The role of tear pH values and Cu-cofactor of lysyl oxidase activity in the pathogenesis of keratoconus

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This article presents a detailed analysis of copper compounds migration in the corneal stroma. Biochemical conditions of the middle periphery of the cornea have been found to inhibit copper ions transition to the center part of the cornea in patients with keratoconus due to increased tear alkalinity. Low concentrations of dichlorocuprate (I) ion in the center of the cornea lead to inactivation of lysyl oxidase, an enzyme that catalyzes collagen cross-linking, and thus promote the genesis of keratoconus. An association of tear pH values and copper distribution in the cornea has been established, which offers new opportunities in pathogenic treatment of keratoconus.

Key words: keratoconus, tear, enzymes, lysyl oxidase, copper, chalcophile elements, microelements.

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There are different theories of keratoconus etiology and development. Lately the genetic etiological theory has been gaining weight. But despite considerable research, no complementary marker for the disease has been found yet. Many researchers note the change in activity of the regulatory enzymes, that cause both destruction and formation of the structural elements of corneal stroma.

Scanning electron microscopy of corneal stroma altered by keratoconus, shows the absence of cross-links in stroma collagen. Most researchers share the opinion that it is the principal morphological marker of tissue abnormality leading to gradual material yielding and subsequently to keratectasia.

Collagen cross-links are formed as a result of interaction of the newly formed tropocollagen produced by keratocytes into the intercellular space, and the Cu-dependent enzyme lysyl oxydase. Although the decrease in the enzyme's activity is indubitable, no certain genetic signs accountable for this decrease have been found yet.

The aim of this research was to conduct a clinical and experimental study of the tear pH level and investigate its role in the intrastromal copper ion migration as a defining factor of lysyl oxydase activity.

Materials and methods

Electron paramagnetic resonance spectra (EPR-spectroscopy) were collected from the tissue samples of 7 cadaver corneas showing no signs of keratoconus, and 8 corneal disks removed during the penetrating keratoplasty (PKP), 3 of which had a clinically perceptible Fleischer ring.

EPR-spectrometer Varian E-112 was used with the following parameters of spectra gathering: 20 mW, 9 Ghz, room temperature. Based upon these parameters, the resonance line was scaled according to the g-factor, which gave us the opportunity of using the spectrum as a characteristic one for revealing some of the compounds and establishing their chemical valence.

X-ray diffraction analysis was also conducted for 2 cadaver corneas and 2 corneal disk specimens removed during PKP for keratoconus. The X-ray Figure was received using an XRD analyzer DRON-3M. The elemental composition of the same tissue material was established using an X-ray fluorescence spectrometer "Respect" (XRF-analysis). It was determined separately for the central part of the cornea and for the Fleischer ring zone.

PH measurement of the basal tear in the rivus lacrimalis area was made with contact electrode (potentiometer "HANNA Instruments") for 33 patients (33 eyes) aged 15—34 with a confirmed diagnosis of stage II—IV keratoconus.

The same test was conducted in a group of 56 patients (56 eyes) aged 16—28 with clinically normal anatomical and biomechanical properties of the cornea. This group was divided into two subgroups based on patient's history of smoking. This criterion was chosen bearing in mind some literature data concerning a possible correlation of smoking and keratoconus.

Results and discussion

XRF analysis of the tissue samples derived from 7 cadaver corneas with no signs of keratoconus and keratoconus corneas removed during PKP, showed a well-marked difference in chalcophile elements content. The analysis showed the presence of small amount of copper and zinc ions in the stroma material of cadaver corneas with no signs of keratoconus, while the corneas removed during PKP performed for keratoconus revealed an accumulation of copper ions in the Fleischer ring zone (about 500—600 times the normal value) and at the same time, lack (at

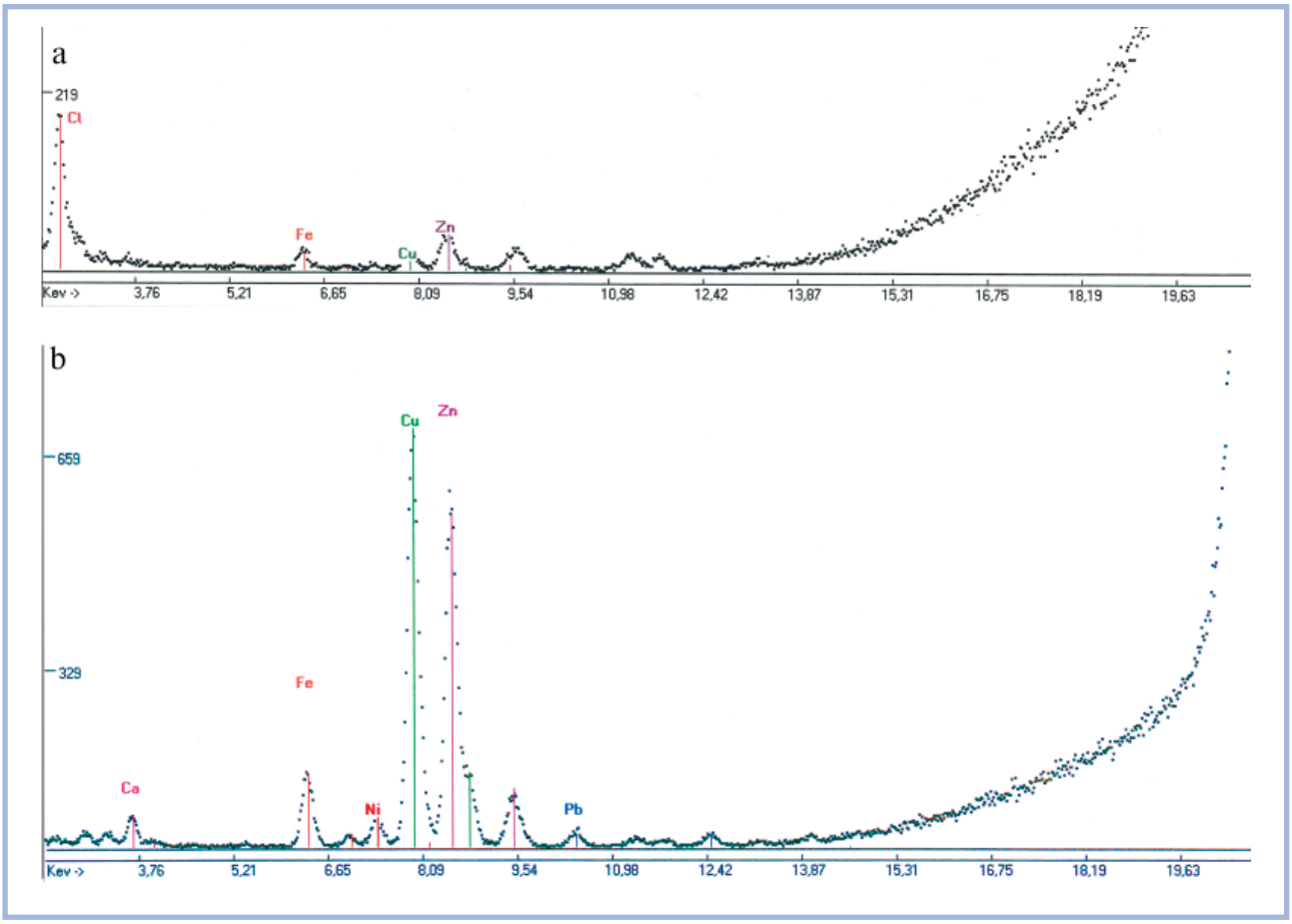

Fig. 1. **Typical X-ray fluorescence spectra of the corneal substance (abscissa axis — quantum re-emission energy; ordinate axis — fluorescence intensivity)**

a — typical XRF spectrum of a normal cornea; b — typical XRF spectrum of the Fleisher ring zone in a cornea removed during a keratoconus surgery.

the quantification level) of this microelement in the central part of the cornea.

All chalcophile elements and ferrum in patients with keratoconus displayed a similar pattern, accumulating in the Fleischer ring zone.

Typical XRF-spectra are shown in **Figure 1**. XRF-analysis data is presented in the table.

EPR-spectroscopy of the cornea material from the Fleischer ring zone made it possible to find a divalent form of copper **(Fig. 2)**, whereas the absence of peaks (dispersion halo) on the X-ray Figure indicated that this substance was in amorphous state **(Fig. 3)**.

Direct pH measurement of the basal tears showed unequal pH levels in different groups **(Fig. 4)**.

It was established that pH value of basic tears in keratoconus patients ranged 7,6-7,8; in the healthy non-smokers group — 7,2—7,6; in the smokers group — 7,0—7,2.

According to these results, the tear pH value may be considered as one of the possible factors influencing the pathogenesis of keratoconus.

Modern-day perception of physiological processes of the human cornea allows us to draw a following scheme of fluid and chemical compounds migration in the stroma.

Normal work of the corneal endothelium cells (CE) creates a water-content gradient in the protein rich matrix of the corneal stroma. The least hydrated area of the stroma is the posterior part adjoining the Descemet's membrane. Simultaneously with dehydration, resulting from the work of CE cells, an opposite process takes place owing to high hydrophilic property of collagen. Incessant redistribution of interstitial fluid from more hydrated areas leads to its constant replacement in the posterior part of the stroma.

Corneal stroma has a distinct anisotropic (stratified) structure; therefore, its resistance to physical water flow is measurably lower in the lateral direction, as compared to the transverse direction. This leads to the characteristic pattern of capillary and intrastromal fluid physical flow **(Fig. 5)**.

This implies that the peripheral part of the stroma only receives nutrition by means of fluid filtration from the lim-

Chemical composition of the cornea based on the XRF-analysis data (all values are presented in weight percent on a dry matter basis)

Cornea tissue sample from:		Fe, %wt	Cu, %wt	Ca, %wt	Zn, %wt	Ni, %wt	Pb, %wt
The Fleischer ring zone	max	0.0821	0.616	0.61	0.138	0.0012	0.005
	min	0.0303	0,551	0,40	0,047	n/a	n/a
Central part of a keratoconus cornea	max	0.0079	n/a	0,99	n/a	n/a	0.012
	min	0.0040	n/a	0,74	n/a	n/a	n/a
Whole normal cornea	max	0.0018	0.0011	n/a	0.023	n/a	n/a
	min	0.0009	0.0011	n/a	0.011	n/a	n/a

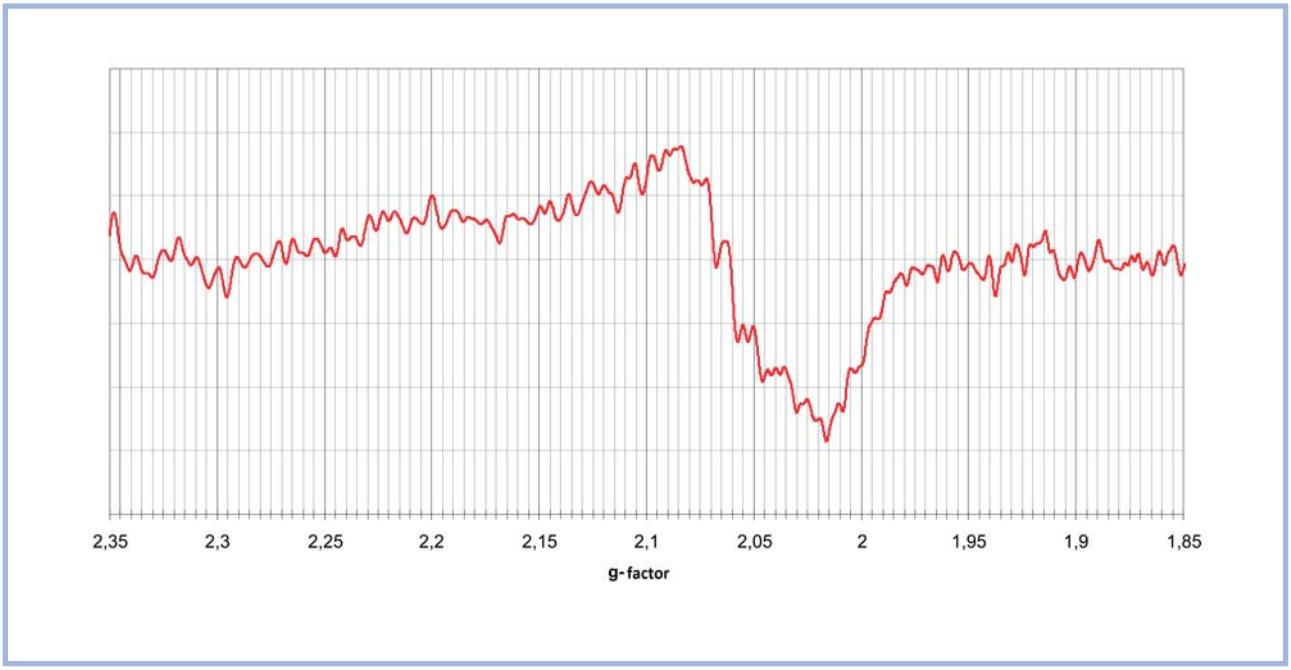

Fig. 2. **EPR-spectrum of the corneal disks removed during the penetrating keratoplasty.**
The characteristic asymmetrical spectrum profile matches that of Cu(II), c $g^{\perp}$ =2,065.

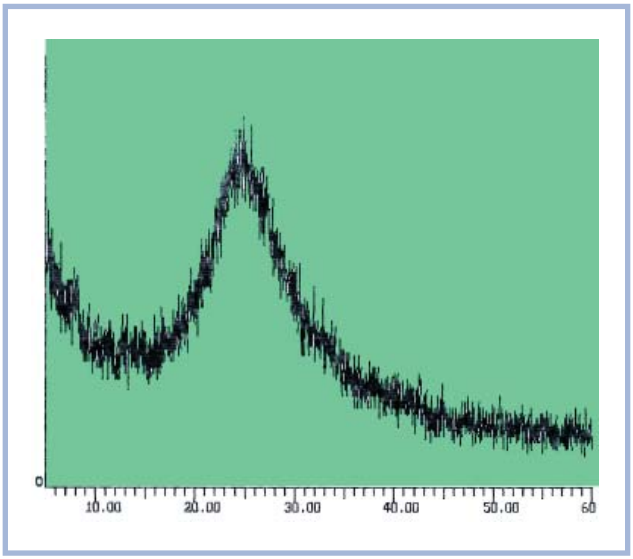

Fig. 3. **X-ray dispersion halo, characteristic of amorphous corneal matrix in the Fleischer ring zone (abscissa axis — angle of rotation of the sample, ordinate axis — intensity on the detector)**

bal region (from the periphery to the centre), whereas the central part is likely to have a substantial share of the tear components contribute to its nutrition. The penetration of the tear fluid into stroma is restricted by a tight Bowman's membrane, which is hardly penetrable for large organic molecules. Meanwhile, smaller ions penetrate it with sufficient ease, and can influence the pH value of the interstitial fluid in the central part of the cornea **(Fig. 5)**.

It should be noted, that the absolute values of H_2O mass transfer are lower than the unbound ions diffusion rate. Thereby the direction of physical water flow doesn't normally have a significant influence on the metal ions migration, with an exception of the cases when one of the further described physicochemical barriers is formed.

Copper ion migration in a healthy human cornea and in a cornea with keratoconus

According to direct measurements, the oxidation-reduction potential (Eh) of normal corneal stroma is near neutral (0V), which makes the stroma practically impenetrable for large albuminous compounds.

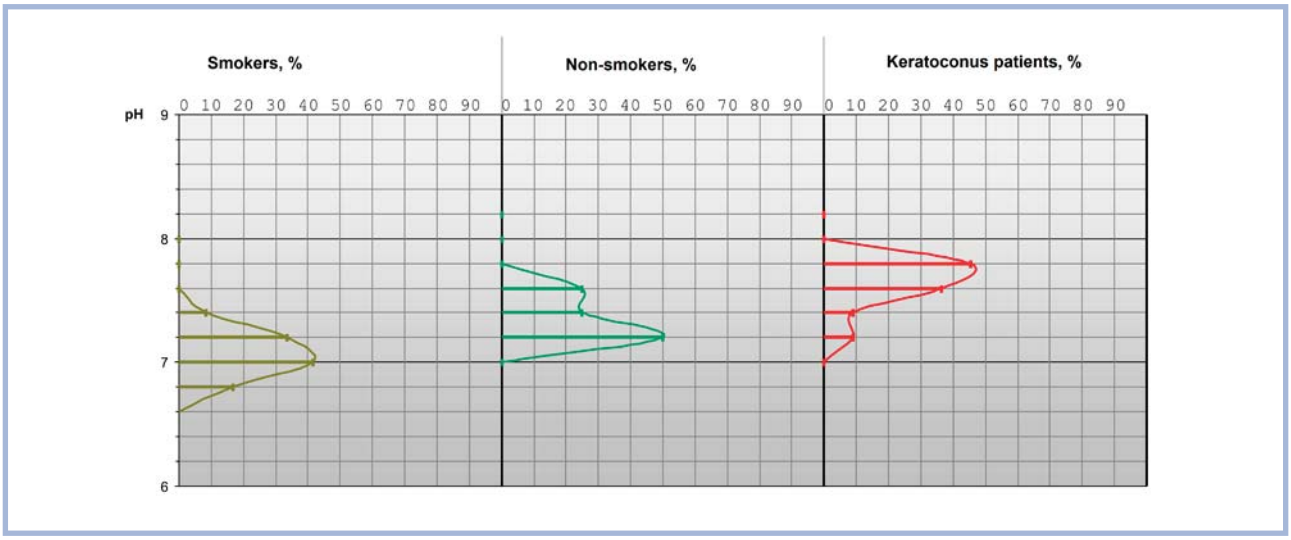

Fig 4. **Various tear pH prevalence in the following groups: healthy smokers, healthy non-smokers, keratoconus patients.**

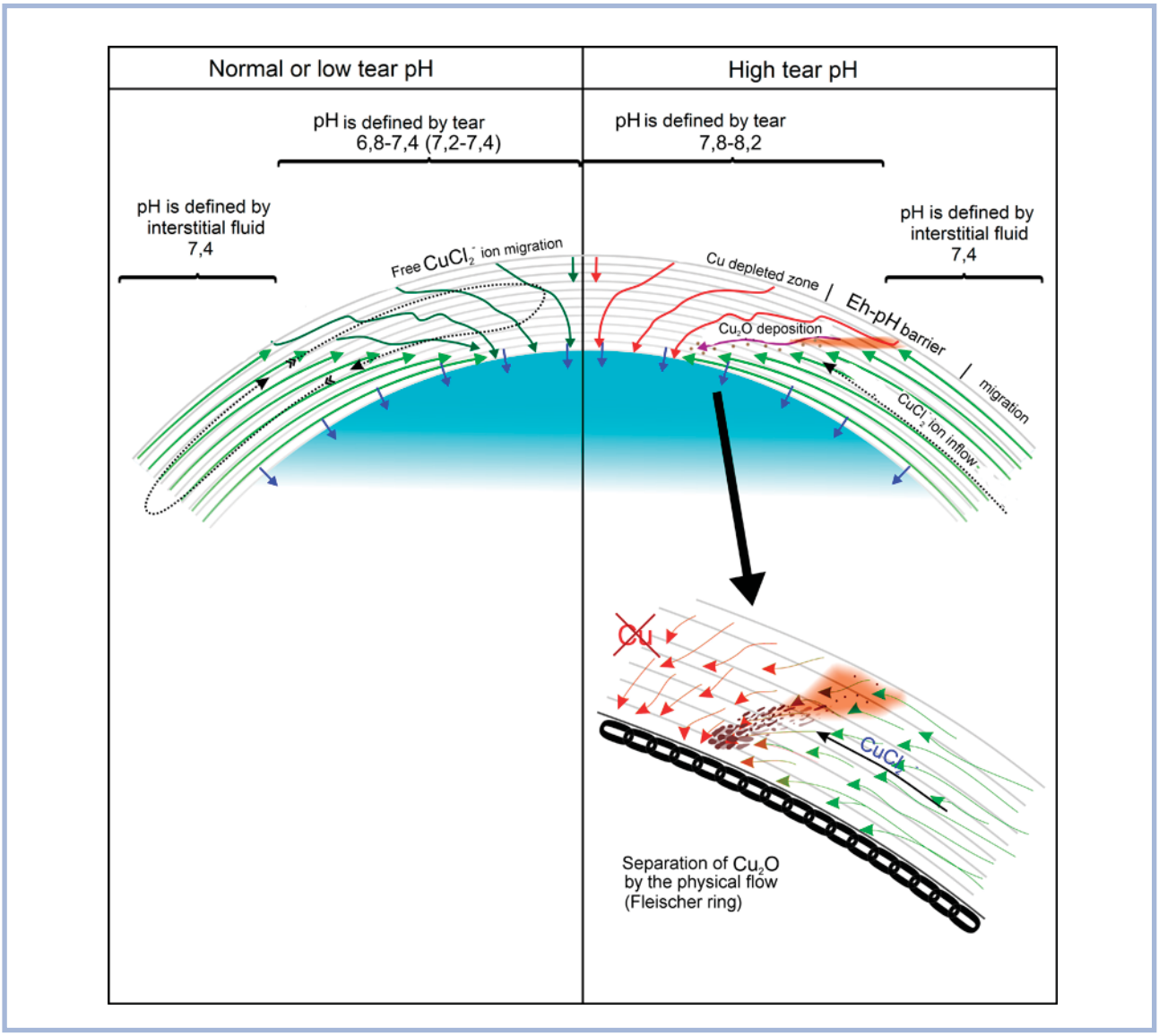

Fig. 5. **Migration of copper in the corneal stroma.**

Therefore, biochemical and physical conditions created in the stroma let us assume that most part of the copper passes into the cornea from the limbal region in the form of a mobile complex dichlorocuprate (I) ion [$CuCl_2$]. Physicochemical stability zone of copper in this form is shown on Eh-pH-diagram **(Fig. 6)**.

XRF analysis of a normal cornea **(Fig. 1)** reveals detectable amounts of chloride in association with chalcophile elements, which also supports the finding of copper in the aforementioned complex.

When copper gets from the limbal region into the stroma, its further migration to the central part of the cornea is regulated by the presence or absence of physicochemical Eh-pH-barriers **(Fig. 6)**.

As follows from the Eh-pH-diagram, copper migration capacity can be constrained by a high pH value of the tear fluid. The diagram shows real values of the basal tear pH level in the rivus lacrimalis area (according to the data of our own measurements).

When copper gets to the edge of the area, where pH values are determined by the high alkalinity of the tear, it forms an insoluble compound with oxygen (Cu_2O) and loses its ion diffusion mobility. Copper oxide segregates and, due to the physical flow of the intrastromal fluid, for some time continues its migration in the form of colloidal particles until its mobility is completely lost.

Terminating this process, Cu2O is unloaded in the circular zone near the Descemet's membrane where it partially changes into the divalent form (CuO), perceptible on the EPR-spectra **(Fig. 2)**.

In case of normal tear pH values, copper doesn't change its ionic form, therefore, its migrational capacity both from the periphery to the central part of the cornea and in the opposite direction doesn't decline. In this way, a

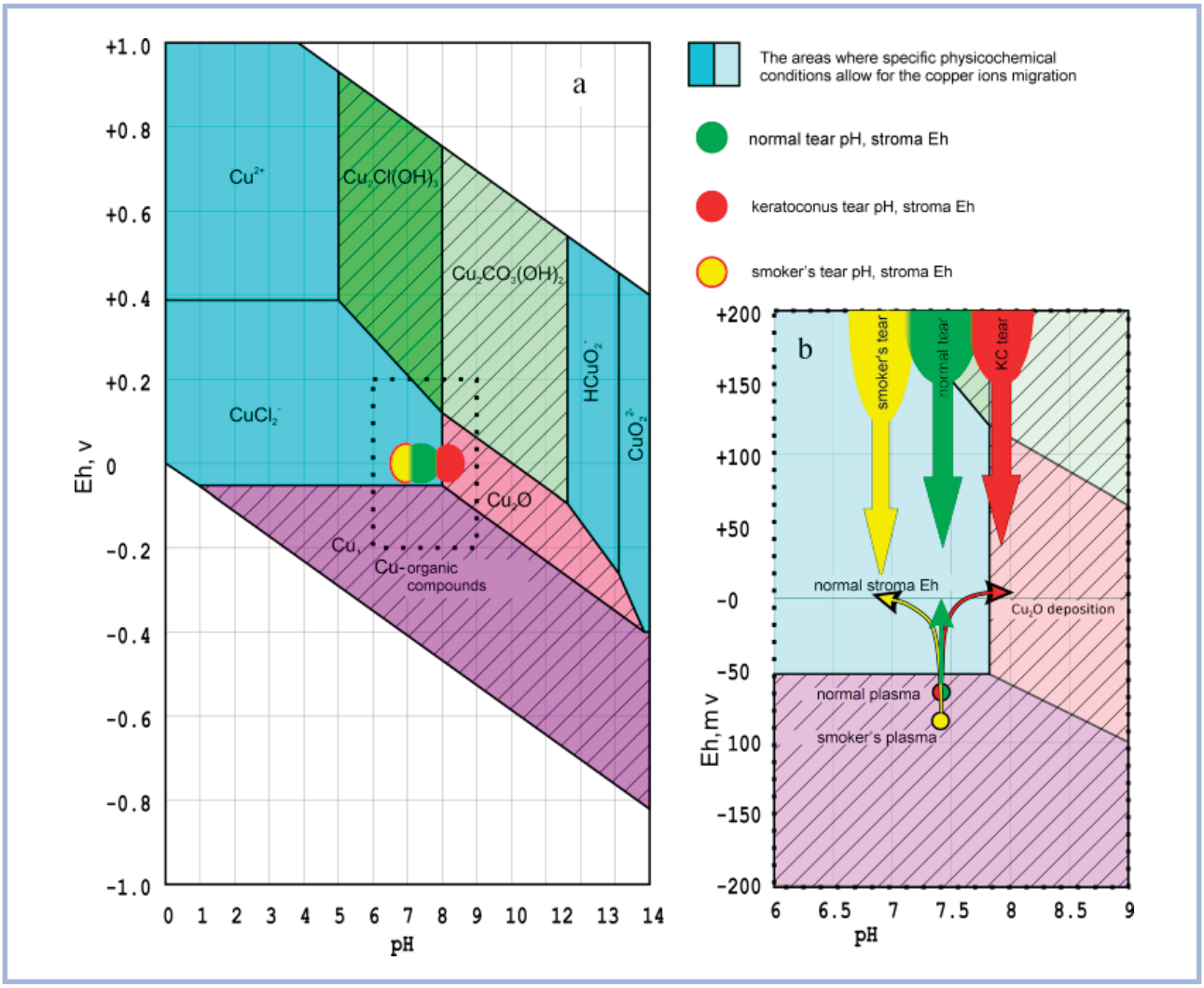

Fig. 6. **Copper compound stability in the system (NaCl, O_2, CO_2) under normal conditions (explanation in the text). Ion balance (CO_3^{2-}, HCO_3^-, H_2CO_3) was calculated in equilibrium with air [8].**

a — Biochemical stability fields for a full range of conditions; b — A detailed trend of modification of conditions for copper during its migration to the central region of the cornea.

healthy cornea maintains a constant concentration of the trace element.

In a healthy cornea the concentration of divalent copper lies below detection limit of EPR spectroscopy (~10^{-3} ppm). Cu (II) concentration is sufficient for attaining paramagnetic resonance spectra only in case of its pathologic accumulation due to constant influx from the limbal region and deposition on the physicochemical barrier in the Fleischer ring zone.

Copper ion depletion of the central part of the cornea leads to lysil oxydase inactivation, absence of cross-links in newly formed collagen and keratoconus genesis.

The role of copper in the formation of the Fleischer ring.

XRD study confirms that copper oxide precipitated in the Fleischer ring zone stays amorphous instead of recrystallization even in cases of long-standing keratoconus **(Fig. 3)**.

Amorphous copper oxide is an efficient sorbent for iron compounds, which accumulate in it and get oxidized to their Fe3+ form. The presence of iron oxides (hemosiderin) makes this zone visible being known as the Fleischer ring.

However, a mixture of oxidic and protoxidic copper compounds (Cu20 • CuO) form a separate reddish-brown pigment. Therefore, some of the clinically visible Fleischer rings are probably formed without iron compounds. In such cases the anterior part of the stroma in the Fleisher ring zone is the most distinctively colored one, because the pigment in that area hasn't undergone the final segregation yet and is still mostly in dissipated form.

Whenever the Fleischer ring is clearly visible in advanced stages of keratoconus, and when it cannot be visualized, EPR-spectroscopy still confirms the presence of divalent copper **(Fig. 2)**. This proves that accumulation of copper is primary to hemosiderin.

XRF analysis confirms the predominance of copper ions over iron compounds in the Fleisher ring zone **(Fig. 1**, **Table)**.

The role of external factors

Smoking is one example of external factors that can influence the tear pH value. Tobacco smoke lowers the tear pH, which in case of a regular exposure can increase copper ion mobility in the cornea. Thus smoking can have a possible inhibitory effect on keratoconus genesis, even in case of genetic predisposition to increased tear alkalinity. This explains, in particular, the paradoxical 95% negative association of keratoconus and smoking, noted by some researchers.

Formerly in medical literature keratoconus was described as a disease primarily found in the peoples of Mediterranean, the northern part of the Arabian Peninsula, south of Caspian Lowland, Crimea, Caucasus and Adriatic, although this fact might seem rather controversial, since there is no genetic affinity between many ethnic groups populating these lands.

In landscape classification, the territory that includes the Arabian Peninsula, north of Egypt, North and Western Caspian Sea region and Mediterranean, is known as the north arid zone. A unique feature of that region is a significant amount of carbonate dust present in the air, which forms an efficient pH-buffer, increasing tear alkalinity. This may present a plausible non-genetic explanation of high incidence of keratoconus in that specific region.

At present, a lot of external factors can contribute to the increase of tear pH value and potentially add to the keratoconus prevalence, such as industrialization, wide use of reinforced concrete constructions that leads to an alkaline dust content in the air, and almost complete lack of contact with high-ash fumes (wood fire, stove heating, thermal power stations) in our everyday life.

Conclusion

The results of this study show an insufficiency of copper ions in corneal stroma of keratoconus patients. This presents an indirect proof of the Cu-cofactor role in activating lysyl oxidase, an enzyme that is responcible for cross-linking formation of collagen in stroma.

High tear pH values that lead to ion diffusion and mobility restriction in stroma is the evident reason for copper deficiency in the central part of the cornea.

Inherent variability of tear pH values and in particular higher tear alkalinity associated with keratoconus may probably have genetic predisposition. But at the same time, tear pH can apparently be modified by external factors, which opens wide perspectives for developing new approaches to keratoconus prevention.