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Does non-activated platelet-rich plasma (PRP) enhance fat graft outcome? An assessment with 3D CT-scan in mice

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Summary *Background:* The adjunction of autologous platelet-rich plasma (PRP) is emerging as a promising approach to enhance the long-term survival of fat grafting, but there are still insufficient data on its efficacy. The aim of this in vivo study was to assess the effect of the addition of non-activated PRP on fat graft outcome.
Methods: Human adipose tissue mixed with 20% of non-activated PRP was injected under the scalp skin of nude Balb/cAnNRj mice and compared to grafted fat mixed with 20% of saline. The fat graft volume was analyzed by a computed tomography scan until day 90 and immunohistochemistry was then performed to assess adipocyte viability and graft revascularization.
Results: At day 90, the volume of fat graft was not enhanced by PRP compared to the saline control group. However, immunohistochemistry showed that PRP significantly increased the fat graft area occupied by intact adipocytes compared to the saline group (72.66% vs. 60.78%, respectively; $p < 0.05$). Vascularity was also significantly higher in the PRP group compared to the control group (6695 vs. 4244 $CD31^+$ cells/μm^2, respectively; $p < 0.05$).
Conclusion: The adjunction of non-activated-PRP to fat grafts significantly increased adipocyte viability and tissue vascularity. However, in contrast to other studies adding activated-PRP, non-activated-PRP did not increase residual fat graft volume until day 90. Further studies are

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therefore needed to understand whether PRP has a positive effect on fat graft volume. As 3D computed tomography scan is a reproducible and precise technique, it should be used to analyze fat graft volume changes over time.

Introduction

Fat grafting has become a frequently used technique in plastic, reconstructive and aesthetic surgery to reconstruct soft tissue defects after oncologic surgery, congenital deformity or traumatic injury, as well as for rejuvenation purposes. Autologous fat is readily obtainable with minimal patient discomfort and low donor site morbidity while permitting substantial soft tissue reconstruction with aesthetic and natural results.[1] The main disadvantage of fat grafting today is the limited rate of graft survival. Therefore, repeated procedures are often required to achieve the desired results. According to the literature, the residual volume of fat graft at 3 months ranges from 20% to 90% of the injected volume, depending on the vascular quality of the recipient bed, the amount of fat injected, and the technique of fat preparation and injection.[1-3]

Many efforts are being made to enhance the residual volume of fat grafts. The technique described by Coleman significantly increased the survival rate of grafted adipocytes by using a gentile manual harvesting technique. He also introduced the concept of the injection of small aliquots of fat distributed in the tissue to increase the contact of grafted cells with the oxygen source.[8] Although this technique significantly improves the survival of grafted adipocytes, further investigations are aimed at defining new approaches to enhance fat graft revascularization in order to further increase fat graft survival and volume disposition. Effective neoangiogenesis of the fat graft is one of the most crucial aspects of its long-term survival.[5,7]

Several strategies have been considered in this direction to improve graft maintenance: fat graft enrichment with stromal vascular fraction[9] or stem cells,[10] adjunction of growth factors such as vascular endothelial growth factor (VEGF),[11] insulin[12] or erythropoietin.[13] Most of these approaches have showed promising results, but due to their complexity and costs, they did not gain popularity with clinicians.

Autologous platelet-rich plasma (PRP) is emerging as a promising approach to enhance fat graft survival.[14,15] PRP is concentrated plasma obtained from the patient's own blood that contains high concentrations of platelets compared to whole blood. Through the activation of their alpha-granules, the platelets contained in PRP provide growth factors and cytokines, which are recognized to play a pivotal role in tissue regeneration.[16] At present, PRP is used in clinics for joint diseases in sports medicine, to enhance wound healing, or in anti-aging treatments.[17] However, it is unclear whether PRP should be used in a non-activated form or be activated by the adjunction of calcium gluconate and/or thrombin. The "artificial" activation of the platelets in PRP results in an immediate, substantial and uncoordinated release of growth factors within hours after application, but limited in time.[18]

Several animal studies have demonstrated the positive impact of PRP on fat graft outcome.[4,6,19-25]. All studies, except one, activated PRP with the adjunction of calcium or thrombin before adding it to the fat graft. In rabbits, Rodriguez-Flores et al. and Pires et al. assessed the influence of activated PRP on autologous fat grafts to the upper lip or to the subcutaneous tissue of the ear, respectively. Rodriguez-Flores et al. observed fewer inflammatory reactions and cysts compared to PRP-free groups, but volume maintenance was not assessed.[22] Pires et al. concluded that activated-PRP significantly increases adipocyte survival and angiogenesis in the fat graft. They observed also that the fat graft weight was higher 6 months after grafting than in PRP-free groups.[24]

Nakamura et al. assessed the influence of calcium chloride ($CaCl_2$)-activated PRP on autologous fat transplants in dorsal subcutaneous pockets in rats. They showed that activated-PRP significantly increased fat transplant weight, viable adipocytes and blood vessel numbers at 1 and 4 months compared to the PRP-free control group.[23] Oh et al. studied the influence of $CaCl_2$- and thrombin-activated PRP on human fat transplants in nude mouse scalps. They concluded that fat weight and vascularity were significantly greater in the activated-PRP group at 10 weeks with less cysts and fibrosis compared to the PRP-free group.[19] The only study concluding that PRP does not have any effect on fat graft volume maintenance, adipocyte survival or vascularity used non-activated PRP (nPRP) in a nude mice model.[21] Recently, Hersant et al. published the unique study comparing the efficiency between nPRP and $CaCl_2$-activated PRP on fat grafting in a rat model. The authors showed that $CaCl_2$-activated PRP is significantly more effective than nPRP to increase adipocyte viability at 3 months, but without any influence on vascularity. However, they did not assess the volume maintenance of fat graft.[25] In most clinical studies, PRP was also activated and the majority of studies demonstrated a positive effect on fat graft maintenance.[26,27]

We previously demonstrated that a media supplemented with nPRP was more effective to enhance the survival and proliferation rate of ASCs in vitro than activated-PRP. In contrast to activated-PRP, more than 50% of non-activated platelets were still intact and secreted growth factors at day 10 of culture.[28] These results are in line with Kakudo et al. who demonstrated that even though platelets activated with thrombin and $CaCl_2$ induced an immediate 100-fold increase in growth factor concentrations in the culture media over non-activated controls, this high rate

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of growth factors had less effect on human ASCs and fibroblast proliferation in vitro than nPRP.[29] Using an animal model of wound repair, we have previously showed that nPRP is more effective than thrombin-activated PRP to enhance fibroblast differentiation and migration, thus promoting angiogenesis and, ultimately, stimulating wound repair.[30]

In this in vivo study, we hypothesized that nPRP would deliver growth factors to the fat graft over time in an orchestrated fashion, promoting its engraftment and outcome. We studied the effects of nPRP on the volume up to 3 months using a reliable model including high-resolution micro computed tomography (CT) scans. In addition, we histologically assessed the effect of nPRP on adipocyte survival and neovascularization of the fat graft.

Materials and methods

Human tissue harvesting and handling were performed according to World Medical Association Declaration of Helsinki (June 1964) and a protocol approved by local ethical committee of human research (no. 10-253). All animal experiments were performed in full compliance with regulatory principles and protocol approved by local ethical veterinary committee (license no. 1065-3681-1).

Human adipose tissue harvesting

Adipose tissue was collected and purified according to the Coleman technique from the abdominal subcutaneous tissue of consenting patients who underwent abdominoplasty.[8] Fat tissue was manually harvested with a 3 mm cannula (Mentor, Santa Barbara, CA) connected to 10 mL Luer-Lok syringes (BD Biosciences, Franklin Lakes, NJ). The fat tissue was isolated from blood, oil, and liquid after 3 min centrifugation at 1200 G.

PRP preparation

PRP was prepared with specific tubes containing 1 mL of sodium citrate as anticoagulant agent and a specific gel separating platelets and plasma from other blood components (i.e., red and white blood cells). In brief, 8 mL of peripheral blood was collected in a Regen-BCT tube (RegenKit; Regen-Lab, Le Mont-sur-Lausanne, Switzerland) from the same patient donating the fat and centrifuged for 5 min at 1500 G. The plasma and platelets accumulated above the gel layer, while the red and white blood cells were collected at the bottom of the tube under the separator gel. Plasma containing high yield of platelets was homogenized by returning the tube five times to obtain 4 mL of nPRP.

Animal models

Eleven-week-old female Balb/cAnNRj mice weighing 23–25 g were purchased from Janvier Labs (France). During all experiments, housing and husbandry were performed in respect of animals' welfare according to international and national recommendations. Before each experiment, mice were anaesthetized with an inhalation of isoflurane 5% for induction and 3% for maintenance of anaesthesia. Human harvested and purified adipose tissue was mixed with either 20% saline as a control or 20% of nPRP. This concentration was chosen according to our previous in vitro experiments, which demonstrated that the adjunction of 20% of nPRP to ASCs offered the highest proliferation rate.[19] Fat was injected with an 18 G needle through different tunnels to the subcutaneous layer on the scalp of each mouse. Each animal received randomly 0.5 mL of fat mixed with either saline or nPRP.

Micro-CT-scan of fat grafts

To assess the volume of the injected fat grafts, 6 mice underwent a high-resolution micro-CT-scan (Triumph microPET/SPECT/CT system [Gamma Medica/Trifoil]) at days 1, 7, 14 and 30: and 3 mice 90 days after fat grafting. Mice were anaesthetized with 2% isoflurane over a heating mattress (30 °C) during the CT-scans. Images were obtained at 80 kVp and 160 μA; 1024 projections were acquired during a 360° rotation with 3.5 and 2.0 magnifications for head and back scans, respectively. The Triumph XO software, which uses a back-projection engine, was used to reconstruct the CT-scans with a reconstruction matrix of 512 and voxel sizes of 0.095 mm for head scans or 0.170 mm for back scans. Reconstructed CT-scans were then re-orientated using the plugin Vivid (Gamma Medica/Trifoil) for Amira (FEI) and exported as DICOM files for high-resolution visualization and analyses of the animals. The software Osirix (Pixmeo, Geneva, Switzerland) was used to analyze the datasets and generate images and videos. Fat graft volumes were obtained by manually drawing regions of interest on 2D slices and computed as 3D volumes.

Immunohistochemistry

Animals were sacrificed at 30 and 90 days after transplantation (3 animals per time point, per condition). At each time point, the fat graft was harvested by careful removal from surrounding tissue, fixed in formaldehyde, and embedded in paraffin for histologic assessment. 5-μm-thick sections of the harvested samples were prepared and immune-stained by the following primary antibodies: anti-human vimentin (dilution 1:50; Dako, Glostrup, Denmark) and anti-perilipin (dilution 1:200; Abcam, UK) for staining intact adipocytes; anti-human CD 31 (dilution 1:10; Dako, Glostrup, Denmark) for staining endothelial cells; and Hoechst (dilution 1:1000; Life Technologies, Carlsbad, CA) for nucleus detection. The following secondary antibodies were used for double fluorescence staining: Alexa fluor 588 conjugated goat anti-human immunoglobulin (dilution 1:1000; Santa Cruz Biotechnology, Heidelberg, Germany) and Alexa fluor 488 conjugated donkey anti-rabbit immunoglobulin (dilution 1:1000; Invitrogen, Carlsbad, CA). Serial sections were obtained and stained with hematoxylin and eosin for the validity of graft appearance. For perilipin, secondary antibodies were applied at a dilution of 1/250

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(goat anti-rabbit IgG1 + IgG2a + IgG3). Detection of secondary antibodies was carried out using the rabbit OmniMap kit (Ventana Medical Systems, Tucson, AZ), based on the conversion of diaminobenzidine to a dye with multimeric horseradish peroxidase. Intact and living adipocytes were identified as vimentin-positive cells with an intact cell membrane marked by perilipin contouring a lumen of 50–150 μm diameter. To evaluate the proportion of intact adipocytes in fat graft tissue, we measured the fraction of the total area surface of the histochemistry slide (mean value of 3 slides per condition) occupied by perilipin- and vimentin-positive cells (values expressed as a percentage). The remaining surface was considered to be fibrotic tissue or disintegrated adipocytes.

Data analysis

Data are presented as mean ± SEM. The *t*-test was used for comparisons between groups. A p value of <0.05 was considered to indicate statistical significance.

Results

Platelet and blood cell counts

Mean platelet concentration of the obtained nPRP was $1.2 \times 10^6 \pm 120.6$ platelets/μL,significantly higher than whole blood ($1.44 \times 10^5 \pm 80.36$ platelets/μL). All platelets over the gel separator were re-suspended in 4 mL of plasma and the final mean platelet concentration of nPRP used was $2.41 \times 10^5 \pm 20.36$ platelets/μL. Conversely, the mean white blood cell concentration was significantly lower in nPRP than in whole blood ($0.72 \times 10^3 \pm 0.11$ cells/μL vs. $4.87 \times 10^3 \pm 0.46$ cells/μL, respectively). The mean red blood cell concentration was similarly reduced ($0.03 \times 10^6 \pm 0.004$ cells/μL vs. $3.86 \times 10^6 \pm 0.21$ cells/μL).

Macroscopic analysis of fat graft

The injected fat appeared as a single mass under the scalp on days 30 and 90. Although the fat volume appeared macroscopically similar under both conditions (saline and nPRP), more vessels were visible in the nPRP condition (Figure 1).

Micro-CT-scan assessment of fat graft volume

CT-scan analysis showed that the volume of grafted tissue in both conditions decreased over time from days 1 to 90 (Figure 2(a)). A comparison of the two groups showed that nPRP did not change significantly the volume of the grafted tissue at any time point. When compared to day 1, the maintained volume measured at day 90 was 62.48% in the control group and 60.9% when nPRP was added (Figure 2(b)).

Immunohistochemistry

The adipocyte viability analysis showed a trend towards an increase of the percentage of the total surface occupied by intact and living adipocytes (i.e. vimentin- and perilipin-positive cells) from 30 to 90 days in both groups. (Figure 3(a)) At day 30, the difference between the control and nPRP group was not statistically significant (52.33 % vs. 57.41%, respectively; $p > 0.05$). At day 90, fat graft combined with nPRP showed a significantly higher area occupied

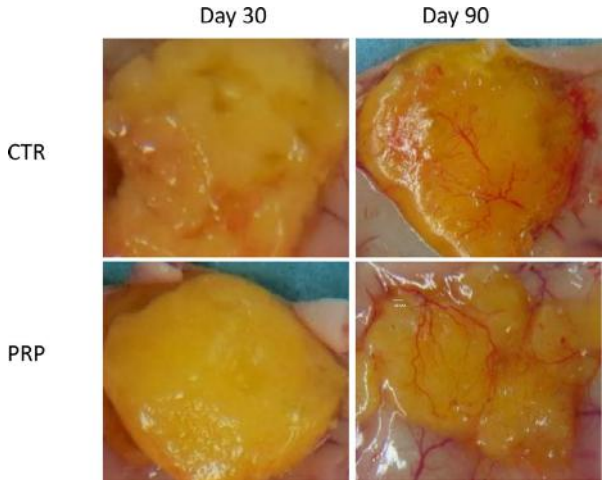

Figure 1 Macroscopic representative view of grafted fat on the scalp of mice on day 30 and 90 after grafting, showing more vascularization with 20% PRP. CTR: fat + 20% saline, PRP: fat + 20% non-activated-PRP.

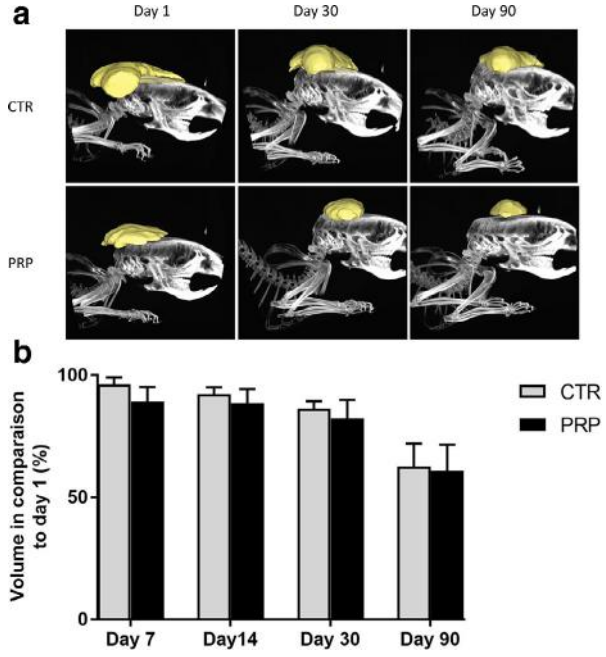

Figure 2 The CT-scan analysis of fat graft volume. (a) 3D images of CT-scan on the scalp on the 1, 30 and 90 after fat grafting with our without PRP. (b) Quantification of 3D fat graft volume does not show any significant difference between CTR group and non-activated-PRP group. Results are quantified as a % of volume present on day 7, 14, 30 and 90 compared to day 1. CTR: fat + 20% saline, PRP: fat + 20% non-activated-PRP ($n = 6$ for day 7, 14 and 30; $n = 3$ for day 90).

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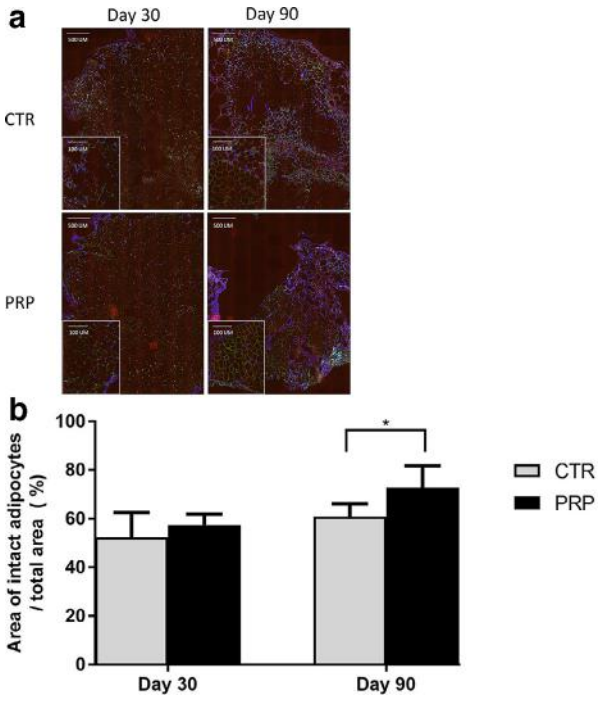

Figure 3 Immunohistochemistry analysis for intact adipocytes: (a) cells marked with anti-perilipin in green and Hoechst in blue on day 30 and day 90 for CTR and PRP (500 μm and 100 μm magnification). (b) quantification of the portion of the surface occupied by intact cells to the total surface. The gray bars represent CTR group and the black bars represent PRP group. ($n = 3$) (*$p < 0.05$).

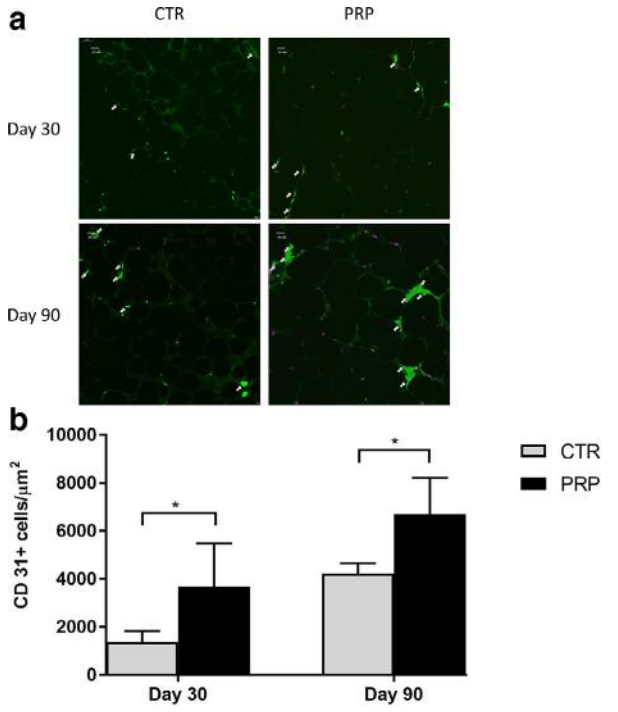

Figure 4 Imunohistochemistry analysis for neoangiogensis: (a) endothelial cells marked with anti-human CD31 (green, highlighted with arrow) in the grafted fat on day 30 and day 90, for CTR and PRP groups on the scalp in 50 μm magnification. (b) Quantification of the portion of the surface occupied by CD31-positive cells (pixels quantification) to the total surface. The gray bars represent CTR group and the black bars represent PRP group. ($n = 3$) (*$p < 0.05$).

by intact adipocytes (72.66%) compared to the control group (60.78%; $p < 0.05$) (Figure 3(b)).

CD31 staining showed that vascularization increased significantly when nPRP was added to the graft (Figure 4(a)). On day 30, CD31-positive cell concentration was 1373+/− 448 nuclei/μm^2 in the control group vs. 3673+/− 1809 nuclei/μm^2 in the nPRP group ($p < 0.05$). The CD31-positive cells increased in both groups over time and reached 4244+/− 427 nuclei/μm^2 on day 90 in the control group with a further significant increase when nPRP was added (6695+/− 1519 nuclei/μm^2; $p < 0.05$) (Figure 4(b)).

Discussion

Our findings show that the adjunction of nPRP to fat grafts does not increase their residual volume when measured by CT-scan at day 90, despite a significant increase of adipocyte viability and tissue vascularity.

Graft volume maintenance over time is a key criterion when evaluating the outcome of fat grafts. However, apart from Por et al., none of the previous in vivo studies assessed the volume of fat graft when PRP was added. Some of them, by weighting with a balance, demonstrated that the fat graft weight is improved when nPRP was added.[19,23,24] This lack of volume assessment in the literature is probably due to technical limitations. In the beginning of our study, we attempted to weight the fat graft sample with a balance and to measure the fat graft volume with a water-emerging system. After a few animal samplings, we noticed that these techniques were not accurate to assess the fat graft maintenance. This inaccuracy was due to the low volume and weight of samples (<0.5 mL), and the small changes that we assessed. Moreover, the dissection of samples from surrounding subcutaneous tissue was not reproducible. Therefore, for our study we decided to use a 3D CT-scan technique. It has been shown previously to be an accurate approach to evaluate fat volume in rodents.[33,34] To the best of our knowledge, this is the first time that this technique has been used to assess the evolution of fat grafts in animal experiments. It is a more reproducible and precise technique than *ex vivo* weighing[33,34] with a balance or volume estimation with a water-emerging technique as reported in previous studies. Moreover, it allows following the same animal over time, without the need to sacrifice animals at each time point, thus respecting the "3R" principles of veterinary welfare. We suggest using this in vivo CT-scan analysis to measure the volume of fat graft over time in future studies.

The histology of the nPRP fat grafts showed that the percentage of the area occupied by intact adipocytes was significantly higher (72.66%) compared to the control group (60.78%; $p < 0.05$) at day 90. Interestingly, our rates are much higher than those observed by Hersant et al. At 3 months following fat grafting on the back of rats, the percentage areas occupied by viable adipocytes in the fat graft

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was 24% in the activated-PRP group compared to 14% in the nPRP and 13% in the PRP-free group ($p = 0.05\%$).[31] The important differences between the two studies could be related to several factors. First, Hersant et al. injected larger amounts of fat (1 mL) than in our study (0.5 mL) and this large fat graft volume could provoke a greater "necrotic zone" with more cysts and less viable adipocytes. The second factor is the receptor site of graft chosen. As the skin of the back is more mobile than the scalp, the rate of fat resorption could be higher.

In both Hersant et al. and our study, the area not occupied by intact adipocytes (vimentin- and perilipin-positive cells) was covered by fibrosis, cell debris or oily cysts at 3 months. These cell debris and oil cysts, resulting from dead adipocytes and ASCs will possibly be resorbed by macrophages and induce fibrosis later as demonstrated by Eto et al.[5]. This would reduce even more the residual fat graft volume. As we observed a significantly lower proportion of these unwanted tissues in nPRP fat grafts, it is conceivable that a longer follow-up period could show a lesser decrease in fat graft volume with the addition of nPRP. However, due to the limitations of our animal model (e.g. high mortality of rodents related to age, fat grafting rejection due to xenograft) the effect of PRP on fat volume could not be assessed for more than 90 days. A longer follow-up with another animal model is required to verify this assumption for nPRP, e.g. autologous fat in a rabbit model as performed by Pires and Rodriguez.

Vascularization, marked by CD31-positive cells, significantly increased in nPRP-treated grafts on days 30 to 90 compared to the control group. Most likely, the improvement of angiogenesis was due to growth factors, such as VEGF and platelet-derived growth factor secreted by platelets present in nPRP. Other studies have also demonstrated vascularity enhancement with PRP.[19,23,24] Tissue vascularization is one of the key elements for fat graft take and maintenance. Immediately after transplantation, the grafted fat tissue undergoes a state of acute ischemia. Initially, cells survive via the plasmatic diffusion of nutrients and oxygen from surrounding tissues. Neovascularization starts from approximately 48 h after transplantation by the ingrowth and reconnection of capillaries and vessels between the recipient bed and the graft.[4] Yoshimura et al. reported that when fat is transferred to the receptor site, adipocytes survive for 24 h and ASCs up to three days under ischemic conditions.[32] A part of the graft, which is not vascularized within the first three days, will become necrotic and form oily cysts, fibrosis and calcifications[5-7] and subsequently provoke a reduction of the grafted fat volume. Therefore, it is primordial that neovascularization for adequate oxygenation of cells takes place by day three.[32] Although vascularity was enhanced in our study on days 30 and 90, in contrast to other studies with activated-PRP, nPRP did not show any effect on fat graft volume maintenance. This difference could be explained by the fact that PRP activation with calcium or thrombin, provoking an immediate secretion of granules' contents (e.g. cytokines, growth factors), could be more effective in accelerating neoangiogenesis during the first days when the presence of oxygen is most crucial in fat grafting. Even though angiogenesis was improved in our experiments, the neovascularization took place possibly too late when PRP was not activated.

Conclusion

In contrast to our previous studies on wound repair and stem cell cultures where we conclude that nPRP is more effective than activated-PRP, we were unable to demonstrate that nPRP increases fat graft residual volume at 3 months. This difference could be explained by the fact that these applications require a lower concentration of growth factors, but a longer time of PRP action, compared to fat grafting. Therefore, before clinical translation, further studies are needed to compare the effect of activated PRP to non-activated PRP for fat graft angiogenesis and maintenance, by using a standardized fat graft volume analysis such as a 3D CT-scan.

Acknowledgments

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Conflict of interest

Regen BCT tubes were generously provided by Regen Lab SA (Le Mont-sur-Lausanne, Switzerland).

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bjps.2018.12.039.

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