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SCIENTIFIC REVIEWS

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MODERN METHODS OF EXPERIMENTAL EVALUATION OF BIOMECHANICAL PROPERTIES

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The review presents a description of methods and results of various experimental studies of corneal biomechanical properties: the technique of tensile testing, the indentation method, and atomic force microscopy. Corneas of experimental animals and humans (in particular, donor eyes and material obtained as a result of keratoplasty) are considered as "sources" of samples. Selective evaluation of individual corneal structures using classical mechanical tensile tests is limited to a certain extent due to the rather small thickness of these structures and, as a consequence, difficulties in fixing the specimen. In real practice, it remains promising to use indentation and AFM, which are more adapted for such studies, on the one hand, eliminating the need for mechanical fixation of the specimen, and on the other hand, providing the possibility of studying various areas and surfaces of the latter.

Keywords: cornea, biomechanical properties, methods of experimental evaluation.

The encyclopaedic interpretation defines biomechanics as "a section of biophysics that studies mechanical properties of biological tissues, individual structures and organs on the basis of models and methods". From the point of view of the tasks to be solved, biomechanical research can be divided into fundamental and applied. In the first case it is a question of determining various indices characterising mechanical properties of tissues, and in the second case - in addition to that, about clinical significance of these properties in terms of potential influence on pathogenesis, methods of diagnostics and treatment of various diseases.

The cornea, being a part of the outer fibrous membrane of the eye, in addition to conducting and refracting light rays, provides the function of maintaining a certain shape of the eyeball, primarily related to biomechanical properties. Besides, it is the cornea that is the zone of "application" of the most widespread in clinical practice aplanation methods of intraocular pressure measurement and the determined indices can also depend on the "biomechanics" of the cornea. Thus, in relation to the cornea, the applied direction of biomechanical research is connected with solving the problems of diagnostics and monitoring of glaucoma, as well as pathological changes in its thickness and shape induced by diseases and surgical interventions [2-3, 5, 14, 15, 22, 26-27, 32, 37, 44].

Methods for assessing the "biomechanics" of the cornea are divided into clinical and experimental methods. Clinical or lifetime (i.e. in vivo) methods are based on the analysis of changes in the corneal shape as a result of some effect, for example, using a McLakov tonometer of different masses or an air jet (elastometry and bidirectional corneal pneumoaplanation, respectively). Experimental methods are based on mechanical tests of isolated corneal specimens (i.e., ex vivo) obtained from experimental animals, from human donor eyes, and as a result of any corneal surgery [74].

To characterise the biomechanical features of a material, such an index as Young's modulus (modulus of elasticity), which characterises the material's resis-

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tance to tension/compression (i.e. stiffness), is used. In this case, the basis of biomechanical tests is the assessment of induced and expressed in varying degrees of deformation of the sample. In modelling experiments, mechanical stretching of specimens up to the moment of rupture, indentation method, and atomic force microscopy are used [4, 8, 15, 52]. The choice of the method of mechanical testing depends significantly on the metric characteristics (in particular, the area and volume) of the samples, which are largely related to the technique of obtaining the latter.

The aim of this review is to analyses modern experimental methods for studying the biomechanical properties of the cornea.

Methods based on tensile stretching of samples. The most common method is uniaxial stretching of biomaterial samples prepared as membranes, films, strips [7, 12, 47, 49]. Corneal fragments in the form of strips can be obtained from its different layers and in different directions [50]. It should be noted that the so-called sample preparation of corneal samples is associated with certain difficulties in terms of achieving a uniform thickness/width of the strip throughout the sample, as well as the use of small sample sizes to reduce the influence of heterogeneity of biomechanical properties of the material on the final results of the study.

During the experiment a strip of material is fixed from both ends in special holders. When fixing the specimens in the holders, it is necessary to ensure simultaneous sTable fixation without damage that may cause the specimen to rupture in the area of the holders [49]. In some cases, holders designed specifically for a particular specimen are used, and in some cases, fixation of the specimen in the holder with adhesive is used.

Before stretching, the specimen is usually preloaded to the minimum detecTable force and the initial length of the specimen is set. In some cases, preload/unload cycles with a small tensile amplitude are used [25]. Pre-cycling of the specimen allows to obtain more reproducible results of mechanical properties measurements. The necessary number of pre-treatment cycles is selected based on the best match of stress-strain diagrams in subsequent cycles.

The specimen is stretched by moving one of the holders at a given speed with the help of both special installations [10, 49] and universal mechanical testing systems [48]. During testing, the movement of the holder and the tensile force are recorded, usually using special load cells. Tensile testing is carried out until the specimen is ruptured. The obtained dependences of relative elongation (ratio of elongation to the initial length of the specimen, in %) on stress (ratio of force to the initial cross-sectional area of the specimen, in kPa or MPa) are used to determine the modulus of elasticity (Young's modulus in tension), elongation at break (maximum elongation) and stress at break (maximum stress). The modulus of elasticity is determined by the slope of the linear stress-strain relationship. The modulus of elasticity characterises the stiffness of the material: the greater the slope of the linear section, the higher the modulus of elasticity and the stiffer the material is. The maximum elongation and stress characterise the strength and resistance to rupture of the material.

The biomechanical properties of the cornea of human and porcine eyes were compared by uniaxial stretching on an Instron device [9] [9]. Similar results were obtained for the modulus of elasticity (value for human and pig corneas 42.814 ± 11.674 and 39.261 ± 11.039 MPa, respectively), which allowed us to conclude that porcine corneas can be used as a substitute for human corneas in various experimental studies according to this criterion.

In keratoconus, a higher strain value (0.45 ± 0.05 N) at lower load (8.2 ± 1.5 N) was observed compared to the data of the control group (0.35 ± 0.03 N at a load of 17.9 ± 0.9 N), as well as a decrease in Young's modulus (156 ± 35 and 376 ± 38 MPa, respectively) [10]. Similar data were obtained in another study [36]. At the same time, when comparing the stiffness of conditionally normal corneal samples (donor eyes) and corneal samples with keratoconus, no significant difference was found [24].

It should be noted that the classical principle of uniaxial stretching (i.e., force application in one direction) does not exclude the possibility that the anisotropy of corneal biomechanical properties due to a certain orientation of stromal fibrils may influence the final result.

A variation of mechanical stretching is the impact on the specimen with the help of liquid (English "inflation test") with the subsequent registration of the dependence of the specimen deviation from the initial position on the level of hydrostatic pressure. The method is more complicated in terms of fixation of the specimen and registration of deformation and requires a strong and hermetic fixation of a disc-shaped corneal specimen around the perimeter. The cornea, thus, as a membrane divides two adjacent chambers, one of which is injected with liquid (physiological solution) to create hydrostatic pressure, simultaneously registering the degree of corneal deviation from the initial position depending on the level of fluid pressure. The deviation is registered using a video camera or more complex methods (laser sensors, special marker objects on the corneal surface). The calculation of the elastic modulus requires special models describing the mechanical behaviour of the membranes.

In a selective study of different layers of rabbit and human cornea by liquid pressure stretching, it was concluded that the stroma was less stretchable than the descemet membrane regardless of species [32]. Another study evaluated the "biomechanics" of animal (cows, pigs, rats) and human descemet membrane in comparison with the anterior capsule of the lens [17]. In human samples, the biomechanical properties of the descemet membrane and the lens capsule were similar in contrast to animal samples, in which the membrane appeared to be stiffer than the lens capsule.

Indentation method. Mechanical testing by means of micro- and nanoindentation is actively used to assess the stiffness of various polymeric materials and metals, and in recent years the method has been actively applied to biological materials, including soft tissues [31, 41, 42]. In the latter case, the characteristic values of deformation using this method are only tens to hundreds of micrometres, so the term "microindentation" is more reasonable. Measurements by the indentation method are carried out both on specialised instruments [31] and with the help of universal measuring systems [21]. The requirements to the sample in such experiments are minimal and include certain surface flatness, sufficient thickness for indentation (hundreds of micrometres) and the possibility of sTable fixation on the surface. When measuring in air, sandpaper is glued to the substrate to prevent sliding and lateral movement of the lower part of the sample on the surface of the substrate. When measuring in liquid, the sample is usually glued to the substrate.

In the process of indentation, the indenter is dipped into the sample surface in a controlled manner. The contact part of the indenter can be made in the form of a cylinder, cone, pyramid, but for the examination of soft samples in order to prevent damage, a sphere of micrometre or millimetre dimensions is more often used. The indenter is made of rigid material (metal, ruby) in order to prevent its deformation during indentation. The

indenter is structurally connected with a strain gauge measuring the force acting on the indenter, as well as with motors for moving the indenter relative to the sample. Vertical movement occurs during indentation, while lateral movement can be used for indentation at different points of the specimen, i.e. for mapping mechanical properties [21, 40].

As a rule, the investigation is started when the contact part of the device is located at a certain height above or in direct contact with the specimen, and indentation is carried out to a given depth or load. In the latter case, the indentation process is carried out at a constant speed of indenter movement until the moment of fixation of the set force by the strain gauge, after which the indenter starts the reverse movement. As a result, the dependence of the force (F) on the penetration depth (δ) is determined, and contact mechanics models are used to obtain the value of Young's modulus (Young's modulus at indentation). For example, for a spherical indenter, the force-indentation relationship is approximated by the following equation corresponding to the Hertz model [41]:

$$F = \frac{4}{3} f(\delta) \frac{E}{1-\nu^2} \delta^{\frac{3}{2}} \sqrt{R},$$

where E is the Young's modulus, v is the Poisson's ratio of the sample (assumed to be 0.5 for most biological samples), R is the indenter radius. For specimens whose thickness is comparable to the indentation depth, a correction for thickness is required in the form of the function f(δ) calculated for this case and known from the literature [18, 24].

The current development of the microindentation method is associated with the use of more complex models that describe, in addition to elastic, viscoelastic and nonlinear behavior of the sample [43]. To do this, a phase is added to the testing process that includes assessing force relaxation or creep by holding the indenter at a constant depth or at a constant force level, respectively.

Using nanoindentation, the biomechanical properties of 17 corneas with keratoconus and 10 conditionally healthy corneas unsuiTable for transplantation were assessed. Nanoindentation was carried out at a depth of 25 μm at a force application rate of 300 μN/min. As a result, a lower elastic modulus was found in keratoconus (23.2 ± 15.0 and 48.7 ± 20.5 kPa) [33].

In another experimental study, the method was used to evaluate changes in the “biomechanics” of the rabbit cornea as a result of corneal crosslinking: after removal of the central zone of the epithelium, the cornea was treated with the photosensitizer riboflavin and UV radiation was applied for 30 minutes at a power of 3 mW/cm2. As a result, a significant increase in corneal stiffness was noted: an increase in Young's modulus by 78.4-87.4%, ultimate stress by 69.7-106.0% and a decrease in ultimate strain by 0.57-78.4% within 8 months observations [53]. In a similar study, after cross-linking, for topographic assessment of changes in the “biomechanics” of the rabbit cornea, 5 indentation zones were identified at a distance of up to 1.5; 1.5 - 3.0; 3.0 - 4.5; 4.5 - 6.0 and 6.0 - 7.5 mm from the central zone of the sample. An increase in the elastic modulus was noted, more pronounced in the central zone [51].

Atomic force microscopy. An atomic force microscope (AFM) is a type of scanning probe microscope that has been widely used in the field of biological research [6]. The AFM imaging process is based on scanning the surface with a special probe called a cantilever. The cantilever is an elastic beam (cantilever) of micrometer dimensions, which at one end is fixed to a special base, and at the free end it is a pointed needle in the form of a pyramid, cone or microsphere interacting with the sample. The tip radius of standard cantilevers ranges from 1 to 100 nm, whereas a microsphere can have a radius of several micrometers. Cantilevers are made of silicon or silicon nitride, usually the beam has a rectangular shape with a length of 100-300, a thickness of 1-10 and a width of 10-50 microns. For optimal light reflection, the top side of the beam is additionally coated with a thin layer of metal (aluminum or gold), which is necessary for the operation of the optical system for recording the cantilever bend, which includes a laser beam directed at the beam and a photodetector consisting of several sections to detect the position of the reflected beam.

AFM operation is based on the force interaction between the cantilever tip and the sample surface. The force acting on the probe from the surface is controlled using the optical system for recording the cantilever bend described above. The signal from the photodetector, usually measured in units of voltage or current, is further converted by calibration into a cantilever deflection signal in nanometers and a force measured in pico- or nanoNewtons. Images are obtained through a process of line-by-line relative movement of the cantilever and the sample under study, called scanning. Individual lines (surface profiles) are added to an array and form the final image. To carry out the scanning process, a piezomotor (scanner) is used, most often made in the form of a tube made of piezoelectric material with electrodes applied to it. When voltage is applied, the piezo tube bends, contracts, or stretches, moving the cantilever relative to the sample with subnanometer precision.

The main mode intended for measuring the physical properties of a sample in AFM is the mode of recording force curves. This mode is very close to testing using nanoindentation. Force curves reflect the dependence of the bending value of the cantilever (the force of interaction between the probe and the surface) on the vertical displacement of the scanner as the cantilever approaches and moves away from the sample, that is, during indentation. However, in addition to indentation itself, local force interactions between the probe and the surface (attractive forces, adhesion) also occur. The process of taking force curves is also called force spectroscopy.

The force acting on the cantilever is calculated in accordance with Hooke's law:

F = kd, where k is the cantilever stiffness coefficient, and d is its vertical deviation from the equilibrium position.

Cantilever stiffness values are provided by the manufacturer within a certain range, including permissible deviations during production, and depend on their geometric parameters and the material from which they are made. To clarify the rigidity values, it is calibrated using the thermal noise method against the background of recording thermal vibrations of the cantilever, the resonance parameters of which depend on the rigidity.

The force curves are further processed using the Hertzian model (in the case of a microsphere as a probe) or other models of contact mechanics. The Young's modulus obtained using AFM may differ from the Young's modulus obtained using indentation methods. When using AFM, the characteristic indentation depths are tens to hundreds of nanometers, which corresponds to the surface layer of the sample. Surface tension (surface energy) can play a large role if present. Biomechanics studies are usually carried out by immersing samples in a layer of liquid (physiological buffer solution), or less often, on completely dried samples. In the first case, a certain influence of capillary forces on the results of mechanical measurements is possible. Drying samples, despite improving the quality of morphological assessment, can lead to a signifi-

cant change in the mechanical properties of biological samples, mainly in the form of an increase in stiffness.

In a number of cases, the AFM method was applied to the study of tissue sections, in particular, those obtained on a cryotome [45, 46]. In this case, the samples are usually not subjected to the stage of chemical fixation; they are quickly frozen in a special medium, after which sections with a thickness of about 10 μm are obtained, transferred to slides, and examined by AFM in liquid.

The AFM method makes it possible to map mechanical properties with high spatial resolution - using a sharp probe on soft tissue of the order of hundreds of nanometers. The disadvantages of the method include the "attachment" of the measured properties to the surface of the sample, and to study the properties of the internal layers it is necessary to perform sections.

It should be noted that AFM significantly expands the possibilities of assessing the "biomechanics" of various layers of the cornea, the objective difficulties of which are primarily associated with the small thickness of the samples. The main directions of research are related to the selective assessment of Young's modulus of different layers of the cornea in normal conditions and after cross-linking in keratoconus, possible changes in the indicator with intraoperative use of dyes, as well as comparison of the biomechanical properties of Descemet's membrane and the lens capsule.

Differences in the elastic modulus of the anterior basement membrane of the epithelium, Bowman's membrane and Descemet's membrane have been revealed [16, 20, 35, 36]. The results obtained in these studies can be presented in the form of the following main provisions:

• Young's modulus of the anterior corneal stroma (281 ± 214 kPa) is significantly higher than the posterior one (89.5 ± 46.1 kPa);

• the Young's modulus of the Bowman membrane is almost twice as high as that of the Descemet membrane (109.8 ± 13.2 and 50 ± 17.8 kPa, respectively);

• the least rigid structure of the cornea is the basement membrane of the epithelium (Young's modulus - 7.5 ± 4.2 kPa).

In a series of comparative studies, rabbit cornea was used as an experimental model for AFM [50]. The average values of elastic modulus for all layers of the cornea (epithelium, anterior and posterior stroma, Descemet's membrane and endothelium) were significantly lower compared to similar indicators for the human cornea. The revealed pattern must be taken into account when using the cornea of rabbits as an experimental model when studying various pathological processes of the cornea and developing modifications of keratoplasty. In an experiment on isolated pig eyes, AFM revealed a statistically significant increase in the average Young's modulus (i.e., an increase in stiffness) in the crosslinking zone [28].

Conclusion. Introduction of the method of bidirectional pneumoaplanation of the cornea into clinical practice has significantly expanded the possibilities of lifetime study of the biomechanics of the eye. At the same time, despite the zone of application of mechanical influence of pnevoaplanation methods (cornea!) and terminological presence of corneal component in the determined indices (corneal hysteresis, corneal resistance factor), taking into account the anatomical integrity of the fibrous membrane, the question of potential influence of biomechanical properties of the sclera on these indices remains open. Besides, the methodology does not allow estimating biomechanical features of different corneal layers, in particular, when studying the mechanism of ectatic corneal diseases and developing modern methods of selective keratoplasty.

As experimental methods of selective corneal biomechanical testing we should consider the technique of sample stretching, indentation method, and AFM, and as sources of samples - corneas of experimental animals and humans (in particular, donor eyes and material obtained as a result of keratoplasty). Selective evaluation of individual corneal structures (e.g., border membranes) using classical mechanical tensile tests is limited to a certain extent due to the rather small thickness of these structures and, as a consequence, difficulties in fixation of the specimen. In real practice, it remains promising to use more adapted for such studies indentation and AFM, on the one hand eliminating the need for mechanical fixation of the sample, and on the other - providing the possibility of studying different areas and surfaces of the latter.

Absolute values of Young's modulus as the main index characterising biomechanical properties of corneal specimens obtained in different experimental studies can significantly differ due to a number of reasons: technology of specimen obtaining and preparation, metric characteristics of the specimen and research algorithm. On this basis, it is relevant to conduct comparative studies involving the determination of relative indicators.

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