• Basic Research •

αB-crystallin mini-peptides support corneal healing *in vitro* and *in vivo* in rabbit model

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Abstract

● **AIM:** To evaluate if topical use of αB-crystallin mini-peptides supports corneal healing following flap surgery.

● **METHODS:** Cultured corneal cells were treated with fluorescent tagged αB-crystallin mini-peptides to assess its internalization. Cultured corneal cells pre-treated with or without the mini-peptides were exposed to H_2O_2 and cell viability was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Elongation of neurites of cultured trigeminal neurones was examined following treatment either with αB-crystallin mini-peptides or protein. Cultured trigeminal neurones were pre-treated either with αB-crystallin mini-peptides or crystallin protein and exposed to H_2O_2 and presence of beading in the dendrites and axons was assessed. Corneal flap surgery was conducted on rabbit cornea and treated topically either with αB-crystallin peptide (0.5 mg/mL thrice daily for 14d) or phosphate-buffered saline (PBS). Corneal healing was evaluated under slit-lamp biomicroscope, mRNA expression of inflammatory cytokines were assessed and the corneas were evaluated by histopathology.

● **RESULTS:** Internalization of αB-crystallin mini-peptides was ascertained by the detection of fluorescence within the corneal cells. The MTT assay revealed that treatment with αB-crystallin mini-peptide reduced cell death induced by H_2O_2 treatment. The mini-peptides did not influence the elongation of trigeminal neurites, but significantly (P<0.05) reduced beading in the neurites. In rabbit eye, the treated corneas showed reduced hyper-reflective zones (P<0.05) and suppression in the expression of inflammatory cytokines. Histopathological examination also revealed reduction of inflammatory response in treated corneas.

● **CONCLUSION:** The αB-crystallin mini-peptides restrict the damage to corneal cells and neurons and aids in corneal healing.

● **KEYWORDS:** corneal healing; αB-crystallin; cyto-protection; neuroprotection

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INTRODUCTION

One of the prerequisites for optimum vision is a clear and transparent optical medium. Fibrosis, which is a consequence of injury, can lead to the formation of blemishes or scars in any part of the body; however, a scarred cornea can be an impediment to vision.

The cornea is the major refractive surface of the eye, and is avascular and transparent. Corneal fibrosis is the second leading cause of global blindness[1]. One of the major factors that help maintain corneal transparency apart from its unique anatomy, that is, arrangement of collagen fibrils and avascularity, and soluble crystallin proteins found abundantly in the cornea. The cornea is the tissue in the body with the most densely populated supply of nerves, which is responsible for its extremely high sensitivity. Corneal sensitivity is largely responsible for its protection, health, and homeostasis. There is a unique interplay between corneal cells and corneal nerves that contributes to the health of corneal cells as well as corneal nerve regeneration. Therefore, complete corneal healing, re-epithelialization, corneal nerve regeneration, and sensitivity restoration are all imperative[2].

The role of αB-crystallin as an anti-inflammatory, antioxidant, and cytoprotective protein has been elucidated in several organs, including the eye[3-5]. *In vitro* treatment of retinal pigment epithelial (RPE) cells with αB-crystallin mini-peptides has demonstrated potential cytoprotective activity[6].

Considering the fact that the cornea is abundantly endowed with crystallin protein aldehyde dehydrogensa 1A1 (ALDH1A1),

ALDH3A1[7] its neuroprotective role, if any, apart from maintaining transparency and ultraviolet scavenging, has never been investigated. We hypothesize that crystallin proteins may render cyto-protection and neuroprotection to the cornea and may prove to be an effective therapy for anatomical and physiological homeostasis.

Given the challenges of delivering protein across plasma membrane, and previous evidence of internalization of mini-peptides from αB-crystallin into RPE cells[6], the present study was designed to evaluate the efficacy of the mini-peptides in restoring corneal homeostasis following surgical injury.

MATERIALS AND METHODS

Ethical Approval All studies were conducted with prior permission from the Institutional Animal Ethics Committee [IAEC/498 dated 6/01/2017] and as per the guidelines of the Association for Research in Vision and Ophthalmology for the use of animals in ophthalmic and vision research.

Culture of Trigeminal Ganglion Cells Following euthanasia, trigeminal ganglia (TGs) were dissected from six Sprague Dawley rats and cultured following method described in literature[8]. The dissected TGs were washed with ice-cold Hanks' Balanced Salt Solution (HBSS) containing penicillin/streptomycin. The tissues were cut into small pieces using spring scissors, placed in HBSS containing papain (40 unit/mL), L-cystein (0.7 mg/mL), saturated $NaHCO_3$ (2 μL/mL), and incubated for 30min at 37°C in a CO_2 incubator. The TG cell suspension was then centrifuged at 1000 rpm for 5min, the supernatant was discarded, and the cell pellet was resuspended in HBSS containing collagenase (4 mg/mL) and dispase (4.7 mg/mL), incubated for 30min at 37°C in a CO_2 incubator, centrifuged at 1000 rpm for 5min, and the supernatant was discarded. The cell pellet was washed with L15 medium [L15 with 5% fetal bovine serum (FBS), 2% 1 mol/L N-2-hydroxyethylpiperazine-N-ethane-sulphonicacid (HEPES), penicillin/streptomycin] by triturating and passing through a 200 mL pipette tip to avoid air introduction. The cell suspension was then centrifuged at 1400 rpm for 7min, the supernatant was discarded, and the cell pellet was resuspended in F12 medium (F12 with 10% FBS, penicillin/streptomycin), seeded on a laminin/poly D-lysine-coated cover slide placed in a 6-well plate, and then incubated at 37°C in a 5% CO_2 incubator. The medium (F12 with 10% FBS and penicillin/streptomycin) was replaced every third day.

Culture of Corneal Keratocyte Cells Following euthanasia, the eyes of six Sprague-Dawley rats were harvested. The corneal surface was scrapped to remove the epithelial and endothelial cells. The corneal rim was excised, cut into small pieces, collected in a 2 mL microcentrifuge tube containing 1 mL of collagenase enzyme solution (3 mg/mL) in serum-free Dulbecco's modified eagle medium (DMEM) with penicillin/streptomycin, and kept in a shaking 37ºC incubator for 30min. The supernatant was collected and kept in a fresh 15 mL tube, and an equal volume of complete DMEM (with 10% FBS, penicillin/streptomycin) was added to it and kept in a 37°C CO_2 incubator. One milli-litre of collagenase solution in serum-free DMEM was added to the tissue and incubated in a shaking 37ºC CO_2 incubator for 60min. After incubation, the supernatant was again collected and kept in a tube containing the previous supernatant, and an equal volume of complete DMEM was added and kept in a 37°C CO_2 incubator. Fresh collagenase solution (1 mL) in serum-free DMEM was added to the tissue and incubated in a shaking 37°C CO_2 incubator for another 60min. The supernatant was collected and stored in a tube containing the previous supernatants, and an equal volume of complete DMEM was added to it. The tissues were then discarded. The supernatant was centrifuged at 1400 rpm for 5min. The supernatant was discarded and the cell pellet was resuspended in 200 μL complete DMEM medium and plated on poly l-lysine-coated coverslips placed in 6-well plates and kept in a 37ºC incubator. After 1h, the plates were flooded with warm complete DMEM (10% FBS and penicillin/streptomycin). The medium was replaced every three days.

αB-Crystallin Peptide Internalization Cultured corneal keratocyte cells were washed twice in sterile phosphate-buffered saline (PBS), 10min each and incubated in serum-free DMEM containing fluorescence-tagged αB-crystallin peptide (Biotech Desk Pvt. Ltd, india customised: Sequence DRFSVNLDVKHFSPEELKVK; at a concentration of 20 nmol/L for 4h at 37°C in a CO_2 incubator in the dark). The following steps were carried out in the dark to avoid fluorescence quenching. The cells were washed twice in sterile PBS (10min each) and fixed in 4% paraformaldehyde (PFA) solution for 20min. The cells were again washed in PBS (10min), mounted on glass slides with a drop of mounting dye containing 4',6-diamidino-2-phenylindole (DAPI), and sealed using a clear nail polish. Cells were viewed under a fluorescence microscope and imaged.

Evaluation of Cyto-Protection by MTT Primary culture of corneal keratocytes was established in 96 well plate. Viability of cells following exposure to H_2O_2 was examined by the MTT assay following the standard protocol. Cells were seeded in each well of a 96-well plate and grown overnight in DMEM. Cells were treated with 20 nmol/L αB-crystallin peptide and incubated at 37°C in a CO_2 incubator for 4h. The cells were washed in sterile PBS, treated with 200 μmol/L H_2O_2 and re-incubated at 37°C in a CO_2 incubator for 24h. The medium was replaced with MTT (0.1 mg/mL in serum-free DMEM) and re-incubated at 37°C for 4h. The reaction was stopped by adding 100 μL DMSO to each well of the plate after removal of the

MTT and media. The absorbance was measured at 570 nm using a Multiskan plate reader.

Immunocytochemistry of Trigeminal Ganglia Cells Cultured TG cells were washed with PBS and fixed in 4% PFA (in PBS) for 20min at room temperature. The cells were then washed twice with PBS (5min each) and permeabilized with PBS with 1% Triton X-100 (PBST) for 90min. The cells were washed three times in 0.3% PBST (10min each) and incubated in PBS containing 5% goat serum albumin (GSA) for 3h for blocking. After discarding the blocking solution, the cells were incubated with primary antibody [Beta III tubulin; 1:100 in 5% GSA solution in PBS] overnight at 4°C in a humidified chamber. The cells were again washed three times with 0.3% PBST for 15min each. Cells were then incubated with a secondary antibody (goat anti-mouse, Alexa Fluor plus 555; 1:500 in 5% GSA solution in PBS) for 4h at room temperature in the dark. After washing three times, 15min each, with 0.3% PBST, cover slides were mounted using mounting medium with DAPI for fluorescent microscopy and sealed using clear nail polish. Cells were viewed under a fluorescence microscope and imaged.

Assay for Neurite Elongation Study Trigeminal neurons were cultured with αB-crystallin peptide (0.077 mg/mL) and αB-crystallin protein (Sigma Aldrich, C7858, 0.077 mg/mL) in low serum F12 (1% FBS, penicillin/streptomycin) medium, and incubated at 37°C in a 5% CO_2 incubator for 4h[6]. The medium was then replaced with fresh F12 medium (10% FBS, penicillin/streptomycin) and incubated at 37°C in a 5% CO_2 incubator. On the third day of culture, neurites were identified following the standard immunocytochemistry (ICC) protocol using beta III tubulin antibody, and the length of outgrowing neurites was assessed by image analysis using Image J software and compared. Totally 20 neuronal cell ICC images were selected randomly from each group of samples. Image J software was then used to measure the length of the selected neurons.

Assay for Neurite Degeneration Study Trigeminal neurons were treated with αB-crystallin peptide (0.077 mg/mL) and αB-crystallin protein (0.077 mg/mL) and incubated at 37°C in a 5% CO_2 incubator for 4h[6]. The cells were then washed twice with HBSS and incubated at 37°C in a 5% CO_2 incubator in low serum media (1% FBS, penicillin/streptomycin) containing 50 μmol/L H_2O_2 for 24h. Neurites were identified following the standard ICC protocol using a beta III tubulin antibody and imaged. Cells were viewed in Image J software and the number of beads was counted for each cell in the most prominent branch for a length of 100 arbitrary unit (AU).

Topical Use of αB-Crystallin Peptides for Evaluating its Healing Potency in Corneal Flap Surgery The study was conducted in adult New Zealand White rabbits (*n*=20) weighing between 2-2.5 kg. The animals were examined to rule out preexisting ophthalmic disorders. Anesthesia was induced using a combination of xylazine HCL (5 mg/kg wt I/M) and ketamine HCL (35 mg/kg wt I/M). The eyes were aseptically prepared for surgery by painting the conjunctiva with 5% povidone iodine. Local anesthesia was induced by topical instillation of proparacaine drops, and mydriasis was achieved by topical drops of tropicamide. A corneal lamellar flap was created following previous procedure[8]. The central cornea was demarcated with a 5 mm trephine, and a partial thickness incision of approximately 0.5 mm was made perpendicular to the corneal surface, tangential to the corneal trephine outline. A straight keratome was used to depress the corneal lip and slowly enter the stroma. A stromal pocket was created, which was further expanded using a lamellar dissector. A vannus knife was used to extend the circumference, leaving the flap undissected at three points around the circumference, forming a hinge. Topical drops of αB-crystallin peptides reconstituted in PBS (0.5 mg/mL) were instilled (one drop 50 mL, thrice daily) for 14d in the cornea of 10 rabbits, while the other 10 rabbit eyes received equivalent volumes and frequencies of sterile PBS.

The cornea of all eyes was routinely examined under a slit lamp biomicroscope for healing (by fluorescein dye test) and fibrosis, and at the end of 14d after slit lamp examination, the animals were humanely euthanized (with an overdose of anesthetic ketamine HCL >200 mg/kg) and the corneal tissue was collected for histopathology and gene expression for inflammatory cytokines interleukin 1 beta (IL1β), tumor necrosis alpha (TNFα), and interleukin 6 (IL6).

Gene Expression by Reverse Transcription-Polymerase Chain Reaction Tissues collected in TRIzol were homogenized, and RNA was extracted using a standard protocol. RNA concentration was determined and cDNA was transcribed using a standard kit protocol. Real-time polymerase chain reaction (PCR) was performed for the genes of interest against the housekeeping gene GAPDH. Relative mRNA levels were calculated using the Delta-Delta-Ct (DD CT) method according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table 1.

Table 1 Sequences of primers used for RT-PCR

Gene	Forward	Reverse
GAPDH	CGAGACACGATGGTGAAGGT	TGCCGTGGGTGGAATCAT
TNFα	AACATCCGGAATGTGGGGAC	GTAGGGCGGTTACCAACA
IL1β	TGCAGGAGCTTTGGGATTCT	TTCTCCAGAGCCACAACG
IL6	GGCTGATAGAAGAAGACGGATG	CCATGCCTGTCCAGAGAT

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TNFα: Tumor necrosis factor alpha; IL1β: Interleukin-1 beta; IL6: Interleukin 6; RT-PCR: Reverse transcription-polymerase chain reaction.

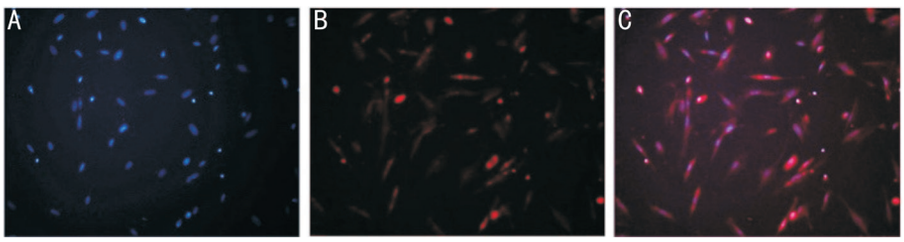

Figure 1 αB-crystallin mini-peptides internalized within the corneal fibroblast Cytoplasmic localization of red fluorescent tagged αB-crystallin mini-peptide was observed in corneal keratocytes following 4h of treatment. A: Nuclear staining by DAPI; B: Uptake of fluorescent tagged αB-crystallin peptide; C: Overlay image.

Histopathology A standard protocol was followed for histopathology and staining. Corneal tissues were dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. Sections of 5 μm thickness were cut on a microtome, mounted on L-lysine-coated glass slides, and stained with hematoxylin and eosin using a standard procedure. Stained sections were examined under a microscope and imaged.

Statistical Analysis Statistical analyses were performed using GraphPad Prism 6. Data are expressed as the mean±standard deviation (SD) or standard error of means (SEM), as indicated. Comparison between groups were done using *t*-test, the confidence level was set at 95% (P<0.05).

RESULTS

Internalization of the Fluorescent Tagged αB-Crystallin Peptide within Corneal Keratocytes Corneal keratocyte primary culture was established from the rat eye, and on the third day of culture, the cells were incubated with 20 nmol/L of fluorescence tagged αB-crystallin peptides. After 4h of treatment, the cells were fixed, stained with DAPI, and viewed under a fluorescence microscope. The cells showed cytoplasmic localization of the red fluorescent tagged αB-crystallin mini-peptide. The obtained result establishes the suitability of these peptides for local therapeutic use in the cornea, owing to their effective cellular uptake, and was thus used for further *in vitro* and *in vivo* studies (Figure 1).

Evaluation of Cyto-Protection to Corneal Keratocytes by αB-Crystallin Mini-Peptides Corneal keratocyte primary culture was established from the rat eye, and on the third day of culture, the cells were incubated with 20 nmol/L αB-crystallin peptides for 4h. The cells were then exposed to 200 μmol/L H_2O_2 for 24h, and the MTT assay was performed. The absorbance was measured and the data was analysed to report that cells pre-treated with αB-crystallin showed significantly (P<0.05) less cell death as compared to those without the peptide pre-treatment. Thus, αB-crystallin peptides provide significant cyto-protection to corneal keratocytes (Figure 2).

Influence of αB-Crystallin on Axonal Growth of Trigeminal Ganglion Cells Trigeminal ganglion primary culture was established, and on the third day of culture, the cells were incubated with either αB-crystallin peptide or protein for 4h.

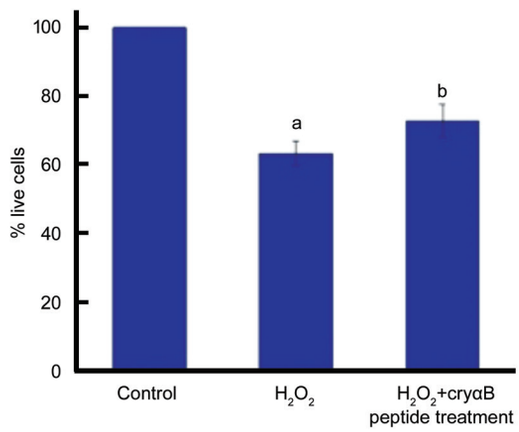

Figure 2 αB-crystallin provides cyto-protection to corneal cells Bar diagram shows percentage cell viability of H_2O_2 exposed corneal keratocyte cells treated with or without αB-crystallin mini-peptides, using MTT assay; cells receiving αB-crystallin peptides show significantly higher cell viability. [a]P<0.05 *vs* normal cells; [b]P<0.05 *vs* H_2O_2 treated cells without peptides treatment. Data presented as mean±SD. SD: Standard deviation.

The cells were fixed, and ICC was performed with beta III tubulin antibody. The lengths of axon from each group of cells were measured and no significant change among the groups was observed. Analysis of the data revealed that neither the αB-crystallin peptide nor the αB-crystallin protein showed any influence on the axonal growth of the neurons *in vitro* (Figure 3).

Influence of αB-Crystallin on Neurodegeneration of Trigeminal Ganglion Cells Trigeminal ganglion primary culture was established, and on the third day of culture, the cells were incubated with either αB-crystallin peptide or protein for 4h. The cells were then exposed to 50 μmol/L H_2O_2 for 24h. The cells were then fixed, and ICC was performed with beta III tubulin antibody. Beads were counted using Image J software for 100 AU in the most prominent branch of neurons (n=20 for each group). Analysis of the data concluded that significantly (P<0.05) reduced beading was observed in neurons treated with αB-crystallin peptides or αB-crystallin protein as compared to control cells exposed to H_2O_2 but not receiving any treatment. Neuronal beadings are bead-like swellings seen on neural branches, such as axons and dendrites, which are formed due to uneven focal accumulation

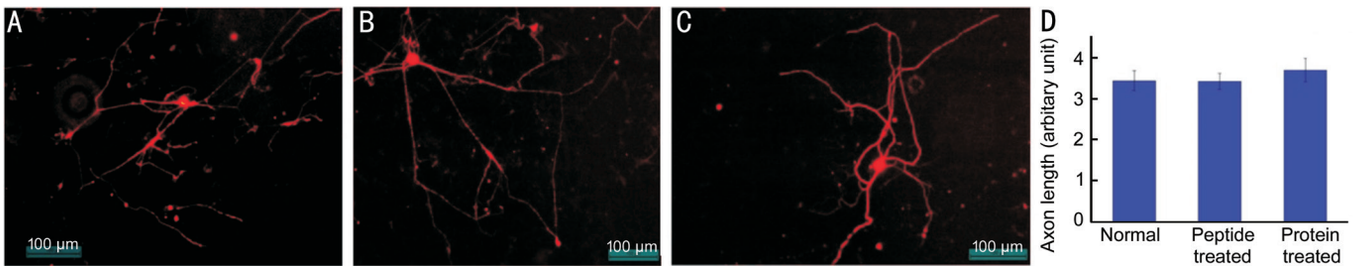

Figure 3 Treatment of trigeminal ganglion cells with αB-crystallin mini-peptide or protein did not adversely influence the growth of axons Representative images show trigeminal ganglion cells cultured with and without αB-crystallin peptides and protein. The neurite lengths of control cells (A) were compared with cells treated with αB-crystallin peptides (B) and αB-crystallin protein (C). Bar graph (D) shows no significant variation (P>0.05) in axon length among the three groups. Data presented as mean±SEM, n=20. SEM: Standard error of mean.

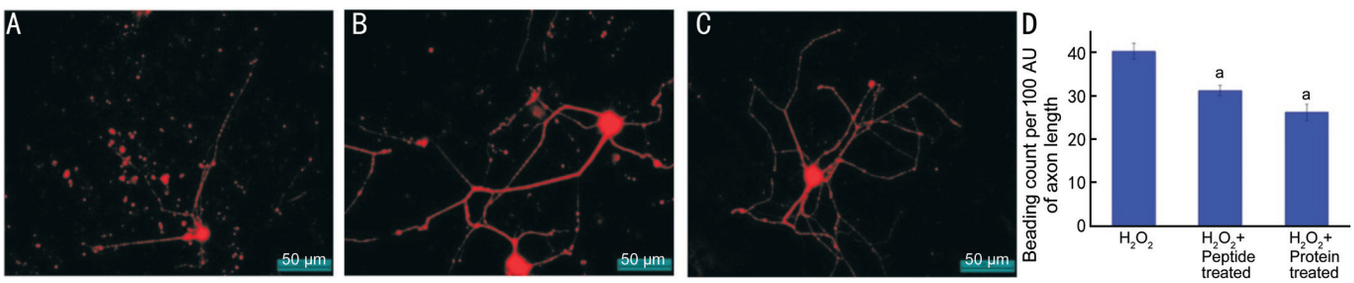

Figure 4 αB-crystallin reduced H_2O_2 induced degeneration in cultured trigeminal cells Trigeminal cells were incubated with 50 μmol/L H_2O_2 and treated either with αB-crystallin peptides (B), or αB-crystallin protein (C) or kept untreated (A). Beads on axon per 100 AU of axon length were counted using Image J software and presented in the bar diagram. Bar graph (D) shows remarkable beading in untreated cells exposed to H_2O_2. Cells receiving treatment with αB-crystallin peptides or αB-crystallin protein showed significantly reduced (P<0.05) number of beads compared to the untreated cells. Data presented as mean±SEM, n=20; ^aP<0.05 *vs* H_2O_2 treated. SEM: Standard error of mean.

of motor and cytoskeletal proteins, gathered by impaired transport of neuronal proteins. Beading formation on neurites is accompanied by gradual neuronal network collapse and is considered a neuropathological sign of neurodegeneration, often preceded by neuronal death. Since increased beading is a classical sign of neuronal degeneration, protection against beading by αB-crystallin peptide and protein can be concluded as a neuroprotective activity of or αB-crystallin in the cornea (Figure 4).

Clinical Findings in Corneal Injury Treated with or without αB-Crystallin Mini-Peptides The corneal injury model was induced as described previously. The injured eyes were either instilled sterile PBS (control) or treated with αB-crystallin peptide topical drops, three times daily for 14d. Eyes were imaged with a slit-lamp biomicroscope at regular time intervals (3, 7, and 14d). There was a distinct zone of hyper-reflectivity in the cornea without αB-crystallin treatment as compared to the treated (Figure 5). The regions of hyper-reflectivity may correspond to hypercellularity or fibrosis, further investigations are warranted.

αB-Crystallin Mini-Peptide Subdues Inflammatory Response in the Injured Cornea Eyes with injured corneas were treated either with αB-crystallin peptide drops or PBS for 14d as described previously. The corneas were collected and reverse transcription-PCR was performed to study the expression of inflammatory cytokines (IL6, IL1β, and TNFα). Expression of all the inflammatory cytokines (IL6, IL1β and TNFα) in the injured corneas were significantly (P<0.05) higher versus normal uninjured corneas. A significant (P<0.05) reduction in the expression of the inflammatory cytokines was observed in αB-crystallin mini-peptide treated corneas versus untreated injured corneas (Figure 6).

Histopathological Evaluation of Corneas Corneas were collected and prepared for histopathological studies using stain hematoxylin and eosin. The histopathological study showed that injured corneal sections without treatment had irregular epithelial healing, infiltration of inflammatory cells, and mid-stromal fibrosis; however, injured corneas that received topical drops of αB-crystallin showed normal epithelial healing, reduced infiltration of inflammatory cells, and reduced fibrosis (Figure 7).

DISCUSSION

This study aimed to investigate the therapeutic efficacy of αB-crystallin mini-peptides in rendering cytoprotective, anti-inflammatory, neuroprotective effects in the cornea, following injury.

As a preliminary assessment of the therapeutic efficacy of αB-crystallin in corneal healing, we first determined its internalization within the corneal cells. We thereby report for the first time, the successful internalization of the mini-

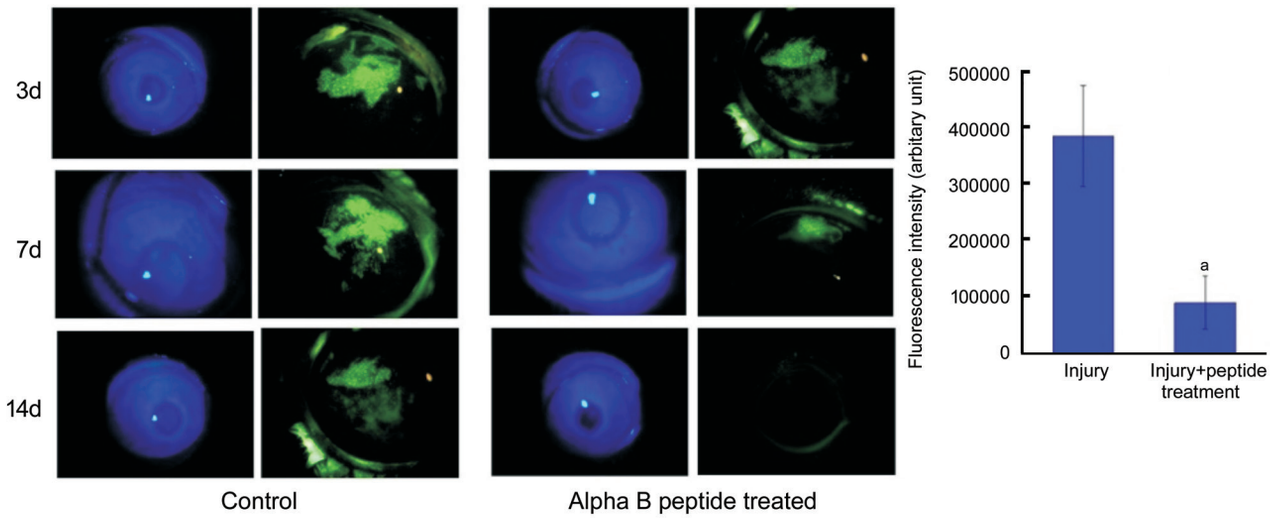

Figure 5 αB-crystallin suppresses corneal fibrosis Slit lamp biomicroscopic pictures under cobalt blue settings or blue free filter recorded at 3, 7, and 14d post corneal injury shows lesser fluorescence intensity in peptide treated corneas compared to untreated corneas. The zone of cellular hyper-reflectivity in the cornea may indicate loss of crystalline protein and increase in fibrotic changes. Bar graph shows significant reduction. Data presented as mean±SEM, *n*=3. Fluorescence intensity was measured using Image J software in arbitrary unit (AU); [a]*P*<0.05, injured and αB-crystallin treated *vs* injured cornea without treatment.

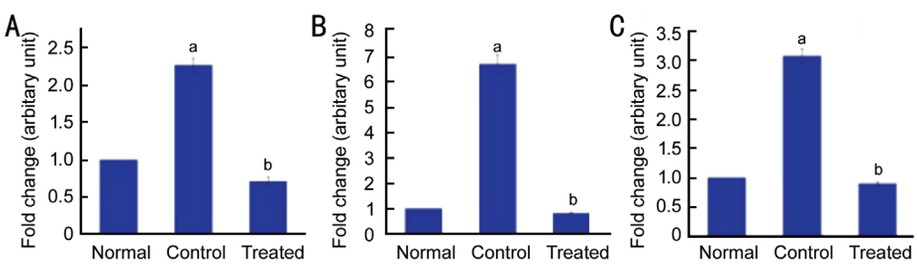

Figure 6 αB-crystallin supresses the expression of inflammatory cytokines following corneal injury Bar graphs show gene expression of various inflammatory cytokines (IL6, IL1β, and TNFα) in corneal tissues with or without treatment with the mini-peptides. Corneal tissues without αB-crystallin peptide treatment, show significant increase in expression of inflammatory cytokines IL6 (A), IL1β (B), and TNFα (C) as compared to normal uninjured cornea. There was significant reduction in the expression of inflammatory cytokines IL6 (A), IL1β (B), and TNFα (C) in treated corneas versus untreated cornea. Data presented as mean±SD, *n*=4, [a]*P*<0.05 *vs* normal uninjured cornea, [b]*P*<0.05 *vs* injured cornea without peptide treatment.

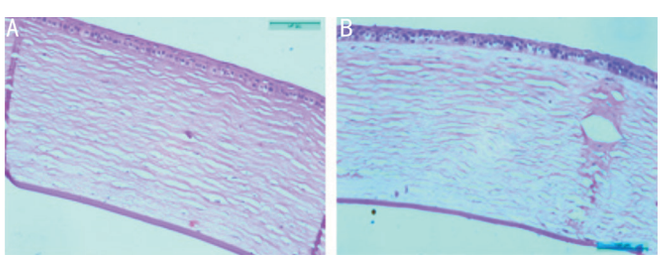

Figure 7 αB-crystallin treatment improved corneal healing Histology of corneal sections treated with αB-crystallin mini-peptides shows inflammatory reaction and mild fibrosis (A). Untreated injured corneal section shows irregular epithelial healing, inflammatory reaction and mid-stromal fibrosis (B).

peptides within the cytosol of corneal keratocytes. A previous study has also demonstrated effective cytosol and nuclear uptake of the mini-peptides in retinal pigmented epithelium (RPE) cells[6]. This encouraged us to use the mini-peptides for *in vitro* experiments and as topical therapy for cornea healing.

When corneal cells were stressed with H_2O_2 and evaluated with or without the mini-peptides, the MTT results showed a significant cytoprotective effect of the treatment. This corroborates with a previous study where similar observation was reported with RPE cells[6]. Cytoprotective activity of αB-crystallin in RPE cells have elegantly been demonstrated in previous studies[9-11].

Neuroprotection, is an integral part of corneal homeostasis. We therefore evaluated the efficacy of the peptides in enhancing the neurite growth of trigeminal neurons. Contrary to previous reports which demonstrated axonal growth and regeneration, using αB-crystallin for peripheral nerve regeneration, we observed no significant influence of mini-peptides on trigeminal neurite elongation *in vitro*[12]. The possible difference in response could be attributed to the structural difference in the corneal nerves, which are unmyelinated versus other myelinated peripheral nerves. Lim *et al*[13] reported

αB-crystallin induced remyelination after peripheral nerve injury. Therefore, a dose-dependent response of αB-crystallin mini-peptides on TG neurons may provide conclusive insights on neurite growth. However, it's worth mentioning, numerous studies have reported neuroprotective role of αB-crystallin in different retinal diseases[14-18].

Neurite beading is a classic sign of neuronal degeneration[19-20]. We observed significant protection of beading in stressed trigeminal ganglion neurites treated with the αB-crystallin mini-peptides. Thanos *et al*[12] provided elegant information on the neuroprotective activity of crystalline proteins in the posterior segment of the eye; however, there has been no previous report on the neuroprotective effect of αB-crystallin on corneal neurons. This observation may lead to new therapeutic applications with the corneal protein.

In an animal model of corneal flap surgery, we observed improved healing of the cornea treated with topical drops of αB-crystallin mini-peptide. The untreated cornea had distinctive hyper-reflective zones. The precise identification of the changes causing incomplete healing was not undertaken in this study. However, the possibility of hyper-reflective cells being dendritic cells which present during the inflammatory stages, cannot be ruled out[21].

Our study shows significant suppression of inflammatory cytokines at mRNA expression levels in corneas receiving crystallin mini-peptide therapy versus the untreated controls. Anti-inflammatory activity of αB-crystallin has been established by providing evidence of its activity in modulating the crosstalk between neutrophils and antigen-presenting dendritic cells, which significantly decreases the production of the proinflammatory factor IL-12p40[22] and its prevention of inflammation in experimental auto-immune uveitis[23-24]. Histopathological examination also demonstrated the reduced infiltration of inflammatory cells in the treated corneas. Nevertheless, further studies are required to decipher the role of αB-crystallin in corneal inflammation.

Interestingly, lens and cornea share similar crystallin protein, which contributes to lens stability and its transparency[25]. To this end the authors to the best of their knowledge have not come across any previous study demonstrating the cytoprotective, neuroprotective, and anti-inflammatory potency of αB-crystallin in the cornea.

The data from this study puts forth the multifaceted role of αB-crystallin in the cornea. Further investigation to explore its role under normal and pathological conditions and its therapeutic efficacy with improvised ocular delivery[26] are warranted.

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