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Expression of matrix metalloproteinases 1, 2, 9, 12 in xenogenic tissues of epoxy-crosslinked bioprosthetic heart valves explanted due to dysfunction

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Aim. To study the expression of matrix metalloproteinases (MMPs) 1, 2, 9, 12 in the leaflets of epoxy-crosslinked bioprosthetic heart valves (BHVs) explanted due to dysfunction and to study the possibility to accumulate these enzymes in xenogenic tissues.

Material and methods. We examined 19 leaflets of 7 mitral and aortic BHVs removed during re-replacement. Tissue sections for microscopy were prepared using a cryotome. Cellular typing and detection of MMP 1, 2, 9, 12 expression in samples were performed using immunohistochemical staining with antibodies to PTPRC/CD45, CD68, myeloperoxidase, and the corresponding MMPs. Analysis of samples was performed by light microscopy.

Results. In 17 studied leaflets from 6 explanted BHVs, sporadic infiltrates consisting of macrophages (PTPRC/CD45+, CD68+) were revealed. A positive staining for MMP 1, 2, 9, 12 was colocalized with immune cell infiltrates. Also, positive staining was observed without cell infiltration. The pericardial BHV removed due to thrombosis 2 days after implantation did not show signs of macrophage infiltration or MMP expression in xenogenic tissues, but the thrombus stained positive for MMP-9 and included a large number of neutrophils positive for myeloperoxidase.

Conclusion. Macrophages and other immune cells infiltrating xenogenic tissues of epoxy-crosslinked BHV are sources of MMPs 1, 2, 9, 12. In addition, MMP-9 can diffuse into BHV leaflets from the blood plasma of patients. The deposition of MMP may contribute to rupture and calcification of the leaflets leading to the implant dysfunction.

Key words: bioprosthetic heart valves, structural valve degeneration, cell infiltration, matrix metalloproteinases.

Relationships and Activities. This study was carried within the program of basic research on the fundamental subject of Research Institute for Complex Issues of Cardiovascular Diseases № 0546-2019-0002 “Pathogenetic rationale for the development of implants for cardiovascular surgery based on biocompatible materials, with the implementation of a patient-centered approach using mathematical modeling, tissue engineering methods and genomic predictors”.

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Today, heart valve prosthesis are the main method of radical treatment of heart defects [1, 2]. More than 200,000 such operations are performed annually in the world, and according to forecasts, by 2050, their number will increase to 850,000, which is associated with the population ageing in developed countries, and therefore, which means an increase in the prevalence of heart valve diseases [3]. Mechanical or xenogeneic bioprostheses (BP) of heart valves are most often used. The latter are made from animal derived tissues stabilized with glutaraldehyde (GA) or epoxides [4]. Optimal hemodynamic parameters and low thrombogenicity distinguish biological from mechanical prosthesis [1, 2]. However, even modern BP are prone to structural degeneration within 10-15 years after implantation, which limits their use in surgical practice [5].

The processes underlying the structural degeneration of BP are poorly understood. The data of modern studies suggest that they may be related on immune mechanisms, partly reminiscent of those involved in the transplant rejection and the development of atherosclerosis [6, 7]. The results of a number of original studies demonstrate the presence of dense macrophage infiltrates co-localized with areas of degenerated biological tissue in the explanted GA-fixed BP [8, 9]. Cells infiltrating BP have been shown to produce matrix metalloproteinases (MMPs) [8, 9]. The latter are zinc-related proteolytic enzymes that catalyze the cleavage of extracellular matrix (ECM) proteins, such as collagens and elastin [10]. Potentially, the deposition of MMPs in BP can contribute to the structural destruction of the prosthetic xenomaterial and the development of hemodynamically significant obstruction or regurgitation caused by calcification or rupture of the leaflets. The data of *in vivo* experiments, indicating that elastolysis promotes the calcification of biological tissue [11], confirm this assumption. Also, high levels of MMP-2 and especially MMP-9 were noted in the tissues of GA-fixed BP, explanted due to rupture [9].

Despite the potentially important role of MMPs in BP degeneration, a limited number of studies have been devoted to the expression of these enzymes in implant tissues [8, 9]. All MMPs present in BP tissues is still unknown. Possible ways of their accumulation in prosthetic valves are also poorly understood. There are no data on the presence of MMPs and their role in the degeneration of epoxy-treated BP in the literature. Thus, the aim of the present study was to assess the expression of MMP-1/-2/-9/-12 in xenogenic tissues of epoxy-treated BP explanted due to dysfunction and to identify related patterns. These MMPs were selected due to the fact that they are the most studied enzymes of the considered family, which are responsible for pathological remodeling of the leaflets' ECM [12].

Material and methods

The material was epoxy-treated mitral or aortic BPs (NeoKor, Russia) removed from 7 patients during re-replacement performed in 2019-2020. A total of 19 leaflets of 7 explanted BPs were examined. Among the latter, 4 samples were represented by xenogenic aortic BPs (KemCor (n=2); PeriCor (n=2)), 3 — pericardial (UniLine (n=2) and TiAra (n=1)). The mean age of patients at primary surgery was 57±11 years. The mean period of BP functioning was 12±8 years, except for the pericardial BP, which was excised 2 days after implantation due to thrombosis. The study was approved by the local ethics committee. All patients signed informed consent.

The material obtained during reoperations after macroscopic analysis was frozen at a temperature of -140° C. To study the cellular infiltration and expression of MMPs, serial sections of 7±1 μm were prepared using a Microm HM 525 Cryostat (Thermo Scientific, Germany). When making sections from each BP, the central part of 2-3 valves from the basis to the free edge was used, as well as areas with degenerative changes. Before staining, the sections were fixed for 10 min at room temperature with 4% paraformaldehyde solution, followed by three times washing (5 min each) in phosphate-buffered saline (PBS) (pH 7,4) on a shaker (Polymax 1040, Heidolph, 25 rpm). Cell typing (markers: PTPRC/CD45, CD68, and myeloperoxidase) and detection of MMP-1/-2/-9/-12 were performed by manual immunohistochemical staining, for which the corresponding primary antibodies (Abcam PLC, UK) were used (Table 1). Immunohistochemical reaction was performed using the NovoLink Polimer Detection System kit (RE7150-CE, Leica Microsystems Inc., USA) according to the manufacturer's modified protocol. First, endogenous peroxidase was blocked with 4% hydrogen peroxide solution (Peroxidase Block) for 5 min. Then the sections were washed twice in PBS and the nonspecific binding of antibodies with 0,4% casein saline solution with ancillary reagents (Protein Block) was blocked for an hour. Primary antibodies were diluted according to the manufacturer's protocol in 1% saline solution of bovine serum albumin. The dilutions used are shown in Table 1. Sections were incubated with antibodies for 20 hours in a closed box at +4° C. Then, the sections were washed three times in PBS and incubated with anti-rabbit antibodies (Novolink Polymer). After three-times washing in PBS, the sections were treated with 0,087% diaminobenzidine solution for 2 min. Then the sections were washed with double-distilled water and placed in hematoxylin for 10 min. Then, the sections were blued in running water (5 min), dehydrated in three changes of 95% ethanol (5 min each) and cleared in 3 changes of xylene (5 min each).

Table 1

Primary antibodies used in the study

Antigens used	Antibody reference numbers	Appointment	Dilution used
CD45	ab10558	Detection of PTPRC/CD45 (pan-leukocyte marker). Identification of immune cells in the BP.	1:3000
CD68	ab227458	Detection of CD68 (macrophage marker). Assessment of BP macrophage infiltration.	1:500
Myeloperoxidase	ab208670	Detection of myeloperoxidase (neutrophil marker). Assessment of the acute inflammatory response to BP.	1:1500
MMP-1	ab52631	Detection of MMP-1/-2/-9/-12. Revealing MMP expression in BP xenogenic tissues.	1:1000
MMP-2	ab92536		
MMP-9	ab38898		
MMP-12	ab52897		

Abbreviations: BP — heart valve bioprostheses, MMP — matrix metalloproteinases.

Samples with immunohistochemical stains were analyzed using an AxioImager.A1 light microscope (Zeiss, Germany). The images were processed using the AxioVision software (Zeiss, Germany).

Intact epoxy-treated xenogenic tissues were used as controls: porcine aortic valve leaflets and bovine pericardium. Also, one section with negative control of primary and secondary antibodies was isolated on each slide.

Results

Macroscopic analysis of the explanted BPs. The xenogenic aortic BPs included in the study had signs of primary tissue failure in the form of leaflet breaks in the commissural region, perforations, and calcification. The reason for re-replacement for these BPs was regurgitation. It is worth noting that the calcifications were insignificant.

Xenogenic pericardial BPs showed no signs of rupture and perforation of the leaflets. One of the three prostheses showed insignificant calcification. In one case, early thrombosis of BP was the cause of reoperation.

Characteristics of cellular infiltration of BPs. Infiltration of xenogenic material by the recipient's cells was detected in 17 leaflets taken from 6 explanted BPs that functioned for 2,5-25 years. In the studied samples, sporadic cellular infiltrates were noted, localized mainly on the surface or in the presurface layers of the xenogenic ECM near the base of the valves. Greater cellular infiltration was noted from the side of the excretory section. In turn, no signs of infiltration of the recipient's cells were found in the two studied valves, taken from the pericardial BP removed due to thrombosis 2 days after implantation. At the same time, the presence of a large number of cells in the thrombus. Aggressive cellular infiltration associated with the penetration of cells deep into the xenogenic material was detected only in the leaflets of aortic BPs, in the presence of large calcifications, perforations, or areas with pronounced dissociation of ECM, near which significant cell clusters were concentrated.

According to the results of immunohistochemical staining, it was found that the majority of cells infiltrating the prosthetic xenogenic biomaterial express PTPRC/CD45 and CD68 (Figure 1). In the leaflets of BP, which functioned for 2 days, these markers were not detected, but the cells in the thrombus were positive for myeloperoxidase (Figure 2). Cells positively stained with anti-myeloperoxidase antibodies were not detected in other samples.

Expression of MMPs in xenogenic tissues of explanted BPs. Immunohistochemical staining of sections with anti-myeloperoxidase antibodies (MMP-1/-2/-9/-12) revealed the studied enzymes in all BP leaflets, with the exception of samples taken from an implant that functioned for two days. A positive staining for MMP-1/-2/-12 was observed exclusively near cell infiltrates, while staining for MMP-9 was observed both in colocalization with cells and in acellular ECM (Figure 3). It is important to note that the intensity of MMP-9 staining did not depend on the presence of recipient cells in the samples and remained high even in their complete absence. The most intense MMP-9 staining was observed for areas with loose tissue, as well as for spongiosa of xenogenic aortic BPs (Figure 4). The leaflets of xenogenic BPs stained for MMP-9 more intensely than those of the pericardial BPs. In turn, the xenogenic biomaterial functioned for two days did not show the expression of the studied enzymes, but the thrombus formed on its surface showed positive staining for MMP-9.

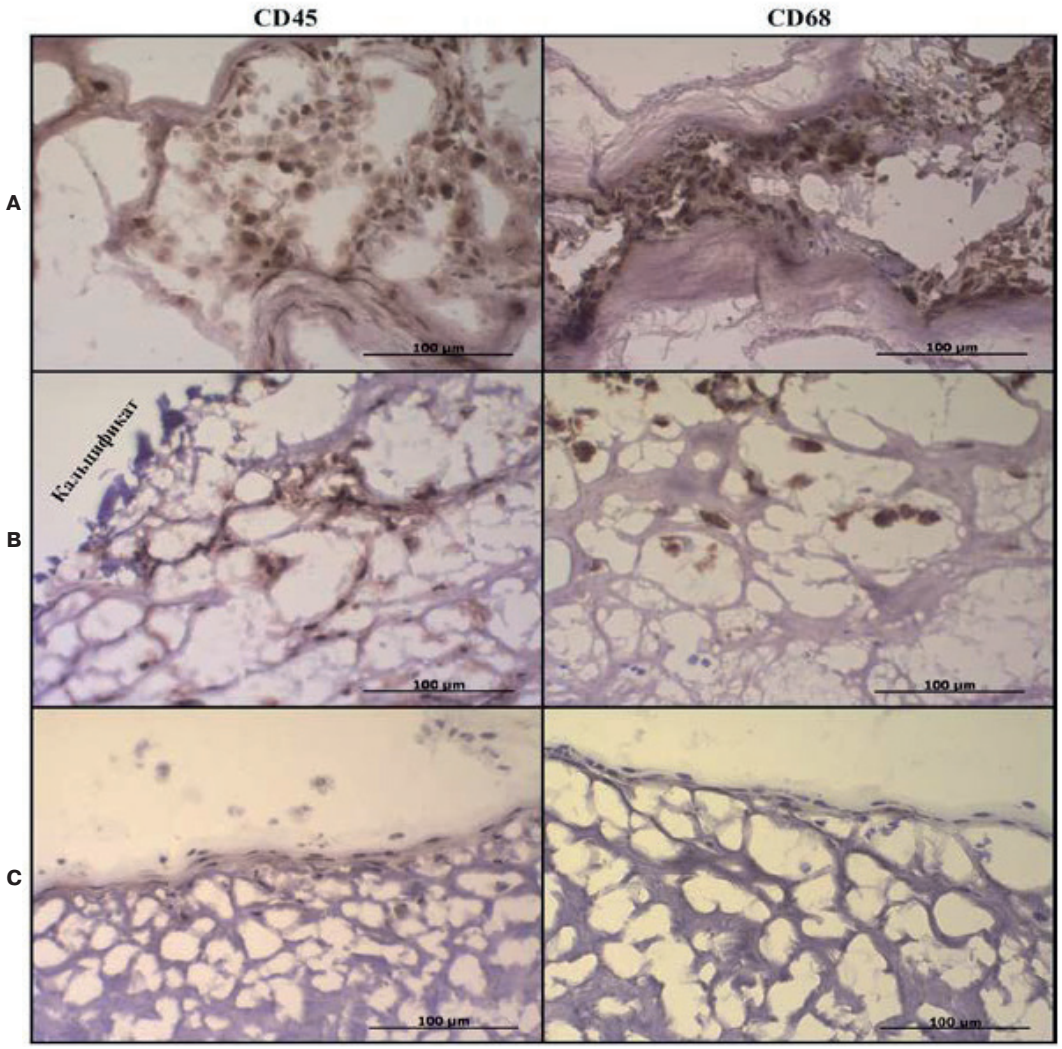

Figure 1. Results of immunohistochemical staining of samples for PTPRC/CD45 and CD68. The densest cellular infiltrates were noted in the loosened surface layers of leaflet ECM in xenogenic aortic BPs (**A**), while inside there were small groups of immune cells located near the calcifications (**B**). No infiltration of the recipient's cells into the depths of the xenogenic pericardial BPs was revealed: cell clusters were present only on the surface of the valves and had a weak staining for the studied markers (**C**).

In all controls, positive staining for PTPRC/CD45, CD68 and myeloperoxidase, as well as MMP-1/-2/-9/-12, was not detected.

Discussion

The results of this study are consistent with the data obtained by other authors on GA-treated BPs [8, 9]. Majority of cells were positive for PTPRC/CD45 and CD68, which indicates macrophage infiltration of epoxy-treated implants and indicates their chronic immune rejection [7]. The leaflets of BP functioned for two days did not show macrophages but contained thrombotic masses with included neutrophils on their surface, which indicates an acute inflammatory response that occurs in the first days upon implantation of any foreign body [13]. Based on the data on staining with anti-myeloperoxidase antibodies, it can be concluded that the main source of MMP-1/-2/-12 are macrophages, while MMP-9 mainly diffuses from the blood plasma. It is important to note that the tendency associated with the accumulation of MMP-9 from plasma was revealed for the first time in xenogenic BPs. Differences in the color intensity for MMP-9 may indicate that the leaflets of xenogenic aortic BPs are characterized by a more pronounced diffusion of this enzyme as compared to pericardial BPs. Apparently, this pattern is due to the looser structure of the ECM of porcine aortic valve leaflets, formed as a result of glycosaminoglycan loss, which are not stabilized by GA and diepoxy compounds [14]. In turn, the bovine pericardium retains a dense structure even after treatment with preservatives, which probably prevents the diffusion

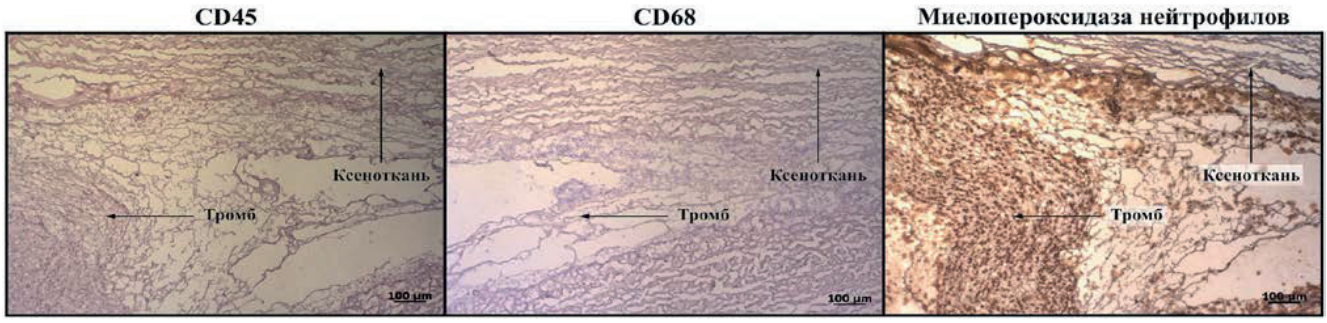

Figure 2. Immunohistochemical staining with anti-myeloperoxidase antibodies of BP leaflets, removed 2 days after implantation, shows the presence of numerous neutrophils in the thrombus formed on the surface of leaflets. At the same time, no cells positive for PTPRC/CD45 and CD68 were found in these samples.

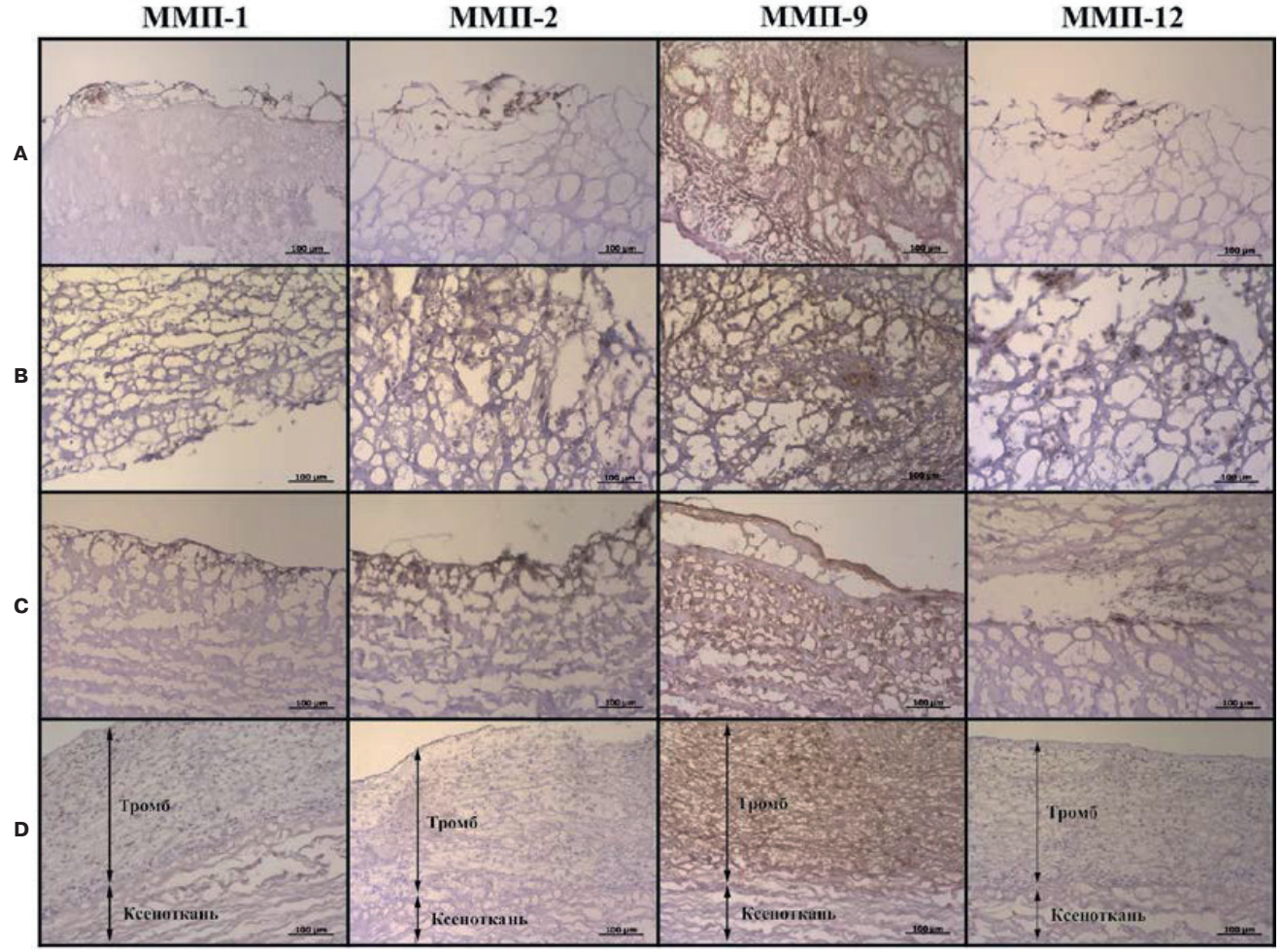

Figure 3. Results of immunohistochemical staining of samples for MMP-1/-2/-9/-12. MMP-1/-2/-12 are colocalized with the cells of the recipient. This pattern is clearly seen in the example of both weakly and strongly infiltrated by cells of leaflets of xenogenic aortic (**A**) (**B**) and pericardial (**C**) prostheses. In turn, staining for MMP-9 is almost independent of the presence of cells in the valves (**A**, **B**, and **C**). This suggests that the main source of MMP-9 is not cells, but the blood plasma of patients. The absence of expression of all MMPs in the xenogenic tissues of the prosthesis removed 2 days after implantation due to thrombosis (**D**), but intense staining of the thrombotic mass on its surface with anti-myeloperoxidase antibodies (MMP-9), confirms the hypothesis of the impregnation of leaflets with this enzyme.
Abbreviation: MMP — matrix metalloproteinases.

of substances from the surrounding liquids. It is noteworthy that GA-treated xenogenic pericardial BPs often require replacement due to calcification-related stenosis, while xenogenic aortic BPs are more prone to leaflet rupture [15]. In the present study, ruptures and perforations of leaflets were also noted for epoxy-treated xenogenic aortic BPs, which was not observed in pericardial BPsD. In

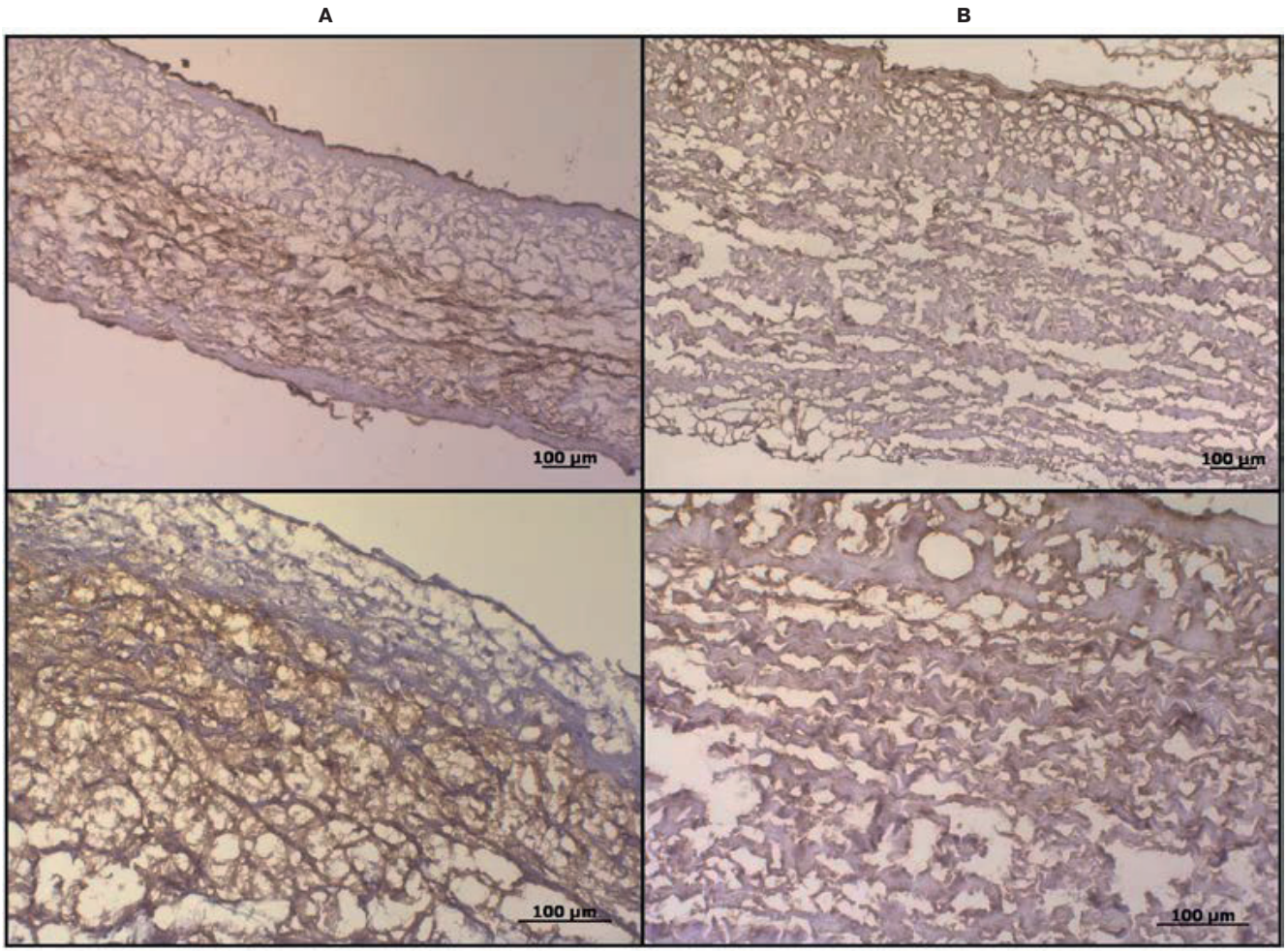

Figure 4. The intensity of staining for MMP-9 is related to the tissue density: the denser and more structured the matrix, the weaker the enzyme signal. This is noticeable when comparing the valves of xenogenic aortic (**A**) and pericardial prostheses (**B**), especially their most intensely stained areas. In the leaflets of xenogenic aortic prostheses, the spongiosa is stained most intensively for MMP-9.

part, this can be explained by the more intensive accumulation of proteolytic enzymes from the blood plasma by the tissues of the latter, followed by the cleavage of collagen fibers of ECM.

Study limitations. The xenogenic aortic and pericardial BPS used in the study are not comparable in terms of the functioning duration and the causes of dysfunctions. The former functioned for 15±6,5 years, the latter for 4±2 years. Thus, more aggressive cellular infiltration and more pronounced staining for MMP-9 of xenogenic aortic BPs as compared to pericardial BPs may be the result not of structural differences in their ECM. More severe tissue wear may be caused by a longer period of functioning. Further studies on comparable samples are needed to confirm the results.

Conclusion

Macrophages and other immune cells infiltrating xenogenic tissues of epoxy-treated BPs are sources of MMPs 1, 2, 9, 12. At the same time, the expression of MMPs is different: MMP-1/-2/-12 are localized exclusively near cell clusters, while high levels of MMP-9 can be detected even in the absence of cell infiltration. This observation suggests that MMP-9 diffuses into xenogenic biomaterial from the blood plasma of patients. The deposition of MMP may contribute to rupture and calcification of the leaflets leading to the implant dysfunction.

Relationships and Activities. This study was carried within the program of basic research on the fundamental subject of Research Institute for Complex Issues of Cardiovascular Diseases № 0546-2019-0002 “Pathogenetic rationale for the development of implants for cardiovascular surgery based on biocompatible materials, with the implementation of a patient-centered approach using mathematical modeling, tissue engineering methods and genomic predictors”.

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