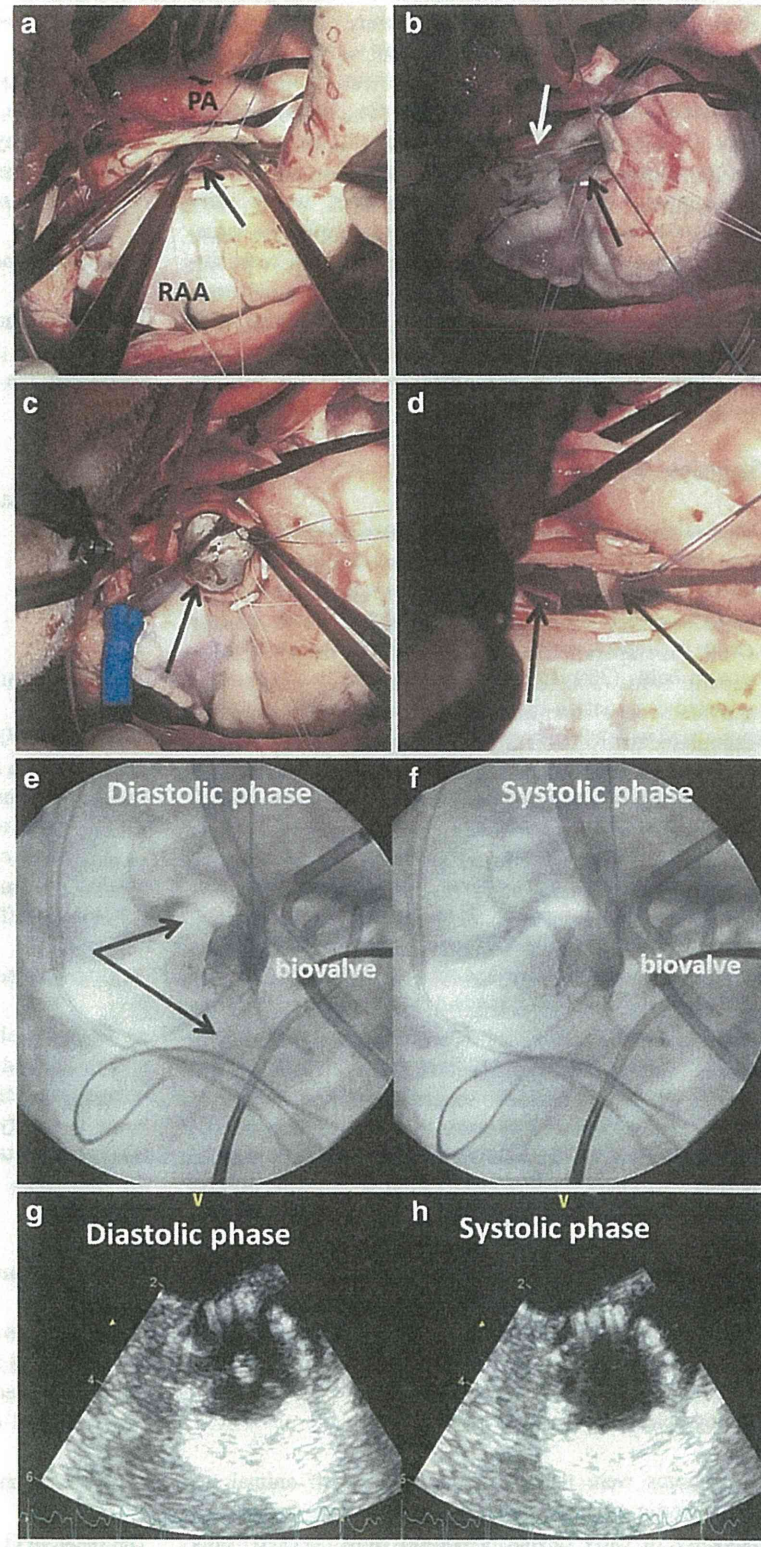

Fig. 2 Sutureless implantation of the stent biovalve (SBV) and the results. Native aortic valve leaflets (*black arrow*) are resected (**a**). Then, the crimped SBV (*white arrow*) is implanted into the aortic annulus, as indicated by three guiding sutures (*black arrow*) (**b**). The 23-mm balloon (*black arrow*) is inserted into the biovalve annulus and inflated with sterile water (**c**). The valve position, leaflet (*black arrows*) conditions, and coronary artery ostia are checked (**d**). Aortogram shows normal coronary arteries (*black arrow*) and less than mild paravalvular leakage (**e–f**). Epicardial two-dimensional echocardiogram indicates adequate valve closure and good coaptation (**g**); the open position provides a large orifice area (**h**). *PA* pulmonary artery, *RAA* right atrial appendage

them for use in serial AVR experiments. Thus, the AVR was actually performed using allograft valves. As described in Fig. 1, specially designed rods with 23-mm balloon expandable stainless stents (Goodman Co., Nagoya, Japan) were implanted in subcutaneous pockets in the valve production animal under general anesthesia. Two months after implantation, we harvested the implants, which were completely encapsulated with abundant connective tissue. After removing the mold, a trileaflet structure with surrounding tubular tissue was strongly adhered to the stent. Excess tissue was trimmed from the valve, and the valve was preserved in 70 % aqueous ethanol. This valve could be crimped to a 10-mm outer diameter and it was attached to its original holder.

Sutureless AVR procedure

Three adult goats (54.5–56.1 kg) underwent sutureless AVR with SBVs. The heart was exposed via a left thoracotomy at the fourth costal bed, and a pressure line was inserted into the brachiocephalic trunk to monitor aortic blood pressure (AoP). In preparation for cardiopulmonary bypass (CPB), a heparin bolus (300 IU/kg) was injected to create a target activated coagulation time of >400 s. The aortic arch was cannulated with a 20-Fr arterial cannula, and a 25-Fr venous drainage cannula was placed into the right atrium. A 14-Fr left ventricle (LV) venting cannula was inserted into the LV. The ascending aorta was then cross-clamped, and cold cardioplegic solution was infused into the aortic root via a root cannula. The ascending aorta was opened using a transverse aortotomy. As shown in Fig. 2, the native aortic valve leaflets were resected, and three guide sutures were placed on the nadirs of the aortic valve annulus of each cusp. Then, a crimped SBV, which was attached to its original holder, was inserted into the aortic annulus position, as indicated by the guiding sutures. The guiding sutures were tied to fix the stent strut to the aortic annulus to secure its position and prevent migration. A 23-mm balloon was then inserted into the SBV annulus, inflated with sterile water to a pressure of 4 atm, and deflated 10 s later. The positions of the SBV, leaflet damage, and the coronary ostia were checked. A running 4-0 polypropylene suture was used to close the aortotomy site, air was purged, and the aortic cross-clamp was removed. The CPB flow was then decreased and eventually stopped. If required, a catecholamine infusion was used to maintain the AoP >80 mmHg.

Angiography

Angiograms were taken after weaning each animal off CPB. Aortic regurgitation, coronary artery status, and incidence of early device migration were checked using aortography.

Epicardial two-dimensional echocardiography

Epicardial two-dimensional echocardiography (Vivid E9, GE Healthcare, Horten, Norway) was performed following the angiography to assess the movement of the leaflets and to measure the aortic valve area (AVA) using echocardiographic planimetry.

Peak-to-peak pressure gradient

The LV pressure was directly monitored by a catheter. We assessed the peak-to-peak pressure gradient (pPG) by subtracting the peak LV pressure from the peak AoP.

Statistics

Quantitative data are presented as mean values ± standard deviations.

Results

Technical feasibility of the sutureless AVR

Sutureless AVR was successfully performed in all goats. There were no leaflet tears caused by crimping or coronary ostia occlusions due to the SBV observed after expanding the stents. All animals could be weaned off CPB following a mean aortic cross-clamp time of 45 min; signs of cardiac conduction abnormalities were not observed after weaning the animals off CPB.

Angiogram findings

Angiograms showed that all the valves were orthotopically located, and that they demonstrated normal opening and closing functionality. The coronary arteries were normally detected in all goats. Less than mild aortic regurgitation was observed in this study, and none of the goats demonstrated detectable central leakage (Fig. 2e–f).

Echocardiogram findings

Epicardial two-dimensional echocardiograms showed smooth leaflet movement with wide opened positions and adequate closed positions (Fig. 2g–h). The average AVA was 2.4 ± 0.1 cm^2.

pPG assessment

The mean pPG was 6.3 ± 5.0 mmHg. The early two goats needed an inotropic support to maintain their AoP over

80 mmHg, after weaning off the CPB. Goat 3 did not require inotropic support.

Discussion

Reducing the immunoreaction to valve substitutes and the engraftment of regenerated valves offers the potential to overcome the disadvantages associated with current bioprosthetic valves. Several TEHV concepts have been reported, and some TEHVs are already clinically approved for use as pulmonary valve substitutes [6, 7]; the TEHVs for aortic valves are still in development. If the functionality and durability of TEHVs can be verified, they may be useful as valve substitutes in all patients with valvular disease, including children and younger patients.

Our biovalve is an autologous tissue valve created using a unique IBTA technology. The IBTA technology does not require special facilities or special management protocols. Thus, the technique is simpler and less expensive than other tissue engineering techniques. Recent advances in valve designs with stents have facilitated minimal invasive approaches in cardiac surgery [13, 14]. We adopted this technology to the biovalve and completed the AVR procedure safely in a goat model. The SBV exhibited good fixation and smooth leaflet movement under systemic circulation. Further, because the stent valve does not require a sewing cuff, the SBV was able to provide a large AVA and demonstrated low transvalvular pressure drop.

Takewa et al. [11] confirmed the long-term behavior of the biovalve in systemic circulation using a conduit type of biovalve in an apico-aortic bypass model. They found a large amount of neovascularization and the infiltration of α-smooth muscle actin-positive cells into the biovalve conduit during histological evaluation. These findings indicate the possibility of biovalve self-maturation and suggest that the biovalve might acquire features such as growth adaptability. Further investigations of the histological features of the autologous biovalve in the orthotopic position and its long-term durability are ongoing.

Conclusion

AVR using a sutureless implantation technique with SBV was demonstrated to be feasible in a goat model. The early valvular function of the SBV as an aortic valve was sufficient. Long-term experiments are needed to evaluate autologous tissue SBV's durability and histological regeneration potential in an orthotopic position.

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Conflict of interest None declared.

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ORIGINAL ARTICLE

Tissue Engineering / Regenerative Medicine

Development of self-expanding valved stents with autologous tubular leaflet tissues for transcatheter valve implantation

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Abstract In this study, self-expanding valved stents were prepared by in-body tissue architecture technology. As molds, plastic rods (outer diameter; 14 or 25 mm), mounted with specially designed self-expanding stents, whose strut was a combination of two wavy rings and three pillars, were embedded into the subcutaneous pouches of goats or beagles for 1 month. Upon harvesting, the molds were fully encapsulated with membranous connective tissues, in which the stent strut was completely embedded. The tubular tissues with the stents were obtained by removing the internal rods. About a half of the tubular tissues as a leaflet part was folded inside the remaining tubular tissues having ring strut as a conduit part. When the overlapped tubular tissues were fixed at the three pillars, two different-sized self-expanding valved stents (internal diameter; 14 or 25 mm) with autologous tubular leaflet tissues were obtained as Stent-Biovales. After shape formation of the leaflets at the closed form, regurgitation rate was approximately 5 and 22 % at pulmonary and aortic condition, respectively. The Stent-Biovalves developed here may be useful as a heart valve for patients undergoing transcatheter heart valve implantation.

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Keywords Biovalve · Stent · Heart valve · In vivo tissue engineering · Self-expanding

Introduction

Although surgical valve replacement improves the quality of life of patients, further progress in the development of artificial heart valves is needed for ideal heart valve replacement. The majority of studies that have retrospectively assessed the effectiveness of existing artificial heart valves suggest that existing artificial heart valves are inefficient and have limited durability [1, 2]. Although mechanical valves have demonstrated long-term durability, they require lifelong anticoagulation therapy [3]. Meanwhile, bioprosthetic valves display relatively good blood compatibility and hemodynamics but limited durability [4].

We have previously reported on the development of autologous prosthetic tissues using "in-body tissue architecture (IBTA)" technology, which is a novel and practical technique in regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in living bodies [5]. To overcome the limitations of existing artificial heart valves, we developed several types of heart valve-shaped autologous tissues based on IBTA, named Biovalve [6–9]. Biovalve, comprised of only autologous tissues mainly with fibroblast cells and collagen fibers, has potential biocompatibility and excellent in vitro mechanical properties such as high burst strength and elastic modulus. Furthermore, Biovalves have demonstrated efficient valvular function as pulmonary and aortic valves, and excellent flow characteristics when assessed on echocardiography, after implantation in a beagle or goat model [10–12].

During the development of the Biovalve, we reported on the "Stent-Biovalve", in which a self-expanding stent strut

was impregnated inside the tissue membrane of the Biovalve. The Stent-Biovalve indicated not only excellent mechanical properties similar to those of the Biovalve, but also flexibility and superelasticity, reflecting properties of self-expanding nitinol stents. The Stent-Biovalves may be useful as heart valves for patients who need minimally invasive heart valve implantation such as transcatheter aortic or pulmonary valve implantation (TAVI or TPVI). Recently, we reported on the development of the Stent-Biovalve based on the innovative concept of the stent eversion method [13]. This technique was highly effective in constructing a robust and completely autologous Stent-Biovalve with adequate in vitro valve function. However, the design of the stent strut was limited to the eversion process.

On the other hand, a tubular membranous valve, the 3F Aortic Bioprosthesis (3F Therapeutics Inc, Lake Forest, CA, USA), was developed [14]. Its valvular function was based on the hypothesis that native heart valves function as simple tubes with sides that collapse when external pressure is applied. Because "form follows function", this hypothesis was theoretically confirmed by implanting a simple tube into the anatomic position of any native heart valve and documenting that under the same anatomic constraints and physiologic conditions as the native valve, the tube would assume the form of a native valve. The valve showed favorable preliminary clinical hemodynamic results.

The purpose of this study was to describe a new method that was based on the concept of the tubular membranous valve, and to evaluate the function of Stent-Biovalves using a pulsatile circulation circuit model simulating aortic and pulmonary conditions. The novel preparation method for the Stent-Biovalve was designed based on the lapel of only leaflet tissue, which was simplified compared with the previously developed stent eversion method and was independent of the strut design. The valvular function of the Stent-Biovalve was examined using an in vitro circulation circuit.

Materials and methods

Animal studies

All animal studies were performed 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) under a protocol approved by the National Cerebral and Cardiovascular Center Research Institute Committee (No. 13034).

Mold preparation

Two different-sized molds (Fig. 1c) were assembled for the preparation of Stent-Biovalves for goats or beagles. The molds were assembled by inserting plastic rods (outer diameter and length: 24 and 55 mm for goats; 14 and 35 mm for beagles, respectively) (Fig. 1b) fabricated by a three-dimensional printer (Projet HD3000, 3D Systems, Rock Hill, SC, USA) in customer-designed, self-expanding stents (internal diameter and length: 25 and 50 mm for goats, 15 and 30 mm for beagles, respectively; thickness of strut: 100 m) (Goodman Co., Nagoya, Japan) (Fig. 1a).

Stent-Biovalve preparation

Nine molds were placed into the dorsal subcutaneous pouches of 3 goats (age, 1–2 years; body weight, 40–50 kg) under general anesthesia induced with ketamine (10 mg/kg) and maintained with isoflurane (1–3 %). After complete encapsulation of the molds with connective tissue, following 1 month of embedding, the implants were harvested (Fig. 1d). The mold was removed from both ends of the developed tubular tissue (Fig. 1e), and was then folded half of the tubular length into the remaining tubular section (Fig. 1h). After sewing 3 commissures of the leaflets and the tips of the stent main body (Fig. 1i), Stent-Biovalves with an internal diameter of 25 mm were obtained (Fig. 1j). The original leaflet length and area were 25 mm and 6.5 cm^2, respectively.

Stent-Biovalves with an internal diameter of 15 mm (Fig. 1l) were similarly prepared in beagles (age, 1 year; body weight, 10–12 kg). Six molds were placed into the dorsal subcutaneous pouches of 2 beagles under general anesthesia induced by intramuscular injection of ketamine (20 mg/kg) and maintained with 1–3 % isoflurane. Stent-Biovalves were obtained after 1 month of similar treatments. The original leaflet length and area were 15 mm and 2.4 cm [2], respectively.

Histological evaluation

The Stent-Biovalves were fixed with 10 % formalin, embedded in paraffin, and sectioned at a thickness of 3–5 μm into longitudinal sections at the conduits and circumferential sections at the leaflets. Specimens were stained with hematoxylin and eosin, Masson's trichrome for collagen, and Elastica van Gieson for elastin.

In vitro valve function

Function of the valves was examined using a pulsatile circulation circuit (LaboHeart NCVC, IWAKI Co., Tokyo, Japan) to simulate aortic or pulmonary circulation. Figure 2 shows a schematic drawing of the circulation circuit. The working fluid was 0.9 % saline. Heart rate was set at 70 bpm and 5–6 L/min. Mean arterial pressure was set at 100 and 15 mmHg for aortic and pulmonary

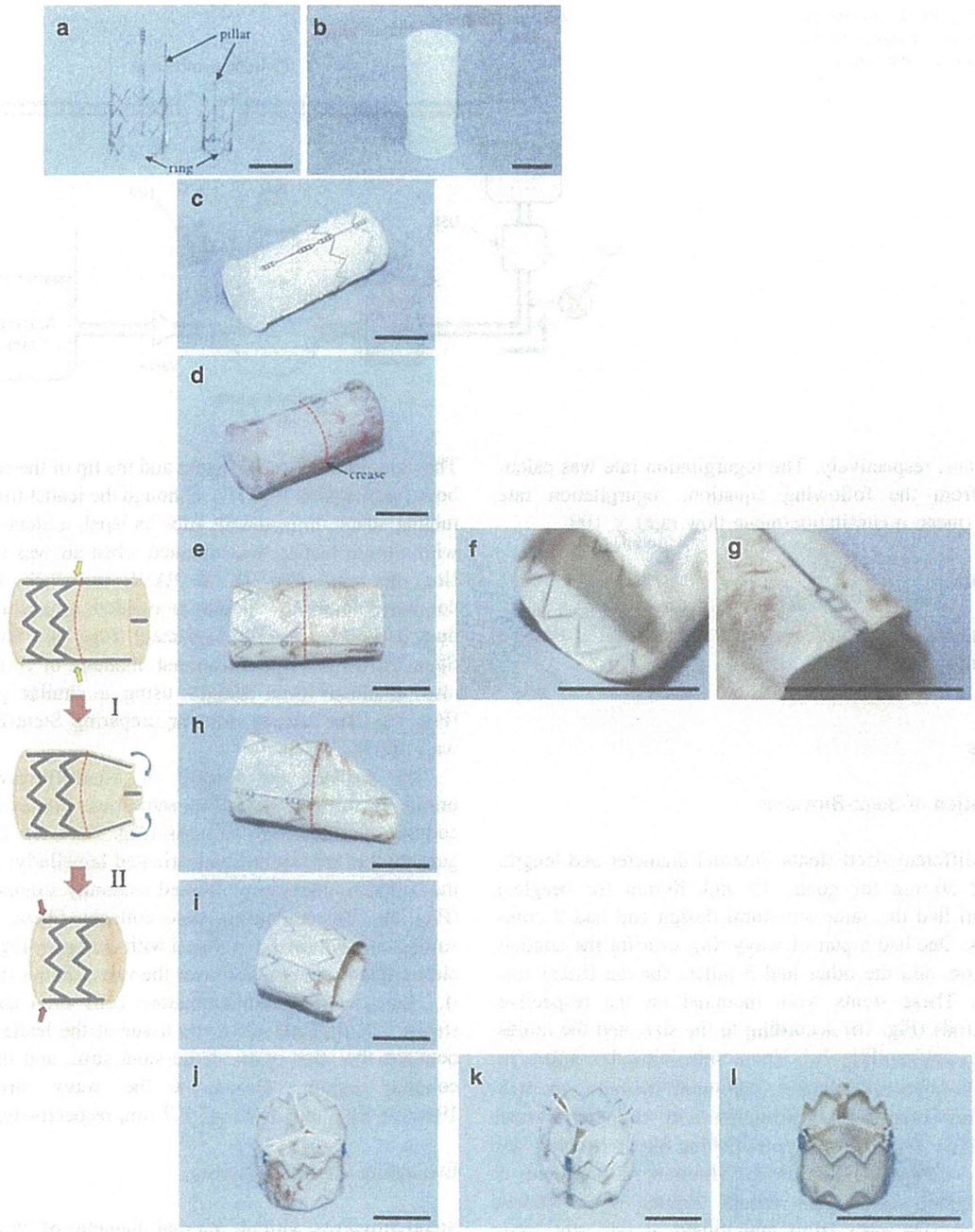

Fig. 1 Photographs of the sequential steps involved in the preparation of Stent-Biovalves. **a** Custom-designed, self-expanding stents. **b** A plastic rod. **c** The mold assembled with the stent and rod. **d** The mold completely encapsulated with connective tissue by embedding into subcutaneous pouches for 1 month. **e** The tubular tissue impregnated with the stent after removing the internal rod. The tubular tissue at the wavy roll part (**f**) and the stick part (**g**). **h** Separating the stent strut at 3 connecting parts (*yellow arrows* in the diagram on the *left*) between the main conduit tube with 2 wavy rings and the leaflet tube with 3 pillars. **i** The conduit tube after turning the tubular tissue inside of the conduit (*blue arrows*). **j** The Stent-Biovalve with an internal diameter of 25 mm obtained by fixation at the commissure portions (*red arrows*). **k** The Stent-Biovalve without a part of the conduit tissue. **l** The Stent-Biovalve with an internal diameter of 15 mm

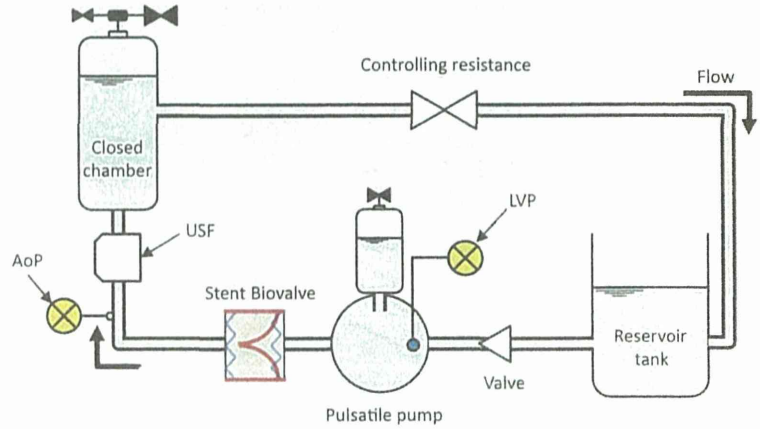

Fig. 2 A pulsatile circulation circuit model designed for the evaluation of valve function

conditions, respectively. The regurgitation rate was calculated from the following equation: regurgitation rate (%) = (mean regurgitation/mean flow rate) × 100.

Statistics

Quantitative data are presented as means ± standard deviations.

Results

Preparation of Stent-Biovalve

The 2 different-sized stents (internal diameter and length: 25 and 50 mm for goats, 15 and 30 mm for beagles) (Fig. 1a) had the same structural design and had 2 components. One had a pair of wavy ring strut for the conduit formation, and the other had 3 pillars for the leaflet formation. These stents were mounted on the respective plastic rods (Fig. 1b) according to the size, and the molds were assembled (Fig. 1c). Upon embedding the molds for 1 month in goats or beagles, each mold including the stent strut was completely encapsulated with connective tissue membrane (Fig. 1d). After removing the inner rod and cutting out the disk-like tissue membrane at both ends of the implants, autologous tubular tissues were obtained (Fig. 1e). The wavy rings and pillars of the strut were completely impregnated in the tissue membrane (Fig. 1f, g). The strut at 3 connecting parts between the 2 wavy rings and 3 pillar parts was separated by cutting (step I in Fig. 1h), and the tubular part with 3 pillars was folded into the remaining tubular part with wavy rings (step II in Fig. 1). In the lapel process, the leaflet tissue membrane could be completely turned inside the conduit tissue without any damage and delamination of both tissues. The tissue membrane and stent remained strongly connected. The commissures of the leaflet and the tip of the stent main body were sewed (Fig. 1i). Although the leaflet tissue had a tubular shape immediately after its lapel, a Stent-Biovalve with closed leaflets was obtained when air was blown inside the tube (Fig. 1j). In TAVI, in which disturbing coronary blood flow should be avoided, a part of the conduit tissue was easily extracted (Fig. 1k). Small-sized Stent-Biovalves with an internal diameter of 15 mm were also obtained from beagles using a similar procedure (Fig. 1l). The success rate for preparing Stent-Biovalves was 100 %.

The leaflets and conduit of Stent-Biovalves were originally formed around the same rod. Almost all extracellular matrixes were collagen (Fig. 3b, e, h). The collagen fiber at the conduit part oriented lamellarly; however, the collagen fibers were oriented randomly around the strut (Fig. 3h). By combining these collagen fibers, the stent struts were completely covered with collagen (Fig. 3e). No elastic fiber was observed over the valve tissues (Fig. 3c, f, i). There were few inflammatory cells even around the stents. The thicknesses of the tissue at the leaflet, formed between the stick parts of the stent strut, and that at the conduit region, formed at the wavy strut, were 194.5 ± 68.6 and 263.6 ± 72.7 μm, respectively.

Evaluation of valve function

Stent-Biovalves with an internal diameter of 25 mm were examined for flow rate waveforms and regurgitation rate at pulmonary and aortic conditions using a pulsatile circulation circuit (Fig. 2). Mean pulmonary and aortic pressures were 15 and 100 mmHg, respectively, under a fixed heart rate of 70 bpm.

Figure 4a shows typical flow waveforms of the originally obtained Stent-Biovalve with the long length of the leaflet tissue of 25 mm under pulmonary conditions. The leaflets opened smoothly, and the maximum flow rate

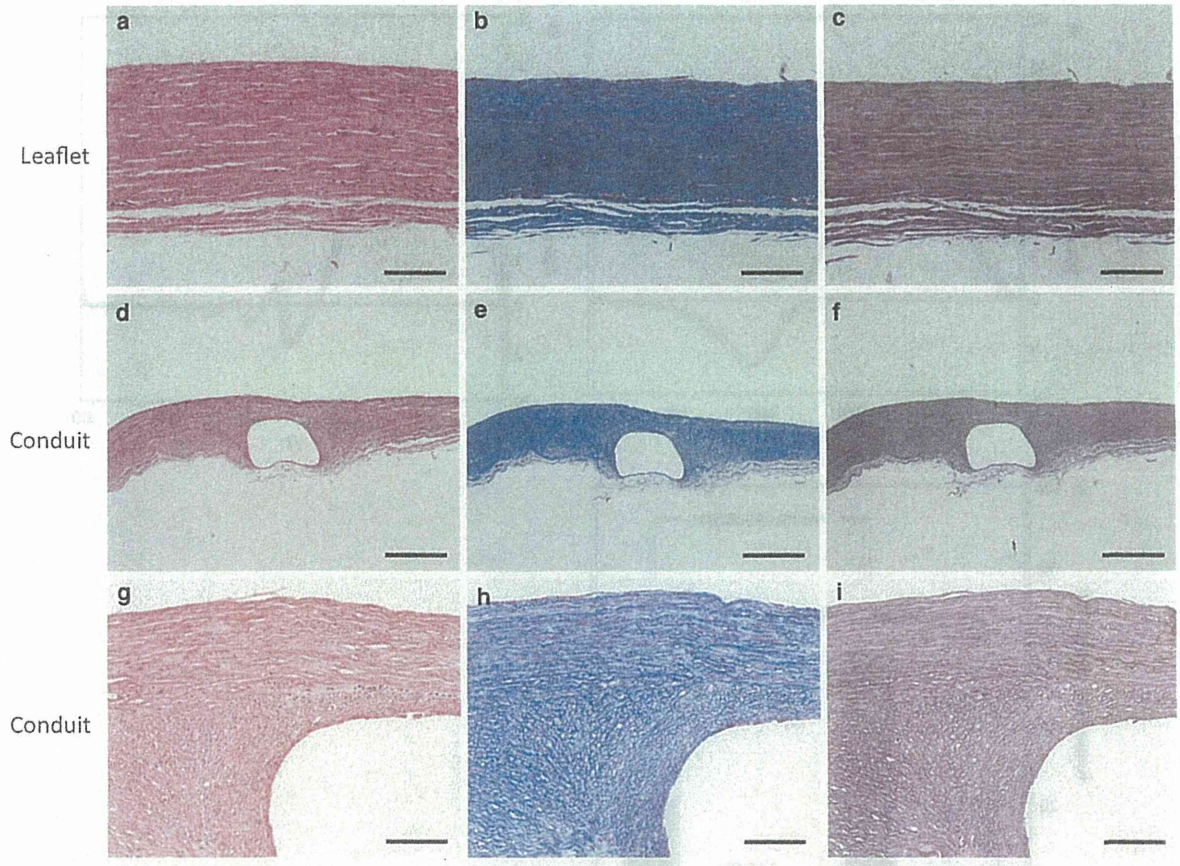

Fig. 3 Histology of the Stent-Biovalves stained with hematoxylin and eosin (**a, d, g**), Masson's trichrome (**b, e, h**), and Elastica van Gieson (**c, f, i**) at a leaflet (**a–c**), conduit (**d–i**), and around the strut (**g–i**). Bar = 100 μm (**a–c, g–i**), 500 μm (**d–f**)

was reached at approximately 25 L/min (the mean flow rate 5.3 L/min). However, delayed leaflet closure occurred, resulting in a high regurgitation rate of approximately 20 %. The leaflet did not close completely at diastole. Therefore, under aortic conditions, the regurgitation rate with the Stent-Biovalve was extremely high (approximately 60 %, Fig. 4d) with low mean flow rate of 2.9 L/min. When the leaflet shape was fixed in the closed form by formalin treatment, the leaflet closed more rapidly (Fig. 4b). An extremely low regurgitation rate (approximately 5 %) under the mean flow rate of 6.0 L/min was noted in the diastolic phase, thus, being almost completely prevented.

Discussion

TAVI has recently become the mainstream minimally invasive heart valve implantation technique [15, 16]. Bioprosthetic xenograft valves are used for TAVI; however, their durability remains a problem. We have developed a Biovalve with a self-expanding stent, named Stent-Biovalve. Stent-Biovalve may be an ideal and useful valve for TAVI. We previously reported a novel method for developing a Stent-Biovalve [13], in which tissue formation occurs outside the mold and with stent reversal occurring eventually. This method renovated the concept of leaflet formation depending on tissue migration via foreign material reaction and significantly improved the leaflet formation rate. In the present study, as an alternative approach for Stent-Biovalve preparation, we designed a novel and simple preparation method.

The mold devised in this study enabled adherence of the entire mold, including the stent strut and adjacent tissue, and enabled us to obtain a wide leaflet tissue. After removing the devised molds from the subcutaneous pouches, the connective tissues were almost homogeneous and completely covered the outer surface of the mold and stent. Tissue formation occurred around the rods, similar to previous Stent-Biovalves, using the stent everting method.

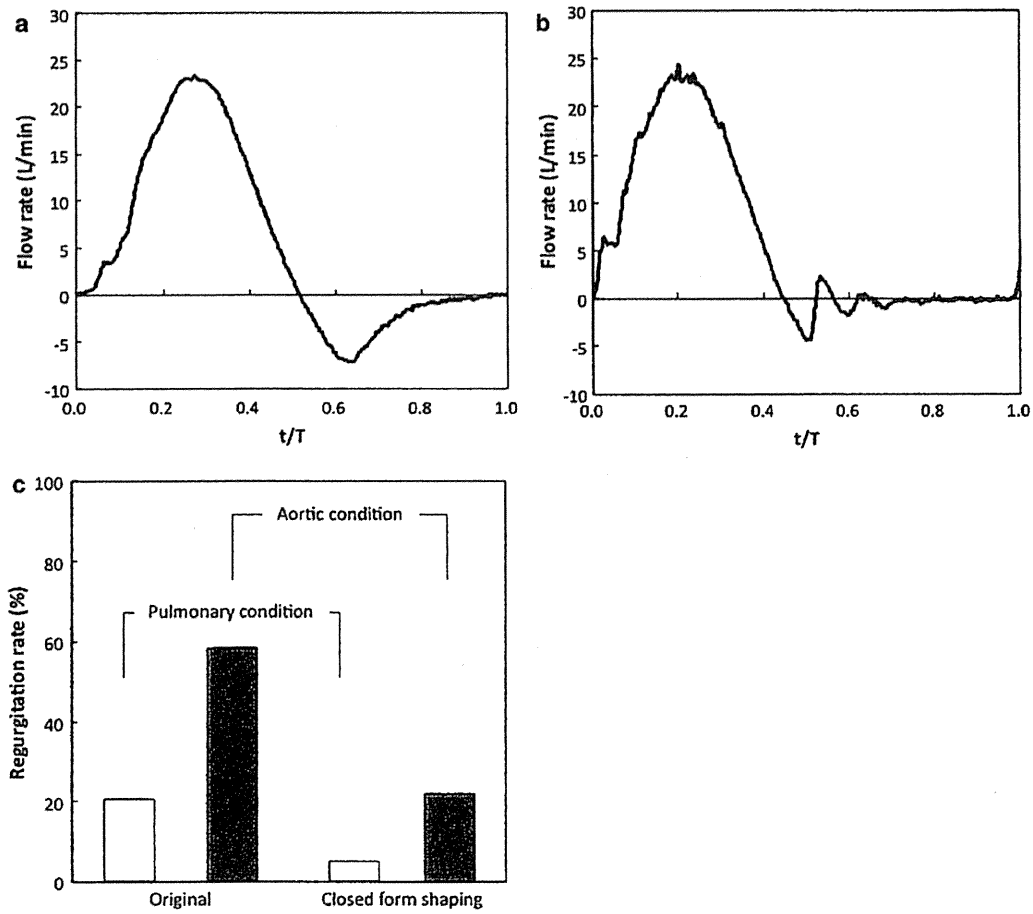

Fig. 4 The flow rate waveforms of Stent-Biovalves with an internal diameter of 25 mm before (**a**) and after (**b**) shape formation of the leaflets at the closed position under a pulmonary circulation condition. **c** Regurgitation rate of Stent-Biovalves with an internal diameter of 25 mm under pulmonary or aortic circulation condition

Indeed, the main component of the tissues was collagen. Therefore, it was considered that the mechanical properties of the formed tissue in both methods were almost the same. In our previous report, the burst strength of the leaflets of the Stent-Biovalves was over 7600 mmHg [10], and the elastic modulus of the leaflets was 2.6 MPa. Furthermore, the connective strength between the Biovalve leaflet and stent was 833.8 gf [13]. Because these values were equal to or more robust than those of native aortic valve leaflets, the Stent-Biovalve may have adequate mechanical properties for heart valves.

The coaptation height, which indicates the margin for preventing regurgitation, reflects the quality of the valve closure [17]. Marom et al. [18] reported the coaptation height and mechanical stress in the aortic cusp tissues during diastole. The >3-mm coaptation height of the leaflet in the aortic valve decreased the regurgitation. In the present study, coaptation height was designed at >3 mm. However, regurgitation function strongly depended on the leaflet shape. In the case of a 25-mm leaflet fixed in the closed form by formalin treatment, the leaflet opened completely and closed snugly, and prevented the backflow of fluid during the closing phase. These results showed that the Stent-Biovalve with an open shape affects the backflow of fluid during the closing phase. In this study, thus, the 25-mm closed leaflet showed that robust tissue and sufficient leaflet length in the Stent-Biovalve can ensure better coaptation of the leaflets and provide functional closure. In the closing phase, leaflet stress and strain are predominantly high at the attachment edge and belly [19]. In the native aortic valve, each leaflet and corresponding sinus constitute a functional structure that has continuity between the leaflets and roots so that the high stresses at the attachment edge can be transferred from the leaflet to the sinus wall [20, 21]. This suggests that the robust leaflet by formalin treatment had almost the same closing

mechanism. However, this method is not directly applicable to our study because the most important advantage of the Stent-Biovalve, that is, its composition of living autologous cells, was lost because of formalin treatment. Therefore, our next challenge is to prepare more robust leaflets of Stent-Biovalve without compromising overall valve performance.

The Stent-Biovalve completely encapsulated the stent inside the autologous tissue. By trimming extra tissue from the Stent-Biovalve devised in this study, the native sinus of Valsalva may be used effectively after implantation. One important role of the sinus of Valsalva is to aid in the opening and closing of the leaflets. The closure of the valve during diastole is largely associated with the vortex flow beneath the leaflets. In the sinus of Valsalva of the native aorta, the vortex flow facilitates the smooth opening and closing of the leaflets by passively pushing the leaflets to close [17, 22]. In the absence of the sinus of Valsalva, the period in which the leaflet opens is longer, and the valve closing velocity is faster [23]. As extra stress plays an important role in the pathogenesis of early structural valve deterioration [20], lower stress magnitude is required because abnormal stress can damage the cusps and reduce valve durability. Valve leaflets without the sinus were subject to greater abnormal stress and underwent bending deformation in the longitudinal direction [23]. That is, the vortex flow can avoid the abnormal stress on the leaflet and decrease the regurgitation volume. A very important aspect is the inherent hemodynamic characteristics of the sinus of Valsalva for closing valve leaflets. In the presence of the native sinus of Valsalva, the Stent-Biovalve may enable a native flow pattern and a smoother valve movement.

Surgical valve replacement is an efficacious treatment for patients with cardiovascular disease. Currently, more than 200,000 aortic valve replacements are performed annually worldwide [24]. The currently available artificial heart valves have limitations regarding their durability. The Stent-Biovalve offers several advantages in safety and efficacy. Regenerative medicine based on strictly defined conventional tissue engineering requires complicated long-term cell culture. On the other hand, the Stent-Biovalve can be obtained by only embedding the mold in subcutaneous pouches based on the "in-body tissue architecture" technique, which is the phenomenon of encapsulation of foreign materials [5]. The process of Stent-Biovalve formation causes little burden on patients, and its clinical use may improve patients' quality of life.

It should also be noted that 0.9 % saline was selected as the working fluid in a pulsatile circulation circuit for examining valve function. The kinetic viscosity of blood is 4.44 m^2/s, which is approximately 4 times that of the saline solution (1.00 m^2/s) [12]. Moreover, the development of vortex flow in the wake of the valve induces platelet activation. The long-term influence of kinetic viscosity and platelet activation by vortex flow should be evaluated in living bodies.

Conclusion

In the present study, a novel preparation method using only the lapel of the leaflet tissues was as an alternative method for the development of the Stent-Biovalve. The Stent-Biovalves were obtained after only 1 month of subcutaneous embedding in a beagle or a goat. The method was simpler than the stent eversion method previously developed [13]. By optimizing the leaflet length and shape, the Stent-Biovalve may be useful as a heart valve in patients undergoing TAVI or TPVI. An animal implantation study is currently ongoing to confirm long-term valve function and durability in vivo. The results will be reported in the near future.

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Conflict of interest The authors declare that they have no conflict of interest.

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Successful implantation of autologous valved conduits with self-expanding stent (stent-biovalve) within the pulmonary artery in beagle dogs

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KEYWORDS
Heart valve replacement;
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Abstract *Objectives:* To evaluate the functionality of an autologous heart valve with stent (Stent-biovalve or SBV) after implantation in the pulmonic valve position in beagle dogs.
Animals: Five beagle dogs.
Methods: A mold with an aperture of a tri-leaflet structure was constructed from a pair of concave and convex rods to which a nitinol (NiTi) stent was mounted. This mold was embedded in a dorsal subcutaneous pouch in beagle dogs for 4 weeks. At

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Self-expandable stent;
Tissue engineering

the time of the removal, the surfaces of the molds were completely covered with connective tissues, tri-leaflet valves were formed and the NiTi stent was tightly connected to the structure.
Results: The mean burst strength of the SBV leaflet was 2710 mmHg (range 2280–3116 mmHg), which was approximately equal to that of the native pulmonic valve leaflet. After implantation in the pulmonary position, the SBV showed good functionality as a pulmonic valve. At 84 days after implantation, the SBV was replaced with autologous fibroblasts and collagenous tissues, and showed organization similar to that of native heart valves.
Conclusion: Stent-Biovalves achieved good valvular function with laminar flow in the pulmonic valve position of beagle dogs.

Abbreviations

3D	three dimensional
αSMA	α-smooth muscle actin
eVG	elastica van gieson
HE	hematoxylin and eosin
NiTi	nitinol
SBV	Stent biovalve

Introduction

Pulmonic stenosis is one of the most common congenital heart diseases in veterinary medicine and has been associated with severe clinical signs including sudden death.[1] Surgical valvulotomy, balloon dilatation and open or closed patch-graft have become the treatment of choice for congenital pulmonic stenosis.[2–7] However, in humans, heart valve replacement is an efficient treatment option for valvular diseases and has become the treatment of choice for patients with obstructive lesions.[8–10] Additionally, less invasive procedures such as percutaneous pulmonic valve implantation have become a mainstream treatment. The hemodynamic performance of the percutaneously placed valve has been shown to be similar to the surgically replaced pulmonic valve. Although valve replacement has demonstrated good efficacy in human patients with pulmonic valvular diseases, there are presently no effective artificial valves available for use in veterinary medicine. The ideal artificial valve should mimic the characteristics of a normal native valve such as hemodynamics, durability, thromboresistance and biocompatibility.[11]

We previously reported the development of an autologous heart valve using the "in-body tissue architecture" technology,[12–15] which had characteristics similar to native heart valves. The autologous heart valve used the "in-body tissue architecture" technology, which was a novel and practical technique for regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in patients. The advantages of this autologous heart valve are the capabilities of self-restoration and growth as well as the lack of immunological rejection after implantation. Most importantly, this autologous heart valve has many sizes and shape options by simply adjusting the mold before implantation. Although this autologous heart valve indicated good hemodynamics in the pulmonic valve position,[16] the main issue was the implantation technique of end-to-end anastomosis. This technique is time consuming and requires well-trained vascular surgical techniques. To overcome this limitation, we developed the autologous heart valve with stent using the "in-body tissue architecture" technology, named stent-biovalve (SBV). Stent-biovalve may simplify the implantation procedure and facilitate the treatment of stenosis. Our eventual goal is that SBV provides the new percutaneous treatment options for pulmonic valve stenosis. The purpose of this study was to develop the SBV and investigate its functionality in a pulmonary position.

Materials and methods

Animal studies

All animals received care according to the Principles of Laboratory Animal Care (formulated by the National Institutes of Health, Publication No. 56-23, received 1985). The research protocols were approved by the ethics committee of National Cerebral and Cardiovascular Center Research Institute (No.12602) and College of Bioresource Science, Nihon University (No. AP11B004).

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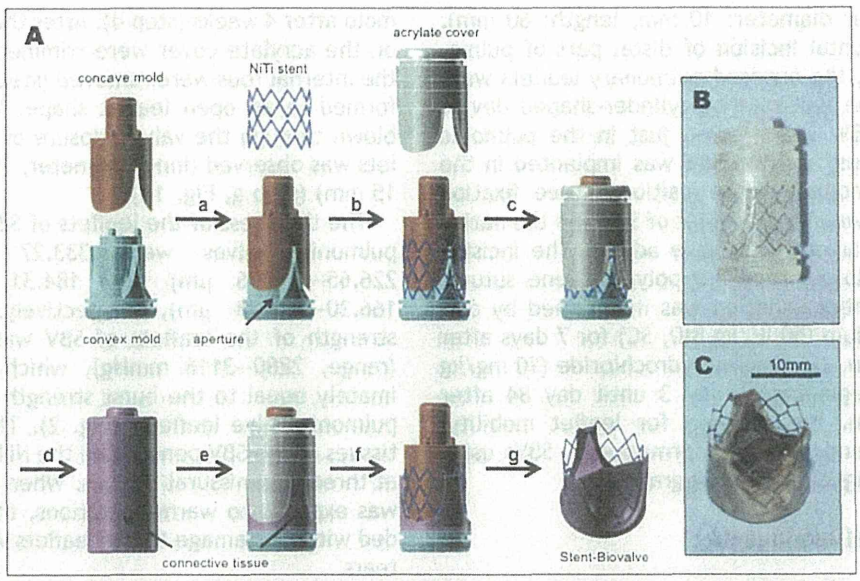

Figure 1 The sequential preparation method of the stent-biovalve. (A) Assembling convex and concave rod (step a), mounting NiTi stent (step b), and capping with an acrylate cover (step c, 1B). After removal from the subcutaneous pouches of beagle dogs, the mold was completely encapsulated (step d), extra tissue membrane was trimmed (step e), and the acrylate cap removed (step f). Then the Stent-Biovalve was obtained (step g, 1C). NiTi; nitinol.

Mold preparation

Stent-Biovalve molds (outer diameter, 17.5 mm; length, 33 mm) were prepared, according to the schematic diagram shown in Figure 1, using the nitinol (NiTi)-stent (inner diameter, 15 mm; length, 15 mm) and 3 kinds of acrylate parts, including a concave mold (outer diameter, 15 mm; length, 27 mm), a convex mold (outer diameter, 17.5 mm; length, 23 mm), and a acrylate cover (outer diameter, 17.5 mm; length, 24 mm). All acrylic parts were prepared using a three-dimentional (3D) printer.[e]

Stent-biovalve preparation

The molds were embedded in the subcutaneous pouches of beagle dogs. Pre-anesthetic medications included atropine sulfate (0.025 mg/kg IM), midazolam (0.3 mg/kg IV), butorphanol (0.2 mg/kg IV), and cefazolin sodium (20 mg/kg IV). All beagle dogs were oxygenated by mask with 100% oxygen. Anesthesia was induced with propofol (4 mg/kg IV) and maintained with 2% isoflurane. Four weeks after embedding, the molds were removed from the subcutaneous pouches of the dogs under the same anesthetic protocol.

Measurement of burst strength

The burst strength of 3 SBVs was determined by using a specially designed tensile tester apparatus. The leaflet specimens were fixed on a sample folder with a hole (diameter, 2 mm) at its center. The burst strength was determined by measuring the water pressure at the instant of tissue rupture using a pressure transducer[f] as previously described.[16]

Implantation procedure

All beagle dogs were maintained with continuous infusion of butorphanol (0.3 mg/kg per minute) and 1.5% isoflurane, following pre-anesthetic medications. Thoracotomy was performed on the left 4th intercostal space and then the arterial and venous cannulas were inserted into the carotid artery and the jugular vein, respectively. Cardiopulmonary bypass was initiated following heparin administration (400 U/kg), and then cardiac arrest was induced with cardioplegia (10 mL/kg). After the native pulmonic valves were removed, SBV was coated with argatroban for anticoagulation.[17,18] Stent-Biovalve after treatment with anticoagulation was shrunk on ice, and placed in the cylinder-shaped device (inner diameter:

[e] Object 260 Connex, Stratasys Ltd., Rehovot, Israel.

[f] N5901, Nihon-Denki Sanei Co., Tokyo, Japan.

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9 mm, outer diameter: 10 mm, length: 60 mm). After horizontal incision of distal part of pulmonary artery, the exposed pulmonary leaflets were excised. The forefront of cylinder-shaped device including SBV was inserted just in the pulmonic valve annulus, and the SBV was implanted in the native pulmonary valve position. Three fixation sutures between the conduit of SBV and the native pulmonary artery wall were added. The incision part was closed using 5-0 polypropylene suture. Systemic anticoagulation was maintained by dalteparin sodium (50 IU/kg BID, SC) for 7 days after implantation and ozagrel hydrochloride (10 mg/kg BID, PO) beginning at day 3 until day 84 after implantation. We checked for leaflet mobility, cusp tears and thrombus formation of SBVs using transesophageal echocardiography.

Histological evaluation

Stent-Biovalves were fixed with 10% formalin, embedded in paraffin and sliced into longitudinal sections. Specimens were stained with hematoxylin and eosin (HE), Masson's trichrome and Elastica van Gieson (eVG) for structural evaluation of the SBVs. Immunofluorescence staining was performed with anti αSMA mouse monoclonal antibody[g] at 1:100 dilution and anti mouse IgG antibody[h] at 1:1000 dilution. Specimens were examined by fluorescence microscopy with a Nikon microscope.[i]

Statistics

All data were expressed as median and range.

Results

Preparation of stent-biovalve

The stent-Biovalve was prepared by assembling acrylate rods with a NiTi stent (Fig. 1). A pair of concave and convex acrylate rods was assembled (step a) with a small aperture for a tri-leaflets shape. The assembled rod was then mounted on a NiTi stent (step b) and capped with another acrylate cover (step c, Fig. 1B). The mold was then removed from the subcutaneous pouch of the beagle dogs. Connective tissues encapsulated the mold after 4 weeks (step d). After the extra tissues on the acrylate cover were trimmed (step e) and the internal rods were removed (step f), a SBV was formed in an open leaflet shape. When air was blown through the valve, closure of the SBV leaflets was observed (inner diameter, 15 mm; length, 15 mm) (step g, Fig. 1C).

The thickness of the leaflets of SBVs and native pulmonic valves were 333.27 μm (range, 226.65–518.86 μm) and 184.31 μm (range, 166.30–191.78 μm), respectively. The burst strength of the leaflets of SBV was 2710 mmHg (range, 2280–3116 mmHg), which was approximately equal to the burst strength of the native pulmonic valve leaflets[16](Fig. 2). The connective tissues of the SBV penetrated the NiTi stent tightly at three commissural regions. When the NiTi stent was exposed to warm conditions, the SBV expanded without damage to the leaflets without cuspal tears.

Implantation in the pulmonic valve position

Five male beagle dogs were enrolled in this study (median age, 594 days; range, 493–1099 days; median body weight, 10.5 kg; range, 7.5–11.0 kg). The SBV was initially maintained in a low temperature environment (iced water) and a specially designed cylinder-shaped device was used to aid with the precise placement and implantation in the pulmonic valve position. After horizontal

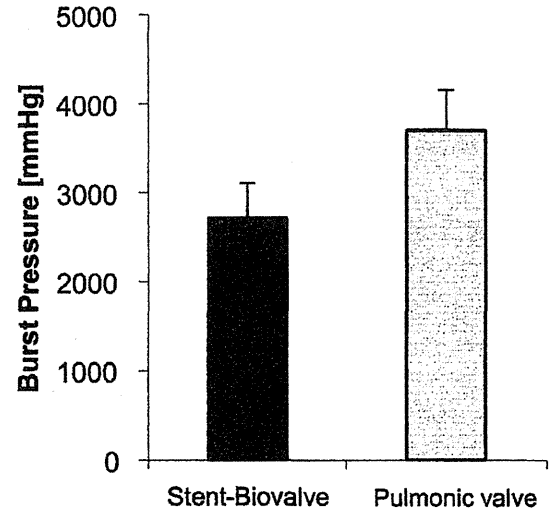

Figure 2 Burst pressure of the stent-biovalves and native pulmonic valves. The burst pressure of the stent-biovalves (n = 3) was 2710 mmHg, which was approximately equal to the burst strength of the native pulmonic valve leaflets (n = 3).

[g] Anti-alpha smooth muscle Actin antibody [1A4] (ab7817), Abcam plc., Cambridge, UK.
[h] Alexa Fluor® 594 rabbit anti-mouse IgG (A-11062), Life Technologies Corporation, Carlsbad, USA.
[i] ECLIPSE-Ti, Nikon Corporation, Tokyo, Japan.

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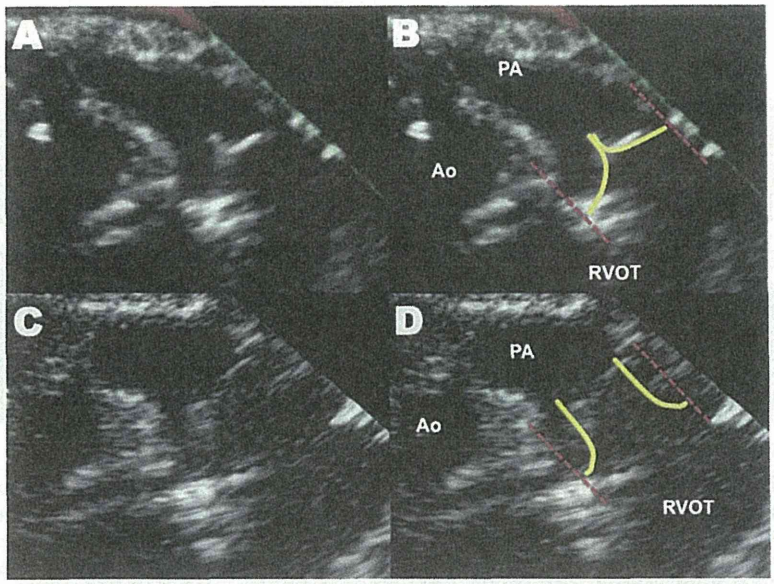

Figure 3 Movement of Stent-Biovalve leaflets in pulmonic valve position. Stent-Biovalve leaflets movement by echocardiography after implantation. The leaflet was smoothly closing (A, B) and opening (C, D). Yellow and red lines show leaflet and stent, respectively. The visible thrombus and cuspal tears were not detected by echocardiography. PA; pulmonary artery, RVOT; right ventricular outflow tract, Ao; aorta.

incision of the distal part of the pulmonary artery, the exposed pulmonary leaflets were excised. The SBV was inserted just in the pulmonic valve annulus. Immediately after implantation, because of the animal's higher body temperature, SBV self-expanded, and coaptation to the pulmonary arterial wall was observed. The success rate associated with primary deployment of SBVs was 100%. Each SBV leaflet revealed functional opening (Fig. 3A, B) and closing similar to the native pulmonic valve motions (Fig. 3C, D). Peak pulmonic valve velocity and pulmonary regurgitation velocity were 1.1 m/s and 0 m/s (before implantation) respectively, and 2.9 m/s and 1.9 m/s (84 days after implantation), respectively (Fig. 4). The visible thrombus and cuspal tears were not detected by echocardiography. At 84 days after implantation, all beagle dogs were euthanized and SBVs were harvested.

Histological evaluation

The lumen and leaflets of SBVs consisted of fibroblasts and collagenous tissues, as well as native pulmonic valve leaflets (Fig. 5A, B, E, F). No abnormal collection or infiltration of inflammatory cells was observed. Staining with HE showed that the surface of the SBV leaflets was covered with endothelial-like cells (Fig. 5A). A layer rich in elastin fiber was identified in the native pulmonic valve leaflets compared with the SBV (Fig. 5C, G). Stent-Biovalves after implantation showed diffuse staining of αSMA on the surface of the leaflets (Fig. 5D). In native pulmonic valve, the αSMA positive cells were confined to the subendothelial layer (Fig. 5H).

Discussion

In humans, pulmonic valve implantation has been shown to minimize right ventricular pressure and volume overload for congenital heart diseases.

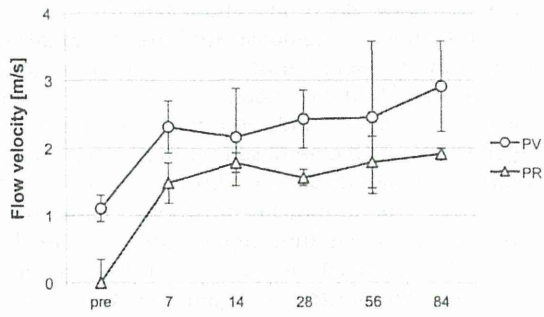

Figure 4 Flow velocity of Stent-Biovalve after implantation. Peak pulmonic valve velocity and pulmonary regurgitation velocity were 1.1 m/s and 0 m/s (before implantation) respectively, and 2.9 m/s and 1.9 m/s (84 days after implantation), respectively.

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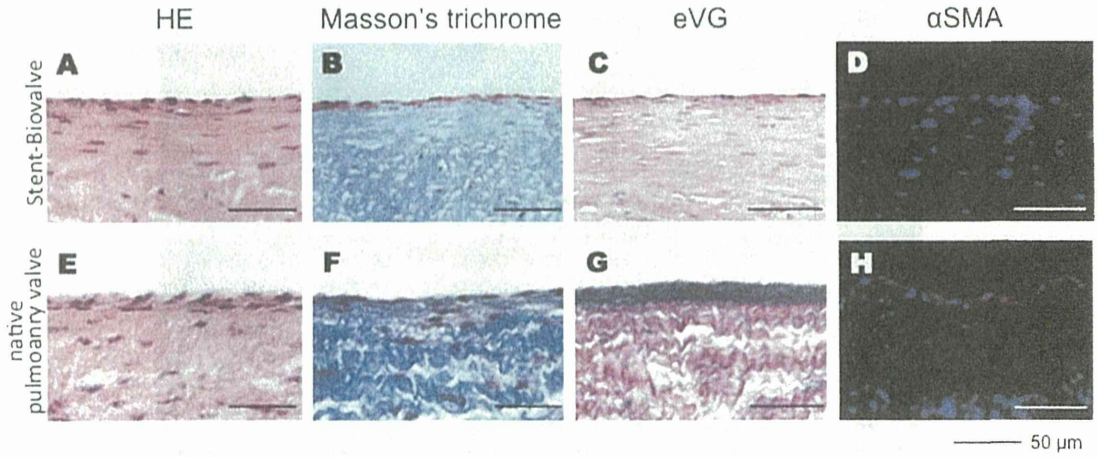

Figure 5 Histology and immunohistochemistry of the harvested Stent-Biovalve and native pulmonic valve. Histology of the Stent-Biovalve leaflet (A–D) and native pulmonic valve leaflet (E–H) were stained with hematoxylin and eosin (HE; A, E), Masson's trichrome (B, F) and Elastica van Gieson (eVG; C, G). Immunohistochemical staining showed the expression of α-smooth muscle actin (αSMA; D, H). Red and blue indicate the fluorescence of αSMA and DAPI stain/nuclei. The stent-biovalve consisted of fibroblasts and collagenous tissues as well as native pulmonic valve leaflet (A, B, E, F). By the 84 days of implantation, endothelial-like cells had progressed on the leaflet surface and extended. The layer rich in elastin fiber was identified in native pulmonic valve leaflets, compared with the stent-biovalve (C, G). Stent-biovalves after implantation showed diffuse staining of αSMA on the surface of the leaflets. In contrast, the α-SMA positive cells were confined to the subendothelial layer in native pulmonic valve (D, H). Bar = 50 μm.

Although valve replacement demonstrates good results for patients with valvular disease in people, there are no such treatment options in veterinary medicine. In veterinary medicine favorable short-term outcomes have been reported with balloon valvuloplasty, valvulectomy, pulmonic valve commissurotomy and open or closed patch-graft, but each treatment option has some limitations. We report SBV implantation as an alternative treatment option. The ideal artificial valve should mimic the characteristics of a normal native valve in terms of hemodynamics, durability, thromboresistance and biocompatibility. The ability to easily manufacture many sizes corresponding to each individual dog is also important.[11]

In this study, we demonstrated the implantation of the SBV by using "in-body tissue architecture" technology. "In-body tissue architecture" technology is a novel and practical technique for regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in recipients. The autologous prosthetic tissue using this technology has biocompatibility because the tissue is comprised of various autologous cells including fibroblasts, collagenous tissues and elastic fibers. Additionally, SBV can be fabricated in a wide range of shapes and sizes to suit the needs of individual dogs. A 3D printer can manufacture the mold corresponding to each dog. Furthermore, the self-expanding stent has excellent mechanical properties such as symmetrical expansion in both the radial and axial orientations.[19] Stent-Biovalve indicates not only tolerability to the burst pressure but also flexibility and superelasticity corresponding to the ability of the stent to self-expand. These features allow the stent to withstand the surgical implantation procedure without damage to the leaflet. Additionally, the self-expanding feature prevents migration of the stent from the optimal location. The 3 months patency results of the transplanted SBVs were considered excellent.

Metallic stents such as NiTi stents potentially induce stent thrombosis. Although several researchers have successfully attached endothelial cells on the surface of the materials in-vitro to prevent thrombosis,[20,21] the problem remains that the endothelial cells changed to different cells in vivo.[22] In our study, the endothelial-like cells, fibroblasts and elastic fibers were observed in SBV after implantation and the stent struts were covered with autologous cells. The interactions between functional endothelial cells and their underlying extracellular matrix are crucial for basic cell functions including migration, attachment and proliferation.[23] Functional endothelial cells form the vascular endothelial layer and also take part in angiogenesis and tissue regeneration.[24] Stent-biovalves, with potential biocompatibility may lead to properties of the native

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pulmonic valve including antithrombotic ability after implantation. This advantage is immensely important as the dogs do not necessitate anti-coagulation therapy after implantation. Therefore the valve replacement may be an effective treatment option for young dogs that need reparative procedures at early ages with severe congenital heart diseases.

In this study, SBV was implanted in the pulmonic valve position without damage at macroscopic levels to the tissues. The success of the SBV based on "in-body tissue architecture" technology indicates the potential capabilities as an artificial heart valve in veterinary medicine. However, further development is still necessary. We directly implanted the SBV in the pulmonic valve position during cardiac arrest by using cardiopulmonary bypass. Transcatheter application of SBV may need to be developed in the future. Additionally, we have only histological data about the endothelial-like cell of SBVs after implantation. For clinical use of the SBV, longer follow-up studies will be required to confirm continuing hemodynamic benefits such as a lack of myocardial dysfunction and pathological changes.

Conclusion

This is the first report of the functional capabilities of the SBV as a pulmonic valve in beagles after implantation. We have successfully developed a completely autologous heart valve with a self-expanding stent (SBV) by using "in-body tissue architecture" technology. The next step is to perform in-vivo implantation of the SBV to investigate its long-term durability. We expect that SBV is a promising technique for heart valve replacement.

Conflicts of interest

Drs. Takeshi Mizuno, Masashi Mizuno, Kayoko Harada and Masami Uechi are employees of Japan Animal Specialty Medical Institute inc., JASMINE Veterinary Cardiovascular Medical Center.

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