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Improved Fat Graft Survival by Different Volume Fractions of Platelet-Rich Plasma and Adipose-Derived Stem Cells FREE

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Abstract

Background

The success of soft-tissue augmentation is offset by the low survival rates of grafted fat tissue. Research shows that adipose-derived stem cells (ASCs) and platelet-rich plasma (PRP) are beneficial to tissue healing.

Objectives

To evaluate the long-term effects of different volume fractions of PRP combined with ASCs on fat graft.

Methods

ASCs were isolated from human fat tissue, and PRP was obtained from human blood. Cell count kit-8 and real-time polymerase chain reaction (PCR) were used to evaluate the influence of PRP (0%, 10%, 20%, and 30%; volume/volume [v/v]) in medium on ASC proliferation and adipogenic differentiation, respectively. A novel lipoinjection consisting of granular fat, PRP, and ASCs was subcutaneously transplanted into nude mice. The grafts were volumetrically and histologically evaluated 10, 30, 60, and 90 days after transplantation.

Results

The addition of PRP improved ASC proliferation. Expression of adipogenic-related genes, peroxisome proliferator-activated receptor-γ, lipoprotein lipase, and adipophilin were up-regulated in PRP-induced ASCs. Compared with other groups, granular fat grafts formed with 20% (v/v) and 30% (v/v) PRP significantly improved residual volumes. More intact adipocytes and capillary formation, but less vacuolization, were observed in the 20% (v/v) and 30% (v/v) PRP groups at 30, 60, and 90 days. However, no significant difference was observed between the 20% (v/v) and 30% (v/v) PRP groups in retaining fat grafts and improving histology.

Conclusions

Fat grafting with 20% (v/v) PRP and ASCs constitutes an appropriate transplantation strategy for improving graft survival and provides a potential approach for soft-tissue restoration in plastic and reconstructive surgery.

Topic: polymerase chain reaction, stem cells, adipocytes, cell count, genes, graft survival, lipoprotein lipase, mice, nude, peroxisome, reconstructive surgical procedures, residual volume, survival rate, tissue transplants, adipose tissue, blood capillaries, histology, transplantation, fat transplantation, soft-tissue augmentation, platelet rich plasma, adipophilin, ambulatory surgery center, lipomodeling, soft tissue

Issue Section: Original Articles

Soft-tissue defects frequently occur and are caused by congenital malformations, traumatic damage, and tumor resection. They not only lead to disfigurement, but also cause psychological problems;[1,2] hence, successful soft-tissue reconstruction is highly desirable. In 1893, Neuber first used upper-arm fat to build up a depressed area of the face.[3] Because it is abundant, relatively cheap, easy to obtain, host compatible, and nonimmunogenic, free fat transplantation has become the most popular method for soft-tissue restoration. The American Society of Plastic Surgeons reported that use of soft-tissue fillers increased 13% from 2012 to 2013.[4] However, clinical attempts of free fat transfer are hindered because of resorption, which normally leads to 20% to 80% (volume/volume [v/v]) of graft loss.[5,6] Inadequate revascularization following transplantation is the main reason for graft resorption.[1,7,8] Many approaches have been tried to enhance long-term retention of fat grafts;[9-11] however, few are convenient and effective over time. Therefore, further improvements are needed.

Platelet-rich plasma (PRP) is generally obtained by repeated differential centrifugation of whole blood which separates red blood cells from plasma and platelets.[12,13] PRP includes platelets, plasma, and a full complement of clotting and growth factors.[13-15] Evidenceshowing that PRP can enhance healing and regeneration of various tissues, such as bone,[13,15,16] skin,[17] nerve,[18] and tendon,[19] has increased. In recent studies, Nakamura et al[20] and Oh et al[21], investigated the effects of PRP on fat grafting. However, the dosages of PRP were not consistent, and the study results were contradictory.[20-22]

Interest has increased in utilizing stem cell-based therapy to regenerate adipose tissue. Adipose-derived stem cells (ASCs) are available in great quantities and are readily harvested by minimally invasive liposuction.[23,24] Compared with bone marrow stromal cells (BMSCs), ASCs have a higher proliferation rate and stronger adipogenic, osteogenic, neurogenic, and endothelial differentiation potential.[25,26] Additionally, ASCs are powerful and conspicuous angiogenic stem cells that promote neovascularization.[27-30] These qualities show the potential of ASCs as seed cells in adipose grafting and adipose-tissue regeneration.

In the current study, we investigated the synergistic effects of PRP and ASCs on adipose transplantation and determined the optimal volume fraction of PRP in granular fat. In addition, the influence of the volume fractions of PRP on ASC proliferation and

adipogenic differentiation was investigated in vitro. Furthermore, a composite of granular fat, PRP, and ASCs was transplanted into nude mice to determine the optimal dosage of PRP in fat grafting in vivo.

METHODS

The experimental protocol was approved by the institutional review board and Animal Care and Use committee of Sichuan University, China. Oral and written informed consent was obtained from all human donors and volunteers.

Cell Culture, Phenotypic Characterization, and Multipotentiality

ASCs were extracted from healthy abdominal subcutaneous adipose tissue of a 28-year-old female donor, who underwent skin transplantation in West China Hospital, Sichuan University. ASCs were harvested through the enzyme digestion procedure, as described by Zhu et al.[26] In brief, adipose tissue was extensively washed with phosphate-buffered saline (PBS) and minced finely after removal of fascia debris and vessels. Resultant tissue was digested with 0.15% (weight/volume [wt/v]) collagenase (type 1, Hyclone, Logan, Utah) at 37°C for 20 minutes by gentle agitation. After centrifugation at 200 g for 10 minutes, the cell pellet was collected, resuspended in alpha minimum essential medium (α-MEM; Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone) and incubated at 37°C; 5.0% carbon dioxide with the medium was replaced every 3 days. The primary cells were cultured for 7 to 8 days until 90% confluency before being passaged.

After reaching 90% confluency, ASCs (P4) were harvested and examined by use of flow cytometry for surface protein molecule expression. Briefly, after being detached with trypsin (0.25%, wt/v; Hyclone), ASCs were stained with fluorescent isothiocyanate-conjugated anti-CD14 (CD14-FITC), phycoerythrin-conjugated anti-CD29 (CD29-PE), CD31-FITC, CD34-FITC, CD44-FITC, CD45-FITC, CD49d-PE, CD105-PE, and CD106-PE (BD Biosciences, San Jose, California). After 3 washes with PBS, cells were resuspended in prechilled (0°C) PBS and the absorbance was analyzed using a standard Becton-Dickinson FACSAria instrument (BD Biosciences).

ASCs (P4) were cultured in α-MEM supplemented with 10% (v/v) FBS and then were induced using induction medium (Table 1). The multipotency of ASCs was determined, as described by Zuk et al.[31] Briefly, for adipogenic differentiation, cells were cultured for 2 weeks before the formation of lipid droplets was determined by Oil Red O staining; for osteogenic differentiation of ASCs, cells were cultured in osteogenic medium for 3 weeks, and accumulation of mineralized calcium nodules was assessed using Alizarin red staining; for neurogenic differentiation, cells were analyzed by immunocytofluorescence for the expression of neural cell markers neurofilament (NF; Millpore, Boston, Massachusetts) and βIII-tubulin (Abcam, Boston, Massachusetts).

Table 1.

Induction Medium

Inductive Conditions	Media	Supplements
Control	α-MEM	10% (v/v) FBS
Adipogenic culture	α-MEM	10% (v/v) FBS, 2 mM glutamine, 100 U/mL penicillin/streptomycin, 100 mM ascorbic acid, 0.5 mM methylisobutylxantine, 0.5 mM hydrocortisone, 60 mM indomethacin
Osteogenic culture	α-MEM	10% (v/v) FBS, 5 mM glycerophosphate, 100 nM dexamethasone, 50 mg/mL ascorbic acid
Neurogenic culture	α-MEM	10% (v/v) FBS, 2% (v/v) dimethyl sulphoxide, 200 mM butylated hydroxyanisole, 25 mM KCl, 2 mM valproic acid, 10 mM forskolin, 1 mM hydroxycortisone, 5 mg/mL insulin, 2 mM-glutamine

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Preparation and Evaluation of PRP

Fresh whole blood (400 mL) was received as a gift from Red Cross Blood Bank of Chengdu with donor consent. Citrate-phosphate-dextrose (CPD) was used as anticoagulant. The platelets in whole blood were determined automatically using a hematology analyzer (Sysmex XS-800i, Sysmex, Japan). According to a traditional two-

step preparation, as described by Nagata et al,[32] the 400 mL whole blood was aliquoted to 20 mL per centrifuge tube (20 tubes). After centrifugation at 200 g for 10 minutes, 2 mL plasma was extracted from each tube. The remaining blood was diluted two times with PBS before it was gently added to the tubes containing lymphocyte separation medium (4 mL/tube), leaving an obvious boundary between the two solutions. After centrifugation at 400 g for 20 minutes, solutions were separated into four parts, including plasma, mononuclear cells, lymphocyte separation medium, and red blood cells from top to bottom, respectively. The mononuclear cells were extracted, washed twice with PBS, and resuspended in the plasma obtained previously to obtain PRP. The platelets in each PRP tube were also determined by a hematology analyzer (Sysmex XS-800i, Sysmex, Japan).

Effects of PRP on ASCs in Vitro

For in vitro tests, PRP was activated with thrombin-dissolved 1% (wt/v) calcium chloride solution. The mixture was allowed to undergo maximum clot retraction at 4°C overnight before centrifugation at 3000 g for 10 minutes. The PRP supernatant was harvested and stored at -80°C.

Cell count kit-8 (CCK-8; Dojindo, Japan) was used to quantitatively evaluate effects of the volume fractions of PRP on ASC proliferation. ASCs (P4, 5×10^3 cells per well) were seeded in a 96-well plate. Five groups were prepared (n = 6), namely, α-MEM; α-MEM + 10% (v/v) FBS; α-MEM + 10% (v/v) PRP; α-MEM + 20% (v/v) PRP; α-MEM + 30% (v/v) PRP. After being cultured for 1, 2, 3, 4, 5, 6, and 7 days, 10 μL CCK-8 solution was added to each well and incubated for 3 hours. The absorbance at 450 nm was determined using a spectrophotometer (Thermo VARIOSKAN FLASH, Thermo, Waltham, Massachusetts).

ASCs (P4) were cultured for 3 weeks by α-MEM supplemented with different volume fractions of PRP (0%, 10%, 20%, 30%; v/v). ASCs induced by adipogenic medium (listed in Table 1) were used as the positive control. Total RNA extraction of ASCs in each group was achieved using RNAiso Plus (TaKaRa, Otsu, Japan) following manufacturer instructions. Reverse transcription was performed using PrimeScript® RT reagent Kit (TaKaRa, Otsu, Japan). cDNA samples were subjected to PCR amplification with primers (Table 2) for peroxisome proliferator-activated receptor-γ (PPARγ), lipoprotein lipase

(LPL), adipophilin, and glyceraldehyde-phosphate dehydrogenase (GAPDH) using the ABI PRISM® 7300 Fast Real-Time PCR System (Applied Biosystems, Foster, California). The primer sequences were: forward PPARγ 5′-GTGCAGGAGATCACAGAGTATGC-3′, and reverse 5′-CTCGGATATGAGAACCCCATCT-3′; LPL primers 5′-CTAAGGACCCCTGAAGACACAGC-3′ (forward) and 5′-GGCACCCAACTCTCATACATTCC-3′ (reverse); forward adipophilin 5′-GGGTAGAGTGGAAAAGGAGCAT-3′, and reverse 5′-GATGTTGGACAGGAGGGTGTG-3′; GAPDH 5′-CTTTGGTATCGTGGAAGGACTC-3′, reverse GAPDH 5′-GTAGAGGCAGGGATGATGTTCT-3′.

Table 2.

Primers for Real-Time PCR

Gene	Primers (F = forward; R = reverse)	Size (bp)	NCBI No.
PPARγ	F:5-GTGCAGGAGATCACAGAGTATGC-3 R: 5-CTCGGATATGAGAACCCCATCT-3	153	NM_138711.3
LPL	F:5-CTAAGGACCCCTGAAGACACAGC-3 R: 5-GGCACCCAACTCTCATACATTCC-3	152	NM_000237.2
Adipophilin	F:5-GGGTAGAGTGGAAAAGGAGCAT-3 R: 5-GATGTTGGACAGGAGGGTGTG-3	143	X97324.1
GAPDH	F:5-CTTTGGTATCGTGGAAGGACTC-3 R:5-GTAGAGGCAGGGATGATGTTCT-3	132	NM_002046.3

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Granular Fat Transplantation

The abdominal subcutaneous adipose tissue obtained from a different woman who received liposuction was washed twice with PBS, dried with gauze, and diced using ophthalmic scissors to produce granular fat, which can pass through an 18-gauge angiocatheter needle. The granular fat tissue was aliquoted into 1-mL syringes (0.3 mL per syringe). According to the volume ratio of PRP to granular fat, four composites were prepared: the 10% (v/v) PRP group (granular fat +10% [v/v] PRP + 5×10^5 ASCs); the 20% (v/v) PRP group (granular fat +20% [v/v] PRP + 5×10^5 ASCs); the 30% (v/v) PRP group

(granular fat +30% [v/v] PRP + 5×10^5 ASCs); and the control group (granular fat +5×10^5 ASCs).

Twelve nude mice (weight 25-30 g, 6 weeks old) were housed and isolated throughout the experimental period. The four dorsal subcutaneous sites of each mouse were taken as recipient areas for four graft composites. The composites were mixed with thrombin-dissolved 1% (wt/v) calcium chloride solution at a volume ratio of 6:1 and immediately and subcutaneously injected using an 18-gauge angiocatheter needle and a 1.0-mL syringe. All mice were housed in the same cage under standard husbandry conditions.

Specimen Collection and Analysis

Mice (n = 3) were sacrificed at days 10, 30, 60, and 90 following implantation. Implanted adipose grafts were collected and the volumes were recorded in a blinded fashion using the liquid overflow method. Specimens were fixed in 4% paraformaldehyde, dehydrated in increasing gradient of ethanol solutions before being embedded in paraffin. A piece of untreated fat was fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections with a thickness of 4 μm were stained with hematoxylin and eosin (H&E). Images of the histologic sections were examined microscopically (Leica DMI6000, Leica, Germany) at magnification of 100× and 200×. Cells with a diameter less than 100 μm were considered intact adipocytes. To quantitatively analyze the area of intact adipocytes, vacuole/cyst, fibrosis, and inflammation,[33] images (n = 8) from each group (100× magnification) were examined using Image-Pro Plus (Media Cybernetics, Bethesda, Maryland). The number of mature capillaries was counted at 200× magnification.

Statistical Analysis

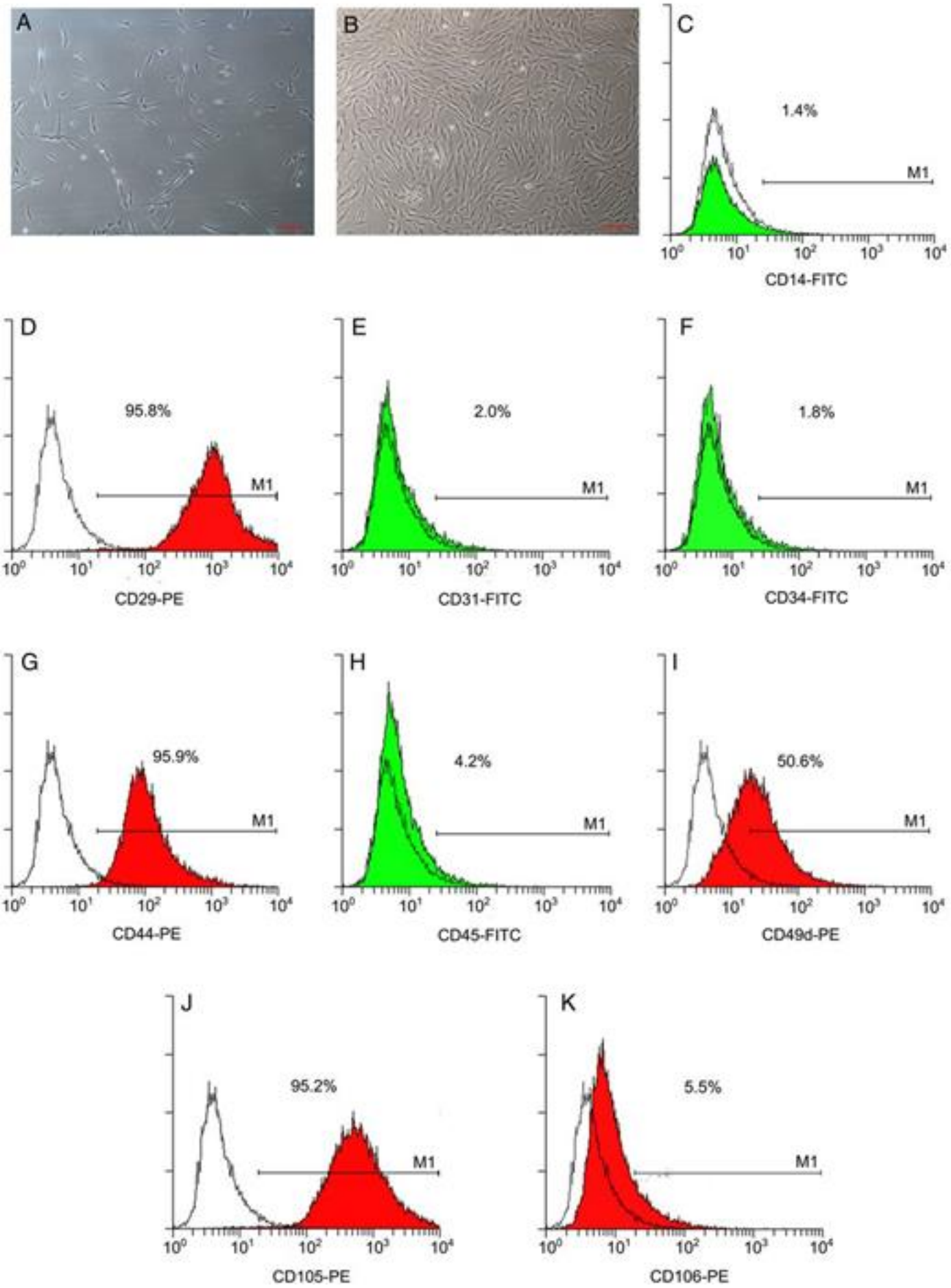
Data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Student-Newman-Keuls post hoc test (SPSS version 17.0; SPSS, Chicago, Illinois). Statistical significance was defined as $P < .05$.

RESULTS

ASCs Culture and Determining Multipotency

The spreading ASCs showed spindle-shaped morphology (Figure 1A). Once passaged, cells proliferated and reached 90% confluency within 3 to 4 days (Figure 1B). Flow cytometry analysis indicated that ASCs were negative for CD14, CD31, CD34, CD45, and CD106 but positive for CD29, CD44, CD49d, and CD105 (Figure 1C-K). After 2 weeks of culturing ASCs in adipogenic medium, intracellular lipid-droplets formation was confirmed by Oil Red O staining (Figure 2A). For osteogenic cultures, ASCs were induced into osteogenic lineage as seen by Alizarin red staining (Figure 2B). ASCs underwent morphologic changes (Figure 2C) and were positive for neurogenic markers NF (Figure 2D) and βIII-tubulin (Figure 2E) under neurogenic conditions.

Figure 1.



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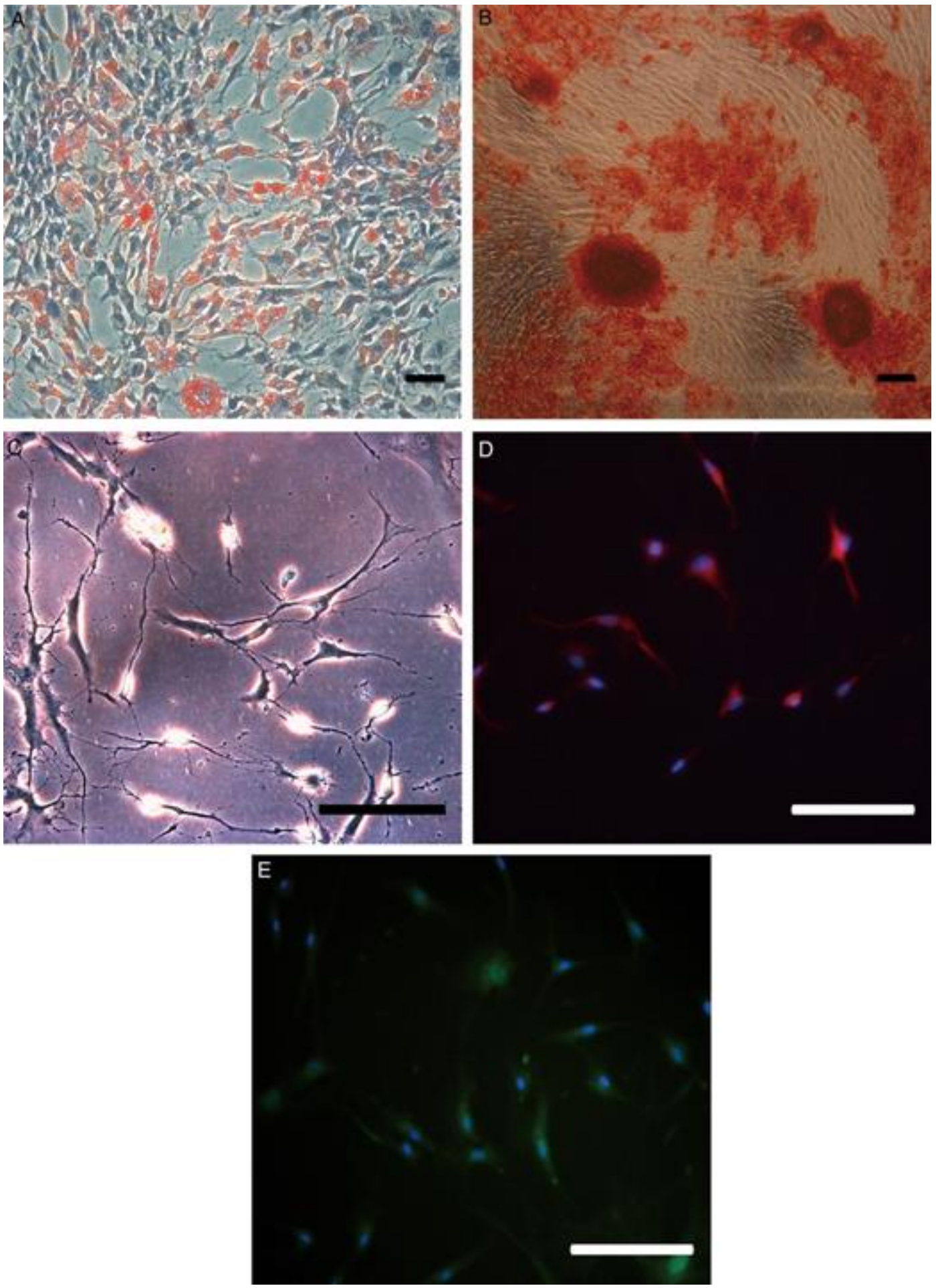
Characteristics of adipose-derived stem cells (ASCs). (A) ASCs (P1) on cell culture flask (day 1), showing spindle-shaped morphology. (B) ASCs (P1) reached approximately 90% confluency after 4 days. (C-K) Flow cytometry analysis of the ASCs (P4). (C) ASCs were stained negatively for CD14 (1.4%). (D) ASCs expressed cell surface antigen CD29 (95.8%). (E) ASCs were stained negatively for CD31 (2.0%). (F) ASCs were stained negatively for CD34 (1.8%). (G) ASCs expressed cell surface antigen CD44 (95.9%). (H) ASCs were stained negatively for CD45 (4.2%). (I) ASCs expressed cell surface antigen CD49d (50.6%). (J) ASCs expressed cell surface antigen CD105

(95.2%). (K) ASCs were stained negatively for CD106 (5.5%). Scale bar = 50 μm. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Figure 2.



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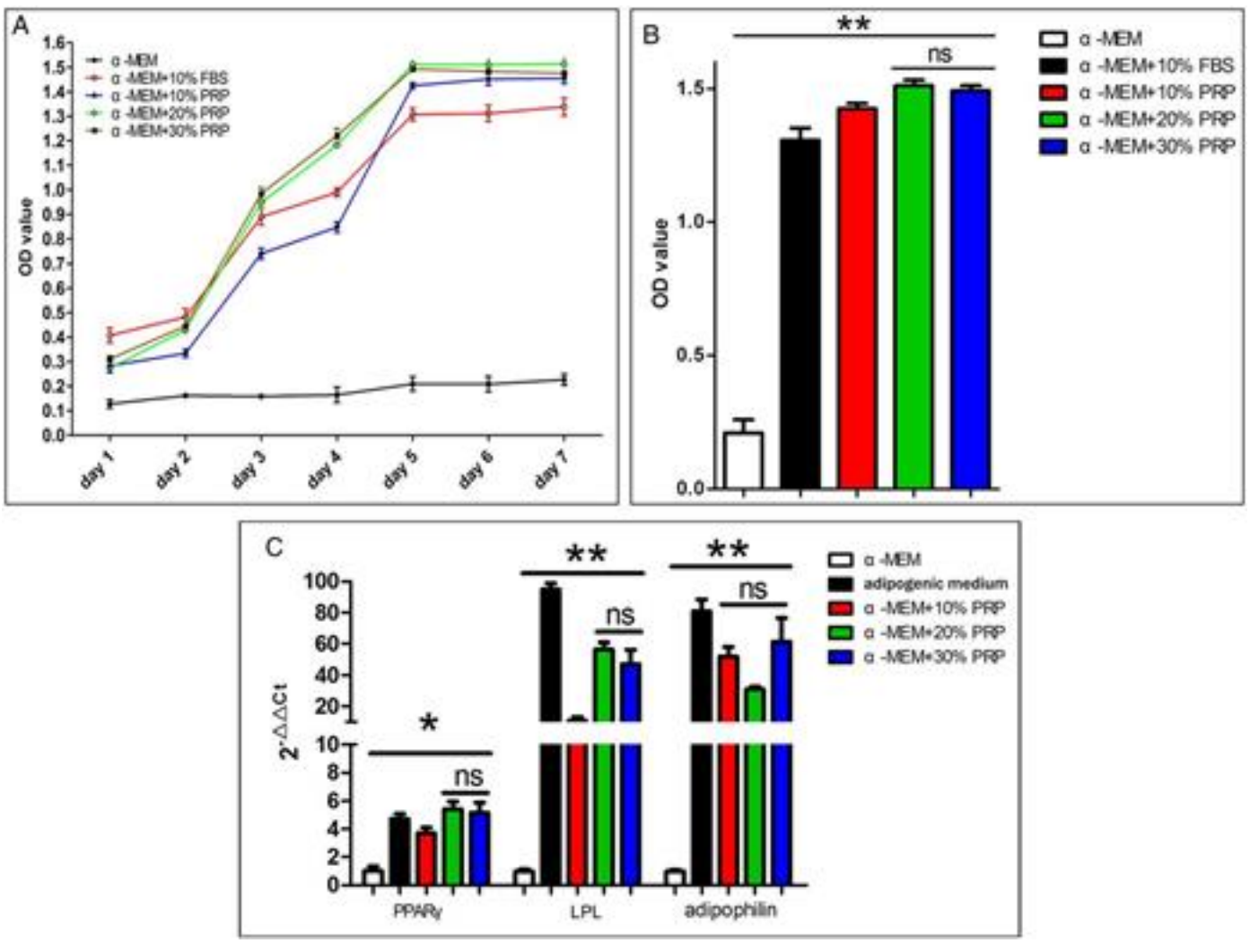
In vitro differentiation of adipose-derived stem cells (ASCs). (A) After 14 days of adipogenic culture of ASCs, the

formation of lipid droplets was confirmed by Oil-red O staining. (B) After 21 days of osteogenic culture of ASCs, deposition of calcium was confirmed by Alizarin red staining. (C) Morphologic changes in ASCs after neurogenic culture. (D) The ASCs expressed neurofilament under neurogenic conditions. (E) The ASCs expressed βIII-tubulin under neurogenic conditions. Scale bar = 50 μm.

Effects of PRP on ASCs in Vitro

Mean platelet count in PRP was 1350×10^9/liter and was 159×10^9/liter in whole blood. The effects of PRP on ASC proliferation are shown in Figure 3A. The OD values of each group on the fifth day were statistically analyzed (Figure 3B). Compared with the negative group, a significant increase in ASC proliferation was observed when cells were cultured with 10% (v/v) FBS or 10% to 30% (v/v) PRP ($P < .01$). Proliferation of ASCs was significantly improved by PRP ($P < .01$). The beneficial effects of PRP on ASC proliferation were comparable for the 20% (v/v) and 30% (v/v) PRP groups but were significantly better than the 10% (v/v) PRP group ($P < .01$). Real-time PCR analyses indicated up-regulated expression of PPARγ, LPL, and adipophilin in all PRP-induced ASCs (Figure 3C).

Figure 3.



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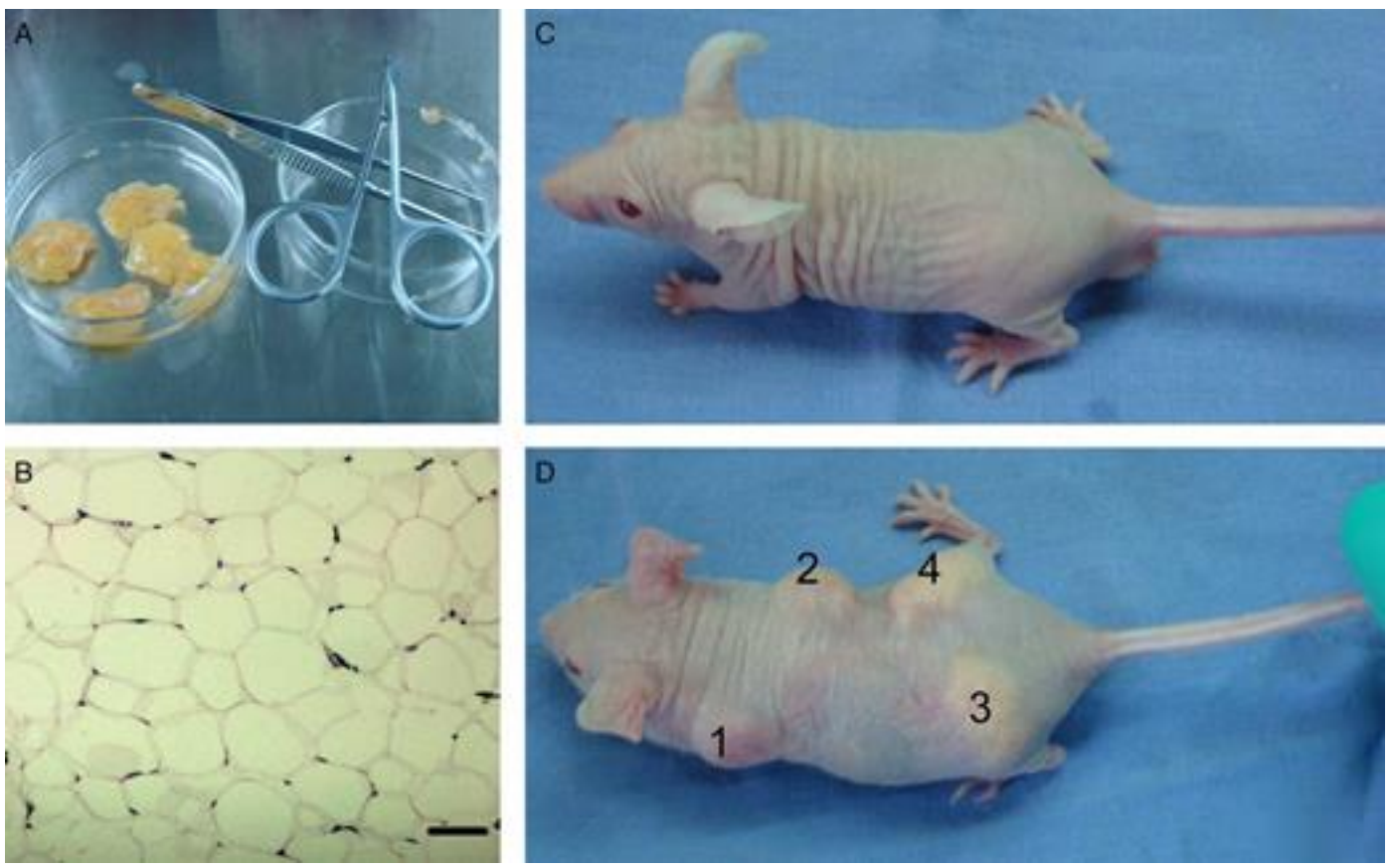
Effects of platelet-rich plasma (PRP) on adipose-derived stem cells (ASCs). (A) The growth curves of ASCs cultured with different volume fractions of PRP. (B) Comparison of optical density (OD) values on the fifth day of ASCs cultured with different volume fractions of PRP. Significant differences were observed in multiple comparisons ($P < .01$), except between 20% and 30% PRP groups. (C) Adipogenic gene-marker analysis of ASCs induced by different volume fractions of PRP for 3 weeks. Real-time PCR data are expressed as mean of $2^{-\Delta\Delta Ct} \pm$ SD; *$P < .05$; **$P < .01$; ns, no significance (Student-Newman-Keuls test). PPARγ, peroxisome proliferator-activated receptor-γ; LPL, lipoprotein lipase.

Visual Inspection of Adipose Grafts

The granular fat tissue was successfully prepared (Figure 4A) and evaluated by histology before implantation (Figure 4B). All nude mice survived the grafting procedures (Figure 4C, D) without obvious complications. Throughout husbandry there were no signs of inflammation, abscess formation, or necrosis in the grafts and adjacent tissues. The comparison of gross observation and residual volume of different samples at different times are shown in Figure 5. All grafts were encompassed by a thin connective tissue, isolating them from host tissues (Figure 5A). Remarkable differences were observed among different groups. For the PRP groups, the grafts seemed larger, with deeper color, and were firmer than those in control groups for all scheduled time points (Figure 5A–E).

Figure 4.



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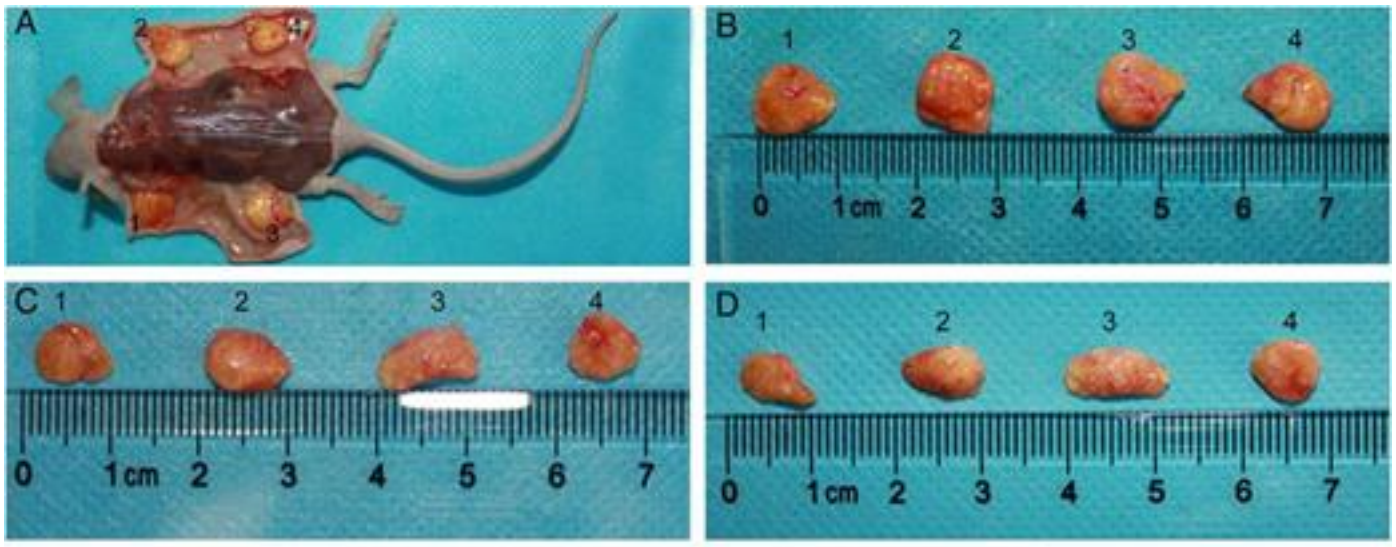
Preparation and implantation of granular fat grafts. (A) Preparation of granular fat tissue from humans. (B) Histology of granular fat tissue before injection. Cells with a diameter less than 100 μm were considered intact adipocytes. (C, D) Granular fat with adipose-derived stem cells (ASCs) and different volume fractions of platelet-rich plasma (PRP) were subcutaneously implanted into nude mice. (C) Preinjection. (D) Postinjection. 1. 10% PRP group. 2. 20% PRP group. 3. 30% PRP group. 4. Control group. Scale bar = 100 μm.

Figure 5.



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Comparison of the size of fat graft residues. (A) Harvest of fat grafts from mice 10 days after injection. (B)

Dimensions of fat grafts 10 days following injection. (C) Dimensions of fat grafts 30 days following injection. (D) Dimensions of fat grafts 60 days following injection. (E) Dimensions of fat grafts 90 days following injection. 1. 10% platelet-rich plasma (PRP) group. 2. 20% PRP group. 3. 30% PRP group. 4. Control group. (F) Comparison of fat grafts residual volume 10, 30, 60, and 90 days after transplantation. At day 10, significant differences were observed in multiple comparisons. At days 30, 60, and 90, significant difference was observed except in the 20% and 30% PRP groups. *$P < .05$; **$P < .01$. ns, no significance. (Student-Newman-Keuls test).

Volume of Adipose Grafts

The residual volumes of injected granular fats decreased over time (Table 3 and Figure 5). There was a PRP dose-dependent increase for survival volume 10 days following implantation. Compared with the control group and the 10% PRP (v/v) group, more residual volume was observed in the 20% (v/v) and 30% (v/v) PRP groups ($P < .05$) at 30, 60, and 90 days; however, there was no significant difference between the two groups (Figure 5F).

Table 3.

Comparison of Fat Grafts Residual Volume (mL)

	Control Group	10% PRP Group	20% PRP Group	30% PRP Group
10 days	0.231 ± 0.012	0.251 ± 0.004	0.267 ± 0.004	0.280 ± 0.003
30 days	0.185 ± 0.010	0.208 ± 0.012	0.226 ± 0.004	0.242 ± 0.009
60 days	0.159 ± 0.007	0.189 ± 0.008	0.214 ± 0.010	0.225 ± 0.013
90 days	0.156 ± 0.005	0.185 ± 0.004	0.204 ± 0.006	0.213 ± 0.007

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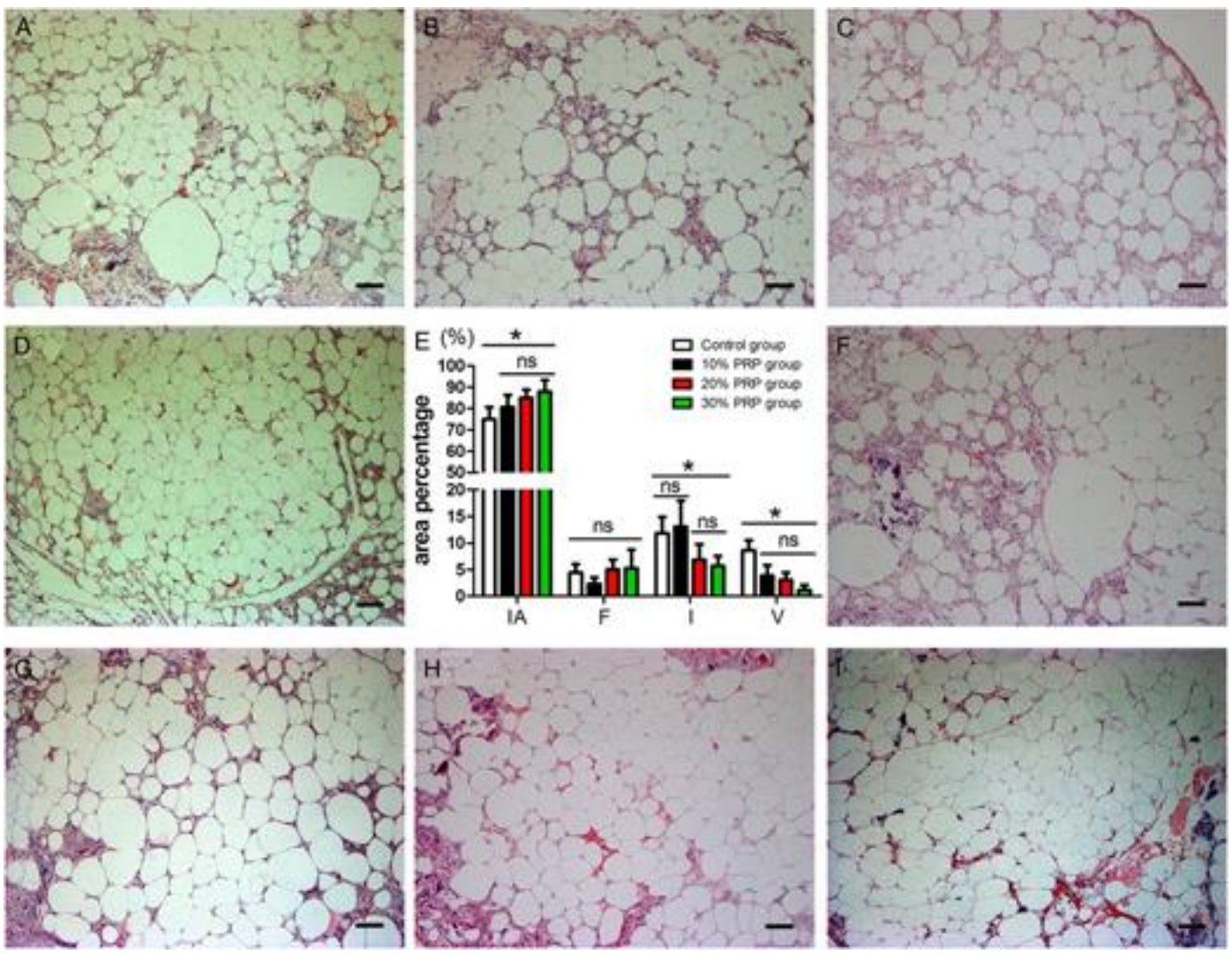
Histologic Observation

Light microscopy revealed the presence of intact adipocytes, nonadipocyte fibrous and stromal tissue, inflammatory tissue, and areas of vacuoles/cysts (Figure 6). Quantitative comparisons (Figure 6E,J,O,T) indicated significantly less intact adipocyte areas and more areas of vacuoles/cysts in the control group than in all PRP groups for all time

points, indicating that PRP was beneficial to the quality of fat grafts. At the 10th day, there was no statistically significant difference in intact adipocyte area for the 10% (v/v), 20% (v/v), and 30% PRP (v/v) groups. On the 30th day, the 30% (v/v) PRP treated grafts retained the most intact adipocyte areas and the least intact areas of vacuoles/cysts. However, histologically, there was no significant difference between the 20% (v/v) and 30% (v/v) PRP groups 60 and 90 days following implantation.

Figure 6.



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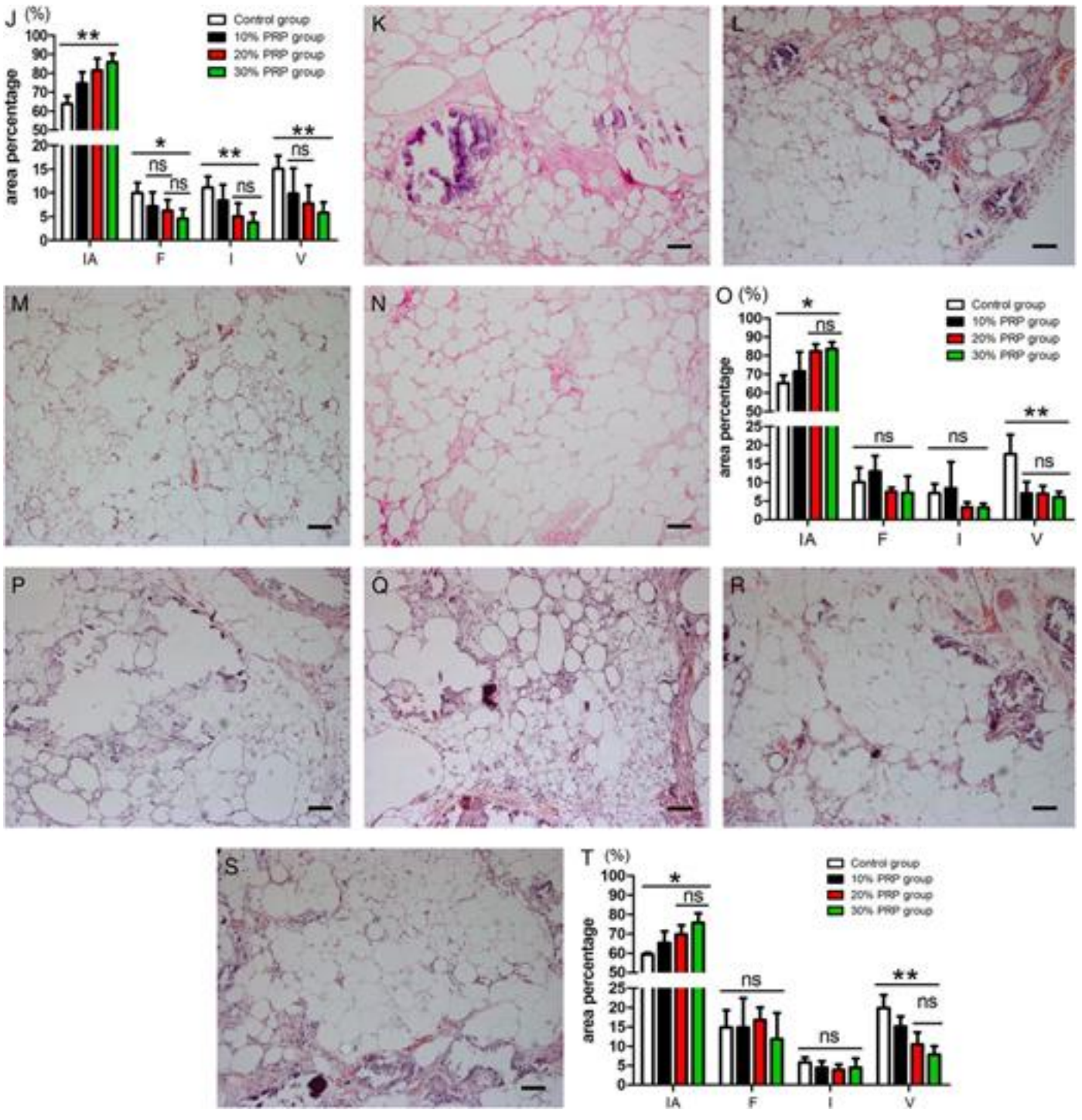
Histologic analysis of fat grafts at 10, 30, 60, and 90 days after transplantation. Tissue was stained with hematoxylin and eosin. (A) Histology of control group grafts 10 days following injection. (B) Histology of 10% platelet-rich plasma (PRP) group grafts 10 days following injection. (C) Histology of 20% PRP group grafts 10 days following injection. (D) Histology of 30% PRP group grafts 10 days following injection. (E) Quantitative comparison of histology evaluation 10 days following injection. (F) Histology of control group grafts 30 days following injection. (G) Histology of 10% PRP group grafts 30 days following injection. (H) Histology of 20% PRP group grafts 30 days following injection. (I) Histology of 30% PRP group grafts 30 days following injection. (J) Quantitative comparison of histology evaluation 30 days following injection. (K) Histology of control group grafts 60 days following injection. (L) Histology of 10% PRP group grafts 60 days following injection. (M) Histology of 20% PRP group grafts 60 days following injection. (N) Histology of 30% PRP group grafts 60 days following

injection. (O) Quantitative comparison of histology evaluation 60 days following injection. (P) Histology of control group grafts 90 days following injection. (Q) Histology of 10% PRP group grafts 90 days following injection. (R) Histology of 20% PRP group grafts 90 days following injection. (S) Histology of 30% PRP group grafts 90 days following injection. (T) Quantitative comparison of histology evaluation 90 days following injection. In panels E, J, O, and T, IA means intact adipocytes area; I means inflammation area; F means fibrosis area; V means vacuole/cyst area. Scale bar = 100 μm. *$P < .05$; **$P < .01$; ns, no significance (Student-Newman-Keuls test).

Figure 6.



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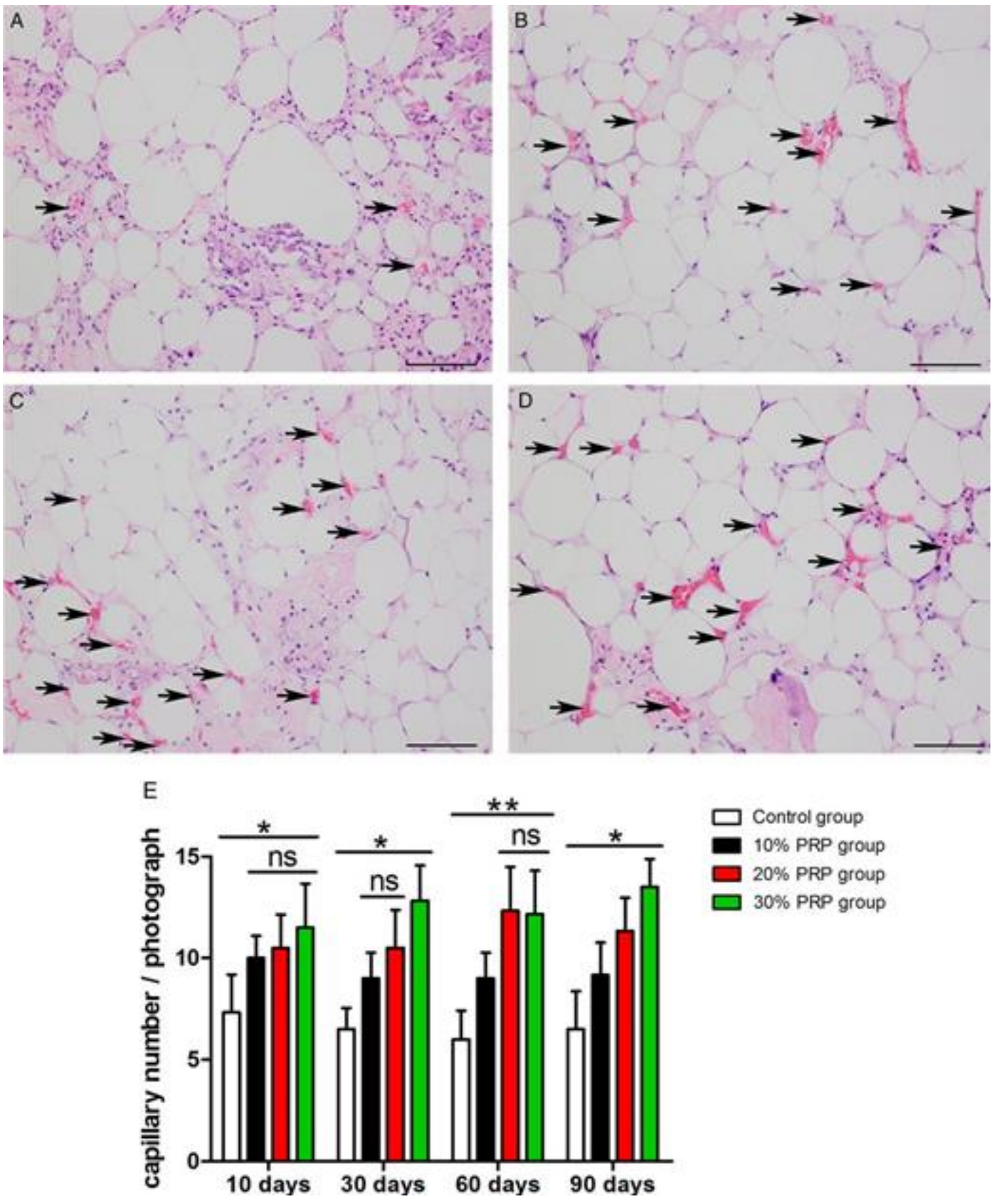
Capillary Numbers

Before injections, the granular fat tissue showed no presence of capillary structures (Figure 4B). Ten days following injection, all grafts became vascularized (Figure 7A-D). Quantitative comparisons (Figure 7E) indicated that capillary formation in all PRP groups was significantly higher than in the control group for all time points ($P < .05$). The 30% (v/v) PRP group showed the greatest number of capillaries at day 30 and day 90 ($P < .05$). These data (Figure 7) indicate that PRP improved neovascularization of grafted granular fat.

Figure 7.



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Vascularization of fat grafts. (A-D) Fat grafts were vascularized 10 days following transplantation. Mature capillaries containing erythrocytes were observed (black arrows). (A) Control group. (B) 10% platelet-rich plasma (PRP) group. (C) 20% PRP group. (D) 30% PRP group. Scale bar = 100 μm. (E) Comparison of mean capillary number of fat grafts sections 10, 30, 60, and 90 days after transplantation. *$P < .05$; **$P < .01$; ns, no significance (Student-Newman-Keuls test).

DISCUSSION

Soft-tissue engineering utilizing adipogenic progenitor cells with the aid of appropriate biomaterial is a new approach to improving adipose graft survival. In the current study, we evaluated the beneficial effects of PRP combined with ASCs on the survival of fat grafts. Furthermore, the volume fraction of PRP in grafts was optimized by the impact of PRP on ASCs in vitro and graft residual volume and histology in vivo.

Fat can be used as graft material in soft tissue reconstruction because of its abundance of ASCs. Matsumoto et al and Zhu et al found that ASCs were important for adipogenesis and revascularization of fat grafts.[34,35] The phenotypes and multipotency of ACS were confirmed in the study. ASCs expressed CD29, CD44, and CD105, indicating the mesenchymal stem cell (MSC) characteristics.[36] Their negative expression of CD14, CD31, CD34, and CD45 markers differentiates ASCs from hematopoietic and endothelial lineage cells.[37] Moreover, unlike bone marrow MSCs, ASCs were positive for CD49d (α4 integrin) and negative for CD106. CD106 is related to hematopoietic tissue, such as bone marrow stroma;[38] negative expression of CD106 confirmed that the origin of the ASCs was nonhematopoietic tissue. Cultured in different conditions, isolated ASCs could differentiate toward the adipogenic, osteogenic, and neurogenic lineages, demonstrating their multipotency and plasticity as seed cells for fat regeneration in soft-tissue reconstruction.

PRP was chosen as a scaffold in this study because of its plasticity and abundance of growth factors. Sommeling et al systematically reviewed studies describing combined use of PRP and fat grafts[39] and found a generally beneficial effect of PRP on graft survival. However the dosages of PRP in those studies were not consistent, leading to contradicting conclusions. For example, with addition of 20% (v/v) PRP in adipose

grafts, Nakamura et al observed enhanced granulation tissue and capillary formation and good maintenance of intact adipocytes over a period of 120 days following transplantation.[20] Oh et al combined 0.7 mL fat and 0.21 mL PRP and transplanted the fat subcutaneously into mice scalps.[21] After 10 weeks, fat graft volume and weight in the PRP group were significantly higher than in the control group, and histologic evaluation revealed greater vascularity, fewer cysts and vacuoles, and less fibrosis in the PRP group. However, Por et al injected the combination of 0.8 mL fat and 0.2 mL PRP into mice scalps and found that PRP had no effect on improvement in free fat graft survival.[22] These results indicated that the viability of transplanted adipose tissue may depend on the dosage of PRP in grafts. To validate our speculation, we compared the effects of different volume fractions of PRP on fat grafts in the current study.

Analysis of fat grafts in the presence of PRP revealed that PRP improved retention of grafted fat tissue; this was further confirmed histologically. Although the mechanism underneath is not clear, it may be attributable to the improved viability of ASCs as a result of the addition of PRP in the graft. To determine this, we investigated the influence of PRP on ASCs in vitro and on the survival of overall grafting in vivo. Our CCK-8 results revealed that PRP promoted ASC proliferation in vitro because PRP contains mitogenic factors for human mesenchymal progenitor cells.[40,41] By comparing the proliferation of ASCs with different volume fractions of PRP, we found that incorporating 20% (v/v) PRP in the culture medium exerted the best influence on ASC proliferation biologically and economically. Although PRP concentration was optimized in several studies[42,43] for ASC proliferation, results were conflicting. Kakudo et al observed enhanced cell proliferation with 5% (v/v) PRP in the medium, but little influence was observed with 20% (v/v) PRP.[42] Liu et al suggested that the optimal concentration of PRP in medium was between 10% and 12.5% (v/v).[43] This conflict may be a result of the variations in PRP preparation and evaluation method. Furthermore, when ASCs were induced with PRP, expression of adipogenic genes, such as PPARγ, LPL, adipophilin, were up-regulated. This is consistent with results of previous studies; for example, PPARγ plays a key role in regulating genes related to adipogenesis;[44] LPL is a lipid exchange enzyme during adipogenesis;[45] adipophilin has been shown to correlate with lipid accumulation in cells and tissues.[46] These results indicated that PRP exerted a beneficial effect on the adipogenic differentiation of ASCs in vitro. This may be attributable to the effect of transforming growth factor in PRP, which induces the

differentiation of mesenchymal progenitor cells into adipocytes.[47,48] Increased graft residual volume, more adipocytes, and less vacuolization were observed in 20% (v/v) and 30% (v/v) PRP-treated grafts in vivo, which was consistent with the in vitro results. This implied that addition of PRP promoted proliferation and differentiation of ASCs and enhanced retention of the transplanted graft.

An increased graft survival rate may result from the beneficial effect of PRP on angiogenesis. Neovascularization in the early stage of transplantation is essential to maintaining the viability of implanted cells and newly formed tissues.[8,10] Activated PRP contains concentrated growth factors,[12] including platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor. These factors are mitogenic and chemotactic in angiogenesis.[49,50] In the current study, more capillary formation was observed in 20% (v/v) and 30% (v/v) PRP-treated grafts than in others ($P < .05$), showing the beneficial effect of PRP on promotion of neovascularization of grafts. Improved neovascularization further contributed to retention of grafted tissue, which was confirmed histologically. However, no statistically significant difference was observed between the 20% (v/v) PRP and 30% (v/v) PRP in regard to retaining fat grafts and improving histology. We hypothesized that 20% (v/v) PRP had supplied sufficient growth factors for cell and tissue survival. Considering the cost, 20% (v/v) PRP combined with ASC seems to be the best choice for fat graft. Additionally, the activated PRP presents as gelation and could be easily processed into a desired shape; therefore, the plasticity of PRP allows graft injection into recipient sites, which minimizes patient discomfort, risk for infection, scar formation, and cost of treatment.

There are some limitations to these findings, and several problems must be solved before the results can be applied in the clinic setting. For instance, this study suggests that the optimal volume fraction of PRP in granular fat is 20% (v/v). To prepare 20% (v/v) PRP for fat transplantation, a large amount of whole blood is required. In the current study, the 1.0 mL prepared PRP could be extracted from 5.0 mL of human blood. Moreover, the study did not evaluate the levels of growth factor in PRP. Because variation in preparation and evaluation method often leads to different results, it is crucial to validate and standardize the methods before use of PRP in clinical practice. Another limitation is that the number of mice in the study was too small; a larger sample size must be included for further study. Additional studies will be performed in the

future; however these findings will direct better clinical treatment.

CONCLUSIONS

PRP facilitated proliferation and adipogenic differentiation of ASCs in vitro, and the optimal volume fraction of PRP in medium was 20%. The addition of a mixture of 20% (v/v) PRP and ASCs improved the retaining volume and histology of grafted material in vivo, which provided a potential solution to the problem of resorption during soft-tissue reconstruction and plastic surgery.

Disclosures

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