

# Three-Month Outcomes of Aortic Valve Reconstruction Using Collagenous Membranes (Biosheets) Produced by in Body Tissue Architecture in A Goat Model: A Preliminary Study

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## Research article

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# Abstract

**Background:** Autologous pericardium is widely used as a plastic material in intracardiac structures, in the pulmonary artery, and in aortic valve leaflets. For aortic valve reconstruction (AVRec) using the Ozaki procedure, it has produced excellent clinical results within a 10-year period. In-body tissue architecture (iBTA), which is based on the phenomenon of tissue encapsulation of foreign materials, can be used to prepare autologous prosthetic tissues. In this preliminary study, we examined whether biosheets can be used as valve leaflet material for glutaraldehyde-free AVRec by subchronic implantation experiments in goats and evaluated its performance compared with glutaraldehyde-treated autologous pericardium for AVRec.

**Methods:** Biosheets were prepared by embedding molds for two months into the dorsal subcutaneous spaces of goats. Allogenic biosheets (n=4) cut into the shape of the valve were then implanted to the aortic valve annulus of four goats for three months without glutaraldehyde treatment. Autologous pericardium (n=4) was used as a control. Valve function was observed using echocardiography.

**Results:** All goats survived the three-month study period. In biosheets, the leaflet surfaces were very smooth and, on histology, partially covered with a thin neointima (including endothelial cells). Biosheets were more thoroughly assimilated into the aortic root compared with autologous pericardium.

**Conclusions:** For the first time, biosheets could be used for large animal AVRec without glutaraldehyde fixation. In this limited preliminary study, it was found that biosheets had superior engraftment and regeneration performance as an aortic valve leaflet material compared with autologous pericardium.

## Background

Valvular insufficiency due to heart disease requires frequent reconstruction using mechanical valves, biological valves, or autologous pericardium valves. Autologous pericardium is widely used as a plastic material in intracardiac structures, in the pulmonary artery, and in aortic valve leaflets. For aortic valve reconstruction (AVRec) using the Ozaki procedure, autologous pericardium is used after fixation with glutaraldehyde. This has produced excellent clinical results within a 10-year period [1]. This method has almost no immunologic reaction, possessing the characteristics of its natural counterparts. However, fixation with glutaraldehyde is required for this method. Glutaraldehyde fixation limits growth adaptability. Tissue engineering applies methods of scientific engineering to create viable structures. In-body tissue architecture (iBTA), which is based on the phenomenon of tissue encapsulation of foreign materials, can be used to prepare autologous prosthetic tissues [2]. This technology has been successfully applied to the engineering of cardiovascular tissues, of vascular grafts as biotubes, or of heart valve-like tissues as biovalves. Biosheets, which are iBTA-induced membranous tissues, have been applied to the cornea [3], as well as to the repair of the diaphragm [4], esophagus [5], and trachea [6]. The studies that have reported on this technology indicated the possibility of tissue regeneration, self-repair,

and growth adaptability. This technology could have potential for use as an alternative aortic leaflet material in AVRec.

In this study, we examined whether biosheets can be used as aortic valve leaflet material for glutaraldehyde-free AVRec by subchronic implantation experiments using goats and evaluated its performance and histology compared with glutaraldehyde-treated autologous pericardium for original AVRec.

## Methods

### Animals procedures

Eight adult Saanen goats (body weight,  $51.1 \pm 6.4$  kg) were purchased Inoue Farm from (Gunma, Japan). An approval/permission from the farm owner to use the animals was not necessary. Animals were housed one in each cage under the same conditions, with dark-light cycles of 12 h and constant temperature of  $24 \pm 2$  °C with ad libitum access to food and water. The animals had olfactory, visual, and auditory contact with the other goats. Animals were distributed randomly into different groups. All animals were cared in accordance with the "Guide for the Care and Use of Laboratory Animals", published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Oita University Animal Ethics Committee approved the protocols (OITA-R022001) used in the present study. AVRec was performed in eight goats using different materials: biosheet or glutaraldehyde-fixed autologous pericardium.

### Preparation of Biosheets

The mold with an alternating pattern was assembled by inserting a silicone tube into a stainless steel pipe and fixing both ends with caps so that the tube is placed at the center. Hence, the gap constituting the tissue formation space between the silicone tube and the stainless steel pipe was 1 mm. The stainless steel pipe had many line slits. Biosheets were prepared by embedding molds in the goats' dorsal subcutaneous spaces under general anesthesia (Fig. 1b-1). Two months after the implantation of the molds, these biosheets were extracted while preserving the collagenous tissue membrane (Fig. 1b-2). Excess tissues were cut from the sheets, and the sheet thickness was 1 mm. The tubular tissues formed in the molds were removed and dried overnight. Biosheets (5×7 cm) were obtained by cutting the tubular tissues in the longitudinal direction and then soaking them in a 70% ethanol solution.

### Preparation of Autologous Pericardium

Adult goats underwent AVRec using autologous pericardium. The heart was exposed via a left thoracotomy at the third costal bed. The autologous pericardium was harvested. The pericardium was excised at least 5×7 cm (Fig. 1a-1). The excised pericardium was then treated with 0.6% glutaraldehyde solution with saline for 10 minutes. The treated pericardium was rinsed thrice using a saline solution for 6 minutes.

### Aortic Valve Reconstruction

Cardiopulmonary bypass (CPB) was initiated following heparin administration (400 U/kg). The aortic arch was cannulated with an 18 Fr arterial cannula, and a 25-23 Fr two-stage venous drainage cannula was placed into the right atrium. The ascending aorta was cross-clamped, and then cardiac arrest was induced with cardioplegia. The ascending aorta was opened using a transverse aortotomy. After resection of the native aortic valve cusps, the distance between each commissure was measured using the Ozaki sizer. Both the biosheet and the autologous pericardium were cut to a leaflet shape using the Ozaki template corresponding to the measured size (Fig. 1a-2, 1b-2). The annular margins of the leaflets were attached to each annulus with 4-0 monofilament sutures (Fig. 1a-3, 1b-3). A 4-0 monofilament suture was used to close the aortotomy. The CPB was weaned off gradually and terminated. Epicardial and transesophageal echocardiography was performed. The left ventricular-aortic pressure gradient (LV-Ao PG) was measured, and aortic regurgitation and LV wall motion were assessed. Finally, the chest wall was closed. We did not use anticoagulants or antiplatelet drugs after the surgery. In this study, the implanted biosheets originated from the same goats. The biosheets were immersed in 70% ethanol prior to aortic valve reconstruction (AVRec). Epicardial and transesophageal echocardiography were performed under general anesthesia 3 months after AVRec. Animals were sacrificed by intravenous injection of KCL. The hearts were harvested as samples for morphological observation.

## **Histological Evaluation**

The aortic biosheet valve extracted specimens were fixed with 10% formalin, embedded in paraffin, sliced into longitudinal sections, and finally stained with hematoxylin–eosin (H&E), Elastica-van Giessen, Masson's trichrome (collagen stain), and von Kossa (calcium stain). In addition, a few sections of the biovalve were also stained for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) using immunohistochemical techniques; the protein was detected using monoclonal antibodies (Abcam, Cambridge, UK).

## **Results**

All goats survived for three months after AVRec treatment with biosheets (n=4) and glutaraldehyde-fixed autologous pericardium (n=4) under smooth movement of the leaflets with little regurgitation, widely opened positions, and adequately closed positions.

### **Echocardiographic Analysis**

#### **Autologous pericardium group**

Epicardial echocardiograms showed smooth leaflet movement with a peak pressure gradient averaging  $15.6 \pm 13.2$  mmHg. In addition, epicardial echocardiograms showed trivial aortic regurgitation (n=2) or none (n=2). Upon removal three months after implantation, the autologous pericardium leaflets had turned light brown, but there was minimal change in size or shape in all cases. The macroscopic appearances of the valve leaflets were similar to that of the native heart valve ones. No thrombus was observed on any luminal surfaces of the valves (Fig. 2a). Commissural cuts were not observed. None of the valves showed infectious endocarditis.

## Biosheet group

The average peak pressure gradient was  $14.7 \pm 9.8$  mmHg. Epicardial echocardiograms showed smooth leaflet movement (Fig. 3a-d), but some had regurgitation (none: n=2, mild: n=1, moderate: n=1). Upon removal three months after implantation, there was little change in size or shape in all biosheet leaflets. The harvested biosheets became transparent, similar to native aortic valve leaflets, and no thrombus was observed on their surfaces (Fig. 2b). A slight dissection at the commissure was observed in one biosheet leaflet (Fig. 2c), and another one had infectious endocarditis (Fig. 2d).

## Histological Examination

The valve leaflets of both the biosheet and the pericardium were thickened compared to a normal native aortic valve, and the surface was smooth in both materials. The surface was covered partially with a thin neointima (Fig. 4a-1, b-1). Both valves consisted of numerous collagen bundles, but the original pericardial fibrous tissue was intact (4a-1, 2, 3 and 4). The collagen bundles of pericardial autograft leaflets were intermingled, but those of the biosheet were extremely regular. The thickness was remarkable at the base, because it was attached to the aortic wall by the suture whereby tissue reaction may easily occur. At the base, the presence of myofibroblasts was confirmed in both sets of valve leaflets by  $\alpha$ -SMA immunostaining; however, there were more myofibroblasts in the biosheet than in the pericardial autograft leaflets (Fig. 4a-4, b-4). Although the materials had assimilated to an extent at the suture line of the aortic annulus as a tissue reaction, the boundary between the pericardial autograft leaflets and the reactive tissue was still clearly visible (Fig. 4a-4). With regard to the biosheet, the boundary was unclear, and it seemed that the new reactive tissue was replacing the attached biosheet (Fig. 4b-4). There were no calcifications observed (Fig. 4a-5, b-5).

## Discussion

Valvular insufficiency due to heart disease requires frequent reconstruction with the use of mechanical valves, biological valves, or autologous pericardium valves. Autologous pericardium is commonly used as a plastic material in intracardiac structures. For AVRec using the Ozaki procedure, autologous pericardium is used after glutaraldehyde fixation. This has produced excellent clinical results, but glutaraldehyde fixation limits growth adaptability. However, for young patients, there are concerns regarding the insufficient amount of the pericardium in reoperation cases, the decreased coaptation due to growth, and the undetermined durability of the pericardium [7].

Some papers have reported that biosheets based on iBTA can be reconstructed in a manner analogous to that of native tissue, for example, as cornea, trachea (biosheet), and blood vessels (biotube) [8, 9]. The technology based on iBTA have also been applied to heart valves (biovalve and stent-biovalve) [10, 11] and stent grafts (bio stent graft) [12]. The papers that reported on this technology indicated the possibility of tissue regeneration, self-repair, and growth adaptability.

To our knowledge, this is the first time that tissue-engineered valve leaflets have been successfully implanted as aortic valves using goats as a large animal. It was amazing that all goats survived for three months after implanting biosheets without requiring reinforcement treatment.

Biosheets were more thoroughly assimilated into the aortic root than the autologous pericardium. On histology, a greater number of smooth muscle actin-positive cells (myofibroblasts) had infiltrated into biosheet leaflets than in the autologous pericardium. This indicates the possibility of biosheet self-maturation, suggesting that the biosheet might acquire features such as growth adaptability. Moreover, it seems that the biosheet had started to assimilate into the heart tissue.

On the other hand, it was unfortunate that one biosheet leaflet had a slight cut at the suture with the aortic annulus. It is possible that the biosheet used had insufficient strength, or it may not have had long-term durability due to an unidentified defect. Therefore, in our other research, we investigated the physical properties of biosheets. A biosheet is formed in a mold, and thus, depends on the opening pattern of the mold which functions as a cell entry port. In the study of the tensile strength of biosheets according to the mold pattern (alternating pattern or parallel pattern) [13], and we compared the tissue stress of the circumferential direction with that of the longitudinal direction. The biosheets with an alternating pattern had low anisotropy. Therefore, the selection of appropriate biosheets as candidate materials for the substitution of the aortic leaflets should be taken into consideration in our future work. Unfortunately, one of biosheet model subjects developed infectious endocarditis. Nevertheless, chronic animal models may develop infectious endocarditis as one of the complications of cardiac surgery; we believe the infection was never caused by the biosheet.

In the technology based on iBTA, the recipient's body is used as a bioreactor. This technique has several advantages. For example, the tissue prostheses can be easily and safely fabricated in a wide range of shapes and sizes to suit an individual recipient by changing the mold design. Most importantly, this technique does not require complex in vitro cell management procedures or exceptionally clean laboratory facilities, which are extremely expensive and time consuming. Moreover, because the biosheets are completely autologous, they are expected to have little calcification with long-term implantation and to possess better growth potential when compared with mechanical valves or biological valves. Consequently, biosheets might be an ideal material for AVRec.

One of the most important features of iBTA-induced tissue is its ability to regenerate.

The current study demonstrated a few experiments using the biosheet. We are now increasing the number of experiments after careful selection of suitable biosheets for implantation, and investigations on the histological features of further long-term durability are ongoing.

## **Limitations of the study**

Although this study included large animal models, the number of animals used might not be enough to generalize the results. Also, as one of the biosheet models required cutting of tissues, we used thin

biosheets, which may have affected the strength of the biosheet. In the future, the quality of biosheets must be confirmed before implantation.

As one of the remedies to select biosheets with an appropriate thickness, we developed a system that can measure the total thickness of this material using optical coherence tomography (OCT, IVS-2000, Santec, Aichi, Japan). On the other hand, the surgical environment will improve, including clean treatment of biosheets to prevent infection. In addition, checking the number of bacteria before AVRec surgery and using clean biosheets can help avoid such complications. Lastly, our limitations must be considered while applying the results of this study for clinical use.

## Conclusions

An iBTA-based autologous connective sheet, biosheet, can be used in AVRec without requiring glutaraldehyde fixation. Biosheet in this experiment indicated the possibility of high tissue regeneration, suggesting optimal clinical advantage. Hence, this study can be considered as a preliminary study on the use of biosheets in AVRec.

## Abbreviations

AVRec: aortic valve reconstruction

iBTA: in-body tissue architecture

CPB: Cardiopulmonary bypass

LV-Ao PG: left ventricular-aortic pressure gradient

H&E: hematoxylin–eosin

$\alpha$ SMA:  $\alpha$ -smooth muscle actin

## Declarations

### Ethics approval and consent to participate

Adult Saanen goats were purchased Inoue Farm from (Gunma, Japan). An approval/permission from the farm owner to use the animals was not necessary. All animals were cared in accordance with the “Guide for the Care and Use of Laboratory Animals”, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and the ARRIVE guidelines. Oita University Animal Ethics Committee approved the protocols (OITA-R022001) used in the present study.

### Consent for publication

Not applicable.

## Availability of data and materials

The datasets that support the findings of this study are available from the corresponding author on reasonable request.

## Competing interests

Yasuhide Nakayama is the employee of Biotube Co., Ltd. Keitaro Okamoto, Tadashi Umeno, Takashi Shuto, Tomoyuki Wada, Hirofumi Anai, and Shinji Miyamoto declare that they have no conflict of interest.

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## Authors' contributions

KO, SM and YN, contributed to the study concept and design. KO, TU, TW and HA, researched and KO and TU interpreted the data. KO drafted the manuscript. KO, TU, HN contributed to data analysis. SM and YN supervised the progress of the project, contributed to the discussion and critically approved the final version of the manuscript.

## Acknowledgements

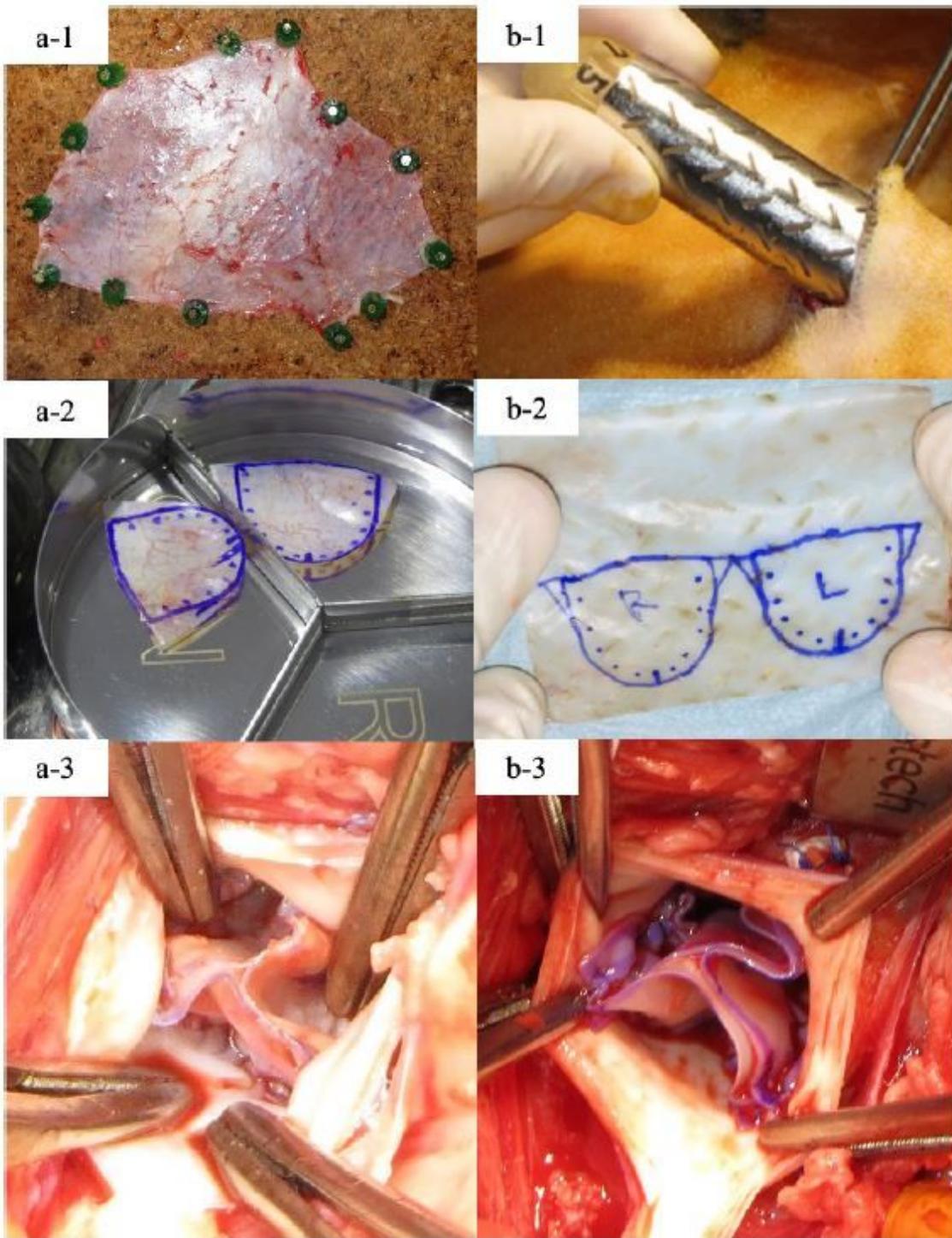
The authors would like to thank Y. Nakashima for excellent technical assistance

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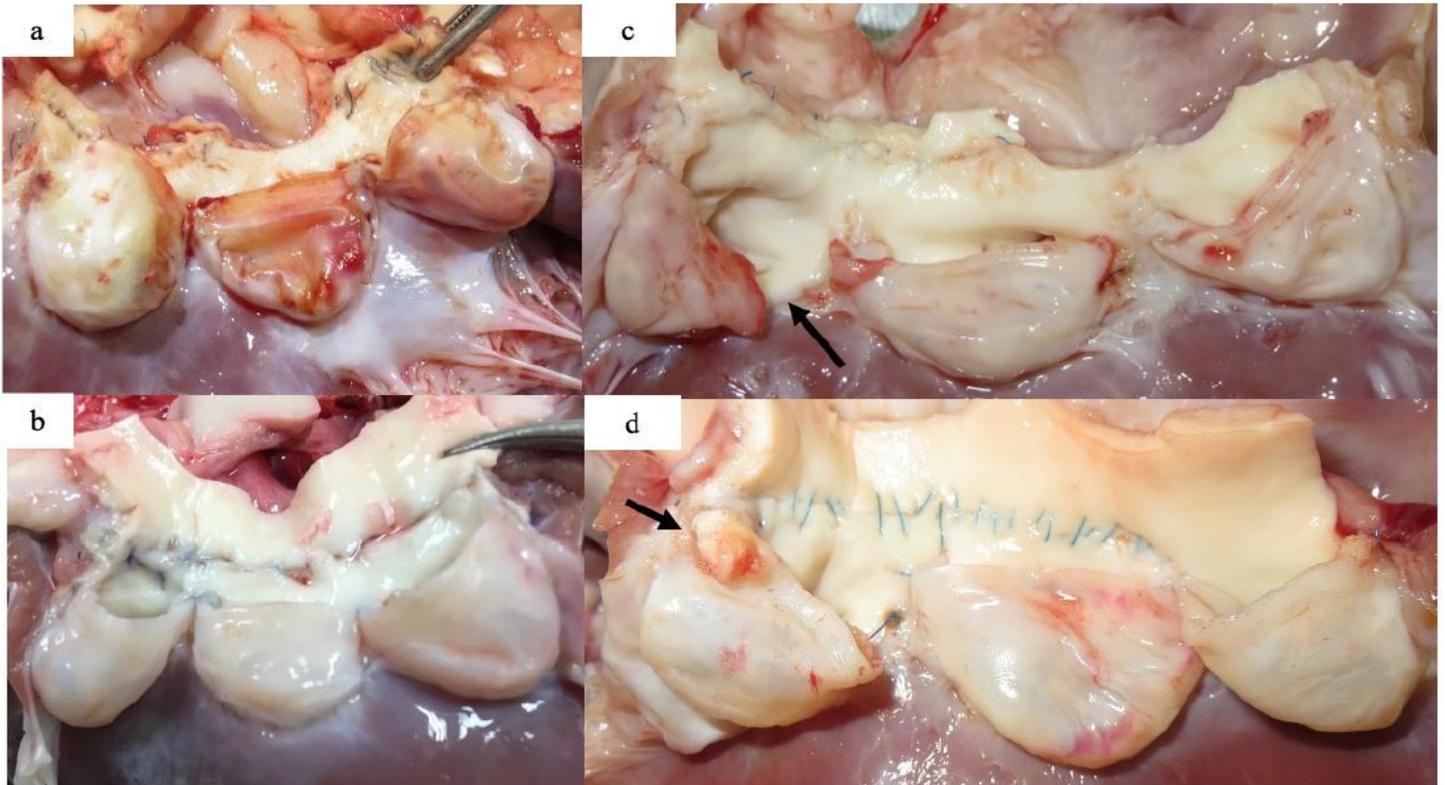
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## Figures



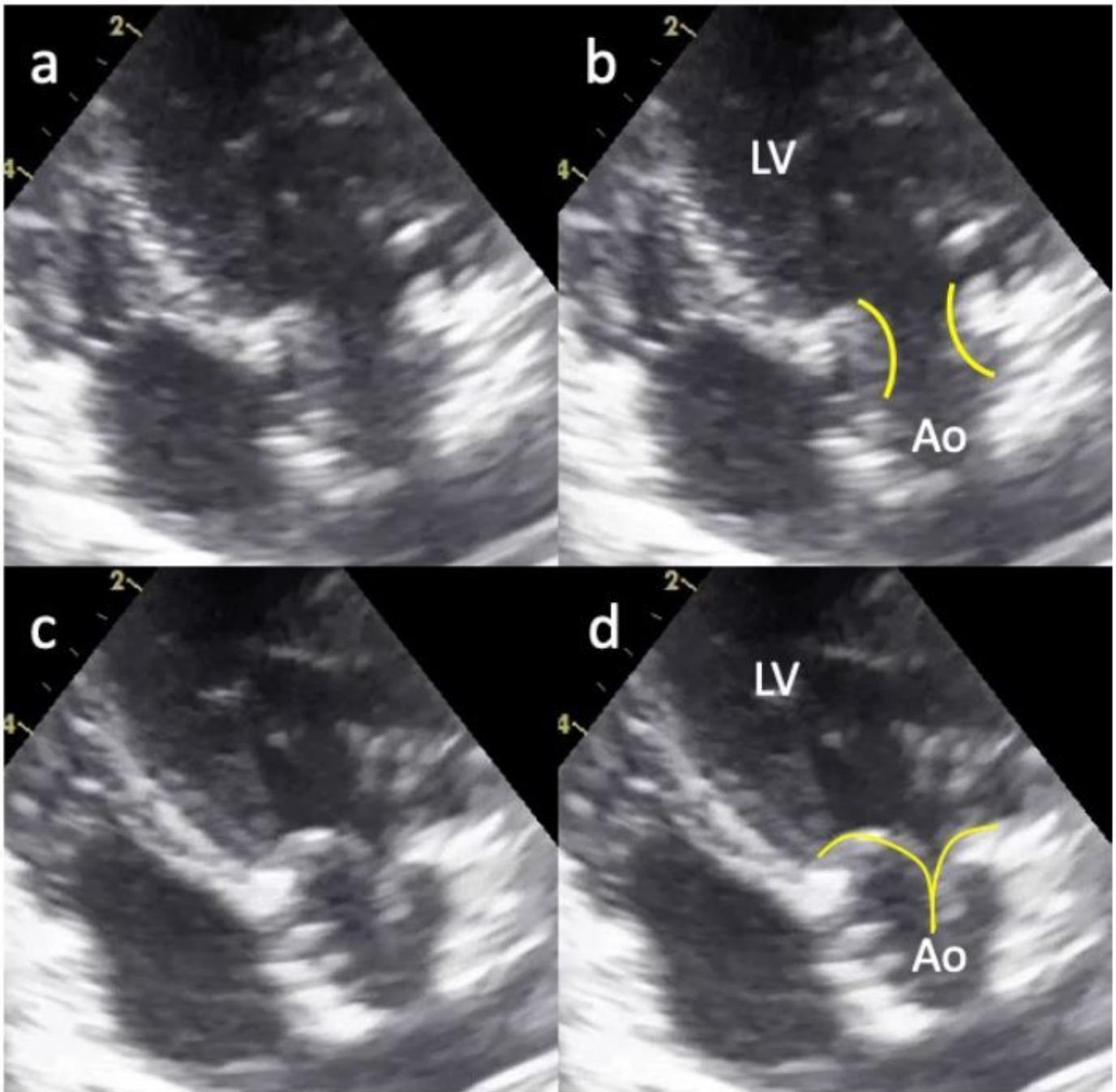
**Figure 1**

The autologous pericardium was harvested (a-1). The autologous pericardium was cut to a leaflet shape (a-2). Three leaflets of the autologous pericardium were sutured with the annulus (a-3). The mold was embedded for 2 months in a dorsal subcutaneous pouch in a goat model (b-1). After 2 months, the molds were encapsulated by the connective tissue membrane that formed thin flexible sheets (b-2). Three leaflets of biosheets were sutured with the annulus (b-3)



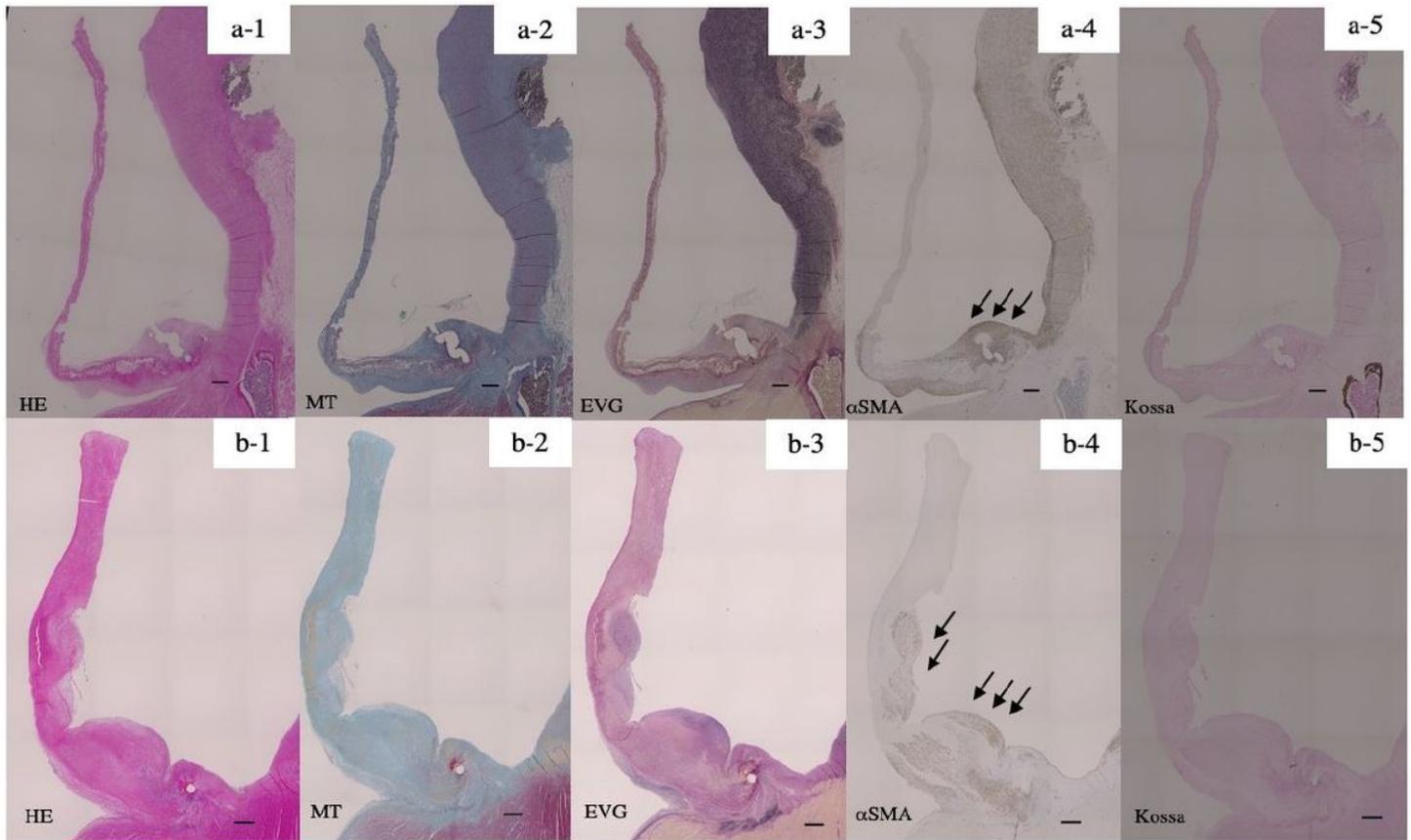
**Figure 2**

Photograph of the harvested valve leaflets of the autologous pericardium (a) and of biosheets (b). Neither the biosheets nor the autologous pericardium showed thrombus formation on any of the leaflet surfaces. Photograph of the harvested valve leaflets of biosheets. One valve had a commissural cut. The arrow shows a commissural cut (c). Another valve had infectious endocarditis. The arrow shows vegetation (d)



**Figure 3**

Movement of the leaflets in the aortic valve position by echocardiography after implantation. The leaflet was smoothly opening (a, b) and closing (c, d). Yellow lines show leaflets. The visible thrombus and cuspal tears were not detected by echocardiography. LV left ventricle, Ao aorta



**Figure 4**

The longitudinal cross sections of valve leaflets of the autologous pericardium (a1-5) and of biosheets (b1-5) obtained 3 months after implantation and stained with hematoxylin–eosin (HE), Masson’s trichrome (MT; collagen stain), Elastica-van Giessen,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), and von Kossa (calcium stain). In the pericardium model, the microscopic findings revealed that the pericardial tissue remained, while the number of  $\alpha$ -SMA-positive cells increased at the base (arrow). In contrast, the biosheet tissue completely fused to the annulus with invasion by  $\alpha$ -SMA-positive cells from at the base to the middle (arrow). Scale bar equals 500 $\mu$ m.

## Supplementary Files

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