IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL PRESENTATION OF APOPTOSIS AND AQUEOUS HUMOR'S NITRIC OXIDE LEVELS IN KERATOCONUS

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ABSTRACT

Objective: *This study aims to demonstrate corneal epithelium and stromal Caspase-9 mediated apoptosis and nitrit/nitrate levels of aqueous humor in cases with keratoconus.*

Material and Method: *Corneal tissues and aqueous humor fluids were obtained from keratoconus cases during Partial Penetrating Keratoplasty and the controls from cadavers within postmortem 24 hours. Caspase-9 and inducible nitric oxide synthase immunreactivities are determined by using immunohistochemical staining techniques. Nitrit/nitrate levels in aqueous humor were determined separately by using Griess reactives combined the Enzyme-Linked Immuno Sorbent Assay (ELISA) method.*

Results: *Apoptotic cells were observed in all epithelial layers. Caspase-9 expression was high in the basal layer and moderate in the upper stroma next to Bowman's membrane. Inducible isoform of nitric oxide synthase enzyme (i-NOS) staining was identified in similar localizations with apoptotic cells in all epithelial layers. Nitric oxide and nitrate levels were significantly low in the keratoconus group (p=0.03 and p=0.01 respectively). There was no difference in nitrit levels between groups (p=0.80).*

Conclusion: *Definitive epithelial apoptosis is a sign that keratoconus is not an isolated stromal disease. I-NOS derived nitric oxide leads to oxidative stress condition related with apoptosis and consequently to keratoconus.*

Key Words: *Keratoconus, caspase 9, nitric oxide, inducible nitric oxide synthase* ***Nobel Med 2013; 9(1): 5-9***

KERATOKONUSLU OLGULARDA APOPTOZİSİN İMMÜNOHİSTOKİMYASAL VE ULTRASTRÜKTÜREL OLARAK GÖSTERİLMESİ VE AKÖZ HUMOR NİTRİK OKSİT DÜZEYLERİ

ÖZET

Amaç: Bu çalışmada, keratokonuslu olgularda kornea epiteli ve stromal hücrelerdeki kaspaz-9 aracılıklı apoptozis ve aköz hümör nitrit/nitrat düzeylerinin saptanması amaçlandı.

Materyal ve Metod: Kornea dokuları ve aköz hümör örnekleri keratokonuslu olgulardan Parsiyel Penetran Keratoplasti yöntemiyle, kontrol örnekler de kadavralardan post-mortem 24. saatte elde edildi. Kaspaz-9 ve nitrik oksit sentetaz immünrektivitesi immünohistokimyasal boyama tekniği kullanılarak belirlendi. Aköz hümördeki nitrit/nitrat düzeyleri de Griss reaktifleri ve ELİSA yöntemiyle saptandı.

Bulgular: Tüm epitel tabakalarında apoptotik hücreler izlendi. Kaspaz-9 ekspresyonu, stromanın Bowman membranı komşuluğunda orta, bazal epitel tabakalarında kuvvetli idi. Uyarılabilir nitric oksit sentaz izoformu (i-NOS) tüm epitel katmanlarında apoptotik hücrelerle benzer lokalizasyon görterdi. Nitrik oksit ve nitrat düzeyleri keratokonuslu olgularda belirgin olarak düşük bulundu (sırasıyla p=0,03 ve p=0,01). Nitrit düzeyleri gruplar arasında farklılık göstermedi (p=0,80).

Sonuç: Belirgin epitelyal apoptozis keratokonusun sadece izole stroma hastalığı olmadığının bir işaretidir. i-NOS kökenli nitrik oksit oksidatif strese neden olarak apoptoza neden olmakta ve sonuçta da keratokonusu ortaya çıkarmaktadır.

Anahtar Kelimeler: Keratokonus, kaspaz 9, nitrik oksit, uyarılabilir nitrik oksit sentetaz **Nobel Med 2013; 9(1): 5-9**

INTRODUCTION

Keratoconus onset is at puberty and runs a progressive course through 3rd and 4th decade.[1] Hypotheses suggest the etiopatogenesis may be abnormally produced free radicals and superoxide radicals, catastrophic effects of peroksinitrate and aldehydes, apoptosis and irreversible cell damage[2]. Recent clinical and experimental studies states the possible relation between keratoconus and magnesium deficiency.[3] Consequently abnormal connective tissue degradation leads to stromal thinning observed in keratoconus.

Contact lens usage leads to hypoxia and stromal acidosis, a source of mechanical-chemical stress for the cornea in keratoconus ethiopatogenesis.[4-6] Low dose of ultraviolet light (UV) causes epithelial reactive oxygen species (ROS) production in the cornea and apoptosis by caspase 3 and 9 activation. Whereas high dose of UV leads to epithelial necrosis.[7,8] Significant epithelial hyperplasia, keratocyte apoptosis in frontal stroma and central corneal thinning demonstrated with pacimete, developed as a result of chronic corneal epithelial trauma.[9] This explains the possible mechanism between eye rubbing accompanied by keratoconus. As to our knowledge, the major source and target of free radicals are the mitochondria where oxidative stress changes the matrix pH to an alchalic environment leading caspase 2 and 9 activation and finally apoptosis.[10]

Nitric oxide is a paracrine mediator which has multiple functions in eyes.[11] Inducible isoform of nitric oxide synthase enzyme (i-NOS) does not exist under usual condition in the cell, contrary to endothelial and neural isoforms. It is expressed probably due to infectious, inflammatory and immunogenic stimulators. The stress factors related to keratoconus (sensitivity to UV, trauma caused by eye-rubbing or contact lens and defects in antioxidant defensive factors) may be other stimulators for i-NOS expression. Oxidative stress, lipid peroxidation, protein oxidation are causes of nuclear cell damage and cell death whether by apoptosis or necrosis.[12]

This study presented the possible relation between keratocyte and epithelial apoptosis and the change of nitric oxide level in aqueous humor in connection with i-NOS expresion. As a sign of oxidative stress, the i-NOS enzyme immunohistochemistry staining in the tissue and nitrit/nitrate levels in aqueous humor were identified. Apoptosis was confirmed by immunohistochemical staining method for caspase-9.

MATERIAL and METHOD

We enrolled 13 patients (6 female and 7 male) who had been diagnosed as kerotoconus and were candidates for a partial penetrating keratoplasty (PPK) at our ophtalmology clinic. Mean age was 26 (19-37 years). No other ocular pathology accompanying keratoconus was observed in any of these cases. During the operation, 0.2 cc aqueous humor was obtained from the anterior camera for determining nitric oxide level. Sick rondels were extracted and fixed in formol solution.

The control group samples (aqueous humor and cornea) were obtained from10 cadavers within postmortem 24 hours. Only cases with no history of ocular operation were selected. The aqueous samples were preserved at -80°C and the rondels at +4°C in formol solution until the time of study.

Nitrit and nitrate levels of aqueous humor were determined seperately by using Griess reactives and the ELISA method. Amount of nitric oxide was determined by calculating the sum of nitrit and nitrate levels. By the help of the standard graphics drawn in accordance with the prepared standards, amounts of nitrit and total nitric oxide were determined. Amount of nitrate was calculated by the formula of $Nox=NO_3-NO_2$.

We used immunoperoxidase techniques[13]. Formalin fixed-paraffin embedded sections firstly deparaffinized and rehydrated. They were boiled in high temperature microwave oven for retrieval stage. After 20 min at room temperature, the tissue was enrolled with a pap-pen (hydrophobic pen). After washing with distilled water and phosphate-buffered saline (PBS), hydrogen peroxide was added drop-wise. After washing with PBS, ultra V block was applied. After an 1-hour application of primary antibodies (Anti i-NOS primary antibody: Neomarkers Fremont CA 1605-R7 USA, Anti-caspase-9 primary antibody: Sigma Aldrich 100K 4852, USA) at room temperature, the samples were washed with PBS and a post-PBS level was applied. After rewashing with PBS the specimens were placed 10 minutes in AEC (3-amino-9-ethyl carbazole) chromogen. Finally the counterstain with Mayer's hematoxilyn was performed for 2 minutes. All slides were evaluated with Leica DM 4000 B light microscope (Wetzlar, GERMANY).

For evaluating electron microscopic sections, we used epoxy resin embedding methods.[14] Samples were fixed firstly with 2.5% gluteraldehyde and 1% osmium tetraoxide afterwise. After washing with graded ethanol series, the samples were embedded in araldite. Semi-thin sections stained with toluidine blue were evaluated under light microscope, and thin sections under electron microscope (Zeiss, 900 EM, Germany). →

RESULTS

The mean age of 13 keratoconus cases was 26 (19-37) and gender distribution was 7 male to 6 female. The mean age of the control group was 68 (48 to 79). The average Sim K value of keratoconus cases was 56.33 (43.3-68.7) dioptri. During detailed inspection, neither ocular pathology likely to influence the level of nitric oxide was present in the study group, nor was hydrops sequela observed. There was no history of contact lens usage, atopy and family history in the study group. Nitrit, nitrate and nitric oxide levels determined in aqueous humor of the control and keratoconus study groups are presented in Table 1 and 2.

Anti-caspase 9 antibody staining demonstrated mild and moderate immunreactivity on the upper and middle layers of corneal epithelium (Figure 1a). However, strong cytoplasmic staining was determined at epithelial cells on stratum basale. Keratocytes of the upper and middle stromal layers showed different grades of apoptosis presenting immunoreactivity, furthermore this reaction was more intensive on the stroma next to Bowman's membrane (Figure 1b,c).

Anti i-NOS immunohistochemical staining samples demonstrates scattered and dens feature on all epithelial layers (Figure 1d), but a mild immunreactivity on keratocytes. Degenerated and vacuolated basal ephitelium was strongly stained, whereas frontal stromal keratocytes showed mild to moderate reactivity (Figure 1e). Surprising was the case with a very intensive i-NOS staining but no keratocyte reaction (Figure 1f).

Under electron microscopic evaluation Bowman's membrane, epithelial layers and stromal structures were shown to be normal in the control group (Figure 2a,c,d).

The keratoconus group had demonstrated classical morphological characteristics of apoptosis which are cellular contraction, nuclear segmentation and margination, chromatine condensation and membrane connected bleps at epithelial cells and keratocytes (Figure 2b,e,f).

Statistical Analysis

Mann-Whitney U test with SPSS 10.0 was used for statistical analysis. The difference between stromal thicknesses of keratoconus and control group was statistically significant (482.74±31.78 and 165.83±64.38 respectively, p=0.0001).

DISCUSSION

Keratoconus is an ectatic dystrophy of the cornea. It progresses with the disruption of Bowman's layer and includes keratocyte apoptosis. Keratocyte apoptosis is triggered by interleukin-1, fas ligand and bone morphogenetic protein 2/4 which are products of damaged epithelia.[15] Wollensak et al. identified chromatine condensation, cellular contraction and apoptotic bodies through the deep stroma of corneal keratocytes following riboflavin/UV-A treatment.[16] In the report of Borderie et al., intensifying of endothelial cell nucleus, reduction in cellular size and subepithelial fibrosis was observed in Fuchs' Dystrophy, which is another dystrophic disorder of the cornea. Keratocytes had not been effected in Fuchs' Dystrophy.[17] In the study of Ziangirova et al. including 28 keratoconus cases, progression to oncosis and apoptosis of the corneal epithelium due to inflamatory ischemia has been reported.[18] Similar findings were observed in our study like apoptosis in stromal keratocytes and basal epithelial cells in keratoconus. →

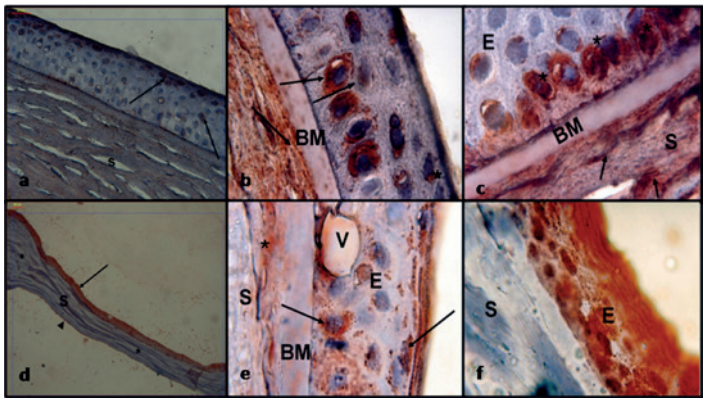

Figure 1: Mild and moderate caspase-9 manifestation on upper and middle layers of epithelium (↑), stromal detachment (S) in a; strong cytoplasmic staining at epithelial cells(↑), more intensive caspase-9 immunreactivity in the stroma (S) next to the Bowman's membrane (BM) in b,c; scattered and intensive i-NOS immunreactivity in all epithelial layers (↑) and mild immunreactivity in keratocytes (S) (▼) in d; degenerated and vacuolated epithelial cells demonstrate strong i-NOS immunreactivity whereas keratocytes do mild to moderate in e; Intensive i-NOS immunreactivity but no keratocyte reaction in f. (a,b,c: caspase-9 immunperoxidase; d,e,f: i-NOS immunperoxidase)

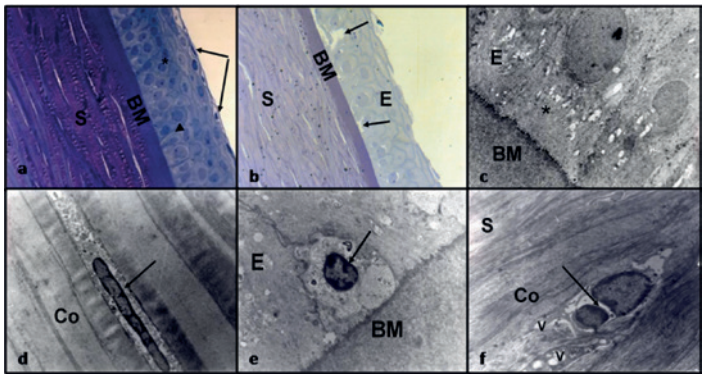

Figure 2: Normal structures of epithelial layers and stroma in control group (↑) in a,c,d: epithelial detachment (↑) in b; cellular contraction (E) in e; nuclear segmentation and margination (↑) in f; chromatine condensation and membrane blebs in epithelial cells (↑) in e; and keratocytes (↑) and vacuolization in collagen fibers (Co) in f. (a,b: toluidin blue staining; c,d,e,f: uranyl acetate and lead citrate)

Table 1: Nitric oxide, nitrit, nitrate levels (micromol/Liter) in keratoconus group determined by gender, age and cornea curvature diameters

Keratoconus	Gender	Age	K1	K2	Max perpendicularity	Nitrit	Nitrate	Nitric Oxide
N1	E	32	55.8	65.1	75.4	5.11	14.27	19.38
N2	K	27	52	59	63	10.76	29.75	40.51
N3	K	24	51	54.4	63.56	5.12	15.19	20.31
N4	E	21	49.8	57.3	68.5	3.68	23.86	27.54
N5	E	28	43.4	51.2	60.3	13.81	7.02	20.83
N6	K	31	53.1	57.3	63.3	7.76	28.29	36.05
N7	E	19	46.8	43.3	63	18.33	17.13	35.46
N8	E	34	74.7	68.7	67	18.99	9.52	28.51
N9	E	25	55.2	59.4	62.8	9.71	17.58	27.29
N10	K	32	64.5	66.8	74	3.66	24.21	27.87
N11	K	21	62.2	67.8	81.85	5.71	24.76	30.47
N12	K	29	51.4	54.9	70.5	11.21	22.27	33.48
N13	E	37	49.6	52.7	66.4	4.19	23.72	27.91

Table 2: Nitric oxide, nitrit ve nitrate levels determined in control group (micromol/Litre)

Control	Age	Nitrit	Nitrate	Nitric oxide
K1	53	9.31	24.12	33.43
K2	66	4.69	31.75	36.45
K3	79	7.32	33.21	40.53
K4	48	5.52	25.14	30.66
K5	61	6.83	40.42	47.26
K6	57	13.32	29.49	42.81
K7	75	5.31	28.88	34.29
K8	59	6.91	34.56	41.47
K9	64	6.14	25.41	31.55
K10	73	8.29	30.74	39.03

The relation of contact lens usage and keratoconus ethiopatogenesis has been mentioned before. Chronic corneal trauma results in significant epithelial hyperplasia, frontal stromal keratocyte apoptosis and pachymeter displayed central corneal thinning. Mechanic debridgement induced keratocyte apoptosis of corneal epithelium can be prevented by topically applied z-VAD-FMK (caspase inhibitor).[19] Under hypoxemic condition, the caspase-8 activity in corneal epithelial cell culture is suppressed, whereas the activity of caspase-9 which is the startpoint of mitochondria-mediated apoptosis seems to be increased.[20] Similarly, ischemia and exotoxins lead to an increase in caspase-9 activity and antiapopitotic bcl-2 protein expression in retinal cellular cultures[21]. Ganglia cell loss due to intraocular pressure augmentation is another point related with caspase-9 activation[22]. Photoreceptor death in experimental retinal detachments also occur with the activity increase of caspase 3 and 9.[23]

These results indicate that oxidative stress factors like hypoxia and ultraviolet light activates the mitochondrial pathway leading apoptosis in keratoconus cases. The major source and the target of free radicals is the mitochondrium, where oxidative stress alkalinize the matrix pH and activate caspase 2 and 9. This was the reason why we studied caspase 9 activity in our study.

We demonstrated epithelial apoptosis which was more intensive in stratum basale than in the upper and middle layers of epithelium. Caspase 9 staining was more dense in the cytoplasm of epithelial cells. This may indicate that activated caspase 9 crosses to cytosol from the mitochondria. We showed that apoptotic keratocytes next to Bowmann's membrane were stained much more than the keratocytes on the upper and middle stroma. Ephitelial apoptosis was demonstrated as well as keratocyte apoptosis, leading to a conclusion that keratoconus is not an isolated stromal disease. Keratocyte apoptosis is triggered by substances releasing from damaged cells like interleukin-1, fas ligand and bone morphogenetic protein 2/4.[15] In a study conducted with keratoconus and simple keratocyte cultures, TNFAIP-6 (tumor necrosis factor alpha induced protein-6) was expressed much more by damaged cells and a decrease in the level of IGFBP-5 (insulin like growth factor binding protein-5) was observed.[24] Especially TNFAIP-6 levels play an important role in explaining the mechanism of stromal thinning.[25]

Nitric oxide is a paracrine mediator which has many different functions in eyes.[11] Special conditions lead to produce the inducible isoform of nitric oxide synthase enzyme.[12] The cytotoxic effects of nitric oxide develops via peroxinitrit, which is formed by the reaction with superoxide anions.[26] Peroxinitrit reacts to the phenolic ring of tyrosine aminoacids transforming them to stable nitrotyrosine components.[27] Recent studies reported significant i-NOS reaction on the epithelium, stroma and endothelium and the strong similarity with nitrotyrosine staining.[28] Fuchs' distrophy and bullous keratopathy expresses i-NOS reaction as well, whereas the lack of nitrotyrosine staining indicates the insufficient degradation of free radicals and reactive oxygen products in keratoconus. We calculated the amount of nitric oxide by determining nitrit and nitrate levels in the aqueous fluid. Nitric oxide and nitrate levels of aqueous fluid were significantly lower compared to the control group, whereas no difference in the nitrit level was observed. The low levels of nitric oxide and stable end products can be explained by the fact of i-NOS stained areas in keratoconus. It could be more helpfull to determine nitrate and nitrit (end products of nitric oxide) levels in teardrop or nitrotyrosine (stable form of peroxinitrit) levels in the cornea. As →

a matter of fact, nitrotyrosine immunohistochemical staining in keratoconus cornea and similar located i-NOS reaction has been established in another study.[29] Nitric oxide levels in aqueous humor also rise because of uveitis and cataract, from which the former situation is a disorder of blood aqueous barrier.[30]

CONCLUSION

Consequently, significant epithelial apoptosis indicates that keratoconus is not an isolated stromal disease. The similarity of staining localizations of i-NOS and apoptotic cells suggested the possible role of nitric oxide, leading to oxidative stress and apoptosis in keratoconus cases. The levels of nitric oxide, nitrit and nitrate could be influenced by the age difference between study and control groups. As keratoconus is an ephitalial-stromal disease, nitric oxide levels in aqueous humor may not indicate any pathology. Determining nitric oxide level in the teardrop or displaying the nitrotyrosine immunohistochemical staining in tissue will supply this subject.

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