

# In Vitro Effects of Ascorbic Acid on Corneal Collagen Cross-Linking in Keratoconus

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## Abstract

**Purpose:** To assess the efficacy and safety of ascorbic acid in the treatment of keratoconus by increasing the number of “anchors” that bond collagen fibers together in human in vitro cornea using electron microscopy.

**Methods:** In this semi experimental study keratoconus cornea is divided into six equal parts after keratoplasty. Two doses of ascorbic acid (10-3 mg/ml and 10-4 mg/ml) in two time ranges of 4 hours and one week after the treatment are applied to analyze the deviations of cross-linking. A part of the cornea is considered as control sample (without ascorbic acid) for different times. Various parts are randomly assigned to different studied doses. Each section of cornea is evaluated using electron microscopy. Friedman and Wilcoxon tests are used for data analysis. The value of significance level was set at 0.05.

**Results:** The average distance between collagen fibrils are measured after the treatment with two different doses of ascorbic acid for two different time ranges. These results showed that higher doses

of ascorbic acid and longer treatment time led to lower distance between collagen fibrils of cornea ( $p < 0.001$ ). This implies the better strength of the cornea. Apoptosis and vacuolization were not observed in keratocytes by electron microscopy after treatment with ascorbic acid.

**Conclusions:** Results showed that ascorbic acid strengthens the cornea and decreases the distance between collagen fibrils (consequently increase cross-linking). Therefore, the efficacy of ascorbic acid is observed by more recovery through increasing its doses and passing time.

**Key words:** Ascorbic acid, Cross-linking, Cornea, Keratoconus, Keratocytes

## Introduction

Cornea is the anterior and transparent segment of the eyeball. There are five layers from anterior to posterior of cornea: epithelium, bowman, stroma, Descemet's membrane and the endothelium. Stroma, constitutes 90% of corneal thickness, and consists of collagen fibrils blades intussusception (anchors). Keratocytes, as important formation cells of stroma, produce collagens and their apoptosis and necrosis cause structural weakness of corneal tissues (1).

Keratoconus (KC) is a bilateral, progressive, degenerative and non-inflammatory pathology of corneal ectasia(2). It usually starts at puberty. Irregular astigmatism, corneal thinning and progressive myopia and protrusion are characteristics of keratoconus that lead to decreased visual acuity(3). The prevalence of KC in the general population is reported to be around 1/2000 often affecting young patients(4). The etiopathogenesis and pathogenesis of keratoconus is unknown but it is likely a multifactorial disease (5). There are changes observed in corneal collagen organization (6), structure (7), intercellular matrix, necrosis and apoptosis of keratinocytes (8). Furthermore, a reduction is observed in cross-linking chemical bonds between collagen fibers inside the stroma for keratoconic corneas as compared to normal ones (9). This implies structurally weakened keratoconus corneal tissue (8).

There was not any effective way to stop progressive keratoconus so that about 21% of keratoconus patients need corneal transplantation (10). Conventional methods only improve the refractive errors and the loss of vision of corneal ectasia such as spectacles, rigid contact lenses, scleral, mini scleral and hybrid lenses, photorefractive keratectomy, intrastromal corneal ring, epikeratoplasty, phakic intraocular lens, keratophakia, keraflex (11) and penetrating keratoplasty. It should be noted that these methods do not remedy keratoconus and cannot stop the progression of this disease (2, 12).

Corneal collagen cross-linking is introduced for the treatment of progressive keratoconus(13). The first research of photobiology was conducted to increase the resistance of stromal collagen in the 1990s (14). The procedure of corneal collagen cross-linking is a combined action of chemical and physical factors which consisted of UVA ray at 370 nm (15) and a photosensitizing substance (riboflavin or vitamin B2) (8). Corneal rigidity is increased by corneal collagen cross-linking (16). This treatment is based on the changes of biomechanical properties of stromal collagen and cause pathogenesis of keratoconus (10).

Investigations show that oxidative stress is effective in KC (5, 17). Oxidative stress leads to disorder in the normal state of the cell and destruction of all cellular components including proteins and collagens resulted in a toxic effect by free radicals (18). Vitamin C (ascorbic acid) and vitamin E ( $\alpha$ -tocopherol) react with free radicals. It is strongly accepted that the antioxidant virtues of these vitamins

are responsible for their biological activity (18). Ascorbic acid is a basis for normal collagen formation (19). It is a main component in the synthesis of hydroxylysine and hydroxyproline in collagen (20). Hydroxylysine is important for formation of the intermolecular collagen cross-linking (21). Furthermore, ascorbic acid plays an important role in collagen biosynthesis (22, 23).

In this study, the distance between collagen fibrils are measured because of the study of cross-linking was qualitative and there were no plans or software to count cross-linking. The consistency of cornea is increased by reducing the distance between collagen fibrils. The purpose of this pilot study was to assess the efficacy and safety of ascorbic acid in the treatment of keratoconus by increasing the number of "anchors" that bond collagen fibers together (the corneal cross-linking strengthen the cornea). This study can be considered as a basis for the future conservative treatment of keratoconus.

## Materials and Methods

This semi experimental study was performed on patients attending the cornea clinic at Negah hospital in Tehran, Iran. The participants of this study were patients with confirmed diagnosis of progressive keratoconus who had an operation of penetrating keratoplasty. Two keratoconus corneal buttons (7-8.5mm) were obtained from 25 and 26-year-old patients treated with 10-3 and 10-4 mg/ml ascorbic acid (chemical formula: C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>, molecular weight (MW): 176.13 gr/mol and Art No: F413727). Two corneal buttons were sliced individually with a microtome to six equal sections. One section was stored in optisol and 10-3 mg/ml ascorbic acid. Another section was stored in optisol and 10-4 mg/ml ascorbic acid for 4 hours. Furthermore, one randomized section was just soaked in optisol as a control sample. Three samples of cornea were considered to be examined after one week as witness groups: one at optisol with 10-3 mg/ml ascorbic acid, one at optisol with 10-4 mg/ml ascorbic acid and the last one at optisol.

### Transmission Electron Microscopy

After the treatment with ascorbic acid, corneal buttons with their controls were prepared for transmission electron microscopy (TEM) (Zeiss EM 900). All sections were immersed in 2% glutaraldehyde. Sections of corneal button were embedded in hard resin after being fixed and dehydrated for transmission electron microscopy. 50–70-nm ultrathin sections were prepared and mounted on copper grids when the semithin sections stained with toluidine blue were observed.

Grids containing color samples were prepared to observe by transmission electron microscopy. EMC software V.16.8 was applied to study respective areas. In order to select images: magnifications of 7000 and 12000 were used to assess keratocytes (vacuolization and evaluation keratocyte membrane), the magnification of 30,000 was applied to assess the distance between collagen fibrils. 150000 sections of each sample were prepared by microkeratome and about 30 images of each section was taken. Finally,

10 images were selected to analyze keratocytes, cross-linking and the distance between collagen fibrils.

The distance between collagen fibrils was measured by AutoCAD 2007 software. In each case, the distance between the fibrils was randomly measured at 100 points to obtain an average. The average of different 100 points were randomly obtained for three cases which were almost same without any considerable difference. Images were passed to two medical geneticists to study keratocytes according to apoptosis, necrosis, the existence of vacuolization in them, shrinkage and freshness. They separately commented on freshness and keratocytes apoptosis rate in different doses of ascorbic acid at different times of treatments. It should be mentioned that the mean comments of these two medical geneticists are reported.

Considering that the control and treated groups were prepared from two corneas and comparisons between samples relevant only to each of these two corneas, there was no difference between comparisons in terms of clinical and demographic parameters (normalized). Studied variables did not follow a normal distribution based on the result of Kolmogorov-Smirnov test. Therefore, non-parametric tests were used for data analysis. Wilcoxon test was applied to compare the interrelated variables. Furthermore, Friedman test was used to assess deviations over time. The first type of error was considered about 0.05 to make decisions about significant deviations. All statistical analyzes were performed using SPSS v.16.

## Results

Measurements of the average distance between collagen fibrils of two corneas after the treatment with two different doses of ascorbic acid at 4 hours after treatment and one week after treatment showed that increasing the dose and passing time, reduce the gap between collagen fibrils (Table 1 - next page). It should be noted that the changes in the measurement of control samples showed the reverse trend. Friedman test showed a significant effect of time on the change in distance between collagen fibrils according to the comparison of repeated measurements for the following conditions: without intervention over three times, intervention with  $10^{-3}$  mg/ml ascorbic acid at fourth hour, intervention with  $10^{-3}$  mg/ml ascorbic acid in one week, intervention with  $10^{-4}$  mg/ml ascorbic acid at fourth hour and intervention with  $10^{-4}$  mg/ml ascorbic acid in one week (Table 2 - page 129). All comparisons are statistically significant in case of mutual conditions for changes in maintenance dose and time and also in comparison with witness group for both corneas (Table 3- page 130). In the first cornea, 98% and 95% of keratocytes were healthy and unchanged with  $10^{-3}$  mg/ml and  $10^{-4}$  mg/ml ascorbic acid after 4 hours, respectively. 87% of keratocytes were healthy and unchanged in the control sample after 4 hours. 78% and 79% of keratocytes were healthy and unchanged with  $10^{-3}$  mg/ml and  $10^{-4}$  mg/ml ascorbic acid after one week, respectively. 72% of keratocytes were healthy and unchanged in the control sample after one week. In the second cornea, 88% and 92% of keratocytes

were healthy and unchanged with  $10^{-3}$  mg/ml and  $10^{-4}$  mg/ml ascorbic acid after 4 hours, respectively. 83% of keratocytes were healthy and unchanged in the control sample after 4 hours. 75% and 78% of keratocytes were healthy and unchanged with  $10^{-3}$  mg/ml and  $10^{-4}$  mg/ml ascorbic acid after one week, respectively. 78% of keratocytes were healthy and unchanged in the control sample after one week.

## Discussion

In the present study, we investigated the effect of ascorbic acid on the increase of corneal strength.

Significant changes in the distance between collagen fibrils were found for the samples by doses of  $10^{-3}$  and  $10^{-4}$  mg/ml ascorbic acid in 4 hours. Both samples showed greater changes for mentioned doses in one week. This indicated the impact of ascorbic acid and longer time of treatment on the strength of cornea and the reduction of distance between collagen fibrils (consequently the increase of cross-linking). The dose of  $10^{-3}$  mg/ml ascorbic acid had a greater effect in comparison with  $10^{-4}$  mg/ml ascorbic acid in one week.

Collagen cross-linking leads to new covalent bonds between the collagen strings. These bonds affect the biomechanical strength and stiffness of cornea. Furthermore, they also change the corneal refractive index at multiple locations in cornea and reduce its adverse effects on vision. The gradual increase of visual acuity implies the efficacy of collagen cross-linking in cornea and its modifying factors. Wollensak et al. (24) studied the effects of riboflavin and UVA rays on 23 eyes with advanced keratoconus. They showed the best corrected vision was raised by  $1.65 \pm 1.5$  lines in vision chart for 65% of patients (24).

Sander et al. (25) also conducted a study on the corneal collagen cross-linking in 60 eyes with advanced keratoconus. They concluded that the best corrected vision was increased about  $2.04 \pm 1.4$  lines in eye chart (25). Caporossi et al. (26) reported the increase of 6 3.6 and 1.66 lines of eye chart in corrected and uncorrected visual acuity for 10 patients at 6 months after treatment, respectively (26). Pinelli (27) showed that uncorrected and corrected visual acuity has been increased by 2 and 1.8 lines in the eye chart at nine months after collagen cross-linking on 10 patients with advanced keratoconus (27). Raiskup et al. (28) studied 272 patients with advanced keratoconus. They showed that the best corrected vision in 53 % of patients was increased more than one line in the eye chart at the first year after collagen cross-linking while it did not change for 20 % of patients (28).

Our results showed that ascorbic acid with different doses and at different times was very less destructive at keratocyte in comparison with optisol. These were inconsistent with cell cultures derived from keratocyte of pigs, the damage threshold for UVA ray in combination with riboflavin 0.0025 was determined 0.4 mw/cm<sup>2</sup> which was 10 times lower than the threshold of UVA ray alone (29). In another study

	Cornea1						Cornea2					
	10 <sup>-3</sup> AA+ 4hours	10 <sup>-4</sup> AA 4hours	Control 4hours	10 <sup>-3</sup> AA 7 days	10 <sup>-4</sup> AA 7 days	Control 7 days	10 <sup>-3</sup> AA 4hours	10 <sup>-4</sup> AA 4hours	Control 4hours	10 <sup>-3</sup> AA 7 days	10 <sup>-4</sup> AA 7 days	Control 7 days
Mean	24.67	26.61	61.86	13.84	14.68	74.73	19.70	19.84	50.94	10.06	11.76	79.65
SD (mean)	.56	.66	.80	.43	.53	.88	.68	.96	.85	.74	.80	1.26
Median	24.85	26.65	61.78	13.91	14.72	74.65	19.48	19.87	51.07	10.14	11.75	79.69
Minimum	23.00	25.13	60.17	12.75	13.78	72.64	18.18	17.69	48.26	7.95	9.30	76.95
Maximum	25.71	28.11	63.89	15.04	17.78	77.28	21.72	21.71	52.86	12.07	13.85	82.46
Percentiles	25	24.42	26.00	13.59	14.28	74.43	19.28	19.27	50.25	9.73	11.28	78.91
	50	24.85	26.65	61.78	13.91	74.65	19.48	19.87	51.07	10.14	11.75	79.69
	75	25.01	27.05	62.46	14.03	74.97	20.16	20.46	51.46	10.40	12.25	80.34

• Ascorbic Acid

Table 1: The distance between collagen fibrils of cornea for two distinct patients at different time and treatments

Cornea1						Cornea2					
	Number	Average	Standard deviation	P-value of Friedman		Number	Average	Standard deviation	P-value of Friedman		
Control/ 4 hours	100	61.86	.80	<0.001	Control/ 4 hours	100	50.94	.85	<0.001		
10 <sup>-3</sup> AA*/4hours	100	24.67	.56		10 <sup>-3</sup> AA/4 hours	100	19.70	.684			
10 <sup>-3</sup> AA/ 7 days	100	13.84	.430		10 <sup>-3</sup> AA/ 7 days	100	10.06	.74			
Control/ 4 hours	100	61.86	.80	<0.001	Control/ 4 hours	100	50.94	.85	<0.001		
10 <sup>-4</sup> AA/ 4 hours	100	26.60	.66		10 <sup>-4</sup> AA/ 4 hours	100	19.84	.96			
10 <sup>-4</sup> AA/ 7 days	100	14.68	.53		10 <sup>-4</sup> AA/ 7 days	100	11.74	.80			

\* Ascorbic Acid

**Table 2: Comparison of changes in measurements during different times based on Friedman's nonparametric test between the two corneas**

Cornea1				Cornea 2				
	Number	Average	Standard deviation	P-value	Number	Average	Standard deviation	P-value
Control/ 4 hours	100	61.86	.80	<0.001	Control/ 4 hours	50.94	.85	<0.001
10 <sup>-3</sup> AA* / 4 hours	100	24.67	.56		10 <sup>-3</sup> AA/ 4 hours	19.70	.68	
Control/ 4 hours	100	61.86	.80	<0.001	Control/ 4 hours	50.94	.85	<0.001
10 <sup>-4</sup> AA/ 4 hours	100	26.60	.66		10 <sup>-4</sup> AA/ 4 hours	19.84	.96	
Control/ 4 hours	100	61.86	.80	<0.001	Control/ 4 hours	50.94	.85	<0.001
Control 7 days	100	74.73	.88		Control/ 7 days	79.65	1.26	
10 <sup>-3</sup> AA/ 7 days	100	13.84	.43	<0.001	10 <sup>-3</sup> AA/ 7 days	10.06	.74	<0.001
10 <sup>-4</sup> AA/ 7 days	100	14.68	.53		10 <sup>-4</sup> AA/ 7 days	11.76	.80	
Control/ 7 days	100	74.73	.88	<0.001	Control/ 7 days	79.65	1.26	<0.001
10 <sup>-4</sup> AA/ 7 days	100	14.68	.53		10 <sup>-4</sup> AA/ 7 days	11.76	.80	
Control/ 7 days	100	74.73	.88	<0.001	Control 7 days	79.65	1.26	<0.001
10 <sup>-3</sup> AA/ 7 days	100	13.84	.43		10 <sup>-3</sup> AA/ 7 days	10.06	.74	
10 <sup>-3</sup> AA/ 7 days	100	13.84	.43	<0.001	10 <sup>-3</sup> AA/ 7 days	10.06	.74	<0.001
10 <sup>-3</sup> AA/ 4 hours	100	24.67	.56		10 <sup>-3</sup> AA/ 4 hours	19.70	.68	
10 <sup>-4</sup> AA/ 4 hours	100	26.61	.66	<0.001	10 <sup>-4</sup> AA/ 4 hours	19.84	.96	<0.001
10 <sup>-4</sup> AA/ 7 days	100	14.68	.53		10 <sup>-4</sup> AA/ 7 days	11.76	.80	
10 <sup>-3</sup> AA/ 4 hours	100	24.67	.56	<0.001	10 <sup>-3</sup> AA/ 4 hours	19.70	.68	<0.001
10 <sup>-4</sup> AA/ 4 hours	100	26.61	.66		10 <sup>-4</sup> AA/ 4 hours	19.84	.96	

\* Ascorbic acid

**Table 3: Comparison of various average measurements of distance between collagen fibrils based on nonparametric Wilcoxon test for two corneas**

on keratocytes in pigs, Wollensak et al. (30) showed that the threshold of cellular damage of keratocytes were 0.5 mw/cm<sup>2</sup> and 5 mw/cm<sup>2</sup> for a combination of UVA ray and riboflavin 0.1% and UVA ray, respectively (30). The reduction in keratocytes for the depth of 270-350 micron inside stroma were observed by clinical investigations with the help of confocal microscopy on 10 corneal cross-linking. However, it was completely restored by the adjacent cell mobilization after 6 months (26, 31).

The low number of samples and different measurements of the distance between collagen fibrils were the weaknesses of our study. The novelty of this work was to study the effects of ascorbic acid on the increase of corneal collagen cross-linking.

## Conclusion

The purpose of the current study was to determine the effects of ascorbic acid on corneal collagen cross-linking in keratoconus in vitro. Corneal collagen cross-linking is explained as the most promising novelty in the treatment of progressive keratoconus in recent years. We found statistically significant increasing in the resistance of stromal collagen. Results showed that ascorbic acid can strengthen the cornea and decrease the distance between collagen fibrils (consequently increase cross-linking). Therefore, the efficacy of ascorbic acid is observed by more recovery through increasing its doses and passing time. It is noteworthy that this study can be a useful preliminary to future researches.

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