Tear Film and Serum Prolidase Activity and Oxidative Stress in Patients With Keratoconus

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Purpose: To determine and compare the serum and tear film prolidase activity (PA) between patients with keratoconus and healthy subjects. Also, we aimed to evaluate the serum oxidative stress level and the correlation with serum PA in patients with keratoconus.

Methods: This prospective, comparative clinical study included 31 patients with keratoconus and 33 age-matched and sex-matched control subjects. All participants underwent a detailed ophthalmologic examination. Serum and tear samples were obtained from all participants. Tears and serum PA and serum oxidative stress markers were measured.

Results: No significant differences in demographic characteristics were detected between groups ($P > 0.05$). The serum PA was significantly lower in the keratoconus group than in the control group (895.6 ± 198.7 vs. 1145.9 ± 285.4 U/L, $P < 0.001$). A tear film comparison showed that PA was lower in the keratoconus group than in the control group; however, this difference was not significant (3075.4 ± 672.2 vs. 3225.8 ± 903.2 $U \cdot L^{-1} \cdot g^{-1}$ protein, $P = 0.45$). Oxidative stress markers, such as total oxidant status and oxidative stress index, were found to be significantly higher in the keratoconus group ($P < 0.001$).

Conclusions: The serum PA was found to be lower in patients with keratoconus than in the controls. Additionally, serum oxidative stress markers were found to be higher than those of the controls. Thus, prolidase and systemic oxidative stress may have a role in the pathogenesis of keratoconus.

Key Words: keratoconus, prolidase, oxidative stress

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Keratoconus is the most common corneal ectasia associated with progressive irregular astigmatism and results in mild to marked visual impairment. It usually appears in the second decade of life and affects both genders and all ethnicities; the prevalence is 8.8 to 229 per 100,000 worldwide.[1,2] Despite intensive research on its pathophysiology, the underlying mechanisms remain poorly understood. Recent studies suggest that a combination of genetic, biochemical, biomechanical, and environmental risk factors, such as eye rubbing, atopy, inflammation, and oxidative stress, play a role in its pathogenesis.[3–11]

Oxidative damage has been suggested to be the origin of the pathophysiology in keratoconus.[4,7–10] The cornea is predisposed to oxidative damage as a result of ultraviolet radiation, atopy, and mechanical trauma.[4] Decreased levels of antioxidant enzymes, such as aldehyde dehydrogenase, catalase, and superoxide dismutase, have been reported in keratoconic corneas.[8–10] The accumulated reactive oxygen species could potentially damage the corneal components.[4] Furthermore, increased levels of proteases and other catabolic enzymes or decreased levels of proteinase inhibitors can initiate the degradation of the corneal collagen content observed in keratoconus, which might be associated with oxidative stress.[12–16]

Prolidase is a manganese-dependent matrix metalloproteinase (MMP) that removes imidodipeptides containing a C-terminal proline or hydroxyproline, catalyzing the final step of collagen degradation and playing an important role in collagen metabolism, matrix remodeling, and cell growth.[17,18] There is a strong correlation between increased collagen turnover and prolidase activity (PA).[17]

Studies have indicated that oxidative stress plays a role in the pathophysiology of keratoconus.[7–10] Type 1 collagen is the principal collagen component of the cornea, comprising approximately 80% of all corneal proteins, and type I collagen contains 25% proline or hydroxyproline.[1,19] It is likely that prolidase is involved in the pathogenesis of keratoconus because it plays an important role in recycling proline from imidodipeptides for the resynthesis of collagen.[20] Hence, the tear film might be a better indicator of the ocular surface. Therefore, we aimed to determine and compare the PA of serum and tears between patients with keratoconus and healthy subjects. Also, we aimed to evaluate the serum oxidative stress markers and the correlations of the serum and tear film PA in patients with keratoconus.

MATERIALS AND METHODS

Subjects

This prospective clinical study was conducted on consecutive patients with keratoconus (study group) and healthy volunteers (control group) who were seen in the Ophthalmology Department of the Medical Faculty Hospital, Harran University, between January 2013 and March 2014. All participants gave

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informed consent. The study protocol was approved by the Ethics Committee of Harran University.

The study group consisted of 31 patients with keratoconus. The difference between the mean inferior and superior power in the cornea was used to verify the diagnosis of keratoconus.[1] Using the mean keratometric readings obtained from computed topography, patients with keratoconus were classified as mild [45 diopters (D)], moderate (45–52 D), or severe (>52 D).[21] The control group consisted of 33 healthy, age-matched and sex-matched volunteers who had only a mild refractive error or were admitted for routine examination.

Subjects with a history of smoking, alcohol consumption, any other ocular pathology or surgery, or the presence of a systemic inflammation or disease (eg, diabetes, uncontrolled hypertension, thyroid disease, or renal or hepatic dysfunction) were excluded from the study. Obese subjects (body mass index >30 kg/m^2) or those currently on antiinflammatory or antioxidant therapy were also excluded.

All participants underwent a complete ophthalmic examination including corrected visual acuity measurement, slit-lamp biomicroscopy examination, applanation tonometry, funduscopic examination, and computerized topography (Tracey Technologies LLC, Houston, TX). Central corneal thickness was recorded using an ultrasound pachymeter (Sonomed-Pacscan 300; Escalon Medical Corporation, NY). In the study group, eyes with a more advanced stage of keratoconus (eyes with a steeper maximum keratometry) were selected for tear sampling.

Serum Collection

Venous blood samples were obtained from all participants to evaluate the serum oxidative stress markers and PA. Blood samples (10 mL) were drawn from the antecubital vein in the morning after overnight fasting. One aliquot was reserved for routine laboratory tests, whereas 1 aliquot was transferred to polypropylene tubes and centrifuged at 3000*g* for 10 minutes at 10 to 18°C. The serum layer was separated and stored at −80°C until used for analysis. No preservative substance was added because of the comparative nature of the study.

Tear Film Collection

A minimum of 5 to 7 μL of tears was collected from the inferior cul-de-sac of both eyes using a glass microtube (ISOLAB; Vitrex Medical, Herlev, Denmark). Special care was taken not to touch the ocular surface during tear collection to prevent reflex secretion of tears. In patients with keratoconus, tears were collected from the worse eye as the values of those were used for analysis. Soon after collection, the tears were stored at −80°C until used for analysis.

Analysis of Serum Oxidative Stress Parameters

Measurement of serum oxidative stress parameters, including total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI), were performed as described previously.[22]

Total Antioxidant Status

The TAS was measured in samples spectrophotometrically using commercial kits (Rel Assay Diagnostics). Results were expressed as millimoles Trolox equivalent per liter.

Total Oxidant Status

The TOS was measured in samples spectrophotometrically using commercial kits (Rel Assay Diagnostics). Results were expressed as micromolar hydrogen peroxide per liter (μmol H_2O_2 equivalent/L).

Oxidative Stress Index

The OSI was defined as the percentage ratio of TOS levels to TAS levels. The results were expressed as arbitrary units.

Prolidase Enzyme Activity

PA was determined by a photometric method based on the measurement of proline levels produced by prolidase.[23] Serum and tear samples (100 mL) were mixed with 100 mL of serum physiological. Then, 25 μL of the mixture was preincubated with 75 mL of the preincubation solution (50 mmol/L Tris–HCl buffer pH 7.0 containing 1 mmol/L glutathione, 50 mmol/L $MnCl_2$) at 37°C for 30 minutes. The reaction mixture, which contained 144 mmol/L Gly-Pro, pH 7.8 (100 mL), was incubated with 100 mL of preincubated sample at 37°C for 5 minutes. To stop the incubation reaction, 1 mL glacial acetic acid was added. After adding 300 mL Tris–HCl buffer, pH 7.8, and 1 mL ninhydrin solution (3 g/dL ninhydrin was melted in 0.5 mol/L orthophosphoric acid), the mixture was incubated at 90°C for 20 minutes and cooled with ice. Absorbance was then measured at a 515-nm wavelength to determine proline by the method proposed by Myara et al.[24] This method is a modification of the method of Chinard.[25] Intraassay and interassay coefficients of variation (CVs) of the assay were lower than 10%. The serum results were expressed as units per liter. Microprotein levels were determined by the Lowry method in the tear samples.[26] The tear samples results were expressed as units per liter per gram protein.

Statistical Analysis

All analyses were performed using SPSS (Statistical Packages for Social Sciences) for Windows version 17.0 (SPSS, Chicago, IL). All data were expressed as mean and SD. The distribution of data was tested using the Kolmogorov–Smirnov test. The differences between quantitative parameters were analyzed using the independent *t* test data with a normal distribution, whereas the Mann–Whitney *U* test was used to analyze skewed data. The Pearson correlation test was used to assess the relationships between parameters. $P < 0.05$ was considered statistically significant.

RESULTS

Thirty-one patients with keratoconus were enrolled in the study group, and 33 patients were enrolled in the control group. The demographic characteristics and laboratory data of

the patients are given in Table 1. The demographic characteristics did not differ significantly between groups. Compared with the control group, the keratoconus group had a significantly higher steepest keratometry value and a lower central corneal thickness (for both $P < 0.001$).

The serum TOS was significantly higher in the keratoconus group than in the controls ($P < 0.001$), whereas the serum TAS did not differ significantly between the 2 groups ($P = 0.17$). The OSI was also significantly higher in the keratoconus group ($P < 0.001$) (Table 1).

The serum PA was significantly lower in the keratoconus group than in the controls ($P < 0.001$). The PA was also lower in tears of the keratoconus group compared with the controls, although this difference was not significant ($P = 0.45$) (Table 1, Fig. 1).

In our study group, there were no mild keratoconus, 11 moderate cases (35.5%), and 20 severe cases (64.5%). As shown in Table 2, there were no significant differences in the TOS, TAS, OSI, serum PA, and tear film PA between the moderate and severe keratoconus groups ($P = 0.85$, 0.10, 0.61, 0.95, and 0.40, respectively).

We did not observe any correlation between the serum or tear film PA and oxidative stress markers ($P > 0.05$). There was also no correlation between the serum and tear film PA ($P = 0.45$). However, there was a significant positive correlation between the serum PA and central corneal thickness ($P < 0.001$) and a significant negative correlation between the serum PA and the steepest keratometry value ($P < 0.001$). Regarding the oxidative stress markers, the steepest keratometry value was positively correlated with TOS and OSI (both $P < 0.01$).

DISCUSSION

Keratoconus was once thought to be a noninflammatory condition. However, recent studies have shown that oxidative stress or chronic inflammation could stimulate the release of interleukins from the corneal epithelium.[3,4,6,9] The main clinical feature of keratoconus is the progressive stromal thinning, which results from the degradation of corneal collagen. These features are attributed to the degradation of the extracellular matrix, which is 70% collagen.[1] Therefore, much of the research on keratoconus has concentrated on proteases and interactions among MMPs, cathepsin, and inflammatory molecules in its pathogenesis; however, the results have been inconsistent, and the evidence, on balance, weak.[3,6,12–16]

In this study, we found that the serum PA was significantly lower in patients with keratoconus than in the controls. Very recently, lower serum PA in patients with keratoconus was also reported.[27] Prolidase plays a role in the resynthesis of collagen from collagen breakdown products.[20,28,29] Increased serum PA has also been reported in diseases in which collagen accumulation and extracellular matrix thickening are involved in the pathogenesis.[30–32] In contrast, serum PA was reported to be low in degenerative diseases, such as degenerative myopia and ascending aortic aneurysm.[33,34] Comparably, we observed a significant correlation between a lower serum PA and a decreased central corneal thickness. In our study group, the keratoconus was advanced, so we have no information on the PA during the early stages of the disease. Kılıç et al[27] also found low serum PA in keratoconus, even in the early stages of the disease.

Since the composition of tear fluid might better reflect the processes that take place on the ocular surface, the role of tear film components in keratoconus has been widely investigated.[3,6,13,14] Balasubramanian et al[13] reported increased expression of collagenases (MMP-1 and MMP-13), stromelysin (MMP-3), and matrilysin (MMP-7) in the tears of patients with keratoconus. Abalain et al[14] found increased levels of collagen degradation products in the tear film of patients with keratoconus. In this study, we evaluated the PA in the tears of patients with keratoconus and found that the tear film PA was lower in the keratoconus group, comparable to the serum values, but the difference was not significant. Furthermore, we did not find a significant correlation between the serum and tear film PA.

The exact mechanisms underlying the pathogenesis and progression of keratoconus remain unknown. However, oxidative stress is thought to play an important role in the pathogenesis of keratoconus.[4,7–10] We evaluated serum oxidative stress markers in our study because PA has been reported to be closely associated with the oxidative stress level.[35] In our study, TOS and OSI were significantly higher in patients with keratoconus than in the controls. The TAS was higher in the keratoconus group than in the controls, but the difference was not significant. Comparable to our results, Toprak et al[7] found that the serum TOS was significantly higher in patients with keratoconus, but they did not find a significant difference between the keratoconus and control groups in terms of the serum TAS. Contrary to these reports, Kılıç et al[27] did not observe an increase in oxidative stress markers in keratoconus. When we consider the disease severity, mild cases constituted approximately one third (32%) of the study group in Kılıç et al. In contrast to the serum PA, which was found to be low in all stages of keratoconus, systemic oxidative stress markers might be influenced significantly only in advanced stages. However,

TABLE 1. Demographic and Biochemical Data of the Patients

	Keratoconus Group (n = 31)	Control Group (n = 33)	*P*
Age, yrs	22.6 ± 9.3	25.1 ± 7.9	0.26*
Sex, female/male	13/18	17/16	0.44†
Body mass index, kg/m^2	22.9 ± 3.7	22.8 ± 3.3	0.89*
Steepest keratometry, D	54.0 ± 4.4	43.7 ± 1.1	<0.01*
Central corneal thickness	460.9 ± 39.5	554.7 ± 28.4	<0.01*
Serum			
TOS, μmol H_2O_2 eq/l	32.0 ± 9.3	20.6 ± 5.9	<0.01*
TAS, mmol Trolox eq/l	1.1 ± 0.2	1.1 ± 0.1	0.17*
OSI, arbitrary unit	2.9 ± 0.8	1.9 ± 0.6	<0.01*
PA, U/L	895.6 ± 198.7	1145.9 ± 285.4	<0.01*
Tear film			
PA, $U \cdot L^{-1} \cdot g^{-1}$ protein	3075.4 ± 672.2	3225.8 ± 903.2	0.45*

Values are represented as mean ± SD.
*Student *t* test.
†χ^2 test.

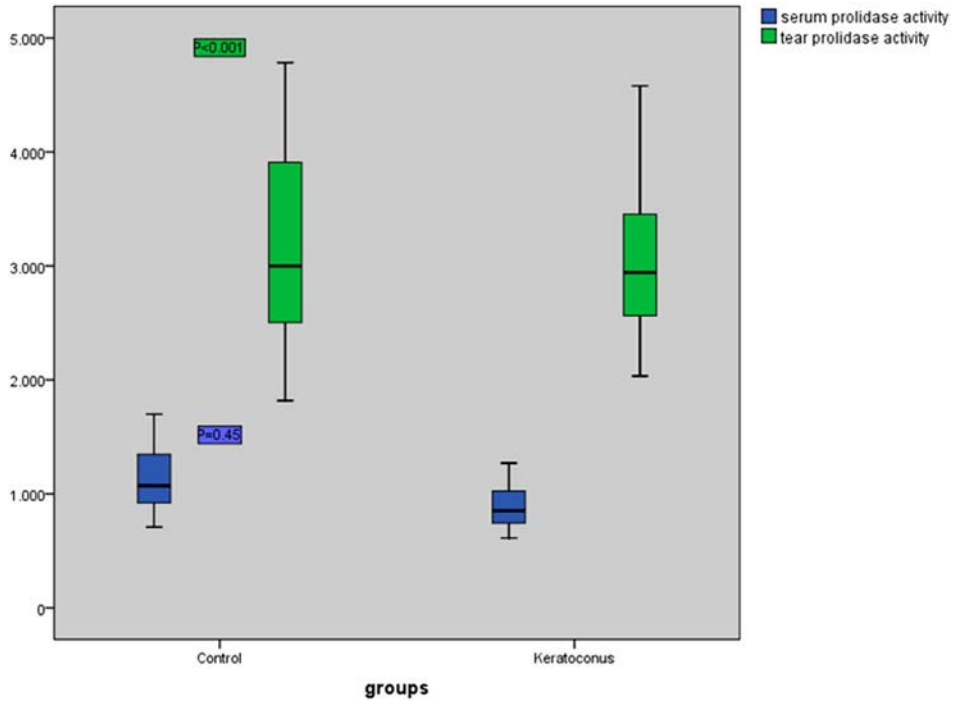

FIGURE 1. Tears and serum PA in the keratoconus and control groups expressed as a boxplot graph. Serum PA was significantly lower in the keratoconus group than in the control group ($P < 0.001$). However, the difference between groups was not significant in tear film PA ($P = 0.45$).

in our study, which included advanced keratoconus, we observed a significant positive correlation between oxidative stress markers (TOS and OSI) and the steepest keratometry value and a significant negative correlation between the serum PA and steepest keratometry value.

The major limitations of this study were the relatively small sample size and failure to evaluate early keratoconus. Another limitation was the difficulty in determining the exact origin of the measured PA. The extent of the corneal contribution to the serum pool of PA remains unclear. We tried to overcome this by evaluating tear film PA at the same time. Although, not statistically significant, the PA was also lower in the tears of patients with keratoconus.

In conclusion, we found that the serum PA was significantly lower in patients with keratoconus than in the controls, whereas oxidative stress markers were found to be significantly increased in these patients. Further investigations of prolidase are necessary; especially, a direct investigation of the PA in the corneal tissue might provide better information.

TABLE 2. Biochemical Data of the Patients in Different Groups of Keratoconus

	Moderate Keratoconus (n = 11)	Severe Keratoconus (n = 20)	*P**
Steepest keratometry, D	49.3 ± 1.6	56.6 ± 3.2	*<0.01*
Serum			
TOS, μmol H_2O_2 eq/l	32.7 ± 8.9	31.6 ± 9.6	0.85
TAS, mmol Trolox eq/l	1.2 ± 0.2	1.1 ± 0.2	0.10
OSI, arbitrary unit	2.8 ± 0.8	2.9 ± 0.8	0.61
PA, U/L	827.8 ± 221.1	932.8 ± 180.3	0.09
Tear film			
PA, $U \cdot L^{-1} \cdot g^{-1}$ protein	3181.5 ± 595.0	3017.1 ± 719.0	0.40

Values are represented as mean ± SD.
*Mann–Whitney *U* test.

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