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Original Article

In vitro and *in vivo* blood compatibility evaluation of a novel Chinese valved bovine pericardium patch

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Abstract: Researchers over the world are discovering ideal materials with favorable biocompatibility for valved conduits to treat congenital heart disease. Blood compatibility should be fundamentally considered for vascular devices. At present, autologous pericardium shows the best blood compatibility. Therefore, we employed the autologous pericardium as a control (control group) to compare and explore the *in vitro* and *in vivo* blood compatibility of a novel Chinese valved bovine pericardium patch with superior fatigue durability and diverse types (treatment group). Both patches were implanted into small fat-tail sheep to replace the antetheca of right ventricular outflow tract (RVOT) and pulmonary trunk through surgery. All animals survived to the predetermined time without any complications. Operation time, cardiopulmonary bypass time, and aortic cross clamp time in the control group were significantly longer than those in the treatment group ($P < 0.05$). Good healing status in the both planted areas was observed without any thrombosis or infarction. There was no significant difference in *In vivo* blood compatibility indicators including red blood cells injury, blood routine, and thrombosis which were investigated by free hemoglobin (HGB) determination, automatic blood cell analyzer, and naked eye/microscope observation, respectively ($P > 0.05$). Results of *in vitro* compatibility indexes including hemolysis, complement activation, plasma soluble P-selectin, and dynamic clotting which were evaluated through spectrophotometry and ELISA kits were also exactly similar between the two groups ($P > 0.05$). Therefore, this Chinese patch exhibited wonderful blood compatibility and might be developed as a medical device for RVOT reconstruction.

Keywords: Blood compatibility, bovine pericardium patch, congenital heart disease, RVOT reconstruction, autologous pericardium, biological compatibility

Introduction

Every year, 100-150 thousand infants with congenital heart disease (CHD) are born in China and CHD with a morbidity of 6.7‰ heads the list of birth defects. At present, over 60% of cardiac surgery every year in China is to treat CHD. Popularization of interventional closure devices and iatrotechnics makes most patients with non-purple clamp type of CHD such as patent ductus arteriosus (PDA), atrial septal defect (ASD), and ventricular septal defect (VSD) can receive timely and satisfactory treatments. However, most of patients with complex CHD (usually accompanied with purple clamp) including pulmonic stenosis or stresia, tetralogy of Fallot, transposition of the great arteries, persistent truncus arteriosus, and double outlet right ventricle have to receive right ventricular outflow tract (RVOT) reconstruction [1]. Although RVOT reconstruction can greatly improve operation effects and postoperative long-term living quality, the present treatment is far from satisfactory because of the unavailability of necessary repair devices. In order to cure these patients, it is extremely urgent to develop feasible surgical repair devices. As a result, researchers over the world are turning to discover ideal materials for valved conduits.

In the past, valved conduits used in the surgery were generally autoallergic capsula cordis, artificial materials such as Gore-Tex patch, allogeneic aortic valved conduits, heterograft valved conduits, and so on [2]. However, on account of the source limitation of these materials, size mismatch, and reconstruction difficulty of valve structure, RVOT reconstruction has become the challenges for cardiac surgery. In 1964, Kirklin JW's group first employed non-valved pericardium conduits to establish a connection from right ventricle to pulmonary artery in a patient with pulmonary artery atresia [3]. Subsequently, many other researchers utilized homograft and

heterograft conduits to complete RVOT reconstruction [4-6]. In 1992, Ichikawa Y used epoxide to modify bovine jugular vein with natural venous valve and developed earliest bovine jugular vein valved conduits for RVOT reconstruction [7]. Then various valved conduits based on bovine jugular vein were produced by many companies such as VenPro and Medtronic. Inner diameter of the common big bovine jugular vein is 18 mm, but generally inner diameter of adult RVOT is 22-26 mm. Thus, this size mismatch may generate hidden troubles of second operation when the children grow up. Moreover, regarding pulmonary stenosis rather than stresia, doctors can widen the pulmonary artery antetheca and reserve a part of child's own pulmonary artery wall, which will promote the regrowth of original tissues and minimize the impact on the children. Therefore, there are four issues in the blood flow reestablishment of extracardiac conduits. First, it is difficult to obtain suitable materials of homograft valved conduits. Second, aorta and pulmonary artery conduits of animals are easy to be calcified and aged if without chemical modification. Thirdly, the size limitation of bovine jugular vein is unable to resolve the problem of conduits replacement when children grow up. Last, regurgitation of pulmonary artery is found in autologous pericardium and the tailoring of valved pocket within short time during operation is extremely difficult; besides, autologous pericardium is inapplicable in second radical operation. Thus, medical conduits with easy obtainment and favorable biocompatibility are urgently necessary to be developed in present cardiovascular tissue engineering field.

In recent years, bovine pericardial tissues are considered as the promising and optimized substitute materials of pulmonary artery reconstruction and repair for their abundant sources, convenient availability, simple management, easy storage, and long life. In this study, the RVOT reconstruction materials provided by Beijing Balance Medical Co., Ltd., China are patented bovine pericardium with modification treatment and artificial valves. These materials were modified with glutaraldehyde and then carboxyl groups in tissues were crosslinked with hydroxyl chromium. All commercially available heterograft biomaterials should be modified with glutaraldehyde because crosslinking between glutaraldehyde and free carboxyl groups of collagen molecules in tissues can reduce immunogenicity. As tripling the amount of amino groups, these carboxyl groups distribute not only in collagen molecules but also in tissue matrix. Therefore, the multiplex crosslinking can highly suppress the immunological rejection and enhance the anti-calcification ability and fatigue durability. Valve structure establishment is another innovation point of this device, which substitutes natural pulmonary valve in function. This valve structure with superior fatigue durability and diverse types can be tailored according to the specific growth of pulmonary artery in surgery.

When biomaterials contact with human tissues or blood, host response harming human bodies and material reaction damaging the materials may further initiate toxic reaction, inflammation, or thrombosis, so biomaterials must be biocompatible in physiological environment [8]. Blood compatibility should be fundamentally considered for a vascular device. At present, autologous pericardium shows the best blood compatibility. Therefore, here we employed the autologous pericardium as a control to compare and explore the blood compatibility of a novel Chinese valved bovine pericardium patch which was developed by Beijing Balance Medical Co., Ltd., China. *In vitro* and *in vivo* blood compatibility examinations including blood routine, hemolysis, complement activation, plasma soluble P-selectin, red blood cells (RBC) injury, *in vitro* dynamic clotting were performed according to medical devices standards ISO 10993-4 and GB/T 16886.4 to provide theoretical foundation of biocompatibility for further animal tests and even clinical trials.

Materials and methods

Materials

Valved bovine pericardium patch (Beijing Balance Medical Co., Ltd., China), Heparin Sodium (Tianjin Biochem Pharmaceutical Co., Ltd., China), Dopamine (Shanghai Harvest Pharmaceutical Co., Ltd., China), Sodium Bicarbonate and Normal saline (China Resources Double-Crane Pharmaceutical Co., Ltd., China), $CaCl_2$ solution and Penicillin (Harbin Pharmaceutical Group Holding Co., Ltd., China), ELISA kits (Elabscience Biotechnology Co., Ltd., Aachen, Germany), Amobarbital Sodium (Shanghai New Asia Pharmaceutical, China), Midazolam and fentanyl (Jiangsu NHWA Pharmaceutical Co., Ltd., China), Vecuronium bromide (Shanxi Powerdone Pharmaceutics Co., Ltd., China), Pr-

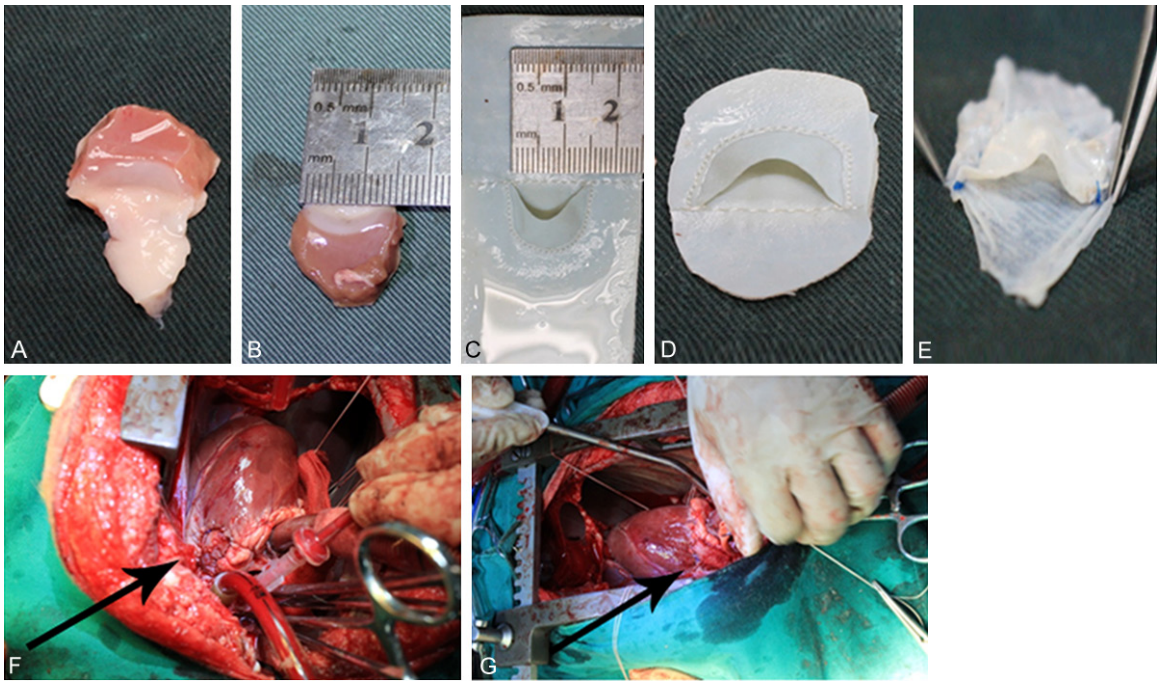

Figure 1. Extracorporeal circulation establishment of the sheep. A: Pulmonary trunk wall, part of pulmonary valve ring (containing a pulmonary valve), and front wall of RVOT were down removed with fusiform excision through pulmonary trunk; B: The length of valve ring; C: According to the measuring result, we chose valved bovine pericardium patches with similar size of valved pocket in the treatment group; D: Bovine pericardium was cut into fusiform shape around the valved pocket; E: Valved autologous pericardium was manually prepared according to measured size and cut into fusiform shape; F and G: They were continuously sutured onto RVOT and pulmonary trunk with 5-0 prolene; Arrow in F showed the autologous pericardium; Arrow in G showed the valved bovine pericardium patch.

ocaine (Beijing YOKON Pharmaceutical Co., Ltd., China), Protamine (Duoduo Pharmaceutical Co., Ltd., China).

Animal model establishment

Animal information: Fifteen normal male small fat-tail sheep (40-50 kg) were obtained from Animal Care Center of Beijing Anzhen Hospital Affiliated to Capital Medical University, China. All animals were raised with free food and drink, but fasted 12 h before operation. They were randomly divided into two groups: treatment (n = 9, valved bovine pericardium patch) and control (n = 6, autologous pericardium). Study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Beijing Anzhen Hospital Affiliated to Capital Medical University, China.

Anesthesia, trachea cannula, and mechanical ventilation: Pentobarbital sodium (3%) was intramuscularly injected at 30 mg/kg. Animals were fixed in the supine position on an operating table. Ear artery was punctured and continuous invasive arterial blood pressure measurement was maintained with a monitor (V24, Philips, China). Ear vein was punctured and injected with midazolam at 0.2 mg/kg and fentanyl at 2-5 μg/kg. Endotracheal intubation was performed via oral cavity and assisted ventilation was given by a breathing machine with 16-20/min of respiratory rate, 8-10 ml/kg of tidal volume, and 40-60% of fraction of inspired oxygen. Vecuronium bromide was administrated at 0.2 mg/kg once. Midazolam (0.4 g/l) was continuously administrated at 2-5 ml/h and fentanyl was discontinuously administrated at 2-5 μg/kg with a microinfusion pump to keep an appropriate depth of anesthesia. Acid-base balance was corrected by adjusting the parameters of breathing machine on the basis of arterial blood gas analysis during operation.

Extracorporeal circulation: Chests were opened by median sternotomy with sternum saw. Pericardium tissues were dissociated and segmentd of 10 × 15 cm were cut off and immersed in normal saline. Heparin sodium was intravenously injected at 3 mg/kg. Aortic intubation (21 Fr, Medos, Germany) was performed. Venous drainage catheters were intubated via

auricula dextra and postcava (32 Fr, 34 Fr, Youfu, China). After sufficient aerofluxus, cardiopulmonary bypass (CPB) catheters (Kewei, Dongguan, China) were connected with an extracorporeal circulation unit (Sarns 7400, Sarns/3M, USA), Sarns temperature control and monitor system (Terumo, USA), and membrane oxygenator (Xijing-II, Xijing, China). Priming solution was balanced salt solution at 20-25 ml/kg. When activated clotting time (ACT) exceeded 400 s, extracorporeal circulation was started and 50-80 mmHg of mean perfusion pressure was maintained. Right atrium was dissected and left ventricular vent catheter was inserted via interatrial septum. Perfusion needle was inserted into ascending aortic root and superior and inferior vena cava was interdicted. Rectal temperature was lowered into 30°C. Ascending aorta was interdicted with occlusion clamp and perfused with 4°C of crystalloid potassium cardioplegia (formulation: Normal saline 450 ml; 15% KCl 14 ml; 25% $MgSO_4$ 2 ml; 5% $CaCl_2$ 1.5 ml; 1% procaine 20 ml; 5% $NaHCO_3$, 20 ml) at 15 ml/kg. Ice water at 0°C was poured into pericardial cavity. When there was no electrical activity in electrocardiogram, pulmonary trunk was dissected and pulmonary trunk wall, part of pulmonary valve ring (containing a pulmonary valve), and front wall of RVOT were downward removed with fusiform excision through pulmonary trunk (**Figure 1A**). During operation, we measured the length of valve ring (**Figure 1B**). According to the measuring result, we chose valved bovine pericardium patches with similar size of valved pocket in the treatment group (**Figure 1C**). Bovine pericardium was cut into fusiform shape around the valved pocket (**Figure 1D**). In the control group, valved autologous pericardium was manually prepared according to measured size and cut into fusiform shape as well (**Figure 1E**). Then they were continuously sutured onto RVOT and pulmonary trunk with 5-0 prolene (**Figure 1F** and **1G**). Venting was performed and the incision of interatrial septum was sutured with 5-0 prolene. After declamping and autologous blood perfusion, the heart regained beating automatically and rectal temperature was gradually recovered to 35°C. Then the incision of right atrium was sutured. Cardiopulmonary bypass assistance was conducted with gradually reduced flow until stop. Protamine was administrated to neutralize heparin sodium. Cardiopulmonary bypass was removed. Drainage tube of No. 24 was placed in fifth rib of the left border of sternum and connected with closed drainage bottle. Lastly, the chest was closed. Penicillin of 1.6 million units was intravenously instilled during operation [9, 10].

Postoperative management: Respiration was maintained with a breathing machine (900-C, Siemens, Germany) at a mode of synchronized intermittent mandatory ventilation (SIMV). Dopamine was pumped by a micro pump at 5 μg/kg/min. Parameters of breathing machine and dosage of vasoactive agent were adjusted according to the results of arterial blood gas analysis. Ventilator weaning was dependent on the circumstances. After removal of endotracheal intubation, animals were sent back to the cages. The next day, drainage tube was removed. Animals were fasted for 12 h after operation. Intramuscular injection of 0.8 million units of penicillin were performed routinely for 3 days at a frequency of twice per day. Anticoagulants were not administrated after operation.

Blood compatibility test in vivo

RBC injury: Blood samples were taken on 1 day before operation, 1 day, 3 days, and 7 days after operation through ear vein and then free hemoglobin (HGB) was determined [11]. In brief, phosphate buffered saline (PBS) and chromogen buffer were first prepared and stored at 4°C in the dark. Then the free HGB was separated from the whole blood and quantitatively determined to obtain HGB standard stock solution (10 g/l) and standard working solution (100 mg/l). Concentrated RBC was added into test tubes at 3 ml per tube. The tubes were centrifuged for 3 min at 3000 rpm and the supernatant was collected. The test solution (0.02 ml) was sufficiently mixed with 1 ml of chromogen buffer and 0.2 ml of 1% H_2O_2 for 20 min at room temperature. Normal saline served as the blank control. Then absorbance was measured at 492 nm on a microplate reader (MK3, Thermo, USA). The content of free HGB was calculated accordingly.

Blood routine: Blood samples were taken on 1 day before operation, 1 day, 3 days, 7 days, and 1 month after operation through ear vein. Blood routine examination was conducted on an automatic blood cell analyzer (BC-6900, Mindray, China).

Thrombosis: Animals were euthanized 1 month after operation and samples were collected. Briefly, pentobarbital sodium (3%) was intramuscularly injected at 30 mg/kg. When anesthesia appeared, heparin sodium (3 mg/kg) was intravenously injected via ear vein. After 5 min, femoral artery was dissociated and transected. Its distal end was occluded and a vacuum extractor was inserted into the proximal end. The blood was extracted. When the heartbeat and the respiration of animals stopped, the chest was opened along the left fourth rib and adhesive tissues were carefully dissociated. Full heart, its contiguous ascending aorta, and pulmonary trunk were taken out. Full lung was taken out as well. The recovery status in the planted area of patches, whether thrombosis occurred in and around valved pocket of patches, and whether infarct lesions appeared in lung tissues were observed by naked eyes. After HE staining, tissues were observed with a light microscopy to evaluate whether the thrombosis and infarct lesions were generated around the patches and in the distal lung tissues.

Blood compatibility test in vitro

Hemolysis: Autologous pericardium of 5 × 5 cm and 20 ml of autologous blood were collected from the animals. Segments of 5 × 5 cm were also cut from valved bovine pericardium patches of different 15 lots. Samples of autologous pericardium and valved bovine pericardium patches were placed in test tubes and immersed in 5 ml of normal saline. Normal saline and distilled water served as negative and positive control, respectively. All tubes were incubated for 30 min in 37°C of water bath. Fresh anticoagulated blood diluted with normal saline (0.1 ml) was added into each tube. After gently mixing, the tubes were incubated for another 60 min in water bath. The blood mixtures in tubes were collected and centrifuged for 5 min at 4°C and 2500 rpm. Absorbance of the supernatant was analyzed at 545 nm on a spectrophotometer (Eppendorf, Germany). Hemolysis rates were calculated accordingly.

Complement activation: Aforementioned autologous pericardium and valved bovine pericardium patches were cut into 1 × 1 cm of segments and loaded into wells of cell culture plates. Plasma (1 ml) was added into each well. The wells without preload of samples served as blank control. The plates were incubated for 30 min at 37°C and 5% CO_2. Complement components 3a, 5a, TCC, Bb, and 4d in the plasma were determined with ELISA kits E-EL-S0291c, E-EL-S0257c, E-EL-S0485c, E-EL-S0321c, and E-EL-S0410c, respectively, according to the manufacturer's instructions for use.

Plasma soluble P-selectin: Aforementioned autologous pericardium and valved bovine pericardium patches were cut into 5 × 3 mm of segments. Then samples were introduced into test tubes and immersed in 5 ml of normal saline. Normal saline and distilled water served as negative and positive control, respectively. All tubes were incubated for 30 min in 37°C of water bath. Fresh anticoagulated blood diluted with normal saline (0.1 ml) was added into each tube. After gently mixing, the tubes were incubated for another 60 min in water bath. The blood mixtures in tubes were collected and centrifuged for 5 min at 4°C and 2500 rpm. Supernatant was collected for P-selectin determination with a P-Selectin/CD62P/GMP140 ELISA kit GD-X3609.

Dynamic clotting: Autologous pericardium and valved bovine pericardium patches were placed in bottom center of small beakers. After incubation for 5 min at 37°C, 0.25 ml of anticoagulated blood was added into the center of each sample. After another 5 min at 37°C, 0.02 ml of $CaCl_2$ (0.2 mol/l) was infused into the blood. Then start timing and shake the beaker for 1 min to mix the blood and $CaCl_2$. Five time points with an interval of 10 min were predetermined. Distilled water (50 ml) was added in the beaker and then the beaker was shaken for 10 min. Supernatant was collected for absorbance measurement at 540 nm.

Statistical analysis

All data were repeated at least three times and presented as mean ± standard deviation (SD). Statistical analysis was carried out using ANOVA with a SPSS 18.0 software (SPSS Inc, Chicago, IL, USA). Significant differences were considered at $P < 0.05$.

Results

Valved bovine pericardium patches were convenient and efficient in RVOT reconstruction

All animals survived to the predetermined time without hemorrhage, allergy, infection, pulmo-

Table 1. Comparison of the operation time, cardiopulmonary bypass time, and aortic cross clamp time during operation

Groups	Operation time (min)	Cardiopulmonary bypass time (min)	Aortic cross clamp time (min)
Treatment (n = 9)	126 ± 13.2*	73 ± 8.6*	33 ± 6.8*
Control (n = 6)	151 ± 15.6	98 ± 11.5	58 ± 7.4
**P* value	0.005249	0.000328	0.000014

*P < 0.05 vs. the control group.

Table 2. Free HGB assessment

Free HGB (g/l)	Pre-operation	Post-operation		
		1 day	3 days	7 days
Treatment group (n = 9)	0.011 ± 0.003#	0.146 ± 0.043*,#	0.083 ± 0.012#	0.015 ± 0.009#
Control group (n = 6)	0.009 ± 0.001	0.158 ± 0.039*	0.079 ± 0.015	0.013 ± 0.011
#*P* value	0.093	0.592	0.576	0.705

**P* value (Treatment group) = 0.000000. **P* value (Control group) = 0.000003. *P < 0.05 vs. the pre-operation; #P > 0.05 vs. the control group.

nary embolism, or other complications after operation. During operation, hemorrhage volume of all animals was 85 ± 12.6 ml. As shown in **Table 1**, operation time, cardiopulmonary bypass time, and aortic cross clamp time in the control group were significantly longer than those in the treatment group (P < 0.05), suggesting solider convenience and higher efficiency of valved bovine pericardium patches in RVOT reconstruction.

Blood compatibility test in vivo

Valved bovine pericardium patches didn't cause RBC injury: The RBC injury was evaluated by determining the content of free HGB in plasma. As demonstrated in **Table 2**, as compared with the pre-operation, the contents of free HGB on 1 day after operation were sharply elevated in both groups (P < 0.05). However, they were gradually reduced in following days. Their contents on 7 days after operation were reduced to low levels which were similar to the contents in pre-operation. Moreover, there was no significant difference in the contents of free HGB between the treatment and control groups in all time intervals (P > 0.05), demonstrating their similar effects on RBC.

Valved bovine pericardium patches didn't induce an abnormality of blood routine: The blood routine results were depicted in **Table 3**. We found that the contents of RBC, HGB, and platelets (PLT) decreased on 1-3 days after operation in comparison with pre-operation. Their contents were smallest on 3 days after operation. Then they recovered to normal levels on 7 days after operation. However, white blood cells (WBC), neutrophil (NEUT), and NEUT rate (NEUT%) just showed opposite variation tendencies from pre-operation to post-operation. Likewise, their elevated levels were reduced to normal on 7 days after operation. Interestingly, there was no significant difference in the blood routine parameters between the treatment and control groups in all time intervals as well (P > 0.05), suggesting their similar impacts on blood routine.

Valved bovine pericardium patches didn't induce thrombosis: Thrombosis after operation was investigated by naked eye and microscope (**Figure 2**). There was no adhered thrombus around the sutures. We discovered well healing status in the planted areas of both bovine pericardium patches and autologous pericardium. Thrombosis was not observed in and around valved pockets in both treatment and control groups (**Figure 2A**). Infarct lesions did not appear in lung tissues (**Figure 2B**). Both the bovine pericardium patches and autologous pericardium were comprised of fibrous connective tissues and infiltrated by scattered lymphocytes and plasmocytes. Focal sutures were revealed and no thrombosis was adhered (**Figure 2C**). No infarct lesion was found in distal lung tissues (**Figure 2D**).

Blood compatibility test in vitro

Valved bovine pericardium patches didn't cause hemolysis: Hemolysis rates in both groups

Table 3. Blood routine examination

Blood routine	Treatment group (n = 9)*					Control group (n = 6)				
	Pre-operation	Post-operation				Pre-operation	Post-operation			
		1 day	3 days	7 days	1 month		1 day	3 days	7 days	1 month
RBC (10^{12}/l)	7.73 ± 0.42	6.45 ± 0.39	4.71 ± 0.32	7.19 ± 0.37	8.56 ± 0.64	7.60 ± 0.42	6.54 ± 0.35	4.78 ± 0.24	6.99 ± 0.13	8.72 ± 0.34
HGB (g/l)	126.12 ± 2.90	92.10 ± 2.37	84.83 ± 2.32	126.83 ± 2.86	125.14 ± 1.87	128.33 ± 2.89	93.00 ± 2.65	83.67 ± 1.53	123.67 ± 1.53	126.35 ± 3.11
PLT (109/l)	189.33 ± 5.85	154.00 ± 8.15	134.67 ± 4.41	176.00 ± 5.48	201.25 ± 4.83	188.67 ± 2.08	153.00 ± 3.61	134.00 ± 5.57	176.33 ± 4.16	197.36 ± 5.28
WBC (10^9/l)	8.60 ± 0.42	9.04 ± 0.44	11.82 ± 0.50	8.85 ± 0.43	7.51 ± 0.43	8.64 ± 0.08	9.02 ± 0.13	12.23 ± 0.05	8.83 ± 0.06	8.02 ± 1.41
NEUT (10^9/l)	3.56 ± 1.06	3.77 ± 0.08	4.63 ± 0.07	3.70 ± 0.06	3.63 ± 0.04	3.54 ± 1.10	3.78 ± 0.03	4.68 ± 0.17	3.71 ± 0.06	3.58 ± 0.23
NEUT% (%)	56.06 ± 0.32	58.30 ± 0.34	71.88 ± 0.42	57.74 ± 0.34	56.36 ± 0.65	56.09 ± 0.17	58.36 ± 0.09	71.79 ± 0.17	57.78 ± 0.07	57.08 ± 0.34

P value		RBC (10^{12}/l)	HGB (g/l)	PLT (10^9/l)	WBC (10^9/l)	NEUT (10^9/l)	NEUT% (%)
Pre-operation		0.567	0.17	0.796	0.823	0.973	0.837
Post-operation	1 day	0.656	0.503	0.784	0.916	0.74	0.626
	3 days	0.656	0.302	0.799	0.068	0.52	0.576
	7 days	0.165	0.062	0.902	0.184	0.757	0.74
	1 month	0.586	0.36	0.163	0.425	0.62	0.095

*P > 0.05 vs. the control group.

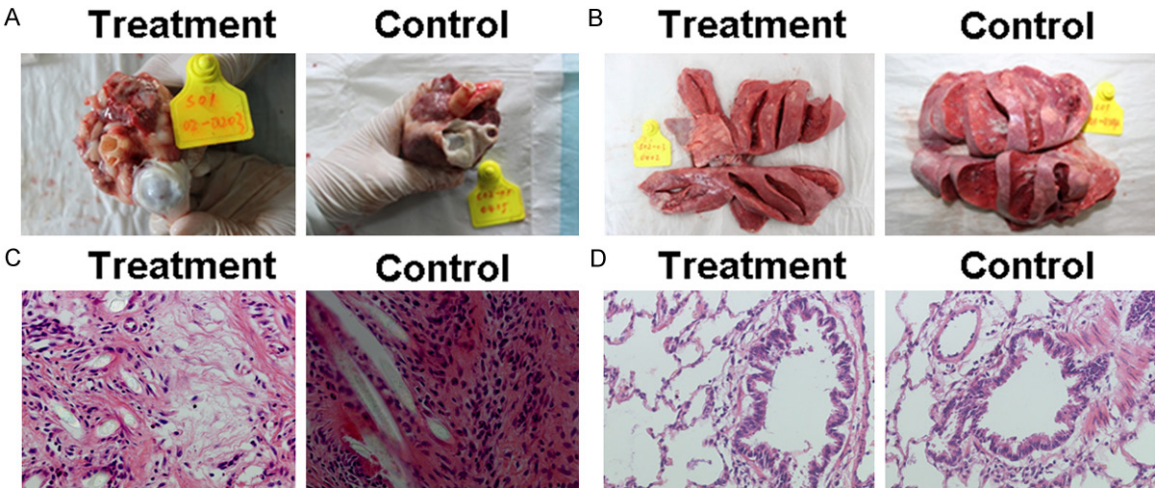

Figure 2. The thrombosis was investigated by naked eye and microscope. A: Thrombosis was not observed in and around valved pockets in both treatment and control groups; B: Infarct lesions did not appear in lung tissues; C: Sutures were revealed in focal lesions and no thrombosis was adhered (HE × 400); D: No infarct lesions was found in distal lung tissues (HE × 400).

Table 4. Hemolysis test (n = 15)

	Treatment group	Control group
Hemolysis rate (%)	1.28 ± 0.14*	1.26 ± 0.06

*P > 0.05 vs. the control group. **P* value = 0.617.

were much less than 5% and there was no significant difference between the two groups (P > 0.05, **Table 4**). It was indicated that both valved bovine pericardium patch and autologous pericardium did not induce hemolysis.

Valved bovine pericardium patches didn't induce complement activation: We determined the contents of complement components 3a, 5a, TCC, Bb, and 4d by ELISA kits and revealed that all of them were exactly similar between the treatment and control groups (P > 0.05, **Table 5**). Therefore, the valved bovine pericardium patch showed similar activity of complement activation to autologous pericardium.

Valved bovine pericardium patches didn't affect plasma soluble P-selectin: We also investigated the content of plasma soluble P-selectin by ELISA kit. Practically, similar contents were found between valved bovine pericardium patch (treatment group) and autologous pericardium (control group) (P > 0.05, **Table 6**).

Valved bovine pericardium patches didn't influence dynamic clotting: In both groups, the absorbance was gradually reduced over time and a relative small absorbance was found at 50 min. Moreover, there was no significant difference in the absorbance at each time point between the treatment and control groups (P > 0.05, **Figure 3**). Slow downward-sloping dynamic clotting curves with long elapsed time were observed in both groups. These results suggested that valved bovine pericardium patch induced similar activity of dynamic clotting to autologous pericardium.

Discussion

Biocompatibility is always an important concern of biomedical materials [12]. In this study, we compared the blood compatibility between valved bovine pericardium patch and autologous pericardium based on a series of *in vivo* and *in vitro* hematological indexes. Interestingly, they showed similarly satisfactory blood compatibility. Autologous pericardium was well known as the most ideal materials in blood compatibility at present. Consequently, this novel Chinese valved bovine pericardium patch demonstrated wonderful blood compatibility.

During the establishment of animal model, the operation time for autologous pericardium was 20-30 min longer than that for valved bovine pericardium patch, because the former operation needed temporary preparation of valved pocket. Although this temporarily prepared valved pocket could prevent backflow, urgent time of operation and great difference in suture techniques of different doctors made it difficult

Table 5. Complement activation test (n = 15)

Groups	3a (ng/ml)	5a (ng/ml)	TCC (ng/ml)	4d (ng/ml)	Bb (ng/ml)
Treatment	46.07 ± 1.23*	4.96 ± 0.39*	5.79 ± 0.49*	24.79 ± 0.43*	7.56 ± 0.36*
Control	47.02 ± 0.20	5.06 ± 0.08	5.87 ± 0.13	24.83 ± 0.10	7.62 ± 0.04
*P value	0.065	0.339	0.546	0.728	0.526

*P > 0.05 vs. the control group.

Table 6. Plasma soluble P-selection test (n = 15)

	Treatment group	Control group
Plasma soluble P-selectin (ng/ml)	1.48 ± 0.09*	1.45 ± 0.06

*P > 0.05 vs. the control group. *p value = 0.292.

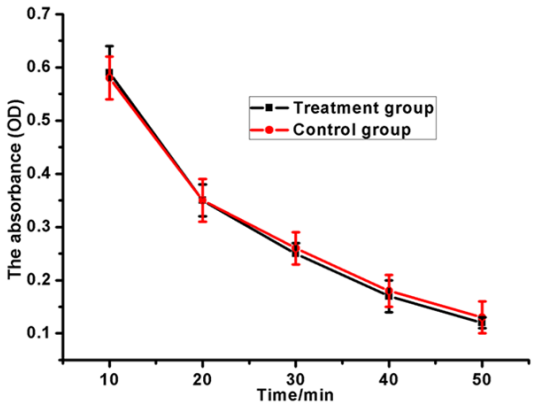

Figure 3. Absorbance in dynamic clotting test. Similar slow downward-sloping dynamic clotting curves with long elapsed time were observed in two groups (n = 15).

to be popularized. The valved bovine pericardium patch had been modified with manual valve. Its abundant sizes could provide the most suitable choice in specific operation accompanied with convenient availability and time-saving.

HGB is a kind of protein being responsible for oxygen transport in higher organisms. In healthy condition, most HGB exists within RBC as intact form and only a very few HGB is freely released from ruptured RBC into the plasma. Free HGB is produced when RBC is damaged by mechanical impact force of blood pulse. If a large number of RBC is damaged, plenty of free HGB will appear in the plasma. Therefore, free HGB in the plasma is a crucial indicator for the RBC damage and the blood compatibility of cardiovascular implants [13]. In patients experiencing cardiopulmonary bypass, spherocytes amount increases and free HGB content in the plasma is also higher than that before operation. The operation implanting devices into circulatory system may enhance the mechanical impact force on RBC and then aggravate RBC damage. This might be the reason for the transient elevation of free HGB on 1 day and 3 days after operation in this study. On 7 days after operation, the free HGB recovered to normal levels. It was implied that operation factors rather than implanted patches were the primary cause of RBC injury. The valved bovine pericardium patch in this study did not generate RBC injury as the autologous pericardium.

In blood routine analysis, RBC count and HGB content represent the hemorrhage volume and the degree of hemocyte damage during operation. In this study, RBC count and HGB content slightly decreased in the first few days after operation and regained preoperative levels on 7 days. In this operation, operators were skillful with small hemorrhage volumes of experimental animals, so all animals did not need blood transfusion after operation. WBC, NEUT, and NEUT% are indicators of infection. They were increased on 3 days after operation and subsequently decreased to preoperative levels. Antibiotics were infused vigorously during and after operation. Clean raising environment was always kept. Thus all animals were raised normally without any infection signs. PLT count indicates *in vivo* blood coagulation. PLT activation induces hemostatic thrombosis. Excessive PLT activation will enhance PLT adhesion, aggregation, and release and increase thrombosis rate. PLT count is greatly significant for the status and function evaluation of PLT aggregation [14]. We found that the valved bovine pericardium patch and autologous pericardium groups showed nearly similar PLT count. In sum, valved bovine pericardium patch was consistent with autologous pericardium in blood routine.

Contact of exogenous materials with *in vivo* blood will stimulate bodies to generate various kinds of biological and chemical reactions.

Wherein, most remarkable variation takes place in coagulation system. Serious abnormality of coagulation function will damage organs function and disturb normal physical state. Intact thrombus is composed of insoluble fibrous protein, deposited PLT, packaged RBC, and RBC fragments [15, 16]. In this study, no thrombus or infarction was found in the implanted areas of patches, sutures areas, and lung tissues. Both valved bovine pericardium patch and autologous pericardium demonstrated excellent anti-thrombosis activity. However, long-term anti-thrombosis effect awaited the future observation.

Hemolysis test is to determine the extent of RBC lysis, which can sensitively show the harmful effect of tested samples on RBC function and metabolism. As an important supplement of *in vitro* cytotoxicity test, hemolysis test plays a crucial role in initial selection of biomaterials. Hemolysis is mainly influenced by the interaction between materials and hemocytes. High hemolysis rate represents great damage of hemocytes [17]. After contact with the blood, hemolytic materials can result in hematoclasis and HGB release; then the elevation of free HGB induces side effect on bodies [18]. Both valved bovine pericardium patch and autologous pericardium showed extremely small hemolysis rates which were much less than 5%. These results conformed to requirements of ISO 10093-4 and GB/T 16886.4 standards.

Contact of biomaterials with blood may activate complement system through classical or alternative pathway. Complement activation products 3a and 5a are anaphylatoxins. As positive polypeptide, components 3a and 5a can cause smooth muscle contraction, provoke mastocytes into releasing histamine, augment vasopermeability, and promote WBC movement. After activation of complement system, antigen-antibody complex is produced and deposited in tissues, which gives rise to pathological changes. Therefore, components 3a, 5a, TCC, Bb and 4d are significant symbols of complement activation [19]. Besides, many factors those cause PLT aggregation can also activate complement system. Complement activation test is hence elected as one of blood compatibility assessments of biomaterials. In this study, no evident difference in the levels of diverse complement components was found between the valved bovine pericardium patch and autologous pericardium. It is well known that complement activation level in autologous blood induced by autologous pericardium is lowest, so the result indicated that this valved bovine pericardium patch possessed wonderful blood compatibility.

P-selectin is a recognized indicator for PLT activation level. Intact P-selectin is derived from PLT surface. When PLT is activated by stimulation of various factors *in vivo* and *in vitro*, P-selectin on the PLT surface will quickly accumulate outside the membrane of PLT. When certain site of complement regulatory protein sequences is digested by specific enzyme, P-selectin is removed from the PLT surface and released into the plasma. Accordingly, a large number of soluble P-selectin is accumulated in the plasma in a short time [20]. Interestingly, we found the similar level of plasma soluble P-selectin between the valved bovine pericardium patch and autologous pericardium. Combining above observations, we demonstrated that effects of valved bovine pericardium patch on PLT activation were extremely similar to autologous pericardium.

Dynamic clotting test is to evaluate biomaterials' influence on coagulation function. Its fundamental is to determine the activated intensity of endogenous coagulation factor and then investigate the effect of materials on clotting time. Slow downward-sloping dynamic clotting curve with long elapsed time suggested superior anticoagulation property of materials. On the contrary, fast downward-sloping dynamic clotting curve with short elapsed time implied inferior anticoagulation property of materials [21]. In this study, the slow downward-sloping dynamic clotting curves with long elapsed time in both groups demonstrated that anticoagulation properties of both the valved bovine pericardium patch and autologous pericardium were superior.

In conclusion, the employment of this valved bovine pericardium patch in RVOT reconstruction was much more convenient and efficient than autologous pericardium. Moreover, this valved bovine pericardium patch showed wonderful *in vivo* and *in vitro* blood compatibility like the well-recognized autologous pericardium. Therefore, this novel Chinese valved bovine pericardium patch was hopeful to be developed as medical device for RVOT reconstruction. Investigations including hemodynamics (antireflux of the valve), histocompatibility, and anti-

calcification will be further performed in larger scale and long term animal tests in future.

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Disclosure of conflict of interest

None.

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