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FEATURES OF IgG ANTIBODY DEPOSITION IN EPOXY-TREATED XENOGENEIC BIOPROSTHETIC HEART VALVES

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Objective: to evaluate changes in the immunogenicity of epoxy-treated xenogeneic bioprosthetic heart valves (BHVs) during *in vivo* function by detecting human IgG antibodies in the biomaterial of explanted samples. **Materials and methods.** Fourteen BHVs explanted during repeat valve replacement were examined. Of these, three valves were removed at 1, 20, and 42 days after implantation, while 11 valves had functioned for periods ranging from 3 to 25 years. Histological sections were prepared from the valve leaflets and analyzed by immunohistochemistry using antibodies against human immunoglobulin G (IgG), as well as Russell–Movat pentachrome staining. Selected leaflet fragments were additionally examined by scanning electron microscopy. **Results.** BHVs removed due to early dysfunction after 1, 20, and 42 days of implantation showed no signs of biomaterial degeneration; however, small thrombi were detected on their leaflet surfaces. In contrast, valves that had functioned for 3 to 25 years exhibited clear features of structural degeneration of the biological tissue, including leaflet tears and extensive calcification. These BHVs were also characterized by moderate macrophage infiltration and a slight increase in pannus on the valve surface. Immunohistochemical staining of histological sections for human IgG revealed intense antibody deposition within the biomaterial of all examined BHVs, irrespective of implantation duration. Positive IgG staining was localized along the fibers of the xenogeneic tissue and was absent in recipient tissues, including thrombi and pannus. **Conclusion.** The biological component of BHVs retains its immunogenic properties even after long-term (up to 25 years) function in the recipient's body.

Keywords: bioprosthetic heart valves, structural valve degeneration, IgG antibodies, immune response, immunohistochemistry.

INTRODUCTION

The gold standard for treating severe valvular heart disease is replacement of the affected valves with biological or mechanical prosthetic valves [1, 2]. In recent decades, xenogeneic bioprosthetic heart valves (BHVs), derived from chemically stabilized animal tissues such as the porcine aortic complex and porcine, bovine, or equine pericardium, have been increasingly adopted for this purpose [3–5]. Compared with mechanical prostheses, BHVs offer the advantage of low thrombogenicity, allowing patients to avoid lifelong anticoagulation therapy and its associated risk of hemorrhagic complications. However, their long-term durability remains limited: approximately 50% of BHVs require replacement within 15 years due to prosthetic dysfunction associated with structural valve degeneration (SVD) [6–8]. Immune-mediated rejection of the implant is considered one of the principal mechanisms driving the development of SVD [9, 10].

Several research groups have reported dense white blood cell (WBC) infiltration in BHV leaflets explanted from patients with SVD [11–14]. In addition to cellular immune components, deposits of humoral immune factors – including IgM and IgG antibodies, the complement component C4d, and the membrane attack complex C5b-9 – have been identified within a damaged valve biomaterial [14–16]. Notably, the contribution of antibodies and complement to SVD pathogenesis has been less extensively studied than that of cell-mediated inflammation [17]. Antibody-mediated mechanisms are typically considered in terms of opsonization of xenogeneic BHV tissues, which facilitates subsequent WBC recruitment [17]. By binding via their Fab regions to residual animal antigens within the biomaterial, antibodies can engage Fcγ receptors on monocytes and other immune cells through their Fc domains, thereby promoting cellular activation and adhesion to the BHV surface [18]. Infiltrating WBCs within the biomaterial subsequently release matrix-degrading factors – including proteolytic and oxidative enzymes, as well as calcium-binding proteins – contributing to progressive degenerative changes in the implant flaps [9, 10].

Valve replacement with xenogeneic BHVs is known to induce a marked (2- to 5-fold) increase in recipient antibody titers directed against specific animal antigens, including galactose-α-1,3-galactoside (α-Gal) and N-

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glycolylneuraminic acid (N-GNA) [16, 19, 20]. Elevated levels of antibodies to both α-Gal and N-GNA have been detected for up to 2–4 years following BHV implantation [16, 19]. However, these initially heightened antibody titers tend to decline approximately 2 years after valve replacement, indirectly suggesting antigen elimination from the biomaterial [16]. Clinical observations are consistent with our previous immunohistochemical findings, which demonstrated gradual degradation of N-GNA within BHV tissue, with near-complete elimination occurring approximately 2.5 years after implantation [21].

Taken together, these findings suggest a decline in the residual immunogenicity of BHVs over time. This observation appears to contradict histological evidence, which shows that the most pronounced WBC infiltration occurs not at early but at later stages of implant function [11–14].

To address this discrepancy, we evaluated changes in BHV immunogenicity by assessing the presence of human IgG antibodies in tissues from explanted xenografts that had functioned for varying durations, ranging from 1 day to 25 years.

MATERIALS AND METHODS

Study samples

The study included 14 epoxy-treated xenogeneic BHVs removed from recipients during repeat valve replacement in the aortic (n = 2) and mitral (n = 12) positions. All prosthetic valves were manufactured by NeoCor CJSC (Kemerovo, Russia), including UniLine (n = 6) and Tiara (n = 2) xenopericardial valves, as well as PeriCor (n = 2) and KemCor (n = 4) xenoaortic implants. Notably, only Tiara prostheses were used for aortic valve replacement.

In this series, three xenopericardial valves were explanted after short-term function (1, 20, and 42 days). The removal of the valve after 1 day was unrelated to prosthesis dysfunction and was performed during emergency surgery due to rupture of the posterior wall of the left ventricle. The Tiara valves functioning for 20 and 42 days were explanted due to frame deformation leading to device failure. The remaining 11 BHVs were removed due to SVD after 3 to 25 years of implantation.

Overall, xenopericardial BHVs demonstrated a shorter functional lifespan compared to xenoaortic prostheses – 68 [45.5–76.5; 34–78] months versus 194 [168.8–240.8; 129–300] months, respectively (data presented as median, interquartile range, and minimum–maximum values: Me [25–75%; min–max]). A detailed description of the studied BHVs is provided in Table.

Histological and immunohistochemical staining

The explanted BHVs were placed in sterile 0.9% sodium chloride solution and transported to the laboratory. After macroscopic examination, the valves were processed for histological and immunohistochemical analysis. Fragments of the valve apparatus were embedded in Neg-50™ rapid tissue freezing medium (6502, Thermo Fisher Scientific, USA), and 6-μm sections were prepared using an HM525 cryostat microtome (Thermo Fisher Scientific, USA). The sections were mounted onto glass microscope slides. Intact BHVs of the UniLine and KemCor models were used as controls.

Table

Key characteristics of the studied bioprosthetic heart valves and their recipients

Case	Patient gender	Patient's age at the time of reoperation, years	BHV model	BHV lifespan
Early dysfunction				
1	Female	71	UniLine	1 day
2	Female	66	TiAra	20 days
3	Male	75	TiAra	42 days
Late dysfunction				
4	Male	72	UniLine	34 months
5	Female	77	UniLine	63 months
6	Male	66	UniLine	68 months
7	Female	37	UniLine	75 months
8	Female	72	UniLine	78 months
9	Female	67	PeriKor	129 months
11	Male	68	PeriKor	182 months
12	Female	70	KemKor	184 months
10	Female	59	KemKor	204 months
13	Male	69	KemKor	221 months
14	Male	60	KemKor	300 months

Note: BHV, bioprosthetic heart valve.

To differentiate the major components of the extracellular matrix within the valve leaflets, Russell–Movat pentachrome staining was performed using a commercial kit (ab245884, Abcam, UK) according to the manufacturer's instructions.

Human IgG was detected in tissue sections using a specific primary antibody (MAA544Hu22, Cloud-Clone Corp., PRC) in combination with the NovoLink Polymer DS immunohistochemical staining kit (RE7150-CE, Leica Biosystems, USA). Sections were first fixed in 4% paraformaldehyde for 10 minutes at room temperature and subsequently washed three times in phosphate-buffered saline (PBS, pH 7.4) for 5 minutes each. Immunohistochemical staining was then performed according to the manufacturer's protocol. The anti-human IgG antibody was diluted 1:16,000 in 1% bovine serum albumin (BSA) in saline. Sections were incubated with the primary antibody in a humidified chamber at 4 °C for 18 hours.

After staining, sections were mounted with VitroGel mounting medium (HM-VI-A250, BioVitrum, Russia) and coverslipped. Negative controls were processed in parallel by omitting the primary antibody.

Light microscopy and image processing

Stained sections were visualized by scanning slides with an MT5300L automated biological microscope (Meiji Techno, USA). Digital image acquisition and processing were performed using QuPath software (v0.6.0) [22].

Scanning electron microscopy

Ultrastructural analysis of explanted BHV leaflets was performed using scanning electron microscopy (SEM). Sample preparation followed a previously established protocol [23]. Briefly, biomaterial fragments were fixed for 24 hours (in two 12-hour cycles) in 10% neutral buffered formalin (B06-003, ErgoProduction, Russia) at 4 °C. Samples were then washed three times in PBS (pH 7.4) for 10 minutes each and sequentially incubated in 1% buffered and 2% aqueous osmium tetroxide solutions (19110, Electron Microscopy Sciences, USA) for 16 and 40 hours, respectively.

Following fixation, tissues were washed four times in PBS (15 minutes each) and dehydrated through a graded ethanol series (50%, 60%, 70%, 80%, and 95%; two changes of 15 minutes per step). Samples were then stained with 2% uranyl acetate in ethanol (22400-2, Electron Microscopy Sciences, USA) for 16 hours. Subsequent dehydration was carried out in 95% ethanol (two 15-minute changes), followed by isopropanol (06-002, ErgoProduction, Russia) and acetone (6-09-20-03-83, EKOS-1, Russia), each for 2 hours. The samples were then impregnated with a 1:1 mixture of acetone and epoxy resin (13900, Electron Microscopy Sciences, USA) for 16 hours, followed by incubation in pure epoxy resin for 24 hours, and finally immersed in fresh epoxy resin at 60 °C for 24 hours.

After resin hardening, samples were ground and polished using a TegraPol-11 system (Struers, Denmark), contrasted with Reynolds lead citrate (17810, Electron Microscopy Sciences, USA) for 7 minutes, and rinsed in double-distilled water (three times for 5 minutes each).

Finally, the specimens were carbon-coated using a Leica EM ACE200 sputter coater (Leica Microsystems, Germany) and examined with an S-3400N scanning electron microscope (Hitachi, Japan) in high-vacuum mode at an accelerating voltage of 10 kV, using backscattered electron detection.

RESULTS

Macroscopic examination of the explanted BHVs revealed no evidence of biomaterial degeneration in the three samples removed during the early postoperative period (1, 20, and 42 days), whereas all remaining valves exhibited characteristic features of SVD.

UniLine xenopericardial valves showed extensive calcification, predominantly localized in the commissural regions, as well as at the base and dome of the leaflets. In xenoaortic valves (PeriCor and KemCor), large calcific deposits were also observed, accompanied by structural damage, including commissural tears and perforations of the leaflet domes.

Moderate connective tissue overgrowth (pannus) was identified at the base of the leaflets and within the commissural regions on the outflow side of BHVs. Pannus was noted in valves that had been in use for more than 3 years.

Additionally, isolated microthrombi were detected on the surface of all examined leaflets, including those from valves explanted at 1, 20, and 42 days. However, no large thrombotic masses or vegetations were observed.

Histological analysis of Russell–Movat pentachrome–stained sections revealed a comparable histopathological pattern in xenopericardial and xenoaortic BHVs. In both implant types, the leaflets were composed predominantly of collagen, with no evidence of elastic fibers or mucopolysaccharides, although minor fibrin deposits were detected on their surfaces (Fig. 1a). Pannus tissue on the leaflets was readily distinguishable due to its high mucopolysaccharide content (Fig. 1b).

Cellular infiltrates were mainly localized to the superficial layers of the biomaterial and along the valve surface (Fig. 1c). In BHVs explanted within 1–42 days, recipient cells were observed only sporadically, whereas valves removed after longer periods exhibited dense cellular clusters. It should be noted that in xenoaortic – but not xenopericardial – valves, foci of cellular infiltration extended into the tissue parenchyma, particularly around

the edges of perforations and large calcific deposits. Ultrastructural examination indicated that these cellular clusters were predominantly composed of canonical macrophages, with occasional neutrophils present (Fig. 1d).

The immunohistochemical analysis revealed intense positive staining for human IgG antibodies in all examined BHVs, independent of their type or duration of implantation (Fig. 2). It is important to note that the positive signal was localized specifically to the biomaterial fibers, whereas microthrombi and pannus on the leaflet surfaces did not exhibit IgG staining, indicating that the antibodies were selectively bound to the biomaterial. In contrast, intact control BHV tissues showed no positive staining.

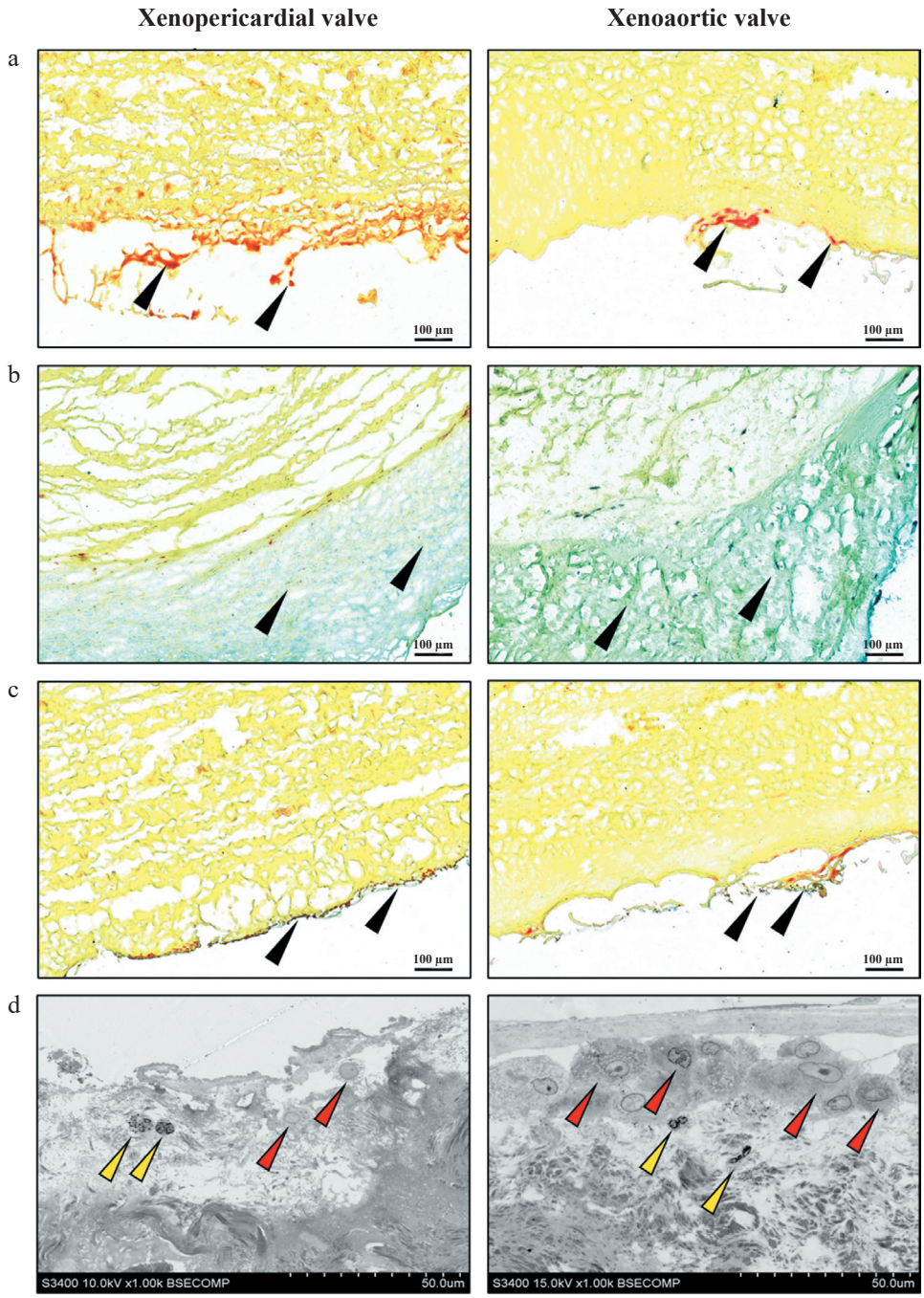

Fig. 1. Movat pentachrome staining of tissues from the studied bioprosthetic heart valves. (a) Leaflets of xenopericardial and xenoaortic valves composed predominantly of collagen fibers (yellow staining), with no evidence of elastin or mucopolysaccharides; but minimal fibrin deposits are present on the surface (red staining; arrows indicate microthrombi). (b) Pannus formed on the surface of the bioprosthetic valve apparatus shows a dense structure composed of collagen fibers rich in mucopolysaccharides (green staining; arrows indicate pannus). (c) As observed in both xenopericardial and xenoaortic valves, recipient cells infiltrate mainly the surface and superficial loose layers of the biomaterial (arrows indicate recipient cells). (d) Electron microscopy demonstrates that the cellular infiltrates within the bioprostheses consist predominantly of macrophages (red arrows) with occasional neutrophils (yellow arrows)

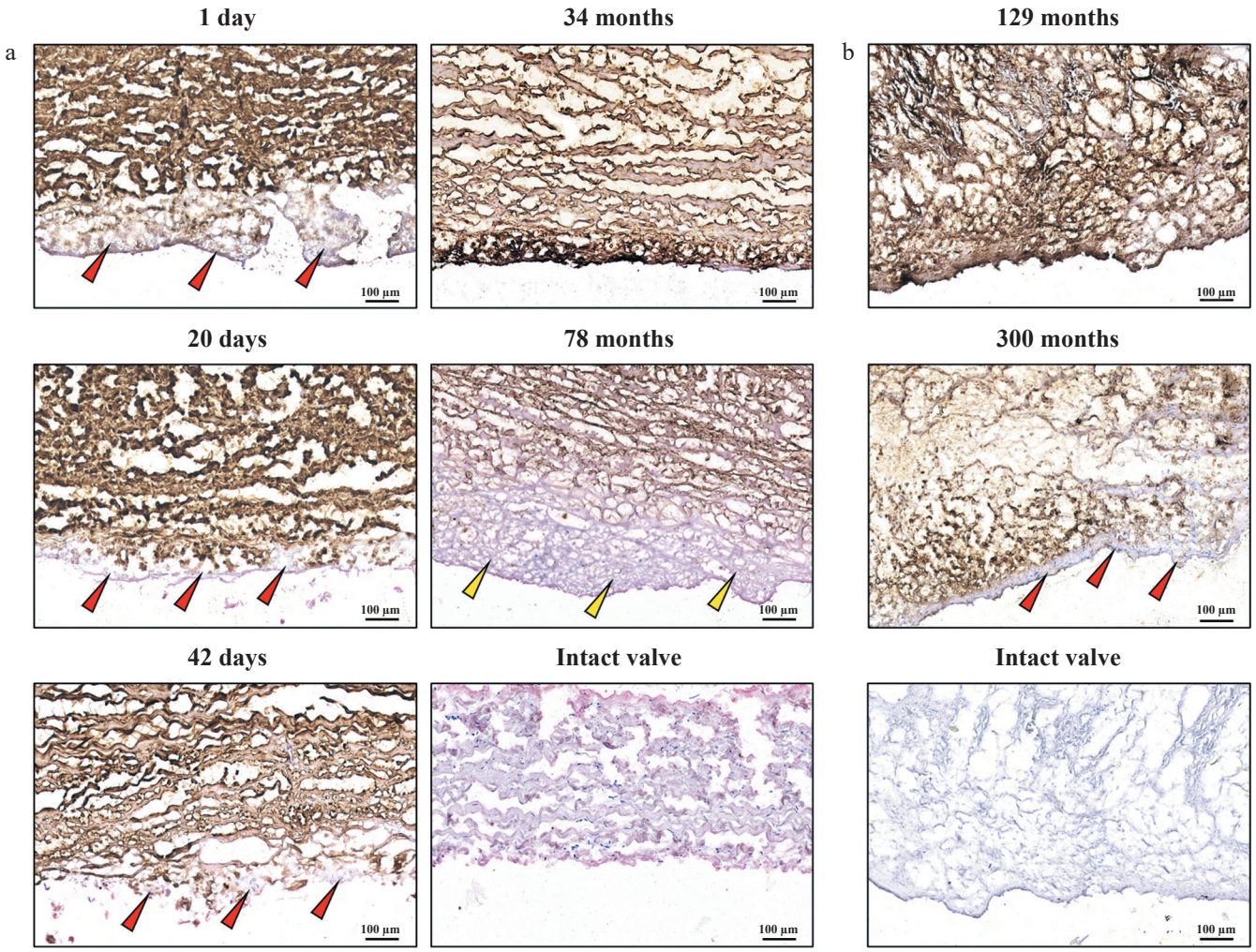

Fig. 2. Immunohistochemical staining of the studied bioprosthetic heart valves. Biomaterial from both xenopericardial (a) and xenoaortic (b) valves explanted from patients was intensely stained with anti-IgG antibodies, regardless of the implantation duration (bioprostheses with the shortest and longest functional periods are shown). In contrast, recipient-derived tissues, including thrombi and pannus, exhibited no positive staining for human IgG (red arrows indicate microthrombi; yellow arrows indicate pannus)

DISCUSSION

In all xenogeneic BHVs examined, the biomaterial contained both human IgG immunoglobulins and clusters of WBCs, primarily macrophages and neutrophils. The pattern of cellular infiltration – particularly the absence of lymphocytes – indicates a nonspecific immune response, consistent with findings from other studies [11–14]. Notably, although recipient antibodies rapidly deposited on the leaflets within the first hours of valve function, cellular invasion occurred much later. Only isolated monocytes and macrophages were observed on BHVs removed in the early postoperative period (1, 20, and 42 days), whereas valves functioning for three years or more exhibited extensive leukocyte infiltrates. These findings suggest that opsonization of chemically fixed xenobiomaterial by antibodies is not the primary trigger for immune cell migration and adhesion to the implant.

An interesting finding of this study is that the deposition of human immunoglobulins in the biological component of BHVs does not diminish over time: all examined valve leaflets showed intense positive staining for human IgG antibodies, regardless of implantation duration. Although previous studies have reported the gradual elimination of certain animal antigens (e.g., α-Gal and N-Gly) in BHVs, our results indicate that the xenobiomaterial remains highly immunogenic even 25 years post-implantation [16, 21]. This persistent immunogenicity is likely due to porcine and bovine collagen stabilized with di-epoxide compounds, which forms the core of the valve biomaterial and may itself act as an antigen, eliciting an antibody-mediated immune response. Additionally, 19 proteins have recently been identified in glutaraldehyde-fixed bovine pericardium that trigger specific antibody production in BHV recipients [20]. Most of these protein antigens are subcellular and appear to derive from residual donor cells [20]. This underscores the importance of the immunogenic role of the biomaterial's cellular component.

The mechanisms by which antibody deposition in BHV tissues accelerates SVD development remain unclear, but the role of the antibody-mediated immune response in valve degeneration has been demonstrated *in vivo*. For example, pretreatment of glutaraldehyde-stabilized biomaterial fragments with antibodies enhanced calcification in mouse, rat, and rabbit subcutaneous implantation models [16, 24–26].

It should be noted that this study is the first to evaluate changes in the immunogenicity of epoxy-treated xenogeneic BHVs over time. However, it has limitations: most importantly, we have not identified the specific xenobiomaterial components that trigger antibody production and binding to BHV tissues. Future studies to identify these antigens and investigate their stability could substantially advance the development of immunologically inert implants.

CONCLUSION

Intensive deposition of human IgG immunoglobulins occurs in epoxy-treated xenogeneic BHV tissues as early as the first day post-implantation. Notably, the capacity of epoxy-treated xenobiomaterials to bind antibodies remains preserved even after 25 years of valve function in the recipient. This indicates that their immunogenic properties persist despite the gradual degradation of certain carbohydrate xenoantigens previously documented.

COMPLIANCE WITH ETHICAL PRINCIPLES

The study adhered to the principles of Good Clinical Practice and the World Medical Association's Declaration of Helsinki. Approval was obtained from the local ethics committee of the Research Institute for Complex Issues of Cardiovascular Diseases (Protocol No. 19, November 6, 2018). All participants provided written informed consent.

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The authors declare no conflict of interest.

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