Aesth Plast Surg (2019) 43:1085–1094
https://doi.org/10.1007/s00266-019-01355-z

ORIGINAL ARTICLE BASIC SCIENCE/EXPERIMENTAL

Effects of Platelet-Rich Plasma on Fat and Nanofat Survival: An Experimental Study on Mice

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Received: 12 December 2018 / Accepted: 5 March 2019 / Published online: 27 March 2019

Abstract

Background Nanofat and fat graft survival is an important clinical problem. The authors of this study investigated whether PRP has an impact on fat and nanofat graft survival and vascularization in a mouse model.

Materials and Methods Fat was harvested from a 50-year-old healthy woman by vacuum suction, and nanofat was obtained by emulsification and centrifugation procedures. PRP was collected after two rounds of centrifugation from an autologous blood sample. Twenty male nude mice were divided into four treatment groups: PRP/nanofat, PRP/fat, saline/nanofat and saline/fat. After 1 month and 3 months, the grafts were extracted and weighed. The microstructure of the fat and nanofat was examined with a scanning electron microscope. HE and immunohistochemical staining was applied to observe neovascularization. Western blot analysis was used to analyse the expression of CD31 and VEGF.

Results In fat tissue, fat cells had normal connections; the fat structure was complete and fibre networks were visible. In nanofat, the extracellular matrix vascular components were visible and their structures were intact. At 1 month and 3 months, the graft weights in the PRP/fat group were significantly higher than those in the other groups. Further, a higher degree of neovascularization was observed in the PRP/nanofat group, and the expression of CD31 and VEGF in the PRP/nanofat group was higher than that in the other groups.

Conclusion PRP can promote nanofat and fat graft survival and vascularization.

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Keywords Platelet-rich plasma · Nanofat · Fat graft · Adipose-derived stem cells · Nude mice

Introduction

Autologous fat grafting technology was introduced in the early twentieth century. After this technology was standardized by Coleman, it has been widely applied in orthopaedics. Fat grafts are applied extensively as soft tissue fillers for breast augmentation surgery, lower limb atrophy [1], depressed scar [2] and so on. It not only has the advantage of good tissue compatibility, but is also feasible for use, abundantly available and cost-effective, so it is widely applied in plastic surgery. However, as the diameter of fat particles is relatively large, these grafts can only be applied as deep-tissue volume fillers [3]. This limitation can be overcome by the latest innovation in this field—nanofat—discovered and reported by Tonnard [4]. Nanofat contains a large amount of adipose stem cells and stromal vascular components, so it can not only be applied as a superficial intradermal filler, but it can also promote tissue regeneration and rejuvenation [4, 5].

During the fat grafting process, the mechanical injury caused by the force of injection and the ischaemic injury

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caused by devascularization minimizes graft survival and causes complications such as liquefaction and necrosis [6, 7]. Numerous techniques and adjuvant materials have been applied to improve the survival of fat grafts, but none of them are very effective. For example, Yi et al. [8] used VEGF gene therapy to improve the survival of grafted adipocytes, and fibroblast growth factor 2 (FGF-2), which can enhance the adipogenic differentiation of adipose stem cells, but its effect on improving adipocyte graft survival is unclear [9]. In addition, the direct use of growth factors carries a certain amount of risk [10, 11]. Platelet-rich plasma (PRP), which contains platelets derived from autologous whole blood, may secrete a variety of growth factors, cytokines, chemokines and other active products. This technique has been found to be safe and is widely applied in plastic surgery, orthopaedics, maxillofacial surgery and soft tissue ulcers [12–15]. Dong [16] injected a PRP and fat mixture into the head and neck of nude mice and found that PRP could improve fat graft survival after 10 weeks.

At present, there are very few reports on the application of nanofat, and particularly, there is no report on the application of PRP mixed with nanofat in animal models. Based on this premise, the present study sought to examine whether PRP can improve nanofat and fat graft survival through its effects on vascularization, based on measurements of graft weight and the findings of histological analyses.

Materials and Methods

Nanofat Preparation

A 50-year-old healthy woman was selected for abdominal liposuction, and her signed informed consent for the same was obtained. The suction area on the lower abdomen was disinfected, draped and marked out. The patient was then placed under general anaesthesia, and a sterile 50-ml syringe was connected to a 4-mm calibre liposuction tube to manually harvest fat under negative pressure (Fig. 1a). The harvested fat was maintained in the vertical position for 10 min to eliminate the underlying blood–water mixture (Fig. 1b). The fat was distributed between two 20-ml syringes through a three-way tube. The syringes were connected through a Luer connector (Fig. 1c). The fat sample was passed through the No. 4, No. 3, No. 2 and No. 1 connectors in sequence for mechanical emulsification. The emulsified fat was divided into 50-ml centrifuge tubes and centrifuged at 2200 *g* for 5 min. After centrifugation, it was divided into three layers: the upper layer contained oil; the lower layer, a blood–water mixture; and the middle layer, nanofat (Fig. 1d).

Scanning Electron Microscopy

The fat tissue and nanofat were placed in separate EP tubes filled with 2.5% glutaraldehyde, washed three times (15 min each time), fixed with 1% citric acid, washed three times (15 min each time), dehydrated through an ethanol gradient (20 min each time), dried in *t*-butanol, vacuum dried and ion plated. The surface morphologies of the fat and nanofat were observed under a scanning electron microscope (S-3400 N; Sato Precision Instrument Inc., Suzhou, CN).

PRP Preparation

A blood sample (40 ml) was voluntarily provided by the same donor. The platelet fraction was separated with a two-step centrifugation process from whole blood. First, the blood sample was centrifuged at 1500 *g* for 10 min to separate the bottom red blood cell layer. The plasma obtained from the upper layer was then centrifuged for 20 min at 3000 *g*, and the supernatant was removed and the platelet fraction resuspended. A total of 5 ml of PRP was collected. PRP was mixed with calcium gluconate at a ratio of 10:1 to fully activate platelets.

Animals

Twenty male nude mice aged 6–8 weeks (weight, 22-28 g) were purchased from the Animal Experimental Center of Southern Medical University. All the experiments were approved by the Animal Care Committee of General Hospital of Southern Theater Command and were conducted in accordance with the institutional guidelines. Mice were divided into four groups according to the treatment they received: 0.5 ml of a PRP and nanofat mixture (1:4, v/v), 0.5 ml of a PRP and fat mixture (1:4, v/v), 0.5 ml of a saline and nanofat mixture (1:4, v/v) and 0.5 ml of a saline and fat mixture (1:4, v/v). The mixtures were administered subcutaneously into the back of the mice for the self-control study (Fig. 2a). The total volume injected in each mouse was 2 ml. Ten nude mice were randomly selected and killed by cervical dislocation at 1 month. This was repeated in the remaining ten mice at 3 months after treatment. The weight of the grafts was measured on an electronic scale and recorded (Fig. 2b). The grafts were then placed, along with their capsule, on a white paper, and images were obtained with a digital camera (Nikon D5300) (Fig. 2c, d). The samples were then divided into two parts of equal weight. One part of the sample was placed in a test tube filled with 4% paraformaldehyde, and the other part was placed in a dry EP tube and stored in a − 80 °C refrigerator.

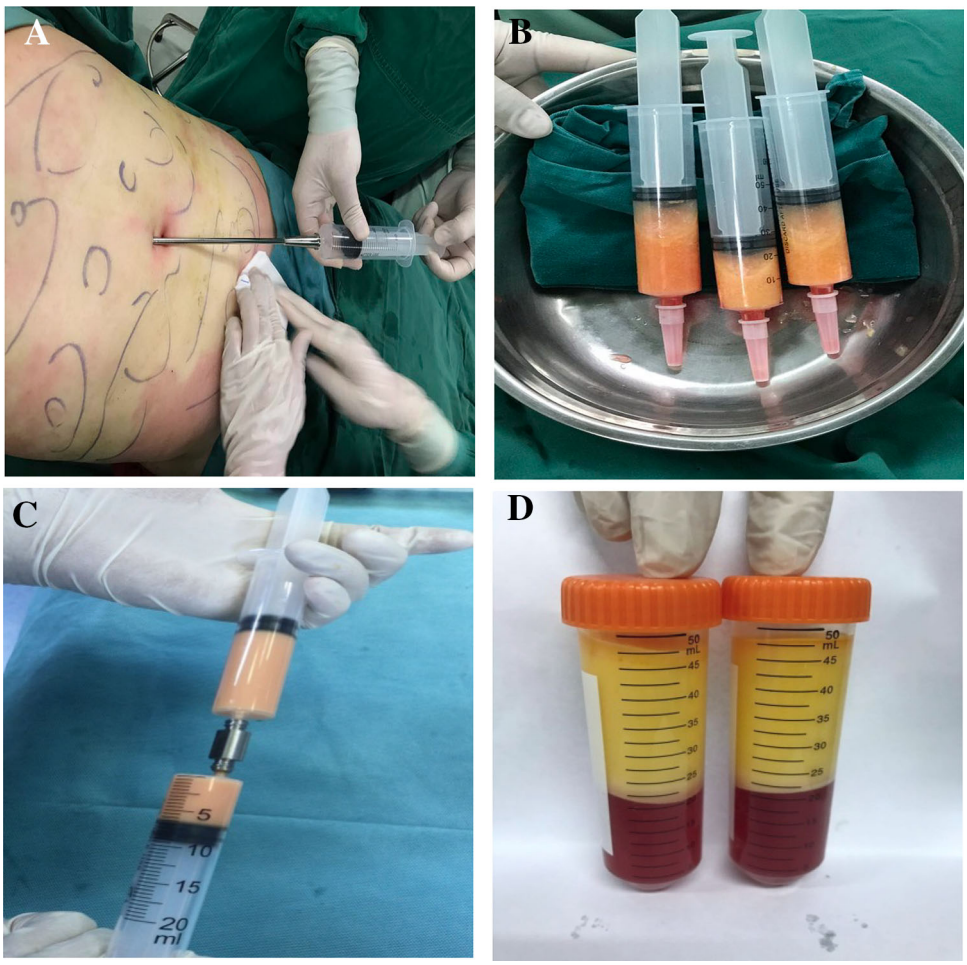

Fig. 1 Process of extracting nanofat. **a** Harvesting of abdominal fat under negative pressure. **b** Vertical positioning of the fat to remove the underlying blood–water mixture. **c** Two 20-ml syringes connected through a Luer connector and passed through No. 4, No. 3, No. 2 and No. 1 connectors in sequence to emulsify the fat mechanically. **d** The upper layer containing oil, the lower layer containing the blood–water mixture, and the middle layer containing nanofat

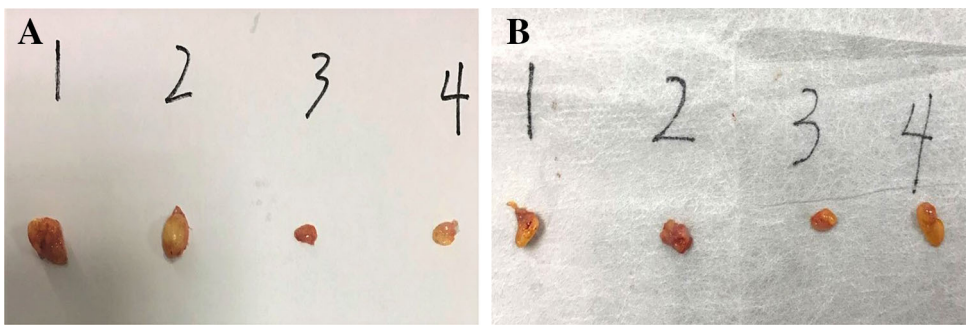

Fig. 2 Weighing of the fat and nanofat grafts. **a** The grafts, along with their capsule, were placed on a white paper and photographed with a digital camera. The sample was weighed on an electronic scale. Weights after 1 month, 1: PRP/fat group, 2: PRP/nanofat group, 3: saline/fat group, 4: saline/nanofat group. **b** Weights after 3 months, 1: PRP/fat group, 2: saline/fat group, 3: saline/nanofat group, 4: PRP/nanofat group

Pathological Staining

The tissues were fixed in 4% paraformaldehyde for more than 36 h. The samples were then gradually dehydrated in a dehydrator (JJ-12 J; Junjie Electronics Inc., Wuhan, CN) according to gradient alcohol. The tissue was embedded in paraffin wax (RM2016; Leica Instrument Inc., Shanghai, CN), sliced to a thickness of about 4 μm, placed in an oven at 60 °C and dried. The dried-out paraffin sections were then dewaxed to water. The sections were stained with haematoxylin (G1005; Google Biotechnology Inc., Wuhan, CN) for 3–5 min, washed with tap water, stained with eosin for 5 min, dehydrated and mounted. The scanning electron microscope was used to observe and take pictures. The images were analysed by the Adobe Photoshop™ CS software.

Immunohistochemistry

The paraffin-embedded sections were deparaffinized to water, repaired with an EDTA–antigen buffer and placed in a 3% hydrogen peroxide solution to block endogenous peroxidase. The sections were lightly dried, and a histochemical pencil was used to draw a circle around the tissue. Then, the tissues were covered with 3% bovine serum albumin for 30 min. The slides were incubated with antibodies against VEGF (GB11034; Servicebio Inc., Boston, MA) and CD31 (Servicebio) in a wet box at 4 °C overnight. Following this, the slides were incubated with the corresponding HRP-labelled secondary goat anti-rabbit IgG (Servicebio) for 50 min at room temperature and DAB solution (Servicebio) was added. The development of colour on the slices was observed under the scanning electron microscope. After a brown colour appeared, the samples were washed with water, counterstained with haematoxylin for 3 min and then dehydrated and mounted. The microscope was used to obtain images, which were analysed with the Image Pro Plus software.

Western Blotting

A portion of the tissue was homogenized in a glass homogenizer for 30 min and centrifuged. The supernatant was collected and the protein concentration was determined with the BCA kit (Beyotime Biotechnology Inc., Shanghai, CN). Separation gum and concentrate gum were prepared. Samples were added into the pore and electrophoresis was carried out. Then the membrane was transferred for half an hour at constant current. The solution was then mixed with 5% skim milk powder for blocking, incubated with antibodies against CD31 and VEGF, washed three times with TBST, incubated with the corresponding HRP-labelled secondary goat anti-rabbit antibody and mixed with ECL solution for blot development. Images of the blot were obtained, and the Image Pro Plus software was used to calculate the grey value of each band and to analyse the results.

Statistical Analysis

All data in this experiment are expressed as mean ± standard deviation ($x \pm S$), and the SPSS 19.0 software was used to process all the data. One-Way ANOVA was used to compare the mean values between the four groups. The SNK-q test was used to compare differences between the four groups. $P < 0.05$ was considered to indicate statistical significance.

Results

Macroscopic Observations

There were no ulceration and necrosis in the injection area in any of the mice. In all four groups, the grafts were spherical and easy to extract. After 1 month, liquefaction and absorption were observed in one mouse each in the saline/fat group, and after 3 months, absorption was observed in one mouse each in the PRP/fat group and PRP/nanofat group and in two mice in the saline/fat group.

At 1 month, the weight of the grafts in the PRP/fat group and PRP/nanofat group was significantly higher than that in the saline/fat group and the saline/nanofat group. At 3 months, the weights of the grafts in the four groups were significantly lesser than that at 1 month. Further, the weight in the PRP/fat group was significantly higher than that in the saline/fat group, but there was no significant difference between the PRP/fat group and the PRP/nanofat group (Table 1/Fig. 3).

Scanning Electron Microscopy

The obtained fat tissue and nanofat were observed by electron microscopy. The microscopic images obtained showed that the fat tissue structure was complete and the fibre network was visible. Fat cells maintained normal connections. The extracellular matrix vascular components of nanofat were visible and the structures were intact. A large number of fat cells were also damaged (Fig. 4).

Pathological Assay

At 1 month, the fat cells were surrounded by numerous vacuoles and exhibited fibrosis in all groups. In the PRP/nanofat group and the PRP/fat group, the number of blood vessels was greater and the number of fibrotic and inflammatory cells was lower than that in the saline/nanofat group and saline/fat group (Fig. 5A1–D1/A2–D2).

Table 1 Comparison of graft weight between the four groups at 1 and 3 months after grafting ($n = 10$, M ± SD)

	1 month	3 months
PRP/fat group	0.247 ± 0.059 g*	0.108 ± 0.034 g*
PRP/nanofat group	0.264 ± 0.051 g*	0.090 ± 0.023 g
Saline/nanofat group	0.130 ± 0.043 g	0.084 ± 0.041 g
Saline/fat group	0.201 ± 0.048 g	0.068 ± 0.013 g

*$p < 0.05$, significantly different compared with the saline/nanofat group and saline/fat group

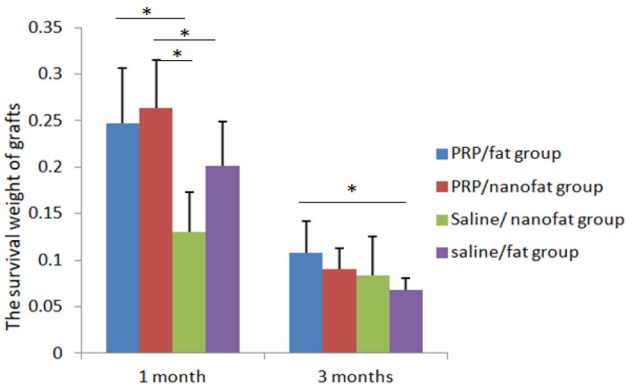

Fig. 3 Analysis of the graft weights. After 1 month, the weight of the grafts in the PRP/fat group and PRP/nanofat group was significantly higher than that in the saline/fat group and the saline/nanofat group ($P < 0.05$). After 3 months, the weight of the grafts in the PRP/fat group was significantly higher than that in the other three groups ($P < 0.05$)

At 3 months, the numbers of inflammatory cells in the four groups were significantly lower than those at 1 month after grafting. A complete vascular lumen was observed in the periphery of the adipocytes, along with a greater degree of fibrosis in all groups. Higher numbers of blood vessels were observed in the PRP/nanofat group and in the PRP/fat group than in the other two groups (Fig. 5A3–D3/A4–D4/B).

Immunohistochemical Assay

The staining results for CD31 and VEGF are indicative of neovascularization. At 3 months, CD31 staining indicated that the numbers of new blood vessels in the PRP/nanofat group and the PRP/fat group were significantly higher than those in the other two groups (Fig. 6a, b, Table 2). VEGF staining showed that the expression of VEGF was also significantly higher in the PRP/nanofat group than in the other three groups (Fig. 7).

Western Blot Assay

At 1 month, CD31 and VEGF protein expression in the PRP/nanofat group was higher than that in the other three groups (Fig. 8a). At 3 months, CD31 and VEGF expression in the PRP/nanofat group and PRP/fat group was higher than that in the other two groups (Fig. 8b).

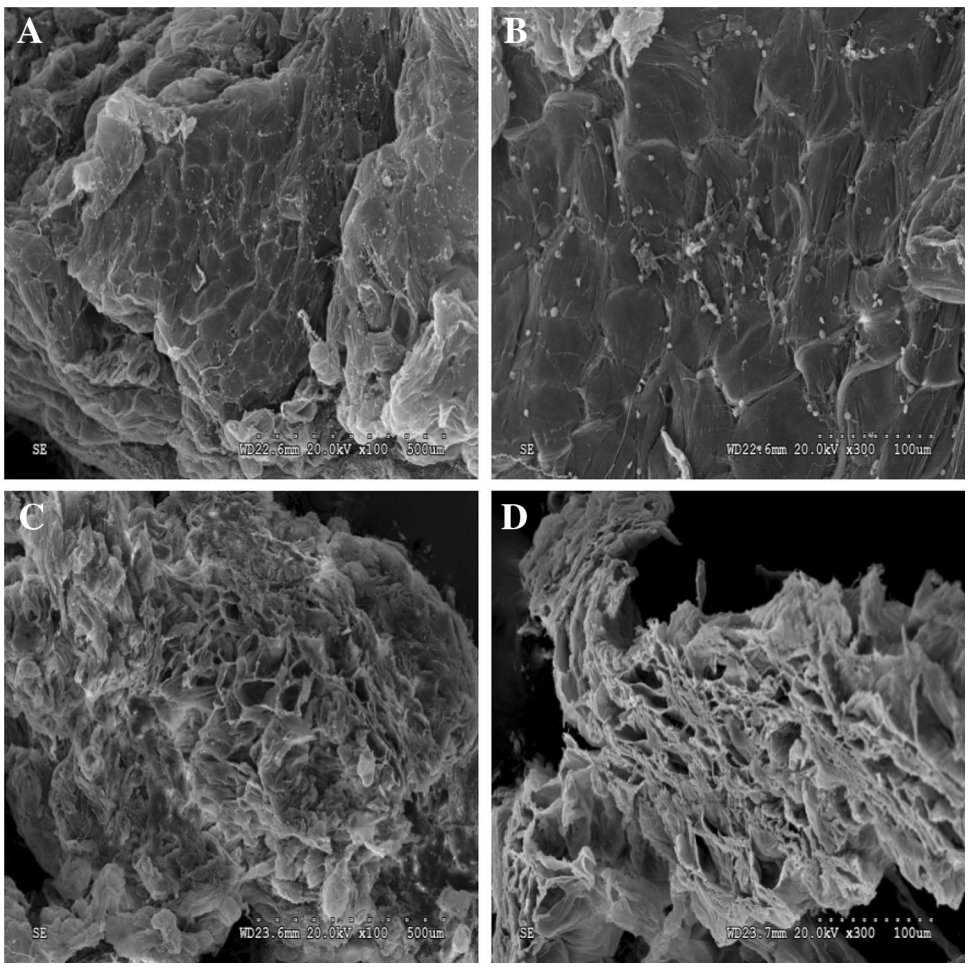

Fig. 4 Ultramicrostructure of the fat and nanofat samples. **a** The structure of the fat samples under 100 ×, **b** structure of the fat samples under 300 ×, **c** structure of the nanofat sample under 100 ×, **d** structure of the nanofat samples under 300 ×. The images were obtained under an electron microscope

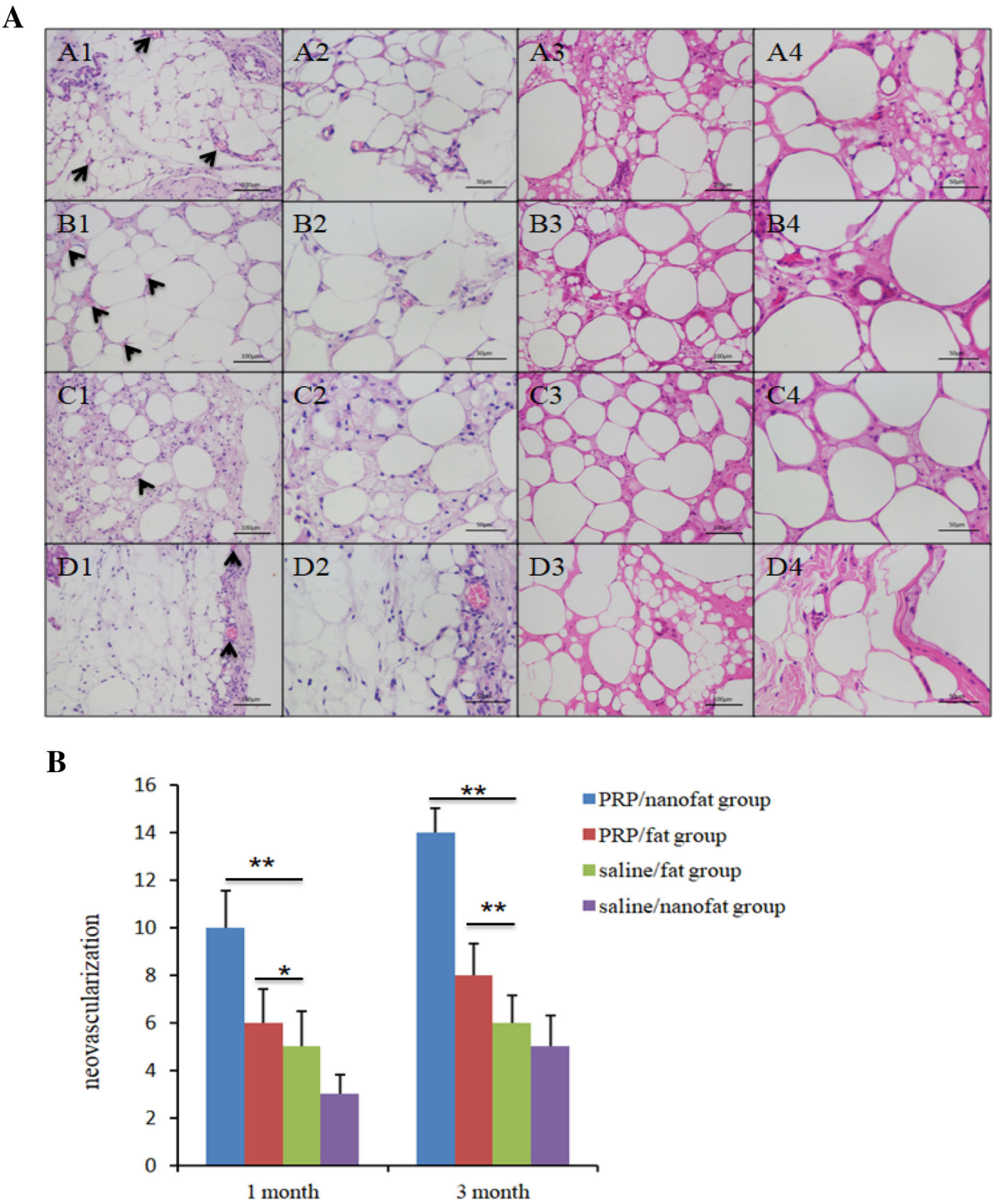

Fig. 5 Pathological assay by haematoxylin–eosin staining. **a** HE staining of the four groups A1/A2: PRP/fat group (1 month), B1/B2: PRP/nanofat group (1 month), C1/C2: saline/nanofat group (1 month), D1/D2: saline/fat group (1 month). After 1 month, more vacuoles and a higher degree of fibrosis are observed around the fat cells. More blood vessels, a lower degree of fibrosis and fewer inflammatory cells are observed in the PRP/nanofat group. (A1–D1, × 200; A2–D2, × 400) A3/A4: PRP/fat group (3 months), B3/B4: PRP/nanofat group (3 months), C3/C4: saline/nanofat group (3 months), D3/D4: saline/fat group (3 months). After 3 months, a complete vascular lumen is observed in the periphery of the adipocytes, along with a higher degree of fibrosis. There were more blood vessels in the PRP/nanofat group and the PRP/fat group than in the other groups (A3–D3, × 200; A2–D2, × 400). **b** Semiquantification of the number of blood vessels under the microscope: A significantly higher degree of vascularization was observed in the PRP/nanofat group and the PRP/fat group than in the saline/nanofat group and saline/fat group (**$P < 0.01$).The arrows in the images indicate blood vessels

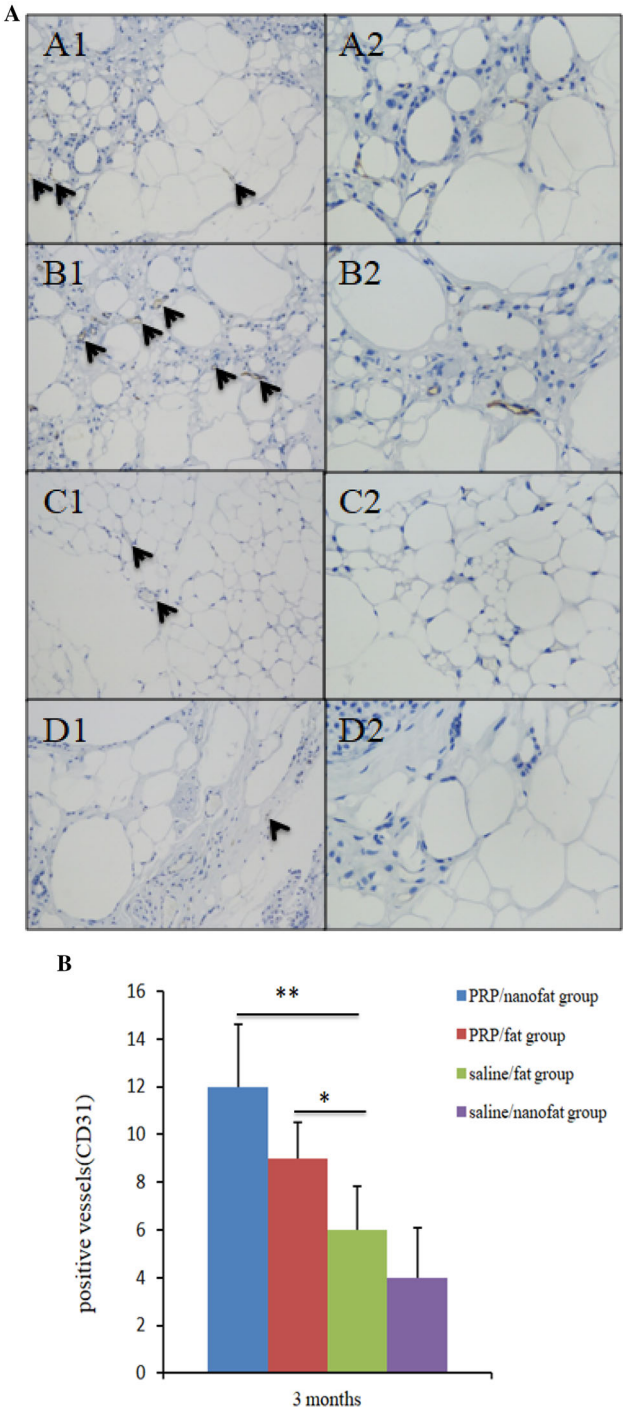

Fig. 6 **a** Immunohistochemical assay for CD31. A1/A2: PRP/fat group (3 months), B1/B2: PRP/nanofat group (3 months), C1/C2: saline/nanofat group (3 months), D1/D2: saline/fat group (3 months) (A1–D1, × 200; A2–D2, × 400). **b** Quantification of CD31 expression. Significant differences were found between the PRP/nanofat group or PRP/fat group and saline/fat group or saline/nanofat group. SNK-q test: $^{*}p < 0.05$, $^{**}P < 0.01$. The arrows in the images indicate blood vessels

Table 2 The number of CD31-positive vessels in the four groups at 3 months after grafting (n = 10, M ± SD)

	PRP/fat group	PRP/nanofat group	Saline/nanofat group	Saline/fat group
3 months	9 ± 1.529*	12 ± 2.610*	4 ± 2.105	6 ± 1.841

*$p < 0.05$, significantly different compared with the saline/nanofat group and saline/fat group

Discussion

In this study, we investigated the different outcomes of administering fat and nanofat along with PRP, and the findings indicated that the nanofat/PRP grafts had a somewhat better treatment effect than the grafts that did not contain PRP. Similar to our results, Yu et al. [17] also transplanted nanofat and fat tissue subcutaneously into nude mice and found that the nanofat/fat and PRP mixtures resulted in higher weight, a higher degree of angiogenesis and better survival.

In the present study, we found that the addition of PRP improved graft weight. In agreement with our findings, Feng et al. [18] also injected granular fat, PRP and ASCs into nude mice and showed that it significantly improved residual volumes. Yu et al. [24] also reported similar findings in nude mice. In addition, in the present study, liquefaction and absorption were mainly observed in the saline/fat group, and the addition of PRP was associated with a decrease in the number of inflammatory cells. In a similar study, Blumenschein et al. [19] transplanted a PRP–fat mixture into rats and found that it reduced necrosis in the graft tissue. All these findings indicate the benefits of adding PRP to fat tissue grafts.

After grafting, the reconstruction of blood vessels plays a crucial role in the survival of the fat tissue. In this study, we used CD31 and VEGF as markers of angiogenesis in the graft tissue. CD31 is a marker protein of vascular endothelial cells, and VEGF is a highly specific vascular endothelial growth factor that promotes the proliferation of vascular endothelial cells and angiogenesis. Studies have shown that VEGF further promotes the survival of fat grafts by promoting angiogenesis and vascularization [20–22] and that VEGF plays a role as a mitogen for vascular endothelial cells, increasing endothelial cell migration and regulating microvascular permeability and vasodilation [23, 24]. In the present study, the expression of CD31 and VEGF in the PRP/nanofat group and PRP/fat group was significantly higher than that in the other groups. Studies have shown that along with the release of various vascular growth factors such as VEGF by PRP, adipose-derived stem cells in nanofat can proliferate and secrete a variety of vascular growth factors under conditions of injury and hypoxia [25, 26]. PRP can also promote the

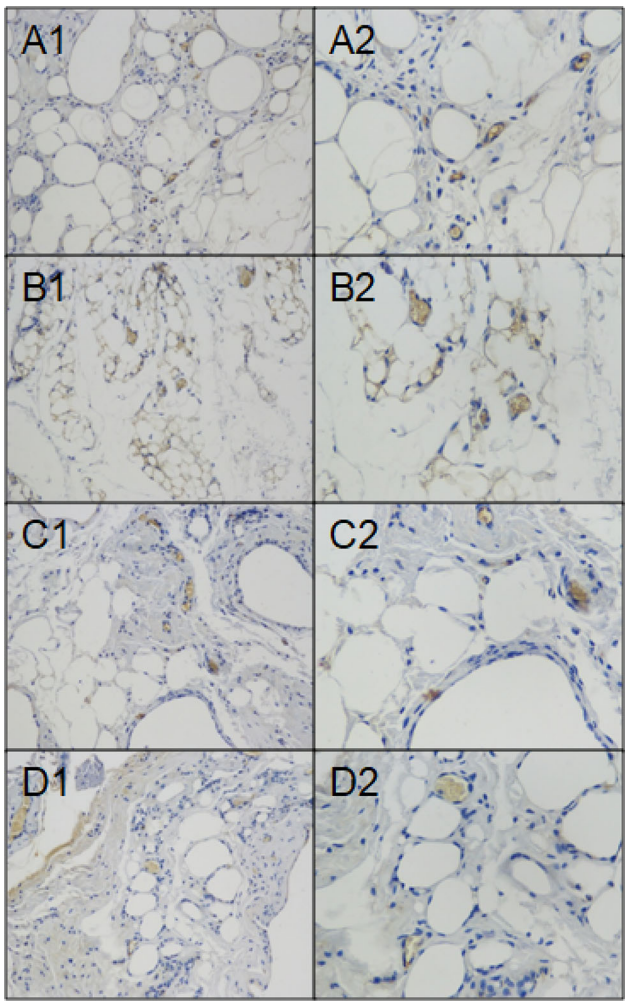

Fig. 7 Immunohistochemical assay for VEGF. A1/A2: PRP/fat group (3 months), B1/B2: PRP/nanofat group (3 months), C1/C2: saline/-nanofat group (3 months), D1/D2: saline/fat group (3 months). (A1–D1, × 200; A2–D2, × 400)

proliferation of adipose-derived stem cells [27, 28]. With regard to nanofat technology, it has been shown that nanofat particles can work in synergy with PRP to release angiogenic products and that PRP is a dose-dependent mitogen for adipose-derived stromal cells [29]. This paracrine effect has expanded the clinical applications of nanofat, which is also beneficial for skin rejuvenation, pigment metabolism and hair growth [30]. Thus, it seems that PRP is a practical, simple and effective means of improving the microvascular environment of new blood vessels, and its addition to nanofat grafts may have great potential in the field of plastic surgery. Therefore, nanofat when combined with PRP may promote angiogenesis and tissue regeneration through paracrine effects.

Other growth factors in PRP play an important role in fat survival. Platelet-derived growth factor, a potent mitogen and known stimulant for murine preadipocytes, could improve fat graft survival when its concentrations were sustained with a gelatine microsphere delivery system [31]. Further, Yuksel [32] evaluated the effects of long-term, local delivery of insulin, insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF) on fat graft survival by using a poly (lactic-co-glycolic-acid)-poly-ethylene glycol (PLGA/PEG) microsphere delivery system and found that the long-term, local delivery of growth factors with PLGA/PEG microspheres has the potential to increase fat graft survival rates. Therefore, nanofat and PRP may be more advantageous for facial rejuvenation, improving skin texture (including improving scars, etc.), promoting collagen deposition and promoting vascularization. Alternatively, in situations where PRP or nanofat is limited, adipose-derived stem cell glue can be applied to accelerate vascularization and improve fat survival. In addition, revascularization of the fat graft depends on the volume and on the interface between the fat graft and the recipient site. In a study by Karacaoglu et al., the volume maintenance of fat grafts placed in subcutaneous, supramuscular and submuscular layers was compared. In the submuscular layer, the fat graft survival rate was higher than that in the other layers and the blood supply was well developed [33]. The main factors that determine the survival of fat transplants may be the graft–recipient interface, vascularization and interstitial hydraulic pressure in the recipient area. Excessive fat droplets exceed the capacity of tissue space, leading to fat necrosis. Nanofat is obtained from fat tissue, by mechanical emulsification and centrifugation, as an emulsion liquid in the form of small drops. The particles of nanofat are much smaller than those of fat. Thus, mixing nanofat with PRP can optimize revascularization and survival.

Different platelet concentrations in PRP lead to different concentrations of growth factors and different therapeutic effects. Harrison et al. [34] activated PRP with thrombin and collagen and found that collagen could maintain the release of growth factors. The activated PRP released a threefold greater amount of TGF-β1, a 2.5-fold greater amount of PDGF-AB and a fivefold greater amount of VEGF than whole blood. Gentile et al. [35] treated alopecia with activated and non-activated PRP. The concentration of PDGF and VEGF in activated PRP was significantly higher than that in non-activated PRP, while hair growth was denser with non-activated PRP. Therefore, it is important that growth factors in PRP are released continuously and their concentration is not too high. Therefore, the standardization of PRP preparation and treatment methods is a pressing issue at the moment.

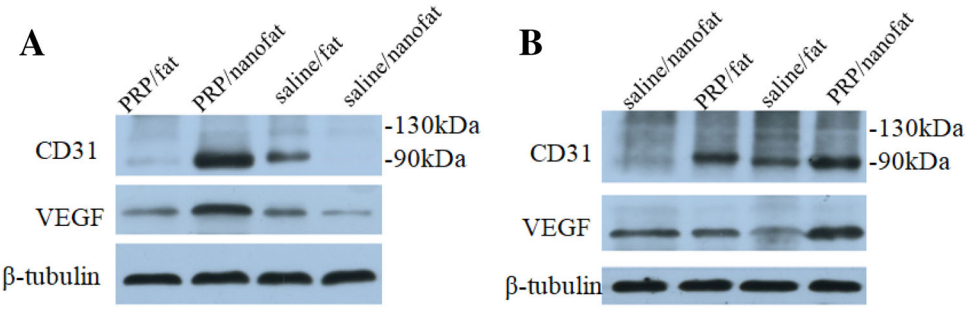

Fig. 8 Western blot analysis of CD31 and VEGF expression at 1 and 3 months. **a** At 1 month, CD31 and VEGF expression in the PRP/nanofat group was higher than that in the other three groups, with the lowest expression level observed in the saline/nanofat group **b** At 3 months, CD31 and VEGF expression in the PRP/nanofat group and PRP/fat group was higher than that in the other two groups

Conclusion

We have explored and compared graft survival and vascularization with PRP/fat and PRP/nanofat at 1:4 ratios in a mouse model. Although the PRP/nanofat treatment was found to be promising in this model, its therapeutic effects may differ according to context and should therefore be explored in other applications. Further, the optimal nanofat-to-PRP ratio for different applications and the PRP preparation procedures still need to be explored and standardized. Therefore, although this study lays the foundation for this technique in an animal model, there is still much work to be done before it can applied in the clinical context.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Nos. 81171812, 81671924 and 81272105), the National Key Research and Development Plan of China (No. 2017YFC1103301), the National Basic Science and Development Program (No. 2012CB518105), Health and Medical Treatment Collaborative Innovation Major Special Projects of Guangzhou (No. 201508020253), the Science and Technology Program of Guangzhou (201508020115) and Science and Technology Project of Guangdong province (Nos. 2014B020212010, 508113150092, 2015A010101313 and 2017A050506011).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest in relation to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Statement of Human and Animal Rights All the experiments were approved by the Animal Care Committee of Guangzhou General Hospital. All applicable institutional and/or national guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee.

Informed Consent A 50-year-old healthy woman was selected for abdominal liposuction from the Plastic Surgery Department of the General Hospital. Her signed informed consent was obtained before the procedure was performed.

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