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EVALUATION OF CALCIFICATION RESISTANCE OF XENOPERICARDIUM TREATED WITH POLYHYDROXY COMPOUNDS

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Calcification of biomaterials used in prosthetic heart valves has been a challenging issue in cardiovascular surgery. The objective of this work is to compare the efficiency of polyvinyl alcohol (PVA) and tannic acid (TA) modification of xenomaterials, pre-stabilized with glutaraldehyde (GA) and ethylene glycol diglycidyl ether (EGDE), in reducing calcification. Analysis of mechanical properties evaluated under uniaxial tension, showed a significant increase in the tensile strength of the test samples compared to the control (unmodified) samples ($p < 0.05$). Additional treatment of GA-fixed tissue with PVA and TA significantly reduced the amount of calcium in the samples implanted into rats for a 60-day follow-up ($p < 0.05$). The level of calcification of samples pre-stabilized with EGDE and treated with PVA and TA did not differ from the control group ($p = 0.063$). Cumulative analysis of the study results demonstrated that the GA-fixed biomaterial modified with PVA and TA can reduce calcium-binding activity and increase strength. This indicates the prospects for clinical application of the proposed treatment methods. This being said, the issue of long-term body response requires further study of the long-term stability of the modified biomaterial under physiologic blood flow conditions.

Keywords: xenopericardium, polyvinyl alcohol, tannic acid, ethylene glycol diglycidyl ether, glutaraldehyde, calcification.

INTRODUCTION

At present, valvular heart disease is tackled by replacing the failed valve with a mechanical or biological prosthesis [1, 2]. Biological substitutes made from animal xenotissues, in contrast to mechanical devices, are characterized by hemodynamics comparable to the native valve and high hemocompatibility [3]. At the same time, xenogeneic material is prone to degradation under enzymatic, chemical and physical effects of the human body environment (both blood and surrounding tissues). For this reason, biological tissue is pretreated with chemical agents that stabilize the structure in order to minimize the body's immune response [4]. The principle of action of chemical stabilizers lies in their interaction with amino groups of collagen, the main protein of the extracellular matrix of the biomaterial, and, as a consequence, formation of additional cross-links in the collagen molecular structure [5, 6]. The most common stabilizing agent in world practice is glutaraldehyde (GA) [7]; however, there are also alternative preservative compounds, such as ethylene glycol diglycidyl ether (EGDE) [8]. Despite the complex treatment of biological prosthesis xenomaterial and the use of modern post-treatments (anticalcium, antithrombotic), it is not possible to achieve complete freedom from dysfunctions comparable to mechanical prostheses – 20–30 years in most cases. In this regard, such valves require replacement after a certain time – their service life is limited, first of all, by the calcification and structural degradation of the biological tissue. There are several hypotheses in publications explaining the mechanism of this process, among which a preservative plays an important role [9]. However, mineralization (calcification) is most likely caused by a complex of events, representing a multifactorial process [10]. To combat the calcification of biological heart valve prostheses, various methods have been proposed, including the use of a new preservative [11], "masking" of free aldehyde groups of glutaraldehyde [12], surface modification with aminodiphosphonates [13], preliminary tissue decellularization to remove alpha-galactose residues, as an element provoking the immune response and calcification [12], filling voids in the collagen space to eliminate potential sites for development of passive mineralization [14], etc. Some of the proposed solutions have already been put into industrial practice, but the problem of calcification has not yet been completely eliminated.

Having analyzed the literature data, we identified a promising area of research – modification of stabilized xenogeneic tissues with bulk polyhydroxy compounds. Such treatment is expected to reduce the calcium-binding activity of the material due to formation of an additional hydrophilic layer, masking of the active groups of the preservative (GA), and filling of voids in the xenotissue

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structure [14, 15]. Two polyhydroxy compounds were chosen as modifying agents: polyvinyl alcohol (PVA, linear structure) and tannic acid (TA, bulky, branched structure).

RESEARCH METHODOLOGY

The object of the study was bovine (cattle) pericardium preserved according to standard methods – 5% EGDE and 0.625% GA. Tannic acid (ACS reagent, Sigma Aldrich, USA) and polyvinyl alcohol Mw 67,000, 88% degree of hydrolysis of acetate group (Sigma-Aldrich, USA) were used as reagents for modifying the xenopericardium in order to reduce calcium-binding activity. The material was modified in two ways: in 5% PVA solution in isotonic saline solution and in 3% TA solution in isotonic saline solution. Exposure lasted for 24 hours at 37 °C and constant stirring in hydrochloric acid as a reaction catalyst.

To confirm the presence of additional hydroxyl groups introduced by the described method in the structure of modified materials, tissue samples (n = 2, for each group) were dried and examined by infrared spectroscopy on an Infralum FT-801 IR Fourier spectrometer (Russia). The analysis was carried out using diffuse reflectance infrared spectra.

Changes in its mechanical properties – ultimate tensile strength, elongation to rupture, and Young's modulus – were used as a criterion for the effectiveness of these xenopericardium treatment methods. For this purpose, samples of the original and modified biomaterial (n = 5, for each study group) were evaluated under uniaxial tension on universal testing machine Zwick/Roell (Zwick GmbH, Germany). Samples were prepared using a specially shaped knife (B083, corresponding to ISO 37: 2017) on a ZCP 020 punch press (Zwick GmbH & Co. KG, Germany). The ultimate tensile strength of biological tissue was determined by the maximum tensile stress before the onset of destruction of the sample (MPa). Elastic-deformation properties were assessed by relative elongation, corrected taking into account the nature of destruction of the samples (%) and Young's modulus (MPa), which was determined in the ranges of small deformations corresponding to the range of physiological load. To measure the thickness of the samples, we used a thickness gauge, TR (ZAO Krasny Instrumentalshchik, Russia) with a ±0.01 mm margin of error (clamping force ≤1.5 N) was used.

The effectiveness of reducing calcium-binding activity was assessed by in vitro and in vivo tests. To determine in vitro the resistance of the original and modified material to calcification, biotissue samples were kept in a solution simulating the physiological environment of the human body. For this purpose, 5 × 5 mm pericardial flaps (n = 5) were placed individually in a 2 mL solution containing sterile medium for growing human and animal cell cultures (DMEM, Sigma-Aldrich, USA), fetal bovine serum (FBS, Sigma-Aldrich, USA), calcium chloride and sodium monohydrogen phosphate. Calcification level was determined after week 2 and 3 of incubation at 37 °C in a CO_2 incubator, the carbon dioxide concentration being 5%. Cryosections of incubated samples, pre-stained with alizarin red S, were examined by light microscopy on an AXIO Imager A1 device (Carl Zeiss, Germany).

Material resistance to calcification was also evaluated in vivo by subcutaneous implantation in male Wistar rats (n = 5) (weight 55–70 g). The follow-up period was 2 months. All interventions were performed under general anesthesia in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS N 123), with the Russian Ministry of Health order No. 199n dated April 1, 2016 "On Approval of the Rules of Good Laboratory Practice" and with Interstate Standard GOST 33044-2014. After the prescribed period, the animals were withdrawn from the study by an overdose of anesthesia. Samples of the biomaterial were explanted, the surrounding tissues were removed and dried to constant weight, after which they were exposed to 50% perchloric acid under heating in order to complete hydrolysis until a clear solution was obtained. Samples diluted with distilled water were examined for calcium content on an icap 6300 atomic emission spectrometer with inductively coupled plasma (Thermo Scientific, USA).

Quantitative data was analyzed using statistical methods in medical and biological information processing software STATISTICA 6.0 (StatSoft, Inc., USA). Using the Kolmogorov–Smirnov test, the distribution model in the samples was determined as nonparametric, as a result of which the results цуку presented as medians (M), quartiles (25% and 75%), minimum and maximum values. The Mann–Whitney U test was used to assess the statistical significance of the differences between the two independent groups; the significance of differences was recorded at a $p < 0.05$ significance level.

RESULTS

Evaluation of mechanical properties

The experiment showed that the ultimate tensile strength of the modified samples in both studied parallels (EGDE- and GA-preserved xenopericardium) was significantly higher than the control values ($p < 0.05$) (Table). In the case of PVA modification, this indicator exceeded the control value by 2.7 and 1.6 times for the EGDE and GA pericardium, respectively. The tensile strength of the TA-treated xenopericardium was 2.4 times higher for EGDE-conditioned material and 1.8 times higher for GA-stabilized material.

In terms of elongation and Young's modulus, no significant differences were found between the groups,

except for the Young's modulus of the EGDE-prefixed and polyvinyl alcohol-modified specimens.

Infrared spectroscopy

The diffuse reflectance spectra obtained for the modified samples (PVA- and TA-treated xenopericardium) revealed changes in the character (width and shape) of the band caused by stretching vibrations of the interacting O–H– bonds in the 3200–3600 cm^{-1} range [16] (Fig. 1). The region of the spectrum obtained for each of the studied samples up to 1800 cm^{-1} contains characteristic bands related to the protein structures of the pericardial tissue, in particular, collagen. As a rule, amide I bands (~1700 cm^{-1}) arise from C=O stretching vibrations associated with N–H bending vibrations. Amide II bands (~1575 cm^{-1}) arise from N–H bending vibrations with C–N stretching vibrations [17]. The bands of C–O–C vibrations caused by the presence of epoxy groups, lie in the 800–900 cm^{-1} range [18]. According to reports, the presence of a carbonyl group determines the appearance of a band in the infrared spectrum of the compound in

Table

Mechanical properties of modified and control materials

Name	Strength, MPa	Relative elongation, %	Young's modulus, MPa
Control (GA)	12.34 (7.75; 9.38; 14.00; 14.63)#	54.12 (45.73; 48.30; 58.75; 60.83)	1.69 (1.12; 1.25; 2.01; 2.13)
Control (EGDE)	3.48 (3.12; 3.14; 4.06; 4.96)*	54.51 (47.38; 47.98; 64.16; 65.79)	1.720 (1.67; 1.68; 1.80; 1.82)
GA + TA	21.67 (20.64; 20.92; 23.11; 23.79)*	61.18 (57.94; 58.78; 64.72; 65.89)	1.66 (1.47; 1.49; 1.72; 1.76)
EGDE + TA	8.48 (4.40; 6.23; 8.75; 9.00)#	48.23 (39.89; 43.35; 62.26; 62.89)	1.63 (1.47; 1.53; 2.00; 2.04)
GA + PVA	19.35 (16.04; 16.36; 24.58; 26.72)*	57.00 (47.87; 49.29; 65.38; 73.25)	1.56 (1.06; 1.29; 1.60; 1.63)
EGDE + PVA	9.55 (5.07; 6.89; 10.50; 10.65)#	58.34 (42.49; 48.80; 65.06; 66.81)	2.04 (1.94; 1.97; 2.38; 2.50)#

* – statistically significantly different from the GA control ($p < 0.05$), # – statistically significantly different from the EGDE control ($p < 0.05$).

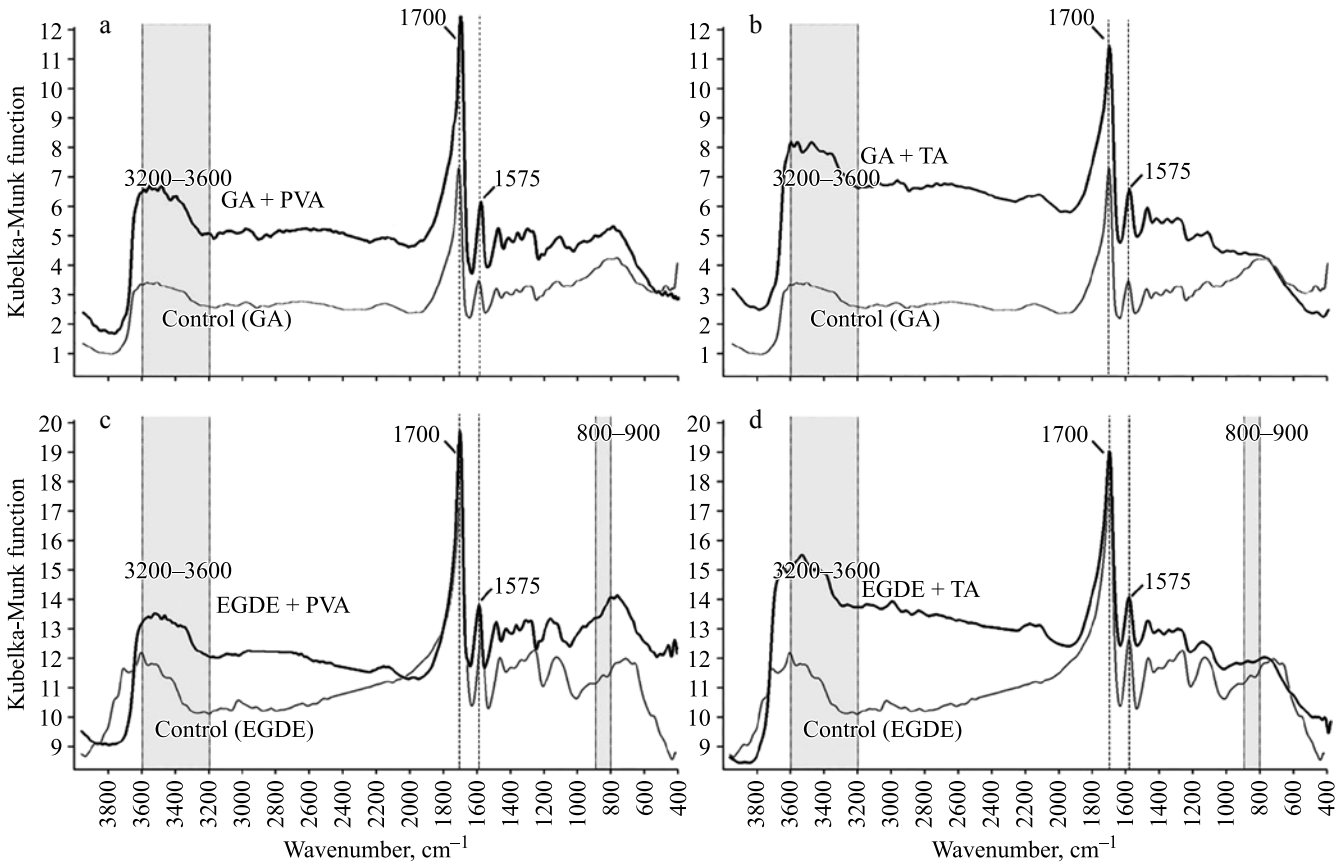

Fig. 1. Diffuse reflectance IR spectra of biomaterial stabilized with EDGE and GA, and modified with PVA and TA: a) comparison of the spectra of GA-pericardium treated with PVA with the control; b) comparison of the spectra of GA-pericardium treated with TA with the control; c) comparison of the spectra of EDGE pericardium treated with PVA with the control; d) comparison of the spectra of EDGE pericardium treated with TA with the control

the 1660–1770 cm^{-1} region [19]. In our case, this band obviously overlaps with the amide I band.

In vitro calcification assessment

Microscopic analysis of slices stained for the presence of calcium and obtained after incubation of samples in solution to simulate accelerated calcification in vitro showed an increase in the resistance to mineralization in all PVA- and TA-modified materials compared with the control groups (Fig. 2). There were no visual differences in the amount of calcium in the PVA- and TA-treated samples. However, a greater propensity to form calcifications was noted for the entire parallel of GA pre-stabilized tissues (Fig. 2).

In vivo calcification

Assessment of calcium content in xenopericardium samples implanted in rats revealed, first of all, a statistically significantly higher level of calcification for the GA pre-stabilized material, $p < 0.05$ (Fig. 3). At the same time, additional treatment of GA-fixed tissue with polyhydroxy compounds (PVA and TA) made it possible to significantly reduce calcium content in the samples, $p < 0.05$. The level of calcification of samples pre-stabilized

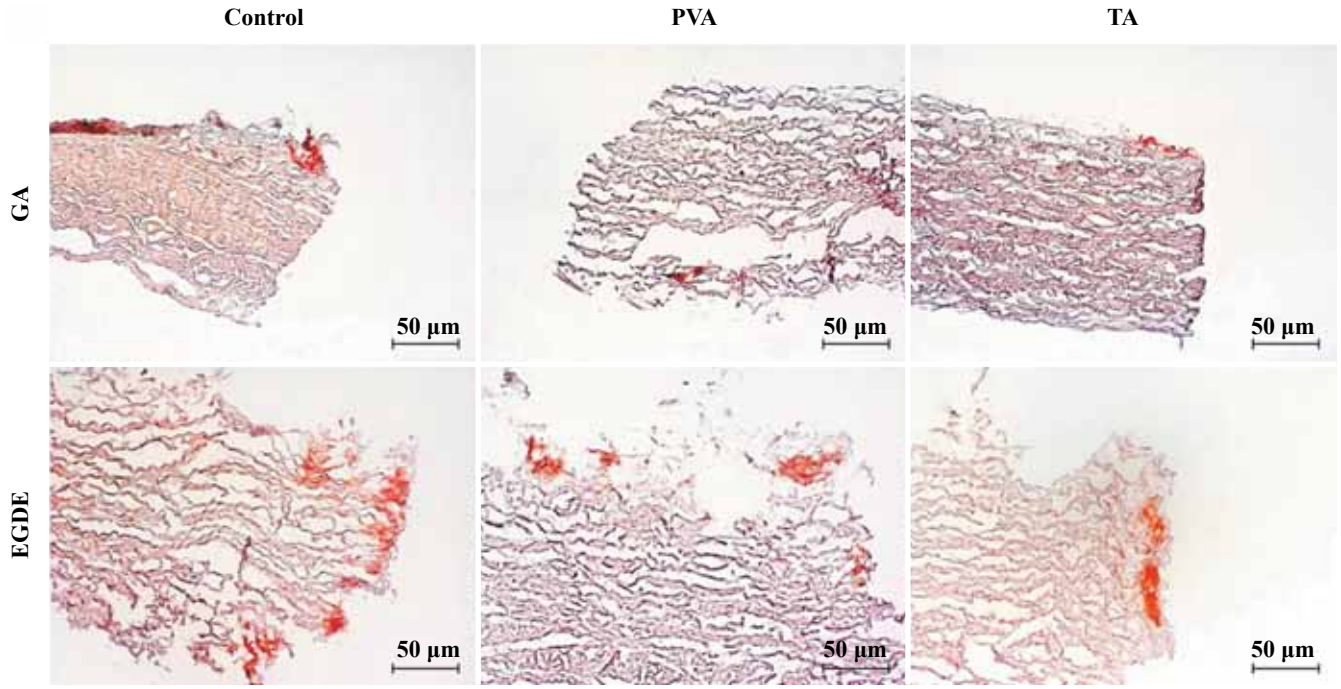

Fig. 2. Histological sections of biomaterial samples, stabilized with EDGE and GA, and modified with PVA and TA. Evaluation of in vitro calcification. 200× magnification

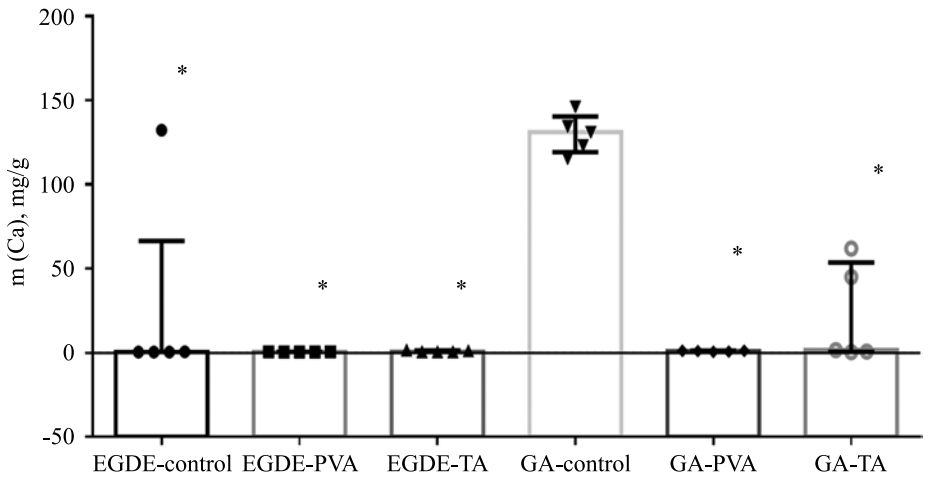

Fig. 3. Calcium content in samples implanted in rats for 60 days
* – statistically significantly different from the GA control group.

with EGDE and treated with PVA and TA did not differ from the control group (p = 0.063).

DISCUSSION OF RESULTS

Modification of xenopericardium with polyhydroxy compounds, pre-stabilized with chemical agents, is based on the principle of interaction of free groups of the preservative with the hydroxyl groups of the modifier. In addition to covalent chemical bonds, polyvinyl alcohol (I) and tannic acid (II) can form less stable hydrogen bonds with amino acid functional groups of collagen molecules, and can be physically absorbed in the voids of the biomaterial and on its surface. Fig. 4 and 5 illustrate the chemical processes occurring during modification using a reaction with polyvinyl alcohol as an example.

Due to the peculiarities of the chemical structure of the main chain and the presence of hydroxyl groups, PVA is a non-toxic and highly hydrophilic compound. Films and hydrogels made on its basis are used, inter alia, in experiments to create a polymeric heart valve [20]. Tannic acid, in turn, has been studied earlier as an individual substance that stabilizes biological tissue [21] and as an additional component in pericardial fixation with glutaric aldehyde [22]. Works performed established a

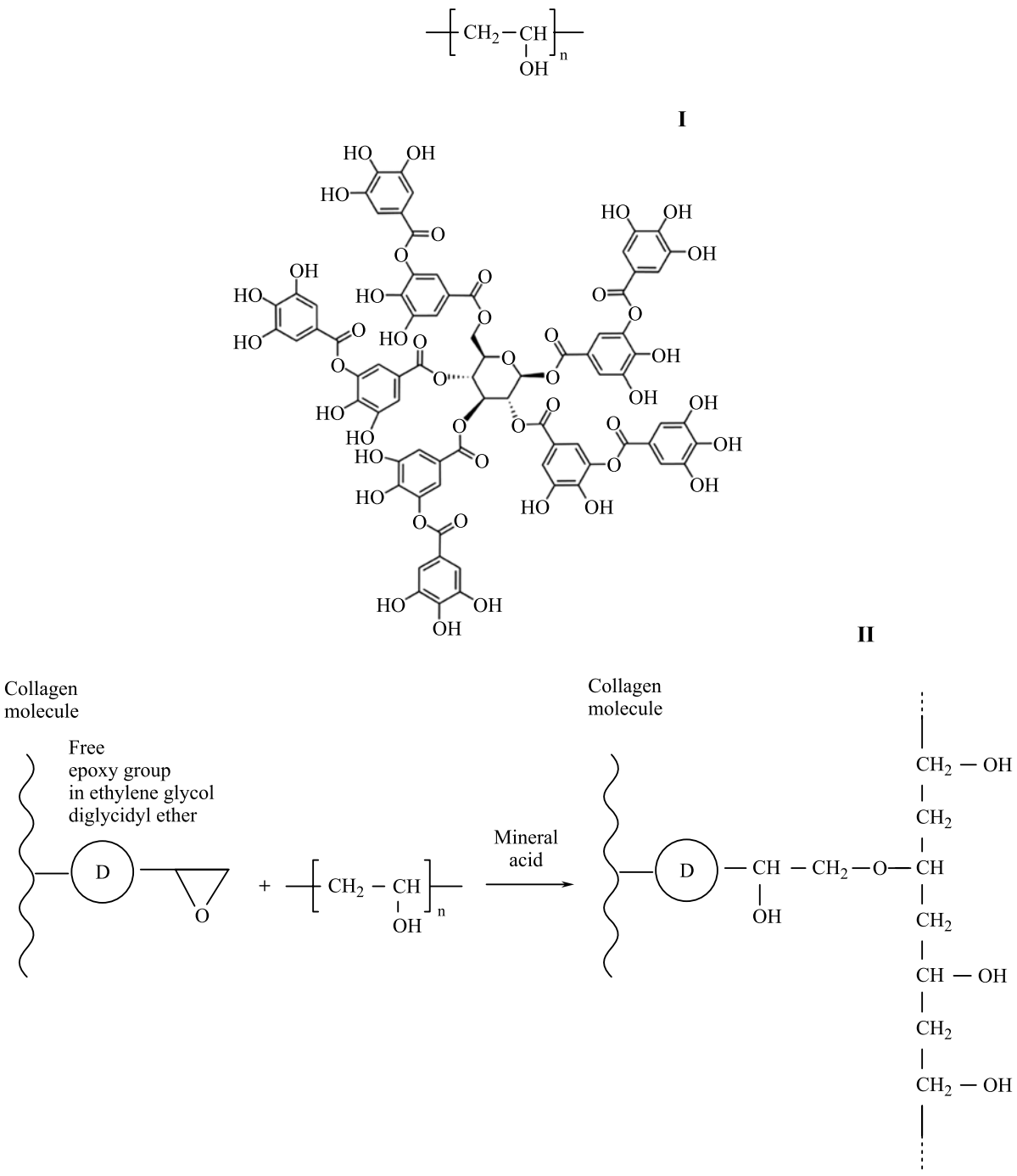

Fig. 4. Interaction between epoxy groups in EDGE-stabilized xenopericardium and hydroxyl groups in polyvinyl alcohol

reduced body's immune response (experiment with rats) and reduced calcium-binding activity of the biomaterial, as well as preserved collagen fibers of biological tissue [21, 22].

Mechanical properties

The experimentally obtained strength values of the samples of the control groups are generally similar to the literature data [23], where, among other things, a higher strength of materials stabilized with glutaraldehyde compared to EGDE was noted, which correlates with the results obtained by us earlier.

Increased strength characteristics of PVA- and TA-modified pericardium may indicate the formation of additional interfibrillar cross-links in collagen [24]. At the same time, the absence of statistically significant differences in strengths between the TA and PVA groups, although it does not allow us to state unequivocally, indicates that these compounds are chemically equivalent. However, the presented chemical interaction scheme reflects only the assumed reaction mechanism; probably, in addition to the proposed explanation, other paths are possible, which is associated with the complexity of the systems under study. Notwithstanding, in the absence of a critical effect on Young's modulus and elasticity of the material modification, one can assert that there is no negative effect on the pericardial structure of the reagents used and the reaction conditions.

Infrared spectroscopy

The change in the nature of the band in the spectral region caused by the O–H stretching vibrations of the interacting hydroxyl groups may be due to the breaking of bonds of unreacted epoxy groups of the preservative, resulting in the formation of new hydroxyl groups, new covalent and hydrogen bonds in the case of EGDE-preserved samples. In this case, direct grafting of polyhydroxyl compounds (PVA and TA) to the surface of the biological tissue makes the main contribution to increased band width. The ambiguity in interpretation of IR spectra is due to the complexity of the composition and structure of the biological structures of the material under study. At the same time, the absence of significant changes in the location of the characteristic bands of the spectrum indicates the preservation of the tissue structure, in particular, tertiary collagen and elastin molecules. Based on the foregoing, it can be argued that modification has no negative effect on the architectonics of biological material.

In vitro calcification

The amount of calcium phosphate precipitate in the in vitro model system is limited and depends on the initial concentrations of the working solution. In this case, a relatively unlimited amount of salt is formed on the implant surface in the blood stream. Moreover, calcium phosphate precipitation in the in vitro system is random compared to pathological calcification. Consequently, the nature of the crystalline mineral phase in the in vitro system is hardly predictable and may differ from in vivo. Besides, there is strong evidence supporting the role of cells in calcification [25]. However, the proposed in vitro model can be used to explain the formation of passive deposits that do not imply cellular involvement [26].

Polyhydroxyl coating formed on the surface of biological materials modified with polyvinyl alcohol and tannic acid is a highly hydrophilic surface, which, under physiological conditions, has a neutral reaction in the case of PVA and weakly acidic for TA. Due to the neutralization of free groups of preservatives capable of provoking calcium accumulation in the tissue, and also due to the filling of voids in the structure of the material [27], such a modification probably partially limits the penetration of calcium and phosphate ions into the tissue, since the existing spaces are potential calcification nucleation centers [14].

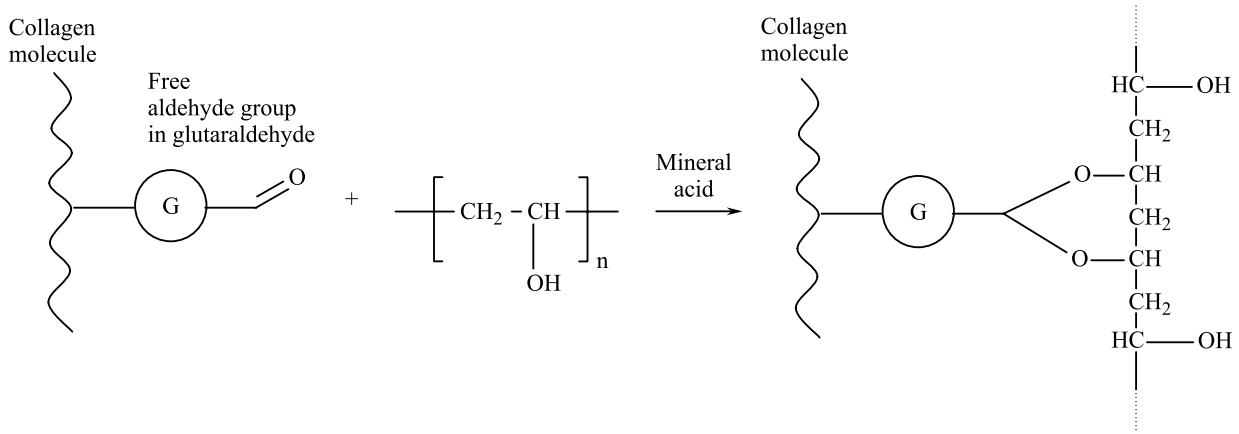

Fig. 5. Hemiacetal formation as a result of interaction between the aldehyde group in glutaraldehyde and two nearest hydroxyl groups in polyvinyl alcohol

In vivo calcification

A decrease in the level of GA-preserved pericardium calcification as a result of PVA and TA modification is probably associated with decreased toxicity of the aldehyde groups of the preservative, and, as a consequence, with reduced inflammatory response. Inflammation is known to precede the development of implanted tissue mineralization [28]. In addition, tannic acid has been reported to be able to bind elastin, which is also a promoting factor in calcification [29]. No similar information about polyvinyl alcohol has been found in publications; so, this issue requires experimental confirmation. Meanwhile, the calcium content in the EGDE group was lower than the same value for the GA control group. However, the presented implantation model for rats does not give a complete picture, since long-term clinical outcomes indicate calcification of biological prostheses of the heart valve preserved by EGDE [8]. For this reason, modification is necessary, and the methods we have proposed in this work may be quite promising as they demonstrate the calcium-binding resistance of the treated tissues.

CONCLUSION

A cumulative analysis of the study results demonstrated the ability of the modified PVA and TA tissues, pre-preserved with GA and EGDE, to reduce calcium-binding activity and increase strength, which indicates the prospects for clinical application of the proposed treatment methods. At the same time, the issue of long-term body response requires further study of the long-term stability of the modified biomaterial under physiological blood flow conditions.

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The authors declare no conflict of interest.

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