Ocular tolerability of chitosan nanoparticles loaded with the antioxidant prodrug 2-oxothiazolidine-4-carboxylic acid

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ABSTRACT SUMMARY

The aim of this study was to investigate the ocular tolerability of 2-oxothiazolidine-4-carboxylic acid (OTZ) loaded chitosan nanoparticles using the Bovine Corneal Opacity and Permeability (BCOP) test, Red Blood Cell lysis and protein denaturation assay along with histological evaluation. The results revealed that the formulation was well tolerated and hasn’t manifested any undesirable effects.

INTRODUCTION

It is paramount that any new eye formulation that is intended for topical ocular application be investigated for potential toxicity/adverse effects on precorneal tear film, conjunctiva, cornea and sclera, as these are the frontline barriers for any formulation administered onto the eye surface. Several assays are described in the literature and some are adopted by the regulatory bodies (EMEA and FDA) to assess the ocular tolerability of a new formulation; amongst these are: Bovine Corneal Opacity and Permeability (BCOP) test, Red Blood Cell (RBC) Lysis and Protein Denaturation Assay, and Hen's Egg Test Chorioallantoic Membrane (HET-CAM).

The BCOP assay is currently used to evaluate the effect of test materials on corneal transparency; it monitors any interference of the test material with the corneal barrier properties. Predicting ocular irritation by using the opacity and permeability endpoints is challenging, especially 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 the treatment with the test substances can provide a more comprehensive assessment of the depth of injury and cellular damage of the three principal layers of the cornea (1). The RBC lysis and protein denaturation assay is used to investigate potential cell membrane damages that may result from the ocular formulation. This assay is carried out by measuring (spectrophotometrically) the hemoglobin leakages from the RBC(2). Finally the HET-CAM, a well-established conjunctival model was employed to study potential conjunctival irritation.(3)

The aim of this study was to investigate the ocular tolerability of OTZ-loaded chitosan nanoparticles using a combination of the BCOP test, histological documentation, examination, and the RBC hemolysis assay.

EXPERIMENTAL METHODS

Formulation of OTZ-loaded chitosan nanoparticles

Chitosan nanoparticles were prepared by the ionic gelation method. Briefly, nanoparticles were generated by adding sodium tripolyphosphate (TPP) to a solution of OTZ and chitosan. The resulting dispersion was subjected to sonication followed by centrifugation. The formed nanoparticles were re-dispersed in water for further investigation.

BCOP Assay

Selected substances were used to evaluate the damage to the cornea, these included, normal saline as a negative control, NaOH solution (0.5% w/v) as an aqueous positive control, acetone as an organic positive control, and the formulated chitosan nanoparticles. Freshly excised bovine eyes were obtained from a local abattoir; the eyes were checked for any corneal epithelial detachment or corneal damage. The eyes were incubated for 10 min in a water bath set at 37±0.5 °C. One drop of saline was then placed onto the corneal surface, and the eyes were incubated for an additional 5 min. The test substance (100µL) was applied to the corneal surface and left for 30 second. This eye washed with saline followed by further incubation for 10 min. The degree of corneal damage caused by the test material was assessed by visual observation of the extent of opacification resulted due to the test material. Further assessment involved staining the exposed corneas with sodium fluorescein solution.
(2% w/v), corneal epithelium injury, visualized using LED light with cobalt blue filter. Finally the analyzed corneas were dissected, fixed and stained with Hematoxylin and Eosin (H&E) following a standard fixing and staining protocol.

**Red Blood Cell Lysis and Protein Denaturation Assay**

Fresh bovine blood was collected from the local abattoir and stored in a citrate anticoagulation buffer. Protocol N°99 (EURL ECVAM DB –ALM protocols) was followed to prepare and isolate the RBC. Test substances include water as a positive control, phosphate buffer saline (PBS) as a negative control and chitosan nanoparticles dispersed in PBS as test the material. The concentration of chitosan nanoparticles was prepared in a range between 1 to 10,000 ppm. The assay was carried out by adding 750 µL of the test material to 250 µL of the RBC sample. After 1 hour of incubation, the samples were centrifuged and the hemolysis degree was determined by measuring the absorbance of the supernatant at 541nm.

**RESULTS AND DISCUSSION**

Figure 1 illustrates the results of the BCOP test. The tested chitosan nanoparticles were found to be devoid of any damaging effects to the corneal epithelium.

![Figure 1 BCOP test results of freshly excised bovine cornea, (A) negative control, (B) aqueous positive control, (C) organic positive control and (D) chitosan nanoparticles.](image)

Figure 2 shows the degree of corneal tissue damage incurred by the positive control (Fig 2B) in comparison with the negative control (Fig 2A) and the test formulation (Fig 2D). There was no evidence of corneal tissue damages caused by the chitosan nanoparticles. On the other hand both positive controls (Fig B and C) showed evidence of corneal epithelial detachment, condensation of the epithelium and stromal swelling. None of these signs were observed in samples that were treated with chitosan nanoparticles.

![Figure 2 corneal histological section results of freshly excised bovine cornea, (A) negative control, (B) aqueous positive control, (C) organic positive control and (D) chitosan nanoparticles.](image)

The prepared chitosan nanoparticles showed no sign of RBC hemolysis, where the total lysis was below 5%. This value is considered acceptable according to the protocol that used (Protocol N°99, EURL ECVAM DB –ALM protocols)

**CONCLUSION**

Results from the current study demonstrate that the chitosan nanoparticles are well tolerated by the ocular surface membranes, thus rendering these systems more attractive topical ophthalmic application for delivering the antioxidant prodrug OTZ to the lens.

**REFERENCES**

