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ORIGINAL RESEARCH

Circumferentially Aligned Electrospun Vascular Grafts Improves Its Vascular Regeneration and Remodeling in vivo

Man Xiong[1,*], Lei Yang[2,*], Xinfang Liu[2], Sidong Luo[2,3], Yeyang Wang[2–4]

[1]School of Nursing, Guangzhou University of Chinese Medicine, Guangzhou, People's Republic of China; [2]Orthopaedic Center, the Affiliated Guangdong Second Provincial General Hospital of Jinan University, Guangzhou, People's Republic of China; [3]The Second School of Clinical Medicine, Southern Medical University, Guangzhou, People's Republic of China; [4]Orthopaedic Center, Zhaoqing Central People's Hospital, Zhaoqing, Guangdong, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yeyang Wang, Email yeyangwyy@163.com

Introduction: Despite the rapid development of small diameter vascular graft (SDVG), the ability of synthetic grafts to facilitate tissue remodeling and regeneration remains an important challenge within regenerative medicine.

Methods: Based on our previous research work, silk fibroin (SF)/fibrin vascular grafts were successfully fabricated using electrospinning technology, and it was demonstrated that the grafts had superior mechanical strength, good cytocompatibility and histocompatibility. This indicated that the vascular graft was an ideal SDVG. We developed SF/fibrin vascular grafts with circumferentially aligned nanofibers to explore some of its properties in vivo.

Results: The graft exhibited randomly arranged microstructure, excellent mechanical properties and compliance properties. These vascular grafts were transplanted into the abdominal aorta of rats, maintaining normal blood flow, vascular patency, and functionality. The M2/M1 ratio value in SF/fibrin grafts increased over time after implantation. Whereas the expression level of inflammatory cytokines initially increased and then eventually reached the normal levels. Moreover, the circumferentially aligned vascular grafts could guide the regeneration of neoarteries, endothelialization formation, enhanced functionality, rapid cellular infiltration and improved extracellular matrix (ECM) deposition, as well as generated more microvessels and fewer calcification.

Discussion: Our research focused on the long-term performance in vivo of SF/fibrin tubular grafts, which could become a new type of SDVG and help guide the development of next-generation vascular grafts.

Keywords: electrospun vascular graft, mechanical, immunoregulation, vascular regeneration and remodeling, vascular tissue engineering

Introduction

Cardiovascular diseases (CVDs) are the primary killers that seriously endanger humans worldwide. Simultaneously, the World Health Organization reports that over 18 million deaths are attributed to CVDs, and this number is projected to rise to roughly 23 million in ten years.[1] In addition, in our country, the number of patients originating from congenital cardiovascular diseases (CCDs) is 200,000 per year.[2] Currently, senior surgeons still unanimously consider that revascularization is the best treatment for severe CVDs and CCDs. Generally, most of the blood vessels used to treat these diseases come from autologous saphenous veins with low immunogenicity. Nevertheless, the disadvantages of autologous vessels, such as lack of tissue donors and anatomical abnormalities, make it difficult to meet the needs of clinical applications.[3] Hence, the advent of tissue engineering artificial vascular scaffolds has had a significant impact on the treatment of various vascular diseases.

In recent years, tissue-engineered vascular grafts have achieved rapid progress.[4] Meanwhile, numerous literatures have reported that artificial vascular grafts with large diameter (diameter >6 mm) have been proven effective in clinical

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applications.[5] Unfortunately, over the past two decades, researchers still have not applied SDVG (diameter < 6 mm) in clinical practice. The reason for this failure is that the vascular graft transplantation in vivo can cause infection, poor long-term patency, intimal hyperplasia, thrombosis, occlusion, calcification and other shortcomings.[6] Although a lot of effort is being spent on improving these weaknesses, the efficient and effective-method has yet to be developed. Thus, we hope to fabricate an SDVG with good mechanical properties and biocompatibility, which remains an important unmet need in medicine.

As we all know, the principle of tissue engineering is to plant bioactive cells on synthetic materials and then culture them in vitro and implant them in vivo to ensure their survival and function. The field of vascular tissue engineering has a bright prospect with the development of tissue engineering.[7] Simultaneously, the main elements of tissue-engineered vascular grafts include cell sources, biomaterials, and fabrication techniques.[8] In fact, appropriate biomaterials can interact with the cells and form functional vascular - like tissue, thereby constructing structures that mimic the natural vessel microenvironment. In the process of vascular remodeling, the ideal vascular graft should have adequate biomechanical characteristics. In the meantime, the vascular scaffold should also be convenient to process and tailorable, as well as resistant to permanent deformation and kinking.[9,10] Excellent scholars have demonstrated that the biomechanical properties of scaffold materials can stimulate macrophage transformation to different phenotypes, which may affect vascular tissue regeneration and remodeling.[11,12] Superior mechanical also plays an important role in vascular smooth muscle cells (VSMCs) of contractile function and ECM deposition.[13] Consequently, our team members speculated that vascular grafts with insufficient mechanical properties may influence the function of vascular reconstruction.

To date, researchers rarely report that artificial vascular grafts have functional properties after successful in vivo transplantation. Immune response of vascular graft in vivo is an urgent problem to be concerned and solved. Meanwhile, macrophages are a very important factor in vascular reconstruction. Many scientists have confirmed that anti-inflammatory macrophages could effectively improve the migration of VSMCs and the proliferation of endothelial cells (ECs), and then it has an active role in the vascular microenvironment.[14–16] Liu et al[17] successfully prepared composite nanomaterials using human placental ECM and poly(ε-caprolactone) (PCL), as well as loaded heparin and IL-4. A series of experiments have demonstrated that the scaffold could regulate tissue cells and promote vascular regeneration. Then, we should consider the cell infiltration of vascular scaffold. Reasonably controlling the porosity of vascular grafts can effectively improve rapid cell infiltration and promote vascular tissue regeneration.[18] Additionally, after successful in vivo transplantation, vascular graft may result in thrombosis, calcification, bleeding, infection, and so on.[19,20] During the process of vascular graft transplantation, we often find these issues, so we need to pay high attention to them.

Currently, many methods such as phase separation, particle leaching, etc. have been developed for manufacturing porous vascular grafts.[21] Unfortunately, the grafts prepared using these techniques cannot induce VSMCs circumferentially oriented regeneration. Many scientists have attempted to prepare controllable topological structures using various methods to induce the VSMCs aligned regeneration.[22,23] Shen et al[24] prepared a synthetic degradable scaffold material with oriented microchannel structure, which could guide VSMCs cultured switch from random orientation to spindle-shaped morphology, and promote the expression of smooth muscle α-actin of VSMCs. However, the vascular scaffold with a single-layer microchannel structure was different from the multi-layer of native arteries. Meanwhile, most studies only conducted in vitro proof-of-concept, and in vivo evidence of effectiveness was still insufficient. Previous studies confirmed that electrospinning technology could accurately control the size and arrangement of nanofibers. Zhao et al[25] prepared electrospun vascular grafts using a mixture of formic acid, PCL, and fibrin to generate functional neoarteries. Inspired by this, we selected SF with superior mechanical properties and fibrin with good biocompatibility as the raw material to prepare electrospun SDVGs with an oriented structure to induce guide VSMCs circumferentially aligned regeneration.

In previous research work, we used electrospinning equipment to fabricate SF/fibrin vascular scaffolds and found that SF/fibrin (25:75) electrospun scaffold had balanced mechanical strength, excellent hemocompatibility, biodegradability and cytocompatibility. Experimental results have showed that the SF/fibrin scaffold was considered as a candidate composite material for SDVG.[26]

Therefore, we hypothesize that SF/fibrin (25:75) vascular scaffolds can be applied to small-diameter artificial vascular synthetic materials. In this study, we mainly transplanted SF/fibrin (25:75) tubular vascular graft into abdominal aorta of SD rats at different time points, respectively. In addition, its mechanical strength, functional effects, vascular cell regeneration, ECM deposition, immunoregulation and calcification were investigated at 1,3 and 5 months.

Materials and Methods

Materials

Bombyx mori silk cocoons were obtained from Guangzhou Chuangseed Company (Guangzhou, China). Fibrin was purchased from Thermo Fisher. Formic acid solution, aspirin, isoflurane, ceftriaxone sodium, 10% KCL, sodium nitroprusside, acetylcholine and adrenaline were purchased from Aladdin Company (Shanghai, China). The mouse anti-CD68 antibody, rabbit anti-INOS antibody, macrophage anti-mannose receptor/CD206 antibody, ELISA kit, anti-endothelial nitric oxide synthase (eNOS) antibody, mouse anti-α-SMA, rabbit anti-calponin 1 antibody and mouse anti-SM-MHC antibody was provided by Sevier Biotechnology Co., Ltd. (Wuhan, China). Hematoxylin, Eosin kit, Masson trichrome kit, Verhoeff van Gieson, Safranin O, von Kossa and anti-CD34 antibody were obtained from Mairuida Co., Ltd. (Beijing, China).

SF/Fibrin Vascular Graft Generation

Up to now, our researchers have successfully completed many similar manufacturing of SF/fibrin vascular grafts through electrospinning.[25–28] Firstly, the Bombyx mori silk cocoons were made into small pieces and 70% soaked them in ethanol. Afterward, we degummed the cocoon with sodium carbonate solution (0.5%w/v) and repeatedly rinsed it with distilled water. Secondly, we dried the as-degummed silk material and dissolved it completely with $CaCl_2$-CH_3CH_2OH-H_2O (molar ratio = 1:2:8) solution. Eventually, we further dialyzed and froze it to obtain a pure SF solution. Subsequently, SF and fibrin with a mass ratio of 25:75 were mixed completely in 98% formic acid to prepare a fixed polymeric concentration of 10% wt solution. Finally, the as-prepared SF/fibrin solution was loaded into a 10 mL syringe and fixed onto the propeller. We prepared SF/fibrin vascular grafts according to the following conditions of the electrospinning equipment (FM1012, Dalian, China): curing distance of 10 cm, a voltage of 20kV, and a flow rate of 1 mL/h. At room temperature, the vascular grafts by ventilated and dried were stored. Similarly, we successfully fabricated SF vascular grafts and considered them as the control group.

Investigation on the Characteristics of the Graft

We investigated the morphology of SF/fibrin (25:75) vascular grafts by scanning electron microscopy (SEM, Hitachi SU8100, Japan). The samples were made to the same size, and their surfaces were rinsed with PBS. Then, the samples were fixed, dehydrated and dried, respectively. We conducted conductive treatment on the tested samples and sprayed gold by sputtering for 30 s. The images were obtained by SEM at an accelerated voltage of 10kV. Furthermore, 50 nanofibers were randomly selected from the pictures. The diameter distribution of vascular grafts was evaluated by ImageJ software.

Vascular Graft Transplantation and Grouping Status

All experiments were approved by Animal Ethics Committee of Affiliated Guangdong Second Provincial General Hospital of Jinan University (Guangzhou, China). We conducted the animal experiments in vivo followed in accordance with the guidelines of the Animal Care and Experiment Committee guidelines. To investigate the performance of vascular grafts, we selected adult healthy SD rats (n = 48) from the animal room of the Affiliated Guangdong Second Provincial General Hospital of Jinan University. Moreover, we have successfully reported similar vascular grafts implanted into SD abdominal aorta.[25,27] The SF vascular grafts (n = 16) were considered as the control group. The SF/fibrin (25:75) vascular grafts (n = 16) were considered as the experimental group. Above all, these SD rats were given oral aspirin to reduce the incidence of thrombosis. All vascular grafts should be immersed in alcohol for sterilization. Meanwhile, all animal surgeries should be strictly aseptic. We anesthetized SD rats with 2% isoflurane by inhalation and

connected anesthesia equipment (Model R500, Ruiwode, China). The abdominal hair with a skin preparation tool was cleaned. After that, we placed the SD rats on the operating table, sterilized them with medical iodophor and covered them with a sterile towel.

We used a surgical knife to make a 5 cm incision in the abdomen of SD rats, carefully protected the blood vessels and nerves, and exposed the abdominal aorta. Then, we successfully blocked the abdominal aortic with a microvascular clip and cut it with a knife. We used 8–0 monofilament nylon sutures to anastomose the vascular graft with the broken ends under the microscope (Model OLYMPUS, Japan) and then closed the rat's abdominal cavity with 2–0 silk thread. After the surgery, we routinely administered aspirin and ceftriaxone sodium drug for treatment. Hereon, we were surprised to find that three rats died accidentally. Survival rate was 93%. The patency of the graft was evaluated under a microscope (Model HGO-P, China). We soaked and stored these samples with 4% paraformaldehyde solution.

Biomechanical Evaluation

Vascular compliance is a measure of the ability of vascular grafts to respond to pressure changes. We anesthetized SD rats with isoflurane drug and fixed them on the operating table. Simultaneously, we used an intelligent non-invasive blood pressure device (Model BP-98AL, softron, China) to detect its systolic and diastolic blood pressure. Finally, we collected the measured data and calculated it by the following formula.

$$\text{Compliance per 100 mmHg } (\%) = \frac{(Rp2 - Rp1)Rp2}{\text{p1} - p2} \times 10^4$$

Where p_1 and p_2 are the high-pressure and low-pressure values (mmHg) of each sample, respectively. The measured inner diameter values of high and low pressure are Rp_1 and Rp_2.

Previously, we reported the biomechanical characteristics of the SF/fibrin vascular material in vitro.[26] Following the previous method, we analyzed the maximum stress, breaking strain, and elastic modulus of each test sample using a universal material testing machine (Model AVIC WDW-5KN, air times, China). Here, we should be evaluate each sample three times successively, as well as obtain its average value and standard deviation.

Examination of the Patency and Functionality of Graft

To clearly investigate the patency performance of different vascular grafts, we evaluated them using a high-resolution animal ultrasound imaging platform (Vevo 3100LT, Visual Sonics, Canada). Meanwhile, we collected their images and recorded the diameter changes of the vascular graft at different transplantation time periods. Here, the red color in these images indicated forward blood flow.

In the past, aortic ring bioassay experiment have been reported in detail by our researchers.[25] We repeatedly washed the vascular graft with PBS and immersed it in buffer solution. To investigate the constriction and relaxation functions of the vascular grafts, we added 10% KCL (50 mm) and sodium nitroprusside (SNP, 1.0×10^{-7}mol/L), respectively. Simultaneously, we also added acetylcholine (Ach, 9μM) and adrenaline (AD, 0.8 mm) to evaluate its endothelial function. These test samples were measured by the force sensors recording equipment (AD Instruments, Australia) and their values were collected.

Immunofluorescence Staining Assay

To obtain three different samples (n = 4), we sequentially sacrificed the SD rat model at 1, 3 and 5 months, respectively. For immunofluorescence staining assay, these samples should be dehydrated, wax embedded, sectioned, and otherwise treated. To evaluate the macrophages cell of three different samples, we stained them with mouse anti-CD68 antibody (1:200), Rabbit anti-iNOS antibody (1:150) and CD206 antibody (1:250). Moreover, we evaluated the expression levels of some inflammatory factors by cytokine ELISA assay. The fluorescence intensity of iNOS of M1 and CD206 of M2 were continuously evaluated in three parallel groups by Image-J software. To obtain the ratio value of M2/M1, we divided the fluorescence intensity of CD206 by the fluorescence intensity of iNOS.

During the 5 months of transplantation, we analyzed the morphology of different vascular grafts by SEM. The samples were treated with fixed, dehydration and dried. The images of different magnification were obtained after the

samples were sputtered with gold. Furthermore, the expression level of ECs on the test samples was evaluated using anti-endothelial nitric oxide synthase (eNOS) antibody. The expression levels of VSMCs on vascular grafts were detected by mouse anti-α-SMA (1:150), rabbit anti-calponin 1 antibody (1:150) and mouse anti-SM-MHC (1:200) antibody. Meanwhile, the nuclei of all samples were stained with DAPI. Finally, we obtained these images with fluorescence microscopy (DMIL, China) and analyzed their data by ImageJ software.

Histological Assessment

For histological analysis assay, we selected SF grafts, SF/ fibrin (25:75) grafts, and native vascular at 5 months for implementation. In addition, these samples were treated with fetal bovine serum and TritonX-100 solution, respectively, and frozen sections were prepared. According to the instructions of the staining kit, the sections of the samples were stained by the H&E staining, Masson trichrome, Verhoeff van Gieson (VVG), Safranin O. At the same time, the sections of the SF grafts and SF/fibrin grafts at a specific time were stained by von Kossa and anti-CD34 (1:100). These pictures were obtained by an upright light microscope (Imager M2M, Zeiss, Germany). Eventually, the data values in the image were calculated using ImageJ software.

Statistical Analysis

The quantitative results of all experiments were established with more than three independent samples. All experimental data were expressed as mean ± SD. For statistical analysis, GraphPad Prism Version 7.0 and ImageJ software Version 1.8.0 were applied for calculation. Unpaired Student's *t*-test used for single comparison. One-way analysis of variance and two-way ANOVA were applied for multiple comparisons. $P < 0.05$ represented statistical differences and ns indicated no significance.

Results

Preparation and Characteristics of SF/Fibrin Vascular Grafts

To successfully prepare tubular vascular grafts with good characteristics, we used SF and fibrin as matrix materials through electrospinning instrument. Figure 1A demonstrated in detail a schematic diagram of the vascular graft preparation and its vascular regeneration after implantation in vivo. The SF/fibrin tubular vascular graft was a length of approximately 10 mm, an inner diameter of 3 mm and a wall thickness of 0.4 mm (Figure 1B). By SEM, we could observe that the electrospun SF/fibrin nanofibers were randomly arranged, appropriate porosity, compacted and the surface of its nanofibers was smooth (Figure 1D and E). The vascular graft with good microstructure was conducted to cell infiltration, adhesion, and proliferation. Furthermore, we randomly selected 100 nanofibers from the picture and calculated relative frequency of the SF/fibrin nanofiber diameter by ImageJ software. We analyzed that the nanofiber diameter of the vascular graft was approximately in the range of (350–450) nm (Figure 1C). Fortunately, the size of the nanofiber diameter of the SF/fibrin vascular graft was completely consistent with our previously validated results.

Electrospun Graft Implantation and Mechanical Properties Evaluation in vivo

At 1 and 3 months, the SF/fibrin (25:75) vascular grafts were different from the native artery. Nevertheless, we observed that the vascular graft at 5 months was extremely similar to the native artery (Figure 2A and B). We repeatedly rinsed the SF/fibrin (25:75) graft with physiological saline until it became bright and resembled a native artery (Figure 2B). Through microscopic instrument, we were surprised to observe that there were differences in the thickness and morphology of the vascular graft lumen at 1, 3, and 5 months and their presence of some arterial-like tissue (Figure 2C). To investigate the performance of vascular grafts, we categorized them into graft viability group, patency group, occlusion group, and acute thrombosis group. In Figure 2D, we found that the values of viability and patency in SF vascular grafts were significantly lower than those in SF/ fibrin vascular grafts. However, the values of occlusion and acute thrombosis in SF vascular grafts were lowest (Figure 2E). Additionally, we observed that the compliance values of SF grafts were greater than those of SF/fibrin grafts at three time points. Meanwhile, the compliance value of SF/fibrin vascular graft also increased gradually over time (Figure 2F). From Figure 2G–I, it can be seen that the values of

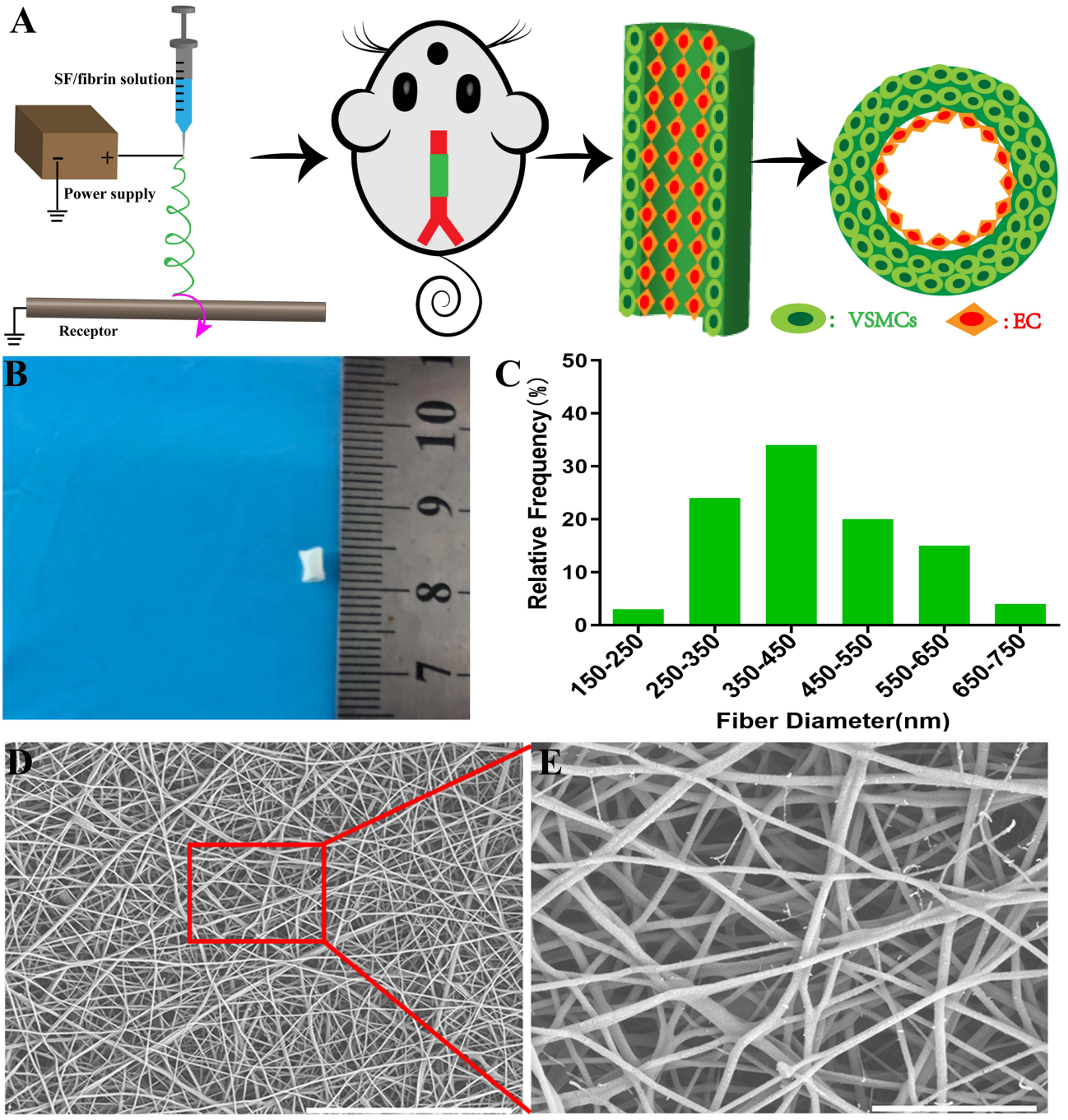

Figure 1 Preparation and characterization of SF/fibrin vascular grafts. Schematic diagram of the fabrication process of vascular grafts and its regeneration in vivo (**A**). Macroscopic view of the SF/fibrin vascular grafts (**B**). Microscopic morphology of the graft under SEM (**D** and **E**). Image of the relative frequency of the SF/fibrin nanofiber diameter by ImageJ software (**C**). Scale bars indicated 50μm in (**D**) and 10μm in (**E**).

maximum stress, breaking strain and elastic modulus of the SF/fibrin graft at 5 months were very analogue to those of the native artery group. The mechanical strength values of SF/fibrin grafts were significantly lower than those of SF grafts at 5 months. Also, the native artery, the SF graft and the SF/fibrin vascular graft at 5 months were statistically significant.

The Patency, Structural Stability, and Regenerative Functionality of Vascular Grafts

We evaluated the patency of SF grafts and SF/fibrin grafts at 5 months using Doppler ultrasound. The results indicated that the two different vascular grafts had good patency and no thrombosis or restenosis, as well as no aneurysms (Figure 3A and B).

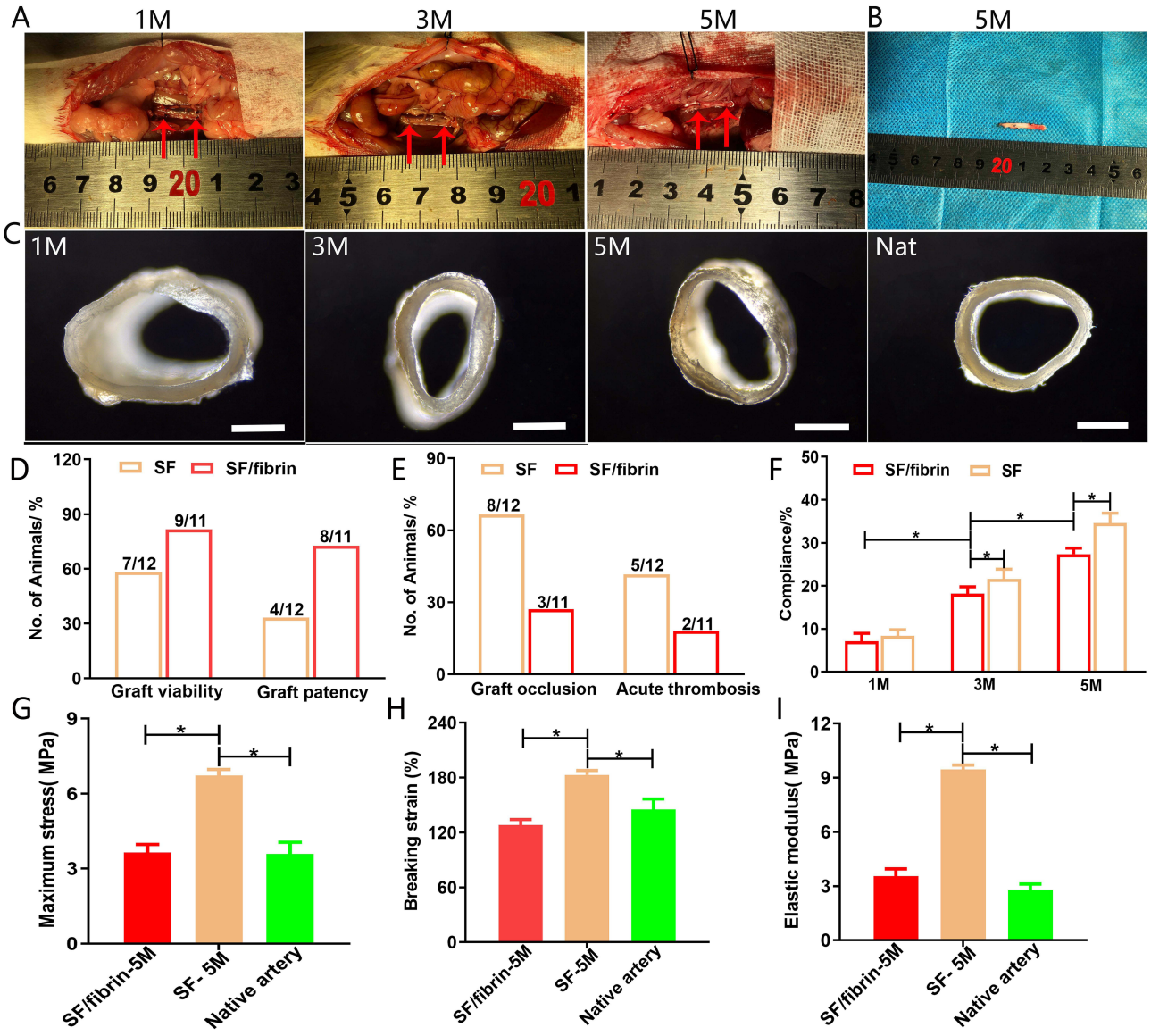

Figure 2 Changes in morphology and mechanical properties of vascular grafts after transplantation in vivo. These images showed SF/fibrin (25:75) grafts were implanted into the abdominal aorta at 1, 3 and 5 months(**A**). The red arrow indicated the anastomotic site. The SF/fibrin (25:75) graft at 5 months were rinsed with 0.9% sodium chloride solution(**B**). The cross-section pictures of the SF/fibrin (25:75) graft after 1, 3 and 5 months(**C**). Quantitative data indicated the performance of vascular grafts in vivo (**D** and **E**). The compliance values of SF/fibrin and SF grafts at 1, 3, and 5 months (n = 3) (**F**). The maximum stress (**G**), breaking strain (**H**) and elastic modulus (**I**) of the grafts were measured (n = 3). Scale bar: 1 mm. ★indicated $P < 0.05$.

Additionally, we examined the diameter changes of the vascular graft in order to investigate its structural integrity in vivo. We observed that SF and SF/fibrin grafts at different time periods (0, 1, 3 and 5 months) maintained a constant lumen diameter by ultrasound equipment. Under the action of hemodynamic, the vascular graft could maintain structural integrity (Figure 3C). Simultaneously, we used aortic ring bioassay assay to detect the function of different grafts at 5 months. From the results, it can be seen that the contraction value of SF/fibrin grafts was much higher than that of SF grafts under the condition of vasomotor agonists (KCL and AD). Nevertheless, the contraction values of the two vascular graft groups were lower than those of the native artery group (Figure 3D). We also found from the results that the relaxation value of SF grafts was significantly higher than that of SF/fibrin grafts, but the relaxation intensity of SF/fibrin vascular graft was similar to that of native artery under the condition of vasodilators (Ach and SNP) (Figure 3E).

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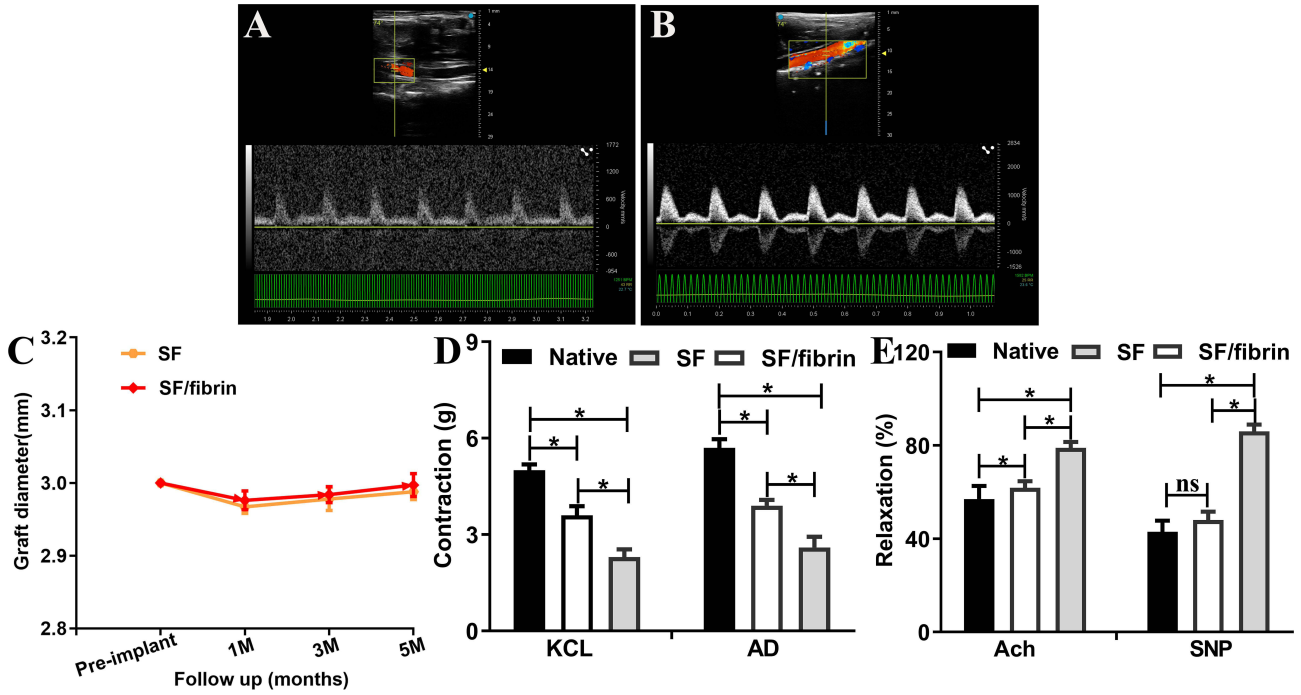

Figure 3 The patency of SF graft (**A**) and SF/fibrin graft (**B**) at 5 months was observed by color Doppler ultrasound. The lumen diameter of the vascular graft was evaluated at different time points using color Doppler ultrasound equipment(**C**). The contraction strength(**D**) and relaxation response(**E**) of three different samples at 5 months were examined (n = 3). ★indicated P < 0.05. ns was not statistically significant.

Expression of Macrophages in SF/Fibrin Graft Remodeling

Actually, macrophages are important participants in vascular remodeling. Macrophages can be divided into pro-inflammatory factor (M1) and anti-inflammatory factor (M2) based on their functions and levels of inflammatory secretion. The most reliable marker of CD68 was used to investigate its distribution of macrophages in SF/fibrin grafts at 1, 3, and 5 months. We observed from the results that the positive expression of all macrophages at 5 months was lower than that at other time points. As the implantation time increased, the number of iNOS positive cells was lower than that of CD206 positive cells. Meanwhile, it can be seen from the immunofluorescence results that the number of positive cells in native artery group was relatively minimal, and it was significantly lower than the positive cells of iNOS and CD206 in other groups (Figure 4A). Moreover, we evaluated the anti-inflammatory/pro-inflammatory macrophages ratio values of different vascular grafts using ImageJ software. The results showed that the anti-inflammatory/pro-inflammatory macrophages ratio of PCL vascular grafts was lower than that of SF/fibrin grafts at 3 and 5 months. Surprisingly, the anti-inflammatory/pro-inflammatory macrophages ratio values of two different vascular grafts also increased with the extension of transplantation time (Figure 4B).

In vascular graft remodeling, M1 and M2 cytokines are crucial elements. To further evaluate the expression level of macrophages, we used ELISA experiments to detect some inflammatory factors in different samples. IL-1β, TNF-α and IL-6 belongs to pro-inflammatory cytokines. The results showed that the secretion of IL-1β, TNF-α and IL-6 inflammatory factors in SF/fibrin grafts increased gradually at 1 and 3 months. Unfortunately, the secretion of three different pro-inflammatory cytokines both significantly decreased at 5 months and until the secretion value was closed to that of native arteries (Figure 4C-E). We observed that the secretion levels of the anti-inflammatory factors IL-4 and IL-10 decreased with prolonged transplantation time. Additionally, the secretion of all inflammatory cytokines in SF grafts were highly expressed at 5 months, except for IL-6 (Figure 4F-G).

Rapid Endothelialization in Different Vascular Graft

Rapid endothelialization can effectively inhibit thrombus formation, thereby ensuring long-term patency of the graft lumen. To investigate the endothelialization of three different samples, these grafts at 5 months were stained with eNOS

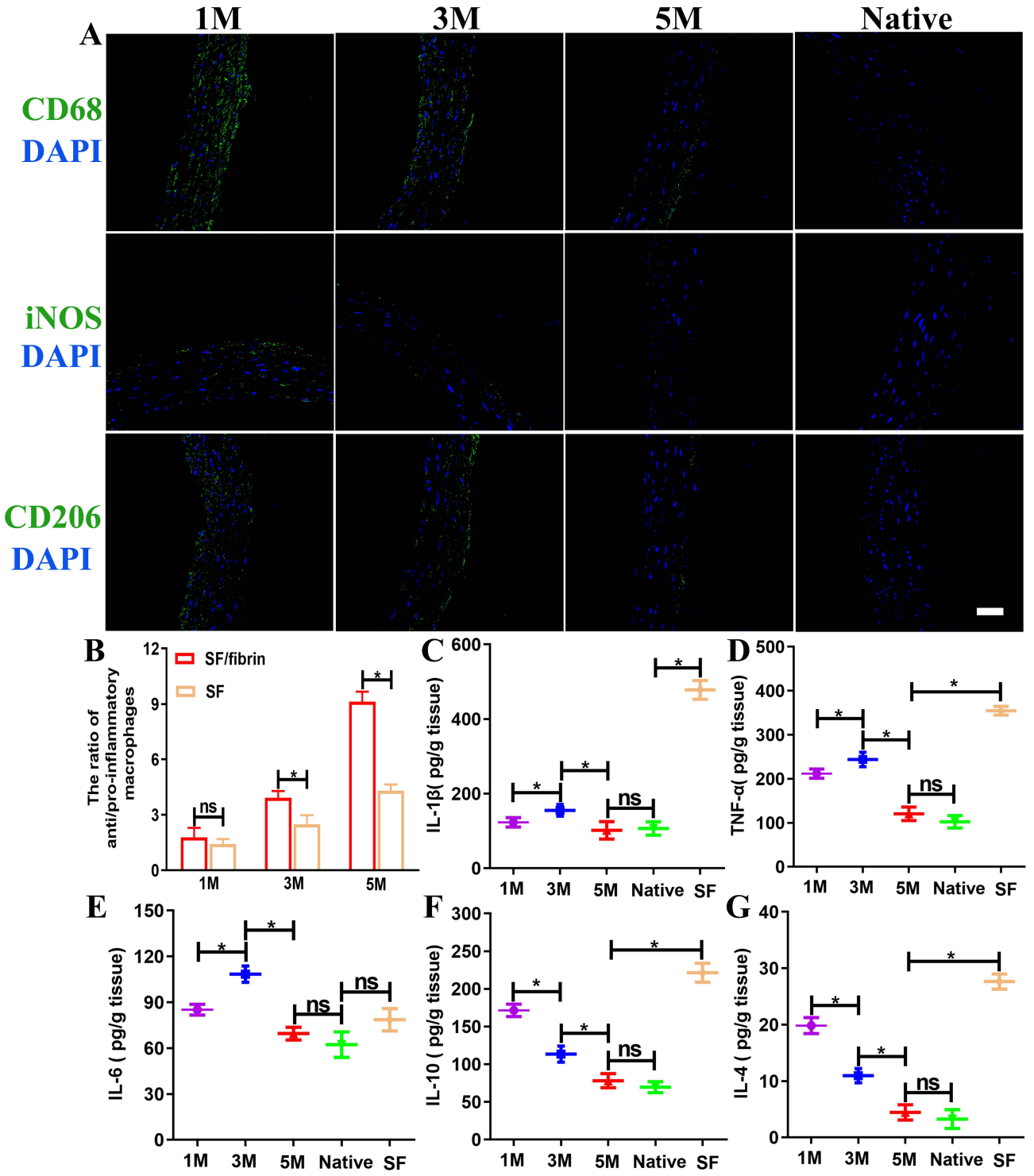

Figure 4 Inflammatory response of vascular grafts at different time periods. Fluorescence microscopy showed the distribution of CD68(macrophages), iNOS(M1), and CD206(M2) in the graft group(**A**). The ratio of anti-inflammatory/pro-inflammatory macrophages in SF/fibrin and SF grafts (n = 3) (**B**). DAPI (blue) indicated cell nuclei. The ELISA kit was used to detect some inflammation cytokines IL-1 β(**C**), TNF-α(**D**), IL-6(**E**), IL-10 (**F**), IL-4 (**G**) (n = 3). Scale bar: 50μm.★indicated $P < 0.05$. ns was not statistically significant.

antibodies and DAPI. The immunofluorescence results confirmed that eNOS positive cells in the lumen of SF/fibrin vascular graft were significantly higher than in the SF graft. Meanwhile, the lumen of SF/fibrin graft at 5 months formed a continuous endothelial layer, which resembled the native artery group (Figure 5A-F). Based on immunofluorescence images, the endothelial coverage of different samples was calculated. We observed that the endothelial coverage of SF/

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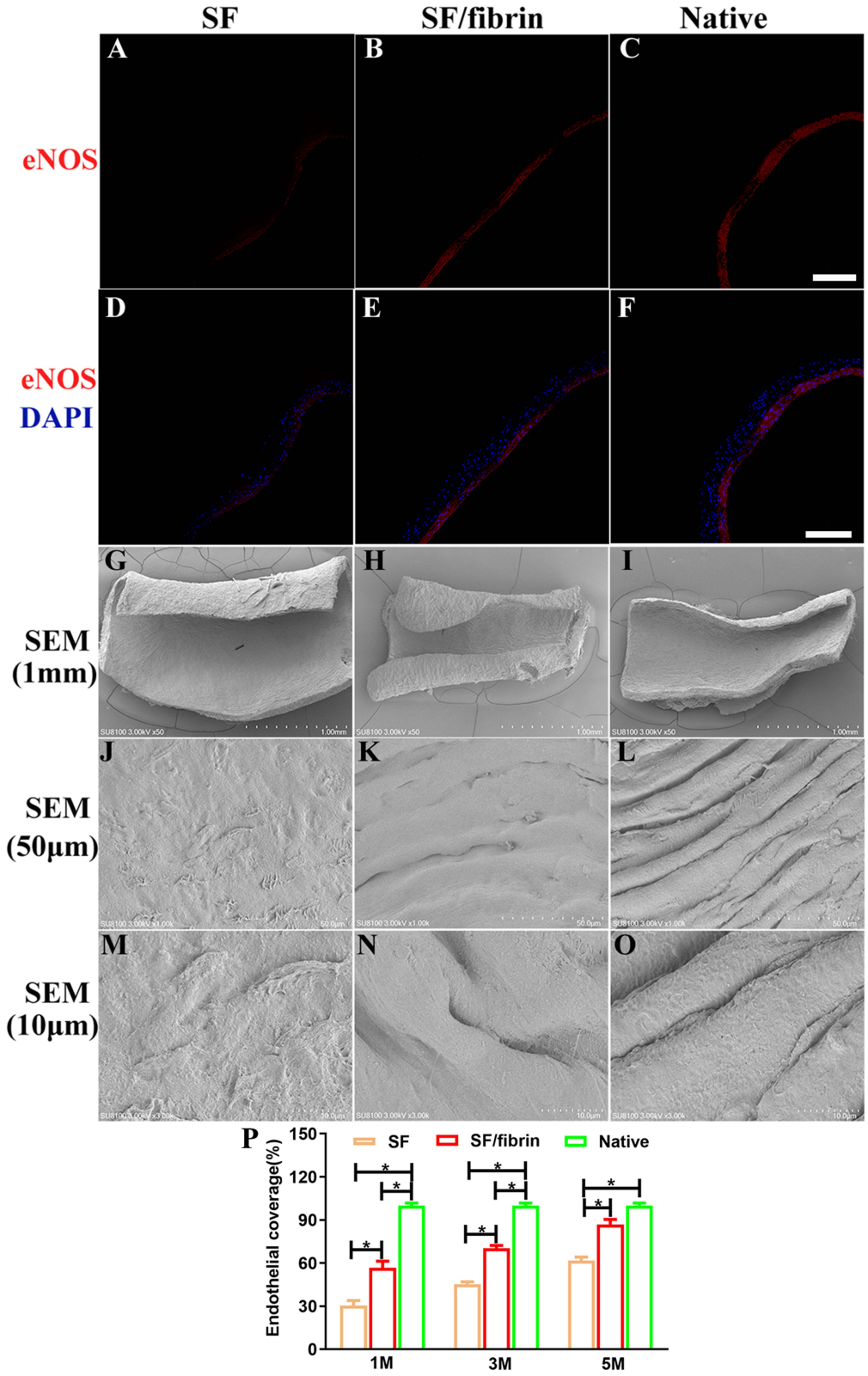

Figure 5 The formation of endothelialization in three different grafts. Immunofluorescence images expressed the distribution of endothelial cells (eNOS, red) in SF grafts (**A** and **D**), SF/fibrin grafts (**B** and **E**) and native artery (**C** and **F**) at the 5 months. DAPI (blue) indicated cell nuclei. SEM pictures of the lumen surface in different vascular graft at 1 mm (**G-I**), 50μm (**J-L**), and 10μm (**M-O**). Endothelial coverage of the grafts was evaluated at different time points (n = 3) (**P**). Scale bar: 100μm. ★indicated $P < 0.05$.

fibrin grafts increased gradually with the extension of transplantation. The endothelial coverage of SF/fibrin was higher than that of SF grafts at three different time periods. The endothelial coverage of SF group, SF/ fibrin group and native group at 1 and 3 months was statistically significant (Figure 5P). Additionally, we further analyzed the lumen structure of the vascular graft by SEM. From Figure 5G-I, it can be seen that the luminal surfaces of three different samples were smooth and clean. Simultaneously, we did not observe thrombosis or platelet aggregation in these images. Interestingly, at 5 months, the number of cobblestone-like cells in the lumen surface of the PCL/fibrin graft (Figure 5K and N) was significantly higher than that in the SF graft (Figure 5J and M), but still lower than in native artery (Figure 5L and O). Here, the cobble stone-like cells indicated ECs. We were surprised to find that the arrangement of ECs and blood stream direction remained consistent in SF/fibrin grafts, and it was similar to native endothelial cells (Figure 5K and L).

Vascular Regeneration of Different Vascular Grafts

To investigate cell infiltration and VSMCs regeneration in different grafts, we stained them with H&E and immunofluorescence. We observed from the results that the cell distribution in the three different samples was oriented in a circumferential arrangement, and the residual degraded grafts were not observed. The wall thickness of SF/fibrin grafts was significantly more than that of SF grafts at 5 months (Figure 6A–C). The number of cells in the grafts was evaluated using ImageJ software. We found that the number of cells in SF/fibrin grafts and native artery was much higher than that of

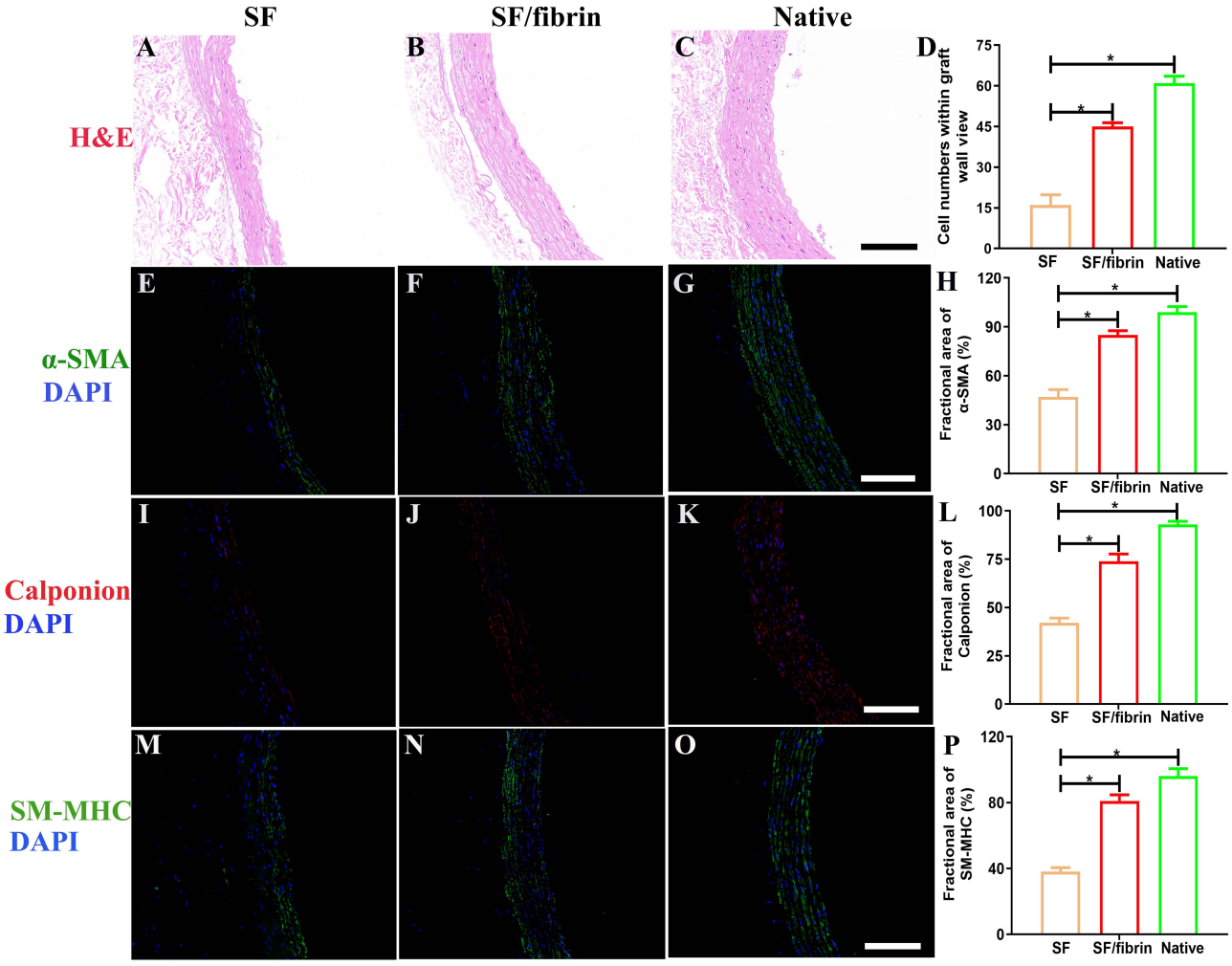

Figure 6 Regeneration of VSMCs within vascular grafts at 5 months of transplantation. Images of three different grafts stained by H&E (**A-C**). The cell numbers within grafts were evaluated by images(**D**) (n = 3). Immunofluorescence images of the α-SMA (green, **E-G**), Calponion (red, **I-K**) and SM-MHC (green, **M-O**) in different grafts. DAPI (blue) indicated cell nuclei. Fractional area of α-SMA(**H**), Calponion (**L**) and SM-MHC(**P**) within the different graft (n = 3). Scale bar:100μm. ★indicated P < 0.05.

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SF grafts at 5 months (Figure 6D). To evaluate the relationship between remodeling of vascular grafts and regeneration of vascular media layer, different samples were stained for α-SMA (early-stage markers of VSMCs) and calponin (middle-stage markers of VSMCs) by immunofluorescence assay. The results showed that the thickness of the smooth muscle layer and the number of α-SMA$^+$ cells in SF/fibrin grafts were significantly higher than those in SF grafts at 5 months (Figure 6E and F). Meanwhile, the number of α-SMA$^+$ cells in SF/fibrin grafts was close to that of the native artery group (Figure 6F–H). Interestingly, we found that the lumen surface of SF grafts only formed a thin layer of smooth muscle media layer (Figure 6E). Furthermore, we observed from the results that the SF/fibrin graft wall and native artery at 5 months had abundant distribution of Calponin$^+$ cells (Figure 6J and 6K). The number of Calponin$^+$ cells of SF graft was lower than the other two grafts (Figure 6I–L). SM-MHC is a late-stage differentiation marker of VSMCs. The results confirmed the high expression of SM-MHC in SF/fibrin graft, which indicated that the grafts had contractile phenotype function (Figure 6N). Meanwhile, there was statistical significance between SF group and SF/fibrin group, and between SF group and native group, respectively (Figure 6M–P).

ECM Deposition in SF/Fibrin Grafts

ECM remodeling can improve hemodynamics and regulate vascular regeneration. To better understand the physiological function of ECM, we investigated the ECM deposition (collagen, elastin and GAG) of vascular grafts at 5 months. The ECM deposition in SF grafts was only sparse, while the ECM deposition in SF/fibrin vascular grafts was denser and more orderly (Figure 7). The results showed the presence of fibrillar collagen and it was arranged in a circumferential appearance within the lumen of all grafts. We observed that the collagen layer of the SF/fibrin neoarteries was comparable to that of native artery (Figure 7A–C). Verhoeff's staining experiments showed that there was a continuous elastic layer in the PCL/fibrin neoartery wall (Figure 7D–F). The safranin O staining experiment showed that the PCL/fibrin neoartery wall had glycosaminoglycans (GAGs), which were located near the luminal side similar to native artery (Figure 7G–I). Simultaneously, the quantitative analysis results showed that the content of ECM deposition (collagen, elastin and GAGs) in SF/fibrin grafts was higher than those in SF grafts at 5 months. Moreover, the content of elastin and GAG in SF/fibrin grafts and SF grafts were lower than those in native artery (Figure 7J–L). At 5 months, the ECM deposition content of different groups (SF group, SF/fibrin group and native artery group) was statistically significant.

The Vascular Density and Calcification of Different Grafts

We assessed the microvessels generation of different vascular grafts at a specific time (1, 3 and 5 months) by CD34 antibody staining. We could see from the results that the vascularization content in SF vascular grafts was much lower than that of in SF/fibrin vascular grafts at 3 months after transplantation (Figure 8A and B). Here, the brown color in the images indicated microvessels with CD34-stained. Meanwhile, we used ImageJ software to detect the vascular density value of the grafts in these images. The results revealed that the vascular density values in SF grafts increased over time. Interestingly, we observed that the vascular density values of PCL/fibrin vascular grafts increased first at 1 and 3 months, and then decreased at 5 months (Figure 8C). Calcification is an important index of vascular graft regeneration. The definition of calcification refers to the transformation of VSMCs into chondrocytes, leading to chondroid metaplasia and then the formation of calcified areas. Calcification can decrease compliance and increase stiffness, which can further cause vascular graft failure in vivo transplantation. Black indicated calcification point, and the darker its color, the more severe the calcification. The calcification of SF/fibrin graft was located around the lumen, and the degree of calcification in the graft was significantly less than those in SF graft. (Figure 8D and E). Furthermore, we also observed that the calcification area of SF/fibrin grafts and SF grafts at 3 months was the highest, compared to those at 1 and 5 months (Figure 8F). The SF group and SF/fibrin group at different time points (1, 3, and 5 months) were considered to have statistical differences.

Discussion

Many researchers have confirmed that SDVG should have excellent biocompatibility, histocompatibility, degradation performance, mechanical strength, and anti-thrombotic.[29,30] Ideally, the vascular graft should be a microstructure relative to native arteries, which can facilitate the conversion of metabolites and the regeneration of ECs and VSMCs.[31]

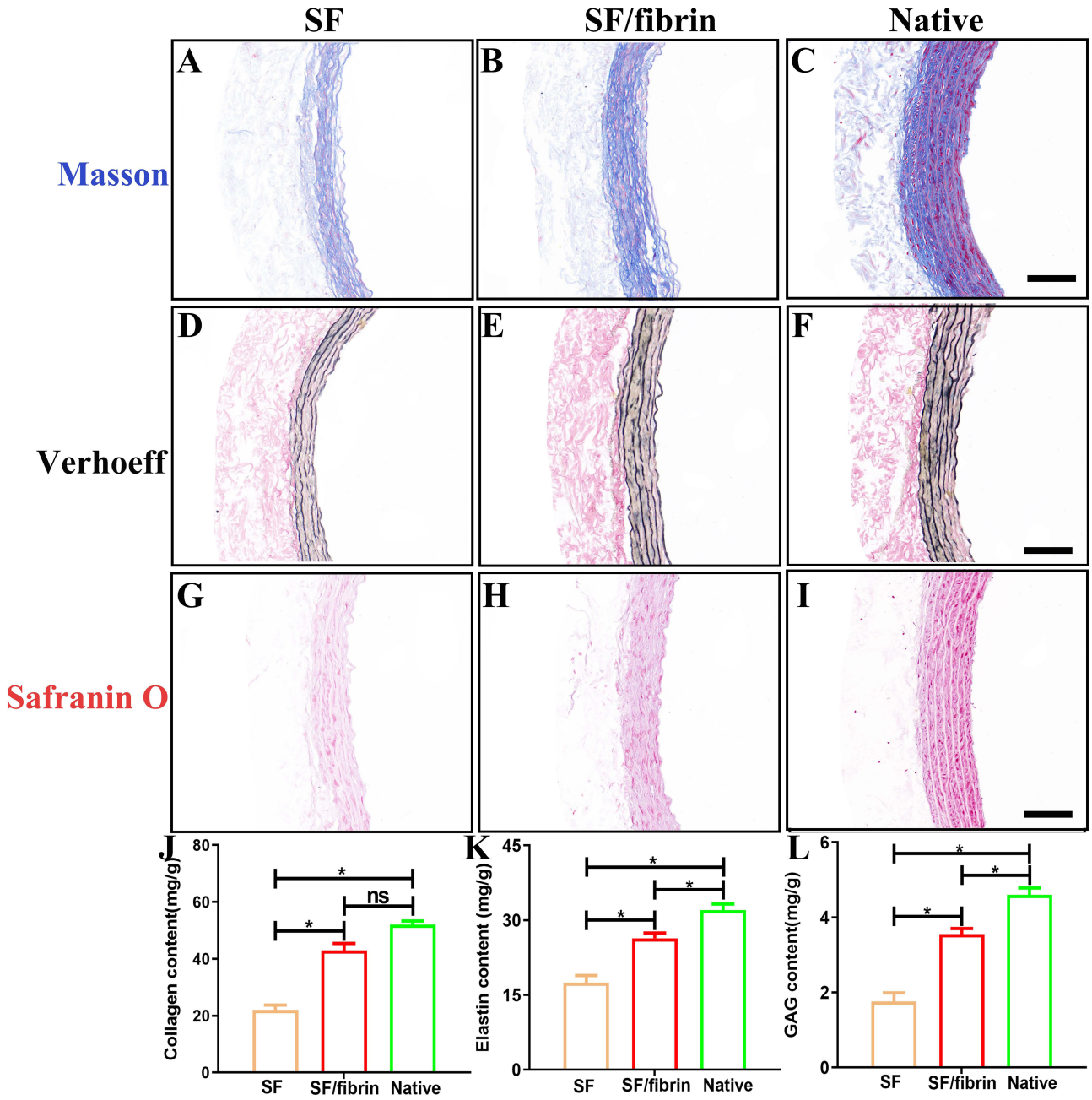

Figure 7 ECM deposition within different grafts at 5 months. Microscopic image of different samples stained by Masson's trichrome for collagen (blue, **A-C**), Verhoeff's for elastin (black, **D-F**) and Safranin O for Glycosaminoglycans (red, **G-I**), respectively. Quantification of Collagen Elastin, and GAGs expression (**J-L**) (n = 3). Scale bar: 50μm.★indicated $P < 0.05$. ns was not statistically significant.

Furthermore, electrospinning is widely recognized as an ideal method for the preparation of vascular grafts. The instrument can better control the diameter and pore size of nanofibers, resulting in a larger specific surface area to benefit the mimicry of natural ECM.[32] In our study, we successfully fabricated SF/fibrin tubular grafts with ideal nanostructures by electrospinning equipment. Subsequently, the vascular graft was transplanted into the abdominal aorta of SD rats at a certain time period (1 month, 3 months and 5 months). We did not observe thrombosis and intimal hyperplasia formation in the lumen of SF/fibrin vascular grafts (Figure 2A-C). However, the structure of the neoarteries at 5-month was corresponded to native arteries. These results demonstrated that SF/fibrin graft was suitable as an SDVG. The indicators of thrombosis and occlusion in grafts have been explored, which is of great significance for the transplantation in vivo of artificial synthetic vascular grafts.[33] Figures 2D and E successfully demonstrated that the

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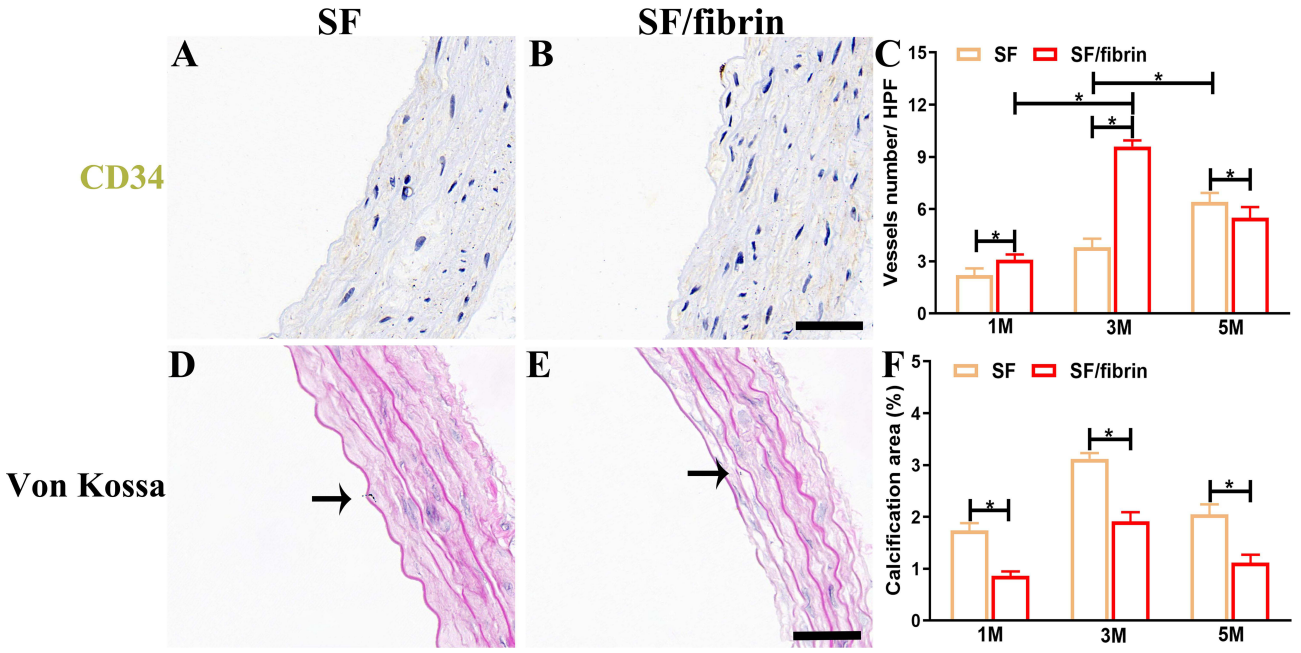

Figure 8 Vascular density and calcification formation in different graft. Images of SF graft and SF/fibrin graft at 3 months stained by CD34 antibody (**A** and **B**). Vessels numbers in grafts at different time points was evaluated (n = 3) (**C**). Images of different samples were stained with Von Kossa reagent (**D** and **E**). Black arrow denoted calcification. The Calcification area of the graft at 1, 3, and 5 months was measured (**F**) (n = 3). Scale bar:50μm.

viability and patency in SF/fibrin vascular grafts were much greater than those in SF grafts, but their occlusion and thrombosis values were lower than those in SF grafts. Moreover, these results were further confirmed by Doppler ultrasound (Figure 3A-B). We analyzed that SF/fibrin grafts with good hydrophilicity and degradation properties were conducted to improve tissue compatibility and physicochemical structure, thereby greatly reducing the incidence of occlusion and thrombosis.

Compliance refers to the change of the volume, length and diameter of the vascular graft wall under the action of blood pressure. The properties of the material, the thickness and structure of the graft wall are the main factors that affect the compliance value of the artificial vascular.[34] Meanwhile, the compliance of vascular grafts promotes mature VSMC phenotype and ECM deposition, and regulates macrophage phenotype. In our study(Figure 2F), we observed that the compliance values of SF/fibrin grafts (18.2 ± 1.58% at 3 months, 27.4 ± 1.36% at 5 months) were much higher than those of native arteries (11%). We analyzed that SF/fibrin grafts with thinner luminal walls and larger pore sizes may have effectively improved their compliance values. Superior mechanical strength is one of the necessary conditions for constructing artificial vascular. The ideal mechanical performance can ensure that the vascular graft in vivo withstand the impact of blood flow, thereby maintaining its patency and stability.[35] The mechanical strength of grafts is closely linked to the formation of ECs, VSMC, ECM deposition and the phenotypic transformation of macrophages. Zhao et al[25] found that the breaking strain, elastic modulus, and maximum stress values of electrospun PCL/fibrin vascular grafts at 9 months were 135 ± 18.98%, 3.12 ± 0.34Mpa, and 3.43 ± 0.425Mpa, respectively. In this study, we could observe from the results that the mechanical performance of SF/fibrin vascular grafts at 5 months and native artery were maximum stress (3.65 ± 0.32Mpa) VS (3.59 ± 0.47Mpa), breaking strain (128 ± 6.1%) VS (131 ± 11.6%), and elastic modulus (3.54 ± 0.41Mpa) VS (2.78 ± 0.33Mpa), respectively. This may be due to the good biomechanical properties of SF itself. These results confirmed that the mechanical properties of SF/fibrin graft could fulfill the requirements of vascular transplantation, and it was significantly better than PCL/fibrin graft. Furthermore, there were no significant differences in the lumen diameter of vascular grafts in vivo at different time periods under Doppler ultrasound instruments. This series of results indicated that the mechanical strength of SF/fibrin vascular grafts in vivo was suitable for the requirements of artificial vascular. We speculated that this may be due to the addition of SF with good mechanical strength, which further enhanced the mechanical properties of the composite graft.

Actually, exceptional researchers have demonstrated that well-designed vascular grafts can effectively regulate the control of phenotypic switching of macrophages in regenerative medicine, thereby achieving ideal vascular regeneration and remodeling of the graft.[36] Meanwhile, appropriate macrophages can effectively improve ECM deposition, ECs and VSMC regeneration ability, which is very important for the vascular grafts neoarteries in vivo.[37] Zhang et al[38] successfully prepared a composite vascular graft based on textile fiber and hydrogel matrix. The experimental results showed that the textile reinforced hydrogel vascular graft could enhance macrophage activation and upregulated the expression of inflammatory factors, thereby greatly improving its endothelial regeneration. In our study, SF/fibrin vascular grafts with a microfiber-oriented arrangement could promote cell adhesion and migration. Simultaneously, the expression level of CD68 positive macrophages in vascular grafts gradually decreased over time (Figure 4A). The remodeling of inflammatory macrophages in SF/fibrin neoarteries at specific time was higher than that in PLCL grafts.[12] Additionally, we were surprised to find that the ratio values of M2/M1 macrophages in SF/fibrin grafts were higher than those in SF grafts at three different time periods (1, 3, and 5 months). This series of results indicated that the sustained and stable degradation of vascular grafts in vivo can lead to changes in mechanical strength, thereby further inducing their macrophage phenotypic transition. At the different stages of inflammation, the phenotype of vascular graft macrophages has different M1 and M2 phenotype. The results suggested that the expression levels of inflammatory cytokines in SF/fibrin neoarteries at 5 months were similar to those in native artery. We speculated that it may be the coordination between M1 and M2 to obtain a stable state of vascular remodeling process.

In the remodeling of vascular graft, regenerated neoartery in vivo should have certain vascular functionality. Zhu et al[39] successfully reported that electrospun PCL vascular grafts showed significant vascular function of contraction and relaxation under the action of drugs. In our studies, we observed that SF/fibrin grafts at 5 months exhibited strong relaxation and contraction function properties under corresponding drug stimulation. However, the functionality of SF vascular grafts was relatively weaker (Figure 3D and E). This functional difference indicated that the regeneration ability of the ECs layer and VSMC layer of SF/fibrin grafts was different from that of SF grafts. The reason for this result may be that SF with slow degradation, poor hydrophilicity and strong substrate stiffness would prevent the function of regenerated vascular. The construction of a complete confluent structure of the endothelial layer can significantly reduce intimal hyperplasia and thrombus formation.[40] Active ECs can stimulate the proliferation and adhesion of SMCs, and then ECM production. In this study, we observed the formation of continuous intact and healthy monolayer of ECs in SF/fibrin vascular grafts at 5 months, but the ECs layer in SF grafts was very thin. At the same time, the ECs in SF/fibrin graft at 5 months were very similar to native endothelium, and their morphology was like cobblestone (Figure 5). The reason for this endothelialization difference may be that the hydrophilic SF/fibrin vascular grafts can promote the migration, proliferation and adhesion of ECs. Meanwhile, the endothelialization regeneration of the scaffold was higher than that of the fiber-angle PCL fiber skeletons vascular scaffold.[9] During the regeneration process, VSMCs with contractile and synthetic phenotype of vascular media can regulate the proliferation of ECs and ECM. Moreover, the mechanical strength, degradation performance and pore size of vascular grafts can influence the biological behavior of VSMCs.[41] In our study, the results showed that the number of α-SMA, Calponin and SM-MHC positive cells in SF/fibrin grafts were higher than that in SF grafts at 5 months (Figure 6). This experimental data successfully confirmed that SF/fibrin grafts formed the neoartery with vascular activity in vivo and completed the phenotypic transition process.

In the process of tissue regeneration, the mechanical strength changes of neoarteries plays a very important role compared to native arteries. Reasonable ECM deposition can significantly increase the mechanical properties of graft.[42] Meanwhile, the ideal pore size and the structure and morphology of vascular materials can influence the regeneration of ECM deposition.[43] In this study, we successfully prepared SF/fibrin vascular grafts that mimic native arteries. Our results showed that the lumen layer of the SF/fibrin graft had circumferential arrangement of ECM deposits, which were similar to native arteries at 5 months (Figure 7). The structural distribution of this vascular graft could promote the formation of ECM deposition (collagen, GAG and elastin), which was closed to the mechanical properties of native arteries. Simultaneously, we also analyzed that the undegraded fibrous neoarteries may be closely related to the mechanical strength of ECM deposition.

The definition of microvessels refers to single vessels or network vessels with permeable lumen. In the process of constructing tissue engineering microvessels, the structure and function control are important problems.[44] In addition,

synthetic materials with microvascular growth can promote the proliferation, diffusion, and adhesion of ECs.[45] In our study, the microvessels values of SF/fibrin vascular grafts at 1 month and 3 months were greater than those of SF grafts (Figure 8A–C). These experimental results indicated that SF/fibrin vascular grafts with functional microvessels may achieve vascular regeneration during in vivo transplantation. For the above results, we analyzed that SF/fibrin vascular grafts with good structure significantly improved microvessels formation, which may be due to the addition of fibrin material with good biocompatibility.

Calcification is an important factor to evaluate the success of vascular grafts. Some research reports have confirmed that calcification of vascular grafts was detrimental to vascular transplantation.[46] Ideal biomaterial design can significantly reduce the formation of calcification due to chronic foreign body reactions. Professional researchers have found that some vascular grafts (for example, polyglycolic acid and polydioxanone) with high cellular infiltration can effectively reduce the risk of calcification.[47,48] Furthermore, the imbalance of macrophage regulatory ability of vascular grafts can increase inflammation associate formation of pathological calcification.[49] In this study, we observed from the results that the calcification area of SF/fibrin vascular grafts was lower than that of SF grafts at three different time points (Figure 8D-F). We analyzed that the reason may be that electrospun SF/fibrin vascular transplantation had a good scaffold material structure, biocompatibility and reasonable regulation of macrophage ability, which significantly improved the formation of calcification.

Conclusions

In this study, the electrospinning technology was used to prepare a circumferentially arranged tubular SF/fibrin vascular graft with good biocompatibility, histocompatibility and appropriate degradability. A series of in vivo experiments successfully confirmed that SF/fibrin vascular graft at 5 months had superior mechanical strength, good patency and functionality similar to native arteries. Furthermore, regenerated SF/fibrin neoarteries could reasonably regulate macrophages, highly expressed VSMCs, effectively promoted ECs, rapid ECM deposition, improved microvessels formation, and rare calcification, compared to SF grafts. In conclusion to this, our study provides a novel strategy for vascular tissue regeneration, and the vascular graft has the potential to become a small diameter tissue engineering vascular graft.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

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Disclosure

The authors declare that they have no conflicts of interest or other associative commercial interests to influence the work submitted and reported in our article.

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