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Surface heparinization and blood compatibility modification of small intestinal submucosa (SIS) for small-caliber vascular regeneration

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Abstract.
OBJECTIVES: This study aims to investigate the small intestinal submucosal (SIS) surface after heparinization with the hypothermia plasma technique, to improve the blood compatibility of SIS, and to explore the possibility of construction of small-caliber vascular grafts with modified SIS scaffolds in vivo.
METHODS: SIS films prepared from jejunums of pigs were processed for surface treatment at different time periods with the argon plasma initiation technique under vacuum, and were then immediately immersed in 4% (m/v) heparin sodium solution for 24-h heparinization. The surface morphologies of heparinized SIS were observed under a scanning electron microscope (SEM). The antithrombogenicity of the modified SIS films was tested by measuring the water contact angle, blood coagulation time, activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), and observation of platelet adherence by SEM. Heparinized SIS films were sewn into a small caliber (3-mm) tube and implanted into the defect of a canine femur by anastomosis as a vascular graft. The efficiency of the SIS graft was evaluated according to the patency for the circulation of blood with Doppler color ultrasonography and hematoxylin-eosin staining.
RESULTS: Heparinized SIS showed a significantly different surface morphology compared with that of untreated SIS. The SIS surface resembles wrinkled film, but the heparinized SIS surface is uniformly coated with microdots, and appears to have a layer of heparin adhesion.
CONCLUSION: Heparin was attached to the SIS surface after hypothermia plasma treatment. Hydrophilicity and antithrombogenicity of heparinized SIS were clearly increased. The heparinized SIS vascular graft showed great potential for replacement of defective small-caliber vessels.

Keywords: Thrombogenesis, SIS, argon plasma, heparin, small-caliber vessels

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1. Introduction

Thrombogenesis is the most common cause of failure in the implantation of tissue-engineered small-caliber vessels [1]. Immobilizing heparin onto the surfaces of vascular scaffolds is often used to improve their blood compatibility [2]. The mechanism of immobilization can be categorized into physical adsorption and chemical integration. The binding force using physical methods is usually too weak to slow the release and prolong the action [3]. With chemical treatments, covalent and ionic linkages are involved, and the former is much more stable than any other binding form of heparinization [4]. However, the bioactivity of heparin will be reduced if its conformation is affected when immobilized at multiple points onto the surfaces of materials [5]. It is difficult to identify materials with both satisfactory mechanical properties and excellent antithrombogenic properties by using chemical treatments. As a novel technology, plasma surface modification is characterized by its high effectiveness and efficiency, non-pollution, easy operation, and ability to improve the properties of material surfaces [6]. Many studies indicate that this method can attach more heparin to the surfaces of polymers than others [7–9]. In the present study, we investigated the method of plasma surface modification and heparinization with covalent linkage on the surfaces of SIS (small intestinal submucosa), and the antithrombogenic properties of heparinized SIS films.

2. Materials and methods

2.1. Preparation of SIS films

The jejunums were collected from farm pigs, frozen for transport, and cleaned within 2 hours. The mucosa and tunica muscularis of the jejunums were scraped off with a knife handle wrapped with bandages, while being continuously rinsed with 40°C water. Then, the SIS were harvested and subsequently processed as described by Abraham et al. [10]. At room temperature, and with the volume ratios of material to solution maintained at about 1:100, the following sequence of procedures was performed. Immerse the SIS in a solution of 10-mmol/L sodium hydrate and 100-mmol/L edathamil disodium at pH 11–12 for 16 h, flush with deionized water, place in a solution containing 1-mmol/L hydrochloric acid and 1-mmol/L sodium chloride at pH 0–1 for 6–8 h, flush with deionized water and soak in phosphate buffer solution with 1-mmol/L sodium chloride for 16 h, flush with deionized water again and immerse in phosphate buffer solution at pH 7–7.4 for 2 h, then after washing with deionized water for 2 h, immerse the SIS in 20% alcohol solution with 0.1% peracetic acid for 8 h and rinse with phosphate buffer solution containing 0.05% sodium azide, reduce the temperature to -80°C gradually to freeze-dry the SIS, and finally sterilize the SIS with 35-kGy γ-rays. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University.

2.2. Hypothermia plasma treatment

Place the SIS films in a plasma processor, and treat under the following conditions. Discharge power = 90 W, Argon gas flow = 20 ml/min, evacuation of background to 0.5 Pa, and treatment times of 0, 2, 4, 6, 8, 10, 12, and 14 s, respectively, in 8 groups. Immerse the films into heparin sodium solution in vacuum immediately after the plasma treatment and keep for 24 h at 37°C. Then, vacuum-dry the films for use.

2.3. Surface characterization and test

The surfaces of treated SIS films were studied with a GSM-5800 scanning electron microscope (SEM) (Jeol Corp. Japan), after drying and coating with platinum in a vacuum. The contact angles were determined by using the sessile drop technique [11] with a contact goniometer with a metering error less than 0.5°.

2.4. In vitro antithrombogenicity assessment

Platelet adhesion test: Anticoagulated whole blood was centrifuged at 1,000 rpm for 10 min to obtain platelet-rich plasma (PRP). This PRP (100 μl) was placed on the SIS films (3×3 cm^2) after preswelling with distilled water (2 ml) and kept at 37°C for 30 and 60 min. Then, the films were flushed with normal saline, stabilized in 2.5% glutaraldehyde fixing solution, dehydrated with alcohol, dealcoholized with acetoacetate, and dried at a critical point of CO_2 in sequence. The morphology of adhered platelets on the films was observed and photographed under SEM.

Determination of prothrombin time (PT), activated partial thromboplastin time (APTT), and thrombin time (TT) [12]. Blood was harvested from healthy donors, and sodium citrate (1:9) was added to prepare blood plasma. Place the films into sample tubes and seal them. Then incubate in a water bath at 37°C for 30 min before the tests. The PT, APTT, and TT were determined using an automated blood coagulation analyzer, and more than 5 samples were tested for each group, and repeated 3 times for each sample to obtain a mean value.

2.5. In vivo antithrombogenicity assessment

Twenty purebred adult dogs were divided equally into 2 groups. During the experiments, dogs underwent general intravenous anesthesia with 2.5% pentothal sodium before the femoral arteries of both sides were dissected and cut off. The treated and untreated SIS films were made into vasiform scaffolds by using interrupted and everted sutures with 6–0 polyglycolic acid noninvasive suture lines, and were anastomosed to the ends of the femoral arteries. The vascular clamps were released to flood the scaffolds with blood. Immediate pulsation and blood flow were observed for 3 h before the incision was closed by layers. The dogs were administered 10 million units benzylpenicillin potassium intramuscularly and 3,000 units heparin sodium subcutaneously for 3 days postoperatively.

2.6. Color Doppler ultrasound

The patency, inner diameters, wall thickness, and blood flow status of the scaffolds were assessed at 1, 3, and 6 weeks, respectively, after the operation.

2.7. Histological evaluation

The dogs were sacrificed 6 weeks after the operation, and the SIS conduits were dissected and harvested. Any thromboses were identified on the inner walls. Further observation was made after hematoxylin–eosin (H–E) staining.

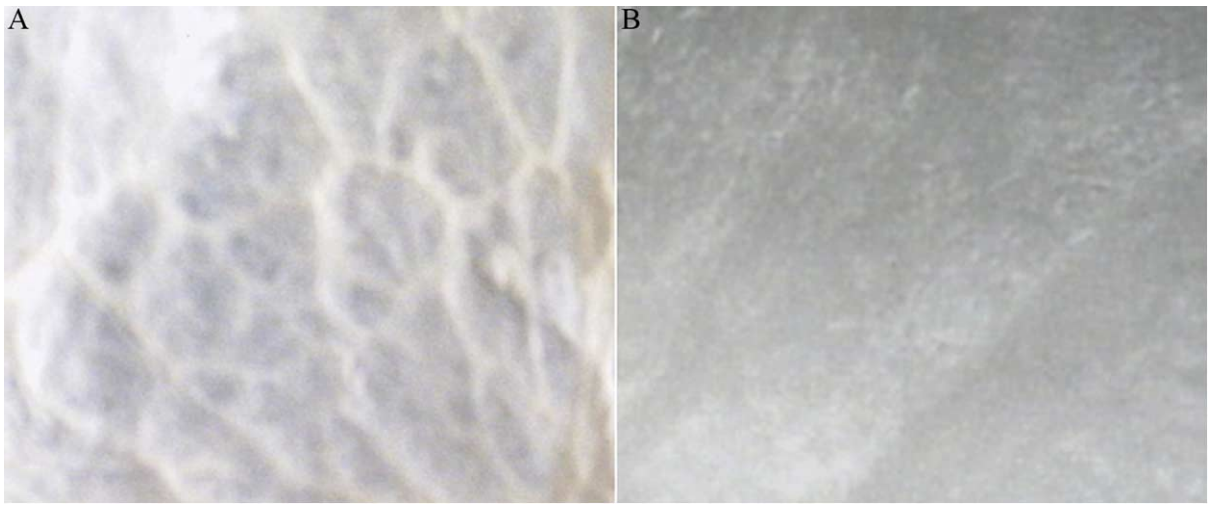

Fig. 1. Macrography of SIS films. (A) BEFORE modification; (B) SURFACE-heparinized with hypothermia plasma initiation technique, showing the niveous combined heparin.

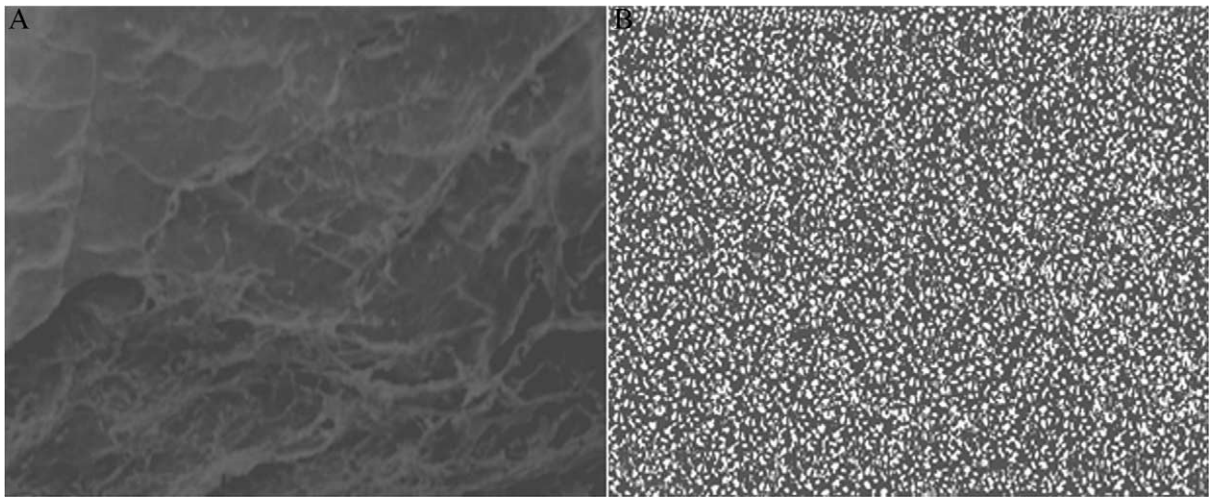

Fig. 2. Scanning electron microscopy photographs of SIS. (A) Untreated group with irregular and gross surface; (B) Modified group with regularly grafted heparin (SEM × 800).

3. Results

3.1. Surface characterization of heparinized SIS films

The surfaces of SIS films had a niveous appearance on macrography (Fig. 1(A), (B)), and showed regular island-like and groove-like changes in SEM photographs (Fig. 2(A), (B)) after heparin was immobilized with hypothermia plasma treatment. The water contact angles were clearly decreased because of the modification, and were 70.6°, 60.0°, and 72.7° when treated for 8, 10, and 12 s, respectively, and reached minimal values when treated for 10 s; the water contact angles of untreated films were 105.3°. Figure 3 shows water contact angle detection on treated and untreated SIS films.

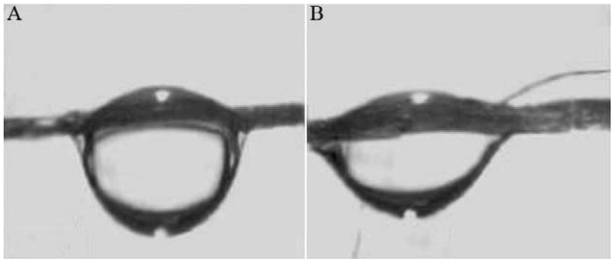

Fig. 3. Water contact angles of SIS films before and after bionic modification, obviously decreased by the treatment.

Table 1
Determination of coagulation time and contact angles of SIS films

No. of Sample	Treating time/s	APTT/s	PT/s	TT/s	Contact angle/d
1	0	30.5	9.8	13.6	105.3
2	2	31.4	10.2	13.8	104.7
3	4	33.7	12.4	17.1	98.2
4	6	56.3	15.6	26.7	81.1
5	8	68.9	29.8	38.6	70.6
6	10	81.5	39.6	50.2	62.0
7	12	70.4	31.1	41.1	72.7
8	14	67.5	30.0	39.9	85.9

3.2. In vitro antithrombogenicity properties

The coagulation time of plasma on untreated and variously treated SIS films is shown in Table 1; when treated for 10 s, the SIS had optimal antithrombogenicity, and the APTT, PT, and TT were most obviously prolonged.

The SEM photographs of platelet adhesion tests are shown in Fig. 4, with a large number of adhered platelets gathering and stretching out pseudopodia in the untreated group, with far fewer activated or deformed platelets in the treated group.

3.3. In vivo antithrombogenicity properties

Animal studies of small-caliber SIS scaffold implantation (Fig. 5) showed that 4 scaffolds were completely embolized within 3 h postoperatively, and obliterated lumens full of thrombi could be seen; the other 6 were also embolized 2 days after the operation, as detected on color Doppler ultrasound (Fig. 6). The 10 scaffolds made of heparinized SIS showed favorable patency in all assessments, including the 3 h observation after blood flow was restarted, and on postoperative color Doppler evaluation at 1, 3, and 6 weeks (Fig. 7); The conduits harvested 6 weeks after the operation showed complete coverage with endothelial cells without any thrombi in the lumens (Fig. 8).

4. Discussion

The carboxyl and other functional groups in heparin molecules can develop esterification with hydroxyl groups under certain experimental conditions with the reaction mechanism as follows. The energy

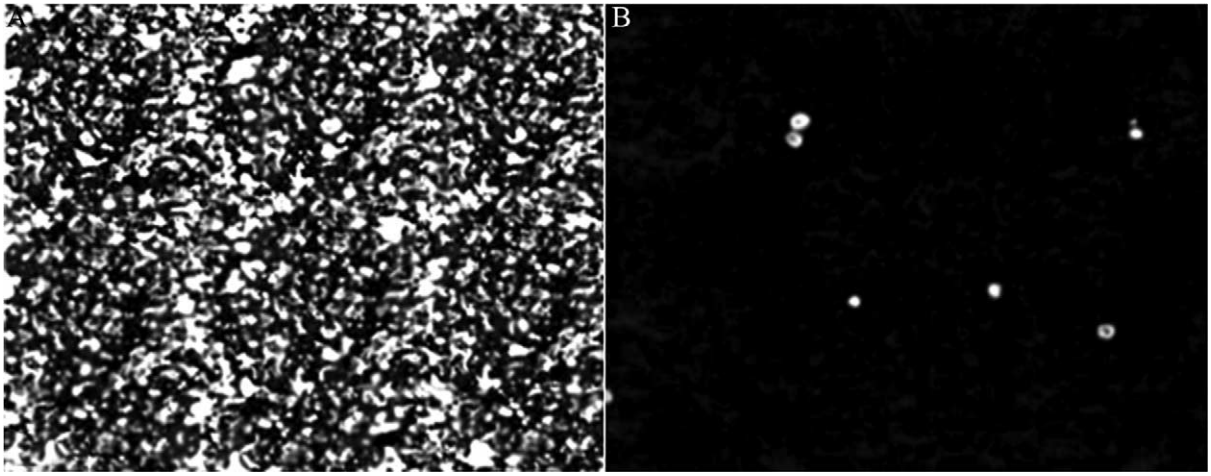

Fig. 4. Platelet adhesion tests of SIS films. (A) Untreated group with dense adhered platelets, which were partly activated to deform and stretch out pseudopodia; (B) Heparinized group indicating a few adhered platelets without any activated or deformed ones (SEM × 1000).

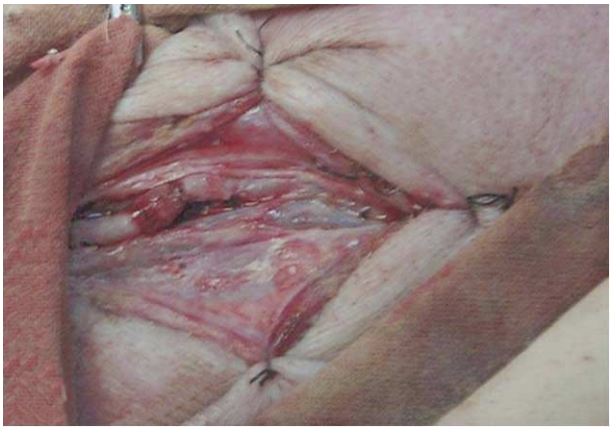

Fig. 5. The small caliber vascular scaffolds made of SIS films were grafted into the canine femoral by anastomosis.

level, 5–9 eV provided by a hypothermia plasma generator, is not high enough to ionize the atoms of giant molecules, but can change them into free radicals by breaking the chemical bonds. When in extensive contact with these radicals, heparin molecules can complete grafting copolymerization [13]. SIS mainly consist of type I and III collagen and a smaller amount of type V and VI [14], with abundant hydroxyl and carboxyl groups, which can produce large quantities of free radicals when stimulated by hypothermia plasma. Therefore, effective covalent bonds can form on the surfaces when the treated SIS films are immersed in a solution of high-density heparin. Tan et al. [15] found that ammonia plasma treated poly (vinyl chloride) can be modified with heparin to effectively reduce platelet adhesion. According to the findings of the present study, the surfaces of plasma-treated SIS films were clearly changed, and the contact angles decreased markedly, indicating increased polarity and hydrophilicity. Because of the introduction of hydrophilic groups onto the surfaces of SIS films, their free energy was increased, which

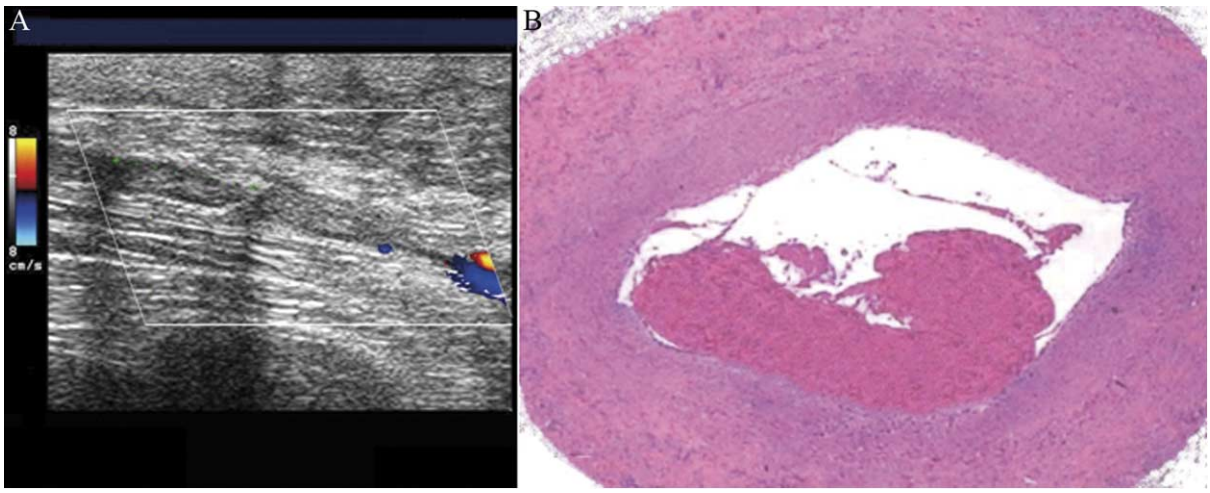

Fig. 6. Patency detection of the small-caliber SIS vascular scaffold without modification. (A) Doppler ultrasound detection 3 days after anastomosis showing the completely embolized lumina full of thrombi; (B) Histological observation of the embolized lumina, with a large thrombus in it (HE × 10).

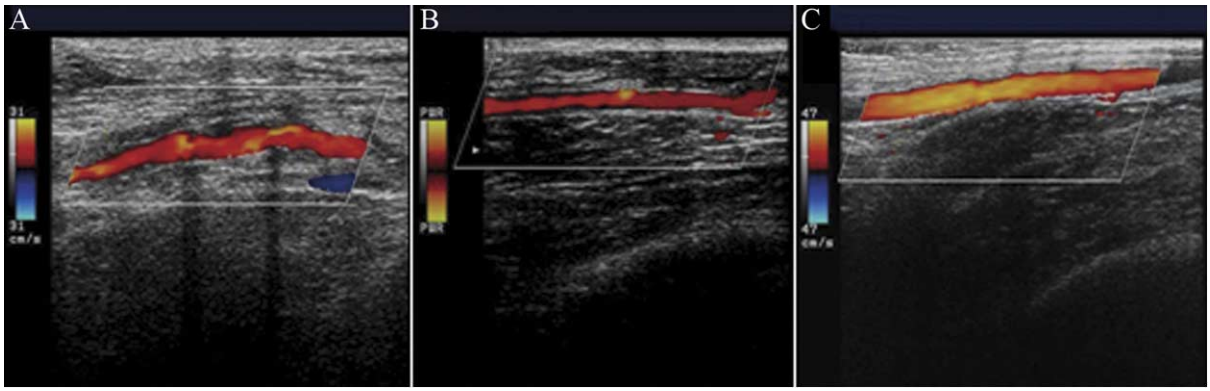

Fig. 7. (A)–(C). Patency detection of the small-caliber SIS vascular scaffold with modification: Doppler ultrasound detection 1, 3, 6 weeks after anastomosis showing the vascular prostheses kept well patency without thrombus.

was of benefit in reducing the reaction between the SIS and blood constituents, thus effectively reducing coagulation caused by platelets and coagulation factors.

For most medical highly-polymerized materials, coagulation may occur to different degrees on their surfaces when blood contacts the surface due to the adhesion and activation of plasma proteins, which can in turn induce the adhesion and activation of platelets and the activation of various coagulation factors [16]. The collagen proteins of SIS films endow them with favorable biocompatibility as well as the extreme ability to promote thrombogenesis. As a modification method for material surfaces, the plasma technique can endow the materials with new physical and chemical properties, including good blood compatibility on the surface, without any influence on their bulk properties [17,18]. Our study revealed that the coagulation time of the plasma-treated SIS was clearly prolonged, compared with that of untreated SIS, and the coagulation time reached its maximum when treated for 10 s; after that, the antithrombogenicity would decline if treated for a longer period. The main reason for this phenomenon

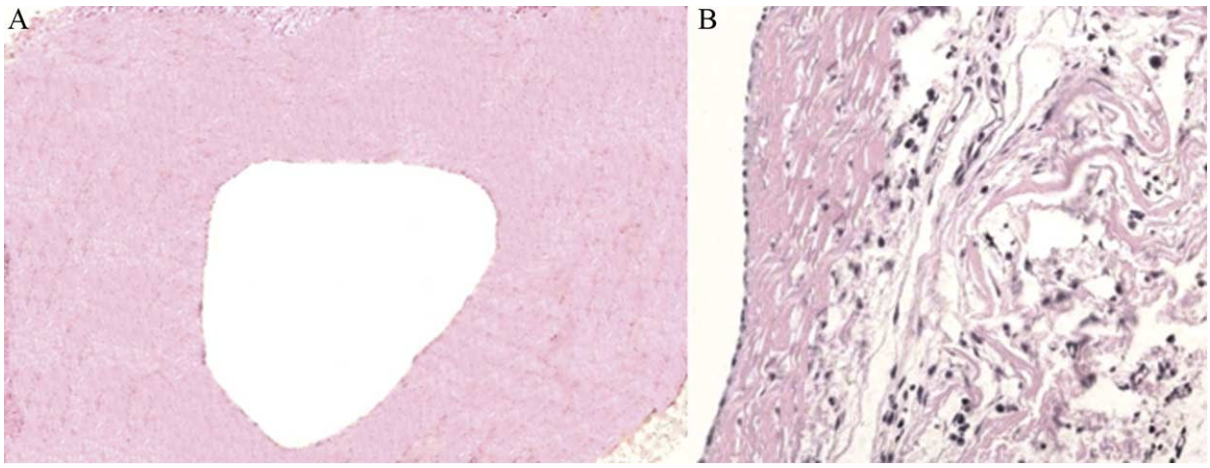

Fig. 8. Histological observation of the small-caliber vascular scaffold made of nano-bionicly modified SIS 6 weeks after anastomosis. (A) HE × 10 showing the grafts having turned into tubular tissue; (B) HE × 40 showing the smooth lumina covered by whole endothelial cells without thrombus.

may be that with increasing processing power, the density of plasma will increase to produce more free radicals. However, the probability of collision quenching between the high-energy particles will increase at the same time [19]. Therefore, at a certain processing power, a relative balance can be reached between the production and collision quenching of the free radicals, and optimal antithrombogenicity can be obtained.

SIS is a commonly used scaffold material for tissue engineering. Baltoyannis et al. [20] prepared moderate-caliber conduits with canine SIS, implanted them into donor dogs, and found that patency persisted one year later. However, when small-caliber tissue-engineered vessels were prepared, thrombi were prone to occur because of the great resistance and low velocity of blood flow [21]. The SIS tissues commonly used at present are from highly heterogeneous sources. Zheng et al. [22] reported that prepared swine SIS films still contained some cellular DNA, and could induce inflammatory reactions with infiltration of lymphocytes when implanted subcutaneously into rats. Therefore, the SIS should be modified with an effective technique to improve blood compatibility when adopted as vascular scaffolds. Sipehia et al. [23] indicated that expanded polytetrafluoroethylene grafts for small diameter blood vessel replacement could acquire good blood compatibility and remain patent for a long time when implanted to bridge the aortas of rabbits after ammonia plasma modification; moreover, the inner lumens were covered completely with endothelial cells 1 month later, while those of untreated controls were embolized in 1 week. In our study, the plasma-modified SIS scaffolds were put into blood circulation to contact platelets and coagulation factors directly under the stress condition of blood flow, with the results indicating that the small-caliber SIS conduits could maintain durable patency and would be covered completely by endothelial cells.

5. Conclusion

Heparin could be immobilized onto SIS films by using the hypothermia plasma initiation technique. The surface characterization and correlation with in vitro and in vivo studies indicated that the modified surfaces provided good and persistent antithrombogenicity, with strong blood compatibility. Ac-

cordingly, the hypothermia plasma technique is an effective method for surface modification, and the heparinized SIS are suitable for small-caliber scaffolds in vascular tissue-engineering suiran. Although the modified SIS exhibits good anticoagulant properties similar to heparin, the next step will require the qualitative analysis of heparin for the SIS surface-immobilized material.

Authors' contributions

Design of the study: FX, BSH, and CYF; data acquisition: BSH, XMM; data analysis: all authors; drafting of the manuscript: FX, BSH, and CYF. All authors read and approved the final manuscript.

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Conflict of interest

All authors have no conflict of interest regarding this paper.

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