Concentrated Growth Factor Enhanced Fat Graft Survival: A Comparative Study

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BACKGROUND Concentrated growth factors (CGFs) belong to a new generation biomaterials that concentrate large number of growth factors and CD34+ stem cells in small volume of plasma.

OBJECTIVE The purpose of this study was to evaluate the impact of the new technique, CGF, on fat graft survival, which compared with platelet-rich plasma (PRP) and platelet-rich fibrin (PRF).

MATERIALS AND METHODS Nude mice received fat graft were divided into PRP group, PRF group, CGF group, and saline. The grafts were volumetrically and histologically evaluated at 4, 8, and 12 weeks after fat grafting. In vitro growth factor levels in PRP, PRF, and CGF were compared using enzyme-linked immunoassay method. Cell count and real-time polymerase chain reaction were used to evaluate the impact of CGF in medium on human adipose-derived stem cell (hADSC) proliferation and vascular differentiation, respectively.

RESULTS Fat graft weight was significantly higher in the CGF group than those in the other groups, and histologic evaluation revealed greater vascularity, fewer cysts, and less fibrosis. Adding CGF to the medium maximally promoted hADSC proliferation and expressing vascular endothelial growth factor and PECAM-1.

CONCLUSION In this preliminary study, CGF treatment improved the survival and quality of fat grafts.

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Fat grafts have become one of the most commonly used methods in the clinical treatment of soft-tissue defects because of its wide range of sources and nonimmunogenic properties. However, the high absorption rate after autologous fat tissue transplantation is a limitation in the development of this technology.[1] Numerous methodologies and algorithms have been suggested to enhance fat graft survival, including the addition of stromal vascular fraction (SVF), growth factors, or platelet-rich plasma (PRP).[2–4] With increasing understanding of fat tissue and the structural changes after the transplantation of adipose tissue, currently, the main cause of the high absorption rate is that the microcirculation can not be established in time, which causes hypoxia adipocyte apoptosis, necrosis, and eventually liquidization of fat.[5] Therefore, many researchers have focused on improving the early vascularization of transplanted adipose tissue to address the high absorption rate of transplanted fat tissue.[6–8]

Growth factors play a critical role in cell migration, cell proliferation, and angiogenesis for tissue regeneration. Several growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, transforming growth factor β1 (TGF-β1), fibroblast growth factors, and others, are contained in platelets, which play an important role in facilitating vascularization.[9] To use these autologous growth factors, numerous methodologies including

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PRP, platelet-rich fibrin (PRF), and concentrated growth factors (CGFs) have been developed.

Autologous PRP is a product derived from fresh whole blood that contains a high concentration of platelets with healing anti-inflammatory and proregenerative properties that permit the body to heal tissue wounds faster and more efficiently.[10] In 2007, Azzena hypothesized that autologous PRP could be used as an in vivo adipocyte delivery system to favor cell survival and to stimulate the early recruitment of microcapillaries to the site of implantation.[11] PRF, as the second-generation platelet product, is safer and more reliable compared with PRP because it has no added anticoagulants or coagulants.

Concentrated growth factors belong to a new generation of biomaterials that concentrate large numbers of growth factors and $CD34^+$ stem cells.[12,13] Concentrated growth factor was obtained from venous blood using a thermostat centrifuge with controlled speeds as proposed by Sacco in 2006.[12] Concentrated growth factor seems to be much more regenerative because it was reported to be used in the field of dentistry, with the aim of promoting new bone formation and researching the impact of gingival tissue.[14–18] Concentrated growth factor is a promising candidate biomaterial for peripheral nerve regeneration.[13]

Concentrated growth factor used for tissue reparation and as a regeneration biomaterial has better attributes than PRP and PRF. The aim of this study was to evaluate the impact of the new technique, CGF, on improving the survival of transplanted adipose tissue.

Materials and Methods

Fat Harvesting and Preparation

Subcutaneous adipose tissue obtained from the abdomen was harvested from a 20-year-old healthy female donor. Informed consent was obtained, and animal procedures were conducted according to a protocol approved by the Ethics Committee of the Sichuan University Health Science Center, Sichuan, China. Samples were placed in a fixed-angle rotor centrifuge and centrifuged at 200*g* for 3 minutes. After centrifugation 3 layers can be observed, namely free lipids, adipose tissue, and aqueous infranatant, which contain blood cells and debris. Discard the free lipids and aqueous layers. Excess liquid and chunks of fascia were removed on sterile gauze, and the collected adipose tissue was divided into four 5-mL tubes with 3 mL of adipose tissue in each tube.

Platelet-Rich Plasma, Platelet-Rich Fibrin, and Concentrated Growth Factor Preparation

Whole blood was collected from the same person. A total of 9 mL of blood was collected with anticoagulant and then centrifuged at 200*g* for 10 minutes. The upper buffy layer with some red blood cells (RBCs) was transferred into a new sterile tube and centrifuged at 800*g* for 10 minutes to separate the PRP from the upper layer platelet-poor plasma. The platelet concentration of whole blood and PRP was measured using an automated hematology analyzer. Platelet-rich plasma was activated just before injection by thrombin activators (1,000 U bovine thrombin in 1-mL 10% calcium chloride) (Figure 1, left).

A total of 9 mL of blood was obtained without anticoagulant and immediately centrifuged at 400*g* for 10 minutes. The blood was divided into 2 layers after centrifugation. The upper layer in the tube was the PRF layer, whereas the translucent light yellow fibrin layer contained platelets and plasma (Figure 1, center).

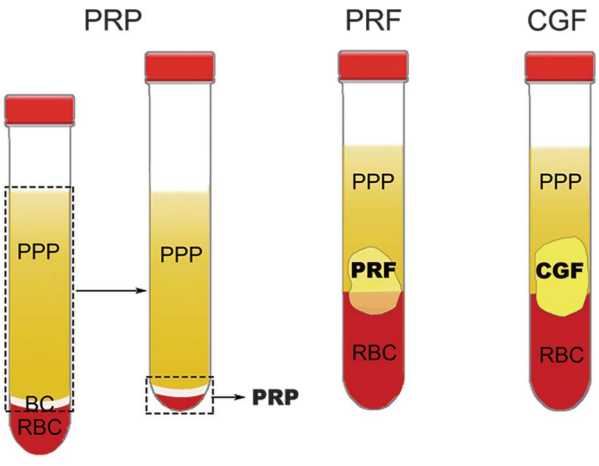

Figure 1. Blood samples after different protocols: (left) platelet-rich plasma (PRP), (center) platelet-rich fibrin (PRF), and (right) concentrated growth factor (CGF). PPP, platelet-poor plasma.

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Concentrated growth factors were prepared according to Sacco's protocol. The tube containing the blood sample was directly placed into the CGF centrifuge (MEDIFUGETM; Silfradentsrl, Santa Sofia, Italy) with a variable speed for 12 minutes according to the instructions. After centrifugation, there were 4 layers: (1) the superior serum layer, (2) the second layer called buffy coat, (3) the third layer containing GFs and stem cells, and (4) the lower RBC layer. Then, the sample was taken out of the centrifuge, and the lower RBC layer was cutoff; the upper layer was CGF (Figure 1, right).

In Vivo Subcutaneous Injection of Adipose Tissue

A total of 1 mL of PRP, PRF, and CGF and normal saline were mixed with 3 mL processed adipose tissue to create the treatments for the 4 groups. Then, 0.2 mL of the treatments were randomly subcutaneously injected into the bilateral fore and hind flanks of 15 male athymic nude mice with an average age of 8 weeks old, ranging from 7 to 9 weeks.

Histology

At the 4-, 8-, and 12-week time points, 5 mice were chosen to be euthanized for gross weight and histological analyses of the lipoaspirate. The dissected fats were immediately fixed in 4% paraformaldehyde solution for paraffin embedding. Sections of 5-μm thickness were cut and stained with hematoxylin and eosin (H&E), and immunohistochemical analysis was performed using anti-CD31 antibody (1:200 dilution). The sections were analyzed using Image Pro Plus software.

Enzyme-Linked Immunosorbent Assay Analysis

Twenty-four hours after preparation, the enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instructions to quantify the concentrations of TGF-β1 and VEGF secreted from PRP, PRF, and CGF treatment groups.

Scanning Electron Microscopy Analysis

Freshly prepared PRP, PRF, and CGF samples were fixed in 4% glutaraldehyde for 12 hours. Then, the samples were dehydrated in graded series of ethanol, subjected to vacuum evaporation and sputter coated with platinum. The samples were observed using a scanning electron microscope.

Human Adipose-Derived Stem Cell Culture

The adipose tissues were harvested from the abdomen of a female who underwent liposuction. The procedure to obtain human adipose-derived stem cells (hADSCs) was previously described. The adipose tissues were washed and digested using 10% collagen type I/PBS solution at a ratio of 1 mL of adipose tissue to 1 mL of enzyme solution, and then, the mixture was incubated in a 37° water bath for 40 minutes with gentle shaking. The adipose tissue was centrifuged at 1,200 rpm for 5 minutes, and then, the supernatant was removed. The cellular pellet and the SVF were added to appropriate PBS solution and centrifuged at 1,200 rpm for 5 minutes. After discarding the supernatant, the SVF was added to general culture media with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA), α-modified Eagle's medium (α-MEM) (Hyclone, Logan, UT), 100-IU penicillin, and 100-mg/mL streptomycin (Solarbio, Bejing). The culture medium was changed every 2 to 3 days.

Groups in the Study

The third passage cells (1.0×10^5 cells) were seeded and incubated with DMEM containing 10% FBS in a T25 flask. After overnight incubation, there were four groups for the experiments: Group I had 4 mL of culture medium, Group II had 1 mL of PRP +3 mL of culture medium, Group III had 1 mL of PRF +3 mL of culture medium, and Group IV had 1 mL of CGF +3 mL of culture medium. The cell medium was replaced every 2 to 3 days for 14 days.

Cell Count

Photographs were taken of each group using an inverted microscope with 5 fields of vision, including central and surrounding, when cells attached on the surface of the culture flask and during the next 7 days of coculture. The magnification of the optical microscope was 200 times. The number of cells was counted using Image J software. Two individuals completed this process in accordance to the same criteria. The average cell number of the 5 fields of vision was used to draw the cell growth curve.

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TABLE 1. Polymerase Chain Reaction Genes and Primer Sequences

Gene Name	*Forward Sequence*	*Reverse Sequence*
VEGF	AGGGAAGAGGAGGAGATGAG	GCTGGGTTTGTCGGTGTT
PECAM-1	GGGTTTGCCCTCTTTTTCTC	CCTGTCAAGTAAGGTGGTGGA
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

VEGF, vascular endothelial growth factor.

Quantitative Real-Time Polymerase Chain Reaction

The RNAiso Plus kit from Takara was used to isolate total RNA according to the manufacturer's instructions. cDNA was prepared using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania, EU). Primer sequences for *VEGF*, *PECAM-1*, and *GAPDH* were designed by the Shanghai Sangon Biotech company, and *GAPDH* served as the internal control. Using the synthesized cDNA as the real-time polymerase chain reaction (PCR) reaction template, quantitative PCR was performed using the fluorescent dye SYBR Premix Ex Taq (RTM: Tli RNaseH Plus) in a Quant Studio TM6 Flex Real-Time PCR System (Life Technologies, Carlsbad, CA). The expression of *VEGF*, *PECAM-1*, and *GAPDH* genes were identified by quantitative PCR using the following primers (Table 1). Triplicate experiments were performed throughout this study. All assays were repeated 3 times to ensure reproducibility.

Statistics

Analysis of variance was used for data analysis to compare differences among the 4 groups. Least Significant Difference *t*-test was used to further compare the differences between 2 groups. A p-value of $<.05$ was considered statistically significant.

Results

Concentrated Growth Factor–Improved Fat Graft Survival

The successful completion of the fat graft was grossly observed at the 4-, 8-, and 12-week time points and

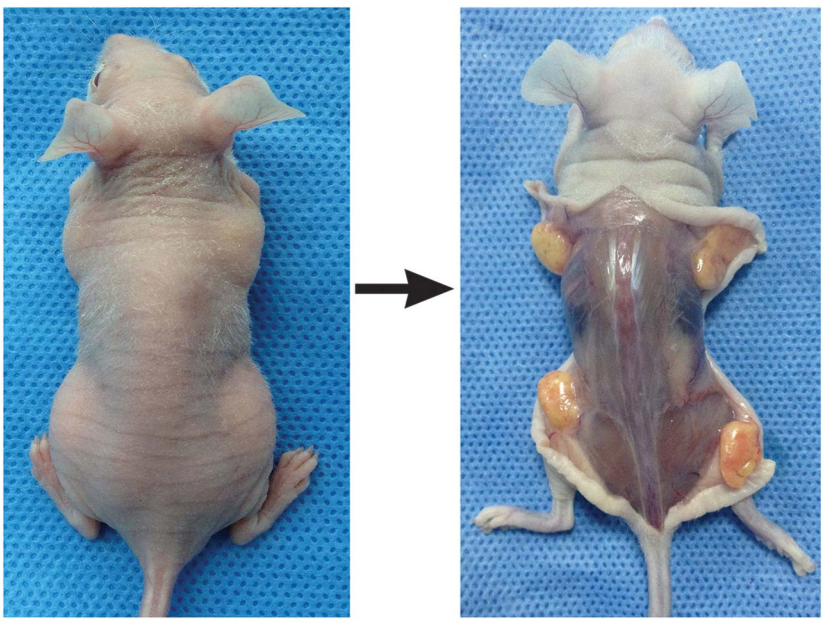

Figure 2. (Left) Processed adipose tissue mixed with platelet-rich plasma (PRP), platelet-rich fibrin (PRF), concentrated growth factor (CGF), and normal saline (NS) was randomly subcutaneously injected into the bilateral fore and hind flanks of nude mice. (Right) The samples were retrieved at 4 weeks.

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wrapped in connective tissue (Figure 2). As time goes on, vascularization of adipose tissue was getting clearness in, generally, vision (Figure 3, left). At the 4-week time point, a greater percentage of the grafts of the CGF group remained when compared with the control group (77.2 ± 8.5% vs 62.4 ± 5.1%, $p < .05$) and the PRP group (68.0 ± 4.7%, $p < .05$). There were no significant differences among the PRF and CGF groups ($p > .05$). However, for the long-term survival rates, we noticed that the statistical discrepancy among the groups began to amplify. At the 12th week, the CGF group maintained a higher fat residual ratio (53.6 ± 6.6%) than that of the other groups (22.0 ± 2.9% in the control group, 30.9 ± 6.0% in the PRP group, and 34.9 ± 3.8% in the PRF group, $p < .05$) (Figure 3, right).

Concentrated Growth Factor Effects on Fat Graft Architecture and Vascularity

Whole-mount histology has shown a well-organized network with capillaries located alongside the adipocytes in normal adipose tissue. The mode of adipocyte diameter was approximately 90 to 110 μm. In this study, adipocyte diameters larger than 120 μm were regarded as fat vacuoles. Hematoxylin and eosin sections of tissues harvested at the 4th week showed significantly more normal adipocytes formed in the CGF group than in other groups ($p < .05$), and fewer fat vacuoles formed in the CGF group than in the control group and PRP group ($p < .05$). In particular, an enlargement of the proportion of fat vacuoles increased, whereas the percentage of normal fat cells decreased at the 8th week. When comparing the proportion of normal adipocytes, fat vacuoles between the CGF group and the control group, the difference was statistically significant. At the 12th week, the CGF group fat graft was significantly more normal adipocytes, with fewer fat vacuoles, than in the control group and PRP group ($p < .05$). In addition, there were no significant differences among the 4 groups in the formation of connective tissue (Figure 4).

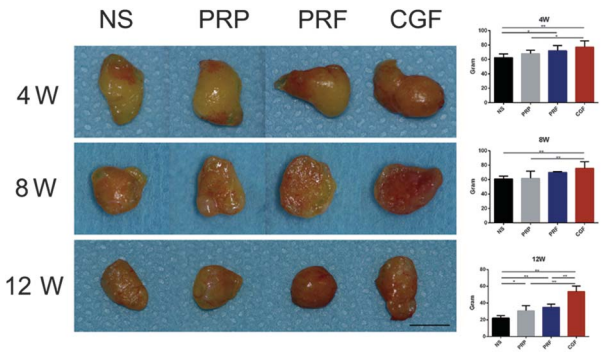

Figure 3. Statistical analysis of the weight of the fat grafts is shown. (Left) The autopsy fat graft specimens from the platelet-rich plasma (PRP), platelet-rich fibrin (PRF), concentrated growth factor (CGF), and normal saline (NS) group at the 4, 8, and 12 weeks. (Right) Significant difference was found between normal saline group and CGF group at 4 and 8 weeks. At 12 weeks, mean graft mass in the CGF group was higher than the others.*$p < .05$ and **$p < .001$. Scale bar = 1 cm.

CD31 staining at the 4th week demonstrated significantly greater vascular density in the control group when compared with that of the CGF- and PRP-treated adipose tissue. Vascular density increased in all groups across the groups. At the 12th week, vascular density in the CGF group was again found to be significantly greater when compared with that of the control group and that of the PRP group (Figure 5).

Platelet Counts, Levels of Growth Factors, and Scanning Electron Microscopy Observations

The number of platelets in whole blood was 220.7 ± 14.6 × 10^9/L, whereas the number was 1901.3 ± 156.5 × 10^9/L in PRP. There were 8.6 times more platelets in PRP compared with whole blood. Vascular endothelial growth factor and TGF-β1 Elisa kits were used to measure growth factors in the supernatants of PRP, PRF, and CGF. Detection results showed that CGF had the maximum content of VEGF and TGF-β1, and the difference between the PRP and PRF groups was statistically significant ($p < .05$) (Figure 6, above).

Images taken using scanning electron microscopy revealed that a mushroom-shaped fiber structure was found in PRP samples. It was unable to determine weather the platelets embedded in the fibrin matrix. In the CGF samples, a much clearer fiber structure tended to form a network and the RBCs trapped within the fibrin matrix. The structure of the PRF samples was intermediate between those of the PRP and CGF samples, and a small number of RBCs adhered to the matrix (Figure 6, below).

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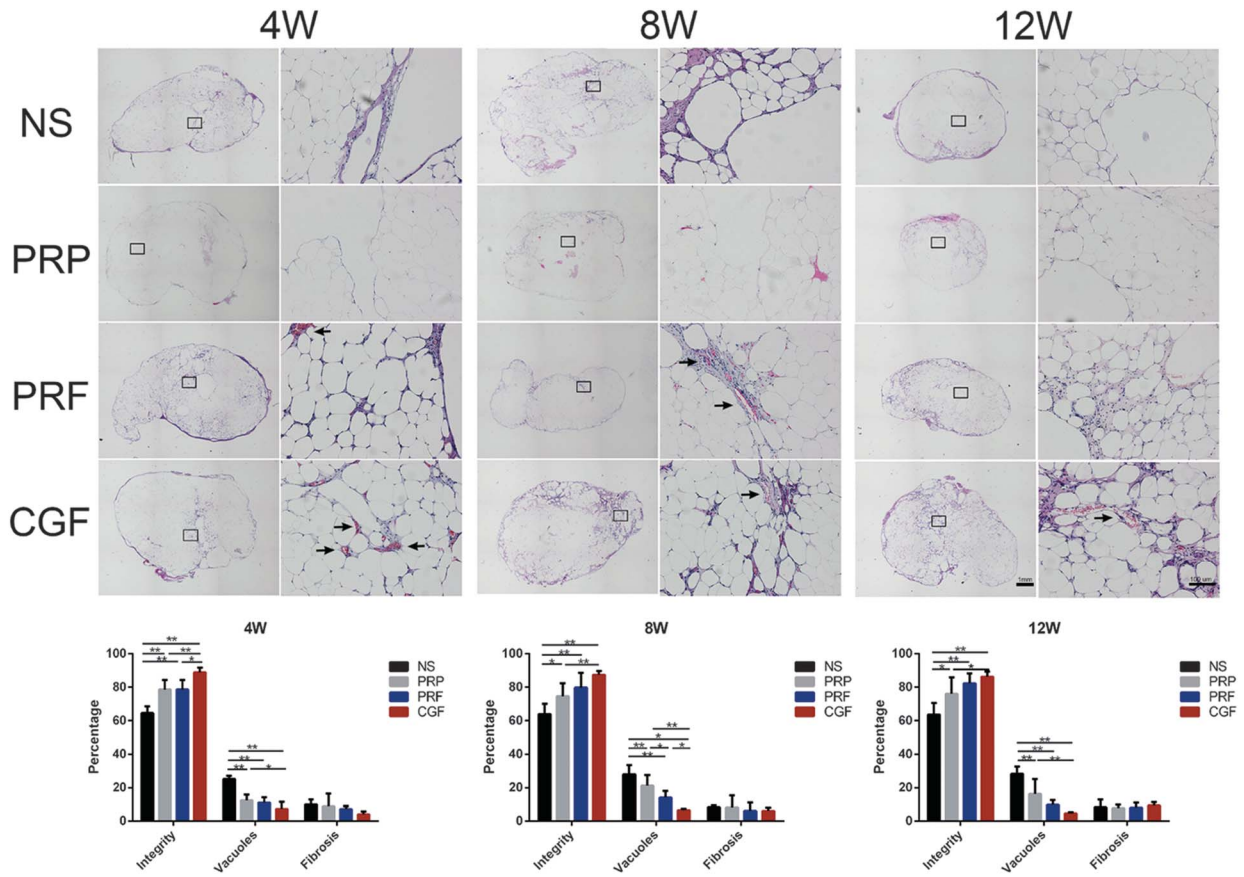

Figure 4. (Above) Histological images of grafts of the 4 groups separately obtained at the 4, 8, and 12 weeks, which were stained by hematoxylin and eosin. (Above, first, third, and fifth columns) The images were adjusted by applying the merge function of Adobe Photoshop CS6 to the original image as a whole. There was no image manipulation and scale bars were added in Photoshop. (Below) The percentage area of integrity, vacuoles, and fibrosis was calculated. Significant difference was found between normal saline (NS) group and concentrated growth factor (CGF) group (*$p < .05$ and **$p < .001$). The arrows indicate vessels.

Effects of Concentrated Growth Factor on Human Adipose-Derived Stem Cell Proliferation and Differentiation

After 7 days of coculture, statistics showed that adding PRP, PRF, and CGF was conducive to an increased growth rate of hADSCs. Comparing the CGF group with the control group and the PRP group, the difference in cell proliferation was statistically significant at day 7 ($p < .05$) (Figure 7, left).

Quantitative real-time PCR (qRT-PCR) data showed that, in cells cultured with PRP, PRF, and CGF compared to groups with general culture media alone, *PECAM-1* and *VEGF* were expressed at higher levels. The expression of *PECAM-1* and *VEGF* in the group

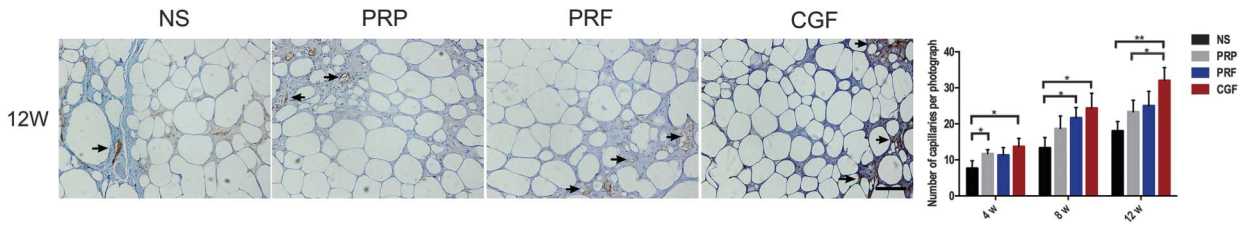

Figure 5. CD31 immunostaining. (Left) Images of grafts obtained at 12 weeks. Neovessels appeared as masses or streaks in the graft and typical vascular lumens containing red blood cells (RBCs) were also seen. (Right) The number of neovessels was calculated in each photograph. Significant difference was found between normal saline (NS) group and concentrated growth factor (CGF) group ($p < .05$). Scale bar = 100 μm. Arrows indicated the neovessels. *$p < .05$ and **$p < .001$. Scale bar, 1 cm.

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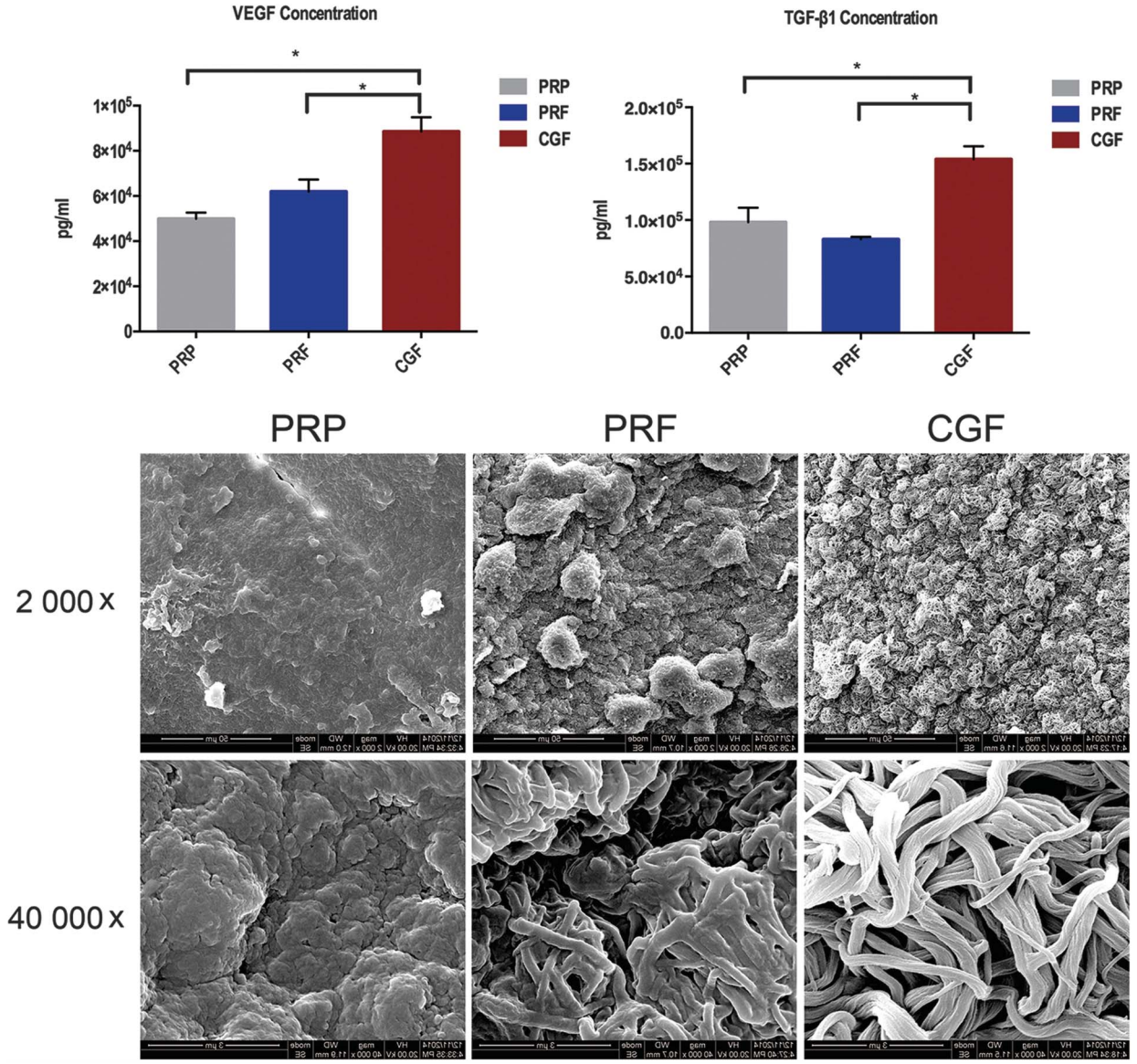

Figure 6. Vascular endothelial growth factor (VEGF) and transforming growth factor β1 (TGF-β1) secreted from platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and concentrated growth factor (CGF). (Above, left) Vascular endothelial growth factor concentration in PRP, PRF, and CGF. (Above, right) TGF-β1 concentration in PRP, PRF, and CGF. Vascular endothelial growth factor and TGF-β1 concentrations were significantly lower in PRP and PRF groups when compared with CGF group ($p > .05$). (Below) The scanning electron microscopy (SEM) photographs of PRP, PRF, and CGF. The top panels represent a low-power magnification (×2000), and the bottom panels show a higher magnification (×40,000). *$p < .05$.

with CGF was expressed at the highest levels compared with that of the groups with PRP and PRF or culture media alone (Figure 7, center, right).

Discussion

Despite the fact that autologous fat grafting has become common application in aesthetic and reconstructive plastic surgery, there is no definitive method to resolve the risk of unpredictable survival and fat necrosis rates. Studies have suggested that oxygen and nutritional supply for fat cells after transplantation are crucial for decreasing apoptosis or necrosis in adipocytes.[19] Clinically, the use of PRPs may be considered a safer and more prudent pathway in cellular-adjunctive therapy, rather than the use of recombinant growth factors or even SVFs or ADSCs, to address the complexities of tissue repair and regeneration because of its current safety profile and its composition of multiple endogenous growth factors and adhesion molecules.[8]

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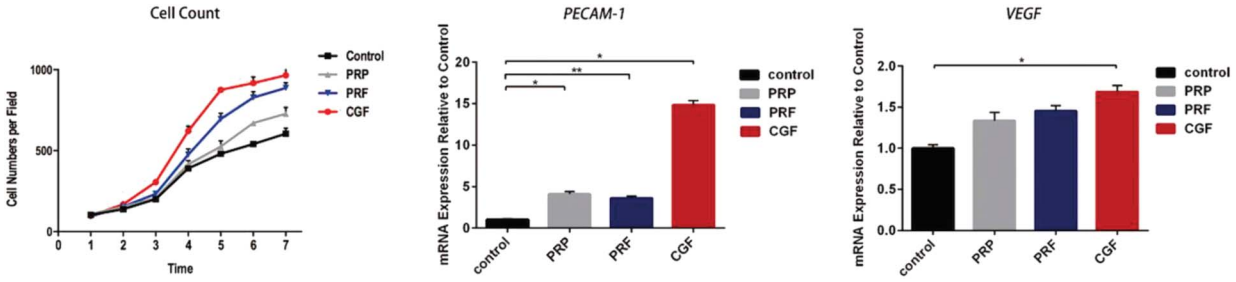

Figure 7. (Left) Human adipose-derived stem cell (hADSC) proliferation in experimental groups. Human adipose-derived stem cell proliferation significantly increased in medium containing platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and concentrated growth factor (CGF) compared with general culture medium. (Center, right) Quantitative real-time polymerase chain reaction (PCR) showed significant increases of *PECAM-1* and *VEGF* mRNA levels in CGF group. Error bars = mean ± SD versus control (*$p < .05$ and **$p < .001$).

Recently, researchers introduced methods using CGF, which forms richer layers of growth factors and provides an enriched fibrin clot. Concentrated growth factor releases growth factors from the adhered platelets and from the fibrin concentrate. Rodella and colleagues studied the biological characteristics of CGF and found that there are CD34-positive cells in the CGF and RBC layers, with a higher number in the CGF layer. Moreover, increasing evidence has shown the role of circulating CD34 in vascular maintenance, neovascularization, and angiogenesis.[12] Although CGF seems to have a better regenerative capacity, as reported in new bone formation and peripheral nerve regeneration, the question remains as to whether CGF increases the success rate of fat grafting. To address this question, we compared CGF with PRP and PRF.

Platelet-rich plasma, as the first method to collect platelets, has been used to assist with fat grafting in experiments and clinical trials. Most fat graft experiments, in which PRP had been activated, have shown that PRP not only increased volume maintenance and blood vessels but also reduced formation of cyst and fibrosis compared with the control groups. However, high concentrations may present limitations when large-volume reconstructions are required, as in the case of breast surgery.[20,21] Therefore, the ratio of 1:3 was selected to use in our experiment.[22] Moreover, experiments had confirmed that the effect of PRF to improve the survival rate of transplanted fat is better than that of PRP.[23] In vivo results after 12 weeks in this study indicated that the CGF group maintained the heaviest mass of adipose tissue compared with that of the other 3 groups. Section staining revealed that the formation of vacuolation and connective tissue in the CGF croup was at a minimum, whereas the neogenesis of capillaries was the highest. We hypothesize that the differences among PRP, PRF, and CGF groups may lead to varied growth factor levels. Therefore, we performed vitro experiments to validate these findings.

From the ELISA tests, we found that the concentrations of VEGF and TGF-β1 were significantly higher in CGF than in PRP and PRF ($p < .05$). There was no significant difference in the growth factor levels in PRP and PRF groups. In the surface morphology analysis of this study, the fibrous structure of CGF was clearer, and the pore structure was more obvious and distinct relative to those of PRP and PRF, which may improve the release of platelets. The results of cocultured hADSCs indicate that the CGF group can promote the proliferation of ADSCs better than other groups.

Vascularization is essential for graft survival, regardless of whether PRP, PRF, or CGF have an effect on the differentiation of hADSCs. To further confirm the results of the differentiation assay, mRNA expression specifically relating to angiogenesis was measured. Quantitative real-time PCR data showed that, in cells cultured with PRP, PRF, and CGF compared to the group with general culture media alone, *PECAM-1* and *VEGF* were expressed at higher levels. The expression of *PECAM-1* and *VEGF* in the group with CGF was higher than that of groups with PRP, PRF, or culture media alone. Furthermore, the increase in *PECAM-1* expression of the CGF group was 15 times more than that of the control group.

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Our findings illustrated that the CGF processing method is able to promote the release of cytokines, thereby promoting hADSC proliferation and differentiation into the vasculature. Vascularization of transplanted fat tissue was improved by CGF, which increases the survival rate of the fat graft. Thus, our study provides the exploration of several mechanisms by which CGF increases fat grafting. However, this study is not without limitations. Therefore, further studies of CGF are needed to elucidate the underlying reasons for the effects on autologous fat transplantation.

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