Acidity and buffering system of ocular surface (according to study of conjunctival sac)

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The conjunctiva, as well as any other mucous membrane, has its own specific pH value, required for maintaining normal functioning of the ocular surface. Measuring tear pH with a glass microelectrode has been the prevailing method used by researches to determine the tear acidity. This method does not rule out factors affecting on the values obtained, such as conjunctival sac zoning, conjunctival tissue acidity, conjunctival epithelium trauma and reflex tear secretion. There are only few studies of a mechanism maintaining the tear pH level in a relatively constant physiological range, in particular the bicarbonate buffering system. **Purpose** — to measure the ocular surface pH and analyze possible mechanisms maintaining its normal acidity, using the developed methods of pH measurement and microchemical analysis of the tear fluid. **Material and methods.** The tear pH measurements of 42 healthy subjects (84 eyes) were carried out using high–sensitivity litmus test strips with the computer color analysis; scanning electron microscopy and electron microprobe analysis with energy dispersive X–ray spectroscopy of 8 tear samples (8 eyes) were performed with the electron probe microanalyzer combined with the EDS detector. **Results**. The pH level among 42 healthy volunteers (84 eyes) varied from 6.30 to 7.23, mean pH value = 6.76; pH mode = 6.74. The mucous secretion was found in the tear samples of 25 involved healthy subjects (28 eyes): the mucous secretion pH level varied from 7.00 to 8.00, mean pH value = 7.26, pH mode = 7.30. Basic inorganic (mineral) components of the tear fluid were chlorine, sodium, potassium and boron. The borate buffering system was considered as a mechanism maintaining the ocular surface acid–base balance. **Conclusion**. The developed pH measurement method provides the reliable and accurate determination of the ocular surface acidity in accordance with the media zoning and heterogeneity, and complex structure of the tear film. In healthy population, the pH levels of the free tear fluid and those of the mucous secretion found in the conjunctival sac significantly differ. Inorganic (mineral) components of the tear fluid predominantly include water-soluble compounds of chlorine, sodium, potassium and boron. The borate buffering system seems to be the most stable constituent of the mechanism maintaining the ocular surface acid–base balance.

Keywords*: pH, acidity, tear, conjunctiva, buffering system, ocular surface.*

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The acid–base balance is a part of human homeostasis. Cells and tissues of the human body are susceptible to pH change: stability of molecular structures and enzyme activity can be maintained only in the tolerable range of pH. The maintenance of the acid–base balance is vital biochemical parameter required for normal physiology, and its change is associated with a wide variety of human diseases [1].

The ocular surface is a widespread medical term used for the group of anatomical structures (the cornea, the conjunctiva, the lacrimal apparatus and eyelids) interconnected as a single anatomo–functional system. The conjunctiva, as well as any other mucous membrane, has its own specific pH value required for maintaining normal functioning of the ocular surface.

According to published data, human tear pH varies from 6.5 to 7.8, and mean value received in some studies varies from 6.93 to 7.45 [2–7]. The ocular surface comfort zone of pH is relatively considered as a range of 6.6–7.8 [8]. The acidity overrange results in eye discomfort common for numerous eye diseases [6, 9, 10]. Physiological pH levels should be taken into account in order to avoid irritation, discomfort or abnormal changes of the ocular surface, when pharmaceuticals, contact lens solution and cosmetics are produced.

Acidity alterations were found in contact lenses wearers, in lacrimal stenosis, keratitis, keratoconjunctivitis sicca , and postoperatively after corneal transplantation [6, 9]. Taking into consideration pH changes seen in different eye diseases, acidity can be regarded as an associated clinical manifestation or a diagnostic criterion.

The tear pH is considered to be a component of keratoconus pathogenesis. Tentative data received during the pilot study, allows making a link between the tendency towards tear alkalization in keratoconic eyes and abnormal allocation of trace elements in keratoconic corneas. Thus, a fundamentally new pathogenetic scheme of keratoconus development was suggested [10].

Determination of the ocular surface pH with a glass microelectrode prevails in medical trials for measuring tear acidity [2–6]. The conjunctival sac contains 6–7 μl of fluid and the tear film thickness is about 6–12 μm – thus, it is really difficult to carry out pH measurement with a glass microelectrode in the immediate region of the tear film, as there is a high probability of a glass microelectrode application onto the conjunctiva. At that, the conjunctival epithelium pH is likely to be measured rather than pH of the free tear fluid.

There are few studies on the subject of the tear composition influence on the tear pH. The bicarbonate buffering system was supposed to be a mechanism maintaining the tear pH level in a relatively constant physiological range [5, 11].

Purpose of the investigation to measure the ocular surface pH and analyze possible mechanisms maintaining its normal acidity, using the developed methods of pH measurement and microchemical analysis of tear fluid.

Material and methods

The tear pH of 42 healthy subjects (84 eyes) was analyzed in 13 males and 29 females, aged 19 to 24. The mucous secretion (more viscous component of the tear) was found in tear samples of 25 healthy subjects (28 eyes; 8 males and 17 females, aged 20 to 24). The pH of the conjunctival fluid was measured in the inferior conjunctival fornix as previously described [12, 13, 14] with high–sensitive litmus test strips with a measuring range of 6.4–8.0 and interval 0.2 pH. The tear fluid was absorbed by one touch of a test strip in the inferior conjunctival fornix. The pH value was determined in 1–4 minutes after the tear sampling, when the test strip color was permanent according to verifying series of activated test strip pH measurements. The typical pH litmus test was supplemented with the computer color analysis to provide objective and reliable data. The computer program developed allows determining pH in several sufficiently colored areas of each test strip. The results were calibrated with buffer standard solutions pH 7.01 and 6.86; reproducibility of the results was ΔpH=0.024.

Crystallography (microchemical analysis) of 8 tear samples (8 eyes of 8 healthy volunteers, 3 males and 5 females, aged 21 to 28) was carried out to investigate possible mechanisms maintaining normal acidity of tear fluid. Samples were taken from the rivus lacrimalis with one touch of a micropipette to avoid the reflex tear secretion. Each sample of the tear fluid was dried out in the container on the surface of a carbon conductive tape under the dust–free conditions at 85% relative humidity at a temperature of 18ºC. Then tear samples were vacuumized and evaporated. Scanning electron microscopy and electron microprobe analysis with energy dispersive X–ray spectroscopy (EDS) was performed using electron probe microanalyzer JXA–8200 (JEOL Ltd., Japan) combined with the EDS detector. Secondary–electron (SE) and backscattered–electron (BSE) imaging, including topography mode (TOPO), were made with further trace elements micro–mapping.

Results and discussion

Ocular surface pH

Earlier studies of the physiological tear acidity showed significant scattering of the data [2–6], which suggests of some kind of systematic deviation in authors' methodologies, having affected received results in general.

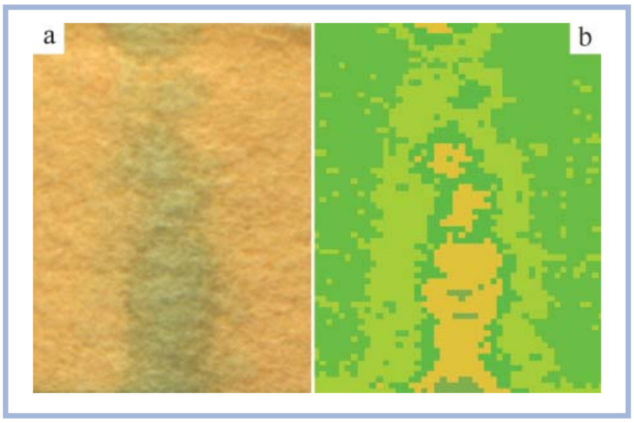

Fig. 1. **The litmus test strip with free tear sample.**

a —test strip, imbued with the tear fluid; b — generalized areas of digital analysis (pH = 6.82).

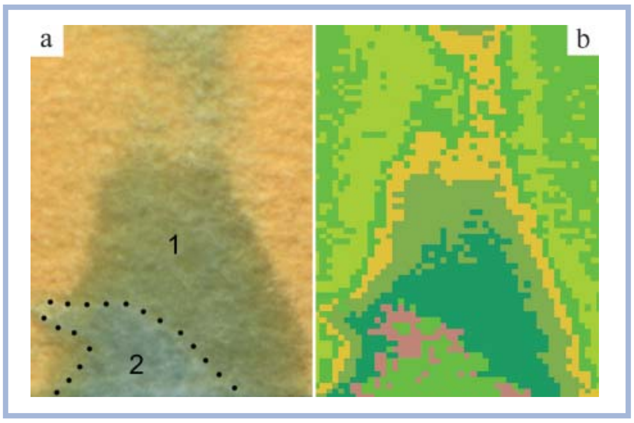

Fig. 2. **The litmus test strip of the sample, containing free tear fluid and mucous secretion.**

a — test strip, imbued with the free tear fluid (1) and the mucous secretion (2); b — generalized areas of digital analysis (the free tear fluid pH = 7.03, the mucous secretion pH = 7.28).

Unlike a glass microelectrode, the litmus test strips reveal the distribution of pH figures within the test strip, and not just peak pH recorded in the conjunctival sac. Furthermore, this method allows to differentiate acidity of free tear fluid (less viscous component of the tear) **(Fig. 1)** from the mucous secretion (more viscous component of the tear) **(Fig. 2)** contained in the inferior conjunctival fornix at the moment of sampling. The filter paper of the test strip is highly adsorptive, this minimizes the time of the sampling, as well as subsequent reflex tear secretion affecting received measurements. Analyzed material of the sample included only secretion found in the conjunctival sac, thus, the conjunctival tissue had no

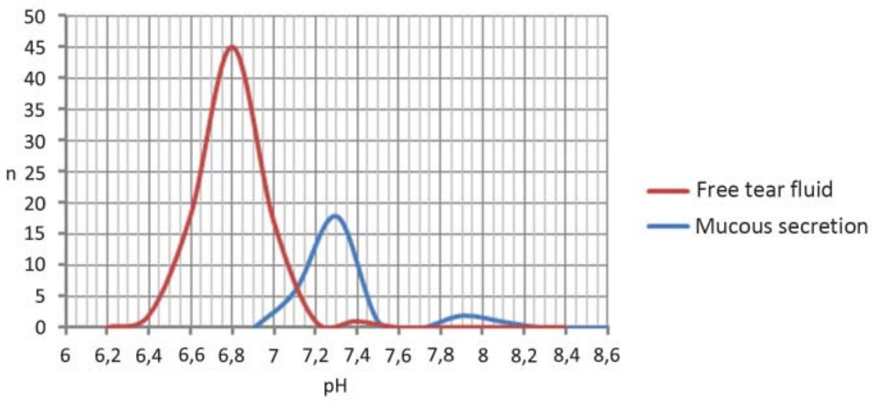

Fig. 3. **pH distribution of the free tear fluid and the mucous secretion.**

impact on the pH determination, in contrast to a glass microelectrode

This study had been primarily designed to obtain the peak pH, registered in the test strip, with no regard to heterogeneity of sample material. The acidity distribution was found to be polimodal (bimodal) while two pH values appeared most often (two modes): 6.74 and 7.30. These data coincide with results shown by other authors [2–7]. There is a possibility that some of investigators gained only one of "our" modes while the other mode was eliminated due to the chosen method of pH determination.

The second digital analysis revealed that samples with high pH levels were quite heterogeneous and contained not only free tear fluid but also mucous secretion found in the inferior conjunctival fornix. The mucous secretion had tendency to be considerably more alkaline, and the free tear fluid had acid reaction. The normal (Gaussian) distribution ($p \leqslant 0.05$) was observed when analyzing the pH of both tear components **(Fig. 3)**. Statistically confirmed significant difference between these two sets of data indicates the great discrepancy of pH between media and zones of the conjunctival sac.

In healthy volunteers the pH range was 6.30–7.23 (mean value – 6.76; mode – 6.74). There were no significant differences between pH obtained in the right (the primarily examined) and in the left (the secondarily tested) eye tear fluid samples. As mentioned above, during litmus test analysis the subgroup of patients was picked out, whose tear fluid samples were found to contain the mucous secretion. The mucous secretion pH level was 7.00–8.00 (mean value – 7.26; mode – 7.30). The pH difference between free tear fluid and mucous secretion was 0.12–1.10 (mean value – 0.51; mode – 0.43). There were no significant differences in age and gender between the whole patient population of this study and the subgroup of patients with the mucous secretion in their tear fluid samples.

The free tear fluid pH measurements determined in this study were similar to those indicating tear tendency to be more acid [6]. The mucous secretion pH values, obtained in this study, correspond to research showing the tear fluid to be more alkaline [2, 5] which can be probably explained by glass microelectrode intrusion into media lying deeper than the tear film, or by reflex tear secretion in response to mechanical stimulation.

On the assumption of the fact, that the intercellular pH is well known to resemble blood acidity equal to 7.28–7.42 [1], the conjunctiva is supposed to be more alkaline in contrast to superficial media. Thereby, the PH distribution in the conjunctiva sac depends on layerwise media zoning. It can be considered a predominant factor affecting the results of contact measuring.

In our opinion, in addition to layerwise media zoning of the conjunctival sac there are also several factors having effect on pH, such as reflex tear secretion and conjunctival epithelium trauma. Stimulation of tear secretion is known to lead to pH change [4]. We observed the reflex tear trend to be notably more alkaline in comparison with the basal tear (unpublished data). Actually, pH determination with a glass microelectrode takes several minutes. That does not rule out the possibility of reflex tear secretion, leading to systematic deviation in obtained values. Moreover, as a rule, the intercellular and intracellular pH values differ markedly from superficial pH of the organ or the tissue, in particular from their secretions [1]. In the process of pH measurement, the mechanical (by micro electrodes) or physical (by potential appearing on the micro electrode surface between the glass and the tested material) trauma of epithelium cells is highly probable, which also leads to unreliable data. In order to obtain the reliable results, it is necessary to avoid

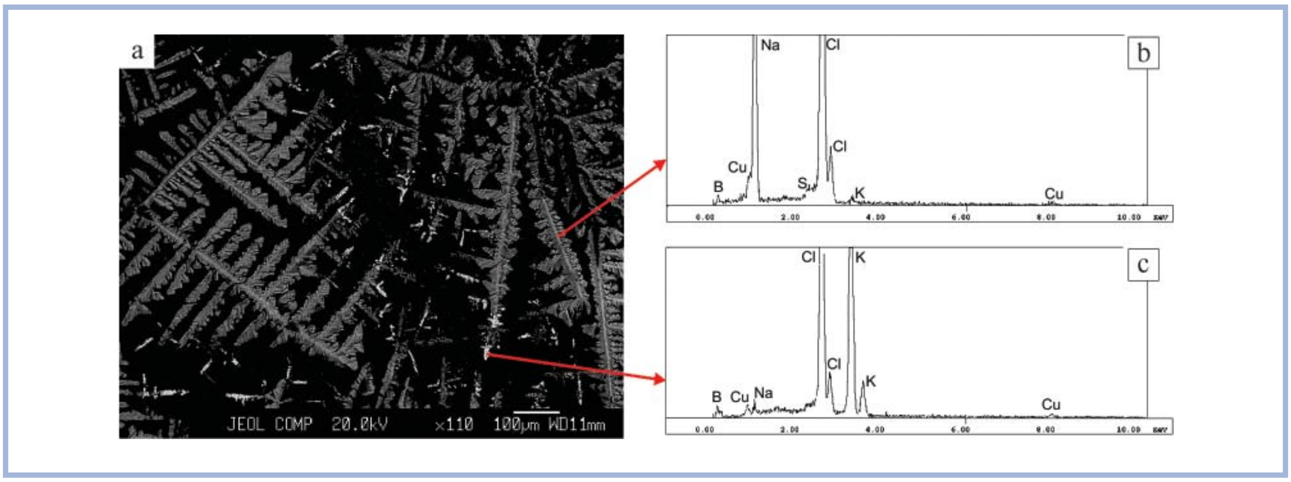

Fig. 4. **Scanning electron microscopy and electron microprobe analysis with energy dispersive X-ray spectroscopy of the evaporated tear fluid sample.**

a — backscattered-electron (BSE) image: NaCl grey crystals and KCl white crystals; b — energy dispersive X-ray spectrum of solid phase with predominant concentration of sodium chloride; c — energy dispersive X-ray spectrum of solid phase with predominant concentration of potassium chloride.

trauma or reflex stimulation of the conjunctiva during pH measurement. All the manipulations should be carried out within the tear film. The pH measurement technique, developed in this study, with litmus test strip and the computer color analysis, allowed minimizing the impact of reflex lacrimation.

Furthermore, in the process of determining acidity, other factors influencing the ocular surface pH, should be taken into account, such as the autonomic (vegetative) innervation and cyclical fluctuations. The tone of the autonomic (sympathetic and parasympathetic) nervous system has great effect on the tear secretion, determining its physicochemical properties [15]. Basal tear secretion was demonstrated to change its acidity during the day: pH levels in the morning and after the periods of prolonged eye closure were found to be more acid than those associated with the waking hours and in the evening [2, 5]. Probably there are some other cyclical changes, for example climatic, environmental or seasonal.

Tear fluid microchemical analysis

The trace elements micro—mapping of the evaporated tear samples revealed organic and inorganic (mineral) compounds of the tear fluid. Inorganic (mineral) components were composed of chlorine, sodium, potassium and boron; trace concentrations of copper, silicon, sulfur, carbon and oxygen were also found. Mineral components of evaporated tear samples included two solid phases: with predominant concentrations of potassium chloride or sodium chloride **(Fig. 4)**. Boron content in solid phase with predominant concentration of potassium chloride was equal to 7.9—26.5 at.% .Its content in solid phase with predominant concentration of sodium chloride varied from 0 to18.0 at.% (with an accuracy of semi-quantitative chemical analysis). Consideration must be given to the fact, that boron concentration can be overestimated in the presence of chlorine.

Some studies have indicated the bicarbonate buffering system as a physiological mechanism of keeping the acid—base balance [5, 11]. Carbonic acid H_2CO_3 acts as a proton H^+ donor while bicarbonate anion HCO_3^- serves as a proton H^+ acceptor (conjugate base):

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-.$$

In this study, crystallography (micro chemical analysis) found no sodium, potassium, calcium or magnesium carbonates or phosphates, which could function as a means of tear pH maintenance. At the same time, significant content of boron (up to 26.5 at.% in potassium chloride phase) suggests of its essential role in tear physiology. Notable boron association with potassium chloride evidences boron presence in the tear fluid as soluble form of boric acids which are orthoboric acid H_3BO_3 (or $B(OH)_3$) or tetraboric acid $H_2B_4O_7$.

Borates, including water dissolved forms, are mainly derivatives of tetraboric acid $H_2B_4O_7$ (i.e. dehydrated orthoboric acid H_3BO_3)[16]:

$$8\ H_3BO_3 \leftrightarrow 10\ H_2O + 2\ H_2B_4O_7$$

In buffering solutions tetraborates are present as a hydrated tetraborate anion $[B_4O_5(OH)_4]^{2-}$ [17]:

$$H_2B_4O_7 + 2\ H_2O \leftrightarrow 2\ H^+ + [B_4O_5(OH)_4]^{2-},$$

where hydrated tetraborate anion acts as a proton H^+ acceptor, and tetraboric acid serves as a proton H^+ donor.

The results received, as well as conclusions made in the study, correspond with theses of General chemistry. The acidity and consequently the pH of the bicarbonate buffering system remain constant only to a certain limit equal to 5.4—7.4 [18]. Therefore, the tear acid—base balance with pH above 7.4 can't be maintained with the bicarbonate buffer solution. However, the borate buffer based on hydrated tetraborate anion has pH = 7.6—9.2 [19].

Although bicarbonate anion HCO_3^- was authentically reported to take place in the tear fluid composition [20], its role in the ocular surface acid–base homeostasis [5,11] is still controversial. In fact, the bicarbonate buffering system represents the water solution of carbonic acid H_2CO_3 and bicarbonate anion HCO_3^- [18]. When pH rises with decreasing acidity and lowering proton H^+ concentration, carbonic acid H_2CO_3 dissociates to form proton H^+ ($H_2CO_3 \leftrightarrow H^+ + HCO_3$ thereby reducing pH to a normal acid–base level. When pH falls with increasing acidity and rising proton H^+ concentration, bicarbonate anion HCO_3^- associates with proton H^+ to form carbonic acid H_2CO_3 ($H^+ + HCO_3^- \leftrightarrow H_2CO_3$), thereby raising pH to a normal acid–base level.

During evaporation, water dissolved carbonic acid H_2CO_3 dissociates forming carbon dioxide and water,

$$H_2CO_3 \rightarrow CO_2\uparrow + H_2O,$$

and water dissolved bicarbonate anions HCO_3^- combined with alkali elements (sodium or potassium) crystallize:

$$HCO_3^- + Na^+ \rightarrow NaHCO_3\downarrow$$

$$HCO_3^- + K^+ \rightarrow NaHCO_3\downarrow$$

Thus, revealed by crystallography (microchemical analysis) absence of glacial bicarbonate salts in the evaporated tear samples is the inferential evidence that proves lack of bicarbonate anions HCO_3^- combined with alkali elements (sodium or potassium), which is the crucial component of the bicarbonate buffering system.

Despite the fact that the bicarbonate buffer was considered as pH maintaining means for ocular surface, its instability must be indicated due to its dependence upon the amount of carbon dioxide in the tear fluid with eyelid opened or closed. That causes a shift in carbon dioxide saturation and chemical equilibrium in the tear [5]. The ocular surface contacts with surrounding environment, and air–tear gas exchange depends on duration of sleep and wakeful periods, as well as eye blinking and air composition. Accordingly, carbon dioxide and bicarbonate anion HCO_3^- content is ever–changing in the tear fluid. This allows to make a reasonable assumption that there ought to be another more predictable, independent and all–sufficient way to keep the physiological pH.

Taking into account all the facts and observations presented in this article, the borate buffering system can be considered to be the most stable constituent of the mechanism maintaining the ocular surface acid–base balance.

However, in this study, boron content in the tear fluid was revealed indirectly by physical method of the research rather than direct chemical analysis. Thus, taking into account small quantity of the analyzed substance, boron presence in the tear cannot be considered fully proven. Therefore this subject requires further investigation by other methods of micro-chemical analysis.

Conclusion

The method developed for pH measurement allows the reliable and accurate determination of the ocular surface acidity in accordance with its media zoning and heterogeneity, and complex structure of the tear film.

In healthy population, the pH levels of free tear fluid and mucous secretion, found in the conjunctival sac, significantly differ.

Inorganic (mineral) components of tear fluid predominantly include water- soluble compounds of chlorine, sodium, potassium and boron.

The borate buffering system seems to be the most stable constituent of the mechanism, maintaining the ocular surface acid–base balance.

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