Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Experimental study of slow-released Angiogenin by silicon micro-needle in improving blood support to multiple-territory perforator flaps in rats

Xiaojun Liu[1], Xue Wang[2], Rui Zhao[2], Yunfeng Liu[2], Wei Chen[3], Jiandong Su[2], Guoliang Shen[1,*]

[1] Department of Burn and Plastic Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215000, Jiangsu, China
[2] Department of Burn and Plastic Surgery, Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou 215000, Jiangsu, China
[3] School of Physics Science and Technology, and Jiangsu Key Laboratory of Thin Films, Soochow University, Suzhou 215006, China

Article Info

Article history:

Received: December 14, 2023
Accepted: March 05, 2024
Published: June 30, 2024

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Abstract

This research aimed to investigate the effect of slow-released angiogenin by silicon micro-needle on angiogenesis in the Choke zone of dorsal multiple-territory perforator flap in rats, as well as its mechanism. Thirty-six adult Sprague-Dawley (SD) rats were randomly divided into control group, model group, and four experimental groups. In model group, slow-release saline through a silicon micro-needle was placed in choke II zone of the flap 7 days before the operation. For rats in four experimental groups, angiogenin was released via micro-needle in the choke I and choke II zones of the cross-zone flap 7 days before and 3 days before flap surgery, respectively. A 12 cm × 3 cm cross-zone perforator flap model was made on the back of all five groups. The flap survival rate in slow-release angiopoietin group was statistically higher than that in model group ($P<0.05$). Angiogenin in choke zone of the flap was increased in slow-release angiogenin group ($P<0.05$). In slow-release angiogenin group, the micro-vessel density was increased and the arteriovenous diameter was decreased, while the arteriovenous diameter was increased in model group ($P<0.05$). The levels of vascular endothelial growth factor A (VEGF-A) and angiotensin 1 (ANG-1) in choke zone were both elevated in slow-release angiogenin group ($P<0.05$). The expression of CD31 was significantly elevated in flaps of experimental groups ($P<0.05$). Micro-needle to slow release Angiogenin can increase the drug concentration in the tissues of the choke zone, promote the vascularization of rat dorsal crossover area perforator flap, reduce the possibility of flap ischemic necrosis, and improve the flap survival rate.

Keywords: Silicon micro-needle, Angiogenin, Angiogenesis, Multiple-territory perforator flap, Choke zone

1. Introduction

Perforator flaps have been one of the main methods for repairing deep wounds and body surface organ reconstructions since their discovery, but large cross-zone perforator flaps are prone to necrosis in the potential donor area (choke II zone) when pedicled with an anatomical resource artery [1, 2]. If the subcutaneous plexus of the perforator flap is reconstructed and transasphyxiated blood flow is formed, the flap flow is usually safe [3-5]. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two examples of pro-angiogenic substances that are involved in angiogenesis. However, the newly built vessels face the risk of structural defects and easy leaks [6-8]. Angiopoietin is also present in normal human circulation in amounts measured in milligrams to promote angiogenesis. However, it can be rapidly degraded *in vivo* by antagonistic molecules, requiring effective slow-release vehicles to increase local concentrations [9]. Currently, many carriers allow the slow release of drugs and hollow silicon microneedles have become an effective choice [10]. The main objective of this experiment was to demonstrate whether slow-released angiogenin through hollow silicon microneedles in the choke zone of a multiple-territory perforator flap in rats could improve blood support by promoting vascularization and to interpret its mechanism.

2. Materials and methods

2.1. Experimental materials

A total of 36 male SD rats (230-270 g each) were provided by Hunan SJA Laboratory Animal Co. Ltd, License No. SCXK (Xiang) 2019-0004. The rats were fed at a temperature of 20-26°C and a humidity of 40-70%.

2.2. Experimental grouping

1) The control group, without any operation or drug treatment

2) The model group, where slow-release saline by hollow silicon micro-needle was administered in the choke II zone for 7 days

3) The experimental group 1 (choke I 3d): silicon micro-needle injections containing angiogenin were admi-

* Corresponding author.
E-mail address: sdfyysgl@outlook.com (G. Shen).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.6.4

nistered in the choke I zone for 3 days.

4) The experimental group 2 (choke I 7d): silicon micro-needle injections containing angiogenin were administered in the choke I zone for 7 days.

5) The experimental group 3 (choke II 3d): silicon micro-needle injections containing angiogenin were administered in the choke II zone for 3 days.

6) The experimental group 4 (choke II 7d): silicon micro-needle injections containing angiogenin were administered in the choke II zone for 7 days.

2.3. Reagents and devices

Silicon microneedle (volume 0.5 ml, provided by the customer, as shown in Figure 1), Angiogenin (Solarbio, P05632), CD31 (GB11063-2, Servicebio), Cy3 Goat Anti-Rabbit IgG (As007, ABclonal), ready-to-use DAPI staining solution (KGA215-50, Keygentec), ultra-clean advanced sealing gel (YZB, BASO), pharmaceutical refrigerator (BYC-310,Biobase), electric thermostatic blast drying oven (HGZF-101-1, Shanghai Yuejin Medical Instrument Co., Ltd.), fluorescent microscope (CKX53, OLYMPUS), microtome (BQ-318D, Bona), thermostat incubator (DHP-9054, Biobase), pipette gun (Research plus 0.5-10 μL, eppendorf), pipette gun (Research plus 20-200 μL, eppendorf), pipette gun (Research plus 100-1000 μL, eppendorf), pressure cooker(YS20ED, Supor), induction cooker (HK-22, Zhongshan Dongfeng Town Hanke Electric Appliance Factory), fume hood, Scott bluing solution (G1865, Solarbio), VEGF-a (AF5131, Affinity), ang-1 (AF5184, Affinity), IgG (H+L) (HRP-labeled Goat Anti-Rabbit (ZB-2301, Zhongshan Golden Bridge), DAB chromogenic kit (CW0125, CWBIO), neutral resin (CW0136, CWBIO), hematoxylin staining (AR1180-1, Bioforce), rat angiogenin kit (2022/01, enzyme-linked), and automatic enzyme-linked immunosorbent assay (ELISA) analyzer (WD-2102B, Liuyi).

2.4. Rat flap model construction

The rats were given general anesthesia by intraperitoneal injection of 1.8% chloral hydrate at a dose of 0.3 mg / 100 g body weight. After successful anesthesia, the back of the rat was shaved, the flap design was drawn, disinfected with iodophor, and a curtain was placed, preferably on the back. The iliolumbar artery was used as the pedicle, the flaps with a length of 9-11 cm and a width of 3-4 cm were designed to pass through the posterior intercostal artery and reach the thoracic dorsal artery. The skin and subcutaneous tissue were cut to the surface of the deep fascia layer according to the design line (Figure 2). The flap was raised, hemostasis was thoroughly stopped, and the posterior costal artery and thoracolumbar artery were lapped to form a cross-zone perforator flap with iliolumbar artery as the pedicle for blood supply. The flap was immediately sutured with 3-0 silk thread. The blocking zones (blocking zones I and II) were marked.

2.5. Flap survival rate

7 days postoperatively, flap color, capillary reaction, floral spots, and necrosis were observed visually. On the 7th postoperative day, the dorsal flap of rats was photographed using the same anesthetic method and imported into Image ProPlus 6.0 image analysis software to measure the flap survival area and calculate the flap survival rate (flap survival rate = viable flap area/total flap area × 100%.

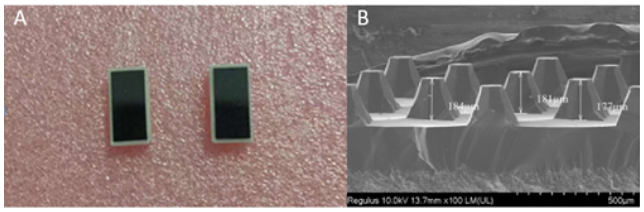

Fig. 1. (A) Microneedles; (B); Microneedle under an electron microscope.

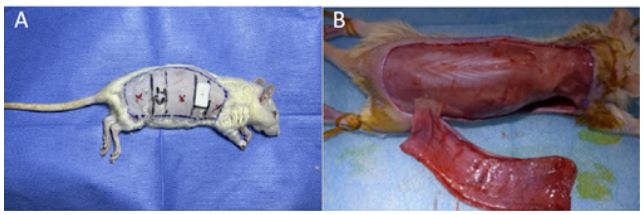

Fig. 2. (A) Microneedle was placed in the choke zone; (B) Flap was formed with the iliolumbar artery as the pedicle.

2.6. ELISA

Ag preparation: 3 ml of N.S was added to the tubes containing defibrillated SRBC and mixed before being centrifuged at 2000 rmp for 5 min. 3 ml N.S was added to the tube and mixed, then 2 ml packed SRBC was taken. We added 2 ml DDW and shattered SRBC and diluted with coating buffer in a ratio of 1:400. Coat Ag: We added 100 μL of Ag to each well of ELISA plate and washed with N.S five times. Prepare the serum: We removed the eyeball of the SRBC-immunized mice and collected the blood into Ep tube, and waited for about 5 min before centrifuging the blood at 12000 rmp for 10 min. Then, supernatant was removed and diluted the serum was into 1:10. Add test serum: We signed and added 100 μL of solution to each well, then put it in a humidified box under 37°C for 45 min. Add secondary Ab: We discarded the solution in ELISA plate and washed the plate 3 times. Then we added 100 μL of HRP-labeled secondary antibody to each well and put it in a humidified box under 37°C for 30 min. Show color: We discarded the solution in ELISA plate and washed the plate 3 times before adding 100 μL of substrate solution to each well so that the color was displayed in the dark for 10 min.

2.7. Hematoxylin and eosin (H&E) staining

Sacroiliacs were performed on animals on POD7 and tissue samples were collected from the flap SCZ (1 × 1 cm) for each group of 6 animals. The samples were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin before being fixed on a microtome and cut into 5-μm-thick slices, which were subsequently stained with an H&E staining kit. We manually counted the number of micro-vessels per unit area (/mm^2) under an optical microscope (× 200 magnification, Olympus, Tokyo, Japan) to calculate the micro-vessel density (MVD). Counts were performed using six random fields from three random sections of each tissue sample.

2.8. Immunohistochemistry (IHC)

Sections from the flap SCZ in each group (n=6) were deparaffinized with xylene and rehydrated in a graded

ethanol series. After washing, endogenous peroxidase activities were blocked with 3% H_2O_2 and antigen retrieval was carried out in 10.2 mM sodium citrate buffer at 95°C for 20 min. After blocking with 10% normal goat serum for 30 min, the sections were incubated with antibodies against CD34 (1:100, Abcam), VEGF (1:200, Abcam), CDH5 (1:100, Abcam), cleaved caspase-3 (1:100, Abcam), SOD (1:100, Abcam), and p62 (1:100, Abcam) overnight at 4°C. Finally, the sections were incubated with horseradish peroxidase-labeled goat anti-rabbit antibody (1:3000, Zhongshan Golden Bridge) and counterstained with hematoxylin. The sections were imaged with a DP2-BSW image-acquisition system (Olympus) at × 200 magnification. Integral absorbance quantitation with Image-Pro Plus v6.0 software was performed to compare CD34-positive blood vessels and protein expression levels. Six random fields were counted from three random sections of each tissue sample.

2.9. Immunofluorescence staining

Skin flap sections (n=6) were deparaffinized and rehydrated in a graded ethanol series and incubated in 10% normal goat serum containing 0.3% Triton X-100 for 30 min at room temperature. After blocking, sections were incubated with primary antibody LC3B (1:100, Abcam) overnight at 4°C, followed by incubation with tetramethylrhodamine-labeled goat anti-rabbit IgG antibody (1:100, Bioworld Technology, Nanjing, China) at room temperature for 1 hour. An anti-fluorescence quenching agent was added after 2 min of DAPI staining. Six random fields were used from three random sections of each tissue sample.

2.10. Statistical analysis

SPSS 19.0 software was used for statistical analysis. All experiments were repeated three times and quantitative results were expressed as mean ± standard deviation (X ± S). One-way ANOVA was used for comparison of quantitative values between multiple groups, and the LSD method for two-way comparisons. Tests were conducted at α=0.05 level and plotted using Graphpad 8.0.

3. Results

3.1. Flap survival rate

Flap survival rate of the different groups was shown in Figure 3. Compared with the control group, flap survival rate of rats was significantly lessened in the model group (P<0.05). Compared with the model group, flap survival rate was significantly higher with Angiogenin in the cross-zone flap choke I and choke II zones after slow release 3 and 7 days before flap elevation (P<0.05).

3.2. ELISA

ELISA detected the content of Angiogenin in skin tissues, as shown in Figure 4. Compared with the control group, the concentration of Angiogenin was significantly lessened in the model group (P<0.05). Compared with the model group, the concentration of Angiogenin in the choke I zone and the choke II zone was significantly raised after 7 days of microneedle injection of Angiopoietin, and the concentrations were higher than 70 ng/ml in the rats (P<0.05), which also suggested that slow-release Angiopoietin could lead to higher local concentrations in tissues without immediate degradation and that increased concentrations of Angiopoietin could have a pro-Angiogenic effect.

3.3. H&E staining

The diameters of veins and arteries were detected by H&E staining, as shown in Figure 5. Compared with the control group, the vein and artery diameters were increased in the model group and decreased in four groups of the choke I and choke II zones after 3 and 7 days of injection, respectively (P<0.05). As the flap had not yet been shaped and blood flow was not yet blocked, the increased

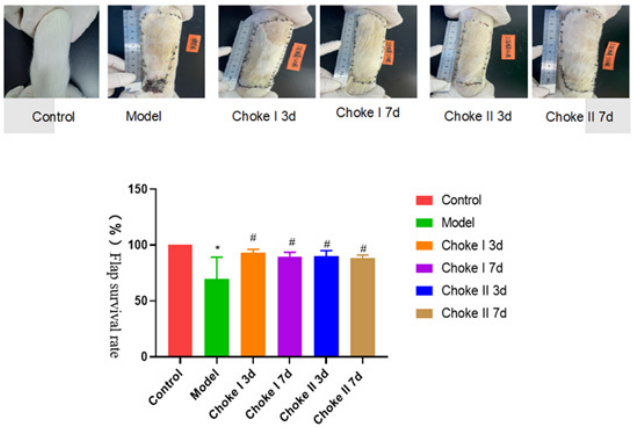

Fig. 3. Flap survival rate (* compared with the control group, P<0.05; # compared with the model group, P<0.05).

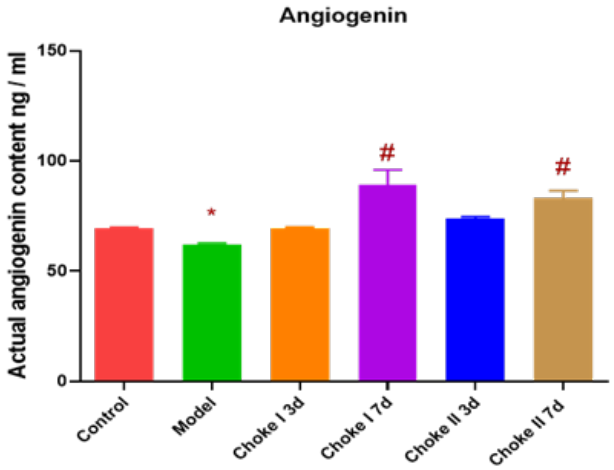

Fig. 4. Angiopoietin concentration in each group (* compared with the control group, P<0.05; # compared with model group, P<0.05).

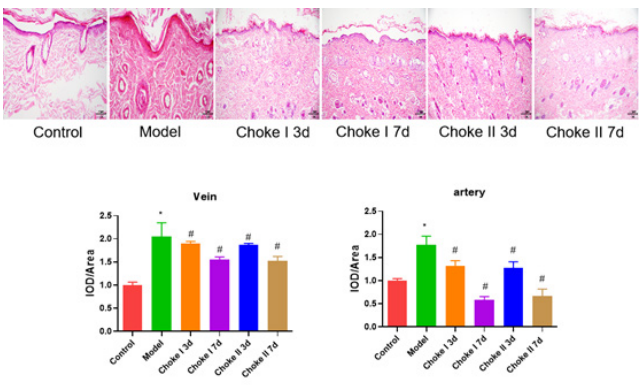

Fig. 5. H&E staining for each group.

vascular density after revascularization led to a reduction in luminal canal diameter rather than expansion. This also suggested that actively increasing micro-vascular density was more effective in improving blood supply than passive immediate lumen expansion due to ischemia after flap formation. The results indicated that it was effective and beneficial to improve the subcutaneous plexus vascular bed and increase the vascular density before the flap formation, which provided a new way for the prefabrication of the flap.

3.4. Changes in VEGF-A and ANG-1 pro-angiogenic factor levels

The expression of pro-Angiogenic factors was detected by immuno-histochemistry, and the results are shown in Figure 6. Compared to the control group, the levels of VEGF-A and ANG-1 in the model group declined (P<0.05). The levels of VEGF-A and ANG-1 were significantly higher in choke I zone and choke II zone after 3 and 7 days of microneedle injection of Angiogenin, respectively, compared to the model group (P<0.05).

3.5. CD31 expression

CD31 expression was detected by immunofluorescence, and the results are shown in Figure 7. Compared to the control group, the expression of CD31 in the model group declined (P<0.05). Compared with the model group, the expression of CD31 was significantly higher in choke I zone and choke II zone after 3 and 7 days of sustained release of Angiogenin, respectively (P<0.05), with the most significant effect in choke II zone after 7 days indicating the effect of promoting vascular proliferation.

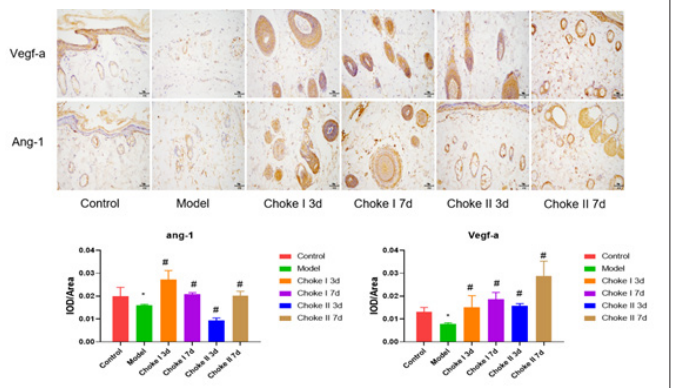

Fig. 6. Levels of VEGF-a, ANG-1 (* compared with control group, P<0.05; # compared with model group, P<0.05).

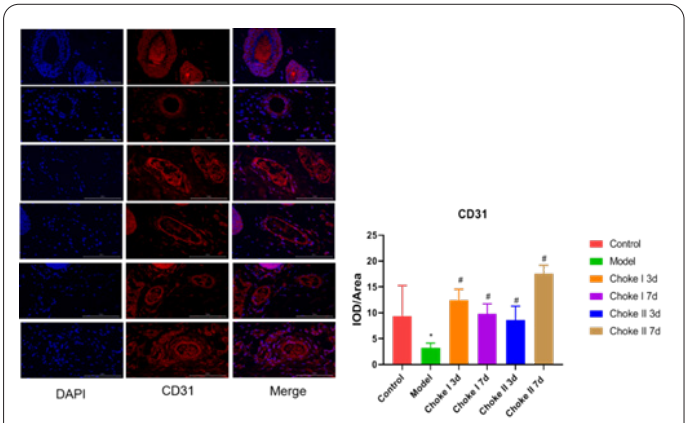

Fig. 7. Vascular proliferation in each group (* compared with control group, P<0.05; # compared with model group, P<0.05).

4. Discussion

Skin flap transplantation is the main method of deep wound repair and body surface organ reconstruction. With the advancement of this technique, perforator flaps have become extensively used for their reliable blood supply without sacrificing major vessels in the donor area and without causing minor muscle destruction [11]. However, the area of a single original arterial perforator flap is usually small. The perforator vessels in a specific vascular body area (known as angiosome) are distributed in a dendritic pattern towards the skin. Between the two adjacent perforasomes, rare micro-vessels restrict the flow of blood from one to another due to their small caliber and inability to expand timely, which is referred to as the choke zone. Therefore, in a multiple-territory perforator flap (also referred to as a cross-zone flap here) containing anatomical donor area, dynamical donor area, and potential donor areas, partial necrosis often takes place at the distal end of the flap after removal [4, 12].

In this experiment, we attempted to increase the density and diameter of blood vessels in the flap to obtain better support, and angiogenesis can play an important role [13, 14]. Angiogenesis is a biological process of growing new blood vessels on top of the original vascular structure, which consists of four typical stages [15]. There are few studies at home and abroad on the effect of related pro-angiogenic factors on the vascular system in the occlusion area of perforator flap. Lubiatowski P et al reported that the use of adenovirus-transfected VEGF gene therapy promoted the improvement of blood flow in the muscle flap. Among them, angiopoitin-1 alone could effectively increase the density of muscle flap capillaries, whereas the combination had no significant effect, suggesting that the vascular neovascularization procedure is complex and its regulation is not only related to the dose but also related to the successive effects of various factors [16]. However, it has not been able to prove whether there is angiogenesis in the choke II zone and what effect it has on the viability of the cross-zone flap. Therefore, the main purpose of this experiment was to screen out factors that could promote angiogenesis and improve the blood flow of the perforator flap by means of neovascularization. In addition, we explored an effective and safe percutaneous drug delivery system that allows local release of drugs in the choked area of the perforator flap.

Angiogenin was selected as an inducing drug because its role in angiogenesis is mainly as below: (1) its chemotactic and aggregation effect on endothelial cells; (2) promoting migration of vascular endothelial cells and formation of tubular structures, increasing the stability of vascular structures; (3) attracting perivascular cells (e.g., peritubular cells) to surround and support endothelial cells, promoting vascular remodeling and maturation, and maintaining the integrity of blood vessels [17, 18]. The mechanism is that angiogenin promotes the degradation of stromal membrane and extracellular matrix by binding to the membrane surface receptor a-actin, so that endothelial cells migrate to the perivascular tissue through the nuclear pathway to proliferate. This will activate the PI3K/AKT/mTOR signaling pathway, stimulate rRNA transcription and ribosome production, promote the proliferation, metastasis and maturation of vascular endothelial cells

and smooth muscle cells. Even though VEGF-induced angiogenesis still requires the involvement of angiogenin [19], angiogenin was introduced as the preferential pro-angiogenic factor in this study.

The perforator vessels emit multiple planar vascular plexuses, such as the subcutaneous plexus and the fascial plexus, on their way from the fascia to the skin. The subcutaneous plexus is the key to keeping the skin flap alive. Therefore, if the subcutaneous plexus can undergo hemodynamic reconstruction and form collateral branches that result in blood flow that can cross the obstructed area, the blood support of the flap can be ensured [4]. Thus, this experiment aimed to promote subcutaneous plexus revascularization and increase microvascular density. Accordingly, a hollow silicon micro-needle with a drug container, a slow-release system, is manufactured because it can painlessly puncture the epidermis and approach the subcutaneous plexus, allowing a sustained release of the drug, increase its local concentration in the dermis and subcutaneous layer, stabilize drug's action, avoid immediate dilution and increase the possibility of angiogenin-induced vascularization. The micro-needle production process has advanced production technology, good mechanical properties and laser perforation at the tip of the micro-needle. It has a height of more than 150 microns and can penetrate the stratum corneum so that the drug is released in the dermis through the micro-needle. Meanwhile, there is no obvious stinging sensation owing to the tip being less than 200 um to touch the nerve ending. It can be removed without residue, and the pure silicon material has no toxic effects on the human body, which facilitates clinical application. It can also be used to vaccine relocation, antitumor exosome delivery [20], antibacterial materials [21] and even micro-particle skin grafts. The micro-needle process will be further improved to increase the drug-carrying concentration and reduce allergy possibility, making it more suitable for extensive clinical applications [22, 23].

In this experiment, a rat dorsal triple-perforator flap was chosen and simulated for humans with multiple-territory perforator flap [24, 25], which could better demonstrate the density variations of blood vessels in the choke zone. This flap model is more in line with human anatomy and clinical application of perforator flap [26] and is consistent with studies of vascular neovascularization.

In this study, an increase in micro-vessel density and a decrease in vessel diameter were found, probably because the tissue was not yet hypoxic and blood flow was not yet altered prior to flap surgery, and if the number of vessels per unit volume increases due to angiogenesis, blood supply requirements could be maintained without expansion of the internal diameter of the vessels. Successively, as a new finding in the present experiment, its mechanism needs to be further elucidated. The present experiment also revealed that both ANG-1 and VEGF-A were elevated. In light of the initiating effect of Angiogenin, both VEGF-A and ANG-1 were involved in the process of neovascularization and play a regulatory role. It is generally believed that VEGF-A can promote EC cell migration and vascular leakage, while ANG-1 can maintain vascular stability [27, 28].

It has been confirmed in some literature that neovascularization requires inflammation [29, 30] or a hypoxic microenvironment [31-33] to be achieved. Previous simple injections were difficult to induce local inflammation, whereas silicon microneedle microarrays in the same plane may cause sterile inflammation in the dermis and subcutis, which may be an important provocation for neovascularization. Angiogenesis has a controlled, effective and graded release in the subcutaneous mass to improve blood flow in the perforator flap, which is the main innovation of this study. Our research is also expected to be applied to tumor therapy through microneedle drug delivery methods (e.g. exosomes, targeted drugs, chemical drugs, etc.) [34-36].

There are several limitations in our study. First, the synergistic effect of VEGF-A and ANG-1 on vascular formation and stability is not explored in this experiment. Besides, the relationship between local inflammation and vascular neovascularization is not explored in this study. All these limitations need to be further confirmed in subsequent experiments.

5. Conclusion

Angiogenin can promote angiogenesis and the sustained release of this pro-angiogenic molecule by hollow silicon microneedle can improve flap's blood support in the choke zone of rat dorsal cross-zone perforator flap, which is beneficial for prefabrication of skin flap. Thus, it can provide some guidance for clinical application of perforator flap.

Acknowledgements

We would like to The First Affiliated Hospital of Soochow University to approval our study.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Municipal Hospital of Suzhou City, Jiangsu Province (approval no. KL901188). All participants provided written informed consent prior to enrollment in the study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

LX conducted the experiments and wrote the paper; WX, ZR, LY, CW and SJ analyzed and organized the data; SG conceived, designed the study and revised the manuscript.

Funding

This work was supported by the Suzhou Science and Technology Project (No. SLJ2021018) and the Municipal Burn Clinic Centre of Suzhou (No. Szlcyxzx202105).

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