犬の僧帽弁閉鎖不全症に対する
既存の根治療法の評価と新規治療デバイスの開発

Evaluation of the existing treatment and development of a novel treatment device for canine mitral valve disease

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Chapter 1

General Introduction

1.1 Mitral regurgitation and mitral valve repair

Mitral regurgitation (MR) due to degenerative mitral valve disease is the most common heart disease in dogs, accounting for 75 to 80% of cardiac disease in dogs[1]. Signs of progressive MR may be challenging to control with medication alone. The median survival time for dogs with severe MR is 6 to 7 months[2, 3]. Mitral valve repair (MVR) is an alternative treatment option for MR and has demonstrated results superior to mitral valve replacement in humans [4, 5, 6]. Surgical intervention to correct MR[7, 8, 9] and long-term survival following valve replacement[8] has been reported in dogs. However, the primary issue associated with mitral valve replacement is the subsequent need for life-long antithrombotic treatment, which is not required following MVR[8]. At present, the long-term outcome in dogs after MVR is poorly documented[7, 9]. Griffith et al concluded that it is difficult to succeed mitral valve repair of the small breed dogs. We succeeded to improve the success rate of mitral valve repair and perform mitral valve repair to small breed dogs consistently. However, the success rate of mitral valve repair is about 90 % that is not enough.

Cardiac surgery with cardiopulmonary bypass (CPB) is well known to cause systemic inflammatory response syndrome (SIRS) in humans, characterized by the activation of cytokines, the complement cascade, free radicals, and nitric oxide.[10, 11, 12] This so-called

post-pump inflammatory response may induce postoperative multi-system dysfunction in the renal, pulmonary, nervous, hepatic, and myocardial systems,[13, 14, 15] which may increase the morbidity and mortality of cardiac surgery[16]. Therefore, it may be difficult for the dogs with severe systemic organ dysfunction to receive mitral valve repair needing cardiopulmonary bypass.

1.2 Transcatheter valve replacement

Since 2002,[17] the less invasive transcatheter aortic valve implantation (TAVI) has been introduced for inoperable and high-risk patients. Recently, with technological advancements, the clinical application of TAVI has extended to intermediate-risk patients.[18] TAVI is expected to evolve further and become more commonly used in the future. Transcatheter valve replacement of atrioventricular valves (mitral valve and tricuspid valve) such as valve in the mitral ring implantation or in annuloplasty ring implantation were clinically performed to the re-operative patient. Transcatheter replacement of the native atrioventricular valves is not a clinical reality yet due to several technical problems arising from the large size and irregular shape of the annulus, potentially resulting in complications with prosthesis fixation and perivalvular leak. Nonetheless, two acute animal studies have been published pioneering feasibility of transcatheter valve implantation in the native mitral and tricuspid position using a custom-made nitinol stent[19, 20] However, using a bioprosthetic valve for TVR has certain disadvantages, since it undergoes progressive degeneration and calcification as it contains no living cells.[21, 22] To overcome these limitations, living heart valves—created by tissue engineering—have been under development. Some heart valves

created by tissue engineering have been successfully implanted in animals.[23, 24] However, these valves require complicated cell management protocols with cell culture in bioreactors under strictly sterile conditions; this procedure is time-consuming and expensive.

1.3 Concept of *in vivo* tissue engineered heart valve

We have previously developed autologous prosthetic tissues by using the "in-body tissue architecture (IBTA)" technology, which is a novel and practical approach for regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in living bodies.9 This technology involves the use of living bodies as a reactor, and does not need expensive facilities or complicated manipulations. We have reported the construction of completely autologous tri-leaflet heart valves, named Biovalves, prepared using this technology,[25-29] which may resolve the abovementioned problems encountered with bioprosthetic valves. (Fig.1) In fact, Biovalves have been demonstrated to have excellent valve function and histological changes when implanted as a pulmonary valve in a beagle model and an aortic valve in a goat model.[30, 31] In these Biovalves, leaflet formation occurs by tissue ingrowth into small apertures in the preparation molds from the connective tissues surrounding the molds. During the encapsulation process, the conduit portion of the Biovalve is completely formed within approximately 4 weeks of embedding, similar to the Biotubes, which are autologous vascular grafts from IBTA. Being created Biovalves with stents for dogs, it may become possible to treat mitral regurgitation with transcatheter mitral valve replacement.

1.4 Research aims

Therefore, the aim of this thesis was to investigate the effectiveness and problems of the existing treatment (mitral valve repair undergoing cardiopulmonary bypass) and develop a novel treatment device for canine chronic mitral valve disease

Chapter 2

Section 1

Long-term outcome in dogs undergoing mitral valve repair with suture annuloplasty and chordaw tendinae replacement

2.1.1 Introduction

Mitral regurgitation (MR) due to degenerative mitral valve disease is the most common heart disease in dogs, accounting for 75–80% of cardiac disease in dogs[1]. Signs of progressive mitral regurgitation may be challenging to control with medication alone. The median survival time for dogs with severe MR is 6–7 months[2, 3].

Mitral valve repair (MVR) developed as an alternative treatment option for MR and has demonstrated results superior to mitral valve replacement in humans[4, 5, 6]. Surgical intervention to correct MR[7, 8, 9] and long-term survival following valve replacement[8] have been reported in dogs. However, the primary issue associated with mitral valve replacement is the subsequent need for life-long antithrombotic treatment, which is not required following MVR.[8] At present, the long-term outcome in dogs after MVR is poorly documented[7, 9]. We present the long-term outcome of 3 small-breed dogs (body weight < 5 kg) after MVR.

2.1.2 Animals, materials and methods

Case 1: An 8-year-old male Maltese weighing 2.4 kg with respiratory distress and cyanosis was referred to the clinic due to pulmonary oedema caused by MR. Clinical examination revealed a grade IV systolic murmur with maximum intensity over the left cardiac apex. The plasma concentration of atrial natriuretic peptide (ANP) was 368 pg/mL (reference range < 100 pg/mL) [32, 33]. A thoracic radiograph showed mild cardiac enlargement with a vertebral heart size (VHS) of 11.0 vertebral lengths (v) and pulmonary oedema. Two-dimensional echocardiography showed mitral valve prolapse and ruptured chordae tendineae. The fractional shortening was 50%. The normalised left ventricular end diastolic diameter was 2.39 (reference range < 1.85 [34]). The left atrium-to-aorta ratio (LA/Ao) in the right parasternal short axis at the level of the heart base was 2.3 (reference range 0.52-1.13[35]). Colour flow Doppler examination revealed severe MR.

Pulmonary oedema was treated with furosemide, candesartan, and bucladesine sodium. The symptoms improved, but the dog presented with pulmonary oedema again, 5 days later. The severe MR and ruptured chordae tendineae rendered symptom management with medication ineffective; thus, MVR under cardiopulmonary bypass was recommended.

General anesthesia[36] and surgery were performed in the same manner for all dogs. Preanesthetic medications included atropine sulfatea (0.025 mg/kg, IM), midazolamb (0.3 mg/kg, IV), fentanylc (5 μg/kg, IV), and cefazolin sodiumd (20 mg/kg, IV). Each dog was then oxygenated with 100% oxygen, which was followed by face mask induction with 5% isofluranee and intubation with an endotracheal tube (internal diameter, 4 to 7 mm). Anesthesia was maintained with 2% to 3% isoflurane in oxygen at 1.5 L/min. During CPB,

anesthesia was maintained by CRI of fentanylc (0.4 μg/kg/min, IV) and propofolf (0.2 mg/kg/min, IV).

During the surgery, heart rate, respiratory rate, rectal temperature, esophageal temperature, arterial oxygen saturation, end-tidal CO2, and isoflurane concentration were continuously monitored by use of a physiologic monitor system.g The right femoral artery was catheterized for measurement of systolic, diastolic, and mean arterial blood pressures, and the femoral vein was catheterized for measurement of central venous pressure, both with a 22-gauge indwelling catheter. Complete blood count, Hct, total protein concentration, activated clotting time, and arterial blood gases were determined as necessary by use of blood samples obtained via the femoral artery catheter. A urinary catheter was placed to monitor urine volume. Cardiopulmonary bypass was provided by a heart-lung machineh with an extracorporeal circuit, 0.5-m2 oxygenator, and heat exchanger.i The CPB circuit was filled with 20% D-mannitolj (5 mL/kg), 7% sodium bicarbonatek (2 mL/kg), heparin sodiuml (500 U), and acetate Ringer's solutionm (120 to 170 mL). Fifty milliliters of whole blood was replaced with priming solution in dogs weighing < 4 kg. After a neck incision, the left carotid artery and external jugular vein were separated and secured with 3–0 nylon sutures. Next, a left thoracotomy was performed in the fourth or fifth intercostal space after intercostal nerve block with bupivacaine hydrochloride. To place a catheter for cardioplegic infusion, the aortic root was elevated and a purse-string suture was applied with a 5–0 polyvinylidene fluoride suture.o Subsequently, heparin sodium (200 U/kg, IV) was administered. After 3 minutes, activated clotting time was measured with an analyzerp and confirmed to be > 300 seconds. An arterial CPB cannulaq was inserted into the carotid

artery in a proximal direction. The arterial line of the CPB circuit was connected to the carotid artery with a CPB cannula. The venous cannular for blood withdrawal was inserted into the right atrium via the left jugular vein and connected to the CPB circuit. A 5F aortic root catheter was inserted at the site of the purse-string suture on the left lateral aspect of ascending aorta. After air was removed from the CPB circuit, partial CPB was initiated and the patient's body temperature was lowered to 30°C (86°F). Blood flow was controlled at 70 to 100 mL/kg/min (31.8 to 45.5 mL/lb/min) by use of the CPB pump. At this time, the anesthetic was switched from isoflurane inhalation to IV administration of fentanyl and propofol. The aorta was occluded with an arterial clamp proximal to the brachiocephalic trunk, and cardioplegic solutions (10 mL/kg [4.5 mL/lb]; cooled to ≤ 4C; Na+, 120 mEq/L; K+, 20 mEq/L; Cl+, 160.4 mEq/L; Mg2+, 32.0 mEq/L; Ca2+, 2.4 mEq/L; and HCO3−, 10 mEq/L) was immediately and rapidly infused into the left and right coronary arteries via the aortic root to produce cardioplegia. The cardioplegic solution was infused every 20 minutes. During CPB, arterial and venous blood pressure, oxygen saturation, and arterial and venous blood gases were monitored.

We approached the left atrium through a fourth intercostal thoracotomy and incised left auricle to approach to the mitral valve. Chordal replacement and semicircular suture annuloplasty were performed for mitral repair (Fig. 2). Artificial chordae tendineae comprising expanded polytetrafluoroethylene (ePTFE) were sutured to the septal mitral valve and papillary muscle. The lengths of the artificial chordae were adjusted to match the level of the mitral annulus. Circumferential suture annuloplasty was performed to reduce the size of the mitral orifice (Fig. 2). The mitral annulus was measured and plicated to provide

good coaptation. ePTFE (CV-6, Gore-Tex®, Tokyo, Japan) and prolene (5-0 prolene, Ethicon, Tokyo, Japan) sutures were used for chordal replacement and suture annuloplasty, respectively.

The left atrium was closed with simple-interrupted and continuous 5–0 sutures. After closing the left atrium, the aortic clamp was released, and spontaneous sinus rhythm was observed. If ventricular fibrillation was observed, a defibrillator was used at 10 to 30 J. The duration of cardioplegia was 60 to 95 minutes. At the return of sinus rhythm, a dobutaminev CRI was started (1 to 5 μg/kg/min), if needed. The body temperature was recovered to 37°C, and the CPB flow was gradually reduced before being completely shut down. Anesthesia was switched to maintenance with isoflurane inhalation.

After recovery from CPB, the catheter for cardioplegic infusion was removed from the aorta. The catheters were removed from the jugular vein and carotid artery. The carotid artery and jugular vein were closed with a continuous pattern by use of 7–0 suture material. The cervical wound was closed with a simple continous pattern by use of 3–0 nylon. After the removal of catheters, protamine sulfate (6 mg/kg, IV) was administered over 30 minutes. Then, a thoracostomy tube was placed and the thorax was closed routinely after confirming that activated clotting time was < 200 seconds. The patients regained spontaneous respiration and reflexes 2 to 3 hours after the end of surgery. Isoflurane inhalation was discontinued, and the endotracheal tube was removed. The total duration of anesthesia was 4 to 6 hours. After removal of the endotracheal tube, the patients were maintained in a cage filled with O2 (25% to 35%) for 12 to 24 hours. Dalteparin sodiumx (25 to 50 U/kg, SC) was administered when chest drainage fluid was reduced to a rate of < 1 to 2 mL/h. The

chest tube was removed 24 h after surgery. Cefazolin sodiumd (20 mg/kg, IV, q 8 h) was administered for 7 days. Laboratory tests were performed every day until discharge. Ozagrel hydrochloridey (10 mg/kg, PO, q 12 h), a thromboxane A2 synthase inhibitor, was administered for 1 month after surgery

The dog was discharged, and the following changes were noted after 1 month: cardiac murmur (grade I), LA/Ao (1.5), VHS (10.2 v) (Fig. 3), normalised left ventricular end diastolic diameter (2.22), and plasma ANP concentration (86 pg/mL). Medication was discontinued, and the cardiac condition remained stable for 5 years after surgery. Five years after surgery, the MR had worsened due to ruptured native chordae tendineae of the mural mitral leaflet.

Case 2: An 11-year-old male Shih Tzu weighing 4.6 kg with respiratory distress was admitted for emergency treatment of refractory pulmonary oedema caused by MR. Clinical examination identified a grade V systolic murmur over the mitral area. Plasma ANP was 154 pg/mL. A thoracic radiograph showed cardiac enlargement (VHS 13.1 v) and pulmonary oedema. Two-dimensional echocardiography showed mitral valve prolapse, ruptured chordae tendineae, and an enlarged left atrium (LA/Ao: 2.8). The fractional shortening was 49%, and the normalised, left ventricular end diastolic diameter was 2.68. Colour flow Doppler examination revealed severe MR.

The dog was treated with furosemide (2 mg/kg, qid), candesartan (0.5 mg/kg, sid), pimobendan (0.1 mg/kg, sid), and bucladesine (0.02 mg/kg/min, CRI). However, the dog did not respond well. With owner consent, MVR was performed on day 27 of hospitalisation.

The surgical procedures were the same as for Case 1.

One month after surgery, the cardiac murmur was grade II; LA/Ao, 1.6; VHS, 11.5 v; and plasma ANP concentration, 104 pg/mL. However, because of moderate residual MR, candesartan (0.5 mg/kg/day) was continued. Two years after surgery, the dog presented with premature ventricular contractions, congestive heart failure, worsened MR, and mitral annular dilatation; torasemide treatment (0.2–0.6 mg/kg/day) was started. Three years after surgery, the dog died due to pulmonary oedema. Post-mortem examination showed that the annular plication suture was partially detached from the mitral valve circumference (Fig. 4).

Case 3: An 8-year-old male Maltese weighing 3.85 kg with respiratory distress was admitted for recurring pulmonary oedema caused by MR. Clinical examination identified a grade IV systolic murmur over the mitral area. Thoracic radiographs showed mild cardiac enlargement with a VHS of 11.4 v and pulmonary oedema. The plasma ANP concentration was elevated (219 pg/mL). Two-dimensional echocardiography showed mitral valve prolapse and ruptured chordae tendineae; however, the left atrium was not enlarged (LA/Ao: 1.3). Colour flow Doppler examination revealed severe MR. The dog was treated with candesartan (0.5 mg/kg, sid), torasemide (0.3 mg/kg, bid), and bucladesine (0.02 mg/kg/min, CRI). Eleven days after hospitalisation, the dog recovered from the pulmonary oedema, but the owner desired that MVR surgery be performed. The surgery was conducted in a manner similar to that in the previous 2 cases.

No residual MR was observed after surgery. One month after the surgery, the following changes were noted: cardiac murmur (disappeared), LA/Ao (1.3), VHS (10.2 v),

and plasma ANP (96 pg/mL). At present (6 years after surgery), the dog is not receiving any cardiac medication and has not shown any clinical symptoms.

2.1.3 Discussion

Favourable long-term (5-year) outcomes were observed in 2 dogs; 1 experienced worsening MR and heart failure 3 years post surgery. Serres and others (2007) reported that the median survival time for dogs with International Small Animal Cardiac Health Council (IASCHC) class III and chordae tendineae rupture was 191 days.[2] Although the medical treatment used in these three cases was unorthodox, these 3 dogs had severe mitral regurgitation with chordae tendineae rupture and these dogs were classified in ISACHC class III. So, the prognosis of these dogs would be poor, but surgical treatment may be associated with longer life expectancy. MVR and mitral valve replacement have been performed successfully in different-sized dogs with MR, including small-breed dogs, [7, 8] indicating that MVR is an effective treatment option for MR in dogs.

Here, chordal replacement was performed at the site of the chordal rupture, and MVR was performed using semicircular suture annuloplasty. The artificial chordae did not rupture in any of the dogs, suggesting that chordal replacement with ePTFE is as effective in dogs[7, 37] as in humans.[38, 39, 40, 41] Chordal rupture—associated with the mural leaflet (unoperated)—occurred after 4 years in Case 1, causing a deteriorating clinical condition and increased MR. Thus, chordal replacement with ePTFE on the septal and mural leaflets may be required to prevent increasing MR due to native chordal rupture.

Annuloplasty was performed with a mitral valve plication suture around the mitral valve. In Case 2, the suture detached, resulting in increased MR. It persisted unchanged in the other 2 dogs. Recurrent annular dilatation may be due to technical error, as a steep learning curve is expected.[42, 43] Aybeck et al. also reported significant and progressive postoperative mitral annular dilatation associated with suture annuloplasty[44]. Therefore, based on these reports, a prosthetic ring was used during annuloplasty, rather than suture annuloplasty, in the subsequent patients. In contrast to suture annuloplasty, prosthetic ring annuloplasty may provide reproducible long-term results, as observed in humans[38] and dogs[7, 9]. Performing circumferential annuloplasty in dogs with MR could also reduce the amount of MR[7, 9, 45]. In human medicine, prosthetic ring annuloplasty is considered the gold standard technique for mitral repair due to its reproducibility and excellent long-term results[44]. Prosthetic rings also allow optimal annular stabilisation and systolic shape presentation but with some functional disadvantages[46, 47, 48]. Moreover, intracardiac prosthetic material is a potential site of haemolysis or thrombosis, while suture annuloplasty is free of these potential sequelae. The selection of the annuloplasty technique remains controversial; the most effective option in dogs needs to be investigated further.

Chordal replacement and circumferential annuloplasty were performed in 8 dogs with severe MR during the same period. Four dogs survived perioperative periods, but we could not follow one dog after discharge from our hospital. Four dogs died in perioperative periods. The causes of death were bleeding, worsening of pulmonary hypertension, ventricular fibrillation after weaning of CPB and low output syndrome after CPB. Although, currently the survival rate in perioperative period is improving[9], the survival rate of this surgery is

about 90%. This is not satisfying result. Therefore, further study to improve the success rate of this surgery is needed.

Section 2

Intra- and Post-operative complications in 47 dogs that underwent mitral valve repair

2.2.1 Introduction

Section 1 shows that mitral valve repair is an effective treatment option for dogs with MR. However, the survival rate of mitral valve surgery is currently unsatisfactory. Cardiopulmonary bypass surgery can sometimes cause postoperative multi-system dysfunction of the renal, pulmonary, nervous, hepatic, and myocardial systems, as well as other life-threatening complications after surgery.[4-6] At our institution, various complications are observed after cardiopulmonary bypass surgery. The success rate of this surgery must be improved by preventing complications. In human medicine, prophylactic treatments are provided to reduce complications after cardiac surgery. However, there are only few reports regarding the complications of open-heart surgery with cardiopulmonary bypass in dogs. Investigating the types and incidence rates of complications can help identify problems in the perioperative period; this can improve the survival rate of mitral valve repair. The aim of this study was to investigate the complications of cardiac surgery using cardiopulmonary bypass in dogs to improve the survival rate.

2.2.2 Materials and Methods

To assess the incidence of intra- and postoperative complications, we retrospectively reviewed 47 cases of dogs that underwent MVP with CPB at the Nihon University Animal Medical Center between August 2006 and September 2007. Mitral valve repair was performed as described in Chapter 2. We retrospectively investigated the incidence and causes of post-operative mortality and morbidity.

2.2.3 Results

Forty-seven dogs [22 males and 25 females, age: 62–175 (123±25) months, bodyweight: 1.8–13.5 (5.7±3.0) kg] were enrolled. (Table 1) The mean age of the 10 dogs that died within 4 months of surgery was 140±21 (range: 115–175) months, which was significantly higher than the age of dogs that survived beyond 4 months post-surgery [119±25 (range: 62–157) months]. The 4-month postoperative mortality rate was 29% for dogs aged 10 years or older and 11% for those younger than 10 years. The causes of death were surgical technical problems (2 cases), thrombosis (4 cases), pancreatitis (2 cases), pulmonary hypertension (1 case), and unknown (1 case). Of the 37 subjects that survived beyond 4 months, 4 had postoperative complications (i.e., 1 case each of thrombotic cerebral infarction, pulmonary infarction, cerebellar infarction, and aspiration pneumonitis).

2.2.3 Discussion

Cardiac surgery using cardiopulmonary bypass can cause various complications, such as

renal, pulmonary, nervous, hepatic, and digestive organ, as well as myocardial dysfunction[4-6]. In this study, the main cause of mortality after mitral valve repair in dogs was related to thrombosis, followed by pancreatitis.

Thrombus formation (including those in the left atrium) was observed in 12 subjects, and was the most frequent cause of postoperative complications and/or death. There appeared to be various causes of thrombosis associated with extracorporeal cardiovascular surgery, such as detachment of endothelial cells by grasping with forceps, foreign substances such as artificial chordae tendineae left inside the heart, and use of heparin, protamine, or proteolytic enzyme inhibitors. Griffith et al. reported that systemic thromboembolism was a main cause of post-operative mortality in dogs who had undergone mitral valve repair.[49] These results suggest that preventing thrombus formation is important for the success of mitral valve repair with cardiopulmonary bypass.

The second most frequent cause of postoperative complications was related to inflammation. A strong systemic inflammatory response occurs due to the release of mediators caused by contact with blood and the extracorporeal surface during CPB, such myocardial ischemia reperfusion secondary to aortic cross-clamping, endotoxemia, and operative trauma.[50, 51, 52] In this study, pancreatitis was the primary cause of mortality associated with inflammation. In human medicine, post-operative pancreatitis after cardiac valve surgery occurs frequently and affects the clinical outcome.[53] Previous studies have reported that splanchnic ischemia, hypoperfusion, systemic inflammatory response, and nonpulsatile blood flow during cardiac surgery may cause pancreatic cellular injury; several risk factors have also been suggested.[54-58] Same phenomenon may occur in dogs. However,

it does not become clear in veterinary clinical cases, yet. To reduce the incidence of inflammation and pancreatitis after cardiac surgery with CPB in dogs, understanding post-pump inflammatory responses and adopting countermeasures are as important in veterinary medicine as in human medicine.

2.3 Conclusion

In conclusion, MVR is an effective treatment option for dogs with MR. Chordal replacement with ePTFE is effective for dogs and provides long-term durability. However, mitral annuloplasty using prosthetic rings may be preferred over suture annuloplasty in dogs. Although the survival rate in the perioperative period is improving[9], the results remain unsatisfactory. Thrombus formation (including those in the left atrium) is the most frequent cause of postoperative complications and/or death. The second most frequent reason for postoperative complications is related to inflammation. These results suggest that prevention of thrombosis and suppression of inflammation are important strategies for improving the surgical outcome of mitral valve repairs. Further study to improve the success rate is needed.

Chapter 3

Plasma cytokine levels in dogs undergoing cardiopulmonary bypass

3.1 Introduction

Cardiac surgery using cardiopulmonary bypass (CPB) causes systemic inflammatory response syndrome (SIRS) in humans, characterized by the activation of the complement cascade and the production of cytokines, free radicals, and nitric oxide.[10-12] This so-called post-pump inflammatory response may induce postoperative multi-system dysfunction in the renal, pulmonary, nervous, hepatic, and myocardial systems,[13-15] which may increase the morbidity and mortality of cardiac surgery.[16] In fact, the results of the study in Chapter 1 suggested that inflammatory response after surgery may increase morbidity and mortality after mitral valve repair under cardiopulmonary bypass in dogs.

The post-pump inflammatory response is triggered by various factors, including surgical trauma, endotoxin, reperfusion injury, and exposure of blood to the surface in the extracorporeal circuit.[10, 11, 16] Several studies have reported elevation of pro-inflammatory (i.e., plasma interleukin-6 [IL-6], interleukin-8 [IL-8], and tissue necrosis factor-α [TNF-α]) and anti-inflammatory cytokines (i.e. interleukin-10 [IL-10]) after CPB in humans.[12, 59]

However, there is no report regarding the inflammatory response to CPB in dogs. The aim of this study was to clarify the inflammatory response to CPB in dogs by measuring pro- and anti-inflammatory cytokine levels, WBC counts, and CRP levels during and after CPB.

3.2 Animals, materials and methods

Cases

Dogs that underwent mitral valve repair using CPB at Nihon University between April 2007 and May 2009 were included in this study. All dogs weighed over 3 kg and did not have any evidence of congestive heart failure (CHF). Dogs with evidence of any systemic diseases were excluded from this study.

Control

Seven healthy female dogs based on physical examination were spayed and included in this study as the control group.

Anesthesia and mitral valve repair

Details of the anesthetic protocol and mitral valve repair procedure were previously described.[60] Anesthesia and surgery were performed in the same manner in all dogs undergoing mitral valve repair. Cardiopulmonary bypass was performed with a cardiopulmonary bypass circuit. After induction of cardiac arrest, a mitral annuloplasty was performed, and the chordae tendineae were replaced with expanded polytetrafluoroethylene chordal prostheses. After closure of the left atrium and declamping to restart the heart, the thorax was closed.

Dogs in the control group were induced by propofol, maintained by isoflurane, and underwent routine ovariohysterectomy. No complications were noted pre- or

postoperatively.

Blood sampling

Blood samples were collected preoperatively (*pre*), 5 minutes after administration of heparin (*heparin +5*), 5 minutes after clamping of the aorta (*bypass +5*), after resumption of the heartbeat (*resume*), 15 minutes after protamine sulfate administration (*protamine +15*), at 3 hours (*End of bypass* [*EB*] *+3hr*), 6 hours (*EB +6hr*), 12 hours (*EB +12hr*), 24 hours (*EB +24hr*), and 48 hours after completion of CPB (*EB +48hr*). Blood samples were taken from an arterial catheter placed in the femoral artery from *heparin +5* to *EB +3hr* and from the saphenous vein or jugular vein at *pre* and after *EB +6hr*.

In the control group, blood samples were collected preoperatively (*pre*), at 5 minutes (*OVH +0hr*), 3 hours (*OVH +3hr*), 6 hours (*OVH +6hr*), and 24 hours after ovariohysterectomy (*OVH +24hr*). Blood samples were taken from the saphenous vein or jugular vein.

C-reactive protein (CRP) measurements and white blood cell (WBC) counts

To obtain CRP measurements, blood samples were mixed with heparin and centrifuged at 12,000 rpm for 120 seconds to isolate the plasma. Plasma concentrations of CRP were analyzed using an immunoserological test[j] (reference range, 0.05–20 mg/dL). The WBC counts were determined from blood mixed with ethylenediaminetetraacetic acid (EDTA) by an automated cell counter[k].

Cytokine measurements

Blood samples for the measurement of cytokines were collected in EDTA tubes and centrifuged at 1000 *g* for 30 minutes at room temperature within 30 minutes after blood collection. The plasma was frozen and immediately stored below –20°C. Blood samples

were thawed at room temperature before measurement. IL-6, IL-10, and TNF-α were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit[1].

Statistical analysis

Statistical analysis was performed with GraphPad Prism® Version 5.0 statistical software (GraphPad Software Inc.; San Diego, California, USA). Data was presented as mean ± standard deviation of the mean (SD). Changes in the parameters with time compared to baseline (*pre*) were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons. Comparison of cytokine levels, CRP levels, and WBC counts between the cases and control group were analyzed using two-way ANOVA followed by Sidak's multiple comparisons. A *P* value less than 0.05 indicated statistical significance. Pearson's correlation coefficient test was performed to examine correlations between peak cytokine levels and the volume of transfusion.

3.3 Results

During this study period, 63 dogs underwent mitral valve repair. Fifty-two dogs were excluded from this study because of pulmonary edema (n = 26), low body weight (<3 kg; n = 14), examiner absence (n = 9), congenital cardiac disease (n = 1), Chiari malformation (n = 1), and renal failure (n = 1). Eight of 63 dogs died during the hospital stay. The causes of death were as follows: intra-operative bleeding, sudden death, aspiration pneumonitis, acute renal failure, pancreatitis, and multiple organ failure. Thus, eleven dogs were included in this study. Clinical characteristics of the dogs are shown in Table 1. All surgeries were

successful, and the dogs were discharged within 2 weeks with no signs of complications (organ failure, severe inflammatory signs such as pneumonia or pancreatitis, low output syndrome, and thrombosis) or infections.

1. Cytokine levels

IL-6 level increased significantly from *protamine +15* (231 ± 284.4 pg/mL, $P < 0.001$) to peak values at *EB +3hr* (271 ± 179.8 pg/mL, $P < 0.0001$) in comparison with baseline (*pre*; 3.96 ± 8.22 pg/mL). Thereafter, IL-6 levels progressively declined to pre-CPB levels by *EB +48hr* (Fig. 5A). Comparisons between groups with repeated measures ANOVA showed higher values in the cases compared with the control group (Time, $P < 0.005$; Groups, $P < 0.0001$; Interaction, $P < 0.05$). With multiple comparisons, IL-6 levels of the cases at *protamine +15, EB +3hr,* and *EB +6hr* were significantly higher than those levels of the control group at *OVH +0hr* (13.5 ± 23.6 pg/mL)*, OVH +3hr* (36.6 ± 32.1 pg/mL), and *OVH +6hr* (20.7 ± 25.3 pg/mL) ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively) (Fig. 7A).

IL-10 levels increased from *resume*, peaked at *protamine +15* (58.2 ± 65.2 pg/mL), and then decreased gradually (Fig. 5B). There were no significant differences among all time points in comparison with *pre* (3.71 ± 5.33 pg/mL). There were no significant differences between the cases and the control group (Fig. 7B).

TNF-α levels displayed a bimodal distribution, peaking at *protamine +15* and *EB +12hr* (0.67 ± 1.63 pg/mL, 0.81 ± 1.99 pg/mL, respectively) (Fig. 5C). However, there were no significant differences among all time points in comparison with *pre* (0.2 ± 0.41 pg/mL). There were no significant differences between the cases and the control group (Fig. 7C).

2. WBC count

WBC counts decreased until *bypass +5* and remained increased until the last time point at *EB +48hr* (Fig. 6A). WBC count at *EB +24hr* (27755 ± 10507/μL) was significantly higher than the control group at *OVH +24hr* (17411 ± 2565/μL) ($P < 0.05$) (Fig. 8A).

3. CRP

CRP levels gradually increased from *EB +3hr* until the end of study at *EB +48hr* (Fig. 6B). There were no significant differences between the cases and the control group (Fig. 8B).

4. Correlations between peak cytokine levels and the transfusion volume

The peak IL-6 concentration was significantly correlated with the transfusion volume (Fig. 9, $r = 0.719$, $r^2 = 0.518$, $P < 0.05$). However, there were no significant differences between peak IL-10 and TNF-α concentrations and total volume transfused.

3.4 Discussion

CPB causes SIRS in humans, and cardiopulmonary bypass influences cytokine levels and complement activation during and after surgery.[10, 11, 16] The present study showed that CPB significantly raised the plasma IL-6 level in dogs, as it does in humans.

IL-6 is involved in a wide variety of biological functions, especially in pro- and anti-inflammatory responses. It acts on B cells to induce differentiation into plasma cells, stimulates the release of hepatic proteins, and is involved in neutrophil-mediated ischemia/reperfusion injury.[15] Therefore, the increase in plasma IL-6 measurements during and after CPB was probably a manifestation of an acute phase inflammatory response.

IL-10 is an anti-inflammatory cytokine that reduces neutrophil adhesion to activated endothelial cells. In infectious diseases and septic shock, IL-10 is synthesized later than

inflammatory cytokines in both T cells and monocytes, suggesting a regulatory role in the later phases of the immune response.[61, 62] In contrast, although not statistically significant, the present study showed that IL-10 levels tended to increase earlier than IL-6. Risnes *et al*.[63] reported a similar phenomenon and suggested that prostaglandin E2 and catecholamines may induce IL-10 secretion. However, the stimulatory mechanism of early IL-10 release occurring independently from systemic proinflammatory cytokine elevation remains unclear.

TNF-α produced by macrophages and monocytes is a proinflammatory cytokine that regulates the adhesiveness of leukocytes through the induction of adhesion molecules.[64] The present study showed that TNF-α levels increased in a bimodal distribution. This result was similar to a previous report in humans.[14] However, there were no significant differences between the values at different time points compared to those preoperatively, which may be due to the low sensitivity of the ELISA kit, as many of the results were below the detection limit.

WBC counts and CRP levels were elevated after CPB compared with baseline, but none of the dogs showed signs of infection. Therefore, these changes may have been caused by the inflammatory response and surgical trauma. The WBC counts and CRP levels were increased for more than 2 days after CPB. This increase suggests that the inflammatory response caused by CPB and surgical trauma continues for at least 2 days.

This study confirms that the levels of inflammatory biomarkers, IL-6, WBC counts, and CRP are elevated after CPB. In human medicine, it is known that the patients who undergo coronary artery surgery using CPB show more severe inflammatory response

after surgery than the patients without the use of CPB.[65, 66] Therefore, it is thought that CPB induces an inflammatory response. In this study, IL-6 levels and WBC counts after CPB were significantly higher than the control group. This result shows that CPB may induce an inflammatory response in dogs as previously reported in humans.

Previous studies reported that transfusion and hemodilution enhanced the inflammatory response during CPB. [67, 68, 69] In this study, the volume of transfusion and IL-6 level had a significant correlation. Since most dogs that undergo mitral valve repair are small breed dogs, to reduce the amount of transfusion, it is important to minimize the priming volume, hemodilution, and blood loss. Frequent administration of cardioplegia causes hemodilution, which increases the number of transfusions. Minimizing the cardiac arrest time may also reduce the inflammatory response.

IL-6 is associated with postoperative renal, lung, and nerve dysfunction and myocardial injury.[15,70,71] In canine systemic inflammatory syndrome and sepsis, plasma IL-6 concentration was shown to be a predictor of outcomes.[72] Therefore, hypercytokinemia facilitates SIRS and may worsen the condition of dogs after cardiac surgery.

Previous studies have demonstrated that increased IL-6 and CRP levels may contribute to myocardial dysfunction, lung inflammation, and intestinal dysfunction during various acute inflammatory conditions.[15, 70, 71] In this study, all dogs were discharged as planned without any complications. However, about 7% of the dogs undergoing mitral valve repair have died after surgery in our institution.[60] The CPB-induced systemic inflammatory response described in this study may increase mortality or be a trigger for postoperative complications. Therefore, countermeasures against the inflammatory response after CPB, such as

improvement of the CPB circuit, administration of steroids or protease inhibitors, reduction of the cardiac arrest time, and minimization of hemodilution, transfusion, and priming volume or development of other radicar treatment for mitral valve insufficiency without cardiopulmonary bypass (e.g. transcatheter valve replacement or transcatheter mitral repair) may be required to improve patient outcomes.

3.5 Conclusion

CPB induced IL-6 release that triggered a postoperative inflammatory response which included elevations in WBC. Such an inflammatory response after cardiac surgery may cause acute lung injury, myocardial dysfunction, renal failure, and multiple organ failure, which ultimately may increase postoperative mortality and morbidity. Therefore, countermeasures against the inflammatory response after CPB or development of other radical treatment for mitral valve insufficiency without cardiopulmonary bypass (e.g. transcatheter valve replacement or transcatheter mitral repair) may be required to improve patient outcomes.

3.6 Study Limitations

Inflammatory pathways not mediated by cytokines (e.g., complement and leukocytes) were not directly examined. Open heart surgery may be more invasive than ovariohysterectomy. Therefore, the difference in cytokine levels and WBC counts might be influenced by the differences in surgery-related stress between open heart surgery and ovariohysterectomy.

This study excluded dogs with CHF or systemic diseases because we wanted to determine the inflammatory response to CPB in dogs. The number of dogs included in this study was

small, but all included cases survived and did not show any complications (organ failure, severe inflammatory signs such as pneumonia or pancreatitis, and thrombosis). Therefore, we could not confirm whether hypercytokinemia affected the outcome of the dogs that underwent CPB. In the future, a larger study is needed to confirm whether hypercytokinemia after CPB contributes to organ dysfunction.

Chapter 4

Preparation of an autologous heart valve with a stent (stent biovalve) using the stent evertion method

4.1 Introduction

The results of previous chapter reveal that countermeasures against the inflammatory response after CPB or development of other radical treatment for mitral valve insufficiency without cardiopulmonary bypass (e.g. transcatheter valve replacement or transcatheter mitral repair) may be required to improve patient outcomes. Since 2002,[17] the less invasive transcatheter aortic valve implantation (TAVI) has been introduced for inoperable and high-risk patients. Recently, with technological advancements, the clinical application of TAVI has extended to intermediate-risk patients.[18] TAVI is expected to evolve further and become more commonly used in the future. Transcatheter valve replacement of atrioventricular valves (mitral valve and tricuspid valve) such as valve in the mitral ring implantation or in annuloplasty ring implantation were clinically performed to the re-operative patient. Transcatheter replacement of the native atrioventricular valves is not a clinical reality yet due to several technical problems arising from the large size and irregular shape of the annulus, potentially resulting in complications with prosthesis fixation and perivalvular leak. Nonetheless, two acute animal studies have been published pioneering

feasibility of transcatheter valve implantation in the native mitral and tricuspid position using a custom-made nitinol stent[19, 20]. However, using a bioprosthetic valve for TVR has certain disadvantages, since it undergoes progressive degeneration and calcification as it contains no living cells.[21, 22] To overcome these limitations, living heart valves—created by tissue engineering—have been under development. Some heart valves created by tissue engineering have been successfully implanted in animals.[23, 24] However, these valves require complicated cell management protocols with cell culture in bioreactors under strictly sterile conditions; this procedure is time-consuming and expensive.

We have previously developed autologous prosthetic tissues by using the "*in-body* tissue architecture (IBTA)" technology, which is a novel and practical approach for regenerative medicine based on the tissue encapsulation phenomenon of foreign materials in living bodies.[25] This technology involves the use of living bodies as a reactor, and does not need expensive facilities or complicated manipulations. We have reported the construction of completely autologous tri-leaflet heart valves, named Biovalves, prepared using this technology, [26-29] which may resolve the abovementioned problems encountered with bioprosthetic valves. In fact, Biovalves have been demonstrated to have excellent valve function and histological changes when implanted as a pulmonary valve in a beagle model and an aortic valve in a goat model.[30, 31] In these Biovalves, leaflet formation occurs by tissue ingrowth into small apertures in the preparation molds from the connective tissues surrounding the molds. During the encapsulation process, the conduit portion of the Biovalve is completely formed within approximately 4 weeks of embedding, similar to the Biotubes, which are autologous vascular grafts from IBTA. However, because tissue

migration into a small aperture is slow in general, leaflet formation in the Biovalve required a longer time than that required for conduit formation.

In this study, to further improve the Biovalve design and to adapt it for transcatheter valve replacement, we attempted to combine the Biovalve design with a self-expandable nitinol stent to construct a "Stent-Biovalve". The preparation mold was designed based on a novel concept, in which the leaflet tissue was formed on the outer side of the mold; the tri-leaflet-shaped valve was obtained by finally everting the stent such that the leaflet tissue existed inside surface of the stent. The valvular function of the Stent-Biovalve was examined by using an *in vitro* circulation circuit. Finally the Stent-Biovalve was implanted in the mitral position in a beagle dog.

4.2 Materials and Methods

Animal Studies

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. 12002).

Mold Assembling

Two kind of stents used were self-expandable (diameter, 14 mm; length, 15 mm or diameter, 20 mm; length, 30 mm), obtained from shape re-memory of E-LUMINEXX

(diameter, 12 mm; length, 100 mm; Bard, Karlsruhe, Germany) by Piolax Medical Devices (Yokohama, Japan) and cutting.

The mold for the Stent-Biovalve was obtained by assembling the stent, a specially designed column-shaped acrylic part (outer diameter, 14 mm; length, 32 mm for 14 mm-sized stent, and outer diameter, 20 mm; length, 46 mm for 20 mm-sized stent), and cylinder-shaped acrylic part (outer diameter, 17 mm; length, 18 mm for 14 mm-sized stent, and outer diameter, 23 mm; length, 34 mm for 20 mm-sized stent) with three slits (width, 1 mm; length, 10 mm for 14 mm-sized stent, and width, 1 mm; length, 15 mm for 20 mm-sized stent). All acrylic parts were prepared using a 3D printer (CONNEX 260, Objet, Rehovot, Israel). After gently everting the stent in ice water, it was mounted on the column-shaped acrylic part and then covered with the cylinder-shaped part for the final mold.

Preparation of Stent-Biovalves

A beagle dog (age, 1 year; body weight, 10 kg) under general anesthesia induced by intramuscular injection of ketamine (20 mg/kg), or a goat (age, 1 year; body weight, 50 kg) under general anesthesia induced with 10 mg/kg of ketamine and maintained with 1–3% isoflurane was used for the Stent-Biovalve preparation. After 4 weeks of mold embedding in the abdominal subcutaneous pouches of each animal, the implants—which were completely encapsulated with connective tissue—were harvested. The fragile, irregular, and redundant tissues around the developed tubular tissue were gently cut, and the impregnated parts were removed from both the ends of the developed capsular tissue. The acrylic cylinder- and

column-shaped parts were removed. The stent, now embedded in connective tissue, also showed 3 flaps of membranous connective tissue on its outer surface. The stent was then everted to its original form in ice water to obtain the Stent-Biovalve, with a tri-leaflet valve on its inner surface.

Measurement of Burst Strength

The burst strength of the leaflet tissues of Stent-Biovalve was determined by using a specially designed apparatus. The specimens were fixed on a sample holder with a hole (diameter, 2 mm) at its center. Saline solution was introduced into this apparatus at a rate of 50 mmHg/s. The burst strength was determined by measuring the water pressure at the instant of the tissue rupture using a pressure transducer (N5901; Nihon Denki Sanei, Inc., Tokyo, Japan).

Histological Evaluation

Some parts of the leaflet of Stent-Biovalve were fixed in 10% formalin solution and embedded in paraffin. Then leaflet sections were cut into pieces 3-5μm thick for hematoxyline and eosin staining. In addition, the histological sections were stained with masson trichrome stain. The composition cell was evaluated and the wall thickness of the TEVGs was measured by microscopic examination of cross sections.

Mechanical test

Measurement of the connective strength between leaflet and stent of Stent-Biovalve were

performed by use of a uniaxial tensile-testing apparatus (Rheoner II; Yamaden, Tokyo, Japan). The connective strength between native aortic valve and conduit of goat were also measured by same way. Each sample was fixed in a sample folder that was specially designed by use of a 3D printer (Projet HD3000; 3D Systems, Rock Hill, SC, USA). The testing speed was 0.05 mm/s until failure, i.e., tissue rupture. Ultimate tensile strength was calculated from the stress-strain curves.

In Vitro Valve Function

Valve function was examined using a pulsatile circulation circuit (LaboHeart NCVC, IWAKI; working fluid, 0.9% saline; mean arterial pressure, 100 mmHg; mean flow rate, 5–6 L/min, Fig. 2). The flow rate, left ventricular pressure, and aortic pressure were measured using an ultrasonic flow meter and pressure meter. The regurgitant ratio and mean flow rate at every 10 bpm from 70 to 120 pulsatile rates were evaluated.

Implantation of the Stent-Biovalve in the mitral position in a beagle dog

A beagle dog (age, 1 year; body weight, 10 kg), under general anesthesia induced by intramuscular injection of ketamine (20 mg/kg) and maintained with 1–3% isoflurane, was used for the Stent-Biovalve implantation. We approached the left atrium through fourth intercostal thoracotomy. The Stent-Biovalve was set into the self-build delivery catheter. A purse-string suture was placed at the left atrium. The position of the mitral annulus was confirmed with angiography. The Stent-Biovalve was implanted in the mitral position by using a delivery catheter through the left atrium. After the chest was closed, the movement

of the Stent-Biovalve at the mitral position was confirmed by echocardiography.

4.3 Results

Preparation of Stent-Biovalves

The two different sized assembled molds (outer diameter of stents, 14 or 20 mm; Fig. 10A) that were embedded in the subcutaneous pouches of the beagle or the goat for 4 weeks showed complete encapsulation with autologous connective tissue (Fig. 10B). The implants could be easily harvested because the developed capsulated tissues and the surrounding subcutaneous tissues were connected only by very fragile, irregular, and redundant tissues, which could be easily removed. The capsulated tissues were dissected to remain the tissue for the leaflets (Fig. 10C). The molds could be smoothly removed from both ends of the implant because there was no adhesion between the molds and the tissues covering the stent (Fig. 10D). The leaflet tissues were strongly fixed at the three commissures. The stents were iced and then inverted inside out. The tissue flaps, which originally existed outside the stent, were thereby converted to inner leaflets; the Stent-Biovalves were thus completely prepared (Fig. 10E). During the inversion, no or little damage occurred to the leaflet tissues and to the connecting tissues between the leaflet and the stent.

Burst Strength

The burst strength of the leaflet was over 7600 mmHg. Two sized Stent-Biovalves with a sufficient coaptation area were thus obtained with outer diameter of 14 mm from a beagle or 20 mm from a goat (Fig. 11).

Histological evaluation

The valve leaflets of Stent-Biovalve mainly consisted of collagen fibers and fibroblasts (Fig. 12). The thickness of the valve leaflet was 579.9 ± 8.2 μm.

Mechanical results

To evaluate the connective strength between Biovalve leaflet and stent, the tensile strength of the Biovalve leaflet and stent was measured (Fig. 13). The connective strength between Biovalve leaflets and stent was comparable to between aortic valve and conduit of goats. The mean values of ultimate tensile strength in each sample were as follows, Biovalve leaflet-stent: 833.8±215.5 gf; aortic valve- conduit of goat: 949.7±186.1 gf.

***In vitro* valve function**

The movement of the leaflets in a pulsatile flow circuit (Fig. 14) was examined using videography. The Stent-Biovalve leaflets closed rapidly and tightly in synchronization with the backward flow in the diastolic phase. In the transition phase of the flow direction, the valve opened smoothly and the aperture ratio of the valve was 89% (Fig. 15A), and coaptation of valve leaflets was optimal (Fig. 15B).

Figure 5C shows the flow rate waveforms of the Stent-Biovalve at 70 bpm. Regurgitation in the diastolic phase was almost completely prevented. The mean flow rate was approximately 5–6 L/min (Fig. 16A), and the regurgitation ratio was approximately 4% for each heart rate tested (Fig. 16B).

Implantation of the Stent-Biovalve in the mitral position in a beagle dog

Implantation of the Stent-Biovalve in the mitral position was performed smoothly and easily by using original delivery catheter (Figure 17) within 1 hour. The Stent-Biovalve leaflets closed and opened smoothly. Although little paravalvular leakage was confirmed, there was

no regurgitant color flow through the Stent-Biovalve. (Figure 18)

4.4 Discussion

Here, we report the successful development of novel construction method for a Stent-Biovalve with robust valve leaflets and favorable *in vitro* function.

In the previously reported design for Biovalves, the molds were designed such that valve leaflets were formed by connective tissue penetrating the apertures in the preparation molds. However, such tissue ingrowth was slow; the formation of the valve leaflets therefore required a long duration and was not always robust.

In the present technique, the structure of the new molds for the Stent-Biovalve ensured successful formation of three valve leaflets, with broad flaps available. The key point of this mold design was that the outer circumferential connective tissue was used to form each valve leaflet. Typically, in a tri-leaflet valve with a diameter of 14 mm, the horizontal leaflet length for adequate coaptation in the closed valve is 14 mm. Using the present method, the leaflet was formed along the acrylic cylinder-shaped part, which had a length of 17 mm, on the outer side of the stent that had a diameter of 14 mm. Thus, the formed leaflet had a length of 17 mm, which is approximately 1.2 times longer than that required for definitive coaptation. Since the area of the leaflet tissues was sufficient large, an extremely low regurgitation rate (~4%) was noted when testing *in vitro* valvular function, much lower than that noted for the previous Biovalve (type IV, regurgitation rate 20%).

Moreover, since the valve leaflets were technically formed in the opening state, each valve leaflet opened smoothly in the systolic phase, resulting in a high aperture ratio of 89%.

In addition, the leaflet tissues were very robust because the tissues were originally formed as a conduit. In our previous Biovalve (type V), the burst strength of the tissue that was formed as a conduit (over 7600 mmHg) was over two times greater than that of the leaflet part (2900 ± 1300 mmHg); however, in the native aortic valve, high burst strength was obtained in both the conduit (over 7600 mmHg) and leaflet parts 6200 ± 1400 mmHg). The connective strength between Biovalve leaflet and stent was comparable to native aortic valve and conduit. Therefore, the strength of the leaflet tissue formed in this Stent-Biovalve was similar to that of the native valve tissue.

In order to facilitate the use of the Stent-Biovalve in TVR, a self-expandable nitinol stent was chosen in this study, which is typically used for transcatheter aortic valve implantation (TAVI) (CoreValve, Medtronic Inc., Minneapolis, USA).[73] For transcatheter implantation, the CoreValve is crimped into a sheathed catheter under low temperature conditions.[74] The valve leaflet for TAVI thus requires adequate durability and a strong tissue connection with the stent for successful crimping into the sheathed catheter. The Stent-Biovalves constructed in the present study showed favorable valve function even after eversion (inside out) under low temperature conditions (iced water). This handling exposed the valve leaflets to conditions that were more severe than crimping. Even after turning the stent inside out, the valve leaflets remained strongly connected with the stent (Fig. 1D), which did not tear apart even by pulling with forceps, as demonstrated in the type IV Biovalve, which was strongly attached to a scaffold material.[29] Therefore, it is highly expected that the valve leaflets of the Stent-Biovalve would have sufficient durability and a robust tissue connection with the stent for crimping into a sheathed catheter for TAVI.

Recently, several studies reported that tissue engineered valved stents were successfully implanted as pulmonic valve *in vivo*.[75-77] These materials may overcome the limitaions of bioprosthetic heart valve prostheses and may be expected further evolution to be applied to clinical use. However, these tissue-engineered valved stents require complicated cell management protocols with cell culture under strictly sterile conditions, and also require decellularized allografts or synthetic scaffold materials to seed autologous cells. In this study, autologous heart valve with stent (Stent-Biovalve) showed favorable valve function, without expensive facilities or complicated manipulations. Stent-Biovalve may overcome the limitations of bioprosthetic heart valve prostheses and have the advantage of other tissue engineered valved stents.

In this study, the pulsatile circuit was designed for actual human aortic valve conditions (mean flow, 5 L/min; mean pressure, 100 mmHg; heart rate, 70–100 bpm); however, saline solution was selected as a working fluid. The kinetic viscosity of blood is 4.4×10^{-6} m^2/s, which is approximately 4 times that of saline solution (1.0×10^{-6} m^2/s). Thus, our circuit did not approximate the viscosity of blood; however, it was considered that the present design achieved an adequate performance of valvular function for a future animal implantation study.

Based on the American College of Cardiology/American Heart Association valvular guidelines 2006,[18] the regurgitant fraction of the present Stent-Biovalve was much lower than the “mild” classification, in fact being present at “trace” levels. Yare et al. reported that 75% of bioprosthetic valves used for TAVI showed trace to mild aortic regurgitation (AR) after implantation; however, such AR did not affect LV structure and function.[78] Previous

biovalves were demonstrated to have favorable valve function and histological changes when used as heart valves in a beagle model[30]; further, biotubes created by in-body tissue architecture technology regenerated arteries within 3 months of implantation.[79] Therefore, although the present evaluation of the Stent-Biovalve was performed *in vitro*, it is expected that this Stent-Biovalve with autologous valve leaflets will show more favorable function *in vivo* than *in vitro*. Although only one pilot animal study was performed here, the Stent-Biovalve showed good valve function at the mitral position in the short study period. We expect that this Stent-Biovalve can be a good treatment device for canine chronic mitral valve disease. Future studies are expected to focus on the implantation of this Stent-Biovalve in animal models to confirm chronic valve function and durability *in vivo*.

4.5 Conclusion

We successfully developed a novel method for efficient construction of a robust, completely autologous heart valve eversion of a self-expandable stent, and named the resulting product the Stent-Biovalve. These Stent-Biovalves were obtained after only 4 weeks of subcutaneous embedding in a beagle dog or a goat. Owing to the large and robust leaflet tissues that were developed in the open-form position, excellent *in vitro* valve function was achieved. As shown in the pilot model in this study, the Stent-Biovalve can be a novel treatment device for canine chronic mitral valve disease. Future studies are expected to focus on the implantation of this Stent-Biovalve in animal models to confirm chronic valve function and durability *in vivo*.

Chapter 5

Conclusions

Mitral regurgitation (MR) due to degenerative mitral valve disease is the most common heart disease in dogs, accounting for 75 to 80% of cardiac disease in dogs. Signs of progressive MR may be challenging to control with medication alone. In this thesis, the problems and effectiveness of mitral valve repair under cardiopulmonary bypass were revealed and autologous heart valves with stents were developed.

Chapter 2

Mitral valve repair under cardiopulmonary bypass was performed in three dogs with clinical signs associated with mitral regurgitation that were not controlled by medication. Mitral valve repair comprised circumferential annuloplasty and chordal replacement with expanded polytetrafluoroethylene. One dog died 2 years after surgery because of severe mitral regurgitation resulting from partial circumferential suture detachment. The others survived for over 5 years, but mild mitral valve stenosis persisted in one. The replaced chordae did not rupture in any dog. Mitral valve repair appears to be an effective treatment for mitral regurgitation in dogs. Chordal replacement with expanded polytetrafluoroethylene is a feasible technique, demonstrating long-term durability in dogs.

Chordal replacement and circumferential annuloplasty were performed in 8 dogs with

severe MR during the same period. Four dogs died in perioperative periods. The causes of death were related to the surgical technique or drawbacks of using cardiopulmonary bypass. Although, currently the survival rate in perioperative period is improving (about 90%), this is not satisfying result. Thrombus formation (including those in the left atrium) was the most frequent cause of postoperative complication and/or death. The second most frequent reason for postoperative complications was related to inflammation. These results suggest that prevention of thrombosis and suppression of inflammation are important strategies for improving the surgical outcome of mitral valve repair. Further study to improve the success rate of this surgery is needed.

Chapter 3

We investigated the time course of pro- and anti-inflammatory cytokine levels during and after CPB and the correlation of the levels with variable parameters of CPB. The study group included 11 dogs that underwent mitral valve repair with CPB at Nihon University between April 2007 and May 2009, and the control group included 7 healthy dogs that underwent ovariohysterectomy. After CPB, plasma levels of IL-6, WBC counts, and CRP levels were significantly higher than preoperative levels, and IL-6 levels in the study group were significantly higher than those in the control group. Peak IL-6 level was significantly correlated with total blood administration. CPB induces a systemic inflammatory response in dogs. Such an inflammatory response after cardiac surgery ultimately may increase postoperative mortality and morbidity. These results demonstrated that to improve outcomes of the patients receiving surgical treatment for mitral valve insufficiency, countermeasures against the inflammatory response after CPB or development of other radical treatment for

mitral valve insufficiency without using cardiopulmonary bypass (e.g. transcatheter valve replacement or transcatheter mitral repair) were required.

Chapter 4

We designed a novel method for constructing an autologous heart valve with a stent, called a stent-biovalve. In constructing completely autologous heart valves, named biovalves, which used in-body tissue architecture technology, tissues for leaflets were formed via ingrowths into narrow apertures in the preparation molds, frequently leading to delayed or incomplete biovalve preparation. In this technique, self-expandable nitinol stents after everting were mounted on an acrylic column-shaped part and partially covered with an acrylic cylinder-shaped part with three slits. This assembled mold was placed into subcutaneous abdominal pouches in beagles or goats for 4 weeks. Upon removing the acrylic parts after harvesting and trimming of capsulated tissues, a tubular hollow structure with three pocket-flaps of membranous tissue rigidly fixed to the stent's outer surface was obtained. Then, the stent was turned inside out to the original form, thus moving the pocket-flaps from outside to the inside. Stent-biovalves with a sufficient coaptation area were thus obtained with little tissue damage in all cases. The valve opened smoothly, and high aperture ratio was noted. This novel technique was thus highly effective in constructing a robust, completely autologous stent-biovalve with adequate valve function. As shown in the pilot model in this study, the Stent-Biovalve will show favorable valve function *in vivo* too. The Stent-Biovalve can be a novel treatment device for canine chronic mitral valve disease. Future studies are expected to focus on the implantation of this Stent-Biovalve in animal models to confirm chronic valve function and durability *in vivo*.

A part of this dissertation is the peer reviewed version of the following article: Mizuno T, Mizukoshi T, Uechi M. Long-term outcome in dogs undergoing mitral valve repair with suture annuloplasty and chordae tendinae replacement. Journal of small animal practice. 2013, 54(2) pp104-107. FULL CITE, which has been published in final form at 10.1111/j.1748-5827.2012.01305.x

A part of this dissertation is the peer reviewed version of the following article:FULL CITE, which has been published in final form at 10.1002/jbm.b.33086

Mizuno T, Takewa Y, Sumikura H, Ohnuma K, Moriwaki T, Yamanami M, Oie T, Tatsumi E, Uechi M, Nakayama Y. Preparation of an autologous heart valve with a stent (stent-biovalve) using the stent eversion method. J Biomed Mater Res B Appl Biomater. 2014. 102(5):1038-1045.

謝辞

本研究をまとめるにあたり、終始、ご指導ならびに御鞭撻を賜りました麻布大学獣医学部外科第一研究室、藤井洋子教授に心より感謝いたします。

また、ご校閲と貴重なご助言をいただきました、麻布大学獣医学部生理学第二研究室、折戸謙介教授ならびに麻布大学獣医学部内科学第二研究室、久末正晴准教授に対し心よりお礼を申し上げます。

また、バイオバルブ作製にあたり、多大なるご指導をいただきました国立循環器病研究センター研究所生体医工学部医工学材料研究室、中山泰秀室長に心より感謝いたします。

本研究の遂行にあたり、多大のご協力を頂きました国立循環器病研究センター研究所人工臓器部、住倉博仁博士、岡山理科大学技術科学研究所、岩井良輔講師、弘前大学理工学研究科、森脇健司助教、神山裕司氏ならびに JASMINE どうぶつ循環器病センターの諸氏に感謝いたします。

最後に、本研究の遂行および論文をまとめるにあたり、終始、多大なるご指導をいただきました上地正実先生に深く感謝いたします。

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Age (month)	123 ± 25	**Cavalier**	11
Sex (male: female)	22: 25	**Shih-tzu**	8
Body weight (kg)	5.7 ± 3.0	**Chihuahua**	8
ISACHC classification		**Maltese**	7
Ia	1	**Pomeranian**	4
Ib	2	**Beagle**	2

II	12	**Other**	7
IIIa	25		
IIIb	7		

Table 1. Clinical characteristics of the dogs.

Sex (male:female)	6:5
Age (months)	
Mean	120.3 ± 18.8
Range	96–145
Weight (kg)	
Mean	7.3 ± 2.2
Range	3–10.7
CPB duration (min)	
Mean	166.3 ± 18.3
Range	112–200
Cross-clamp time (min)	
Mean	118.2 ± 10.1
Range	84–135

Table 2 Clinical characteristics of dogs undergoing cardiopulmonary bypass

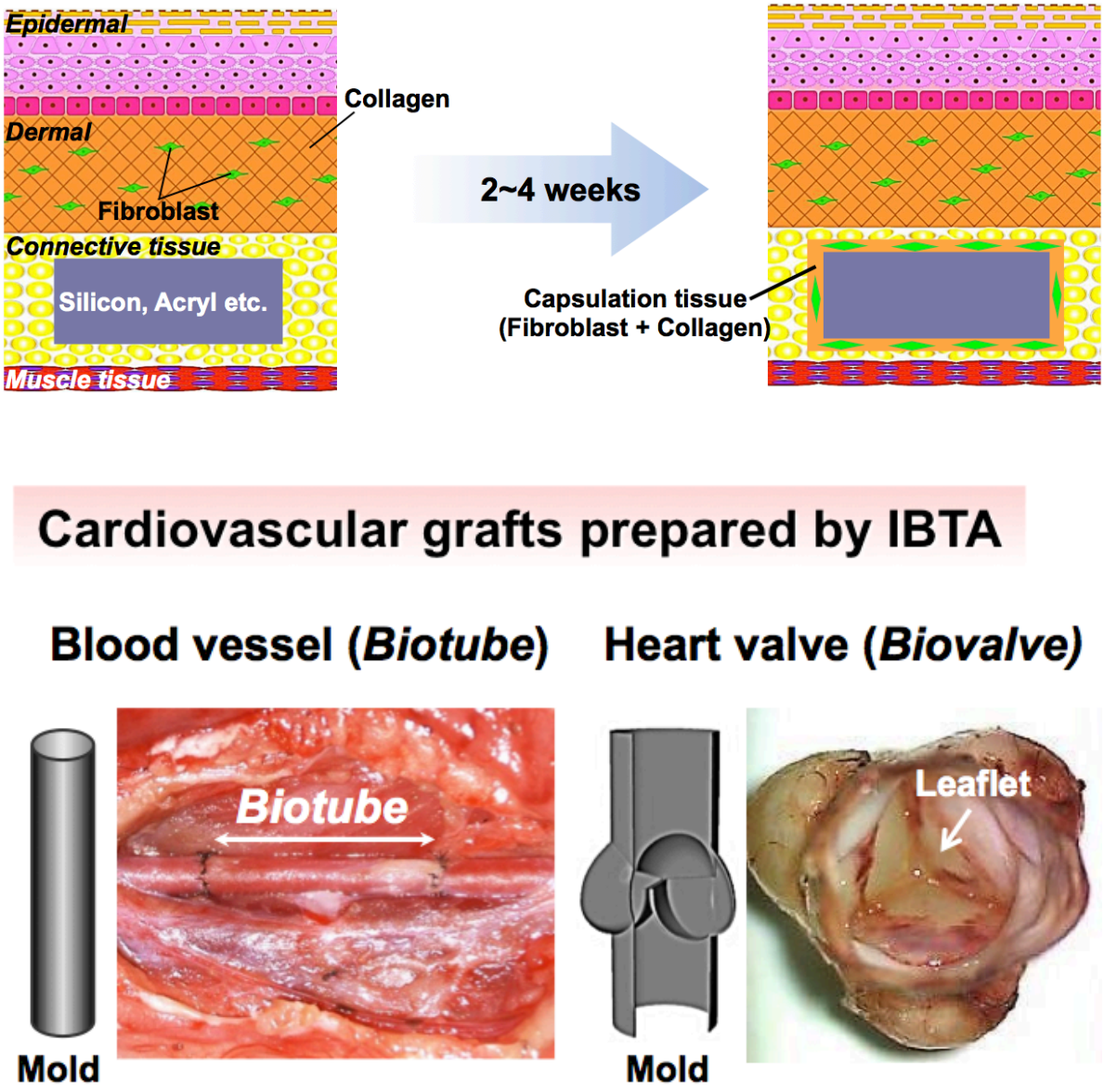


Fig. 1 Cardiovascular tissue engineering by in body tissue architecture technology (IBTA)

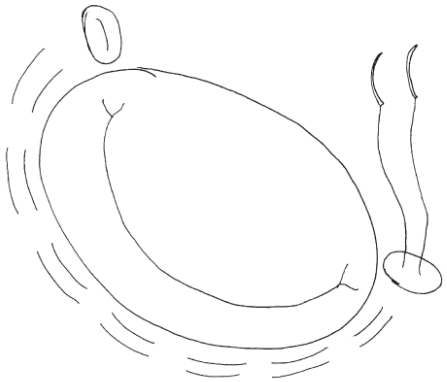


Fig 2. Procedure for circumferential annuloplasty. A double semicircular suture was place in the mitral annulus around the posterior leaflet. The suture was reinforced with pledgets at each commissure

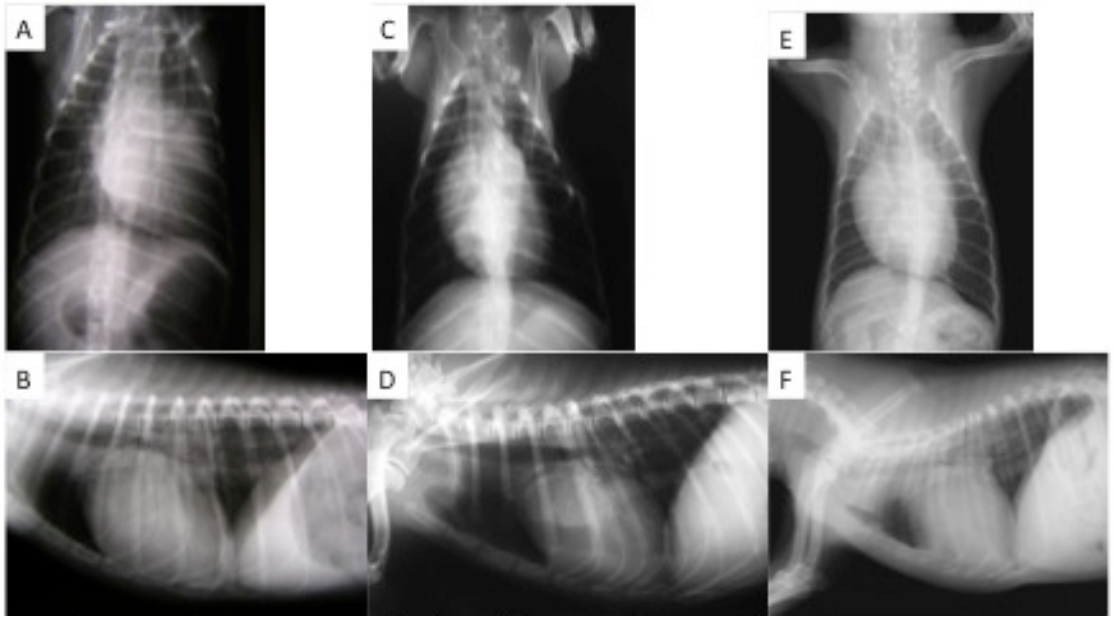


Fig. 3 Thoracic radiography (Case 1)

A, B; Preoperative thoracic radiography, VHS=11v

C, D; Postoperative thoracic radiography, VHS=10.2

E, F; 5 years after surgery, VHS=12.4v

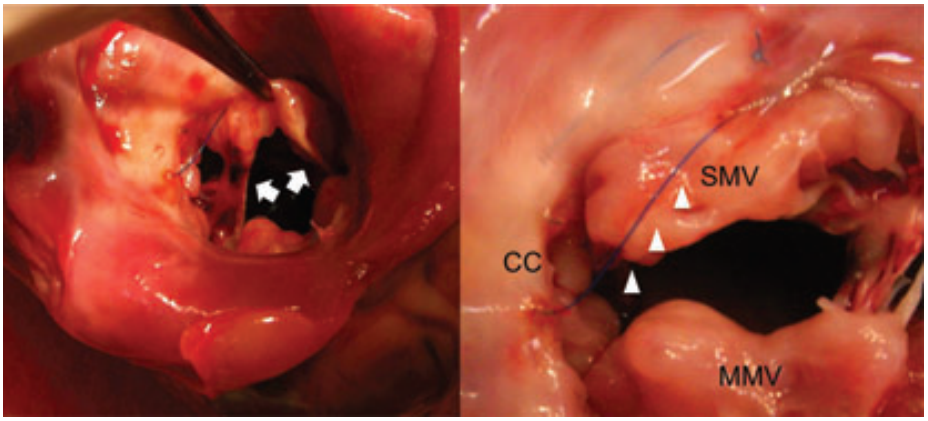


Fig 4. Post-mortem images from case 2. Stability of the artificial chordae (arrow) was confirmed. To the naked eye, the chordae were indistinguishable from the native chordae tendineae. The annular plication suture was partially detached from the mitral valve annulus (arrowhead). SMV, septal mitral leaflet; MMV, mural mitral leaflet; CC, cranial commissure

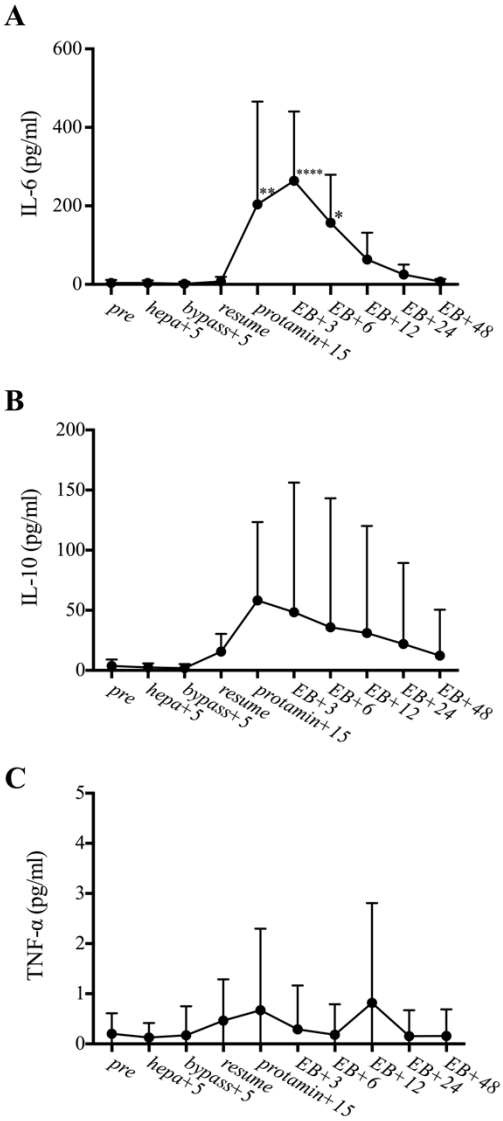


Fig. 5. Plasma cytokine levels before, during, and after CPB. IL-6 levels (A), IL-10 levels (B), and TNF-α levels (C) before, during, and after surgery.

Points of collection are preoperatively (pre), 5 minutes after administration of heparin (heparin +5), 5 minutes after clamp of aorta (bypass +5), after resumption of the heartbeat (resume), 15 minutes after protamine sulfate administration (protamine +15), 3 hours after completion of CPB (EB +3hr), 6 hours after completion of CPB (EB +6hr), 12 hours after completion of CPB (EB +12hr), 24 hours after completion of CPB (EB +24hr), and at 48 hours after completion of CPB (EB +48hr). Error bars indicate the standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.0005$ compared with preoperative levels (pre).

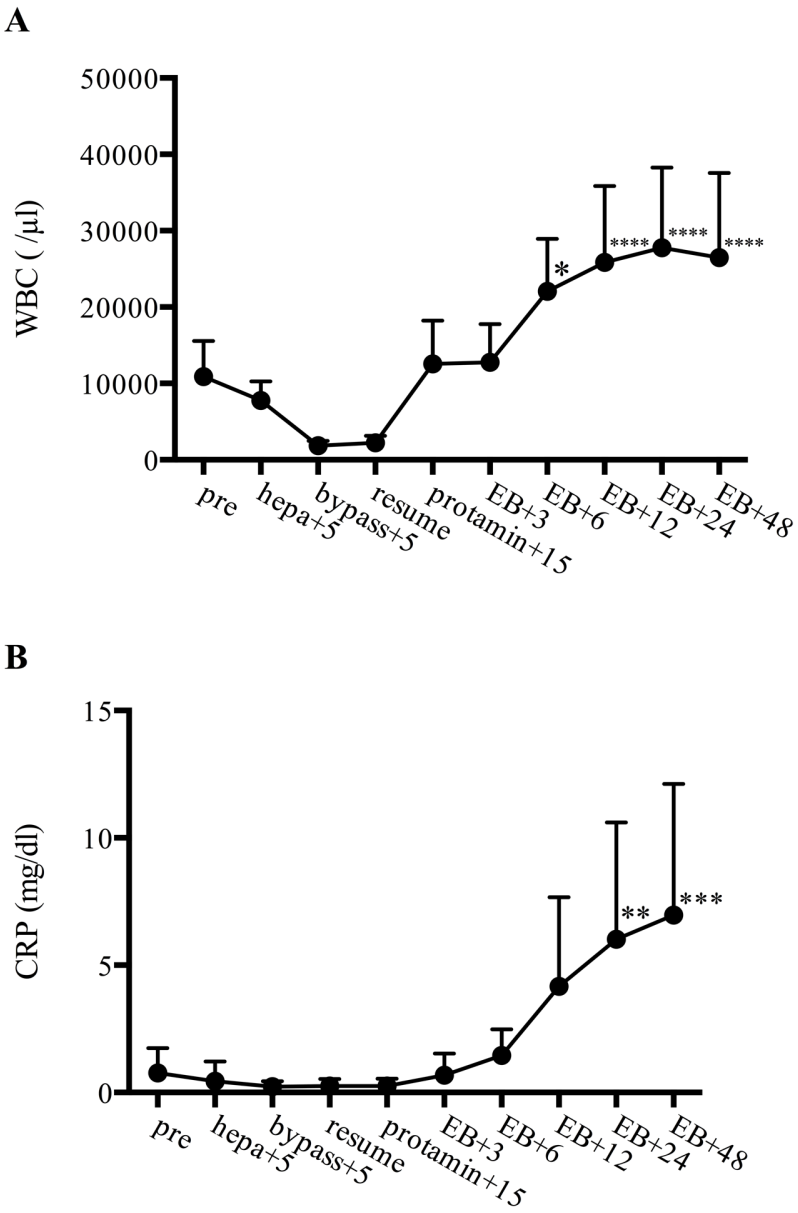


Fig. 6. White blood cell counts (A) and CRP concentrations (B) before, during, and after surgery. Points of collection are pre, heparin +5, bypass +5, resume, protamine +15, EB +3hr, EB +6hr, EB +12hr, EB +24hr, and EB +48hr (Please see text for additional details). Error bars indicate the standard deviation of the mean.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ and **** $P < 0.0005$ compared with preoperative levels (pre).

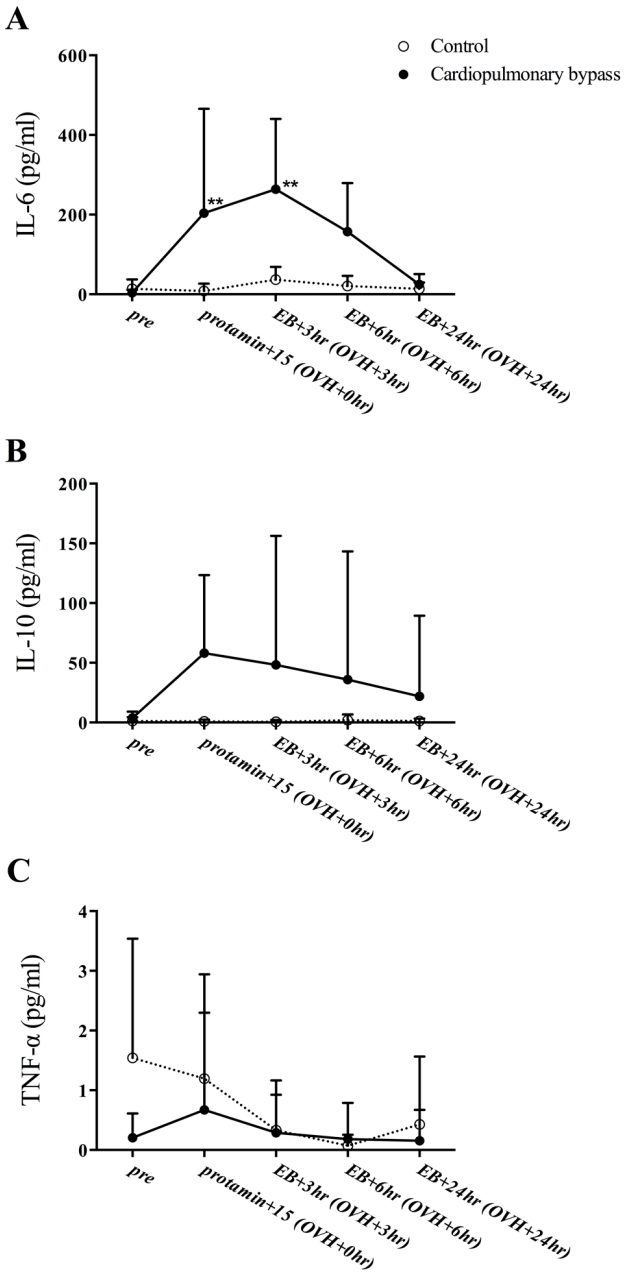


Fig. 7. Plasma cytokine levels of IL-6 (A), IL-10 (B), and TNF-α (C) in cardiopulmonary bypass cases (dogs underwent mitral valve repair) and the control group (dogs underwent ovariohysterectomy) before and after operation. ** Significantly different between the cases and the control group ($P < 0.01$).

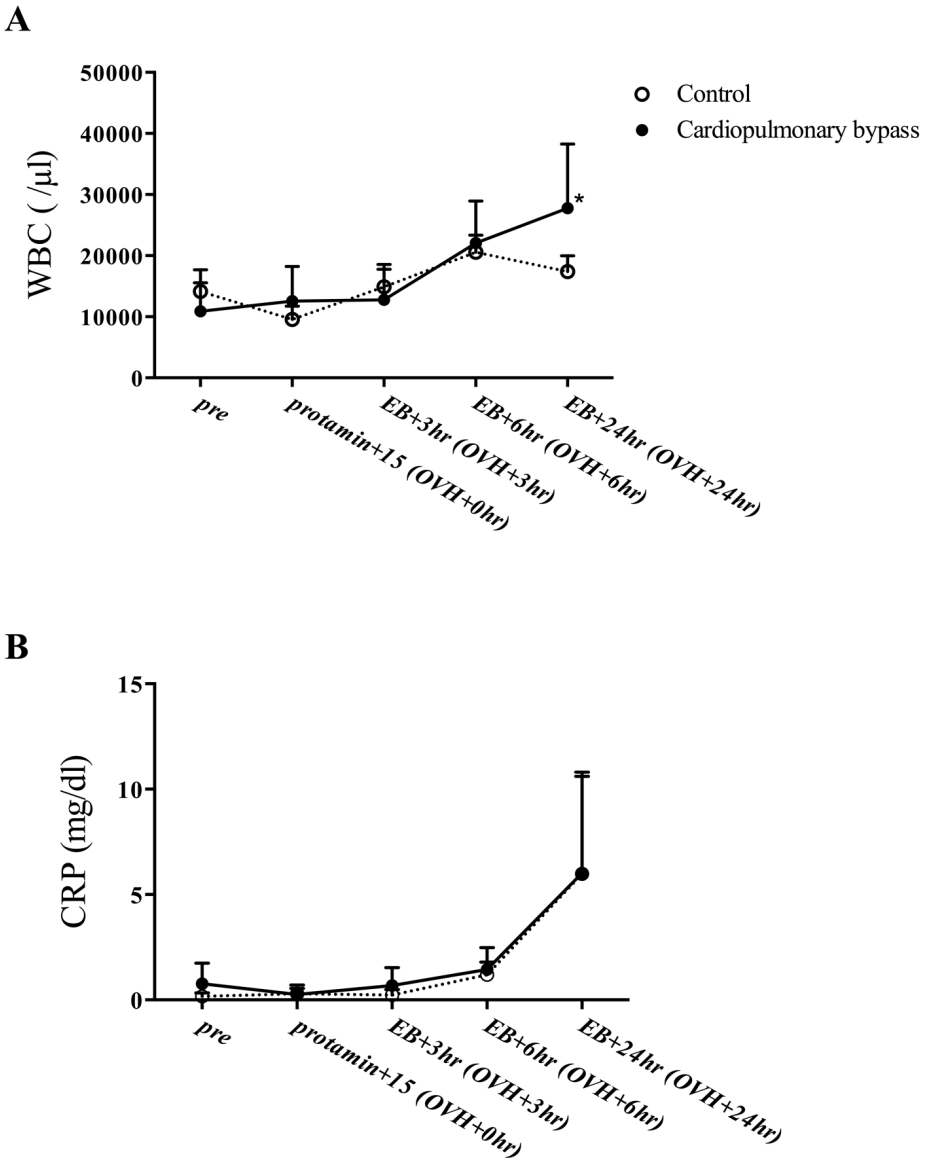


Fig. 8. White blood cell counts (A) and CRP concentrations (B) of cardiopulmonary bypass group and control group before and after operation. * $P < 0.05$ compared with the control group.

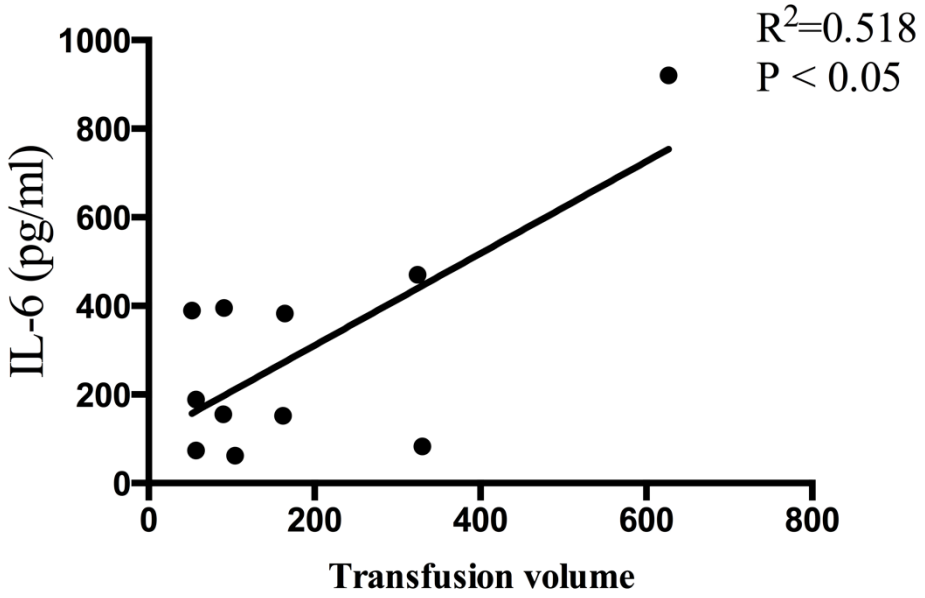


Fig. 9. Relationship between PCV-corrected peak IL-6 level and the transfusion volume (r = 0.719, r^2 = 0.518, P < 0.05).

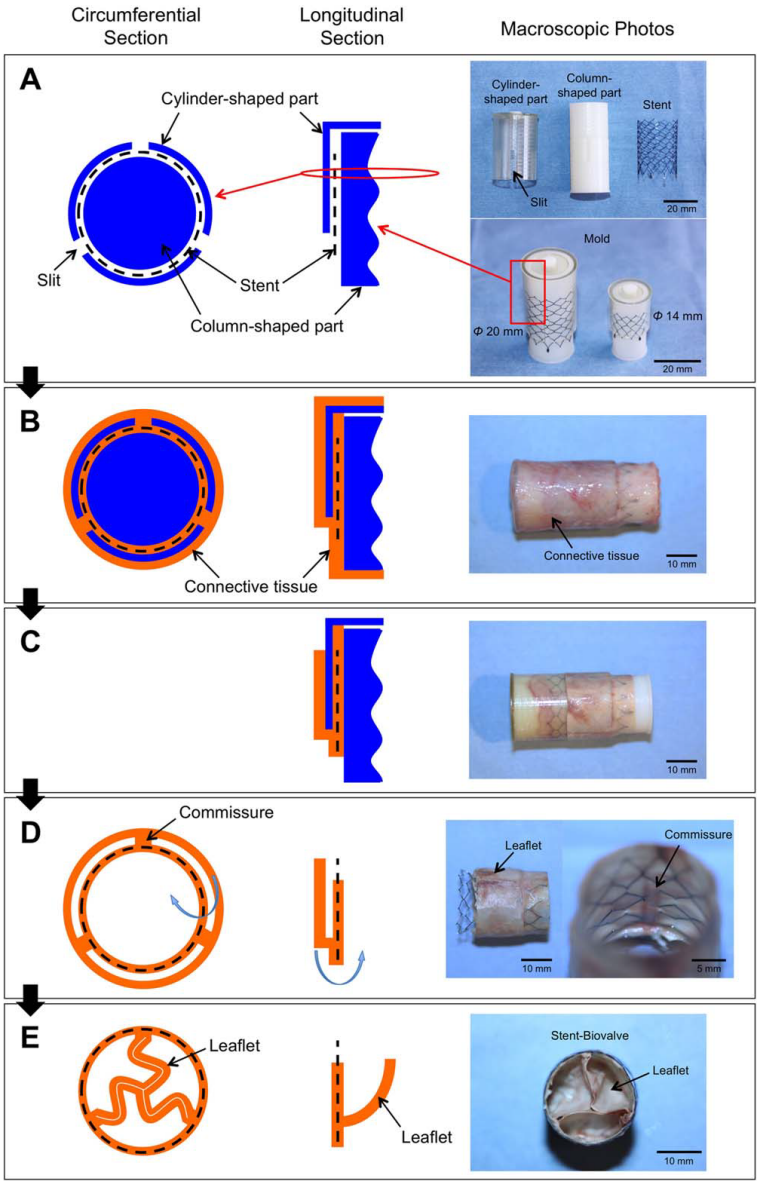


Fig. 10 Preparation process of the Stent-Biovalve using the stent eversion method. A specially designed cylinder-shaped part, column-shaped part, and self-expandable nitinol stent (diameter, 14 or 20 mm) were assembled to prepare molds for Stent-Biovalves (A). After 4 weeks of *in vivo* mold placement, the implants were completely encapsulated with robust connective tissue (B). After trimming the capsulated tissue the three leaflet parts were obtained (C). The cylinder and column parts were removed to create a patent hollow tube partially fixed with the stent (D). Each stent was iced and turned inside out again (E). The three pocket-flaps that were outside the stent now formed a tricuspid valve on the inner surface.

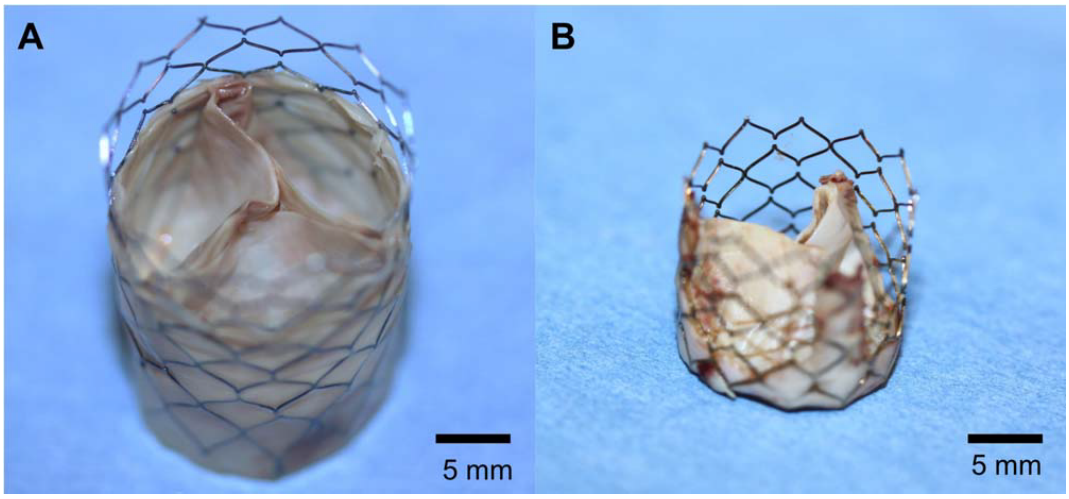


Fig. 11 The obtained Stent-Biovalves with diameter of 20 mm (A) and 14 mm (B).

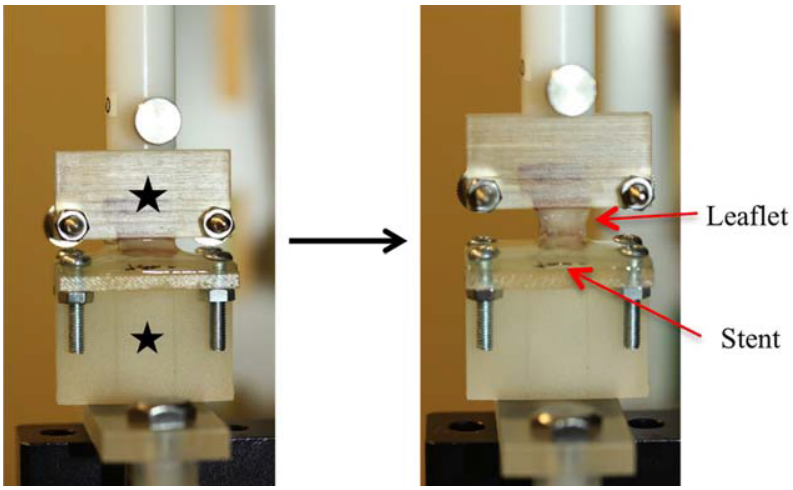


Fig. 12 Schematics of mechanical tests. Sample was fixed in a sample folder (★) that was specially designed by use of a 3D printer.

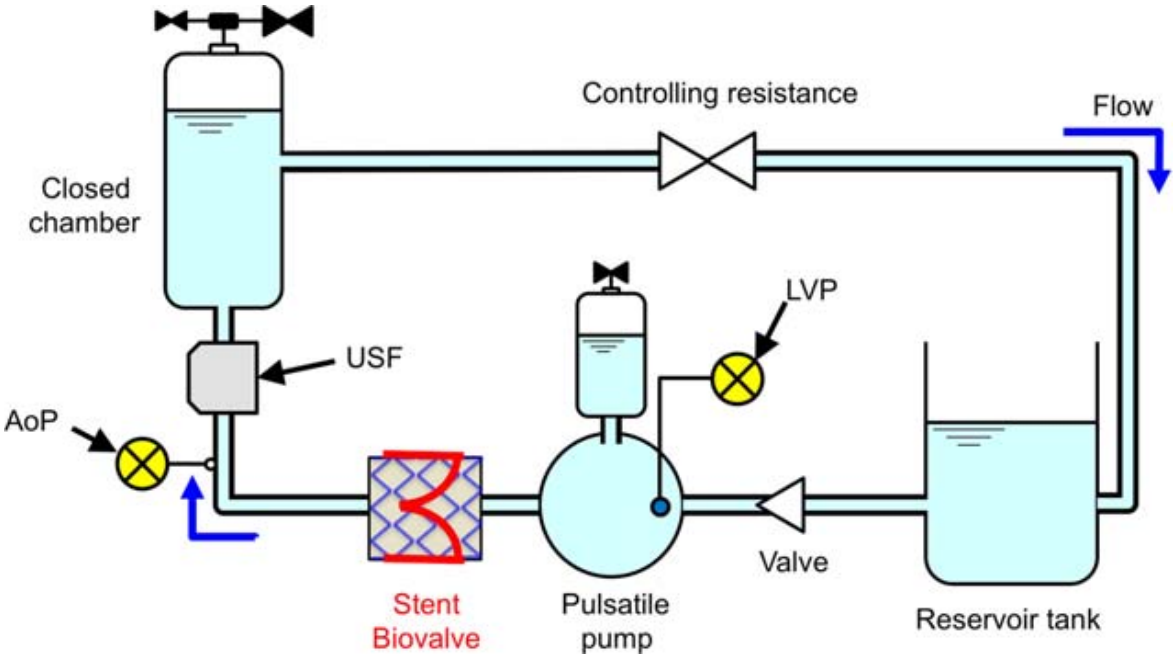


Fig. 13 A pulsatile circulation circuit model designed for the evaluation of valve function.

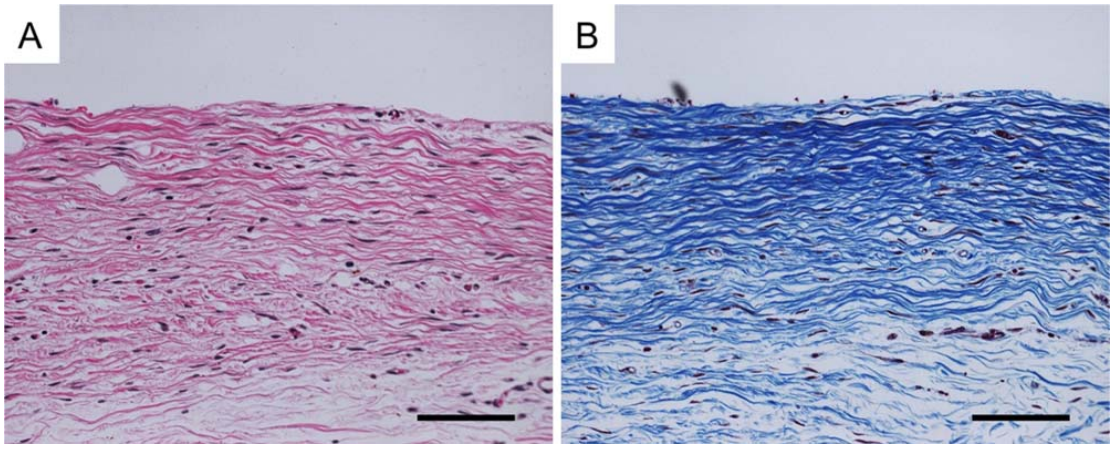


Fig. 14 Histology of the leaflet of stent-biovalve stained with Hematoxylin and eosin staining (A) and Masson's and trichrome staining (B). bar =100 mm.

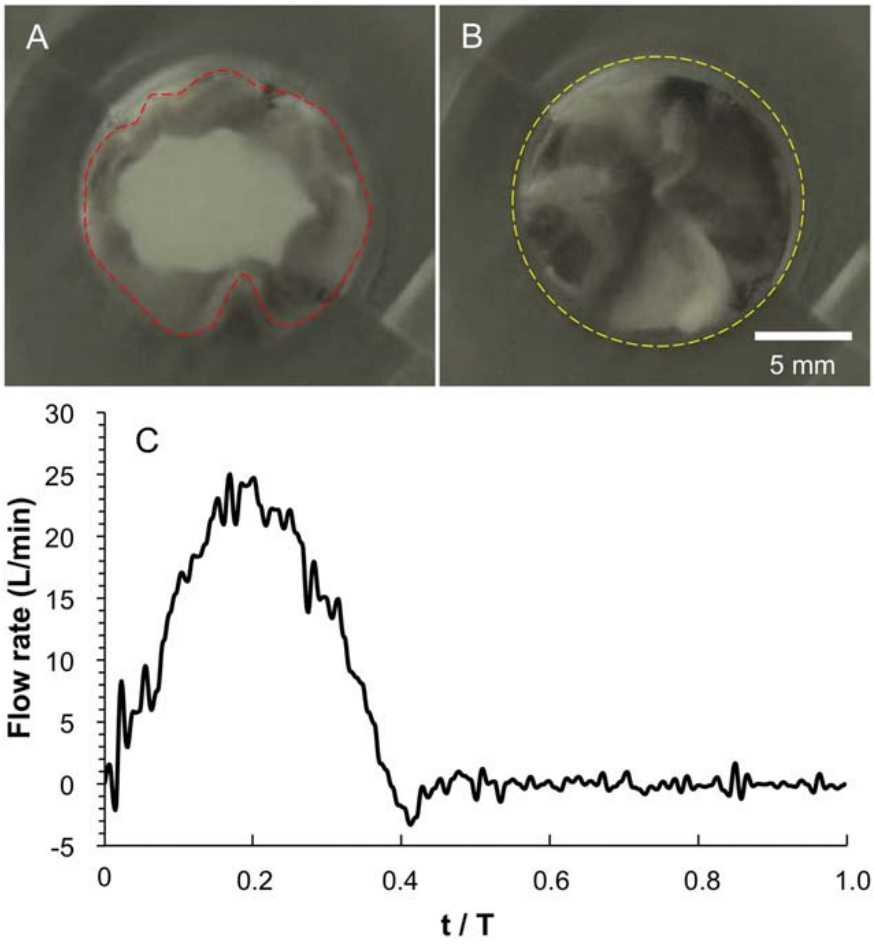


Fig. 15. Macroscopic photos of the opening (A) and closing (B) form of the stent-biovalve in the circuit as shown in Figure 3. The pulsatile flow was 70 bpm, and the mean flow rate was 5–6 L/min. The aperture ratio was 89%. Pulsatile flow waveform in a single cycle of the stent-biovalve.

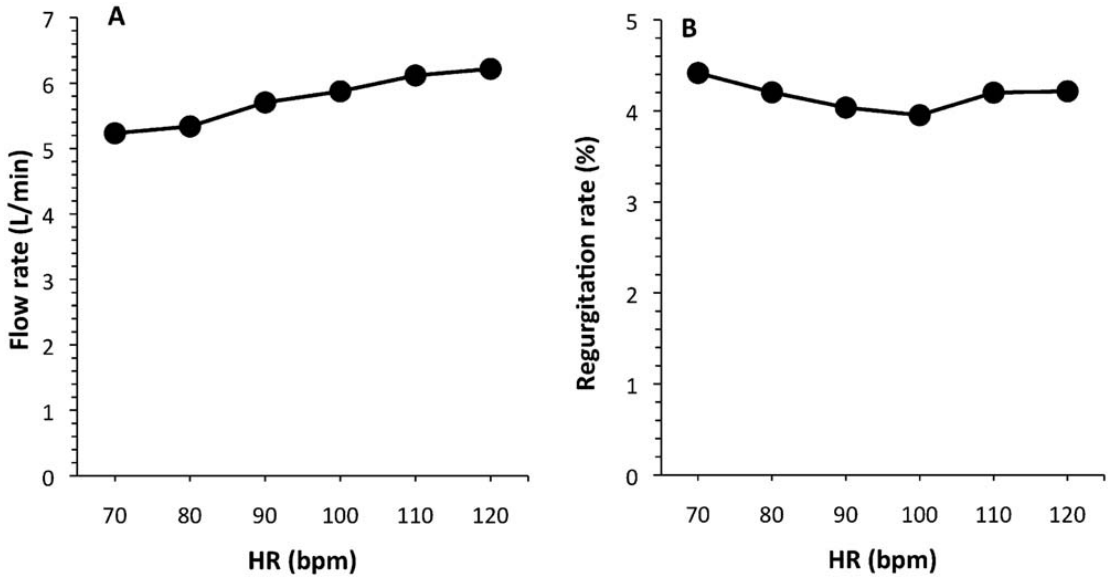


Fig. 16. Mean flow rate (A) and regurgitation rate (B) at every 10 bpm from 70 to 120 bpm.

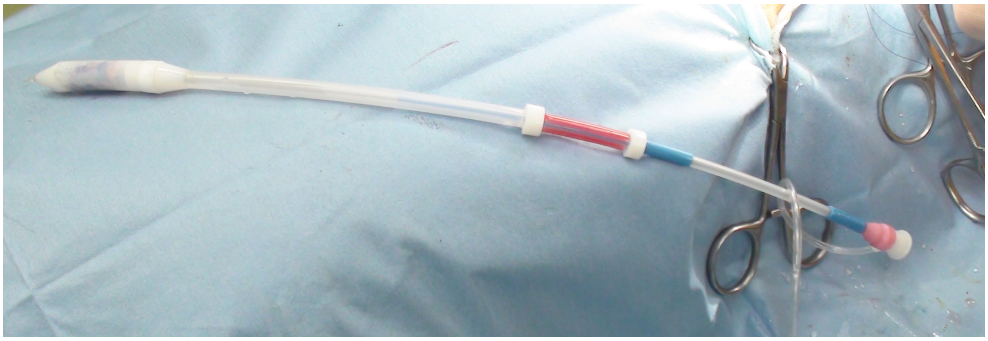
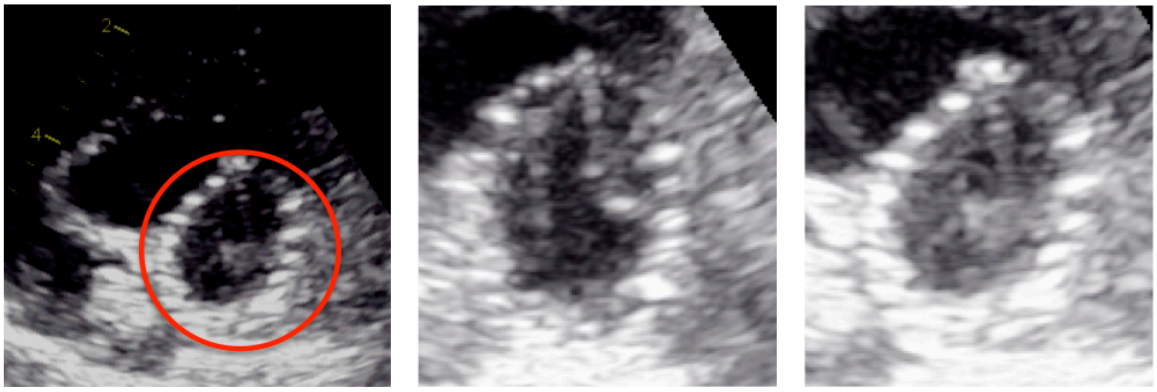


Figure 17 Delivery catheter of the Stent-Biovalve



Opening form Closing form

Figure 18 Echocardiographic images of the opening and closing form of the Stent-Biovalve in the mitral position