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Atomic Force Microscopy in Studying Regeneration of Tissues in Sclera Plasty in Ophthalmology

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Abstract—The atomic force method (AFM) was used to study the processes of repair in the sclera tissue within the grafting zone of a nanostructured biomaterial in a rabbit. The use of the nanostructured placenta has been shown to speed up the penetration of a graft into the sclera and to induce the formation of new connective-tissue structures. Differences have been revealed in the structure of mature and newly-formed collagen fibrils.

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INTRODUCTION

In case of progressive myopia scleroplasty is now performed by the principle of extrascleral enhancement of the outer coat of the eyeball (sclera), using for this purpose both biological material (sclera, meninx fibrosa, aorta, etc.) and artificial materials (collagen-impregnated polyester, silicon gel, etc.).

It is known [1] that sclera has a relatively low metabolic activity and weak regenerative capacities. Therefore, significant importance is now given to the development of methods able to activate the above processes and to allow us to increase the efficiency of scleroplastic surgeries in progressive myopia. Studies into regeneration of biomaterial grafts have shown that with reduced dispersion of a graft (within the micrometer range) the efficiency of its interaction with the recipient tissue increased [2]. Therefore, it is natural to expect that with a graft sizes developing to the nanometric range, biological effects may increase, in particular, acceleration of repair processes in the scleral connective-tissue structures.

Our experimental studies into the repair processes in the area of nanostructured alloplacenta grafting, using methods of standard optic microscopy showed penetration of the placenta nanoparticles into the recipient sclera [3]. However, due to the wavelength limitations, optic methods prevented us from watching the kinetics of the processes occurring as a result of nanosized particles grafting.

The ultrastructural study of the graft applied and the sclera connective-tissue structures after grafting should be of particular interest for understanding the regenerative mechanisms in the area of grafting of nanodispersed biological materials. It is traditional for studying the particulars of the scleral's collagen fibril structure to use transmission electron microscopy (TEM), which has a needed high resolution for observing the ultrastructural specificities of biological objects, in particular, fibrils [1, 4, 5]. However, the application of TEM requires a special prolonged preparation of biological samples (including, such as dehydration (drying), etching, impregnation, freezing, and covering with a conductive layer) when their uncontrolled alterations may take place.

Scanning probe microscope methods, in particular, atomic force microscopy (AFM), allow us to analyze samples of nanosized biological nonconductive materials in their initial state. In particular, this method was used for studying fibrillar [6–10] and subfibrillar [11] structures in air and liquids.

This work was aimed to clarify the mechanisms of interaction between the particles of the grafted nanostructured alloplacenta and the connective-tissue structures of the fibrous coat of the recipient eyeball.

MATERIALS AND METHODS

Our experimental studies were performed on sexually-mature chinchilla rabbits aged 1 to 2 years weighing from 3 to 3.5 kg. The animals were grafted with a biological container capsule sized $(10.0 \pm 1.0) \times (2.0 \pm 0.5)$ mm into their episcleral space under local anesthesia. The container was a segment of a vein of a human umbilical

cord, which was filled with a mechanically activated nanodispersed alloplacenta [12]. Optical tissues of the animals, destroyed in conformity with the Rules for Conducting Works with the Use of Test Animals, were morphologically investigated in 3, 10, and 20 days, and 1, 2, and 3 months after the grafting. The tissues were fixed in a 1%-neutral formalin solution and imbedded in paraffin. The obtained sections of the surgical intervention area were placed on the surface of a slide with subsequent chemical removal of paraffin.

The powdered human placenta was fixed to a polystyrol film obtained by evaporating ethyl acetate from a polystyrol ethyl acetate solution. The film was coated on cytall with subsequent fixation of the powder under UV radiation.

The structure of the powdered human placenta and the surface of histological sections of the surgical area were investigated, using the Ntegra scanning probe laboratory (NT-MDT) and SolverPro (NT-MDT) scanning probe microscope under the semicontact mode in air. We used polysilicon cantilevers HA_NC with resonance frequency 200 kHz, which had a 10-nm radius of rounding and a high aspect ratio. All tests were conducted on the sites marked in the figure with arrows (Fig. 1).

The diameter of collagen structures and the period of their striation (D is the size) were evaluated through analyzing the profiles transversally and along the axis of fibrils on the AFM-images, which were pretreated by the nonlinear filter of high frequencies using the Image Analysis 3 (NT-MDT) software. The height of a step between the gap and the overlap zone of individual fibrils was evaluated analogously.

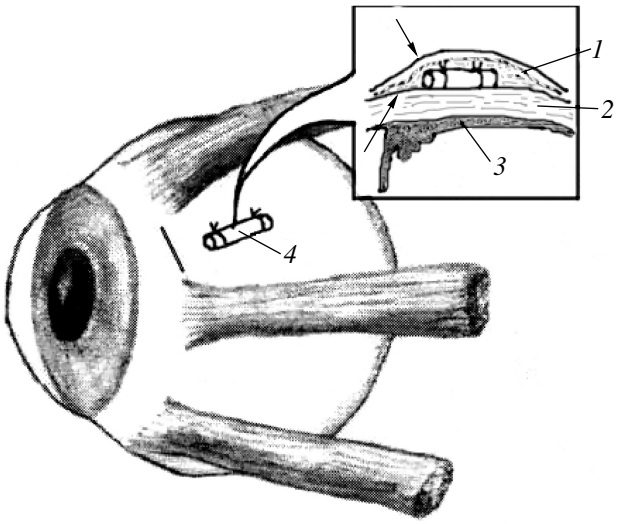

Fig. 1. The eye scheme: *1*—episcleral coat; *2*—recipient sclera; *3*—vascular coat; *4*—biological container with nanostructured alloplacenta. The AFM-investigated sites are indicated by arrows.

RESULTS AND DISCUSSION

The results of studying the mechanically-activated human placenta under the atomic force microscope method showed that after powdering by a ball planetary mill the placenta is transformed into a nanostructured state with granule sizes of 40–100 nm, which form particles, sized 200–500 nm (Fig. 2a). The particles, in turn, are loosely structured as agglomerates sized 2–10 μm (Fig. 2b). Such a strict hierarchy is characteristic of a broad spectrum of materials obtained through mechanoactivation—from organic and inorganic dielectrics to metalceramics [13]. The density of agglomerates in the powdered nanostructured placenta is very low, since the particles they form are only loosely adhered, and they are being destroyed to individual particles by the cantilever-attached needle already under scanning. The granules' characteristic sizes of 40–100 nm are especially well-observed on the section profiles of particles and aggregates (Fig. 2b).

The studies of histological sections in the area of grafting allowed revealing the alterations happening to collagen fibrils in the superficial scleral structures. It was revealed that already in early periods of the experiment (3 days) we can observe a release of nanodispersed material beyond the biocontainer (Fig. 3a). The released nanostructured placenta looked like small-dispersed aggregates, in which the nanosized structure of the placenta's mechanically activated powder was preserved (Fig. 3). Simultaneously, we observed defibrillation of collagen fibrils in the adjacent site of the sclera (Fig. 4a). We can only suggest that this is a result of the effect of the nanodispersed placenta on the connective-tissue structure of the recipient sclera, since we have not recorded such alterations neither on the control sample, nor under earlier studies of sclera tissues [1, 4, and 6]. Later on (20 days) we observed interaction of the nanodispersed biological material with the recipient tissues outside the biocontainer, not only within the area of grafting, but also beyond this area (Fig. 3b).

Within the further periods (2 months) we recorded resorption of the biocontainer's vascular wall. We also recorded the formation of newly-developed connective tissue (Fig. 4b) on the scleral surface within the surgery area, which was close by its density to the intact sclera that was distant from the area of grafting. The sclera's locations with newly-formed connective tissue were investigated under the phase contrast mode, under which the image is formed on the basis of recording the cantilever's phase shift in its interaction with the surface under scanning and this image reflects both topographic specificities and the alterations in the elasticity characteristics of the investigated sample's surface. Fig. 5 gives AFM-images of the same site of the sclera

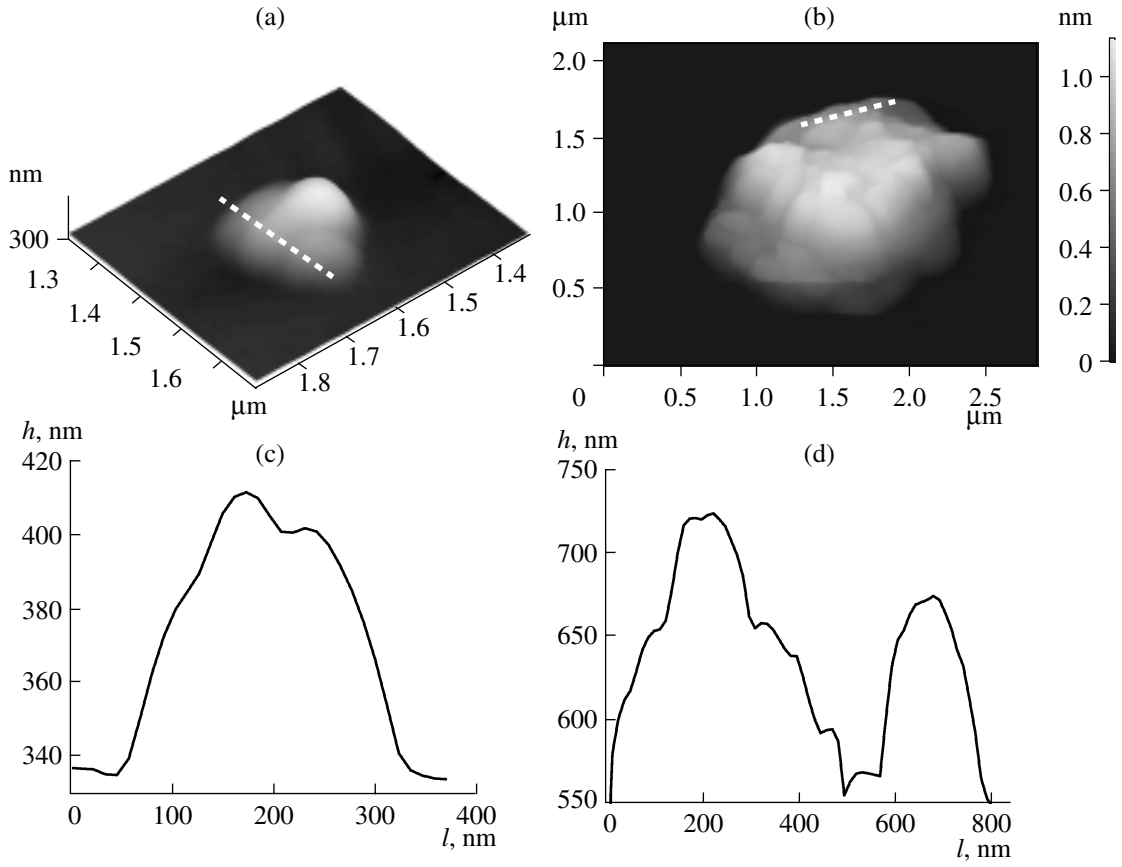

Fig. 2. AFM-images of the particle (a) and the aggregate of particles of mechanically activated human placenta (b); c, d—section profiles along the line a and b.

(the topographic one and under the phase contrast mode). The characteristic structure of collagen fibrils (cross striation) can be seen in both images. However, the image resulted from the phase contrast mode is sensitive to the elastic properties of the surface under study and allows us to reveal differences in hardness between individual collagen fibrils. The dark regions with the well-resolved cross striation are in correspondence with the harder mature collagen fibrils. At the same time, sites with newly-formed fibrils with lesser density have a weakly-resolved cross striation. And the cross-striation periods for mature and newly-formed collagen fibrils, which were defined on AFM-images using Fourier's analysis (Image Analysis 3, NT-MDT) of spectral frequencies, practically do not differ—69.4 nm for mature collagen fibrils and 69.7 nm for newly-formed collagen fibrils. The obtained D-size values agree with the data by AFM [6, 8, and 14] and transmission electron microscopy [4, 5, and 6] for dehydrated collagen fibrils. The height of a step between a gap and an overlap zone for mature fibrils is 28–29 nm, whereas this value for newly-formed collagen fibrils is constant and varies within a broad range of values of 14–24 nm respectively (Fig. 5c, d).

Tropocollagen is known to be the key structural unit of a collagen fibril. The structural studies [15] show that tropocollagen molecules, located nearer to a fibril's periphery, are bound by a greater number of covalent bonds than the central tropocollagen molecules are, due to which, a fibril generally has a solid superficial coating and a softer core. Mutual shadowing of end sites of tropocollagen molecules in their associating into a fibril leads to the characteristic striation of collagen fibrils. It is supposable that the amount of covalent bonds between fibrils (molecules) of tropocollagen for a newly-formed tissue is fewer, therefore, newly-formed fibrils have a lower hardness and a lesser height of a step between a gap and an overlap zone.

After 3 months, a well-shaped connective-tissue capsule is formed in the area of grafting, and the structure of newly-formed tissues differs from the control sample only by the thickness of bundles (Fig. 4c). Individual aggregates of nanodispersed biomaterial are observed at the biocontainer's former location.

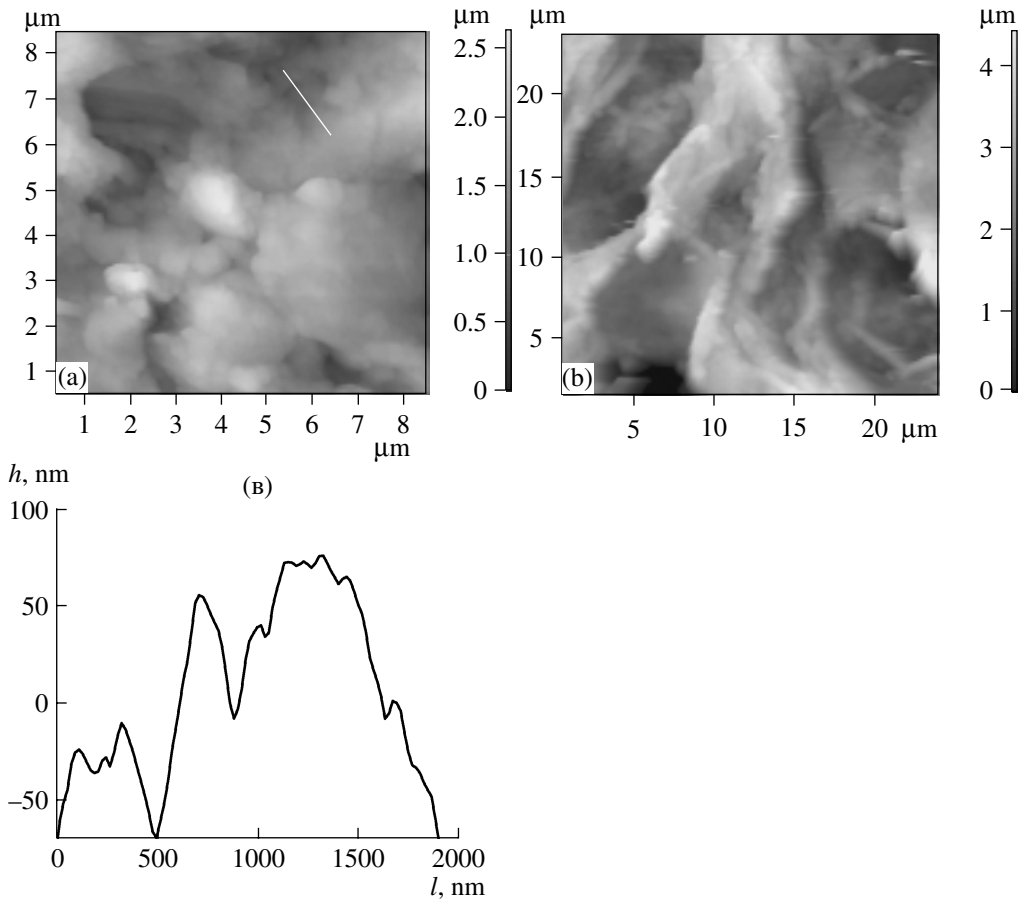

Fig. 3. AFM-images (topographic mode) of the zone of interaction of the nanodispersed biological material with the recipient tissues: a—after 3 days; b—after 20 days; c—the section profile along the line.

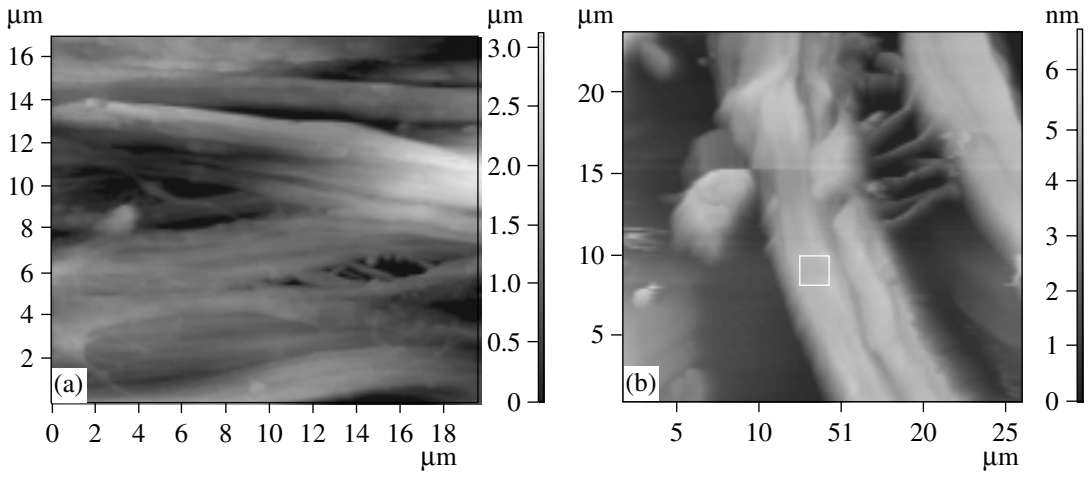

Fig. 4. AFM-images (topographic mode) of the sclera surface in the area of the biocontainer grafting: a—3 days; b—2 months; c—3 months. The area of study with a high resolution is shaded by a square (Fig. 5).

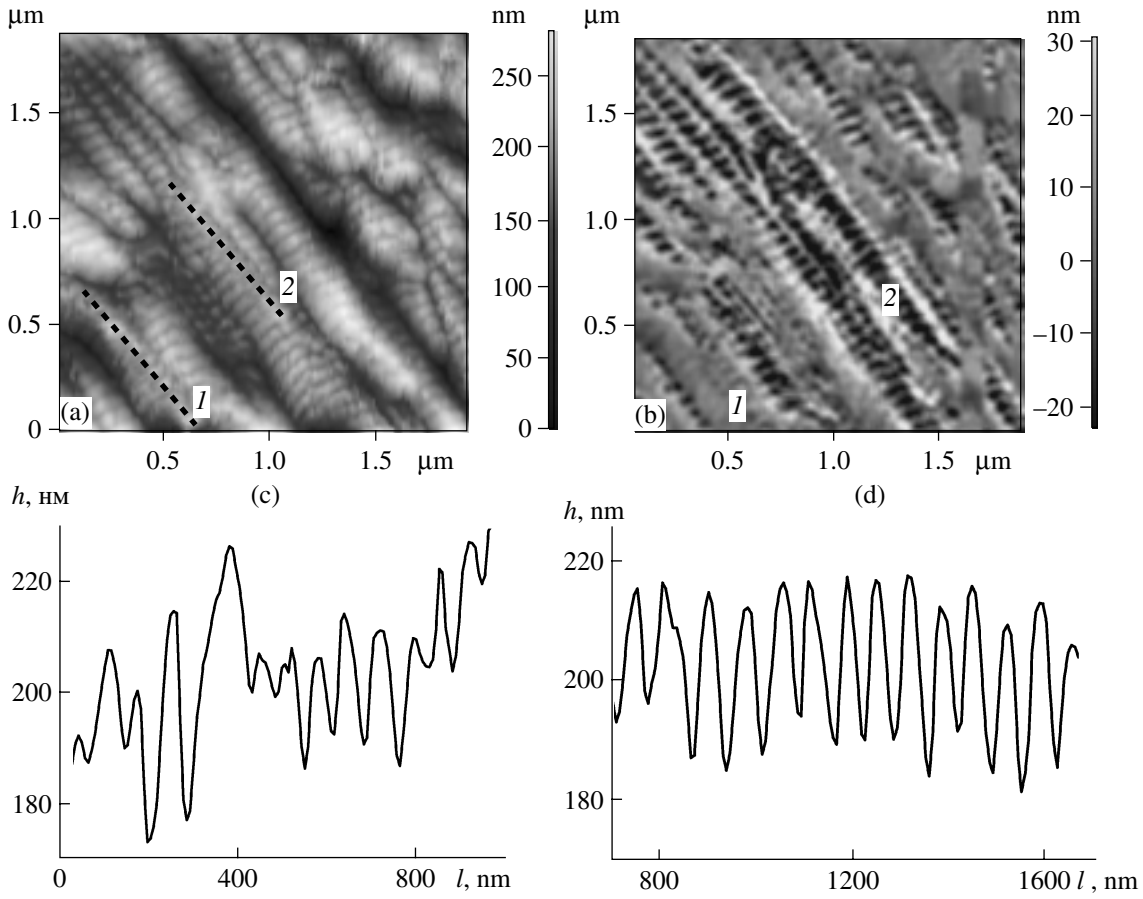

Fig. 5. AFM-images (under the topographic mode (a) and under the phase contrast mode (b) of the sclera site with newly-formed collagen fibrils within the area of grafting: *1*—the newly-formed tissue; *2*—mature collagen fibril. The profiles of the sites of collagen fibrils in regions *1* (c) and *2* (d) respectively.

CONCLUSION

Thus, the conducted studies showed the informative character of the AFM method for investigating the regenerative processes in sclera within the area of grafting of nanodispersed biological materials. AFM helped establish the structure of mechanically activated placenta (used as a graft), evaluate the level of penetration of the donor material into the structures of the recipient eye's connective tissue. We have established the formation of new connective-tissue structures, which is induced by penetration of the placenta's nanoparticles into the depth of the recipient sclera.

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