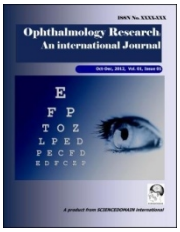

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Apoptosis of Corneal Stromal Cells Induced by Telopeptides: An *In vitro* Study

Vimalin Jeyalatha[1,2], Jambulingam Malathi[1*], Prema Padmanabhan[3] and Hajib N. Madhavan[1]

[1]L&T Microbiology Research Centre, Vision Research Foundation, Sankara Nethralaya 41, College Road, Chennai, 600 006, India.
[2]Birla Institute of Technology and Science (BITS), Pilani-333 031, Rajasthan, India.
[3]Department of Cornea and Refractive Surgery, Medical Research Foundation, Sankara Nethralaya 41, College Road, Chennai, 600 006, India.

Authors' contributions

This work was carried out in collaboration between all authors. Author VJ designed the study, wrote the protocol, performed the experiments and wrote the first draft of the manuscript. Author JM correction of protocol, data collection and final manuscript approval. Authors PP and HNM helped in literature survey. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Uncontrolled collagen degradation is the characteristics of keratoconus. Degradation of type 1 collagen releases telopeptides. The role of telopeptides on the apoptosis of corneal stromal cells have not been studied so far.
Methods: Human primary corneal stromal cells was cultivated and treated with varying concentrations of synthetic telopeptides (3.012µg, 6.125µg, 12.25µg, 12.25µg, 23.5µg, 47µg and 94µg) and incubated for 24 hours, 48 hours and 72 hours. MTT and TUNEL assay was performed following incubation. The difference between the number of viable cells present in the treated and untreated cells were considered for the analysis.
Results: Primary corneal stromal cells treated with varying concentrations of synthetic telopeptide at 24 hours and 48 hours had no morphological or apoptotic changes, the viability remained 100%. The percentage viability was altered after 72 hours of incubation with the synthetic telopeptide. 47µg/ml, 94µg/ml concentrations of telopeptide showed considerable decrease in the cell viability ($p<0.05$, *t test*).

**Corresponding author: E-mail: drjm@snmail.org;*

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

Keywords: Keratoconus; keratocytes; apoptosis; telopeptides; collagen.

1. INTRODUCTION

Chronic keratocyte apoptosis is associated with pathogenisis of keratoconus. Alteration of keratocyte morphology and reduction of keratocyte density is evident in keratoconus corneas [1,2]. The etiology of stromal thinning may be due to the loss of keratocytes which may be due to the degradation of the extracellular matrix (ECM) by collagenolytic enzymes or by anoikis mediated cell death in which cells loose their anchorage due to ECM degradation [3]. The effect of degradation of ECM is being studied in detail [4] but the effect of the degradation products of the stromal collagen is not been studied.

The specialized tissue cornea constitutes three functionally distinct layers. The epithelium, endothelium, and the collagenous stroma. The cornea is morphologically supported by the collagen composition of the stroma. The homogeneity of the collagen type varies from layer to layer yet 75% of the cornea is composed of type 1 collagen. Type 1 collagen is composed of two α1 chains and one α2 chain, forming a continuous triple helical structure with non-helical telopeptides at both the N-terminal and the C-terminal. C-telopeptides corresponds to the C-terminus region of the alpha-1 chain of type 1 collagen [5]. Degradation of collagen during physiological turnover or during excess proteolytic activity leads to the release of telopeptides into the plasma [4,6]. Presence of C-telopeptide indicates the collagen degradation and it is also used as biomarker in heart diseases. Abalain et al. [7] confers C-terminal telopeptides in tear of keratoconus patients as biomarker for the follow up of progressive keratoconus patients. Thus the current study was designed with a hypothesis that the released telopeptides can contribute to the apoptosis of stromal cells which may eventually lead to stromal thinning.

2. MATERIALS AND METHODS

2.1 Primary Corneal Stromal Cells

Primary culture of stromal cells were established from donor corneas obtained from CU Shah Eye Bank (Sankara Nethralaya, India) and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. In brief, a whole globe was obtained from 27 years male expired of road accident. The cornea was removed from the whole globe and quatered and rinsed with Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Grand Island, NY) containing antibiotics. Manual scraping of epithelium was avoided to reduce the chance of apoptosis of the keratocytes. The central cornea was digested with 3.3mg/ml Collagenase Type 1 at 37ºC under for 45 min to remove the epithelium and the endothelium. Then the stromal explants were placed on to the 6 well plate. Supplemented with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with Penicillin (150units/ml), Streptomycin (0.75%), Ciprofloxacin (0.1%) in 5% -10% CO_2, at 37ºC. The medium was replaced twice weekly for routine maintains.

2.2 Synthetic Telopeptide

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

2.3 Induction of Apoptosis by C-Terminal Telopeptide

The established primary culture of stromal cells were seeded into 96-well plates in DMEM/F12 containing 10% FCS for 24 hours. After the removal of medium, cells were washed with 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 3.012µg, 6.125µg, 12.25µg, 12.25µg, 23.5µg, 47µg and 94µg in 1% FBS. Control assays were performed with untreated cells. The percentage viability of cells were determined using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and the percentage of cells undergoing apoptosis was determined by Terminal deoxynucleotidyl transferase dUTP nick end labeling (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).

2.4 Data Analysis

All experimental data are expressed as mean with +/-standard error of the mean (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 $p< 0.05$.

3. RESULTS

Primary of stromal cells culture established from a single donor cornea (Fig. 1) was subjected to the experiment. Telopeptide treatment was performed on early passages (P1). Percentage viability of telopeptide treated cells were detected by MTT assay.

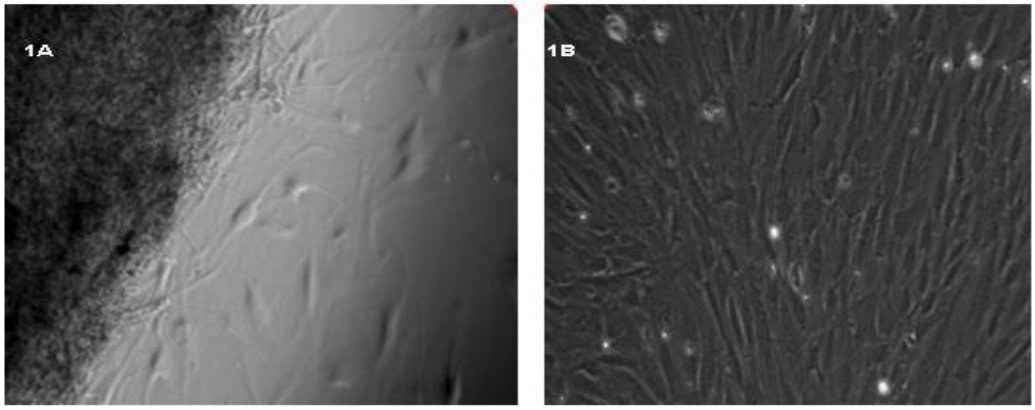

Fig. 1. Primary culture of primary corneal stromal cells

A. Out growth of stromal cells from the explant after 48hours, B. Confluent monolayer of primary corneal stromal cells after 14 days of incubation

Telopeptide of varying concentrations namely 3.012µg/ml, 6.125µg/ml, 12.25µg/ml, 23.5µg/ml, 47µg/ml, 94µg/ml in 1% FBS was used to treat the primary corneal stromal cells at three time points. No significant effect of telopeptide was observed at 24h and 48h of incubation. The percentage of cell viability decreased considerably at 72h of incubation with 94µg/ml, 47µg/ml of telopeptide (Fig. 2) concentrations. To validate the results obtained from the MTT assay, fluorescent TUNEL assay and microscopy based TUNEL assay was performed.

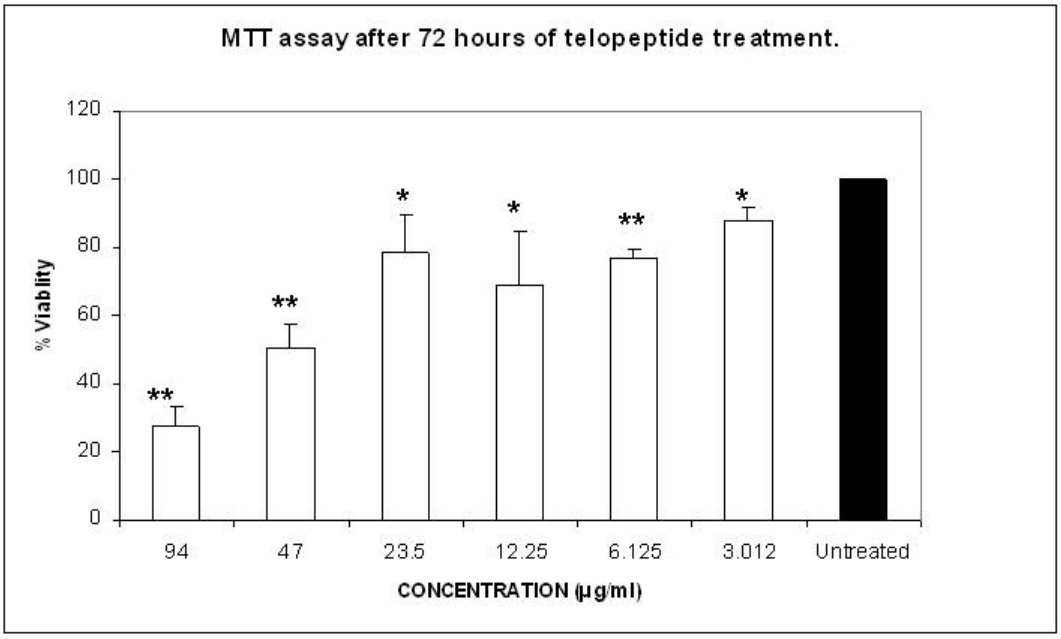

Fig. 2. Dose dependent effect of telopeptide on primary corneal stromal cells viability Human primary corneal stromal cells were incubated with telopeptide 72 hours with varying concentrations of telopeptide. Shown are the percentages of viable cells determined by MTT assay. *p<0.05, **p<0.005

In fluorescent based TUNEL assay the untreated primary corneal stromal cells served as control. Nuclease treated cells served as the positive control. Apoptotic nuclei were only observed in the cells treated with 94µg/ml, 47µg/ml of telopeptide after 72 hours of treatment.

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

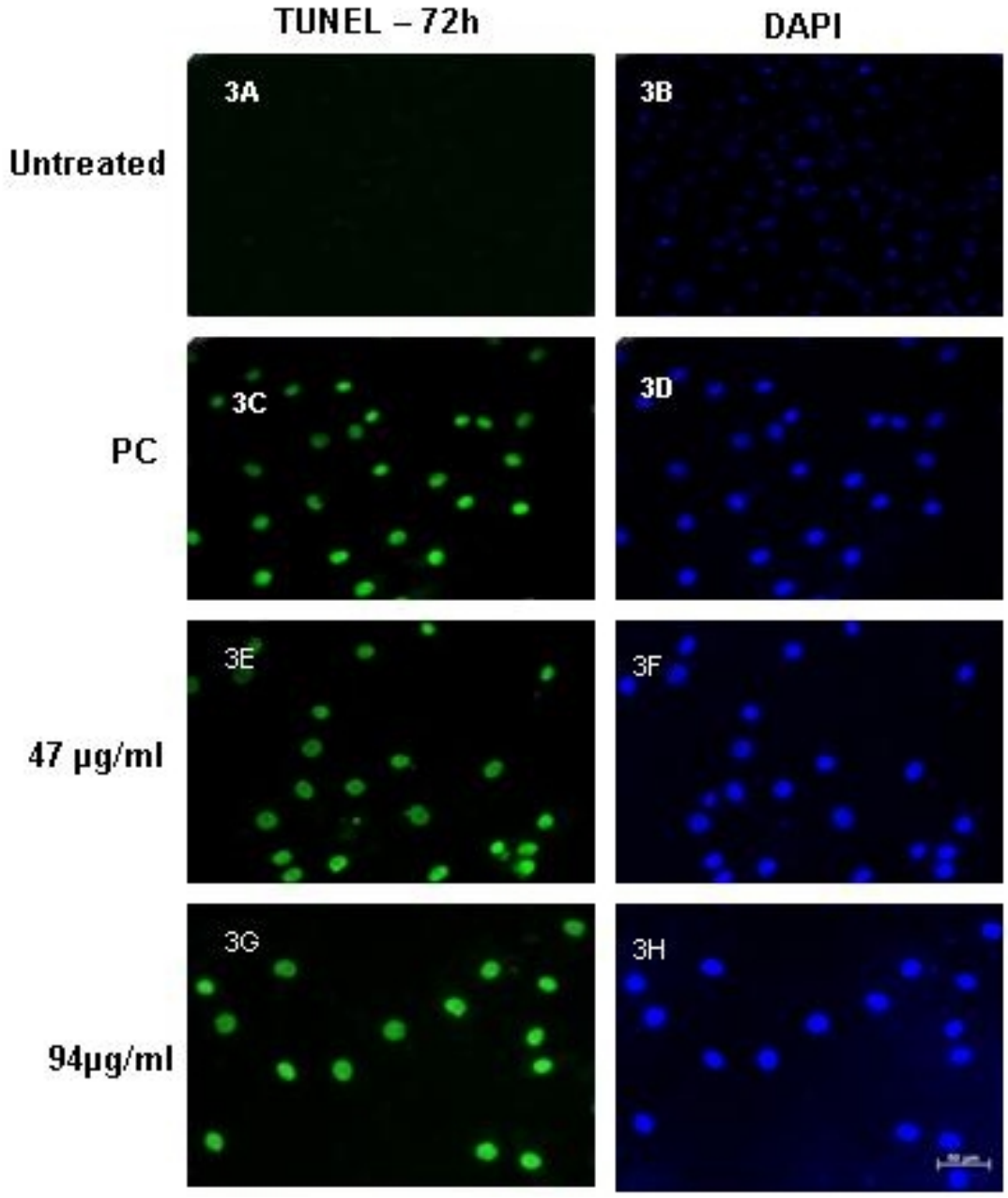

Fig. 3. TUNEL assay performed after 72h on treatment with telopeptides. a. Untreated-negative control (NC) , b. DAPI counter stain of NC, c. Nuclease treated positive control (PC), c. DAPI counter stain of PC, e. Apoptotic cells on treatment with 47µg/ml telopeptide. f. DAPI counter stain, g. Apoptotic cells on treatment with 94µg/ml telopeptide. h. DAPI counter stain

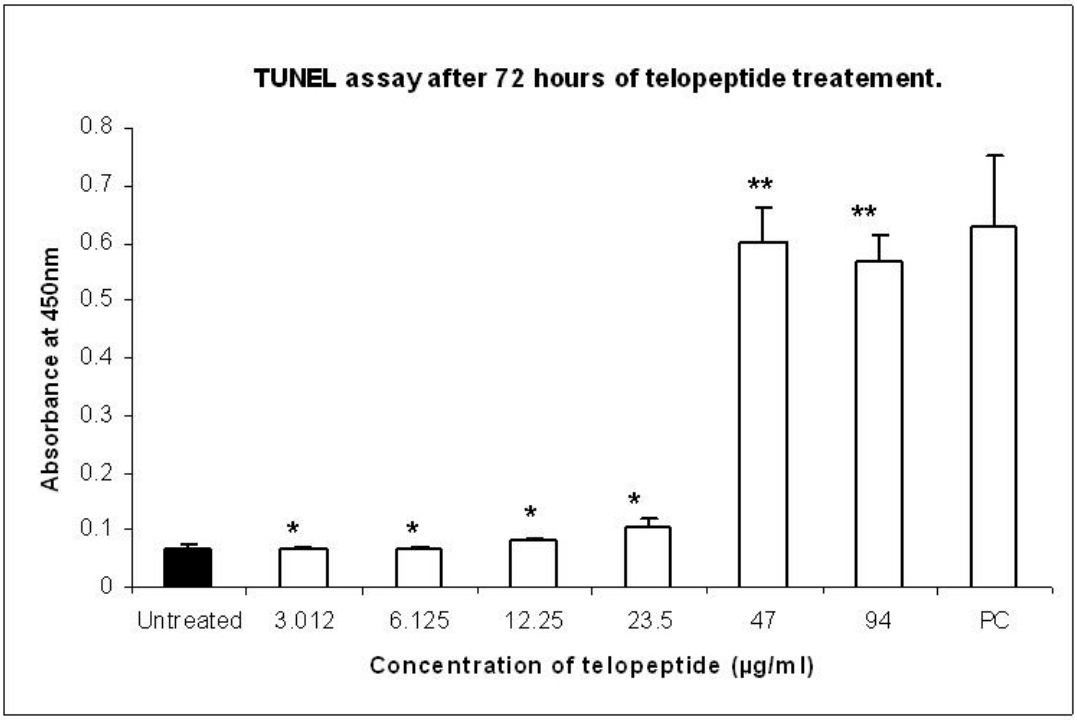

Fig. 4. Dose dependent effect of telopeptide on primary corneal stromal cells viability using TUNEL assay. Human primary corneal stromal cells were incubated with telopeptide 72 hours with varying concentrations of telopeptide. *p<0.05, **p<0.005

4. DISCUSSION

In the current preliminary study, the apoptotic effect of the collagen degradation product – telopeptide *in vitro* 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 met alloproteinase (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 degradation of collagen results in the production of telopeptides. C-telopeptides are carboxy terminal peptides of type 1 collagen.

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

Though the quantification of telopeptides in the stroma of keratoconus patients will be a supporting data to the current study this was not included due to the unavailability of corneal buttons as the treatment modalities like Deep anterior lamellar keratoplasty (DALK) and penetrating keratoplasty are replaced by collagen cross linking procedure [12]. Telopeptides

are used as biomarker used to measure the rate of bone turnover. The effect of telopeptides on the organ system are not studied. Yet the effect of collagen degradation has been studied by von Wnuck et al. [13,14] where in the degraded collagen rapidly activates and leads to the apoptosis of human vascular smooth muscle cells.

Primary Corneal stroma cells were established and was treated with various concentrations of C-telopeptides for 24 hours, 48 hours, and 72 hours. Morphological changes or apoptosis was not observed in the primary corneal stromal cells when treated with the synthetic telopeptide for 24 hours and 48 hours. Apoptosis of primary corneal stromal cells were observed at 72 hours of telopeptide treatment, at concentrations 94µg, 47µg. Based on the study by Abalain et al. normal tears showed 3.10±0.37µg/µg of telopeptide and tears of keratoconus patients showed 8.11±1.52µg/µg of telopeptides. *In vitro* 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.

5. CONCLUSION

The present *in vitro* study evaluates the effect of telopeptide on corneal stromal cells. Similarly, the C-terminal telopeptides released in the tear film can potentially induce apoptosis of the corneal stromal cells and can play a significant role in the pathogenic mechanism and progression of keratoconus *in vivo.*

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kim WJ, Rabinowitz YS, Meisler DM, Wilson SE. Keratocyte apoptosis associated with keratoconus. Exp Eye Res. 1999;69:475-481.
2. Erie JC, Patel SV, McLaren JW, Nau CB, Hodge DO, Bourne WM. Keratocyte density in keratoconus: A confocal microscopy study. Am J Ophth. 2002;134:689-695.
3. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol. 2001;13:555-62.
4. Matthews FJ, Cook SD, Majid MA, Dick AD, Smith VA. Changes in the balance of the tissue inhibitor of matrix met alloproteinases (TIMPs)-1 and −3 may promote keratocyte apoptosis in keratoconus. Exp Eye Res. 2007;84:1125-34.

5. Michelacci YM. Collagens and proteoglycans of the corneal extracellular matrix**.** Braz J Med Biol Res. 2003;36:1037-1046.
6. Dong Fan, Abhijit Takawale, Jiwon Lee, Zamaneh Kassiri. Fibrogenesis & Tissue Repair. Fibrogenesis & Tissue Repair. 2012;5:15.
7. Abalain JH, Dossou H, Colin J, Floch HH. Levels of collagen degradation products (telopeptides) in the tear film of patients with keratoconus. Cornea. 2000;19:474-6.
8. Jun AS, Cope L, Speck C, Feng X, Lee S, Meng H, Hamad A, Chakravarti S. Subnormal cytokine profile in the tear fluid of keratoconus patients. PloS one. 2011;6:e16437.
9. Kaldawy RM, Wagner J, Ching S, Seigel GM. Evidence of apoptotic cell death in keratoconus. Cornea. 2000;21:206-209.
10. Kenney MC, Nesburn AB, Burgeson RE, Butkowski RJ, Ljubimov AV. Abnormalities of the extracellular matrix in keratoconus corneas. Cornea. 1997;16:345-51.
11. Balasubramanian SA, Pye DC, Willcox MD. Are proteinases the reason for keratoconus? Curr Eye Res. 2010;35:185–191.
12. Hashemi H, Seyedian MA, Miraftab M, Fotouhi A, Asgari S. Corneal collagen cross-linking with riboflavin and ultraviolet a irradiation for keratoconus: Long-term results**.** Ophthalmology. 2013;120:1515-20.
13. Karin von Wnuck Lipinski, Petra Keul, Susann Lucke. Degraded collagen induces calpain-mediated apoptosis and destruction of the X-chromosome-linked inhibitor of apoptosis (xIAP) in human vascular smooth muscle cells. Cardiovascular Research. 2006;69:697–705.
14. Ihanamaki T, Salminen H, Saamanen AM. Age-dependent changes in the expression of matrix components in the mouse eye. Exp Eye Res. 2001;72:423–31.

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