



Constructing a heparin-modified penile decellularized scaffold to improve re-endothelialization in organizational reconstruction

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Background: Due to the unique anatomy and complex function of the penis, the reconstruction of penile defect is fraught with great challenges. The current standard methods are limited by numerous complications and insufficient donor sites. While functional vascularized penile tissue engineering offers a novel way to address this problem, revascularization remains the primary concern.

Methods: In this study, a penile scaffold with associated modifications was constructed. The performance of decellularized penile scaffolds (DPSs) was improved by conjugation with heparin (HEP) and reseeding with human umbilical vein endothelial cells (HUVECs). There were three groups according to the modifications, including native DPSs, HEP-DPSs, HEP-HUVECs-DPSs. After perfusing with 1% Triton X-100/0.1% ammonium hydroxide solution, the cellular components were removed. Subsequently, the covalent binding of heparin in the DPSs was performed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide, followed by reseeding with HUVECs. Scaffolds were implanted into the backs of rats and the implanted tissues were harvested at 1, 2, 3, and 4 weeks. Then hematoxylin and eosin (H&E) staining and immunofluorescence assays were performed to assess the degree of angiogenesis.

Results: The native DPSs retained the extracellular matrix and heparinized modification. The H&E results indicated that more HUVECs covered the inner surface of tubular structures in the HEP-DPSs group compared to the native DPSs group. The number of vessels in the HEP-HUVECs-DPSs was significantly increased compared to the control scaffolds at all time points.

Conclusions: These results suggested that, compared to the native DPS, heparin-conjugated scaffolds provided a superior environment for the growth of HUVECs and the modified methods provided a perspective for overcoming the obstacles in tissue engineering of transplantable penile tissues and the establishment of a functional vasculature.

Keywords: Heparin; penile scaffold; tissue engineering; angiogenesis; decellularization

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Introduction

The male genitalia plays an important role in the male psyche (1) and the psychological impact of penile absence is often detrimental. Neoplastic disease, genitourinary trauma, and other diseases can lead to severe penile defects that require reconstruction. This is a major challenge due to the anatomical and functional complexities of the penis (2,3), as well as the limitations imposed by the availability of compatible grafts.

At present, the main forms of penile reconstruction include pedicled skin flaps and free microsurgical flaps, both of which have limited ability to reproduce the complex structure of the penis and are often associated with significant donor site complications (4-6). Although traditional plastic surgery can produce satisfactory outcomes in many cases, it is still laden with significant donor site morbidity, prosthesis extrusion, and catastrophic infections (7,8). In addition, increasingly, more patients with traumatic penis loss and concomitant limb injury, especially wartime injuries, are not suitable for autologous reconstruction due to a lack of sufficient donor sites (9). Organ allogeneic transplantation remains the treatment of choice for the optimum recovery of multi-functional organs (9). However, patients must take life-long immunosuppressive drugs, and the main risks include infections and malignancies. Therefore, there is an urgent need to develop alternative methods to manage penile loss.

In recent years, there have been rapid advancements in the field of tissue engineering (TE) for the replacement of defective tissues. The increasing importance of TE has been observed in different disciplines of regenerative medicine, including urology (10). TE biomaterials can be classified into synthetic polymers, biopolymers, and scaffolds. Synthetic polymers, including poly-L-lactic acid, polyglycolic acid (PGA), and poly-L-lactic-co-glycolic acid, can be manufactured with tailored architectures. Engineered autologous cartilage rods have been used as penile prostheses in rabbit models, but these prostheses did not restore physical function (11). Compared with these fore-mentioned materials, scaffolds have better biocompatibility and lower immunogenicity (12). How to achieve re-endothelialization in a reasonable time to reduce vascular thrombosis and inflammatory stress in scaffolds is a common problem faced by various decellularized scaffolds in practical applications. Especially considering the features of the penis, the vascularization and neuralization of penile scaffold is still a major obstacle. Another problem

is that of thrombosis of the graft *in vivo* (13). Different measures have been implemented for the modification of the scaffold, including heparin (HEP) (14,15) and arginine-glycine-aspartic acid (RGD) peptides (16). Recently, heparin has received increasing attention in modifying decellularized scaffolds due to its pharmacological properties such as anticoagulation and anti-inflammation. Decellularized porcine small intestinal submucosa (SIS) scaffolds with immobilized heparin have the potential to greatly improve anticoagulant activity (17). The use of antithrombotic coating reagents, such as heparin-modified acellular liver scaffold combined with vascular endothelial growth factor (VEGF), to induce angiogenesis has been reported (18). In addition, endothelial cells are considered to have physiological effects in angiogenesis (19) and have been used in many aspects of tissue engineering, including in blood vessels (20,21).

A number of growth factors contribute to a microenvironment that is conducive to tissue repair and regeneration. Furthermore, these growth factors can regulate cellular behavior, including migration, proliferation, and differentiation (22). Among them, the basic fibroblast growth factors (bFGFs) have been shown to be the main regulators of organogenesis and tissue homeostasis (23). The bFGFs are considered to be one of the most potent angiogenesis inducers by stimulating the migration, proliferation, and differentiation of endothelial cells in a dose- and time-dependent manner (24-26). Moreover, bFGFs have also been described as important neurotrophic factors with excellent ability to promote the migration, proliferation, and self-renewal of neural stem cells (27). According to a report (28), growth factors were retained after decellularization. Therefore, this current study investigated the effects on growth factors after reseeding human umbilical vein endothelial cells (HUVECs).

In this paper, the covalent binding of heparin in the decellularized penile scaffolds (DPSs) was performed using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS). Furthermore, the possible mechanisms by which HEP-HUVECs-DPSs promote angiogenesis were explored. The content of VEGF and bFGF in HEP-HUVECs-DPSs was higher compared to native DPSs. Angiogenesis was evaluated *in vivo* by implanting the scaffolds in the back pocket of rats. The HEP-HUVECs-DPSs showed significant cell adhesion ability and angiogenesis. The modified methods provided a perspective for surmounting the obstacles in TE related

to transplantable penile tissue and the establishment of a functional vasculature. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://tau.amegroups.com/article/view/10.21037/tau-22-315/rc>).

Methods

Animals

Sprague Dawley (SD) rats, aged 6–7 weeks and weighing 300–400 g, were obtained from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). All animals were housed in a 12-h light/dark cycle and offered a standard diet. All animal treatments complied with institutional guidelines for the care and use of animals and were approved by the Ethics Committee of the Shanghai Tenth People's Hospital, School of Medicine, Tongji University (No. SHDSYY-2021-3647). A protocol was prepared before the study without registration. HUVECs were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Harvest and decellularization of the penis

After euthanasia, the anticoagulant heparin (100 IU/kg body weight) was injected with into the inferior vena cava to avoid thrombosis. The whole process remained sterile. For better biocompatibility and lower immunogenicity of the scaffold, we performed decellularization to remove the cellular components. A porous scaffold was created by soaking the penis in phosphate buffered saline (PBS) for 1 hour, followed by 1% (w/v) Triton X-100 (Solarbio, Beijing, China)/0.1% ammonium hydroxide (Solarbio) in distilled water for 24 hours until the penile tissue became translucent. The penile tissue was then rinsed with distilled water for 2 hours and reperused with PBS for 4 hours to remove the remaining cell fragments and maintain isotonicity. The cellular penile scaffold with intact extracellular matrix was preserved in PBS at 4 °C.

Analysis of the decellularized penile scaffolds

Specimens were fixed in formaldehyde, embedded in paraffin, and sectioned prior to staining with DAPI (4',6-diamidino-2-phenylindole) and hematoxylin and eosin (H&E). To verify the integrity of collagen I, collagen IV, and fibronectin, the sections were stained with primary anti-

collagen I (1:100; Abcam, Cambridge, UK), anti-collagen IV (1:100; Abcam), and anti-fibronectin (1:100; Abcam), followed by incubation with a secondary antibody (Solarbio). Finally, the sections were observed using an Olympus microscope (Tokyo, Japan).

Heparin immobilization

Binding the heparin to the DPSs was performed as previously described (29). Briefly, the carboxylic acid groups of heparin were activated for 30 minutes using 1 mg EDC and 0.6 mg NHS (EDC/NHS) in 500 mL 0.05M morpholine ethanesulfonic acid buffer (pH 5.6). The DPSs were then rinsed with PBS for 15 minutes, followed by irradiation with ultraviolet light for 2 hours to achieve sterilization. Finally, the DPSs were placed in Dulbecco's Modified Eagle Medium (DMEM) and observed under the microscope after 48 hours to ensure sterility. The tissue sample was placed in 4% paraformaldehyde at 4 °C for 24 hours and subsequently embedded in paraffin. The scaffolds were cut into 5 µm thick slices. Following deparaffinization, the slices were placed in toluidine blue (TB) liquid to dissociate for 30 minutes, followed by rinsing in deionized water. After sealing, images were visualized via microscopy.

Cell culture of HUVECs

HUVECs are considered to have physiological effects in angiogenesis and have been used in many aspects of tissue engineering. Thus, we wanted to explore the role of HUVECs to promote angiogenesis and the changes in growth factors in penile scaffold. The HUVECs suspension was cocultured with the DPSs in 48-well culture plates. The cell suspension was replaced every 2 days to ensure that the cells had sufficient nutrients. The stability of the HUVECs was assessed by EdU staining (Beyotime Biotechnology) according to the manufacturer's instructions. In addition, the viable cells in each scaffold were examined via H&E staining after two weeks.

The levels of VEGF and basic fibroblast growth factor (bFGF) from the scaffolds

To determine the effects of the HUVECs on the scaffolds after implantation, the content of VEGF and bFGF in the HEP-HUVECs-DPSs and the native DPSs was evaluated. The growth factors were isolated from the scaffolds as

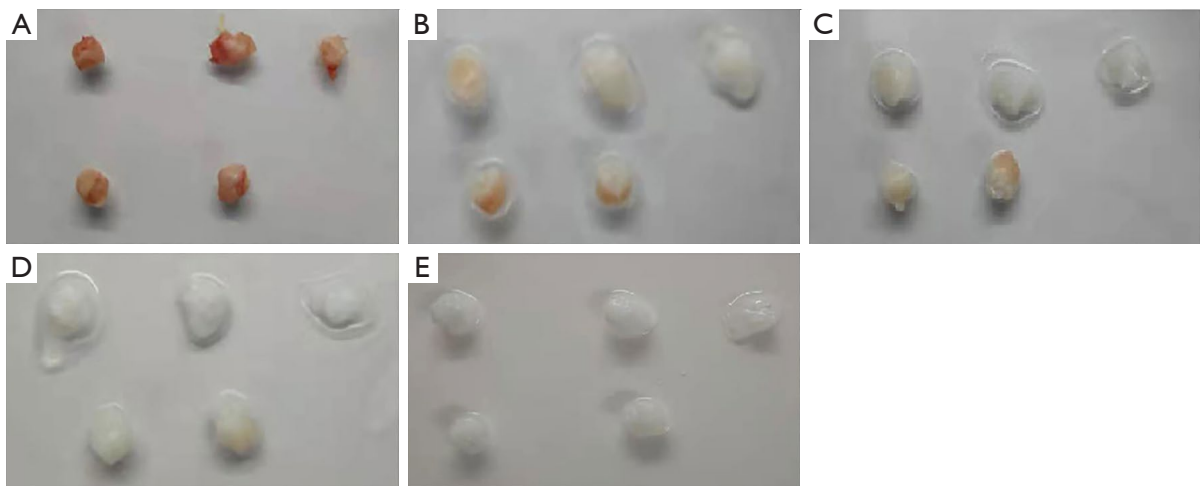


Figure 1 Penile decellularization at (A) 0 hours; (B) 6 hours; (C) 12 hours; (D) 16 hours; and (E) 24 hours after perfusion with 1% Triton X-100/0.1% ammonium hydroxide.

previously described (30). Samples were analyzed using an ELISA kit (Quanzhou Ruixin Biological Technology Co.LTD, Quanzhou, China) according to manufacturer's instructions.

Assessment of angiogenesis in the penile scaffolds in vivo

The DPSs were implanted into the dorsal subcutaneous fascia of SD rats (n=3), and the implanted tissues were harvested at 1, 2, 3, and 4 weeks. The tissue samples were placed in 4% paraformaldehyde at 4 °C for 24 hours, before embedding in paraffin. The scaffolds were cut into 5 μm thick slices and stained with a primary anti-CD31 antibody (Santa), followed by incubation with secondary Alexa Fluor 488-conjugated goat anti-mouse/anti-rabbit antibodies (Solarbio). The sections were then counterstained with DAPI (Sigma) for 10 minutes.

Statistical analysis

The Student's *t*-test was performed for all comparisons in this study. Differences were considered significant if the *P* value was less than 0.05.

Results

Characteristics of the scaffolds

After perfusion with 1% Triton X-100/0.1% ammonium hydroxide solution, the DPSs gradually became transparent

(Figure 1). To verify the decellularization effect, H&E (Figure 2A), DAPI (Figure 2B), and scanning electron microscopy (SEM) (Figure 2C) staining were performed. H&E and DAPI staining clearly showed no cell residue on the decellularized scaffold (Figure 2D,2E), and the collagen matrix was continuous without any breakage. The SEM showed that the extracellular matrix (ECM) was intact, and no residual nuclei were observed (Figure 2F). Importantly, immunofluorescence staining (Figure 3) revealed that collagen IV (Figure 3A,3D), collagen I (Figure 3B,3E), and fibronectin (Figure 3C,3F) were all well preserved after decellularization. In addition, TB staining demonstrated that the cross-linking of the heparinized scaffold was successful (Figure 4). After heparinization, the heparinized scaffold exhibited TB-positive staining throughout the entire cellular matrix, while no TB staining was observed in the unmodified DPSs (Figure 4A,4C).

Recellularization of the DPSs

Following 2 weeks of culture, the scaffolds were harvested and analyzed. H&E staining showed that the number of HUVECs in the heparinized DPSs (Figure 4D) was significantly higher than that in the native DPSs (Figure 4B). There was a significant difference in the VEGF and bFGF content between the HEP-HUVECs-DPSs and the native DPSs (Figure 4E). These results suggested that the reseeded HUVECs may promote angiogenesis and cell differentiation by secreting cytokines. The HUVECs were co-cultured with native

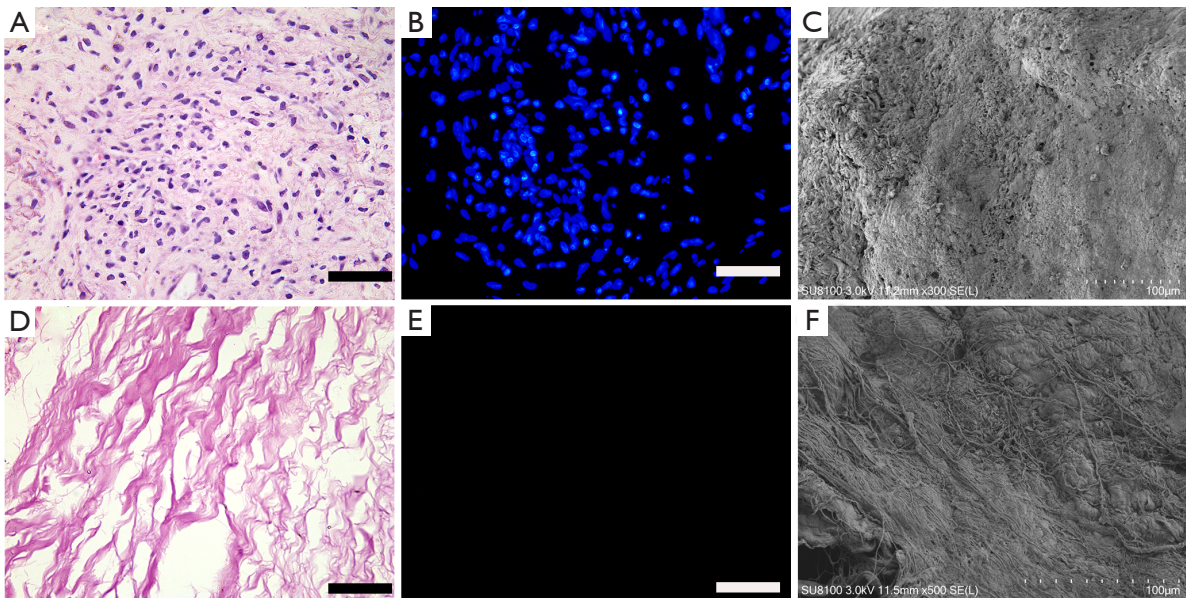


Figure 2 Analysis of the decellularization procedure. (A,D) H&E staining clearly shows the absence of any cell residue on the decellularized scaffold. (B,E) DAPI staining shows the absence of any cells after decellularization. (C,F) SEM shows that no residual cells were observed. Scale bar =100 µm. H&E, hematoxylin and eosin; DAPI, 4',6-diamidino-2-phenylindole; SEM, scanning electron microscopy.

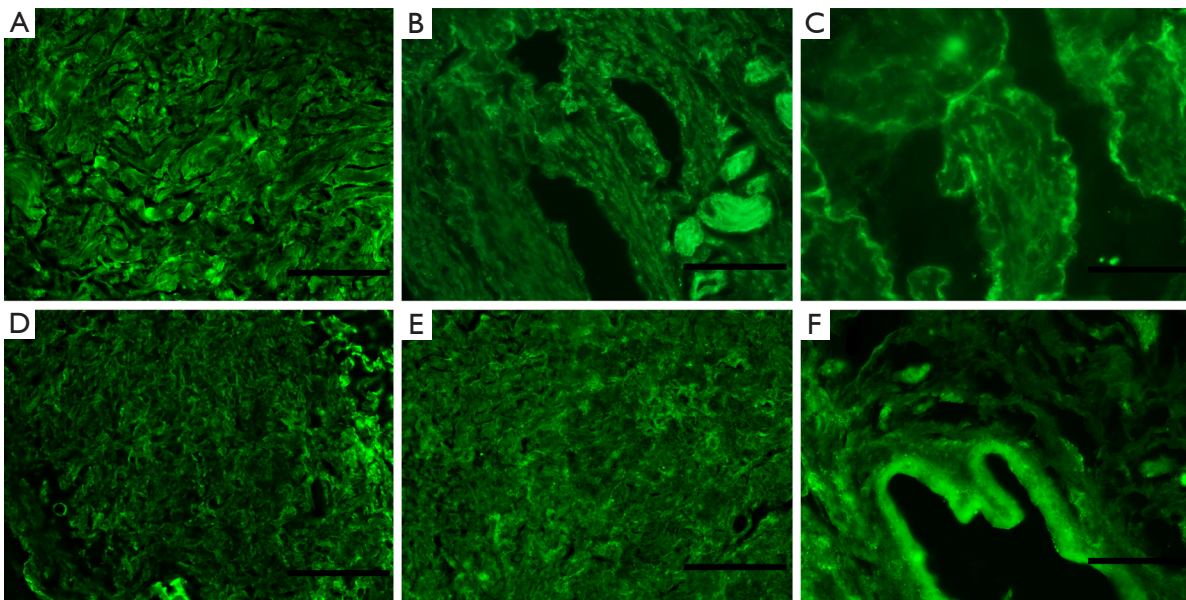


Figure 3 Characterization of the extracellular matrix via immunofluorescence staining for (A,D) collagen IV; (B,E) collagen I; and (C,F) fibronectin. Scale bar =50 µm.

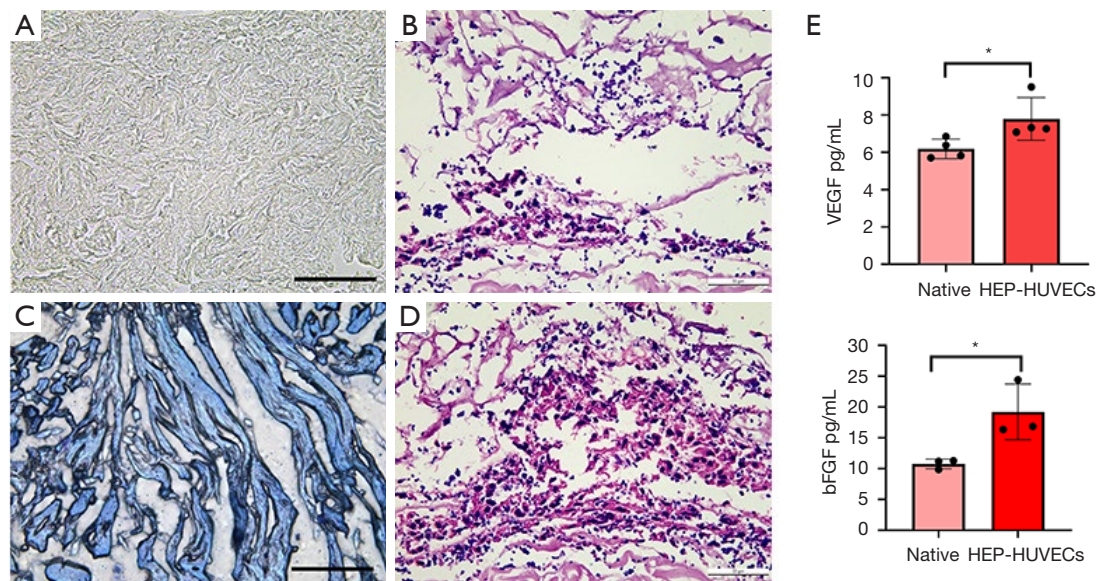


Figure 4 The effects of recellularization in the penile scaffolds. (A,C) Toluene blue staining; (B,D) hematoxylin and eosin staining of HUVECs; and (E) ELISA analysis. Scale bar =50 μ m. *, $P < 0.05$. bFGF, basic fibroblast growth factors; VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.

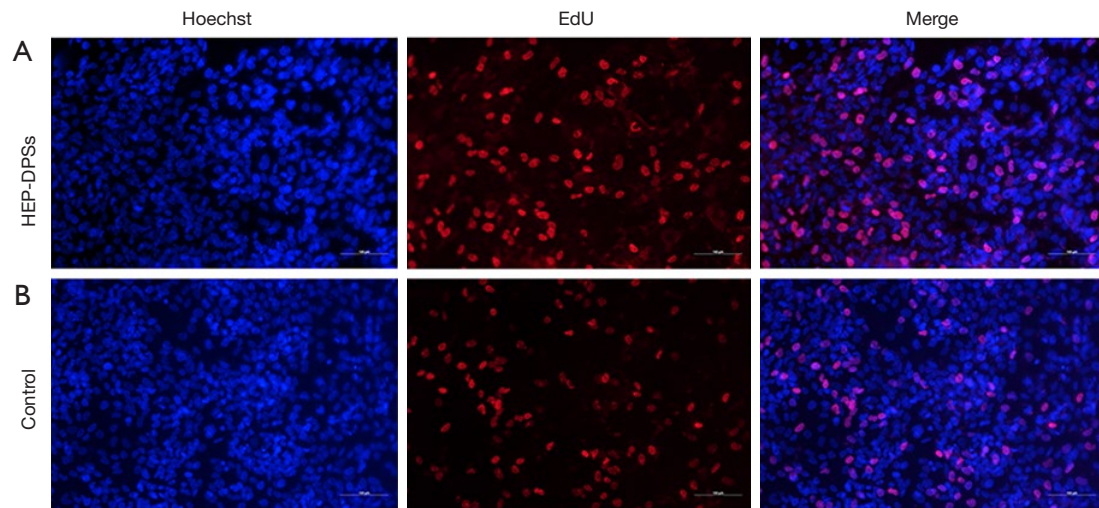


Figure 5 EdU staining showing the proliferation of HUVECs in (A) the HEP-DPSs and (B) the control. Scale bar =50 μ m. HEP, heparin; DPS, decellularized penile scaffold; HUVECs, human umbilical vein endothelial cells.

DPSs and heparinized DPSs and assessed by EdU staining to determine the cytocompatibility *in vitro* (Figure 5). The proliferation observed with the HEP-DPSs was higher than that of the native DPSs. These results demonstrated that the HEP-DPSs showed better biocompatibility for the proliferation of HUVECs.

Angiogenesis in the rat model

The HEP-HUVECs-DPSs, HEP-DPSs, and untreated scaffolds were implanted into the backs of the rats. The body weights of the rats were recorded and presented in Table S1. Samples were harvested at 1, 2, 3, and 4 weeks after implantation and evaluated by H&E

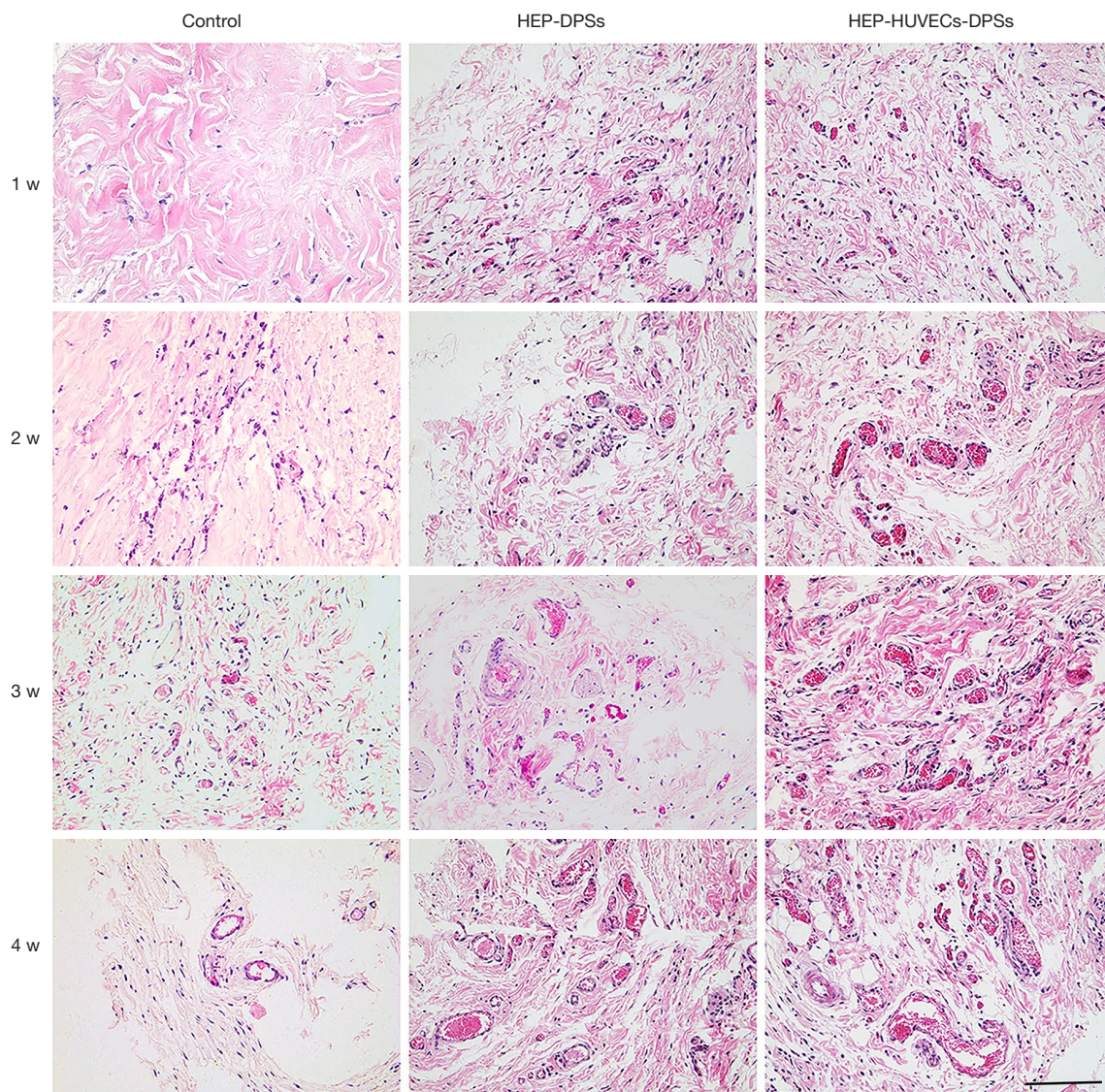


Figure 6 The angiogenesis of endothelialized control, HEP-DPSs and HEP-HUVECs-DPSs *in vivo*. Hematoxylin and eosin staining show the blood vessels that have formed in the scaffolds. Scale bar =100 μ m. HEP, heparin; DPSs, decellularized penile scaffolds; HUVECs, human umbilical vein endothelial cells; w, week.

staining (Figure 6) to assess the degree of angiogenesis. Direct observation suggested that all scaffolds had good biocompatibility (Figure 7A,7B). H&E staining showed small neovascularization in the scaffolds. During the first week, blood vessels started to appear inside the HEP-HUVECs-DPSs and HEP-DPSs, while this was not observed in the untreated scaffolds. Small blood vessels did not appear in the control scaffolds until the third week. At the same time, the number of blood vessels in the HEP-HUVECs-DPSs significantly increased and the structure

was more complete and more mature. Interestingly, the degree of endothelialization in the HEP-HUVECs-DPSs was superior to that of the HEP-DPSs and the untreated scaffolds.

The expression of CD31 was used to accurately quantify the angiogenesis of the re-endothelialized scaffolds. The number of blood vessels of the immunofluorescence staining was similar to that observed with the H&E staining (Figure 7C). There were significantly more vessels in the HEP-HUVECs-DPSs compared to the HEP-DPSs and

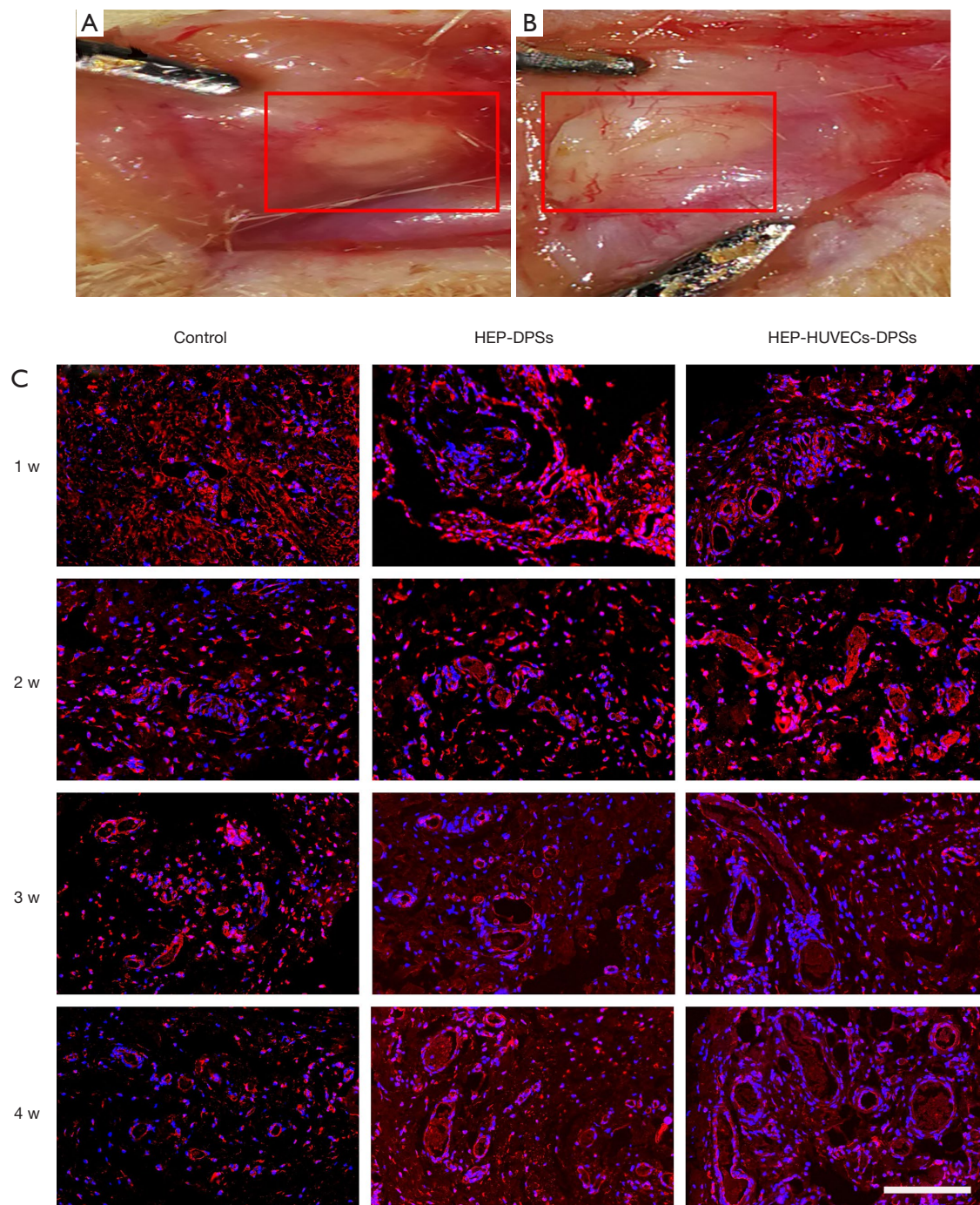


Figure 7 The degree of angiogenesis *in vivo*. (A,B) A whole view of the implants after implantation. (C) Immunofluorescence staining of CD31 (red) and DAPI (blue). Scale bar =100 μ m. HEP, heparin; DPSs, decellularized penile scaffolds; HUVECs, human umbilical vein endothelial cells; w, weeks.

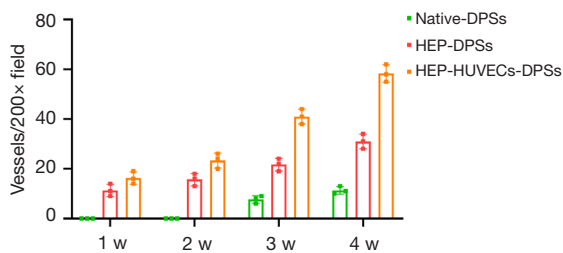


Figure 8 The blood vessel densities of the three groups ($P < 0.05$). HEP, heparin; DPSs, decellularized penile scaffolds; HUVECs, human umbilical vein endothelial cells; w, weeks.

the control scaffolds at the same time points. Random fields of view were selected for counting and statistical analyses. The quantitative results revealed that there was a significant difference in the number of blood vessels in the HEP-HUVECs-DPSs compared to the HEP-DPSs and the control scaffolds ($P < 0.05$; *Figure 8*).

Discussion

At present, penile reconstruction involves the use of a variety of free or pedicle flaps, with or without an inflatable penile prosthesis or penile implant (31,32). However, high infection rates, implant erosion, and donor site morbidity have prompted the search for alternative solutions. TE is increasingly being applied in various disciplines, including urology. Bioengineered penis for transplantation is becoming an alternative remedy for patients struggling with penile deficiency. It not only provides three-dimensional spatial structure, but also promotes cell migration and metabolism by interacting with the cell matrix (33). Unfortunately, the weak vascularization rate of the matrices and the lack of effective cell reimplantation methods present major obstacles to further clinical application.

To surmount these problems, researchers have applied different methods to modify the scaffold, including the use of heparin (29) and GRGDSPC peptides (34). Heparin acts as an antithrombotic agent by regulating the reaction with antithrombin, and is a widely used anticoagulant (35). Heparin can be immobilized in different ways, such as ion binding, covalent binding through multi-point conjugation, or end-point binding (36). Not only does heparin promote the angiogenic potential (37), but it also enhances adhesion and growth of reseeded cells (38). Therefore, heparin may be useful in promoting angiogenesis, strengthening adhesion, and promoting the growth of HUVECs.

Cell growth requires an adequate blood supply, otherwise necrosis may occur due to ischemia. Poor vascularization remains a major challenge in manufacturing an artificial organ. The decellularization process can damage endothelial cells and blood can induce thrombosis by direct contact with the ECM. Hence, not only does re-endothelialization reduce the probability of thrombosis but it also facilitates the formation of new blood vessels. Researchers have suggested various methods of cell reimplantation to promote vascularization. In this investigation, we covalently cross-linked heparin and reseeded HUVECs to the DPSs to promote vascularization. The scaffolds were harvested at 1, 2, 3, and 4 weeks after implantation and new blood vessels were formed. In addition, the possible mechanisms by which HEP-HUVECs-DPSs promote angiogenesis were investigated, with a focus on the role of growth factors. The levels of VEGF and bFGF were significantly higher in the HEP-HUVECs-DPSs compared to the native DPSs. In addition, the HEP-HUVECs-DPSs had more blood vessels, which may be attributed to the differences in the levels of growth factors. Therefore, the methods used herein provide a potential solution for penile tissue engineering.

In conclusion, these results suggested that, compared to the native DPSs, the HEP-DPSs provided a superior environment for the growth of HUVECs. The significant number of blood vessels observed in the *in vivo* implantation studies suggested that the HEP-HUVECs-DPSs were effective and deserve further investigation in penile tissue engineering. However, this is only the first step and there are still many hurdles to overcome before successful clinical application. In particular, smooth muscle cells (SMCs) are indispensable in penile tissue and further experiments will explore the effects of reseeded SMCs and HUVECs on DPSs, which may promote erection and facilitate intercourse. Restoring the main functions of the penis including erection and sexual intercourse is the ultimate goal. The erectile response was elicited by electrical stimulation of the cavernous nerves and quantified by calculating the max intracavernous pressure (ICP) and the ratio of max ICP to mean systemic arterial pressure (max ICP/MAP) (39). This will also be the main content of our next experiment.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://tau.amegroups.com/article/view/10.21037/tau-22-315/rc>

Data Sharing Statement: Available at <https://tau.amegroups.com/article/view/10.21037/tau-22-315/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tau.amegroups.com/article/view/10.21037/tau-22-315/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal treatments complied with institutional guidelines for the care and use of animals and were approved by the Ethics Committee of the Shanghai Tenth People's Hospital, School of Medicine, Tongji University (No. SHDSYY-2021-3647).

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Supplementary

Table S1 The changes in rat body weight following transplantation with decellularized penile scaffolds

Group (n=6)	0 w	1 w	2 w	3 w	4 w
Control	99.83±4.32*	142.23±4.35	191.34±6.65	251.35±6.13	304.52±6.31
HEP-DPSs	96.76±5.21	140.13±7.14	195.21±6.11	254.58±7.46	306.26±7.52
HEP-HUVECs-DPSs	97.56±5.13	139.43±6.34	193.29±5.62	256.63±5.45	301.58±6.68
P value	0.5446	0.7122	0.5637	0.3784	0.4295

*, body weights are shown in grams as mean ± standard deviation. HEP, heparin; DPSs, decellularized penile scaffolds; HUVECs, human umbilical vein endothelial cells; w, weeks.