# Dose-dependent effect of α-connexin carboxyl-terminal peptide treatment on nitrogen mustard-induced corneal injuries in an in vivo rabbit model

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## ABSTRACT

Nitrogen mustard (NM) is a less toxic analogue of the potent vesicant sulfur mustard (SM) that is safer to work with in laboratory settings and has produced well-characterized in vivo and ex vivo rabbit models of ocular injuries that parallel SM-induced injuries. Eyes are extremely sensitive to vesicants. Exposure may result in epithelial degradation and disruption of the cellular junctions in the corneal epithelium and endothelium, thus impairing cell-cell communication and causing inflammation and corneal edema, in a dose- and duration-dependent fashion. Alpha-connexin carboxyl-terminal (aCT1) peptide is a connexin43 mimetic peptide that has shown anti-inflammatory, regenerative, and anti-scarring properties via the stabilization of gap junctions (intercellular communication) as well as tight junctions (intercellular contacts) of epithelial and endothelial cells and a coordinated reduction in hemichannel activity.
Thus, evaluation of the potential therapeutic effect of high dose (HD; 5mM) and low dose (LD; 0.2mM) aCT1 treatment every 12h beginning 2h post NM exposure for 7 days post NM exposure was performed. Rabbits were divided into 4 groups: Group 1 (left eye: untreated control; right eye: NM), Group 2 (left eye: vehicle; right eye: NM+vehicle), Group 3 (left eye: vehicle; right eye: NM+aCT1 LD), and Group 4 (left eye: vehicle; right eye: NM+aCT1 HD). Clinical parameters (corneal opacity, ulceration, and neovascularization) were assessed at day 3 and 7 post NM exposure. Biological (epithelial degradation, epithelial-stromal separation, blood vessels density, and inflammatory cell count) and molecular (COX-2, MMP-9, and VEGF expression) parameters were studied at day 7 post NM exposure.
Results indicated that aCT1 treatment reduced NM-induced corneal injuries in a dose-dependent manner. The most significant alleviating effects of aCT1 HD were decrease in NM-induced corneal thickness (51% reversal) and decrease in NM-induced inflammatory cell infiltration (78% reversal). A complete reversal in the blood vessel count was also observed with both aCT1 doses. In conclusion, though more studies are required to definitively characterize aCT1's efficacy, these data support aCT1 administration in the in vivo ocular rabbit model to alleviate NM-induced corneal injuries.

## INTRODUCTION

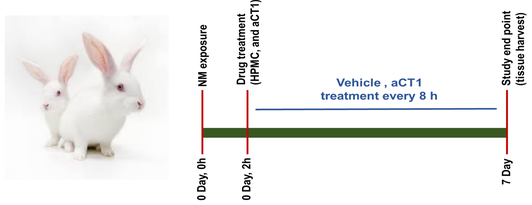
- Sulfur mustard (SM), a vesicating chemical warfare agent (CWA) causes severe injuries to several organs like skin, respiratory organs, and ocular tissue. To understand the mechanism of action and to find the effective therapeutic drug treatment against SM-induced injuries, nitrogen mustard (NM), an analogue of SM, has been used in the research lab to induce the injuries to the ocular tissue.
- Mechanism of action: SM (bis-[2-chloroethyl] sulfide) and NM [2-chloro-N-(2-chloroethyl)-N-methylethanamine oxide] are bifunctional alkylating agents which, upon exposure to the skin, respiratory and ocular tissues, bind with the biologically important molecules, including proteins, lipids, and nucleic acids. SM-/NM-exposure elevates the reactive oxygen species (ROS) levels, which results in loss of homeostasis between ROS and glutathione (ROS scavengers). These alkylating agents' interaction with the DNA causes formation of nonfunctional DNA adducts, resulting in DNA damage that is the major cause of lesions, directing the cytotoxicity that eventually leads to cell death.
- NM-exposure to the ocular tissue causes severe injuries leading to clinical symptoms, corneal ulceration, neovascularization, corneal opacity, and lacrimation starts to appear 2-6 h post exposure.
- Histopathological analysis of the exposed ocular tissue has confirmed the increased total corneal thickness, decreased corneal epithelial thickness, inflammatory cell infiltration, loss of keratocytes in the stromal region, increased epithelial-stromal separation and increased blood vessel numbers in the cornea.
- At molecular level, the expression of cyclooxygenase 2 (Cox-2), matrix metalloproteases (MMP-9), and VEGF (an angiogenic marker) proteins are increased post-NM exposure.
- MMP-9 is an important matrix metalloprotease that plays an important role in the remodeling of the tissue because of its ability to degrade extracellular material. It was hypothesized that MMP-9 exerts its effects on the epithelium by cleaving one or more components of cell-cell junctions and triggering anoikis.
- In this study, α-connexin carboxyl-terminal peptide (aCT1) is used for the treatment of nitrogen mustard induce ocular injuries.
- aCT1 is a peptide that enhances the junctional activity of Connexin 43, which is a member of very known gap junctional protein family connexin. Gap junctions are the channels that link the cytoplasm of two cells. Gap junctions are the means for the exchange of ions ($K^+$ and $Ca^+$), second messengers (like, cAMP, cGMP, and ATD) and small metabolites (glucose), allowing electrical and biochemical coupling between cells. A recent study showed the transfer of small interference RNAs between adjacent cells through gap junctions.
- In this study, aCT1 successfully decreased the total corneal thickness and inflammatory cell infiltration in the nitrogen mustard exposed ocular tissue.

## METHODS

- New Zealand white male rabbits (4-5 lbs and not less than 3 months of age) were obtained from Charles River Laboratories and, inspected, acclimatized for one week in the university's animal house facility.
- Buprenorphine SR was administered (SQ) to the rabbits for **pain managements,** 24 h before the NM exposure. Thereafter, buprenorphine was administered every 72 h of the first Buprenorphine SR injection.
- **For anesthesia**, rabbits were administrated ketamine + xylazine (IM) and followed with 2% isoflurane throughout the procedure. Retro-bulbar block using Bupivacaine was also carried out for pain relief.

### Treatments Groups:

**Group A**: left eye was exposed with normal saline for 5 minutes; right eye was exposed with NM (25 µL of 1% NM) for 5 min.
**Group B**: left eye was exposed with normal saline (0.9% saline) for 5 min; right eye was exposed with NM (25 µL of 1% NM) for 5 min. After 2 h NM exposure, both the eyes were treated with vehicle hydroxypropyl methylcellulose (HPMC; 0.5% in normal saline).
**Group C**: left eye was exposed with normal saline (0.9% saline) for 5 min; right eye was exposed with NM (25 µL of 1% NM) for 5 min. After 2 h NM exposure, both the eyes were treated with aCT1 low dose (LD) (0.2 mM, 1 drop).
**Group D**: left eye was exposed with normal saline (0.9% saline) for 5 min; right eye was exposed with NM (25 µL of 1% NM) for 5 min. After 2h NM exposure, both the eyes were treated with aCT1 high dose (HD) (5.0 mM, 1 drop).




- One quarter of the harvested tissues were then fixed in 10% formalin and processed further for sectioning and staining (H&E, IHC). Rest three quarters of the tissue were snap frozen and stored in -80°C for western blotting and RNA analysis.
- **H&E stained tissues were used for histopathological evaluations**: total corneal thickness, epithelial thickness, inflammatory cells density, keratocyte cells death, epithelial-stromal separation and blood vessel count were measured in the corneal tissue. For total corneal thickness, 8-10 randomly selected regions were measured at 40X magnification. Epithelial thickness was measures from 8-10 epithelial regions throughout the corneal length at 400x magnifications. Four to five measurements were taken from each region. Epithelial-stromal separation was measure throughout the length of the cornea.
- **Inflammatory cells, blood vessels and keratocytes cell death were measured in the stromal region**. The hematoxylin and eosin (H&E) stained rabbit cornea sections were evaluated microscopically for the number of keratocytes and blood vessels in the stroma (Data presented is in 1.25 $mm^2$ section of the cornea) . Also, the number of inflammatory cells and blood vessels were quantified ~ 8 $mm^2$ area of the cornea. The density of inflammatory cells was scored as 1, < 50; 2, 50-100; 3, 100-500; 4,>500 inflammatory cells.
- **Immunohistochemistry (IHC) staining** was performed for the COX-2, MMP-9 and VEGF molecular analysis. Staining was evaluated in 8-10 regions of each tissue for each molecule

## RESULTS

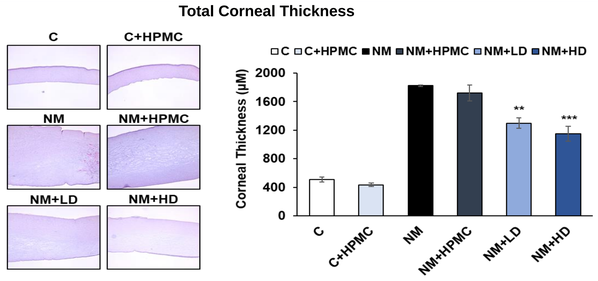



**Figure 1.** Efficacy of aCT1 peptide treatment on total corneal thickness in the ocular tissue exposed with NM. Data presented are mean ± SEM (n=3). * p<0.05; ** p<0.005; *** p<0.001 in comparison with NM.

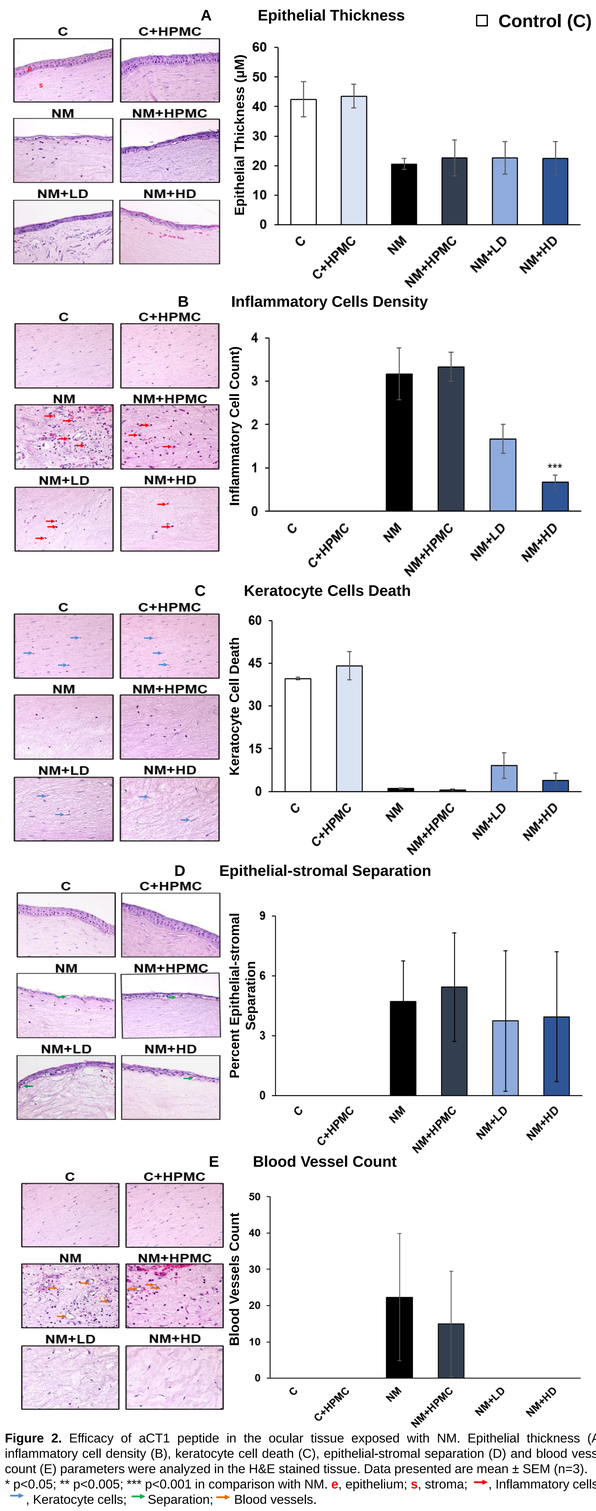



**Figure 2.** Efficacy of aCT1 peptide in the ocular tissue exposed with NM. Epithelial thickness (A), inflammatory cell density (B), keratocyte cell death (C), epithelial-stromal separation (D) and blood vessel count (E) parameters were analyzed in the H&E stained tissue. Data presented are mean ± SEM (n=3). * p<0.05; ** p<0.005; *** p<0.001 in comparison with NM. e, epithelium; s, stroma; →, Inflammatory cells →, Keratocyte cells; →, Separation; →, Blood vessels.

## RESULTS

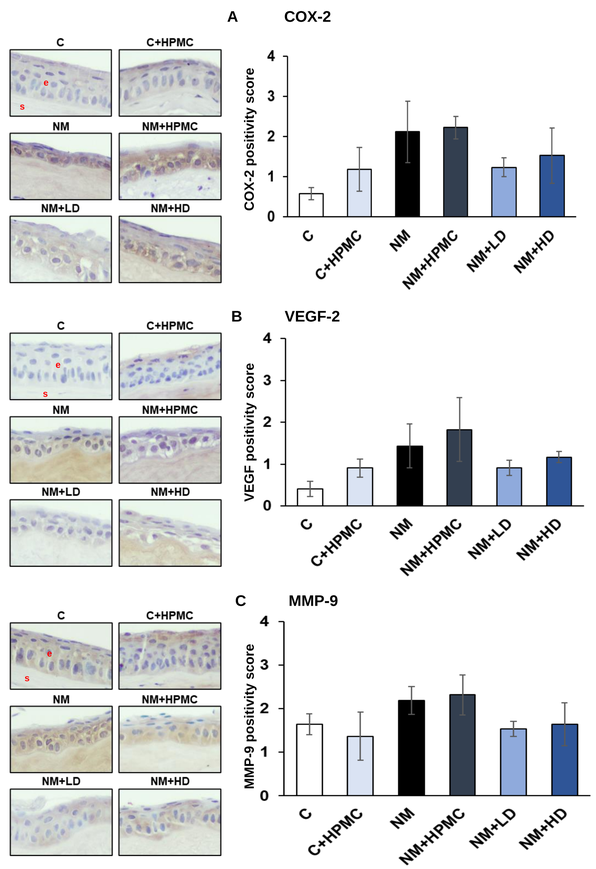



**Figure 3**. Efficacy of aCT1 peptide in the ocular tissue exposed with NM is measured by quantifying the NM injury markers COX-2 (A), VEGF (B) and MMP-9 (C) in the corneal Epithelial layer. The brown colored cytoplasmic staining for VEGF was scored as positivity score in 10 randomly selected fields (400x magnification). This intensity of brown color was scored as 0 (no staining), +1 (weak staining), +2 (moderate staining), +3 (strong staining), and +4 (very strong staining). Data presented are mean ± SEM (n=3) * p<0.05; ** p<0.005; *** p<0.001 in comparison with NM. e, epithelium; s, stroma.

## SUMMARY

- aCT1 peptide high does treatment in the NM injured ocular tissue reduced the total corneal thickness as well as inflammatory cells infiltration in the tissue. In the others parameters (biological markers) the effects were non-significant, probably owing to the variability of the data.
- At the molecular levels, COX-2 (an inflammatory marker), MMP-9 (member of matrix metalloproteases family), and VEGF (an angiogenic marker) expressions decreased in both low dose and high dose treatments, though the effect is not significant.
- Further studies are required to confirm the efficacy of aCT1 peptide compound in SM/NM induced ocular injuries.

## ACKNOWLEDGMENT

**Funding:???**