

1 Apoptosis of corneal stromal cells induced by telopeptides: An *invitro* study

2 ABSTRACT

3 **Background:** Uncontrolled collagen degradation is the characteristics of keratoconus.
4 Degradation of type 1 collagen releases telopeptides. The role of telopeptides on the apoptosis of
5 corneal stromal cells have not been studied so far.

6 **Methods:** Human primary corneal stromal cells was cultivated and treated with varying
7 concentrations of synthetic telopeptides (94µg, 47 µg, 23.5 µg, 12.25 µg, 6.125 µg and 3.012 µg)
8 and incubated for 24 hours, 48 hours and 72 hours. MTT and TUNEL assay was performed
9 following incubation. The difference between the number of viable cells present in the treated
10 and untreated cells were considered for the analysis.

11 **Results:** Primary corneal stromal cells treated with varying concentrations of synthetic
12 telopeptide at 24 hours and 48 hours had no morphological or apoptotic changes, the viability
13 remained 100%. The percentage viability was altered after 72 hours of incubation with the
14 synthetic telopeptide. 94µg/ml, 47 µg/ml concentrations of telopeptide showed considerable
15 decrease in the cell viability ($p < 0.05$, *t test*).

16 **Conclusion:** The current study aims to evaluate the effect of telopeptides on primary corneal
17 stromal cells *invitro* and results revealed that the synthetic telopeptide had apoptotic effect.
18 Similar phenomenon can be exhibited in keratoconus stromal cells.

19 KEYWORDS:

20 Keratoconus

21 Keratocytes

22 Apoptosis

23 Telopeptides

24 Collagen

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30 **Introduction:**

31 Chronic keratocyte apoptosis is associated with pathogenesis of keratoconus. Alteration of
 32 keratocyte morphology and reduction of keratocyte density is evident in keratoconus corneas.
 33 ^[1,2]. The etiology of stromal thinning may be due to the loss of keratocytes which may be due to
 34 the degradation of the extracellular matrix by collagenolytic enzymes or by anoikis mediated cell
 35 death in which cells loose their anchorage due to ECM degradation^[3]. The effect of degradation
 36 of extracellular matrix (ECM) is being studied in detail but the effect of the degradation
 37 products of the stromal collagen is not studied.

38 The specialized tissue cornea constitutes three functionally distinct layers. The epithelium,
 39 endothelium, and the collagenous stroma. The cornea is morphologically supported by the
 40 collagen composition of the stroma. The homogeneity of the collagen type varies from layer to
 41 layer yet 75% of the cornea is composed of type 1 collagen. Type 1 collagen is composed of two
 42 $\alpha 1$ chains and one $\alpha 2$ chain, forming a continuous triple helical structure with non- helical
 43 telopeptides at both the N-terminal and the C-terminal. C-telopeptides corresponds to the C-
 44 terminus region of the alpha-1 chain of type 1 collagen^[4]. Degradation of collagen during
 45 physiological turnover or during excess proteolytic activity leads to the release of telopeptides
 46 into the plasma ^[5,6]. Presence of C-telopeptide indicates the collagen degradation and it is also
 47 used as biomarker in heart diseases. Abalain *etal* confers C- terminal telopeptides in tear of
 48 keratoconus patients as biomarker for the follow up of progressive keratoconus patients ^[7]. Thus
 49 the current study was designed with a hypothesis that the released telopeptides can contribute to
 50 the apoptosis of stromal cells which may eventually lead to stromal thinning.

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53 **Material and methods:**

54 **Primary corneal stromal cells:**

55 Primary culture of stromal cells were established from donor corneas obtained from CU Shah
 56 Eye Bank (Sankara Nethralaya, India) and managed according to the guidelines in the
 57 Declaration of Helsinki for research involving human tissue. In brief, a whole globe was
 58 obtained from 27 years male expired of road accident. The cornea was removed from the whole
 59 globe and quatered and rinsed with Dulbecco's modified Eagle's medium (DMEM)/F-12
 60 (Gibco, Grand Island, NY) containing antibiotics. Manual scraping of epithelium was avoided to
 61 reduce the chance of apoptosis of the keratocytes. The central cornea was digested with 3.3
 62 mg/ml Collagenase Type 1 at 37 °C under for 45 min to remove the epithelium and the
 63 endothelium. Then the stromal explants were palaced on to the 6 well plate. Supplemented with
 64 Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with Penicillin
 65 (150units/ml), Streptomycin (0.75%), Ciprofloxacin (0.1%) in 5% -10% CO₂, at 37°C. The
 66 medium was replaced twice weekly for routine maintains.

67 **Synthetic telopeptide:**

68 The sequence of C-terminal telopeptide was obtained from protein database UniProt. The
 69 synthetic octapeptide EKAHDGGR (Accession No: P02452) was synthesized by GeneScript,
 70 USA.

71 **Induction of Apoptosis by C-terminal telopeptide:**

72 The established primary culture of stromal cells were seeded into 96-well plates in DMEM/F12
 73 containing 10% FCS for 24 hours. After the removal of medium, cells were washed with
 74 phosphate-buffered Saline (PBS). The synthetic peptide was reconstituted in water at a stock

concentration of 9.4mg/ml. the cells were treated with diluted synthetic peptide at a final concentration of 94μg, 47 μg, 23.5 μg, 12.25 μg, 6.125 μg, 3.012 μg in 1% FBS. Control assays were performed with untreated cells. The percentage viability of cells were determined using MTT assay and the percentage of cells undergoing apoptosis was determined by TUNEL assay TiterTACS™ Colorimetric Apoptosis Detection Kit (Trevigen Inc. Gaithersburg, Catalog No: 4822-96-K) and TACS® 2 TdT Fluorescein Kit (Trevigen Inc. Gaithersburg Catalog No: 4812-30-K).

Data Analysis:

All data are expressed as mean $\pm$ SEM. Experiments were performed three times. Experimental and control conditions significance was determined with Student's *t* test. The level of significance was set at $p < 0.05$.

Results:

Primary culture of stromal cells were established from whole globe obtained from a single donor cornea (Figure 1). Telo peptide treatment was performed on early passages. Percentage viability of telo peptide treated cells were detected by MTT assay.

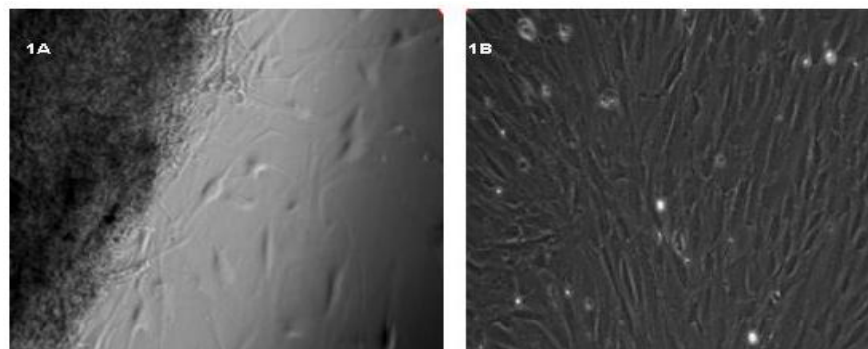


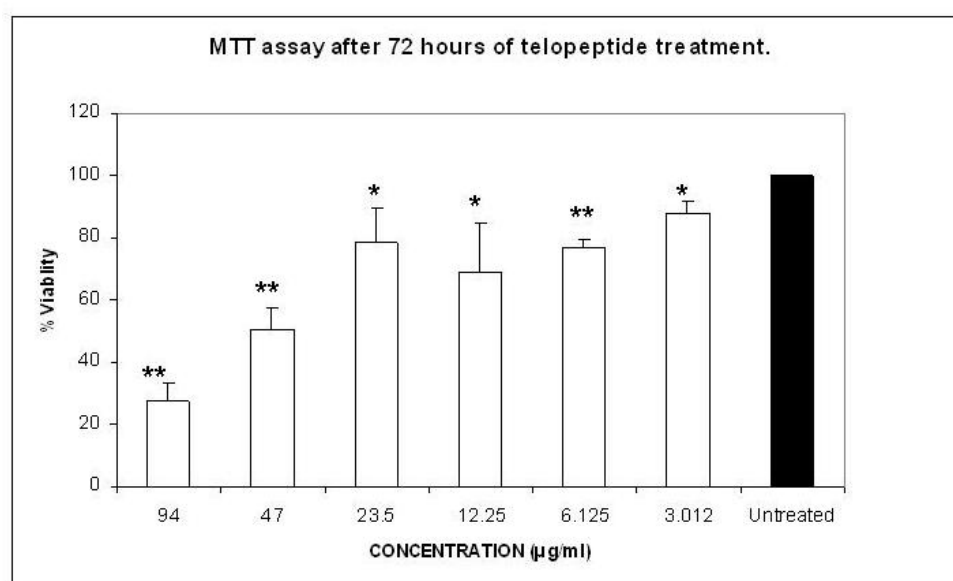
Fig 1: Primary culture of primary corneal stromal cells.

A. Out growth of stromal cells from the explant after 48hours,

B. Confluent monolayer of primary corneal stromal cells after 14 days of incubation.

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95 Telo peptide of varying concentrations namely 94 $\mu\text{g/ml}$, 47 $\mu\text{g/ml}$, 23.5 $\mu\text{g/ml}$, 12.25 $\mu\text{g/ml}$,
 96 6.125 $\mu\text{g/ml}$, 3.012 $\mu\text{g/ml}$ in 1% FBS was used to treat the primary corneal stromal cells at three
 97 time points. No significant effect of telo peptide was observed at 24h and 48 h of incubation. The
 98 percentage of cell viability decreased considerably at 72h of incubation with 94 $\mu\text{g/ml}$, 47 $\mu\text{g/ml}$
 99 of telo peptide (Figure 2) concentrations. To validate the results obtained from the MTT assay
 100 fluorescent TUNEL assay and microscopy based TUNEL assay was performed.



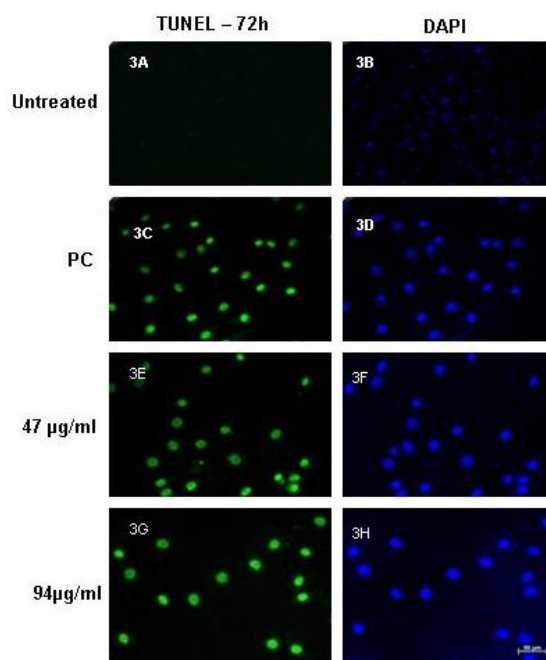
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102 **Fig 2:** Dose dependent effect of telo peptide on primary corneal stromal cells viability. Human
 103 primary corneal stromal cells were incubated with telo peptide 72 hours with varying
 104 concentrations of telo peptide. Shown are the percentages of viable cells determined by MTT
 105 assay. * $p < 0.05$, ** $p < 0.005$.

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107 In fluorescent based TUNEL assay the untreated primary corneal stromal cells served as control.
 108 Nuclease treated cells served as the positive control. Apoptotic nuclei was only observed in the
 109 cells treated with 94 $\mu\text{g/ml}$, 47 $\mu\text{g/ml}$ of telo peptide after 72 hours of treatment.

110 (Figure 3). Colorimetric TUNEL assay quantified the apoptotic cells on telopeptide treatment.
 111 Concordant results was observed in the colorimetric TUNEL assay. A dose-effect relationship
 112 could be obtained between the telopeptide concentration and the apoptotic cells (Figure 4).



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114 **Fig 3:** TUNEL assay performed after 72 h on treatment with telopeptides.

115 A. Untreated- negative control, B. DAPI counter stain of NC, C. Nuclease treated positive
 116 control, D. DAPI counter stain of PC, E. Apoptotic cells on treatment with 47 µg/ml
 117 telopeptide. F. DAPI counter stain, G. Apoptotic cells on treatment with 94 µg/ml
 118 telopeptide. H. DAPI counter stain.

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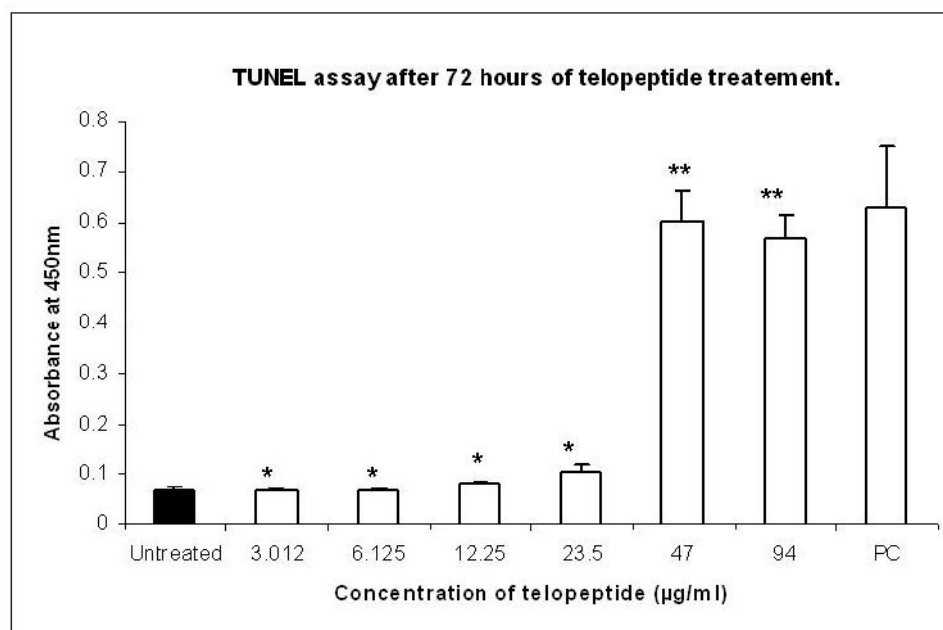


Fig 4: Dose dependent effect of telopeptide on primary corneal stromal cells viability using TUNEL assay. Human primary corneal stromal cells were incubated with telopeptide 72 hours with varying concentrations of telopeptide. * $p < 0.05$, ** $p < 0.005$.

Discussion:

In the current preliminary study, the apoptotic effect of the collagen degradation product – telopeptide *invitro* was experimented. Pathogenicity of keratoconus is of unknown etiology. Recent studies have suggested that the proinflammatory marker like IL-6, TNF- α and the matrix metalloproteinase (MMP)-9 plays a major role in the pathogenicity of keratoconus^[8] The stroma is mainly composed of keratocytes and these cells manufactures and maintains the protein and the collagen content of the corneal stroma. Apoptosis of keratocytes contributes to the alteration in the density of stroma^[9, 10]. Studies on keratoconus cornea emphasize the uncontrolled process of collagen degradation by the collagenolytic enzymes leading to stromal thinning^[11]. The

134 degradation of collagen results in the production of telopeptides. C-telopeptides are carboxy
135 terminal peptides of type 1 collagen.

136 Study by Abalain *etal* has quantified the amount of telopeptides present in the tear of
137 keratoconus and normal eyes. The tear film of keratoconus eye showed 2.5 fold increase of
138 telopeptide concentration when compared to the tear film of normal eyes ^[7]. The current study
139 was designed to elucidate the effect of the telopeptides on primary Corneal stroma cells.

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141 Though the quantification of telopeptides in the stroma of keratoconus patients will be a
142 supporting data to the current study this was not included due to the unavailability of corneal
143 buttons as the treatment modalities like Deep anterior lamellar keratoplasty (DALK) and
144 penetrating keratoplasty are replaced by collagen crosslinking procedure [12]. Telopeptides are
145 used as biomarker used to measure the rate of bone turnover. The effect of telopeptides on the
146 organ system are not studied. Yet the effect of collagen degradation has been studied by von
147 Wnuck *etal* where in the *degraded collagen* rapidly activates and leads to the apoptosis of
148 human vascular smooth muscle cells^[13].

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150 C- telopeptides sequence was obtained from Uniprot and synthesized from Genescript. Primary
151 Corneal stroma cells were established and was treated with various concentrations of C-
152 telopeptides for 24hours, 48 hours, and 72 hours. Morphological changes or apoptosis was not
153 observed in the primary corneal stromal cells when treated with the synthetic telopeptide for 24
154 hours and 48 hours. Apoptosis of primary corneal stromal cells were observed at 72 hours of
155 telopeptide treatment, at concentrations 94µg, 47 µg. Based on the study by Abalain *etal*
156 normal tears showed $3.10 \pm 0.37\mu\text{g}/\mu\text{g}$ of telopeptide and tears of keratoconus patients showed

8.11 $\pm$ 1.52 $\mu\text{g}/\mu\text{g}$ of telopeptides. *Invitro* treatment with physiological concentrations of telopeptide quantified by the above study did not show any effect of telopeptides on primary corneal stromal cells. It can also be hypothesized that all the telopeptides synthesized by collagen degradation in keratoconus cornea need not be released into the tear. Thus the quantified physiological concentration of C-terminal telopeptide may be low. This could be the reason why the physiological concentration of telopeptides of keratoconus patients did not match the results of the current study.

Conclusion:

Excessive collagen degradation by the collagenolytic enzymes results in the release of collagen degradation products like C-telopeptides that are released into the tear film and these telopeptides released may also play a vital role in the apoptosis of corneal stromal cells *invivo*.

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