Early Results of Novel Bovine Pericardial Patch Using Comprehensive Anticalcification Procedure in a Swine Model

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We evaluated the short-term safety and effectiveness of our comprehensive anticalcification procedure in swine model. Our comprehensive anticalcification procedure consisted of four steps, including decellularization with sodium dodecyl sulfate and tritonX-100, space filler treatment with polyethylene glycol (PEG), glutaraldehyde cross-linking with organic solvent, and detoxification with glycine. We simultaneously implanted both the commercially available bovine pericardial patch (Supple Peri-Guard) and novel bovine pericardial patch processed by the comprehensive anticalcification procedure into the main pulmonary artery in seven pigs. Every pig underwent a cardiac angiography and was killed on the postoperative day 28. The extracted patches were stained with hematoxylin and eosin. All pigs survived for 4 weeks without any complication. Cardiac angiography showed the absence of leakage and structural problem. Neointimas were formed evenly without intimal hyperplasia. There were no significant differences in the degree of inflammation, necrosis, and calcification between the novel and commercially available patch ($p = 0.450$, $p = 0.317$, $p = 0.999$). Novel bovine pericardial patch using comprehensive anticalcification procedure was similar to existing cardiovascular patch in early surgical results in a swine model. The comprehensive anticalcification procedure could facilitate appropriate bioprosthetic properties of the bovine pericardium. *ASAIO Journal* 2016; 62:100–105.

Key Words: pericardium, calcification, tissue engineering

Bovine pericardium is widely used for a variety of cardiovascular surgeries, as heart valve substitutes and intracardiac and vascular patch materials.[1] However, dystrophic calcification remains an important drawback of the xenograft. Therefore, there have been many studies to decrease calcification and improve the durability of xenograft.[2–5] We have been involved in the study of various issues of xenograft tissue engineering,[6–9] such as decellularization, space filler treatment, cross-linking, and detoxification. On the basis of these studies, we developed a comprehensive anticalcification procedure and produced novel bovine pericardial patch using this technique. In this study, the short-term safety and effectiveness of our comprehensive anticalcification procedure were evaluated in a comparative study of the novel and commercially available bovine pericardial patch in a swine implantation model.

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Materials and Methods

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (IACUC No: BA1306-130/049-01, SNUBH animal research project No: 64-2013-049).

Novel Bovine Pericardial Patch

This study was designed to compare the two bovine pericardial cardiovascular patches, *i.e.*, the novel patch (NP) and commercially available patch (CAP). The NP is a new complete product that is produced with a comprehensive anticalcification procedure at a manufacturing facility that is certified for good manufacturing practice (**Figure 1**). The size is 4×3 cm and thickness is 0.3 ± 0.1 mm. The CAP was Supple Peri-Guard made by Synovis Surgical Innovations and has been used for various surgical procedures, including cardiac and vascular surgery. Our comprehensive anticalcification procedure consisted of four steps, including decellularization with sodium dodecyl sulfate (SDS) and tritonX-100, space filler treatment with PEG, glutaraldehyde (GA) cross-linking with organic solvent, and detoxification with glycine. The comprehensive anticalcification procedure is described later.

Comprehensive Anticalcification Procedure

Fresh bovine pericardia obtained from a local slaughterhouse were placed in phosphate-buffered saline (PBS; pH, 7.4) and immediately transported to our laboratory. All chemical processings were performed at the International Organization for standardization (ISO) class 5 and ISO class 7 cleanroom. After removing the excess fat and damaged tissue, they were rinsed with normal saline. Disinfection was achieved by treating the bovine pericardia with phosphate buffered saline (PBS) (pH, 7.3–7.5) containing 0.1% peracetic acid and 4% ethanol for 2 hours at 4°C. Sodium hydroxide was used for prevention of bovine spongiform encephalopathy, and propylene oxide was used for final sterilization. After completion of all process, aseptic condition was confirmed by sterility test.

Decellularization. Decellularization was conducted in four steps. First, the tissues were treated with a hypotonic buffer solution that contained 0.25% SDS for 24 hr and rinsed with washing solution for 1 hr. Second, the tissues were treated with

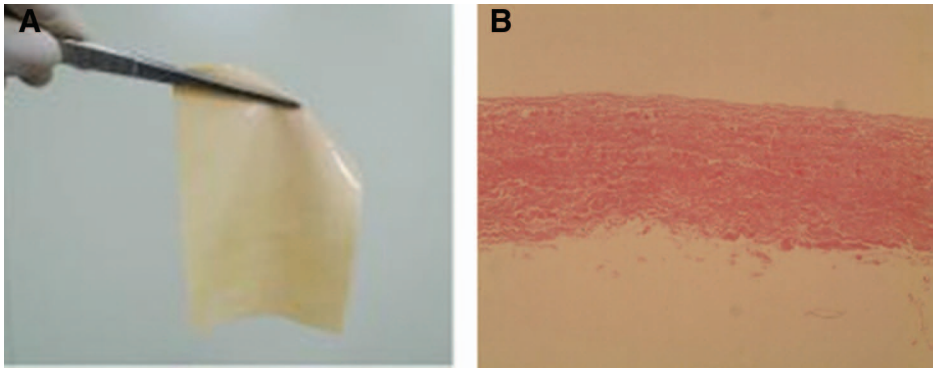

Figure 1. A: Gross appearance of novel patch (3 × 4 cm). **B**: Microscopic view of novel patch. full color online

hypotonic buffer solution that contained 0.5% tritonX-100 for 24 hr and washed with normal saline for 1 hr. Third, they were treated with isotonic-buffered solution for 24 hr and rinsed with washing solution for 1 hr. Fourth, the tissues were treated with hypertonic-buffered solution for 3 hr and PBS for 1 hr. After decellularization, the tissues were preserved in PBS. All processes were performed at 4°C. The chemical compositions of buffered solution and washing solution were as follows.

1. Hypotonic buffered solution: distilled water 1,000 ml; tris-HCl 0.01 mol/L; ethylenediaminetetraacetic aicd (EDTA) disodium salt dihydrate 0.05%; neomycin trisulfate 50 mg; pH 8.0.
2. Isotonic buffered solution: distilled water 1,000 ml; tris-HCl 0.05mol/L; NaCl 0.15mol/L; EDTA disodium salt dihydrate 0.05%; neomycin trisulfate 50 mg; pH 8.0.
3. Hypertonic buffered solution: distilled water 1000mL; tris-HCl 0.1mol/L; NaCl 1.2mol/L; pH 8.0
4. Washing solution: distilled water 1,000 ml; EDTA disodium salt dihydrate 0.05%; neomycin trisulfate 50 mg.

Space filler treatment. Between the third and fourth step of decellularization, the bovine pericardia were treated with PBS that contained 30% PEG 1,000 molecular weight for 24 hr at 4°C to fill the interstitial space.

Glutaraldehyde crosslinking. Bovine pericardial tissues were cross-linked with 0.5% glutaraldehyde (GA) solution for 3 days and additionally cross-linked in 0.25% GA solution with 75% ethanol and 5% octanol for 2 days. Finally, the tissues were fixed with 0.25% GA for 7 days. All processes were performed at room temperature.

Detoxification. After the completion of GA cross-linking, the tissues were treated with PBS that contained 0.2M glycine for 24 hr at room temperature.

Operation

We implanted the NP and CAP into the main pulmonary artery in seven crossbred pigs (57.7 ± 2.3 kg). The implantation sites were the proximal and distal part of the main pulmonary artery. The site was alternated between pigs.

Under general anesthesia, each pig was placed in the right lateral decubitus position, and a left thoracotomy was performed through the third intercostal space. On heparin injection, the proximal part of the pulmonary artery was partially clamped and excised. A cardiovascular patch was implanted using 5-0 polypropylene. The other cardiovascular patch was implanted on the distal part of the pulmonary artery in the same manner. Each patch was circular, with a 10-mm diameter (**Figure 2**).

Postoperative Care

Cefazolin and ketoprofen were injected postoperatively for 3 days, and anticoagulants were not administered. Blood samples were taken from each pig on the operation day and

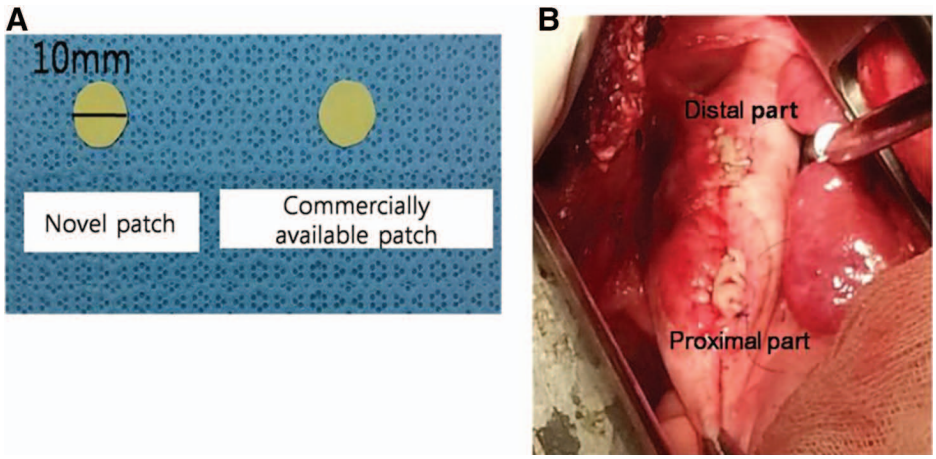

Figure 2. Main pulmonary artery state after patch implantation. full color online

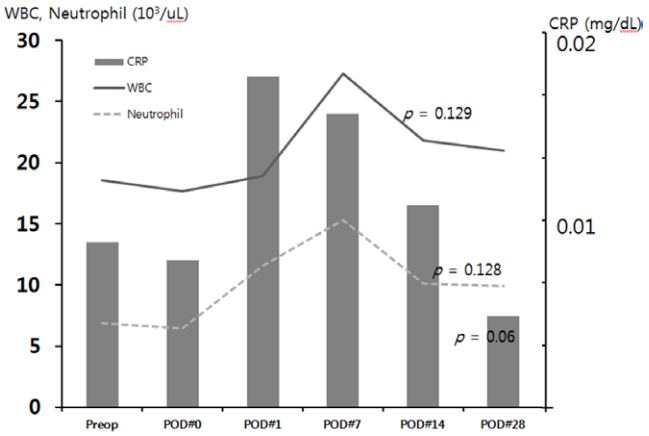

Figure 3. Results of blood sample test. CRP, c-reactive protein; POD, postoperative day; WBC, white blood cell.

postoperative day 1, 7, 14, and 28 to measure complete blood cell count (CBC) and c-reactive protein (CRP). Postoperative care for the pigs was provided by our institution's veterinarian.

Cardiac Angiography and Histology

After heparin injection, the pigs were killed on the postoperative day 28, and the patches were harvested for macroscopic observation and histological study. Cardiac angiography was performed to evaluate the implanted patch areas before killing the pig.

The extracted patches were stained with hematoxylin and eosin and observed for inflammation, necrosis, and microcalcification. We quantified each condition for subsequent statistical analyses; the grading was by our hospital's pathologist.

Statistical Analysis

Statistical analyses were performed using the SPSS software package (version 19.0, SPSS Inc., Chicago, IL). The data were expressed as mean ± standard deviation. Repeated measured analysis of variance was used to compare the changes in blood sample test, and the Wilcoxon signed rank test was used to compare the changes in microscopic findings between the two patches. A p value < 0.05 was considered statistically significant.

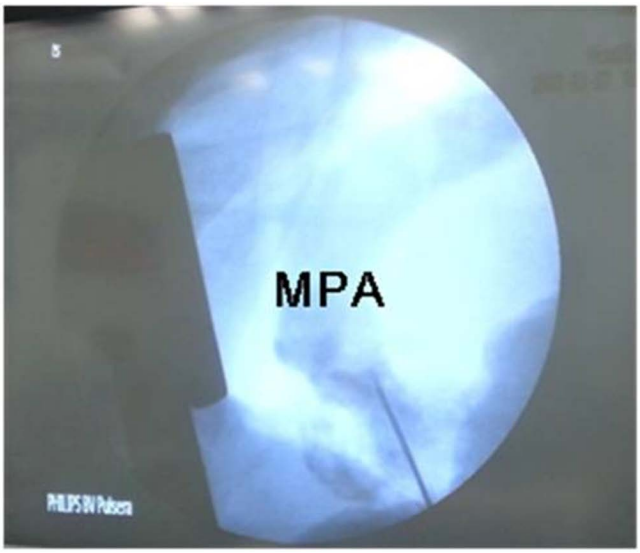

Figure 4. Results of cardiac angiography. Blood flow was very smooth, and there was no structural problem. MPA, main pulmonary artery. full color online

Results

All seven pigs survived for 4 weeks without any complication. All pigs maintained good food intake, and weights increased from 57.7 ± 2.3 kg to 61.9 ± 4.8 kg.

Blood Sample Test

We checked CBC and CRP before and after surgery as a screening procedure for postoperative infection and other problems. Postoperative white blood cell (WBC) and neutrophil counts showed a tendency to increase on the postoperative day 7 and decrease on the postoperative day 14 and 28. There were no significant differences in the changes of WBC, neutrophil count, and CRP value ($p = 0.129$, $p = 0.128$, and $p = 0.06$, respectively, **Figure 3**).

Cardiac Angiography and Macroscopic Findings

Cardiac angiography demonstrated that there were no leakages or structural problems in the implanted patch areas. The blood flow of main pulmonary artery was very smooth in all pigs (**Figure 4**).

There was no gross hematoma formation on the outer surface of the implanted patch areas. Two pigs developed observable thrombi on the inner surface of the implanted patches. One case was observed at a distal-located NP, and the other was developed at a distal-located CAP. The size was 4 × 2 mm in NP and 7 × 5 mm in CAP. The thrombus on the CAP was approximately four times larger than the thrombus on the NP (**Figure 5**).

Histologic Findings

The extracted patches were stained with hematoxylin and eosin to identify the degree of inflammation, necrosis, and microcalcification (**Figure 6**). All inflammatory reactions were localized near the patches. Neointimas were formed evenly with good re-endothelialization except in one case. This one case was in the CAP with an observed thrombus (**Figure 6**).

For quantification, we classified inflammation into six distinct grades according to infiltration extent of inflammatory cells; 0 = absent, 1 = minimal (infiltration extent ≤ 0.1 mm), 2 = mild (0.1 mm < infiltration extent ≤ 0.3 mm), 3 = moderate (0.3 mm < infiltration extent ≤ 1.0 mm) and focal (one site), 4 = moderate (0.3 mm < infiltration extent ≤ 1.0 mm) and diffuse (more than one site), and 5 = marked (1.0 mm < infiltration extent). Necrosis was classified into three grades (0 = absent, 1 = present [<50%], 2 = present [≥50%]), and microcalcification into two grades (0 = absent and 1= present). There were no significant differences in inflammation and necrosis between the two groups ($p = 0.450$ and $p = 0.317$), and microcalcification was observed in all patches. Comprehensive evaluation of patch was performed by calculation of a total score that is sum of the inflammation, necrosis, and microcalcification grades

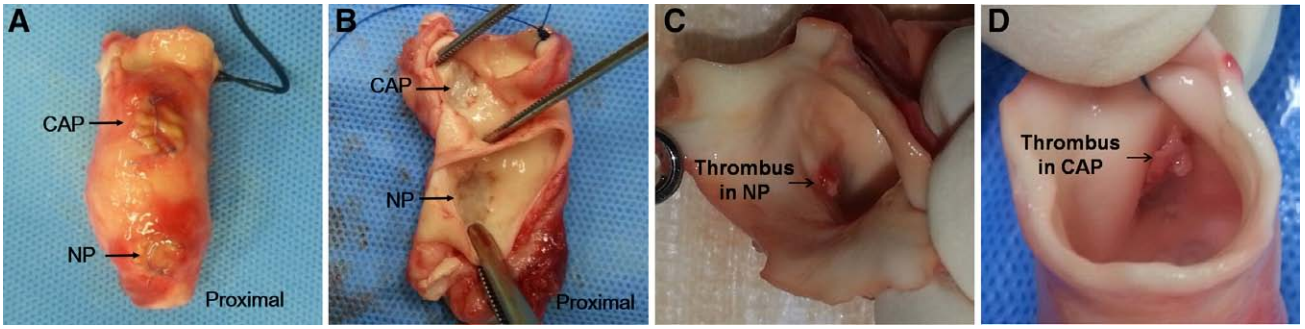

Figure 5. Gross findings of extracted pulmonary artery in (**A**) and (**B**). There was no gross hematoma formation on the outer surface. Thrombus was observed at distal located novel patch (NP) (**C**) and distal located commercially available patch (CAP; **D**). The thrombus on the CAP was approximately four times larger than the thrombus on the NP. full color online

in each patch. The total score was not significantly different between the two patches ($p = 0.581$, **Table 1**).

Discussion

Xenograft tissues, such as bovine pericardium or porcine aortic valve, are widely used in the field of cardiovascular surgery. These xenografts require chemical processing to reduce immune or inflammatory reactions and to increase mechanical endurance for implantation in the human body. Glutaraldehyde has been used as a representative cross-linking agent. However, GA cross-linking has known drawbacks, such as dystrophic calcification and cytotoxicity.

On the basis of our previous studies, we developed a comprehensive anticalcification procedure using decellularization with SDS and tritonX-100, space filler treatment with PEG, GA cross-linking with organic solvent, and detoxification with glycine. Although the mechanisms of calcification of GA cross-linked xenograft are not fully understood, the causes of calcification are known to be related to tissue phospholipids, free aldehyde groups of GA, and residual antigenicity.[10] Therefore, the comprehensive anticalcification procedure targeted the removal of phospholipids and free aldehyde groups, as well as the reduction of the antigenicity of the xenograft.

Calcification of cardiac xenograft is initiated primarily within nonviable connective tissue cells that have been devitalized.[11] In this study, antigenicity was reduced by the decellularization procedure that was performed in four steps using hypotonic solution, 0.25% SDS, and tritonX-100. We previously demonstrated that the stepwise use of SDS and tritonX-100 produced

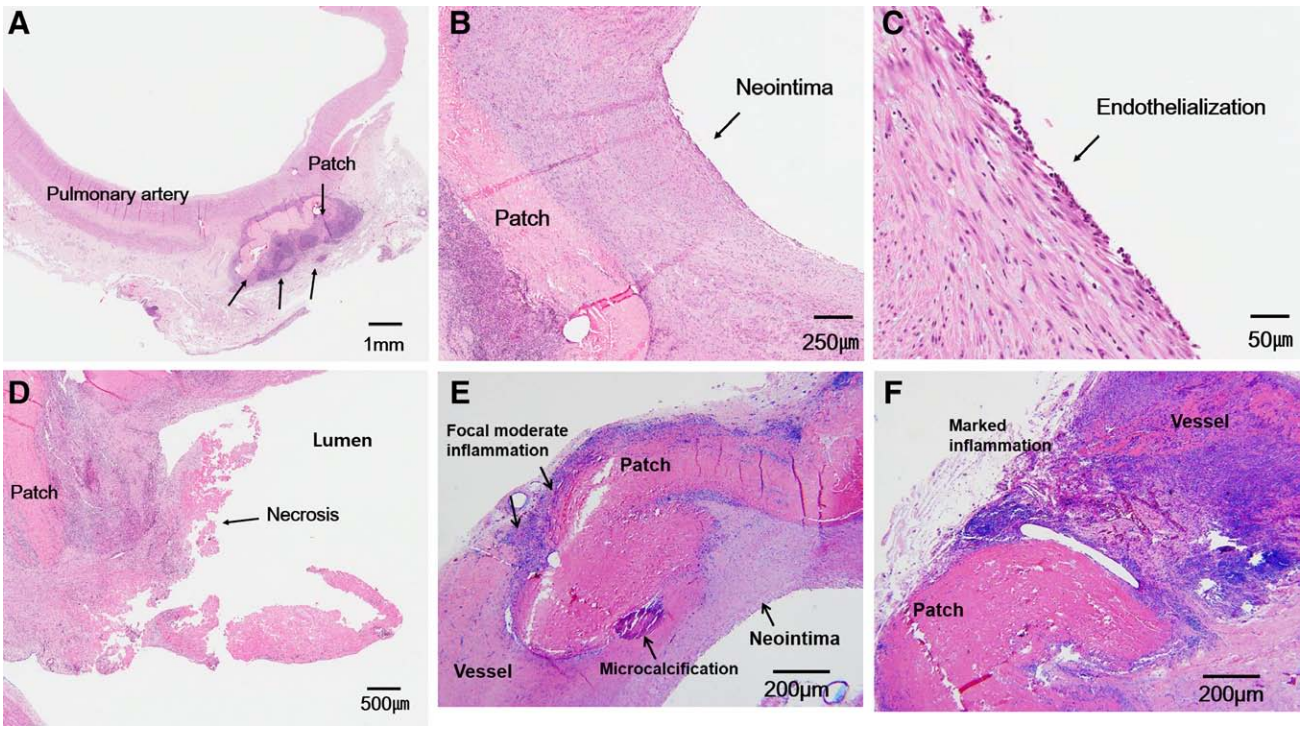

Figure 6. Microscopic views of extracted patches. Inflammation was restricted around the patch (**A**). Neointima was formed evenly with good endothelialization (**B** and **C**). Among seven cases, the one commercially available patch (CAP), which was observed with thrombus, was found with necrosis instead of endothelialization (**D**). Grading examples of novel patch (**E**) and CAP (**F**) were shown (**A**: ×10, **B**: ×20, **C**: ×40, **D**: ×200, **E** and **F**: ×100). full color online

Table 1. Quantification of Degree of Inflammation, Necrosis, and Microcalcification

	No 1	No 2	No 3	No 4	No5	No 6	No 7	Total	*p*
Inflammation									
NP	3	3	5	3	5	5	5	4.14 ± 1.07	0.450
CAP	5	5	5	4	3	5	5	4.57 ± 0.79	
Necrosis									
NP	0	1	0	0	0	1	1	0.43 ± 0.53	0.317
CAP	0	1	0	0	0	1	0	0.29 ± 0.49	
Microcalcification									
NP	1	1	1	1	1	1	1	1.0	>0.999
CAP	1	1	1	1	1	1	1	1.0	
Total score									
NP	4	5	6	4	6	7	7	5.57 ± 1.27	0.581
CAP	6	7	6	5	4	7	6	5.86 ± 1.07	

Inflammation grades: 0 = absent; 1 = minimal (infiltration extent ≤0.1 mm), 2 = mild (0.1 mm < infiltration extent ≤0.3mm), 3 = moderate (0.3 mm < infiltration extent ≤ 1.0 mm) and focal (one site), 4 = moderate (0.3 mm < infiltration extent ≤1.0 mm) and diffuse (more than one site), and 5 = marked (1.0 < infiltration extent).

Necrosis grades: 0 = absent, 1 = present (<50%), and 2 = present (≥50%).

Microcalcification grades: 0 = absent and 1 = present.

CAP, commercially available patch; NP, novel patch.

good decellularization, and a high concentration of SDS could cause structural alteration by compromising collagen integrity.[12,13] Using α-galactosidase in the decellularization process decreases the immune response and prolongs durability of a xenograft. Despite our earlier reports on α-galactosidase treatment,[14–16] we were unable to use α-galactosidase in the comprehensive anticalcification procedure because of low cost-effectiveness. The use of α-galactosidase for decellularization would lead to a more effective anticalcification procedure in the bovine pericardium.

Phospholipid is known to play an important role in calcification of the xenograft. Calcium-containing extracellular fluid is assumed to combine with phosphorus in the membrane-associated phospholipid of dead xenograft cell membrane and form calcium phosphate crystals.[11] Treatment with organic solvents, such as ethanol, octanol, and octanediol, are known to reduce the phospholipid content and prevent calcium phosphate nucleation.[6] We used a short- and long-chain alcohol combination organic solvent. Pathak *et al*.[17] reported that bovine pericardium treated with a short- and long-chain alcohol combination shows a reduction in phospholipid content. We further demonstrated the GA cross-linked bovine pericardium with a combination of short- and long-chain alcohol had a superior anticalcification effect.[6] Our previous studies showed that GA and organic solvent treatment had better mechanical durability than GA treatment alone and did not cause a loss in the tensile strength, elasticity, and thermostability.[18,19]

Amino groups can improve protein cross-linking and neutralize toxicity of free aldehyde groups.[20] Residual-free aldehyde groups easily combine with serum Ca^{+} and result in tissue calcification.[21] Many studies showed that detoxification of GA cross-linked xenograft using various amino groups, such as homocysteic acid, L-glutamic acid, and L-arginine, was effective in reducing tissue calcification. We used glycine, the simplest form of all amino acids, for detoxification in the anticalcification procedure. Our previous study demonstrated that postfixation treatment with glycine strongly prevented calcification of GA-fixed bovine pericardium in the rat subcutaneous implantation model.[8]

Our comprehensive anticalcification procedure included space filler treatment using PEG. Otherwise, the Supple Peri-Guard used in the control group was not treated with space filler. Filling interstitial void spaces in GA-treated tissue with macromolecular substance likely has a preventive effect on tissue calcification.[22] Although the mechanisms of space filler treatment are not fully understood, it is believed that reaction of macromolecules with free aldehyde groups of GA causes their inactivation and forms a barrier that prevents the release of residual GA.[23,24] We previously demonstrated that GA cross-linking with PEG as space filler was an effective anticalcification method in the rabbit intramuscular implantation model.[7]

Vasudev *et al*.[25] reported that PEG substantially inhibited deposits of calcium and platelet–tissue attachment. They hypothesized that PEG treatment modifies or masks the platelet receptor sites on collagen and reduces platelet density on the surface. Thus, it is likely that PEG treatment not only has an anticalcification but also has an antithrombogenic effect through prevention of platelet attachment. We believed that this property of PEG would cause the prevention of thrombus formation and thromboembolic events. This study showed that the NP had smaller thrombus formation compared with the CAP that was not treated with a space filler.

This study had four main findings that indicated the safety and effectiveness of our comprehensive anticalcification procedure. First, postoperative infection did not occur in all pigs. Second, we could not find any structural problem related to the implanted patches. Third, neointima was evenly formed in the implanted NP. Fourth, there were no significant differences in the degree of inflammation, necrosis, and microcalcification between the two patches. These findings indicated that the NP was similar to the CAP in terms of early surgical results in a swine model.

This study had limitations that must be noted. First, the number of pigs enrolled in this study might be too small, and implantation period was too short to draw definite conclusions. Second, it is possible that the patch size was too small to affect structural changes, such as aneurysms or suture dehiscence. Furthermore, considering mechanical failure was exaggerated in high pressure systems, a definitive conclusion on the mechanical durability of the NP could not be reached. Third, our study did not reach to show the superiority on the NP.

But, our comprehensive anticalcification procedure included the space filler treatment and showed the advantages in terms of endothelialization and thrombus formation. Therefore, we need a large sample and well-designed long-term study to demonstrate more definitive effect of our comprehensive anticalcification procedure.

Conclusions

Our comprehensive anticalcification procedure was refined based on our previous studies and included the space filler treatment. This study showed that novel bovine pericardial patch using comprehensive anticalcification procedure was similar to existing cardiovascular patch in early surgical results in a swine model. Although thrombus formation was observed in both groups, the one on the CAP was approximately four times larger compared with the thrombus on the NP. Furthermore, the thrombus-formed CAP failed to achieve endothelialization. Therefore, our comprehensive anticalcification procedure involving decellularization, space filler treatment, GA cross-linking, and detoxification was a good method to facilitate appropriate bioprosthetic properties of the bovine pericardium.

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