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Polyvinyl alcohol improves resistance of epoxy-treated bovine pericardium to calcification *in vitro*

A.E. Kostyunin✉, M.A. Rezvova, T.V. Glushkova, D.K. Shishkova, A.G. Kutikhin, T.N. Akentyeva, E.A. Ovcharenko

Research Institute for Complex Issues of Cardiovascular Diseases, 6 Sosnovy Blvd., Kemerovo 650002 Russia

✉Corresponding author: Alexander E. Kostyunin, Cand. Sci. (Biol.), Research Fellow, Laboratory for Novel Biomaterials, Department of Experimental Medicine, Research Institute for Complex Issues of Cardiovascular Diseases, rhabdophis_tigrina@mail.ru

Abstract

Background. *Around half of bioprosthetic heart valves become dysfunctional 15 years postimplantation because of structural valve degeneration notable for the degradation and calcification of the prosthetic tissue. Protection of bioprosthetic heart valves from structural valve degeneration requires innovative materials, science approaches including enveloping of the bioprosthetic heart valves into the polymer sheath.*

Aim. *To develop a polyvinyl alcohol sheath for improving resistance of bioprosthetic heart valves to calcification.*

Material and methods. *Bovine pericardium fixed with ethylene glycol diglycidyl ether was incubated with distinct concentrations of polyvinyl alcohol (5, 10, 12, or 15%) with the following freezing and thawing to perform cryotropic gelation. Surface and structure of unmodified and polyvinyl alcohol-modified bovine pericardium have been investigated by*

fluorescence microscopy and scanning electron microscopy, whilst tensile testing was carried out by uniaxial tension test. Haemocompatibility was assessed through the measurements of haemolysis and platelet aggregation/adhesion upon the contact of donor blood with the samples. Resistance to calcification was tested by incubation of the samples in calcium and phosphate supersaturated (10 µmol/L) cell culture medium for 3 and 6 weeks with the following tissue lysis and colorimetric measurement of Ca^{2+} *ions.*

Results. *Using cryotropic gelation, we obtained a polyvinyl alcohol-coated and filled bovine pericardium matrix. Out of all polyvinyl alcohol concentrations, 12% polyvinyl alcohol solution sealed pores and hollows within the bovine pericardium (what was not achieved using 5% or 10% poly(vinyl alcohol) solutions) and demonstrated the best processability as compared to 15% polyvinyl alcohol solution. Cryotropic gelation did not deteriorate durability, elasticity, or haemocompatibility of bovine pericardium. After 3 and 6 weeks of the incubation in calcium-supersaturated solution, polyvinyl alcohol-modified bovine pericardium contained 5- and 3-fold reduced amount of calcium compared to unmodified bovine pericardium.*

Conclusions. *Enveloping of bovine pericardium into polyvinyl alcohol increases its calcification resistance, retains its tensile properties and haemocompatibility, and can be considered as a promising approach for the modification of bovine pericardium during the manufacturing of bioprosthetic heart valves.*

Keywords: bioprosthetic heart valves, bovine pericardium, cryotropic gelation, polyvinyl alcohol, calcification

Conflicts of interest. The authors declare no conflict of interest.

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AHD, acquired valvular heart disease

BHVs, bioprosthetic heart valves

FM, fluorescence microscopy

IPRP, intact platelet-rich plasma

MP, poly(vinyl alcohol)-**m**odified bovine **p**ericardium

PRP, platelet-rich plasma

PVA, polyvinyl alcohol

SEM, scanning electron microscopy

UP, **u**nmodified bovine **p**ericardium

Rationale

Acquired valvular heart defects (AHDs) have been one of the main causes to decrease the working capacity, quality of life, and life expectancy, especially in old age [1]. Replacement of affected heart valves with mechanical and bioprosthetic heart valves (BHVs) is a radical method of treating AHDs [2]. The latter favorably differ from mechanical analogues by low thrombogenic potential, enabling recipients to obviate the need for lifelong anticoagulant therapy [3]. At the same time, up to half of BHVs are subject to dysfunction developments after 15 years of functioning due to the structural degeneration of their biological component [4, 5]. This feature

limits the possibility of using BHVs in the surgical correction of AHDs, in particular in patients younger than 65 years, whose life expectancy exceeds the mean longevity of BHVs [2].

The results of recent studies have demonstrated that an important role in the development of structural valve degeneration is played by the imbibition of various substances circulating in the blood of recipients into the BHV biomaterial [6]. In particular, the entry of calcium ions, calcium-binding proteins, and proteases into the BHV leads to calcification and rupture of the valvular apparatus [6]. Accordingly, a promising trend in the search for a way to increase the BHVs durability is to develop a modification that would prevent the accumulation of calcium ions and chemically aggressive compounds in the biomaterial.

The solution to the stated problem can be innovative methods of biotissue processing based on filling its internal structure with polymer gels [7]. Gels form a physical barrier that prevents the entry of substances dissolved in the blood into the interfibrillar space of the biomaterial and their interaction with collagen fibers [7].

Aim. The aim of the study was to develop an original polyvinyl alcohol (PVA) sheath for bovine pericardium used in BHV manufacture to protect it from calcification. In the course of achieving the goal, we applied the method of PVA cryotropic gelation followed by an assessment of the internal and surface structure of the modified biomaterial, as well as its mechanical, hemocompatible, and anti-calcification properties.

Material and methods

Modifiable biomaterial and modifying agent

KemPeriplasNeo pericardial flaps (KPi7080M, ZAO NeoKor) of 0.6–

0.7 mm thick were used as the material for modification. This type of biomaterial is used in manufacturing the bovine pericardial BHVs clinically used in the Russian Federation [8]. Unmodified pericardial flaps were used as controls.

Linear PVA with the molecular mass of 89–98 kDa and the degree of hydrolysis of acetate groups more than 99% (341584, Sigma Aldrich) was used as a modifying agent. The most important advantages of PVA-based gels, which determined the choice of a specific polymer for the purpose of the study, included their stability in water and biological fluids, comparability with biological tissues in terms of tensile properties, non-toxicity for cells, and resistance to nonspecific adsorption of proteins [9–11].

Modification of bovine pericardium with polyvinyl alcohol

The processing of the bovine pericardium with PVA aimed at obtaining a composite material: we expected the formation of gel that would form a film on the surface of the biomaterial and fill the interfibrillar space in its thickness. In this case, the method of PVA cryotropic gelation was used forming the gel and its bind to the collagen matrix of the biological tissue.

To select the optimal conditions for modifying the bovine pericardium, four types of modifying aqueous solutions were prepared, differing in the PVA (5%, 10%, 12%, and 15%) concentration. They were prepared before the start of modification in the following way: a weighed portion of PVA was taken, and added to distilled water upon heating to 95°C and constant stirring until a homogeneous transparent solution was obtained.

The bovine pericardium flaps (at the rate of 1 cm^2 of biomaterial per 1 ml of liquid) were kept in solutions cooled to room temperature for 24 hours

with constant stirring for their effective impregnation with PVA. Next, the samples were removed from the solutions and frozen at -40°C for 24 hours, after which they were incubated for 12 hours, first at -5°C, then at 5°C to form a PVA gel. Finally, the samples were left for 30 minutes at room temperature.

The obtained samples of the modified biomaterial were washed from PVA residues in physiological saline for 24 hours and prepared for testing.

Study of the biomaterial surface microstructure

The surface structure of the biomaterial was studied by means of scanning electron microscopy (SEM). For this, 3 fragments of modified and control bovine pericardium of 1 cm^2 with in area were prepared and lyophilized for 24 hours in a FreeZone 2.5 Plus unit (Labconco) at a temperature of -40°C and a pressure of lower than 0.04 mbar. Then the samples were mounted on tables and a conductive (Au-Pd) coating was formed on their surface by ion sputtering using the EM ACE200 system (Leica microsystems). The biomaterial surface was visualized using a S-3400N scanning electron microscope (Hitachi) under high vacuum conditions at an accelerating voltage of 10 kV in the secondary electron mode.

Study of the biomaterial internal microstructure

For this purpose, the specimens of bovine pericardium fragments of 1 cm^2 in area were cryo-cut using the Microm HM525 NX Cryostat (Thermo Fisher Scientific) to obtain the sections of 14 and 6 μm thick (for SEM and FM, respectively) that were transferred onto glass slides.

During SEM sample preparation, the slides with the sections applied

onto them were washed for 2 hours in bidistilled water, and the procedures described in the previous section (lyophilization, mounting, sputtering, and SEM visualization) were performed.

To additionally confirm the presence of PVA in the pericardium thickness, the FM method was used with preliminarily staining the sections with Mayer's hematoxylin and eosin according to the standard technique. AxioImager.A1 microscope (Carl Zeiss) and AxioVision image processing software (Carl Zeiss) were used for visualization.

Assessment of the biomaterial mechanical properties

The mechanical properties of the samples were evaluated by uniaxial tension in accordance with GOST 270-75. Ten fragments of modified and control bovine pericardium each were prepared using a special knife (B083 according to ISO 37 standard) for testing on the ZCP 020 punching press (Zwick GmbH & Co. KG). Testing was carried out on a Zwick/Roell testing machine (Zwick GmbH & Co. KG) with a transducer providing a nominal force of 50 N. The tensile strength of the specimens was assessed by the maximum tensile stress, taking into account the cross-sectional area of the working segment; the stress-strain properties were assessed by the relative elongation adjusted for the nature of the specimen destruction and Young's modulus determined in the ranges of small deformations.

Assessment of biomaterial hemocompatibility

In accordance with the requirements for medical devices in contact with blood (GOST R ISO 10993.4), we assessed the degree of platelet aggregation and adhesion, as well as the hemolysis degree after blood contact with PVA-modified bovine pericardium. Blood sampling for all

experiments was performed from one apparently healthy volunteer in Improvacuter tubes containing buffered sodium citrate solution (9NC 0.129M). Platelet-rich plasma (PRP) was obtained from fresh citrated blood by centrifugation for 10 minutes at 1200 rpm.

Platelet aggregation was assessed on a semi-automatic ARAST 4004 4-channel analyzer (LABiTec). Platelet-poor plasma obtained by centrifugation of citrated blood for 15 minutes at 3000 rpm was used to calibrate the instrument. The duration of contact between the modified and control bovine pericardium specimens (7 per group) of 0.25 cm^2 in area and 250 μL of PRP before measurement was 3 minutes, after which the 5`-diphosphate (AG-6, AGRENAM) platelet aggregation inductor, was added to the latter in the concentration of 20 μmol/L Intact PRP (IPRP) was used as a control. The aggregation intensity after 5 minutes of measurement was expressed by the values of the maximum aggregation percentage, the period before the aggregation onset after the addition of the inductor, the slope of the aggregation kinetic curve, and the time to reach the maximum percentage of aggregation.

To assess the degree of adhesion and transformation of platelets, 3 specimens each of modified and control bovine pericardium of 0.25 cm^2 in area were taken, incubated for an hour at 37° C in 300 μl of PRP. Further, in order to remove non-adhered PRP components, they were washed three times in a phosphate-buffered saline solution (pH 7.4) followed by their fixation in 2% glutaraldehyde overnight. Then the specimens were washed in distilled water and prepared for SEM according to the previously described technique.

To assess the degree of erythrocyte hemolysis, 5 specimens each of modified and control bovine pericardium of 5 cm^2 in area were placed in

sample bottles, added with 10 ml of saline, and placed in a thermostat at 37 °C for 2 hours. Normal saline and distilled water were used as negative and positive controls, respectively. After incubation, 200 µl of fresh citrated blood was added to each sample bottle and again kept in a thermostat at 37 °C for an hour. After incubation, the solutions were taken from the sample bottles into plastic tubes and centrifuged for 10 minutes at 2800 rpm to precipitate erythrocytes. The optical density of the obtained solutions was measured at a wavelength of 545 nm on a GENESYS 6 Spectrophotometer (Thermo Fisher Scientific). The degree of hemolysis was determined by the formula (Dt-Dne)/(Dpe-Dne) × 100%, where Dt denoted the optical density of the sample incubated with the specimen, Dne is the optical density of the sample added to saline solution, Dre is the optical density of the sample after complete hemolysis. Absent hemolysis was defined as the arithmetic mean of the optical density when measuring the physiological solution with citrated blood; complete hemolysis was defined as the arithmetic mean of the optical density when measuring distilled water with citrated blood.

Assessment of the biomaterial susceptibility to calcification

In order to study the susceptibility of the modified and control bovine pericardium to calcification, the corresponding specimens were incubated in a solution simulating the body physiological environment with an increased level of calcium and phosphate ions. Thus, 10 biomaterial fragments of 0.25 cm^2 in area from each group were placed individually in 2 ml of a solution containing 10 mmol of calcium, where they were incubated for 3 and 6 weeks at 37 °C in a carbon dioxide incubator. To prepare the solution, we used 1.655 ml of sterile nutrient medium for growing cell cultures (D0697, Sigma-Aldrich), 0.20 ml of fetal bovine serum (F2442, Sigma-Aldrich,

USA), 0.05 ml of calcium chloride, and 0.1 ml of monohydrogen phosphate sodium.

The quantitative content of calcium in the studied samples was determined by the spectrophotometric method. For this, biomaterial fragments were lyophilized for a day and their weight was measured. The specimens were then subjected to hydrolysis in 0.5 ml of 65% perchloric acid in a sand bath (150–180°C) until complete dissolution. The volume of the resulting mixture was adjusted to 5 ml with sterile water for injections. The calcium content in the solution was determined on a Multiskan Sky Spectrophotometer (Thermo Fisher Scientific) at a wavelength of 575 nm using a commercially available Calcium Assay Kit (ab102505, Abcam) according to the manufacturer's protocol.

To assess the typical locations of microcalcifications, 2 cryosectioned samples of 6 μm thick from each study group were used. Then the cryosectioned samples were stained with alizarin red C according to the standard technique and examined by light microscopy on an AxioImager.A1 microscope (Carl Zeiss).

Statistical analysis

Statistical processing of the data obtained in the course of the above experiments was performed using GraphPad Prism 8 software (GraphPad Software). The data distribution type was determined by the Kolmogorov–Smirnov test. Since the distribution in the groups was different from normal, the data are presented as median, percentile, minimum and maximum values. Intergroup comparison was performed using the Kruskal–Wallis test corrected for multiple comparison (FDR approach); intergroup differences were considered statistically significant with the maximum acceptable

probability of rejecting the correct null hypothesis of $p<0.05$.

Results

Microstructure and mechanical properties of polyvinyl alcohol-modified bovine pericardium

The surface of unmodified epoxy-treated bovine pericardium had the structure typical of the pericardium: the smooth serous surface consisted of densely packed collagen fibers with pores ranging in diameter from 200 nm to 15 μm, while the fibrous surface consisted of loosely arranged collagen fibers (Fig. 1). The internal structure of the unmodified bovine pericardium was loose with large spaces between the fibers (Fig. 2).

In turn, gel was present on the surface of the PVA-modified biomaterial, forming a loose partial (when using a PVA solution at a concentration of 5%) or dense monolithic (when using PVA solutions at a concentration of 10% or more) coating with a cellular structure (Fig. 1). The internal structure of the PVA-modified bovine pericardium showed a tendency to denser filling of the interfibrillar space with gel as the polymer concentration increased from 5 to 12% (Fig. 2). We did not reveal any differences in this parameter between the biotissue samples treated with 12% and 15% PVA solutions.

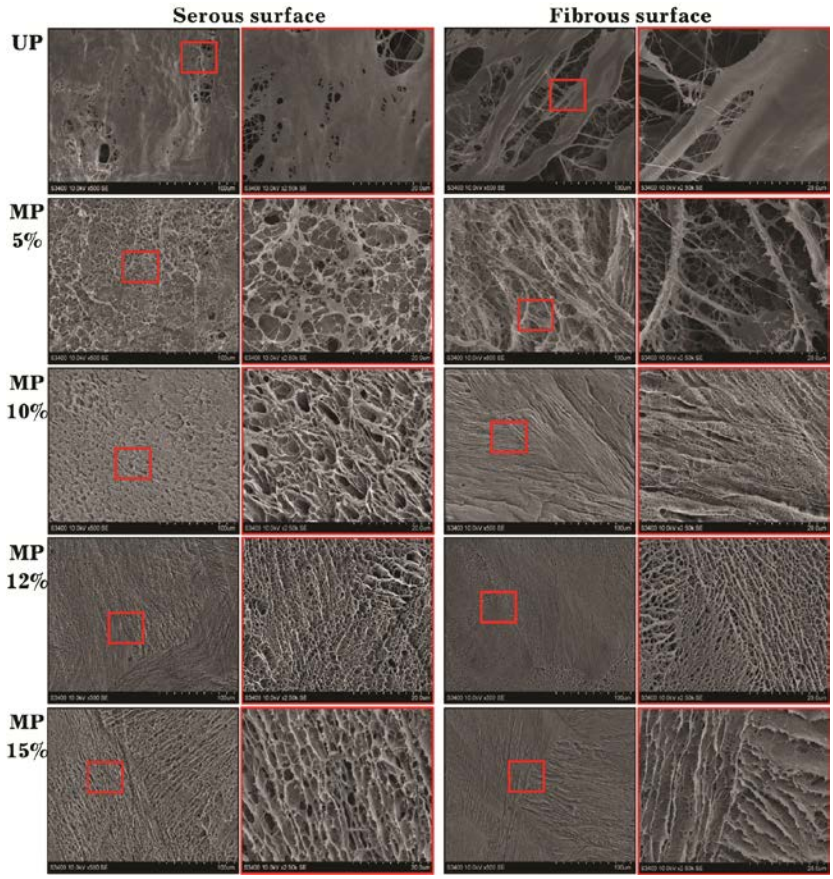

Fig. 1. Electron microscopy examination of the surface of unmodified (UP, top row) and polyvinyl alcohol-modified (MP, other rows) bovine pericardium. 5%, 10%, 12%, and 15% are concentrations of polyvinyl alcohol

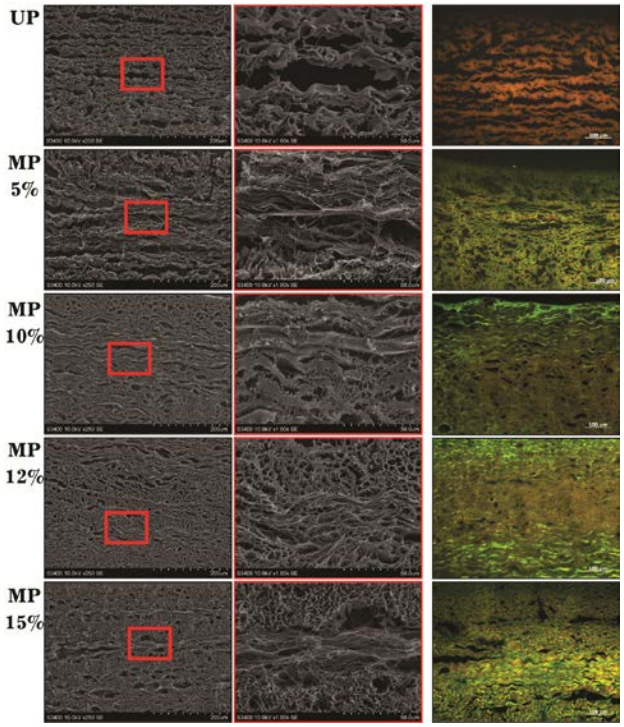

Fig. 2. Structure of unmodified (UP, top row) and polyvinyl alcohol-modified (MP, other rows) bovine pericardium. Images in the right column confirm the successful cryotropic gelation (green colour) within the collagen fibers (orange colour) at haematoxylin and eosin staining and subsequent fluorescence microscopy. 5%, 10%, 12%, and 15% are concentrations of polyvinyl alcohol

The results of testing the mechanical properties showed that the PVA-modified bovine pericardium samples did not have statistically significant differences from the control samples and from each other in terms of durability, elongation, and Young's modulus, regardless of the modifying agent concentration ($p>0.05$) (Fig. 3).

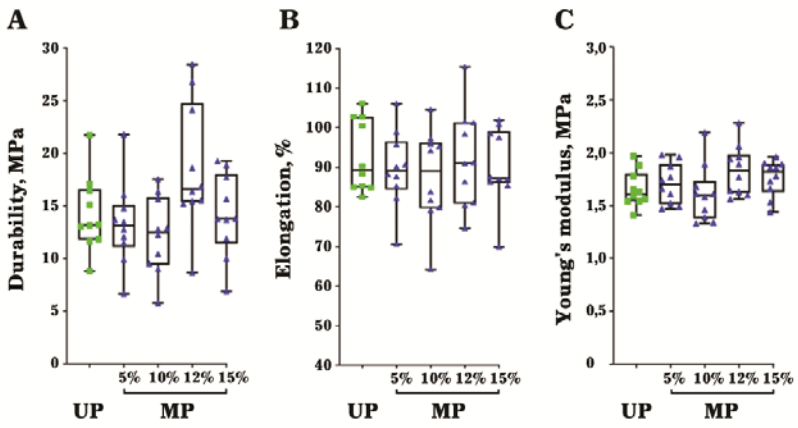

Fig. 3. Tensile properties of unmodified (UP, left) and polyvinyl alcohol-modified (MP, right) bovine pericardium. A – durability (ultimate tensile strength, MPa), B – tensile elongation at break (%), C – tensile elasticity (elastic modulus, MPa). 5%, 10%, 12%, and 15% are concentrations of polyvinyl alcohol

Taking into account the obtained results, a 12% PVA solution was chosen as optimal for further testing hemocompatible and anti-calcification properties of the modified biomaterial. When using less concentrated PVA solutions, we failed to achieve complete and uniform filling of the bovine pericardium interfibrillar space with the gel, while the use of a 15% PVA solution did not demonstrate additional benefits, leading to excessive consumption of the modifying agent.

Hemocompatible properties of the polyvinyl alcohol-modified bovine pericardium

There were no statistically significant differences in the maximum percentage of aggregation between the IPRP and that one incubated with control and modified fragments of bovine pericardium ($p>0.05$) (Fig. 4). Aggregation started earlier in the PRP contacted with control samples than in the IPRP or the one incubated with PVA-modified bovine pericardium ($p=0.002$ and $p=0.01$, respectively statistically significant in both cases).

Meanwhile, the PRP contacted with control samples was characterized by a lower rate of platelet aggregate formation, as evidenced by a smaller slope of the aggregation curve compared to that for IPRP and the one for contacted with PVA-modified biomaterial ($p=0.001$ and $p=0.002$, respectively statistically significant in both cases). Ultimately, despite the differences in the aggregation rate, the time to reach the maximum percentage of aggregation between all samples was the same ($p>0.05$). Thus, we have revealed insignificant differences in the hemocompatible properties between the control and modified samples, affecting the aggregation kinetics. They do not suggest any deterioration of the bovine pericardium hemocompatibility after it has been modified with PVA.

When studying the platelet adhesion on the bovine pericardium surface in the control group, we noted numerous platelets of the 2nd–3rd degree of activation, quantitatively predominating on the outer (fibrous) surface. No platelets were found on the PVA-modified bovine pericardium (Fig. 5).

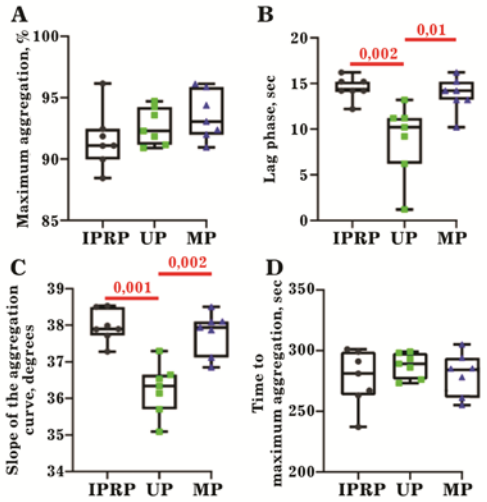

Fig. 4. Haemocompatibility of intact platelet-rich plasma (IPRP, left), unmodified (UP, center) and polyvinyl alcohol-modified (MP, right) bovine pericardium. A – maximum aggregation percent; B – lag phase (time to the beginning of the aggregation curve, seconds); C – maximum slope of the aggregation curve (degrees); D – time to the maximum aggregation (seconds)

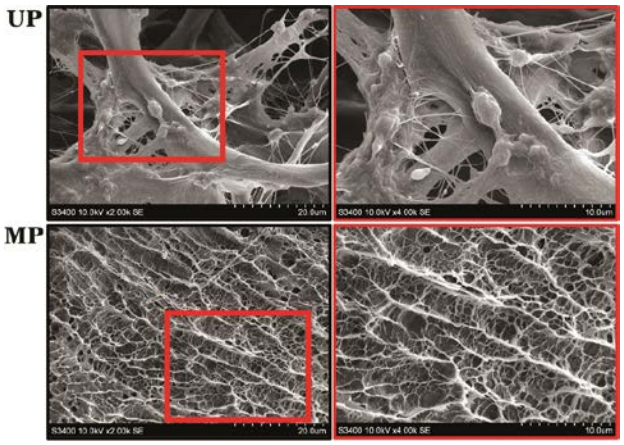

Fig. 5. Electron microscopy examination of platelet adhesion at unmodified (UP, center) and polyvinyl alcohol-modified (MP, right) bovine pericardium. Note the absence of platelets at the surface of polyvinyl alcohol-modified pericardium

The results obtained at analyzing the hemolysis upon the blood contact with the studied biomaterials indicated that both the control and PVA-modified bovine pericardium had no significant effect on erythrocyte lysis. Hemolysis rates for both types of biomaterials were less than 1% (data not shown).

The polyvinyl alcohol-modified bovine pericardium stability to calcification

The calcium content in PVA-modified samples after 3 and 6 weeks of incubation in a solution saturated with the calcium and phosphate ions averaged 1.52 mg/g and 6.89 mg/g of dry tissue, respectively (Fig. 6). Those figures for unmodified (control) bovine pericardium samples were 8.22 and 23.07 mg/g.

The study of patterns of calcification locations in the samples showed that calcification can be visualized as soon as after 3 weeks of incubation in the unmodified bovine pericardium, meanwhile the microcalcifications

formed mainly in the surface layers of the biomaterial along the collagen fibers (Fig. 6). After 6 weeks of incubation, microcalcification spread to the entire thickness of the control samples. As for the PVA-modified bovine pericardium, no calcium presence was detected by light microscopy after 3 weeks of incubation, however, microcalcification was seen after 6 weeks of incubation. We should note that microcalcifications were located mainly near the edge of the biological tissue, which had been dissected while preparing the fragment specimens for testing. Thus, the data obtained indicate the excellent insulating properties of the PVA gel formed in the biomaterial.

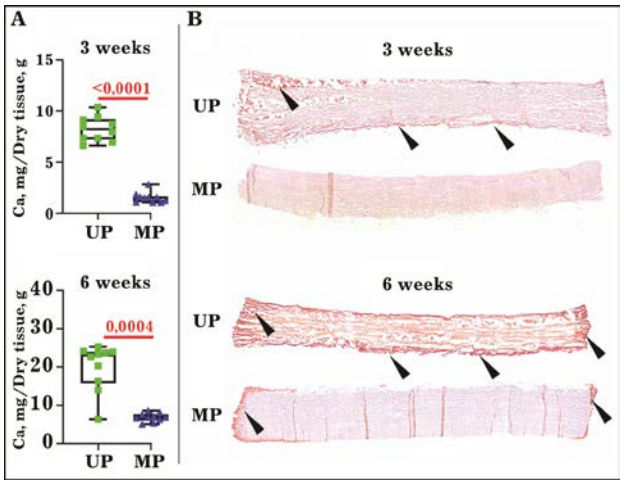

Fig. 6. Assessment of calcification of unmodified (UP) and polyvinyl alcohol-modified (MP) bovine pericardium. A – calcium measurement upon the tissue lysis, B – visualisation of calcification patterns by alizarin red S staining. Arrows indicate the most calcified areas

Discussion

Structural degeneration of BHVs occurs through several mechanisms. These include a fatigue destruction of biomaterial collagen fibers under the action of cyclic loads, as well as the calcification and proteolysis occurring

with the participation of calcium ions, calcium-binding proteins, and proteolytic enzymes [12]. The listed substances accumulate in the biomaterial as a result of their passive imbibition from the recipient's blood and during their secretion by immune cells infiltrating the BHV leaflets [13–15]. It is important to note that there are currently no effective ways to prevent structural degeneration of BHVs [4].

The main areas of research and developments aimed at reducing the rate of BHV structural degeneration include optimizing the design of the valvar apparatus of the implants and their manufacture using a low-immunogenic biomaterial obtained from genetically modified animals or by decellularization of biological tissue [12]. The former approach reduces the mechanical load on bioprosthetic heart valve leaflets, slowing down the fatigue destruction of their collagen matrix, while the latter is designated to reduce the immune response to the biomaterial [12]. Nevertheless, no attempts to prevent the BHV impregnation with substances circulating in recipients' blood have been undertaken so far.

In the future, the modified biomaterial we have proposed will be able to solve the mentioned problem: PVA gel fills the interfibrillar space of the biological tissue and creates a physical barrier for its penetration with the compounds from the blood plasma, including small-sized calcium ions. Thus, the PVA-processing of biomaterial makes it possible to slow down the calcification rate, which has been confirmed by our in vitro experiments. In addition, the PVA sheath formed on the surface of the biomaterial can prevent the deposition of cellular elements (including platelets) on the BHV, inhibiting the implant immune rejection development and improving their thromboresistant properties.

We should note that since BHVs operate in an aggressive mechanical

environment, additional studies are needed to identify the fatigue-strength properties of the modified biomaterial. In particular, it is not yet known whether PVA gel will retain its insulating properties when subjected to cyclic loads. The cytotoxic properties of the PVA-treated biomaterial also require studying, which will allow us to draw conclusions regarding the safety of the considered modification. Finally, the study of antiprotease properties by incubation of PVA-modified bovine pericardium in collagenase will show whether this type of modification is sufficient to slow down proteolytic degradation of the biomaterial in the recipient's body. The above in vitro tests are the next stage of studies planned by our group as part of the implementation of the Russian Science Foundation Grant No. 21-75-10107. In accordance with their results, it will be possible to judge the feasibility of conducting preclinical trials on large experimental animal models and assess the prospects for implementing PVA-modified BHVs into clinical practice.

In the course of the study, based on the method of cryotropic gelation with polyvinyl alcohol, a modification of the epoxy-treated bovine pericardium used in the manufacture of bioprosthetic heart valves was developed. This modification did not worsen the mechanical and hemocompatible properties of the biomaterial, but significantly changed its surface and internal structure: the gel formed a sheath on the bovine pericardium surface, sealing the natural pores of the biological tissue, and also filled the interfibrillar space to its entire thickness. The in vitro testing results demonstrated a significant slowdown of the calcification rate in the polyvinyl alcohol-modified bovine pericardium compared with that in the unmodified one. Thus, the biomaterial processing with polyvinyl alcohol can

be taken as a basis for the development of next-generation bioprosthetic heart valves.

Conclusion

A new method for modifying the epoxy-treated bovine pericardium with a 12% solution of polyvinyl alcohol followed by cryotreatment has been proposed. A composite material was obtained, which demonstrated a 5- and 3-fold statistically significant lower calcium content compared to the control bovine pericardium when they were incubated in a calcinating solution for 3 and 6 weeks, respectively.

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Information about the authors

Alexander E. Kostyunin, Cand. Sci. (Biol.), Researcher of the Laboratory of New Biomaterials, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0001-6099-0315, rhabdophis_tigrina@mail.ru

20%, literature analysis, development of the study design, analysis of the obtained data, writing the working versionof the article manuscript

Maria A. Rezvova, Junior Researcher of the Laboratory of New Biomaterials, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0002-4405-8904, rezvovamaria@mail.ru

20%, development of an original method for modifying epoxy-treated biomaterial with polyvinyl alcohol, analysis of the obtained data, editing the text of the article

Tatyana V. Glushkova, Cand. Sci. (Biol.), Senior Researcher of the Laboratory of New Biomaterials, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0003-4890-0393, bio.tvg@mail.ru

20%, data collection according to the study design assessment of the biomaterial mechanical properties, light, fluorescence and electron microscopy, analysis of the obtained data, editing the text of the article

Daria K. Shishkova, Cand. Sci. (Biol.), Researcher of the Laboratory of Molecular, Translational and Digital Medicine, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0002-1518-3888, shishkovadk@gmail.com

10%, evaluation of the biomaterials hemocompatibility, analysis of the obtained data

Anton G. Kutikhin, Cand. Sci. (Med.), Head of the Laboratory of Molecular, Translational and Digital Medicine, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0001-8679-4857, antonkutikhin@gmail.com

10%, editing the text of the article

Tatyana N. Akentieva, Junior Researcher of the Laboratory of New Biomaterials, Research Institute for Complex Issues of Cardiovascular Diseases, http://orcid.org/0000-0002-0033-9376, t.akentyeva@mail.ru

10%, sample preparation of the studied biomaterials, quantitative assessment of calcium content in the studied biomaterials, statistical processing of the received data

Evgeny A. Ovcharenko, Cand. Sci. (Tech.), Head of the Laboratory of New Biomaterials, Research Institute for Complex Issues of Cardiovascular Diseases, https://orcid.org/0000-0001-7477-3979, ov.eugene@gmail.com

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