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Evaluation of nanoformulated therapeutics in an *ex-vivo* bovine corneal irritation model

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ABSTRACT

Aim: To determine the internalization and protective effects of potential ophthalmic formulations and nanoformulated natural proteins in *ex-vivo* bovine corneal alkali burn model.
Methods: The bovine cornea obtained were subjected to the 0.5 N NaOH insult that induced alkali burn and inflammation as observed in the *in vivo* situation. The toxic effects of the nanoformulation were evaluated in the normal and insult induced cornea using histological analysis. Internalization studies were carried out using *in vivo* imaging and analysis (IVIS, PerkinElmer, USA).
Results: The nanoformulations employed in this study showed no obvious changes in the integrity of the cornea. Further, improvements in the light transmittance and reduced inflammation were observed. The IVIS showed a dose dependant increase in the uptake of the nanoformulations with time.
Conclusion: The nanoformulated bovine lactoferrin and SurR9-C84A (SR9) proteins evaluated in the *ex vivo* bovine corneal irritation model is the first of its kind, and we report here the non-toxic and therapeutic potential of these formulations for topical applications.

1. Introduction

In order to replace the *in vivo* eye test, several efforts have been made to find an apt replacement, and Draize et al., were the first to report several methods for studying irritation and toxic effects of substances applied topically (Draize et al., 1944). The same procedure was also made as a governmentally endorsed methodology and is described in the organisation for economic co-operation and development (OECD) testing guidelines 405 (OECD, 2002). The first *in vitro* alternative methodology to replace the *in vivo* eye test was suggested by Fraizer et al., 1987. *In vitro* methodologies such as isolated rabbit eye (IRE) test proposed by Burton et al., 1981 and bovine corneal opacity (BCOP) assay proposed by Gautheron et al. (1992) have been used for the prediction of ocular irritation of the prototype shampoo formulations (Cooper et al., 2001). The BCOP test is performed to evaluate the response of an excised bovine cornea upon exposure to potential ocular irritants by measuring the measure light transmission and permeability of the corneal layer. Histological evaluations provide a better characterisation of substances that do not produce measurable opacity or permeability changes (Curren et al., 1999). Several other studies have also made use of these assays to determine the pharmacological screening of compounds such as benzimidazoles (with analgesic and anti-inflammatory properties) (Jesudason et al., 2009). A marked reduction in the opacity was reported after treatment with 1% NaOH for mere 30 s to 1 min. On the basis of A570 score, NaOH was classified as a mild irritant that can lead to an opacity score in the moderate to severe irritant range (Ubels et al., 2000). Another study reported evaluation of fifty-six chemicals including alcohols, surfactants and esters using the BCOP assay. An accuracy rate of 69.6% was obtained using the results with a favourable predictive capacity having the accuracy of 71.4% (Hayashi et al., 2012).

Normally, the opacity is measured using a spectrometer or an OP-KIT opacitometer that provides a centre-weighted reading of light transmission through the corneal tissue. However, studies have been performed, showing that a laser-based opacitometer allows the analysis for complete corneal surface and thus, has an improved sensitivity to detect any minute changes in the corneal transparency (Van Goethem et al., 2010). The potential cytotoxicity of nanoparticles is a rising concern on account of their agglomeration, stability issues, dosage frequency, etc. Thus, studies have shown that BCOP and several other assays may be useful to determine the irritation and cytotoxic effects of nanoparticles *in vitro* in non-cancerous cells (Hartung, 2010). Another study has used BCOP test to determine the toxic effects of zinc-oxide (Zn-O)

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nanoparticles and to observe the human skin and eye irritation caused by them. Intriguingly, the results were able to confirm that the nanoparticles had no toxic effects on the normal human cells and non-cancerous bovine corneal cells (Wiench et al., 2012). Some other studies also confirmed that the BCOP assay is the optimum method to recognize the limitations and determine the cytotoxic effects of nanomaterials *in vitro* (Hartung and Sabbioni, 2011). In the present study, we have used the BCOP assay to test the potential cytotoxicity, irritability of a natural milk derived protein, bovine lactoferrin (bLf). In addition, a recombinant dominant negative mutant form of survivin protein (SurR9-C84A also referred as SR9) (Fig. 1) in both nanoformulated and nano-free form. We studied the irritability and internalization potential of poly(lactic-co-glycolic acid) (PLGA) and chitosan nanoparticles (CHNP) in the BCOP model. Apart from this, we have also tested the irritability and cytotoxicity of some commonly used ophthalmic formulations such as mitomycin C and Optrex eye mist.

Mitomycin-C is considered to be very effective in addressing corneal haze targeting the cellular proliferation and induction of apoptosis through its anti-fibrotic effects (Jester et al., 1999; Wilson et al., 2001; Netto et al., 2005). Although it improves clinical condition after PRK and LASIK, it has significant toxicity on corneal keratocytes that will not disappear until 3–6 months (Tae-im Kim et al., 2004). Various other strategies and procedures must be undertaken after application of MMC to minimize its unwanted side effects (Majmuder et al., 2000; Carones et al., 2002). Thus, its safety is of critical importance and therefore, we have included it in our study and compared its effects against the potential ophthalmic drug "Eyecure".

2. Materials and methods

The polymer (PLGA 50:50, inherent viscosity 0.32–0.44 dL/g, M_w 24,000–38,000) Poly (D,L-lactide-co-glycolide) RESOMER® RG 503 H, poly (vinyl alcohol) (M_w 13,000–23,000), low molecular weight chitosan and sodium tripolyphosphate (STPP) were purchased from Sigma Aldrich, Australia. All other solvents and chemicals used in the study were of analytical grade.

2.1. Procedure

The methodology for BCOP assay was adapted from previously published studies (Gautheron et al., 1992; Vanparys et al., 1993). The fresh bovine corneas were obtained from a nearby slaughterhouse and transported to the lab in HBSS. The corneas were isolated carefully using a scalpel from the bovine eye and washed in HBSS within a period of 2 h of enucleation. A similar methodology was proposed by Vanparys et al., 1993 where the corneas were isolated within 3 h after the killing of animals.

An improvement to BCOP assay for more precise measurement of opacity in BCOP assay was suggested by Casterton et al. (1996) who successfully demonstrated the light absorbance at 570 nm (A570) using a spectrophotometer. Hence all eyes were carefully examined and precautions were taken to avoid damage to corneal endothelial side (downwards). The cornea obtained from slaughterhouse were tested for a background opacity at 570 nm using a spectrometer and selected on basis of their absorbance. Any cornea which showed a lot of variance in the A570 reading was not included in the study. Therefore, a consistency in the corneas included in the present study was maintained. The standard BCOP protocol requires the corneal exposure to the testing substance for 10 min after which the cornea is washed and incubated in Eagle's minimum essential medium (MEM) for 3 h at 35 °C (recovery time) before measuring the opacity and permeability.

All the eyes were carefully examined and precautions were taken to avoid damage to corneal endothelial side (downwards). The cornea showing the scratches were, therefore, excluded from the study. All the corneas were rinsed with hank's balanced salt solution (HBSS) and then mounted on the holders (endothelial side downwards). In order to maintain the original shape of the cornea, both sides of the corneal holders were filled with MEM and a background opacity at A570 was also recorded before any treatments.

2.1.1. Nanoparticle synthesis and characterisation

Ionotropic crosslinking method was used for the formation of chitosan nanoparticles, where chitosan was cross linked with STPP. There is a formation of gel beads when negatively charged STPP is added to cationic chitosan (Mi et al., 1999). Generally, the drug can be loaded on to chitosan by two methods, one, where chitosan is mixed with the drug with or without the crosslinker (entrapment method) such that the drug is trapped within the chitosan molecule.

The other method is the incubation of the drug with chitosan (incubation method) where the drug is adsorbed on the surface of chitosan (Kawashima et al., 1985). 2 mg/ml of chitosan was dissolved in an acetic aqueous solution. A little amount of glacial acetic acid was added to bring the pH down to 4.5 to completely dissolve the chitosan. The entrapment method was used for loading of the drug and SurR9-C84A was added drop-wise in a known concentration to the chitosan suspension under constant magnetic stirring at 4 °C. For ionotropic gelation of chitosan, 1 mg/ml of STPP

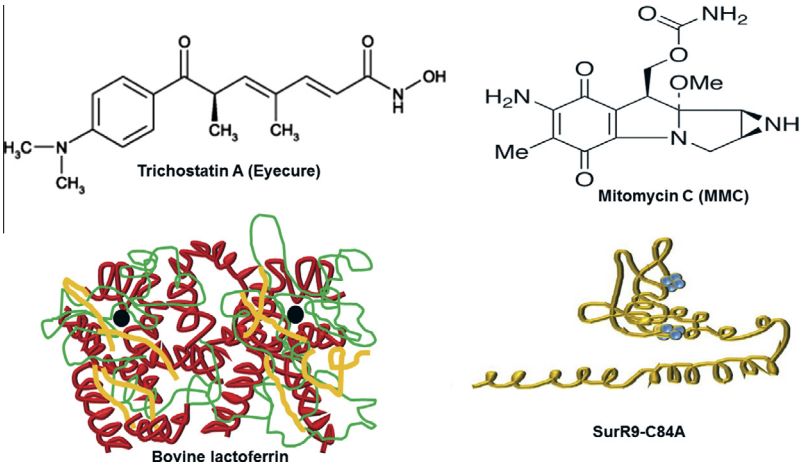

Fig. 1. Structure of chemicals and proteins. Chemical structure of trichostatin-A, MMC and peptide forms of b-Lf and SurR9-C84A (SR9) are represented in the figure.