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Research

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Authors’contributions

ZF, FMM, and GLL performed cell isolation, including isolation of SVF cells and the cultivation of ADSCs. Characterization of the cells was performed by ZF and FMM. GLL, ZXH, and CHY analyzed and interpreted the results, including performing statistical analysis. ZF, FMM and GLL completed the draft manuscript. GLL and WGB revised the manuscript to its final version. All authors read and approved the final manuscript.

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Competing interests

All authors declare that they have no competing interests.

Ethics and approval and consent to participate

Patients provided informed consent. The ethical committee of the Shanghai Changzheng Hospital granted permission to collect the tissue and animal experiment. All procedures were carried out in accordance with the declaration of Helsinki in its latest amendment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Not applicable.

Footnotes

Zhou Feng，Fei Miaomiao and Gong Lunli contributed equally to this work；
Zhu Xiaohai and Cui Haiyan are co-responding author for this work，any questions please e-mail Prof. Zhu and Prof. Cui for detailed information.

Abstract

Background To investigate the role and difference of autologous stromal vascular fraction（SVF）cells and allogeneic adipose-derived stem cells(ADSCs) in full-thickness skin graft.

Methods SVF cells and ADSCs were prepared from the inguinal fat pad of the rats, and full-thickness skin on the back of the rats were transplanted in situ. then, SVF, ADSCs and PBS were injected under the graft. Gross survival, H-E staining, Masson staining, CD31 and VEGF immunofluorescence were observed on 3d, 7d and 14d respectively.

Results the survival rate of SVF group was the best, followed by ADSCs group ($p<0.05$). the H-E and Masson staining structures of the SVF group were better than those of the other two groups under the microscope. CD31 and VEGF expression in SVF group at 7 days and 14 days after surgery was more obvious than that in ADSCs group and PBS group.

Conclusion Subcutaneous injection of autologous SVF or allogeneic ADSCs cells can improve the survival rate of full-thickness skin grafts. In addition, the effect of autologous SVF cells is better than that of allogeneic ADSCs.

【Key words】Adipose-derived Stem Cells（ADSCs）；Stromal Vascular Fraction（SVF）；Full-thickness Skin Graft（FTSG）

Introduction

Skin graft is one of the most indispensable techniques in plastic surgery and dermatology(1).Generally skin grafts are classified as split-thickness or full-thickness grafts. Among them, as containing the entire dermis, full-thickness skin grafts is rich in elastic fibers, glands and capillary tissue structures , with less contraction and better color, appearance, firmness and flexibility after survival, Also, it is more wear-resisting and therefore more suitable to use in facial and load-bearing parts wounds including feet and hands. However,with the increase of skin thickness, it is more difficult to establish the new blood supply, which would result in the poor and delayed survive of the graft.Therefore, it is of great clinical significance to find a method that can promote vascular reconstruction of full-thickness skin graft to improve the survival rate of skin graft and accelerate skin graft healing.

For recent years, increasing attention has been put on adipose-derived stem cells (ADSCs) , which is considered as a source of cells with regeneration potential and has similar characteristics and abilities to mesenchymal stem cells (MSCs)(2-4). And it is theoretically feasible to be a new method to improve the effect of full-thickness skin graft.When deriving ADSCs, aqueous fraction is collected after enzymatic digestion of lipoaspirate, which is composed with ADSCs, endothelial precursor cells (EPCs), endothelial cells (ECs), macrophages, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes among others, knowned as stromal vascular fraction (SVF)(5).It is reported that SVF has similar and even better effects in the area of tissue regeneration compared with ADSCs(6-8). And it is the aim of this article to verify whether ADSCs had a positive effect on rat full-thickness skin graft transplantation, and to compare the effect of SVF cells with that of ADSCs cells.

Methods

Animals and Groups

A total of 26 male SD rats （6-8 weeks old），180-200g were purchased and retained under standard conditions of 12 h light and 12 h dark cycles at 21 ±2℃ and fed with sufficient amounts of food and water in the Animal Center of the Naval Military Medical University. 2 of them were reserved for isolation and culture of ADSCs while the remaining 24 rats were randomly divided into three groups(n=8) and treated with fresh-isolated autologous SVF cells (group A), allogeneic ADSCs (group B) and phosphate-buffered saline (PBS)(group C) after skin grafting as control respectively.

Isolation and Culture of ADSCs

After 2 SD rats were sacrificed, their inguinal fat pads were collected and repeatedly washed with PBS (GIBCO, USA).Then obvious vessels, fibers and other tissues were removed and the pads were repeatedly cut into paste. Equal volume of 0.25% type I collagenase(GIBCO, USA) was added and the paste-like fat was digested for about 50mins, during which it was shaked and blended 2-3 times to make sure fully contacts. Behind that, complete medium, which was composed with high-glucose DMEM medium(Hyclone), 10% fetal bovine serum(FBS, GIBCO ,USA) and 1% Penicillin-Streptomycin(Hyclone, USA),was added to terminate the digestion and the chyle liquid was filtered with a 70um micron strainer. The filtrate was centrifuged at 1200g for 5min and the supernatant was removed. The 1ml complete medium was used to resuspend the cell pellet. Appropriate amount of erythrocyte lysate(SIGMA, USA) was added and incubated at room temperature for 8min. Then the supernatant was centrifuged at 1200g for 5min again. Freshly separated SVF suspension was then obtained by resuspended precipitation in 1ml medium and inoculated in 10cm culture dishes to acquire ADSCs. The medium was changed every 2-3 days, and passaged at a ratio of 1:3 for about 1 week depending on the actual situation. For this experiment, ADSCs of the third generation was used .

Preparation of SVF Cell Suspension

Both sides of the groin fat of rats in group A were incised and the SVF cell suspension was collected according to the above method for later use.

Full-thickness Skin Graft and Cells Transplantation

After weighed and anesthetized with pentobarbital sodium intramuscular injection, rats were kept in prone position and the back hair in the back was removed with electric razor.The back area was fully exposed and disinfected with 75% alcohol. A circular incision with a diameter of about 2cm was made in the middle of the back and the full-thickness skin was lifted and obtained. The it was trimmed with a ophthalmic scissors carefully to remove the panniculus carnosus remained until the dermis could be seen and attentions should be given to prevent excessive clip. The residual panniculus carnosus within the wound was discarded and the underlying sarcomere was exposed. The skin grafts were then turned 180 °horizontally and implanted and immobilized in the wound with 4-0 sutures. Beneath the graft, autologous SVF cell suspension was injected for group A, the allograft third-generation ADSCs cell suspension (about 1 x 10^6/ml) for group B and PBS for group C respectively(Figure 1). “Tie-over dressing” was given and the animals were kept in single cage after operation for at least 3 days.

Observation, Sampling and Examination

At 3, 7, or 14 days after the surgery, the general conditions of the skin grafts were observed and one animal was randomly chosen and sacrificed in each group.The grafts and surrounding cutaneous tissues were cut off and sent for examination. The main observation indexes were listed as follow:1. Hematoxylin and eosin (H&E) staining. 2. Masson’s trichrome staining;3. CD31 and VEGF immunofluorescence: three samples of high-power microscope were randomly selected and their fluorescence OD values were calculated by Image J software(National Institutes of Health, USA). Meanwhile, the OD values of normal rat skin were recorded. For better evaluation, the specific OD values were calculated by ratios of OD values of the samples to that of the normal skin. 4. Skin necrosis rate: the necrotic parts of the grafts as well as area with obviously poor blood supply were marked and the necrosis rates were calculated by Image J.

Statistical analysis

SPSS 20.0 software(IBM, USA) was used for statistical test analysis. t-test was used between paired samples while anova was used between groups. $p<0.05$ was considered statistically significant.

Results

General Conditions (Figure 2)

Necrosis of the full-thickness skin grafts was observed in all three groups, especially the margins of the skin grafts. Comparatively, the necrosis rate was lower and the survival rate of group A was better than those of group B and C. Three days after the operation, some areas were seen obvious congestion, followed by skin necrosis. At 14 days after surgery, as shown in the figure, the grafts from group A had better skin survival, more natural colour as well as tender tactile impression. However, there was also coagulative necrosis around the skin and the shape of the skin graft was changed. Some parts of the graft in group C were dry and deformed and touched harder as well. It was also found that the surrounding skin appeared obvious contraction, just like an "insertion" into the space between the grafts and the wounds, resulting in the upwarping of the peripheral part of the cutaneous tissue as well as necrosis. The gross conditions in group B was between the groups mentioned above, with some necrotic foci in the center of the grafts.

Necrosis Rates of the Full-thickness Skin Graft（Table 1）

Pairwise comparison of the above data (paired sample t-test) :

3d after surery: compared with group C, p=0.00 in group A; Compared with group B, p=0.00 for group A; Compared with group C, p=0.06 for group B; 7d after surgery: p=0.00 compared with group C in group A; Compared with group B, p=0.01 for group A; Compared with group C, p=0.01 in group B; 14 days after surgery: compared with group C, p=0.00 in group A; Compared with group B, p=0.00 for group A; Compared with group C, p=0.06 for group B.

Anova was used between the groups, and F values at the three observation time points of 3d, 7d and 14d were 76.84, 68.30 and 21.08, respectively. $p=0.00<0.05$, all of which were statistically significant.

According to the data, the skin necrosis rates at the three observation points in group A were 25.29±4.54%, 28.18±4.76% and 32.17±6.16%, respectively, which were better than those in group B (46.42±3.95%, 51.86±5.53% and 59.68±5.70%), and those in group C (51.91±5.04%, 62.63±5.33% and 71.51±12.70%), with significant statistical significance. The skin necrosis rate of group B and C was statistically significant at 7d, but there was no significant difference between 3d and 14d postoperatively.

H-E Staining

As shown in Figure 3, 3d postoperative H-E staining (40×) indicated that no obvious abnormalities were seen in the skin grafts in group A(a). The specimen of group B and C showed obvious structures from subcutaneous tissues (b, c).

It was also seen in the H-E staining (40×) 7 days after surgery that group A had satisfied skin growth, obvious interface between the skin and the surrounding skin, and the skin survived without obvious abnormal structure (d). Meanwhile, there was partial dermal papillary edema in the sample from Group B. However, in group C, the biospy was ruptured and necrotic tissues were found. It seemed hard to establish the blood supply. Obvious contraction could be seen at the interface with the surrounding skin, and it grew into the space between the grafts and the wound base, just as an "insertion" (f).

It was found in the H-E staining (40×) 14 days after surgery that group A showed good skin growth without obvious abnormalities (g), as what was seen above. In group B, the "insertion" of surrounding skin slices in the sample of group C on 7d also occurred (h). In the non-necrotic skin of group C, partial epidermis and dermis were separated to form blisters (i).

Obvious epidermal necrosis and inflammatory cell infiltration were observed in skin slices of group C in the H-E staining under the microscope

(400×) on day 14 (j). In addition, edema of the stratum spinosum, formation of blisters, deep epidermis disconnection (k) were seen as well. Moreover, a blister was formed at the epidermal junction with hemorrhage and chronic inflammatory cell infiltration and basal cell edema (l).

CD31 and VEGF Immunofluorescence (Figure 4 and 5)

The results are recorded as table 2,3. Since there was only one specimen in each group at each observation time point and the number of samples was small, no statistical test was performed.

As seen from the table, compared with the other two groups, VEGF and CD31 expressions in group A were more obvious at 7 days and 14 days after surgery.

Masson's trichrome staining

Three days after the operation, Masson staining of each group showed that the samples in group B and C all showed dermal atrophy, collagen thinning, subcutaneous tissues such as hair follicles and fat moving upward, etc., while the samples in group A were relatively normal (Figure 6).

Discussion

Full-thickness skin grafts are a common method in plastic surgery and have played an important role in skin defects of various causes(9, 10), nasal defect repair(11),vaginal reconstruction(12), scar repair(13) or even hernia repair(14). Compared with split-thickness skin grafting, full-thickness skin grafting have better effects. However, the clinical usage of it is limited due to several disadvantages such as difficulty in establishing blood supply and susceptibility to infection(1). Therefore, how to improve the success rate of full-thickness skin graft transplantation has always been the topic of plastic surgery clinicians and researchers.

ADSCs are a type of MSCs, and recent studies have shown that about 5×10^4 to 2×10^5 cells can be extracted from 1g of adipose tissue, which is about 2,500 times of the number of stem cells available in bone marrow tissue(15). Furthermore, compared with that of bone marrow mesenchymal stem cells, the process of ADSCs' isolation would cause less donor site damage, less costs, and less invasive.

During the process of obtaining the ADSCs, SVF cells, a mixture of various cells, would be collected obtained in the process of obtaining ADSC. Besides ADSCs, SVF also contains cell components such as endothelial

progenitor cells, endothelial cells, macrophages, smooth muscle cells, lymphocytes and pericytes(16), making it better than using ADSCs alone at some time. For example, in a study on the recovery of erectile function after nerve injury in rats, the application of SVF can promote the formation of blood vessels and smooth muscle more than the application of ADSCs(7). It is believed by the author that the reason for this result is precisely the other cells contained in SVF except ADSCs. Another advantage of SVF is that it is easier to obtain than ADSCs. It does not need to go through the complicated and highly demanding cell culture in the laboratory, which saves a lot of manpower and material resources and avoids the use of various reagents to the greatest extent, making it safer to use. However, it also needs to be used immediately after harvest, and because of the diversity of its cells, the use of foreign bodies will cause immune rejection, which means that it can only be used for self-use(11). Although ADSCs need some culture, they are Immuno-immunity cell and do not express HLA-DR so that they can be used as an allograft theoretically(17).

In this study, we focused on comparing the effects and differences of autologous fresh isolated SVF cells and allogeneic ADSCs in full-thickness skin graft transplantation. In terms of the comparison of skin graft survival area, it was found that the SVF group at each observation time point was superior to the ADSCs group and PBS group with statistical significance. The necrotic rate of the skin grafts in ADSCs group was statistically lower than that of PBS group at 3d and 14d, but there was no significant difference between them at 7d. It is indicated that the promoting effect of ADSCs on full-thickness skin graft is not as obvious and stable as that of SVF. As we all know, the survival of full-thickness skin grafts is closely related to angiogenesis and revascularization, which can be evaluated by CD31 and VEGF Immunofluorescence. In this study, specific OD value(the ratio of OD value in the samples to that of normal skin) was found higher in SVF group, suggesting that it is more effective to enhance the formation of blood vessels and hence improve the survival rate of the grafted full-thickness skin by injecting autologous SVF cells. It should be noted that in this experiment we only used 1×10^6/ml allogeneic ADSCs, which is enlightened with reference to the study by Juan Wang et al(18). Perhaps it was the insufficient number of ADSCs that caused a weaken effect contracted to SVF cells in promoting angiogenesis and revascularization and even the survival of the grafts. In addition, whether there is an immune response in the practical application of allogeneic ADSCs without antigenicity in theory needs to be further confirmed and excluded in future experiments.

It should be explained that contraction rate of the skin grafts was not included as an observation index in this study. Skin graft contraction is one of

the important observation indexes in free skin grafting, but after observing practically, it was found that the surrounding skin would contract dramatically during the wound healing due to the characteristics of rodents. The space between the grafts and wounds would often be interfered with the “insertion” of the surrounding skin, making it difficult to reconstruct the blood supply and poor healing of the margins of the grafted skin. Since the current formula for skin grafts contraction is calculated by 1- area of the grafts postoperatively /that preoperatively× 100%, it is hard to differentiate contraction and margin necrosis and prone to a relatively serious bias.

Conclusion

Subcutaneous injection of autologous SVF or allogeneic ADSCs cells can both improve the effects of full-thickness skin grafts compared with PBS. However, autologous SVF cells are more effective than allogeneic ADSCs (1 $\times 10^6$/ml). It is still necessary to further study the effects of different concentration of ADSCs and whether there is immunogenicity in practical usage. In the future, optimization and further researches are needed for the animal model of full-thickness skin graft transplantation as well.

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Figures

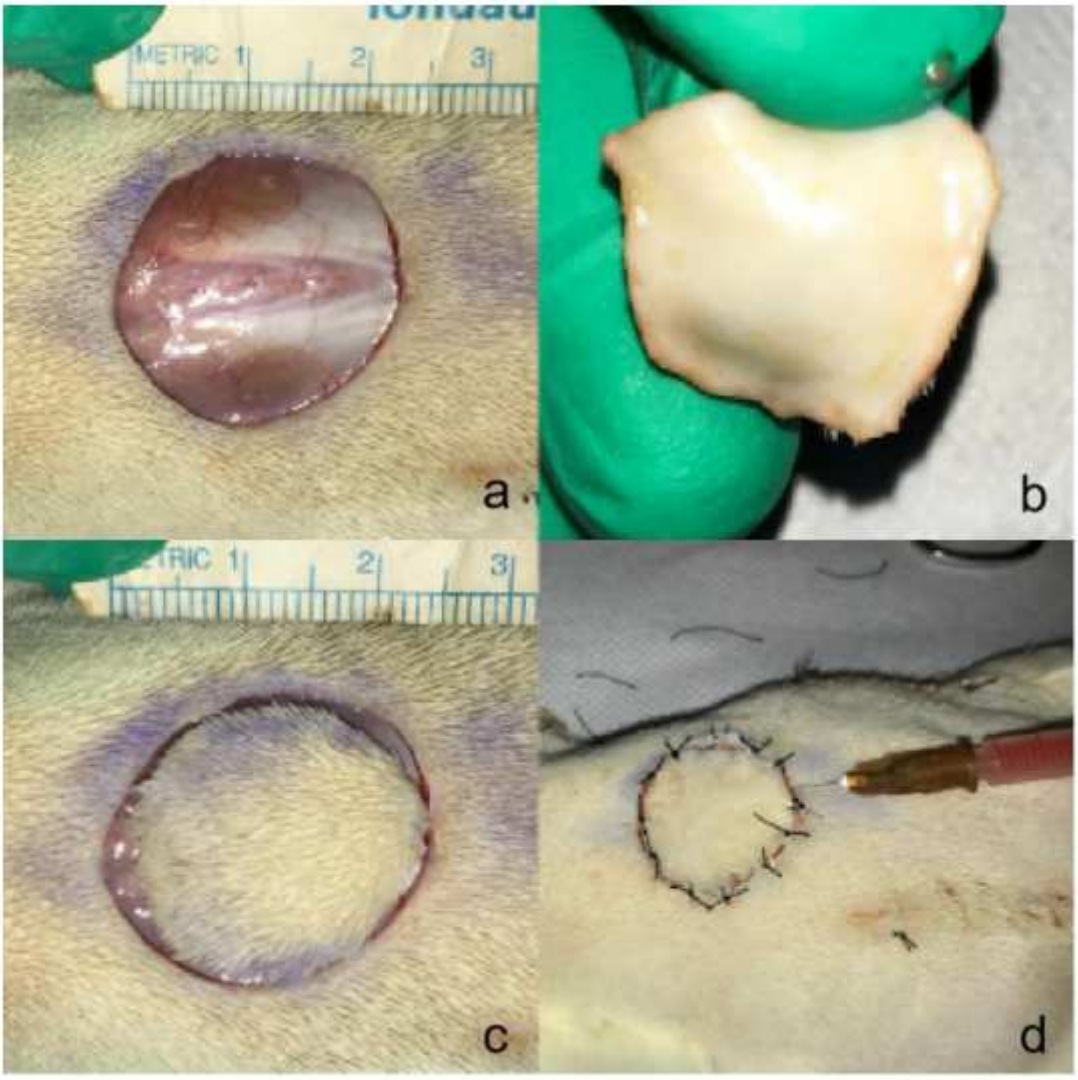

Figure 1

Surgical Procedure: a. A full-thickness wound with a diameter of about 2cm was prepared, reaching deep to the myometrium surface; B. Make full-thickness skin slices from the cut skin; C. Rotated 180°, and implanted in wound; D. Injection of SVF/ADSCs suspension (1ml) between the skin plate and the wound base

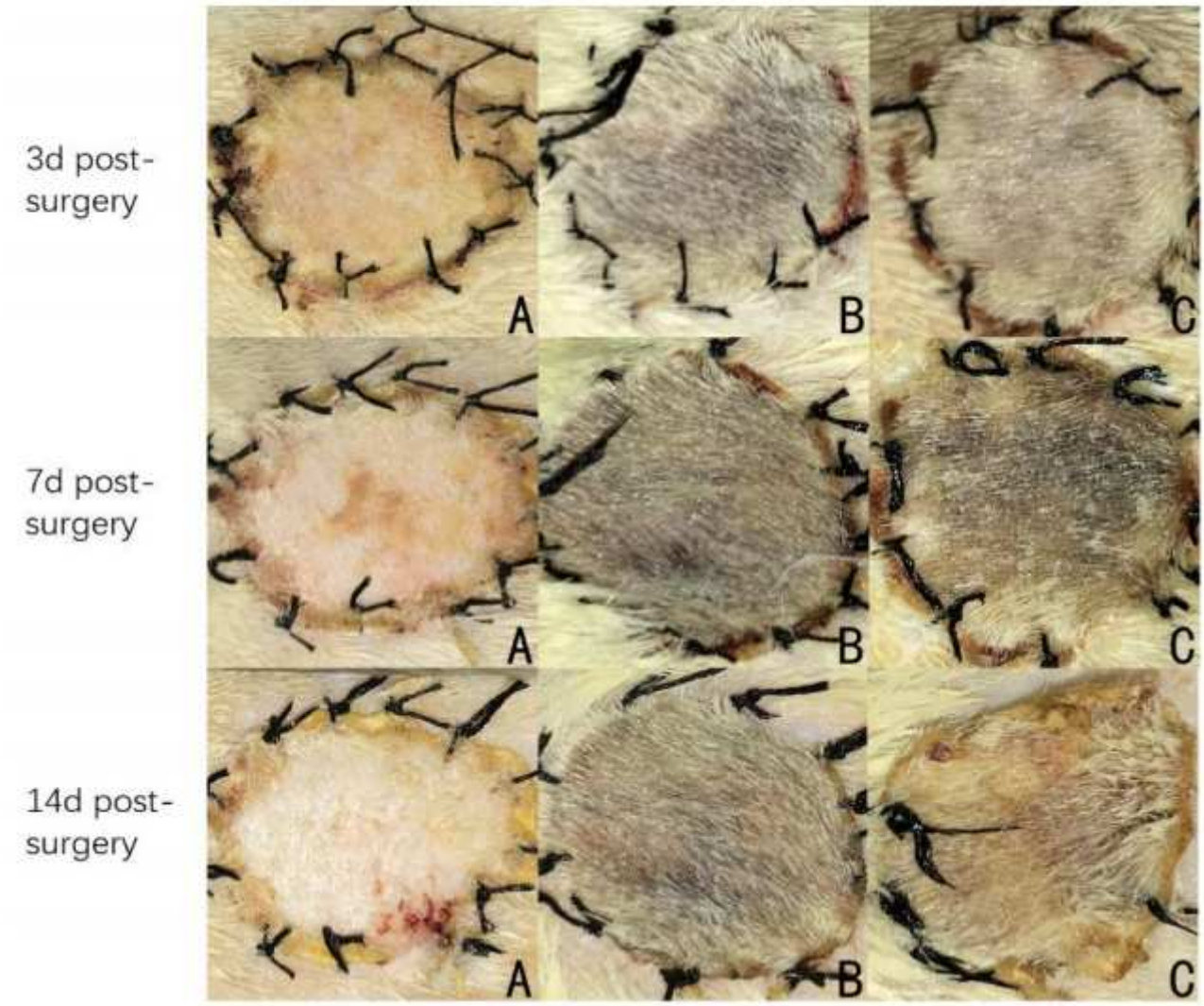

Figure 2

Postoperative Gross Observation: A, B and C represent group A, group B and group C respectively

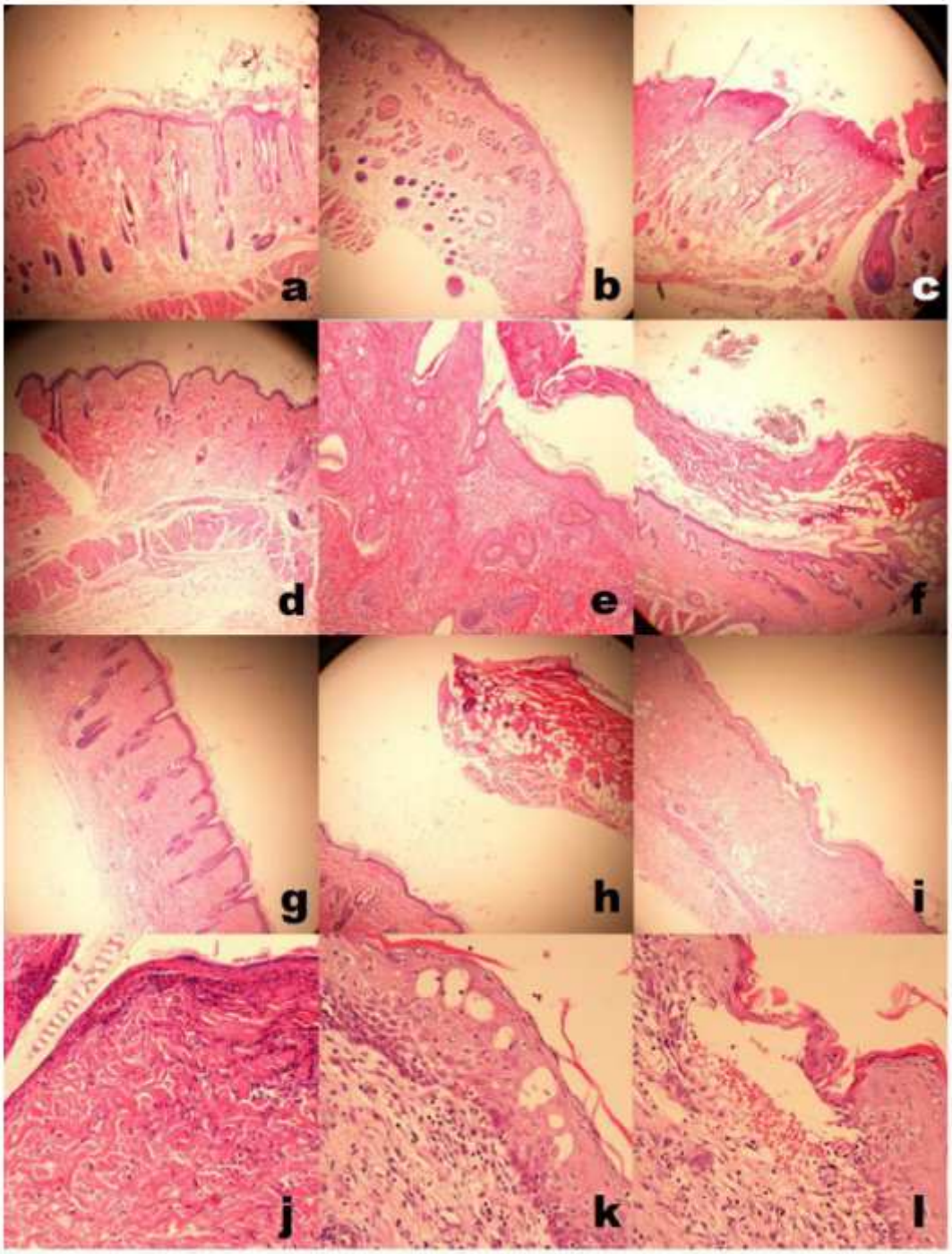

Figure 3

H-E Staining Results: a, d and g represent the results of group A on 3d, 7d and 14d respectively. b, e and h represent the results of group B on 3d, 7d and 14d respectively. c, f and I represent the results of group C on 3d, 7d and 14d respectively. When j, k and l were 14d, the results of group C were 400 times under the microscope

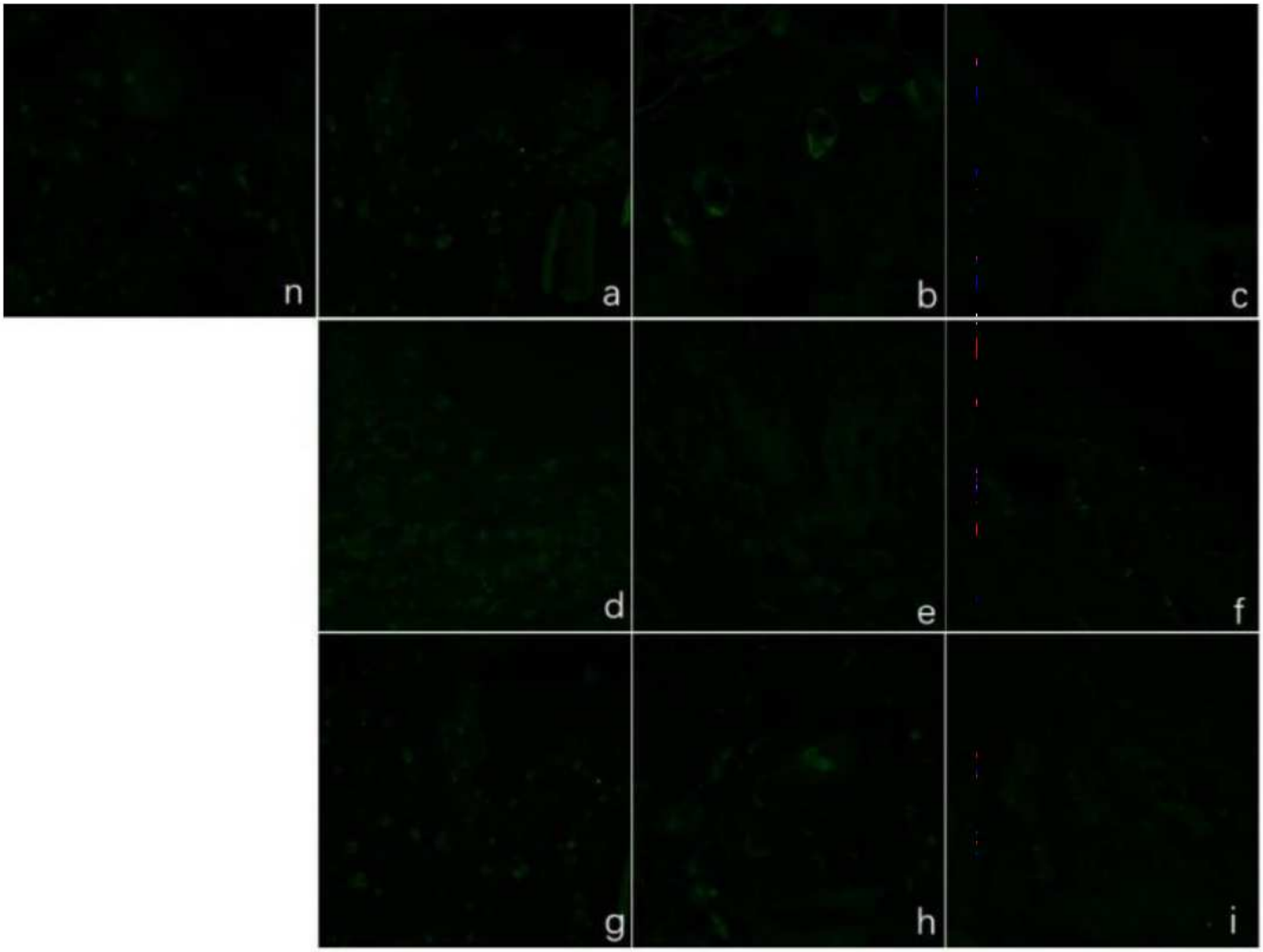

Figure 4

VEGF Immunofluorescence Images: At each observation time point represents normal skin tissue; a, d and g represent the results of group Aon 3d, 7d and 14d respectively. b, e and h represent the results of group B on 3d, 7d and 14d respectively. c, f and I represent the results of group C on 3d, 7d and 14d, respectively

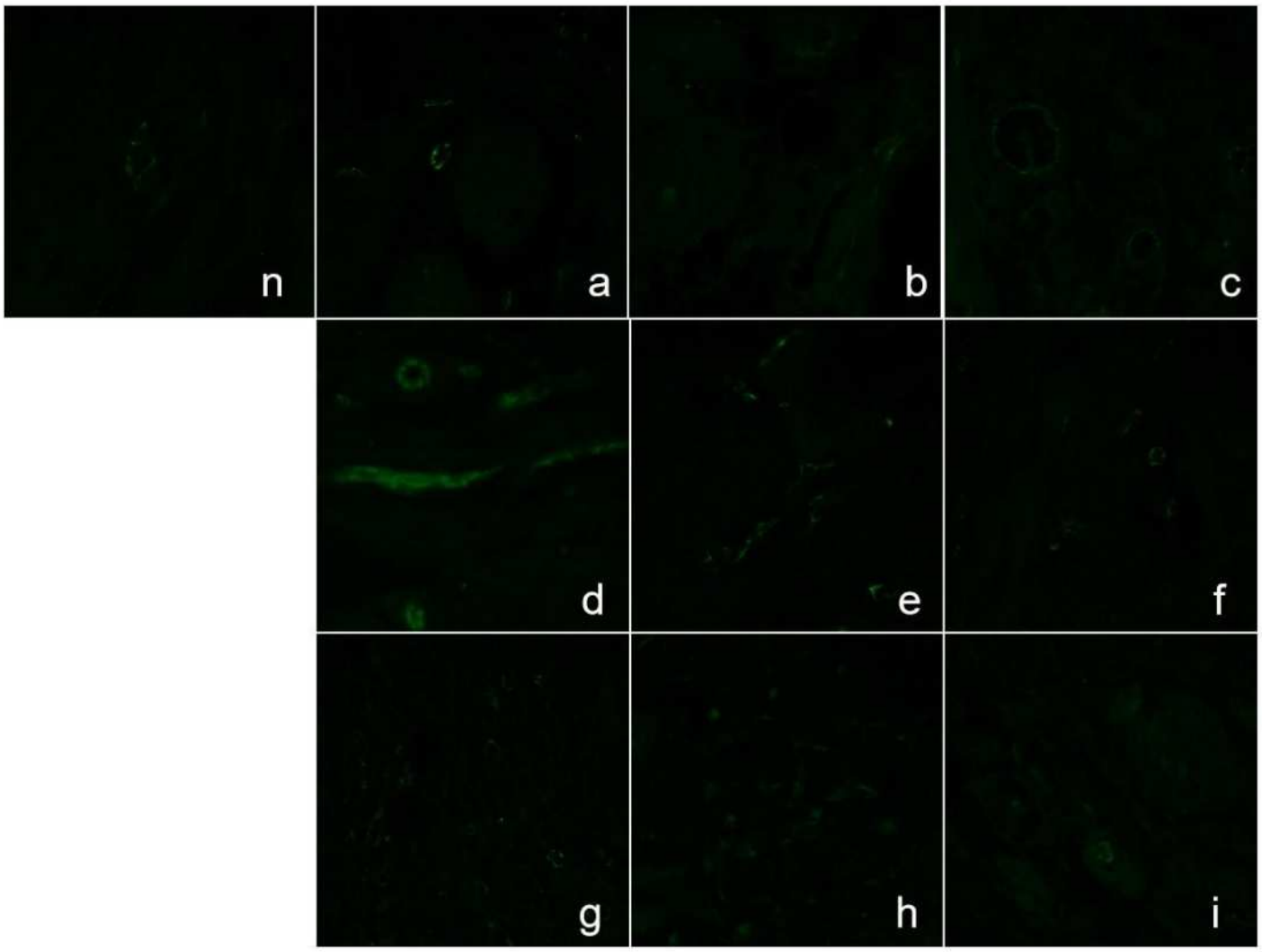

Figure 5

CD31 Immunofluorescence Images: At each observation time point represents normal skin tissue; a, d and g represent the results of group A on 3d, 7d and 14d respectively. b, e and h represent the results of group B on 3d, 7d and 14d respectively. c, f and I represent the results of group C on 3d, 7d and 14d, respectively

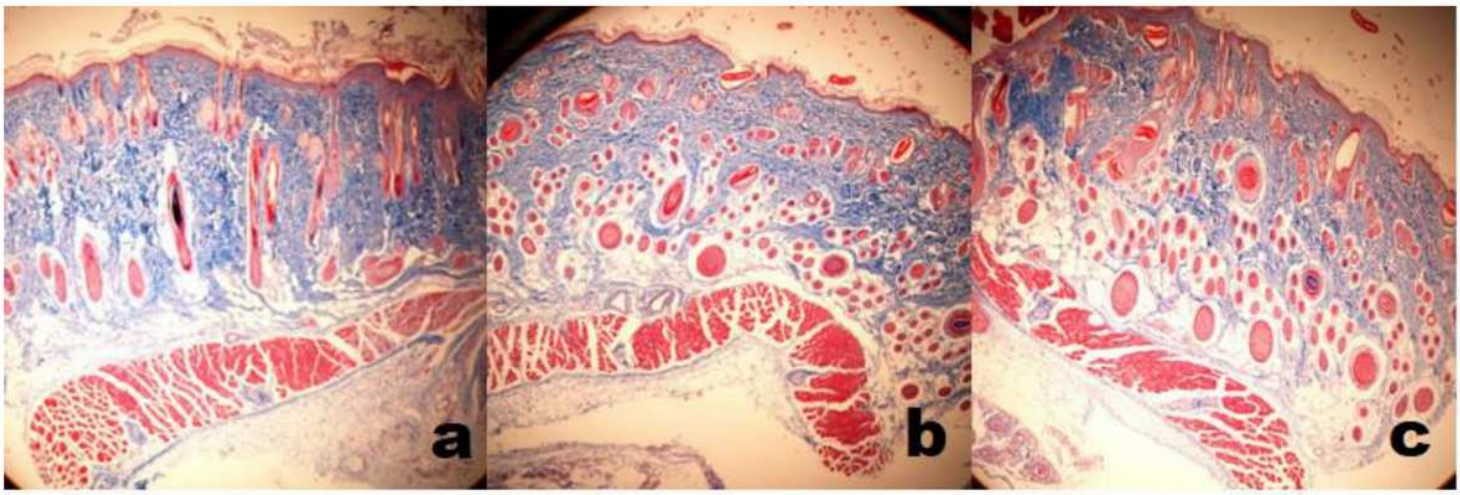

Figure 6

Masson staining results A, B and C represent group A, group B and group C respectively

Supplementary Files

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- Tables.pdf