64	-
F-DCP (30 s)	-	-/-	649 ± 21	-
F-C24 (30 s)	-	-/-	663 ± 15	-
F-CH (30 s)	-	-/-	745 ± 22	-
Control (1 h)	-	-/-	740 ± 39	-
F-S60 (1 h)	-	-/-	621 ± 70	-
F-DCP (1 h)	-	-/-	672 ± 58	-
F-C24 (1 h)	-	-/-	691 ± 12	-
F-CH (1 h)	-	-/-	664 ± 17	-
Control (3 h)	-	-/-	674 ± 91	-
F-S60 (3 h)	-	-/-	720 ± 15	+
F-DCP (3 h)	-	-/-	733 ± 49	+
F-C24 (3 h)	-	-/-	632 ± 14	+
F-CH (3 h)	-	-/-	677 ± 25	+
Control (8 h)	-	+/-	687 ± 73	+
F-S60 (8 h)	-	+/-	702 ± 16	+
F-DCP (8 h)	-	+/-	635 ± 31	+
F-C24 (8 h)	-	+/-	679 ± 28	+
F-CH (8 h)	++	++/-	781 ± 28	+

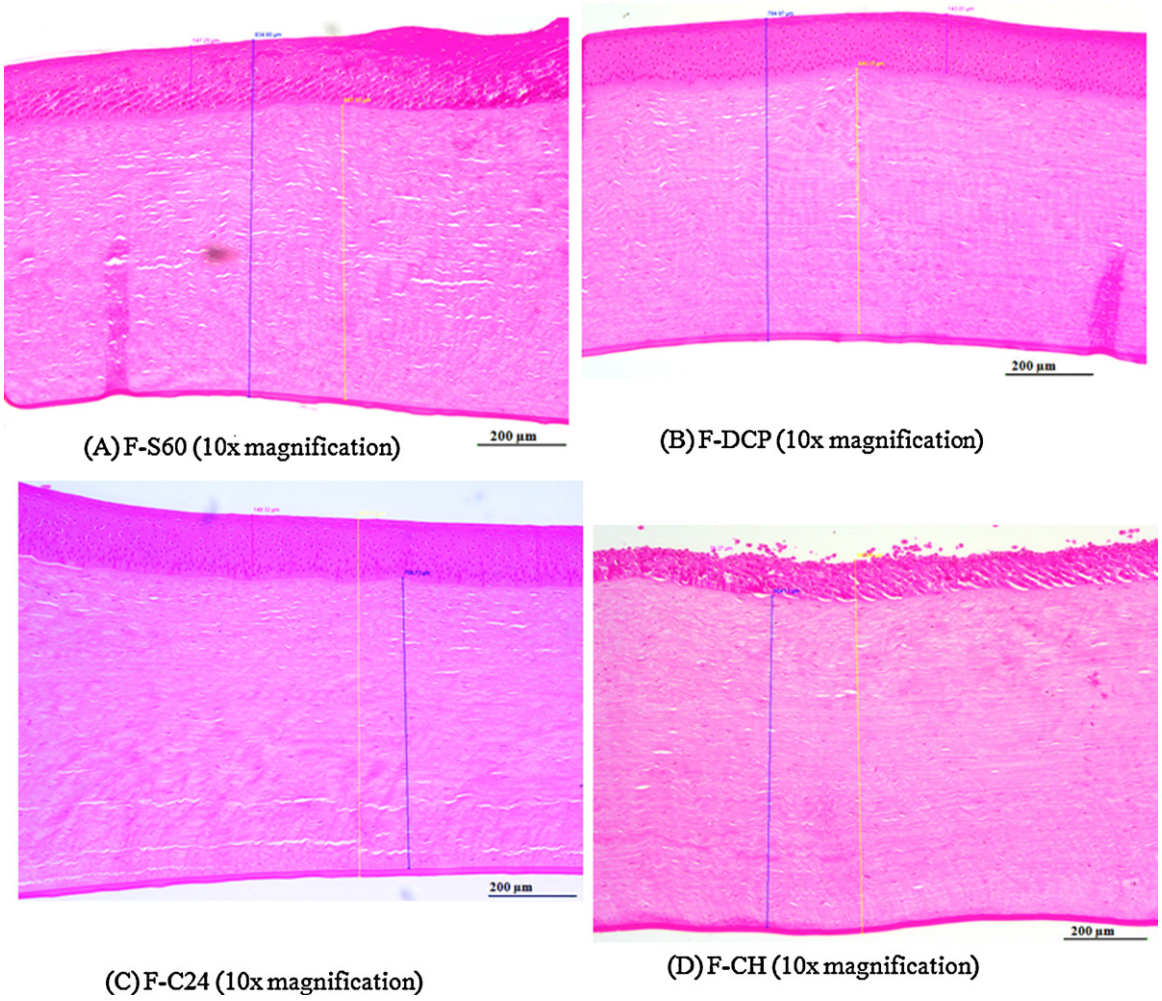

Fig. 7. Photomicrographs (A, B, C and D at 10× magnification) of H&E stained corneal sections treated with the different niosomes for 8 h.

normal ordered array of extracellular collagen matrix fibres. Stromal swelling is directly related to corneal opacity. The goal of scoring the corneal lesions is not to report separately but rather to correlate histological scores with the opacity and permeability values. It is accepted that the greater the stromal oedema, the greater the corneal opacity found (Nishida, 2005). A potential utilisation of histological scoring of the corneal lesions was to quantify the degree of opacity by measuring stromal thickness.

Stromal swelling could be manifested in the form of vacuoles in the organised collagen matrix. As the degree of vacuolisation increases, the overall thickness of the stroma is expected to increase. Lastly, there is no evidence of endothelial damage, as the damage to endothelium would be expected to result from mechanical damage rather than the topical application of test substances.

Fig. 6(left) shows the corneal layers treated with NaOH (0.5 M) and a magnified photomicrograph for the remnants of the epithelium and stroma. Extensive corneal damage was observed for the NaOH treated control. For example, epithelial rupture, saponification and epithelial loss were noticed. Furthermore, the stroma exhibited extensive vacuolisation and swelling. These results correlated well with opacity and permeability scores from the bovine eyes test. The marked opacity and loss of lustre induced by NaOH can be attributed to complete loss of the epithelial barriers.

Fig. 6(right) shows the corneal layers treated with acetone and a magnified photomicrograph for the epithelium layer. Squamous layer vacuolisation, sloughing and wing layer coagulation were observed. The stroma showed a significant increase ($P < 0.001$) in thickness compared with that of saline-treated cornea. The stromal thickness was 2 times that of the negative control-treated. Further, marked stromal collagen matrix condensation and keratocytes condensation were observed. The lower opacity and permeability scores calculated for acetone-treated eyes from the bovine eye test are attributed to coagulation of the epithelium. Consequently, this could decrease the permeability of the epithelium to the fluorescent dye. Hence, acetone-treated corneas should score higher due to this coagulation and condensation effects.

3.3.3. Effect of niosomes upon application on to bovine corneas for up to 8 h

The effect of exposure duration was also studied by applying the prepared niosomes for 1, 3 and 8 h. The treated corneas were dissected, fixed, sectioned, stained and photographed. Table 5 summarises the histological toxicity and stromal thickness for all test substances at the different time points.

After 1 h, all the prepared niosomes showed minimal to no histopathological signs. The epithelium remained intact and no significant differences were noticed between corneal stromal thicknesses of the 1 h and 30 s samples. Similarly, the niosomes did not demonstrate notable differences after 3 h. However, epithelial vacuolisation and slight epithelial swelling was induced only by F-CH.

After 8 h, moderate stromal collagen matrix vacuolisation was exhibited by the prepared niosomes (Fig. 7A–D). On the other hand, marked epithelial cell loss and erosion was induced only by F-CH (Fig. 7D). The stromal oedema induced by the niosomes is likely to

be reversible. The corneas are likely to regain their normal configuration and structure after stopping treatment (Guinedi et al., 2005; Hughes et al., 2004).

4. Conclusion

Four niosomal formulations, their components and NTX were evaluated for corneal and conjunctival toxicity using the HET-CAM test and BCOP assay along with histological corneal examination. These tests have the advantage of being rapid and discriminating. These *in vitro/ex vivo* toxicity tests can be useful for preliminary toxicity screening of surfactant-based dosage forms. They can be a promising alternative for the *in vivo* Draize test. The investigated niosomes can be used in future *in vivo* ocular experiments with some certainty regarding their safety.

Conflict of interest

None.

Acknowledgment

The authors wish to acknowledge the financial contribution of the Culture Affairs and Mission Department, Ministry of Higher Education, Cairo, Egypt.

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