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

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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

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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