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Roles of ephrin-B2$^+$ and CD34$^+$ cells in post-angioplasty pericardial patch repair

Ephrin B2$^+$ and CD34$^+$ hücrelerinin anjiyoplasti sonrası perikard yama tamirindeki rolü

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ABSTRACT

Background: This study aims to investigate whether an endothelial marker ephrin-B2 exists in pericardial patches during post-angioplasty repair and whether CD34$^+$ endothelial progenitor cells are present in the patches after being implanted into the arterial environment.

Methods: Eighteen eight-week-old male Wistar rats were subjected to laparotomy to check the abdominal aorta below renal arteries, to incise it, and then to perform angioplasty by using porcine or bovine pericardial patch. The control group consisted of 18 age- and weight-matched Wistar rats. Angioplasty was conducted by subcutaneously implanting patches in the control group. The patches were taken on one, three, five, and 30 days after surgery and investigated by histological and immunohistochemical assays, immunofluorescent labeling, Western blotting and reverse transcription polymerase chain reaction.

Results: The patches containing collagen were acellular before angioplasty. After implantation of a bovine pericardial patch, the numbers of ephrin-B2$^+$ and CD34$^+$ cells increased significantly; however, the test results for CD68, alpha-actin and von Willebrand factor were negative. There was a monolayer of cells in the inner luminal surface five days after implantation of a porcine pericardial patch. In contrast, ephrin-B2$^+$ or CD34$^+$ cells did not appear in the control group. On the postoperative 30th day, there were ephrin-B2$^+$ and CD34$^+$ cells in the two types of patches.

Conclusion: Ephrin-B2$^+$ and CD34$^+$ cells began to infiltrate pericardial patches soon after implantation. The patches which allow endothelialization during arterial remodeling are potentially applicable to tissue plasty and angioplasty.

Keywords: Angioplasty; CD34; ephrin-B2; pericardial patch; positive cell; repair material.

ÖZ

Amaç: Bu çalışmada endotel belirteci ephrin B2'nin anjiyoplasti sonrasında perikard yamalarında mevcut olup olmadığı ve CD34$^+$ endotel progenitör hücrelerinin arteriyel ortama implante edildikten sonra yamalarda bulunup bulunmadığı araştırıldı.

Çalışma planı: Renal arterlerin aşağısında abdominal aortu kontrol ve insize etmek ve ardından domuz veya sığır perikard yaması ile anjiyoplasti yapmak üzere sekiz haftalık 18 erkek Wistar sıçanına laparotomi uygulandı. Kontrol grubu, yaş ve ağırlık olarak eşleştirilmiş 18 Wistar sıçanından oluşuyordu. Anjiyoplasti kontrol grubuna yamaların subkutan olarak implante edilmesi ile gerçekleştirildi. Yamalar cerrahiden bir, üç, beş ve 30 gün sonra alındı ve histolojik ve immünhistolojik testler, immünfloresan işaretleme, Western blot ve reverz transkripsiyon polimeraz zincir reaksiyonu ile çalışıldı.

Bulgular: Kollajen içeren yamalar anjiyoplasti öncesi aselüler idi. Sığır perikard yama implantasyonundan sonra, ephrin B2$^+$ ve CD34$^+$ hücre sayısı anlamlı düzeyde arttı; ancak CD68, alfa-aktin ve von-Willebrand faktörünün test sonuçları negatif idi. Domuz perikard yama implantasyonundan beş gün sonra, lümenin iç yüzeyinde tek hücre tabakası mevcuttu. Buna karşın, kontrol grubunda ephrin B2$^+$ ve CD34$^+$ hücrelerine rastlanmadı. Cerrahinin 30. gününde iki yama türünde de ephrin B2$^+$ ve CD34$^+$ hücreleri mevcuttu.

Sonuç: Ephrin B2$^+$ ve CD34$^+$ hücreleri implantasyondan kısa bir süre sonra perikard yamalarına infiltre olmaya başladı. Arterlerin yeniden biçimlenmesi sırasında endotelializasyonu sağlayan yamalar, doku plasti ve anjiyoplastide potansiyel olarak kullanılabilir.

Anahtar sözcükler: Anjiyoplasti; CD34; ephrin B2; perikard yama; pozitif hücre; tamir malzemesi.

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Medical materials, as multidisciplinary products of chemistry and biomedical engineering, have been widely used in surgeries,[1] particularly in angioplasty.[2] Synthetic materials, such as vascular stents, artificial blood vessels and vascular patches, have been also applied in modern vascular surgeries.[3] In particular, vascular patches made of polymer materials of biological origin can improve the therapeutic outcomes of surgery and decrease the risk for postoperative stenosis.[4] Being easily operable and highly biocompatible, porcine and bovine pericardial patches reduce bleeding during suturing and allow direct Doppler hemorheological detection[5] and real-time monitoring of postoperative blood flow. Besides, the implanted blood vessels are highly endurable due to satisfactory remodeling and adaptability. In this study, we aimed to investigate whether endothelial marker ephrin-B2 (EFNB2) existed in pericardial patches during post-angioplasty repair and whether $CD34^+$ endothelial progenitor cells (EPCs) were present in the patches after being implanted into the arterial environment.

MATERIALS AND METHODS

All the animal experiments were approved by the Institutional Animal Care and Use Committee of Huaihe Hospital of Henan University and were performed in accordance with the principles of the Animal Research: Reporting *in vivo* Experiments (ARRIVE) guideline.

The overriding principle is expressed as the 3Rs: Replacement, Refinement, Reduction. The documents recommended herein are available in the website of National Centre for 3Rs (http://www.nc3rs.org.uk).

Preoperative preparation

Eighteen eight-week-old male Wistar rats (mean weight: 210±33 g) were selected, continuously anesthetized by isoflurane, and implanted with patches. The flow rates of oxygen and isoflurane during anesthesia were 0.8 L/min and 3 mL/min respectively, and were 0.6 L/min and 1.5 mL/min during surgery, respectively. Porcine and bovine pericardial patches were purchased from the Tissue Regenix (United Kingdom) and Beijing Balance Medical Co., Ltd. (China), respectively.

Surgical methods

Under general anesthesia, the rat limbs were fixed and abdominal hair was completely shaved. The abdominal skin was sterilized with medical alcohol, covered with a sterile towel drape, and cut until the muscle was reached. The intestinal canal was wrapped and pulled rightwards with gauze moistened with saline. The abdominal wall was, then, stretched to expose the posterior peritoneum. The abdominal aorta below renal arteries was isolated, hypodermically injected with heparin, and blocked about 6 mm from the proximal and distal ends with a microvascular occlusion clamp 1 minute later.[6] After the anterior wall of the abdominal aorta was cut with a 3 mm incision and trimmed into an approximate oval one along the blood vessel, on which pericardial patches trimmed into the same shape, it was interruptedly sutured with 10-0 nylon threads. The microvascular occlusion clamp was removed after surgery to recover the blood flow in abdominal aorta and the suture site was compressed with a cotton swab to prevent postoperative bleeding and possible pseudoaneurysms. Subsequently, the abdominal incision was sutured with 5-0 polyester threads and the rats were kept in cages and warmed for rapid resuscitation.[7] Another age- and weight-matched 18 Wistar rats were subcutaneously implanted with patches by using identical methods as the control group.

Postoperative tissue collection

All rats were anesthetized and fixed again using the methods as mentioned above. The skin and muscle in the middle of chest were cut and the anterior wall of the chest was scissored around the rib to expose the heart. Afterwards, the left ventricle was punctured into which about 30 mL of phosphate buffer was perfused after the liver was cut to drain blood, and, then another 20 mL of 10% formalin solution was perfused to fix systemic tissues. After the abdominal cavity was opened, the intestinal canal was removed to expose the peritoneum, and the patched abdominal aorta was scissored, rinsed with normal saline, and stored prior to use.

Histological assay

The collected samples were fixed in 10% formalin, stored in 70% ethanol, fixed in paraffin, and cut into sections. Specifically, the samples stored in 70% ethanol were dehydrated, soaked in a mixture of dimethylbenzene, and ethanol for two hours, and then in dimethylbenzene solution refreshed twice (1.5 h each time). The dehydrated tissue samples were poured into a box containing liquid paraffin and cooled until white blocks appeared, which were then cut into sections and transferred into a 37 °C water bath with an ink brush. Afterwards, the unfolded sections were put onto glass slides with water wiped, dried in a 40 °C oven for about one hour, air-dried, subjected to hematoxylin-eosin staining, and studied under an microscope to count cells[8] and to record the mean number.

Immunohistochemical assay

The glass slides with sections were de-paraffinized in dimethylbenzene-dimethylbenzene-absolute ethanol-absolute ethanol-95% ethanol-90% ethanol-80% ethanol-70% ethanol sequentially (10 minutes in each solution). Subsequently, the sections were washed with water, soaked in 3% H_2O_2 for 10 minutes to remove endogenous catalase,[9] re-rinsed with water, boiled in citrate buffer for three minutes, and cooled down to room temperature (the boiling-cooling procedure was repeated). The sections were, then, rinsed with water and washed twice with phosphate buffer on which tissues were marked with a marker pen. Afterwards, the sections were blocked with 1:10 diluted serum and incubated at 37 °C for 0.5 hours, from which excessive serum was dried with an absorbent paper. After addition of primary antibodies and overnight incubation in a 4 °C refrigerator, the sections were rinsed three times with phosphate buffer, added secondary antibodies, incubated again in a 37 °C incubator for 0.5 hours, rinsed three times with phosphate buffer, added 1:100 diluted SABC (Sigma, CA, USA), incubated in the 37 °C incubator for 0.5 hours, washed three times with phosphate buffer, and counterstained. The sections were then dehydrated in 70% ethanol-80% ethanol-90% ethanol-95% ethanol-absolute ethanol-absolute ethanol-dimethylbenzene-dimethylbenzene (2 minutes in each solution), sealed by dropping vegetable glue, and air-dried.

Immunofluorescent labeling

The glass slides with sections were de-paraffinized in dimethylbenzene-dimethylbenzene-absolute ethanol-absolute ethanol-95% ethanol-90% ethanol-80% ethanol-70% ethanol sequentially (10 minutes in each solution). The sections were, then, washed with water, soaked in 3% H_2O_2 for 10 min, re-rinsed with water, boiled in citrate buffer for three minutes, and cooled down to room temperature (the boiling-cooling procedure was repeated). Subsequently, the sections were rinsed with water and washed twice with phosphate buffer, on which tissues were marked with a marker pen. Afterwards, the sections were blocked with 1:10 diluted serum and incubated at 37 °C for 0.5 hours, from which excessive serum was dried with absorbent paper. Anti-EFNB2, CD31 and CD34 antibodies (1:100 diluted), as primary antibodies, were added into the sections which were, thereafter, incubated overnight at 4 °C in the refrigerator. On the next day, the primary antibodies were discarded, and the sections were rinsed three times with phosphate buffer in a constant-temperature shaker (5 minutes each time), added 1:5000 diluted secondary antibodies in dark (fluorescently labeled antibodies corresponding to the primary ones), and incubated in a 37 °C incubator for one hour in dark. After the secondary antibodies were discarded, DAPI was added onto the marked region, and the sections were sealed with nail polish, and stored overnight in a dark plastic box in a 4 °C refrigerator.[10] Fluorescences were observed in dark under a fluorescence microscope using varying wavelengths.

Western blotting

The thoracic aorta, pericardial patches, and inferior vena cava samples were freeze-dried in liquid nitrogen, ground into powders, evaporated, and mixed with protease inhibitor-containing buffer for 30 seconds, the protein concentrations in which were measured with a UV-vis spectrophotometer. The samples were, then, diluted based on proteins with the same masses, homogenized on an ultrasonic vibrator at 4 °C for one hour to extract and to collect the proteins. Proteins with the same quantities were subjected to electrophoresis and membrane transfer. Anti-EFNB2, CD34, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and heat shock protein 90 (HSP-90) antibodies were used as primary antibodies. Protein signals were detected by Pierce ECL reagent (Western Blotting Kit. Thermo Fisher Scientific, MA, USA).[11]

Reverse transcription polymerase chain reaction (PCR)

Ribonucleic acid (RNA) was extracted with Trizol solution (Thermo Fisher Scientific, MA, USA), purified and quantified with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), and prepared into cDNA. Real-time fluorescent quantitative PCR was performed by using SYBR GreenSupermix as the labeling group. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Statistical analysis

Statistical analysis was performed using PASW version 17.0 software (SPSS Inc., Chicago, IL, USA). The differences between two groups and those among multiple groups were compared by t test and one-way analysis of variance (ANOVA), respectively. A p value of <0.05 was considered statistically significant.

RESULTS

Cell counting

The pericardial patches containing collagen were acellular before angioplasty of the abdominal aorta.

Table 1. Cell counts at different time intervals

No. of visual fields	Cell count (high-power)			
	Blank patch	Postoperative first day	Postoperative third day	Postoperative fifth day
1	0	100	246	344
2	0	88	155	201
3	0	144	179	367
4	0	55	242	288

After implantation of bovine pericardial patches, the numbers of EFNB2+ and CD34+ cells increased significantly ($p<0.05$) in a time-dependent manner in the gaps between collagen fibers. Although the majority of the cells migrated toward the patch middle over time, only a few cells appeared around the subcutaneously implanted patches and remained thereafter (Table 1).

Cell changes in bovine pericardial patches

Cells were observed in bovine pericardial patches on the postoperative one, three, and five days; however, the test results for CD68, alpha (α)-actin and von Willebrand factor (vWF) were negative. Since immunofluorescent staining using anti-CD31 antibody on the three days did not produce positive results, endothelial cells did not exist. On the postoperative seventh day, CD68+, α-actin+ and vWF+ cells became detectable. In addition, the numbers of EFNB2+ and CD34+ cells, as suggested by immunofluorescent staining, increased time-dependently and moved toward the patch center with a large overlapping area. In contrast, no EFNB2+ or CD34+ cells were detected in the control group.

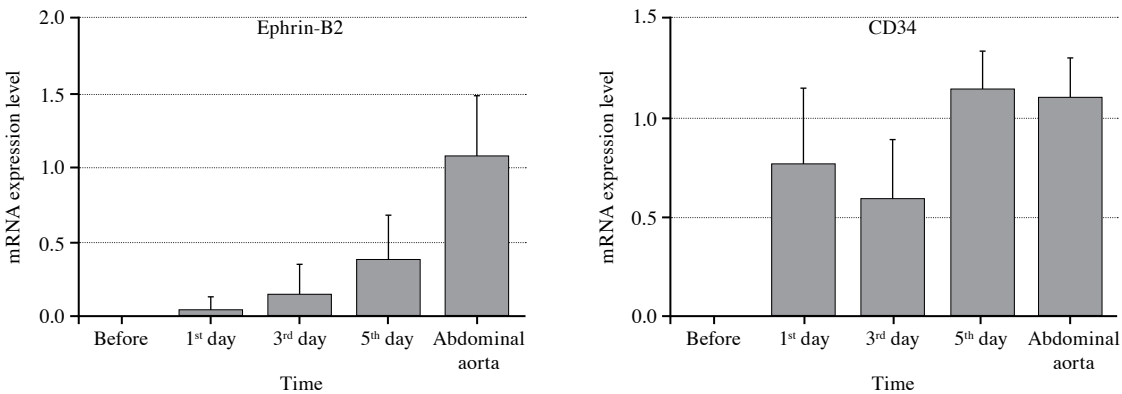

Figure 1. EFNB2 and CD34 mRNA expression levels in bovine pericardial patches. mRNA: Messenger ribonucleic acid.

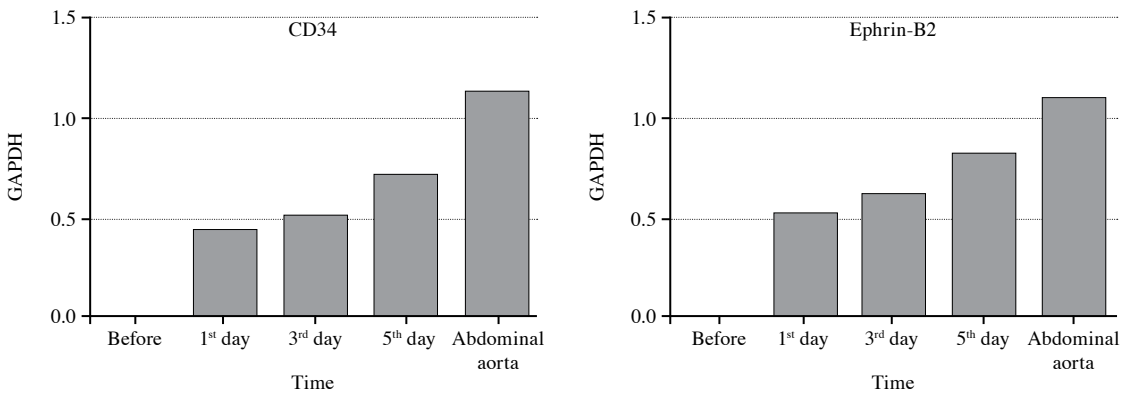

Figure 2. EFNB2 and CD34 protein expression levels in bovine pericardial patches. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

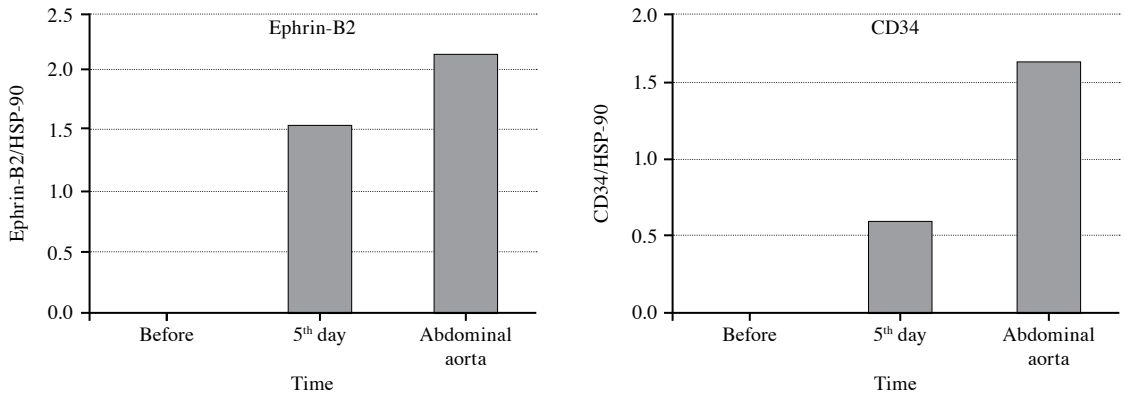

Figure 3. EFNB2 and CD34 protein expression levels in porcine pericardial patches. HSP-90: Heat shock protein 90.

To further verify the results, the patches were taken out, from which RNA was extracted for reverse transcription PCR that showed the levels of EFNB2 and CD34 increased significantly after angioplasty ($p<0.05$) (Figure 1). However, the two genes were not expressed in the control group. Similarly, Western blotting demonstrated EFNB2 and CD34 proteins in the patches (Figure 2), but not in the subcutaneously implanted ones.

Cell changes in porcine pericardial patches

On the postoperative fifth day, dual $EFNB2^+$ and $CD34^+$ cells also appeared in the porcine pericardial patches. In addition, there were EFNB2 and CD34 proteins in the patches (Figure 3).

Comparison between porcine and bovine pericardial patches

There was a thicker layer of neointima 30 days after implantation of porcine pericardial patch than that after bovine patch implantation (Figure 4). Immunofluorescence assays of both patches showed that $CD34^+$ cells increased over time and tended to accumulate in the center (Figure 5). Similarly, $EFNB2^+$ cells, which also increased with an elapsed time, were distributed in the same region as that of $CD34^+$ ones. Furthermore, EFNB2 and CD34 proteins were detected in both types of patches (Figures 6 and 7).

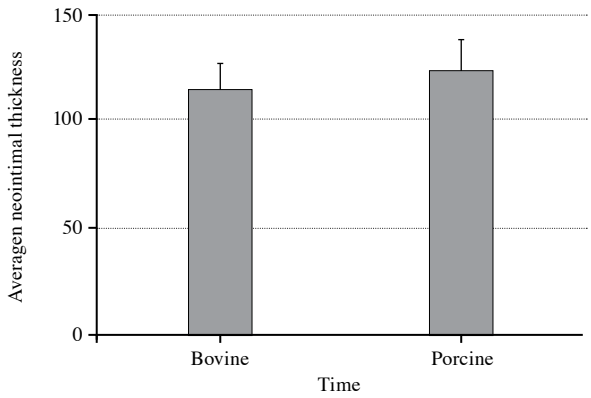

Figure 4. Neointimal thicknesses of porcine and bovine pericardial patches on the postoperative 30th day.

DISCUSSION

In vascular implantation, the shear stress of implants and the migration of cellular constituents during the blood flow should be considered.[12] In particular, endovascular endothelial cells with an intact function and morphology can prevent thrombosis and maintain normal function of the implants.[13] As EFNB2 does not increase along with arterial blood flow dynamics in intravenously implanted endothelial cells, time is needed for the implants to adapt to the specific arterial environment. In our study, five days after implantation of pericardial patches, positive cells began to infiltrate,[14] and EFNB2 and CD34, but not CD68, vWF or α-actin, were expressed. Thus, the patches were found to be adaptable to the arterial environment within a short time after surgery.[15] Also, markers in the arterial endothelial cells originated from initial cells instead of adjoining vascular endothelial cells. On the postoperative 30th day, both porcine and bovine patches underwent endothelialization in the vascular lumen.

Currently, EFNB2 and its receptor EPHB4, which are expressed in arteries and veins, respectively, are commonly used as the corresponding markers.[16] Edema factor protein and EPh receptor-interacting protein, as ligands and receptors,[17] predominantly regulate blood and lymphatic vascular remodeling and endothelial cells, and support cells and smooth muscle cells.[18] It has been previously reported that,

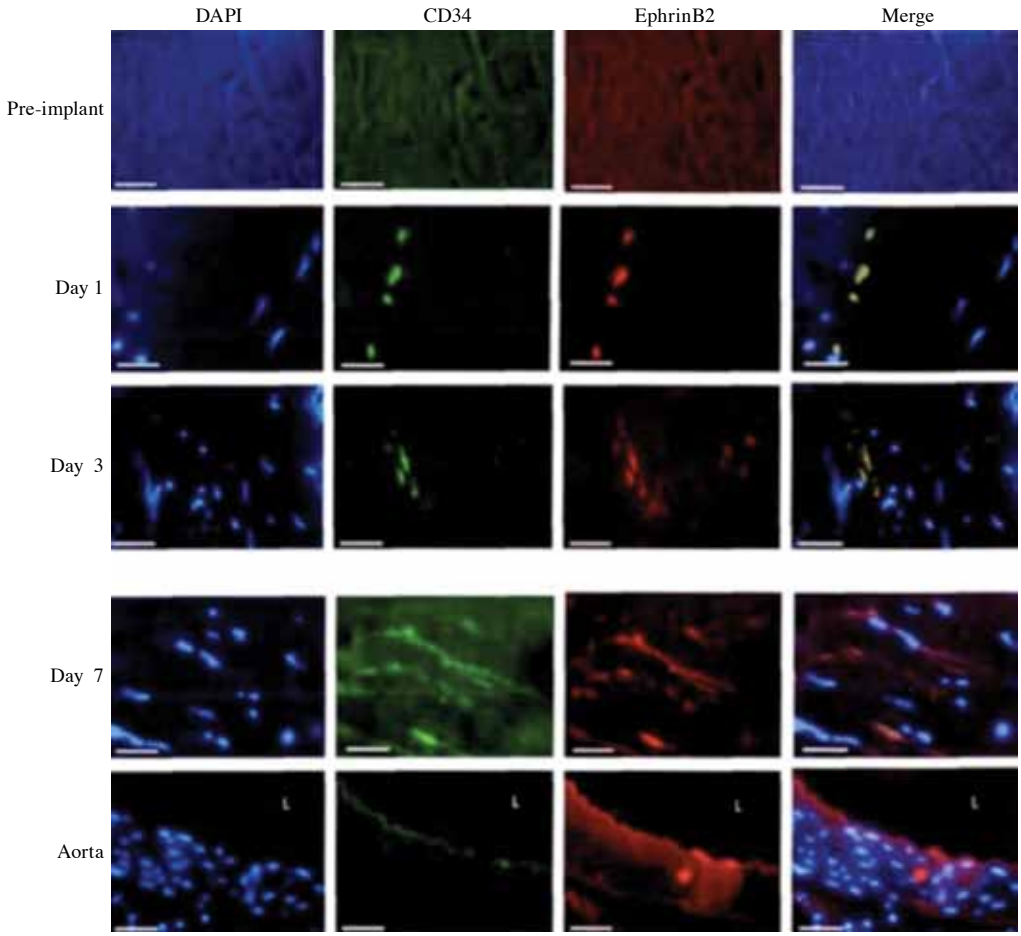

Figure 5. Immunofluorescence assay results.

unlike intravenous implants, pericardial patches can acquire an arterial marker EFNB2, thereby, being accommodated to the arterial environment through cellular infiltration.[19]

On the other hand, EPCs play a crucial role in vascular remodeling. Under ischemic conditions, EPCs can promote revascularization and repair vascular intima.[20] Therefore, they are potentially eligible for treating lower limb ischemic diseases.[21] In this study, we used an EPC-specific marker,[22] i.e. CD34, to find out whether there were EPCs in the patches.

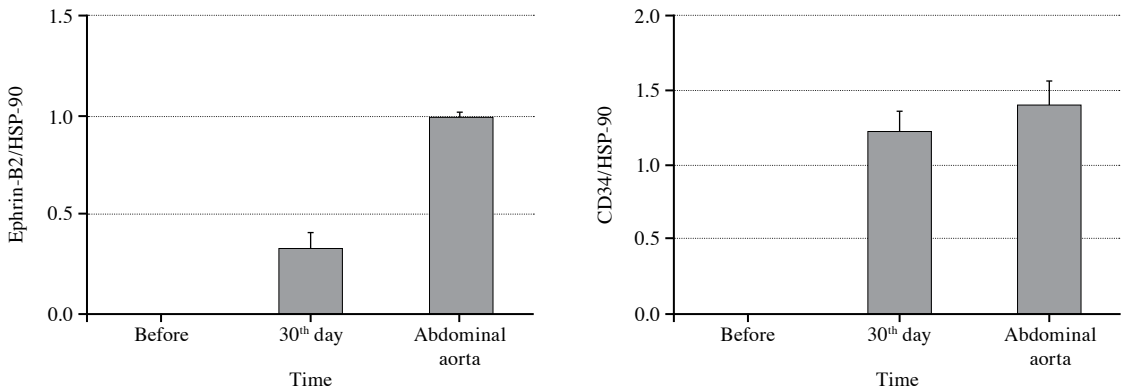

Figure 6. Protein expression levels in bovine pericardial patches detected by Western blotting. HSP-90: Heat shock protein 90.

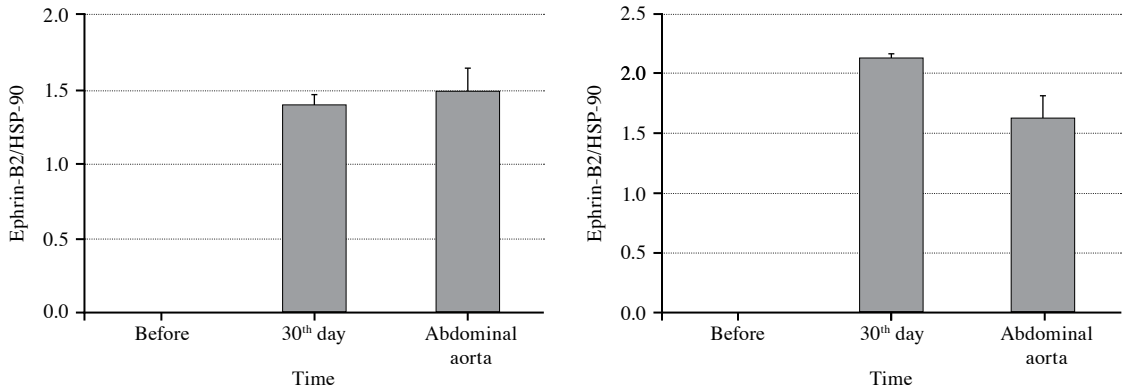

Figure 7. Protein expression levels in porcine pericardial patches detected by Western blotting. HSP-90: Heat shock protein 90.

On the other hand, neither EFNB2$^+$ or CD34$^+$ cells were detected in the subcutaneously implanted patches, probably as they failed to contact with blood flow in the arterial circulatory system.[23]

Although the formation of luminal endothelial cells in porcine patches preceded that in bovine ones, the latter had neointima within 30 days after surgery. In other words, neointimal endothelialization was both allowed, which reduced the risk of thrombosis, infection and pseudoaneurysm,[24] and augmented the patency rate after angioplasty.

In conclusion, implantation of pericardial patches after angioplasty promoted endothelialization, vascular remodeling, and adaptation to the arterial environment.[25] The patches had EFNB2$^+$ and CD34$^+$ cells which were prone to differentiation into arterial endothelial cells, which are potentially eligible materials for vascular tissue engineering.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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