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Review

Role of biochemical factors in the pathogenesis of keratoconus

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Keratoconus (KC) is a corneal disease associated with structural abnormalities in the corneal epithelium, Bowman's layer and stroma and altered concentration of tear components. KC corneas show a different pattern of collagen lamellae than their normal counterparts. Also, a reduction of several collagen types in KC epithelium and stroma was observed. Altered expression and/or activity of lysyl oxidase, a critical enzyme of the biogenesis of connective tissue detected in KC corneas, may weaken covalent bonds between collagen and elastin fibrils, what may lead to biomechanical deterioration of the cornea. Increased activity of matrix metalloproteinases observed in KC may induce the degradation of the extracellular matrix causing damage to the cornea. Oxidative and nitrative stress play an important role in KC pathogenesis and KC corneas are characterized by the disturbed lipid peroxidation and nitric oxide pathways. Malfunctioning of these pathways may lead to accumulation of their toxic by-products inducing several detrimental effects, along with apoptosis of the corneal cells, which may result from the loss of β-actin or increased levels of cytokines, including interleukin-1 and -6. Change in the expression of genes associated with wound healing, including the nerve growth factor and the visual system homeobox 1, may contribute to increased susceptibility of KC corneas to injury. Consequently, biochemical changes may play an important role in KC pathophysiology and, therefore, can be considered in prevention, diagnosis, prognosis and in the therapy of this disease as well.

Key words: keratoconus, cornea, collagen, proteinases, proinflammatory markers, antioxidants

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INTRODUCTION

Keratoconus (KC) is a bilateral and usually asymmetrical ectatic eye disease, in which the cornea assumes a conical shape. Keratoconus typically occurs in teens and progress until the third or fourth decade of life. The signs and symptoms of KC vary depending on stage and severity of the disease. In the initial stage, the disorder has no symptoms, therefore the diagnosis is difficult and only specific tests, such as corneal topography, allow to identify the disease. With the disease progression, patients with KC decrease quality of vision resulting from development of irregular astigmatism and myopia (Rabinowitz, 1998; Romero-Jiménez *et al.,* 2010; Ahmadi Hosseini *et al.,* 2013).

Keratoconus is characterized by a central or paracentral stromal thinning, resulting in alteration in the corneal curvature (Rabinowitz, 1998). A decrease in keratocyte density, a reduction in the number of lamellae and a degradation of fibroblasts in KC corneal stroma were reported (Romero-Jiménez *et al.*, 2010). In addition, changes in the gross organization of the lamellae, and an uneven distribution of collagen fibrillar mass, especially around the apex of the cone, were observed in KC corneas (Meek *et al.,* 2005).

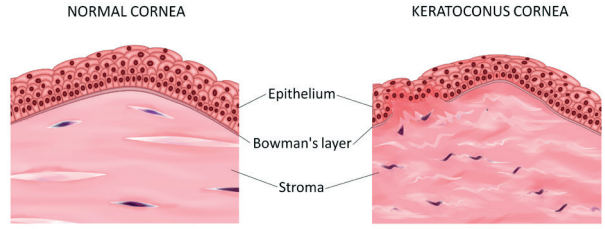

Figure 1. Epithelium and Bowman's layer from normal and keratoconus corneas, showing structural changes, including central epithelial thinning and breaks in Bowman membrane, typical for keratoconus.

Moreover, structural abnormalities were revealed in the central part of Bowman's layer, a condensed layer of collagen, in KC-affected corneas. Sharply edged defects and interruptions in Bowman's layer resulting from collagen bundles separation can be observed in this disease (Fig. 1). Breaks in Bowman's layer are usually filled with collagen derived from the stroma (Rabinowitz, 1998). Keratoconus is characterized by a thinning of the central part of epithelium. A positive correlation was reported between the occurrence of breaks in Bowman's layer and the extent of such thinning (Sherwin & Brookes, 2004). Keratoconus corneas show also a line partially or completely surrounding the cone, called Fleischer's ring (Romero-Jiménez *et al.,* 2010). Other KC typical signs are ruptures and folds in posterior limiting membrane, (Descemet's membrane), that may lead to acute stromal edema, sudden vision loss and pain (Sherwin & Brookes,

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Abbreviations: ALDH3, aldehyde dehydrogenase Class 3; ECM, extracellular matrix; HGF, hepatocyte growth factor; IL-1, interleukin-1; IL-6, interleukin-6; INPPL1, inositol polyphosphate phosphatase-like 1; KC, keratoconus; LOX, lysyl oxidase; MMP, matrix metalloproteinase; MMP-2, matrix metalloproteinase-2; NGF, nerve growth factor; PON1, paraoxonase 1; SNP, single nucleotide polymorphism; SOD1, superoxide dismutase 1; SOD3, extracellular superoxide dismutase; TIMP, tissue inhibitor of matrix metalloproteinases; TrkANGFR, NGF-receptor TrkA; VEGF, vascular endothelial growth factor; VSX1, visual system homeobox 1

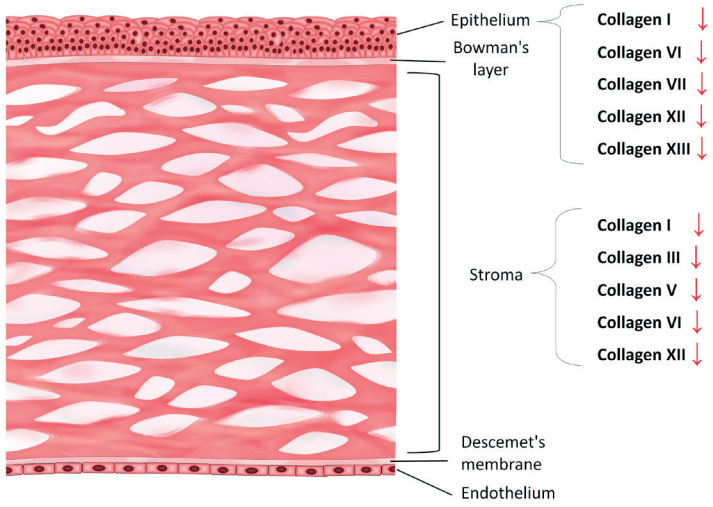

Figure 2. Reduction of collagens in layers of the keratoconus corneas.
Epithelium exhibits reduction in type I, VI, VII, XII and XIII collagens, while corneal stroma has decreased levels of type I, III, V, XII and VI collagens.

2004). Descemet's membrane is a thin cellular layer located between the corneal stroma and endothelium, which is rarely affected in KC, except for pleomorphism, intracellular dark structures and elongation of corneal cells (Sherwin & Brookes, 2004). Keratoconus is manifested by the occurrence of fine vertical lines in the deep stroma and Descemet's membrane, termed Vogt's striae (Rabinowitz, 1998). The disease is associated with superficial and deep corneal opacities that can occur in different stages of this disorder (Sherwin & Brookes, 2004).

Keratoconus is likely a multifactorial disease and several processes, also of biochemical nature, can contribute to its development (Kenney & Brown, 2003; Chaerkady *et al.*, 2013). However, biochemical aspects of KC are not known completely. The corneal thinning in KC is likely caused by the degradation of the extracellular matrix (ECM) components and loss of keratocytes, but the source of these changes has not been recognized yet.

COLLAGEN AND BETA-ACTIN

Results of several studies suggest that the corneal thinning, typical for KC, may be underlined by a decreased amount of total collagen, the main corneal protein, and an alteration in ECM structure in KC corneas (Quantock & Young, 2008; Stabuc-Silih *et al.*, 2009).

In the stroma, collagen is composed of heterotypic fibrils containing mainly collagen type I and V and smaller amounts of type VI, XII, XIII, XIV and XXIV (Michelacci, 2003; Meek, 2009). Type XII collagen was detected in the stroma in the area near to Bowman's and Descemet's membranes, whereas type XIII was found in the posterior stroma (Meek, 2009). Type XVIII collagen is localized in the corneal epithelium and epithelial basement membrane (Michelacci, 2003). The arrangement of the collagen lamellae in KC corneas differs from that in normal corneas (Radner *et al.*, 1998). Collagen fibrils in KC do not show delimited collagen lamellae, but form uniform layers and the interlacing between adjacent collagen layers are decreased or even absent. A disruption in the collagen organization in KC may result from disturbances in the process of formation of corneal extracellular components, resulting in their damage or structural changes in collagen fibers and collagen lamellae (Rabinowitz, 1998). Keratoconus epithelium displayed a reduction of collagen types VII and XII (COL7A1, COL12A1) as well as all three chains of type VI (COL6A1, COL6A2 and COL6A3), whereas KC stroma showed a decreased content of collagen type I, III, V, XII and VI (Cheng *et al.*, 2001; Chaerkady *et al.*, 2013) (Fig. 2). Results of immunological studies suggest an altered expression of collagen type XIII in KC (Määttä *et al.*, 2006). In addition, a decrease in the content of proteoglycans, including decorin, lumican, biglycan and keratocan was observed in KC. These proteoglycans interact with fibrillar collagens, making them biomechanically strong, refractive and transparent (Chaerkady *et al.*, 2013). Furthermore, a decreased concentration of transforming growth factor beta (TGF-β) was observed in KC. TGF-β can interact with several collagen types and proteoglycans. It is also involved in cell junction, facilitating contact between cells and ECM (Runager *et al.*, 2011; Chaerkady *et al.*, 2013). Collagen plays a crucial role in the maintenance of corneal shape and its transparency, therefore disturbance in collagen structure or arrangement may lead to a mechanical weakening of the cornea and vision deterioration (Chaerkady *et al.*, 2013).

Collagen cross-linking is a first-line treatment of KC patients, involving the formation of bonds between collagen molecules, fibres and microfibrils, which strengthen the cornea (Viswanathan & Males, 2012). In its basic form, collagen cross-linking involves activation of riboflavin by UV-A radiation and forming of new bonds between adjoining collagen strands in the corneal stroma. General success of this procedure confirms the importance of collagen content and distribution in the structural relationship in the cornea.

Several genes encoding collagen were considered as candidate genes in KC (Stabuc-Silih *et al.*, 2009; Karolak *et al.*, 2011, Nielsen *et al.*, 2013). The analysis of *COL4A3* and *COL4A4* genes in a Slovenian population showed that P141L, D326Y and G859G single nucleotide polymorphisms (SNPs) in *COL4A3*, P482S, M1237V, V1516V and F1644F in *COL4A4* had a different genotype distribution in KC patients and controls (Stabuc-Silih *et al.*, 2009). Screening of mutations in *COL4A1* and *COL4A2* genes in 15 Ecuadorian families with KC revealed numerous alterations in both genes, but none of variants was associated with familial KC (Karolak *et al.*, 2011). SNPs found near or within the *COL5A1* gene, which encode a component of type V collagen at the 9q34.2-3 region, were associated with corneal thinning in KC (Li *et al.*, 2013). Genome-wide studies identified a KC locus in the chromosomal region 3p14-q13. This region contains α1 chain of type VIII collagen gene (*COL8A1*). However, genetic analyses of *COL8A1* and *COL8A2* genes indicated no pathogenic mutations associated with KC (Aldave *et al.*, 2007; Nielsen *et al.*, 2013). A novel potential KC susceptibility region located at 2q21.3 was recently identified, suggesting the involvement of *RAB3GAP1* gene, encoding Rab3 GTPase-activating protein subunit 1 (Li *et al.,* 2012). Although several correlations between polymorphisms in collagen genes and KC were found, it seems that most abnormalities in corneal collagen structure observed in KC are not associated with variation in collagen genes and, therefore, probably other different genetic factors are involved in the degradation of ECM components.

Biomechanical properties of the cornea depend largely on the corneal collagen cross-linking (Dudakova *et al.*, 2012). Owing to the fact that lysyl oxidase (LOX), a critical enzyme in the biogenesis of connective tissue, catalyzes formation of bonds between collagen and elastin in ECM, *LOX* gene may be considered as a candidate for KC susceptibility. An increased LOX mRNA level observed in KC corneas compared to their age-matched controls confirms this assumption (Nielsen *et al.*, 2013). However, a lower LOX activity in tissue culture medium with KC corneal fibroblasts and a decreased LOX activity in all corneal layers, particularly in the stromal matrix were observed (Dudakova *et al.*, 2012). Analysis of SNPs in *LOX* gene showed an association between its variants and KC both in familial and sporadic cases (Bykhovskaya *et al.*, 2012). Change in *LOX* expression or activity of its product in corneal tissue may cause a decrease in cross-linking of collagen fibers, resulting in a biomechanical weakening of the cornea and contribute to KC development.

It was hypothesized that an increased apoptosis of keratocytes observed in KC corneal stroma might be caused by the loss of β-actin, a non-muscle cytoskeletal protein involved in cell structure, integrity and motility (Karakozova *et al.*, 2006). The KC stroma exhibited a significant reduction of the β-actin gene expression and a complete loss of this protein in KC corneas was observed (Joseph *et al.*, 2012; Srivastava *et al.*, 2006). Loss of β-actin may induce destabilization of the cytoskeleton of keratocytes and their apoptosis leading to a decrease in number of KC corneal cells, but exact effect of changes in β-actin levels is unclear and requires further studies (Joseph *et al.*, 2012).

PROTEOLYTIC ENZYMES AND THEIR INHIBITORS

Degradation of collagen and increased cell death in KC corneas may result from an increased level of proteolytic enzymes, inluding cathepsin-B, -G, -V/L2 and lysosomal enzymes (Fig. 3) (Chwa *et al.*, 2008, Arnal *et al.*, 2011).

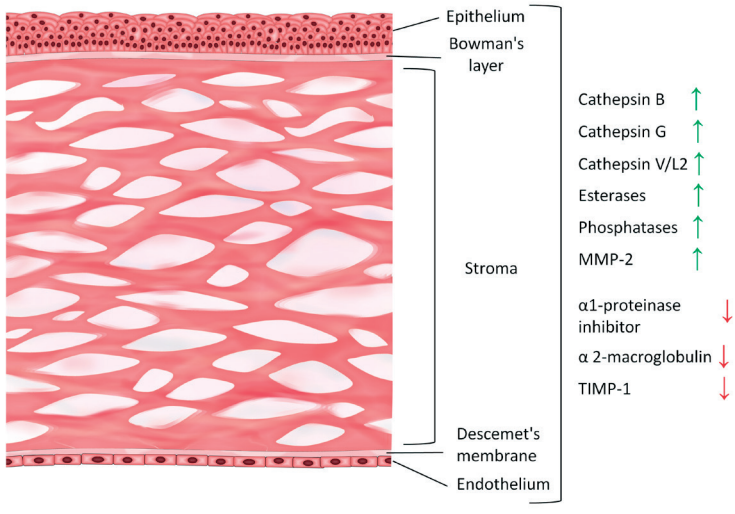

Figure 3. Changes in levels and/or activities of degradative enzymes and their inhibitors in the keratoconus corneas.
Increased levels of cathepsin-B, -G, and -V/L2, acid esterases, acid phosphatases, and decrease in α1-protease inhibitor, α2-macroglobulin and tissue inhibitors of matrix metalloproteinase (TIMP-1) can be observed in KC. In addition, matrix metalloproteinase-2 (MMP-2) activity is increased in KC corneas.

Cathepsins are proteases present in lysosomes that can activate caspases, the main apoptotic executors (Roberg *et al.*, 1999). Enhanced protease activity may contribute to ECM degradation and corneal thinning (Sherwin *et al.*, 2002, Kenney *et al.*, 2005). Moreover, an increased cathepsin activity may destabilize mitochondria and stimulate the formation of mitochondrial reactive oxygen species (ROS), contributing to oxidative stress (Zhao *et al.*, 2003, Kenney *et al.*, 2005). Increased levels of acid esterases, acid phosphatases, and acid lipases were also found in KC corneas (Critchfield *et al.*, 1988). Moreover, a decreased level of two major proteinase inhibitors in the plasma α1-protease inhibitor and α2-macroglobulin was observed in KC epithelium and stroma (Sawaguchi *et al.*, 1990, 1994). The α1-proteinase inhibitor blocks the activity of trypsin, chymotrypsin, elastase and plasmin, whereas α2-macroglobulin inhibits trypsin, chymotrypsin, papain, collagenase, elastase, thrombin, plasmin and kallikrein (Kenney & Brown, 2003).

Matrix metalloproteinase-2 (MMP-2) is the main corneal matrix metalloproteinase and its increased activity was observed in keratoconic corneas (Kao *et al.*, 1982, Brown *et al.*, 1993). However, more recent studies found no alteration in MMP-2 levels in KC (Zhou *et al.*, 1998, Kenney *et al.*, 2005). An imbalance between matrix metalloproteinases and their tissue inhibitors (TIMPs) in KC may influence corneal proteinase activity and may contribute to corneal thinning. A decreased level of TIMP-1 mRNA in KC corneas was observed (Kenney *et al.*, 2005, Kenney & Brown 2003). Keratocyte cultures from KC corneas had higher levels of MMP-2/TIMP-1 compared to cells of normal corneas (Kenney *et al.*, 1994, 2005). TIMP-1 has also anti-apoptotic properties, so keratocyte apoptosis in KC corneas may be associated with abnormal level of this inhibitor (Matthews *et al.*, 2007). Moreover, MMP-14, the membrane-type metalloproteinase that activates MMP-2, was reported to increase in KC corneas (Kenney & Brown 2003). A sequence analysis of *MMP-2*, *MMP-9* and *TIMP-1* genes found no mutation associated with KC (Brown *et al.*, 2004, Joseph *et al.*, 2011). Although increased level of cellular proteases and reduction of their inhibitors might cause the destruction of ECM in KC corneas, the roles of MMP-2 and TIMP-1 in KC are still controversial and require further studies.

CYTOKINES AND GROWTH FACTORS

Keratoconus corneas show a loss of keratocytes in the anterior corneal stroma following epithelial abrasion (Wilson *et al.*, 1996). Reduction of these cells is likely caused by an increased apoptotic cell death in KC corneas (Wilson *et al.*, 1992). An increased number of interleukin-1 (IL-1) receptors in KC keratocytes compared to their normal counterparts was demonstrated (Bereau *et al.*, 1993). IL-1 is produced by both corneal epithelial and endothelial cells (Wilson *et al.*, 1996). Results of an *in vitro* studies suggest that IL-1 induces keratocyte death and negative chemotaxis as well as upregulates hepatocyte and keratinocyte growth factor in corneal stromal fibroblasts (Wise *et al.*, 1994, Wilson *et al.*, 1996). IL-1 can also regulate the expression of metalloproteinases, collagenase and complement factors (Wilson *et al.*, 1996, Girard *et al.*,

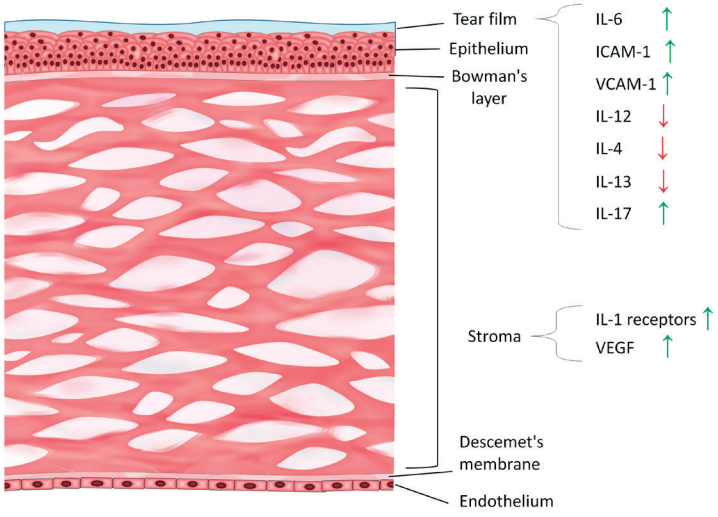

Figure 4. Altered levels of cytokines and growth factors in keratoconus. Keratoconus corneas have increased number of interleukin-1 (IL-1) receptors and enhanced level of vascular endothelial growth factor (VEGF), while keratoconic tear films show decreased levels of interleukin-12 (IL-12), interleukin-4 (IL-4), and interleukin-13 (IL-13), and increased levels of interleukin-6 (IL-6), ICAM-1, VCAM-1 and interleukin-17 (IL-17).

1991). Increased expression of IL-1 receptor in keratoconic corneal fibroblasts may trigger off a higher sensitivity of cells to IL-1 released from the epithelium and endothelium (Wilson *et al.*, 1996). Increased sensitivity of keratocytes to IL-1 may lead to their reduction due to apoptosis and a gradual loss of stromal mass (Wilson *et al.*, 1996). This hypothesis would explain an association between KC and eye rubbing, contact lens wear, and atopy. Chronic contact lenses wearing may cause an increased release of IL-1 in epithelium, facilitating a slow, progressive loss of corneal fibroblasts (Romero-Jiménez *et al.*, 2010). Eye rubbing and atopy may induce similar effect in KC cornea (Wilson *et al.*, 1996). Genetic analysis showed an association between SNPs in the promoter region of the interleukin-1 beta (*IL1B*) gene, a SNP in intron 6 of the IL-1 alpha (*IL1A*) and an increased susceptibility to KC in Korean patients (Kim *et al.*, (2008)). Moreover, a significant association between SNPs in the promoter region of the *IL1B* gene and KC in Japanese patients was shown (Mikami *et al.*, 2013). SNPs in IL-1 receptor antagonist (*IL1RN*) were observed more frequently in familial KC than in normal population (Nowak *et al.*, 2013). These results support hypothesis that IL-1 may play an important role in KC development.

The tear film in KC shows also increased levels of proinflammatory markers, including interleukin-6 (IL-6), ICAM-1 and VCAM-1 (Fig. 4). Eye rubbing in normal subjects leads to increased levels of IL-6 in tear fluids (Balasubramanian *et al.*, 2013). It was suggested that cytokines produced in the cornea or conjunctiva were accumulated and transferred by tears. Inflammatory molecules may induce apoptosis in keratocytes that is the major mode of cell death in KC corneas (Lema *et al.*, 2009). Persistent eye rubbing might induce increased levels and activity of corneal cytokines, which contribute to the development or progression of the disease (Balasubramanian *et al.*, 2013). However, cytokine level in the serum displayed no differences between KC patients and controls, supporting the hypothesis on the lack of association between KC and major systemic inflammation (Jun *et al.*, 2011).

A decreased level of IL-12, IL-4, and IL-13 was observed in keratoconic tear films (Jun *et al.*, (2011)). In contrary, an increase in IL-17 level was detected in some KC cases. IL-4 is involved in the regulation of cell proliferation and tissue homeostasis (Nelms *et al.*, 1999). It was found that IL-4 and IL-13 were involved in the synthesis of collagen type I and III in dermal fibroblasts and intrahepatic cells (Aoudjehane *et al.*, 2008). Reduction of IL-4 in the corneal environment may lead to a decreased survival of stromal keratocytes, induction of oxidative stress and reduced level of collagens observed in KC corneas (Jun *et al.*, 2011). Several results suggest that IL-17 is associated with the production of proteases associated with tissue degradation, so elevated level of IL-17 may induce tissue damage in KC (Cortez *et al.*, 2007; Qiu *et al.*, 2009; Jun *et al.*, 2011).

Cytokines play an important role in the regulation of many processes and an imbalance between pro-inflammatory and anti-inflammatory cytokines may induce cascades of intracellular signaling leading to changes in several cellular processes. Aberrations in levels of some interleukins observed in KC may stimulate an increased production of metalloproteinases, and other proteinases as well as enhance apoptosis in keratocytes, resulting in weakening of the corneal stroma.

Vascular endothelial growth factor (VEGF), which plays an important role in the pathogenesis of several diseases of the eye including age-related macular degeneration, was reported to overexpress in KC corneas and bullous keratophaty, but its expression decreased in diabetic corneas (Saghizadeh 2001). If even the increased level of VEGF in KC results from its involvement in the pathogenesis of the disease, this involvement may not be associated with basic VEGF functions, vasculogenesis and angiogenesis, due to the structure of the cornea supporting its transparent character.

As mentioned above, a reduced expression of transforming growth factor beta (TGF-β) was observed in KC (Saghizadeh *et al.,* 2001). A further work showed a potential usefulness of TGF-β as a marker in severe KC and an importance of TGF-β signaling in the pathophysiology of KC (Engler *et al.,* 2011). Some contradictory results were obtained in the analysis of variability in *TGF-β* genes in different populations – an association was reported in a Chinese subpopulation (Guan *et al.,* 2012), whereas a lack of it was reported in the American one (Udar *et al.,* 2004). Transforming growth factor beta 2 can be found in the aqueous humor, where it plays an important role in the immunological response in the anterior chamber of the eye, and also its increased level was reported in KC eyes (Maier *et al.,* 2007). This can have a prognostic significance for KC patients undergoing penetrating keratoplasty.

ANTIOXIDANTS

Results of some studies indicated aberrant structure and function of antioxidant enzymes in KC corneas. Screening of the superoxide dismutase 1 (*SOD1*) gene in 15 KC families revealed a 7-base deletion in the intron 2 in two KC families (Udar *et al.*, 2006). Moreover, mRNA analysis indicated the presence of two additional

transcript splice variants encoding proteins lacking the active site of the SOD1 enzyme. Superoxide dismutase is an important antioxidant enzyme that metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide (Valko *et al.*, 2006). There are three major types of this enzyme: SOD1 located in the cytoplasm, SOD2 in the mitochondria, and extracellular SOD3 (McCord & Fridovich, 1969; Weisiger & Fridovich, 1973; Marklund, 1982). A deletion in *SOD1* gene may influence structure of the enzyme and was reported to decrease its activity in familial KC corneas (Udar, 2006, 2009). However, later research did not detect changes in *SOD1* gene in both sporadic and familiar KC cases (Gajecka *et al.*, 2009; De Bonis *et al.*, 2011). Results of other studies indicated a decreased level of SOD3 activity in KC corneas (Behndig *et al.*, 2001). Despite the decrease in SOD3 activity in KC, the level of SOD3 mRNA was unaltered in this disease (Kenney *et al.*, 2005). It was shown that the synthesis of SOD3 is reduced by IL-1 in cultured KC stromal cells in contrast to normal keratocytes that showed an increase in SOD3 synthesis (Olofsson *et al.*, 2007). Therefore, the release of IL-1α in the KC corneas may lead to a reduction of SOD3 synthesis in the corneal stroma, which in turn contributes to an increased oxidative stress.

A reduced level of aldehyde dehydrogenase Class 3 (ALDH3) was detected in KC epithelial extracts (Gondhowiardjo *et al.*, 1993). This enzyme plays an important role in the neutralization of reactive aldehydes of the lipid peroxidation pathway (Bhuyan & Bhuyan, 1978; Sophos *et al.*, 2001). However, there was no change in ALDH3A1 RNA levels in KC corneas compared to normal corneas (Kenney *et al.*, 2005). The lack of correlation between mRNA levels of SOD3 and ALDH3A1 and their activities may result from post-translational modifications, changes in enzyme turnover rate or genetic polymorphisms.

A decreased paraoxonase 1 (PON1) activity was observed in KC patients compared to non-keratoconic subjects in a Malaysian population (Poh *et al.*, 2012). PON1 displays antioxidative properties that protect both low- and high-density lipoproteins against oxidation and reduce lipid peroxidation.

Keratoconus corneas were reported to have increased catalase mRNA levels, suggesting that they had elevated cincetrations of H_2O_2 (Kenney *et al.*, 2005; Bhuyan & Bhuyan, 1978; Valko *et al.*, 2006). Altered antioxidant enzymes activities may result in elevated levels of superoxide radicals, hydrogen peroxide, hydroxyl radicals and other ROS, that react with cellular and extracellular compounds including proteins, nucleic acids, and membrane phospholipids (Valko *et al.*, 2006).

Small molecular weight, non-enzymatic antioxidants, including reduced glutathione (GSH), ascorbic acid, cysteine, uric acid and tyrosine, also have a significant role in the regulation of cellular redox status and protection of cells against oxidative damage (Birben *et al.*, (2012)). Keratoconus corneas displayed a decreased glutathione content and total antioxidant capacity (Arnal *et al.*, 2011). Moreover, an increased concentration of tyrosine and uric and a decreased level of glutathione were observed in KC tear film, but the concentration of ascorbic acid did not differ from that observed in control tears (Saijyothi *et al.*, 2012). Alternation in levels of GSH suggests an increased levels of ROS and altered redox status. Glutathione is involved in the neutralization of ROS and maintains some antioxidants, including vitamins C and E, in their reduced forms (Gukasyan *et al.*, 2007). Significant lowering of the glutathione and total antioxidant capacity in KC corneas and tears indicate increased oxidative stress in this tissue that may induce oxidative damage to corneal components.

Reduced level of antioxidants in the cornea may cause the accumulation of cytotoxic products resulting in alteration of various corneal proteins involved in several pathological processes in KC corneas (Fig. 5). Increased levels of reactive aldehydes can disrupt the membranes of lysosomes resulting in the release of their content. In addition, ROS can contribute to altered protein functions leading to a cascade of events, including apoptosis, collagen resorption, tissue degradation, and corneal thinning (Kenney & Brown, 2003).

Changes in antioxidant status of keratoconus corneas support an important role of oxidative stress in the pathogenesis of this disease (Wojcik *et al.,* 2013).

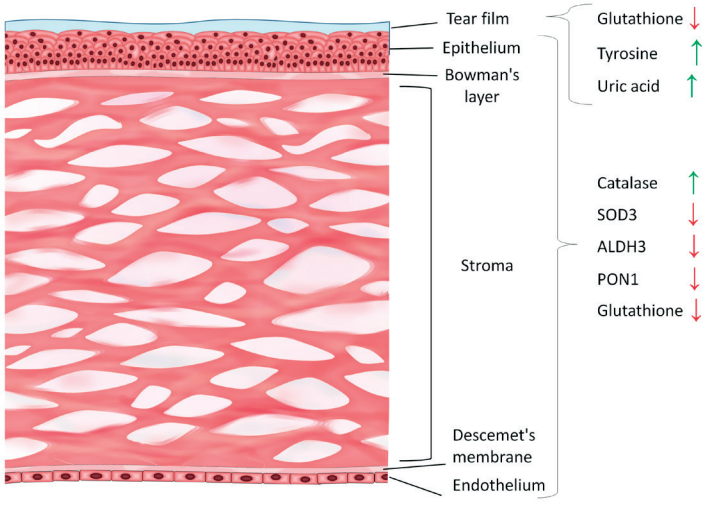

Figure 5. Change in levels and/or activities of antioxidants in keratoconus. Keratoconus corneas have decreased levels and/or activities of extracellular superoxide dismutase (SOD3), paraoxonase 1 (PON1), aldehyde dehydrogenase Class 3 (ALDH3) and glutathione, and increased levels of catalase. In addition, increased concentrations of tyrosine and uric acid along with a decrease in glutathione levels was observed in keratoconus tear film.

CORNEAL WOUND HEALING

It was suggested that KC corneas are more susceptible to injury due to disturbance in wound healing. A lack of NGF-receptor TrkA (TrkANGFR) expression and a significant decreased expression of nerve growth factor (NGF) and p75 neurotrophin receptor (p75NTR) was observed in KC (Lambiase *et al.*, 2005). NGF is an important molecule involved in trophism and wound healing of the cornea. Moreover, NGF influences *in vitro* corneal epithelial-cell proliferation and differentiation and promotes impairment of corneal sensitive nerves. The absence of TrkANGFR expression may be associated with an increase in the Sp3 short isoform(s) and a lack of the Sp3 long isoform. Imbalance between the Sp transcription-factor isoforms may play a role in the controlling of NGF signaling leading to a progression of the disease.

A positive correlation between mutations in the visual system homeobox 1 gene (*VSX1*) and KC occurrence was reported (Stabuc-Silih *et al.*, 2010). *VSX1* gene encodes a transcription factor, which is involved in craniofacial and ocular development (Semina *et al.*, 2000). It was shown that mutations in *VSX1* were associated with anomalous development of the corneal endothelium that may be involved KC pathogenesis. Moreover, a strong association between VSX1 expression and corneal wound healing was found (Barbaro *et al.*, 2006). Variation in *VSX1* gene may impact function of its protein and increase the susceptibility of KC corneas to injury (Dash *et al.*, 2010). It seems that VSX1 may play a role in several pathways of KC pathogenesis and further studies are needed to clarify it (Abu-Amero *et al.*, 2011).

The variability of regulatory RNAs may be important in KC pathogenesis as shown in the case of mutations in *miR-184* gene in familial KC (Hughes *et al.*, 2011). It is suggested that miR-184 plays a role in the repair of corneal injury through the regulation of the expression of the inositol polyphosphate phosphatase-like 1 gene (INPPL1). Deregulation of INPPL1 expression may increase susceptibility of corneal cells to apoptosis and this may underline the involvement of miR-184 in KC pathogenesis.

OTHERS FACTORS

As mentioned above, a correlation between SNPs located near the gene encoding a catalytic subunit of RAB-3GAP and KC susceptibility was shown (Li *et al.*, 2012). RAB3GAP activates the Rab3 GTPase that is involved in the regulation of calcium-mediated hormone and neurotransmitter exocytosis. Rab3 proteins are necessary for functioning and normal structural development of the eye. Alterations in the RAB3GAP may lead to a defect in the vesicular membrane transport. However, the precise explanation of how the loss of RAB3GAP function causes anomalies in the eye remains unknown (Li *et al.*, 2012; Wheeler *et al.*, 2012).

An association between mutations in *DOCK9* gene and familial KC was found (Burdon & Vincent, 2013). DOCK9 (dedicator of cytokinesis 9) activates the cell cycle regulator, CDC42, belonging to the Rho family of GTPases, but its exact role in KC pathogenesis needs explanation.

An association between KC occurrence and SNPs in the promoter region of the hepatocyte growth factor (HGF) gene was reported (Burdon *et al.*, 2011). Results of some studies detected a correlation between HGF and refractive errors, particularly with myopia and changes in HGF levels, may be linked with impairment of visual acuity in KC. In addition, *HGF* gene promoter contains binding sites for the proinflammatory cytokine IL-6, thus it appears that the influence of HGF on KC may be associated with inflammatory pathways (Burdon & Vincent, 2013; Burdon *et al.*, 2011).

CONCLUSIONS AND PERSPECTIVES

Biochemical processes underlying the development of keratoconus seem to play an important role in its pathogenesis. Corneal thinning, which is a clinical hallmark of KC, is associated with the destruction of ECM by increased activity of proteolytic enzymes, including metalloproteinases, and decreased level of their inhibitors. A concerted action of ECM-degrading enzymes and their regulators result in decreased levels of several collagen types and different pattern of collagen lamellae in KC epithelium and stroma. However, deregulation of metalloproteinases and their inhibitors as well as other proteinases, including cathepsins, in the KC cornea should be causatively associated with clinical signs of KC. Imbalance between oxidants and antioxidants, resulting in oxidative stress, a major KC pathogenesis factor, can be underlined by changes in biochemical properties of antioxidant enzymes, often resulted from mutations in their genes. Although we are far from complete understanding all biochemical processes in the cornea, it is clear that modulation of these processes can be important in KC pathogenesis. Therefore, these processes can be considered as diagnostic and prognostic indicators in KC and can be take into account as potential targets in KC therapy.

Biochemical factors are already considered to be important in KC diagnosis, that is supported by deposition of hemosiderin in Fleicher's ring, which appearance belongs to canonical criteria of the disease. This is an example of the contribution of biochemical factors to gross physical changes observed in KC by various imaging procedures, including keratometry, videocartography, the Orbscan II, the Pentacam and aberrometry. Several other biochemical factors are used to distinguish between normal and KC-affected eyes, but they are not directly measured in diagnostic routine. Instead, the consequence of changes of biochemical features typical for KC eyes are visualized. Therefore, better understanding of mechanisms leading to changes visualized with current diagnostic apparatus may lead to the extension of the range of observed changes, and may contribute to work out technologies resulting in new diagnostic tools with a higher resolution and allowing for an earlier detecting of the disease presentation as well as its more accurate prognosis.

Biochemical factors involved in KC pathology and pathogenesis may be exploited to modify properties of KC corneas. Corneal collagen crosslinking with UV and riboflavin is the most significant example supporting this thesis. This procedure is considered as the only one, which may directed the normal pathway of KC into less-devastating route. Although collagen-crosslinking was enthusiastically welcomed in ophthalmological society almost 20 years ago, there is still a lack of large population-based clinical trials on the effectiveness of this procedure (Vaziriani & Basu, 2013). Therefore, further studies on the role of collagen in KC pathogenesis may lead to modifications of collagen-crosslinking procedure, resulting in its better clinical suitability and wider application. This, in turn, may provide more reliable clinical data, contributing to further modification of the procedure.

Although keratoconus was firstly documented in the middle of the 19th century, our knowledge on molecular mechanism underlying pathogenesis of the disease is limited. Biochemical changes in the cornea associated with the course of the disease may be important for KC diagnosis, prognosis and therapy, and thus should be studied to obtain further clinically-relevant information.

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Conflict of interest statement

The authors do not declare any conflict of interest.

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