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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Fructose 1,6-Bisphosphate as a Protective Agent for Experimental Fat Grafting

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ABSTRACT

Fat grafting procedures are considered to be a promising regenerative, cell-directed therapy; however, their survival is mainly influenced by ischemia condition. Fructose 1,6-bisphosphate (FBP), as an intermediate in energy metabolism, has the potential to rescue cells and tissues from hypoxic-ischemic circumstances. In the present study, human lipoaspirates were grafted subcutaneously into nude mice followed by a daily intraperitoneal injection of FBP at different doses for 7 days. Next, the grafts were harvested at different time points till 12 weeks postimplantation and were evaluated for cell viability and function, tissue revascularization and inflammatory cell infiltration using histological analysis, whole-mount living tissue imaging, glycerol 3-phosphate dehydrogenase activity assays, and quantitative analysis of gene expression. The results demonstrated that exogenous FBP administration could attenuate the volume and weight reduction of fat graft; meanwhile, FBP enhanced adipocyte viability and function, increased blood vessel formation, and decreased inflammation. Moreover, in vitro cell experiments showed that FBP could promote adipose-derived stem cell viability and vascular endothelial growth factor (VEGF) mRNA expression in ischemia conditions. Our study indicates that FBP can be used as a protective agent for fat grafting and may be applied in stem cell-based regenerative medicine. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:606–616*

SIGNIFICANCE STATEMENT

Fat grafting has been regarded as a promising biomaterial for regenerative therapy, but the challenge is its survival and longevity after implantation because of ischemia. In the present work, fructose 1,6-bisphosphate (FBP) was demonstrated to improve fat graft survival postimplantation by enhancing adipocyte viability and function, increasing vascularization, reducing inflammatory infiltration, and promoting viability of adipose-derived stem cells (ASCs). The study not only extends the application scope of FBP, which could be used to improve the efficacy of fat graft and other substitute biomaterials, but also provides a more thorough understanding of FBP on ASCs useful for cell-based regenerative medicine.

INTRODUCTION

Adipose tissues distribute throughout the body and play essential roles in storing energy, protection of underlying structures, and imparting a normal human appearance. In recent years, it has also been recognized as a major endocrine organ and functions in metabolism and immune regulation [1]. Soft-tissue defects resulting from congenital defects, trauma, or tumor resection are common in the clinic. Adipose tissue has been successfully used as a regenerative treatment option in many different clinical situations, such as breast augmentation and reconstruction, contour deformities, scars, wound healing, and hair growth. Recent studies have also evaluated it as a therapeutic approach to fight against metabolic disorders through the implantation of fat tissue [2]. In addition, adipose tissue is rich in stem cells (adipose-derived stem cells, ASCs), which may further expand its application scope in regenerative medicine.

Fat grafting has been regarded as a promising biomaterial for soft-tissue reconstruction, because it is readily available; easily obtainable, with low donor-site morbidity; repeatable; inexpensive; versatile; and biocompatible [3]. However, although fat graft provides good results in the early period, it may disappoint the patient and surgeon in the long term because of its reported resorption rate of 10%–90% [4]. Strategies to reduce the reabsorbed proportion of fat have evolved, and they focus on the surgical technique, donor-site selection, supplementation

of stromal vascular fraction or ASCs, and the addition of growth factors. The cell assistant lipotransfer technique through supplementation with ASCs shows superiority in maintaining fat-graft volume and recipient-site contour [5, 6]. Long-term, local delivery of the growth factors insulin-like growth factor-1 and basic fibroblast growth factor also has the potential to increase fat-graft survival rates [7]. Tan et al. have recently shown that the indolamine hormone melatonin promotes survival of fat grafts via reducing inflammation [8]. However, loss of transplanted graft is the result of hypoxia and a lack of nutrients because of ischemia [4, 5] when the implanted tissue does not become fully vascularized to support the metabolic needs of the tissue in the required timeframe. Reducing fat-graft desorption is still an area that needs further improvement, we may consider to find more efficient agent to enhance survival of the substitute graft under ischemia condition.

Fructose 1,6-bisphosphate (FBP) is a principle endogenous intermediate in the glycolytic pathway [9], and it has been reported to exert a protective action in different cell types and tissues during ischemia and hypoxia [10]. For example, pretreatment with FBP provides histologic and functional protection in rats that are subjected to bilateral renal artery occlusion [11]. Moreover, FBP prevents ischemia-induced brain damage, and it exhibits a beneficial effect in patients with coronary artery disease [12, 13]. Currently, the Medical Subject Headings (MeSH) Pharmacological Classification includes FBP as a protective compound in the following categories: neuroprotective agents, immunological factors, cardiovascular agent, and antiarrhythmia agent [10]. The underlying mechanisms of FBP to exert its protective effect are complex and involve different signaling pathways. FBP maintains glycolysis and stimulates the pentose-phosphate pathways by providing an ATP supply under oxygen restrictions. In addition, FBP limits immunological and inflammatory responses via modulating different signaling pathways. All these effects render FBP a complex protective compound that is useful for a wide range of pathological conditions and present new prospects for its clinical use. Could FBP be applied in fat grafts to protect their survival?

In the present study, we investigated the role of FBP in fat transplantation in nude mice and its potential mechanism. We presented the evidence that FBP could improve volume and weight retention and mitigate resorption of the graft postimplantation. Histologically, FBP enhanced adipocyte viability and function, increased vascularization, and reduced inflammatory infiltration in fat grafts. In vitro cell experiments showed that FBP could promote viability of ASCs, which further expanded the application possibility of FBP. The current study not only extends the application scope of FBP, which could be used to improve the efficacy of fat graft, but also provides a more thorough understanding of FBP on ASCs useful for cell transplantation strategy.

Materials and Methods

Samples and Animals

This study was conducted in accordance with the ethics committee of Plastic Surgery Hospital (Institute), Chinese Academy of Medical Sciences (CAMS), Peking Union Medical College. Written informed consent was obtained from each patient for the harvest and use of adipose tissue samples for research purposes. The lipoaspirate that was harvested from the abdominal region via surgical procedures was washed with phosphate-buffered saline, and fat separated from oil and other fluids through centrifugation for 5 minutes at 500*g* was prepared as free fat granules.

Animals

Nude mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). Animal experiments were approved by the Institutional Animal Care and Use Committee of Plastic Surgery Hospital (Institute). The animals were housed individually in polycarbon cages at $20^{\circ}C \pm 2^{\circ}C$ and in a humidity-controlled environment with a 12-hour day/night cycle in the preoperative and postoperative periods. The nude mice were allowed free access to water and food.

Lipotransfer and FBP Administration

The mice were randomly divided into five groups with 15 female mice in each group, which weighed approximately 20 g, and each one received two injections of 500 μl of free fat granules. Fat grafts were transferred to a 1 ml syringe with 16-gauge needle and injected subcutaneously into the dorsum. The mice in the experimental groups were intraperitoneally injected with FBP (Xiya Reagent, Shandong, China) at a dosage of 0.5, 1, 2.5, and 4 mg/g at 2 hours before fat transplantation and once a day for six consecutive days after surgery. As a control group, the mice were intraperitoneally injected with physiological saline, following the same protocol.

Fat-Graft Harvest and Morphological Assessment

Mice were euthanized and sacrificed at 5 days and 2, 8, and 12 weeks after transplantation ($n = 3$ at each time point). Next, the grafts were dissected and weighed. Their volumes were measured according to the formula used in the assessment of implanted tumor volume ($a^2b/2$; a represents width, and b represents length) [14].

Histological Examination

Panoramic view of H&E staining

Each group of samples that were harvested at 2 and 12 weeks in vivo was fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Serial sections were obtained and subjected to H&E staining. Digital images with 20-fold magnifications were captured using an image acquisition system (Olympus, Japan) and integrated into a panoramic view. Intact adipocytes that were distributed at the peripheral and central regions were counted (average from 10 high-magnification fields per section), respectively.

Whole-mount staining of living tissue

Visualization of nonfixed living adipose tissue was performed according to a previously described procedure [15]. Briefly, samples that were harvested at 2 and 12 weeks in vivo were cut into 3- to 5-mm pieces within 2 hours after sampling, incubated with boron-dipyrromethene (Bodipy) 558/568 (Molecular Probes) for 15 to 30 minutes, washed with stroke-physiological saline solution (SPSS) 3 times, and observed under a confocal microscope system (Zeiss, LSM710, Germany). Six to 10 images were vertically acquired at an interval of 5 μm for each low-magnification field of view.

Immunofluorescence staining of Von Willebrand factor, perilipin, and CD68

After being embedded in paraffin, samples that were harvested at 2 and 12 weeks were stained with Von Willebrand

factor (vWF) antibody (1:250, Abcam), and some of them that were harvested after 5 days of implantation were stained with CD68 (1:500, Abcam) and anti-perilipin antibody (1:250, Abcam), followed by staining with Alexa Fluor 488 goat anti-rabbit IgG (1:500, Abcam) as the secondary antibody. Cell nuclei were stained using 4′,6-diamidino-2-phenylindole. Next, vWF-positive regions, which indicated the vessels; perilipin-positive areas, which indicated intact adipocytes; and CD68-positive macrophages were counted under a high-magnification field (average from 10 high-magnification fields per section).

Glycerol-3-Phosphate Dehydrogenase Assay

Samples that were collected at 5 days and 2, 8, and 12 weeks after surgery were subjected to the analysis of glycerol 3-phosphate dehydrogenase (GPDH) activity using a GPDH Assay Kit (Abcam). Briefly, 10 mg of tissues was cut into small pieces, immersed into 200 μl ice-cold GPDH assay buffer, and cell lysis solution was used to examine the activity of the enzyme according to the manufacturer's instructions. One unit is defined as the amount of enzyme required for the consumption of 1 μmol NADH in 1 minute at 30°C. Each assay was performed in triplicate.

In Vitro Culture of ASCs and Ischemia-Mimic Culture Conditions

The adipose tissue was digested by 0.1% (weight/volume) collagenase type I (Sigma-Aldrich, St Louis, MO) at 37°C for 60 minutes with gentle agitation, as described in previous studies [16]. The suspension was filtered through a nylon mesh (100 mesh), followed by centrifugation at 1000 rpm for 10 minutes, and the final pellet was resuspended in culture medium. The cells were plated at a density of 1.5×10^5 cells per centimeter square for culture in mesenchymal stem cell (MSC) medium (ScienCell, Carlsbad, CA) that contained 10% fetal bovine serum (FBS, HyClone, South, Logan, UT) in a humidified 37°C incubator under 5% CO_2.

To analyze the cellular effects of FBP in ischemia (hypoxia and low nutrients), four groups were used for this study. (a) In the conventional-culture group, ASCs were cultured with DMEM containing 10% FBS in a 37°C incubator under 95% air (20% O_2) and 5% CO_2. (b) In the no-FBS group, ASCs were cultured with DMEM without FBS, simulating the state of nutritional deficiencies. (c) In the hypoxia group, ASCs were cultured with DMEM containing 10% FBS under hypoxia conditions in a vacuum bag that was maintained at 37°C under a humidified atmosphere of 5% CO_2, less than 0.5% O_2, and the balance N_2. (d) In the ischemia-mimicking group, the cells were cultured with DMEM without FBS under hypoxia conditions. Under different culture conditions, FBP was applied at concentrations of 0.5 and 1 mM to ascertain its corresponding effects.

Cell Viability, Proliferation, and VEGF mRNA Expression Level Assay

Following treatment of ASCs for 12 hours under different conditions, living and dead cells were distinguished using a live/dead cell-viability assay kit (BioVision, CA). According to the protocol, the media were removed carefully, the staining solution was added, and the cells were incubated for 15 minutes at 37°C. Slides were viewed using a fluorescence microscopy. Viable (green fluorescent) and dead (red fluorescent) cells were recorded from five random microscopic fields for analysis.

Cell proliferation was measured using Cell Counting Kit-8 (CCK-8, Dojindo, Japan), following the manufacturer's instructions. In brief, the cells were cultured under different conditions. Ten microliters of the Cell Counting Assay Kit-8 solution was added to each well, the cells were incubated for another 1 hour, and the absorbance was measured at 490 nm using a spectrophotometer (PerkinElmer EnSpire Multimode Plate Reader, MA).

VEGF mRNA expression levels were assessed through quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis after treatment for 12 hours under different conditions. Experiments were performed at least three times, and the representative data were presented.

Adipogenic Differentiation Assay

The ASCs were seeded at a density of 7.5×10^4 cells per milliliter onto six-well plates (Corning, Tewksbury, MA) and cultured under different conditions as aforementioned in In Vitro Culture of ASCs and Ischemia-Mimic Culture Conditions section for 12 hours before switching to adipogenic medium (AM; growth medium supplemented with 2 nM dexamethasone, 33 mM biotin, 17 mM pantothenic acid, 0.5 mM isobutylmethylxanthine, 5 mM rosiglitazone, and 1 mM insulin (all from Sigma-Aldrich). After 7 days in AM, adipogenesis marker peroxisome proliferator activated receptor (PPAR-γ) was assessed through qRT-PCR analysis.

Gene Expression Analysis

Samples that were collected at 5 days and 2 and 8 weeks in vivo (n = 3 per group for each time point) were harvested, and the total RNA was extracted using the Trizol reagent (Invitrogen), respectively. RNA was reverse transcribed into single-stranded cDNA, according to the manufacturer's protocol (Promega). The expression levels of the selected genes were analyzed via quantitative PCR using a LightCycler 480 system (BD) and a SYBR green kit (Roche Molecular Germany). The forward and reverse primer pairs are shown in Table 1. To normalize mRNA levels, the housekeeping gene of β-actin was amplified as an internal control.

Statistical Analysis

The morphology parameters, cell count, GPDH activity, and mRNA expression level for each group were compared via the one-way analysis of variance test using the SPSS Statistics 17.0 software. Data are presented as the mean ± SEM, and a p value less than .05 was considered to be statistically significant.

Results

Macroscopic Views on Grafted Tissues and Volume/Weight Assessment

After 12 weeks, the fat grafts were harvested, and all presented yellow adipose-tissue-like appearances. Compared with the specimens in the normal saline (NS) group, the fat grafts in the FBP groups appeared as larger volumes (Fig. 1A). Volume analysis showed significantly decreased graft resorption among grafts that were treated with FBP compared with that in the control grafts, beginning as early as 2 weeks postimplantation and continuing up to 12 weeks (Fig. 1B); however, the decrease in graft weight was observed only at 12 weeks (Fig. 1C). At the terminal time point of 12 weeks, the control group maintained less than 20% of the initial grafted volume and weight, whereas the 2.5 and 4 mg/g groups preserved approximately 50% and 60% of

Table 1. Primer sequences used for qRT-PCR

Gene	Forward primers	Reverse primers
β-Actin	ATAGCACAGCCTGGATAGCAACGTAC	CACCTTCTACAATGAGCTGCGTGTG
PPAR-γ	TGGAATTAGATGACAGCGACTTGG	CTGGAGCAGCTTGGCAAACA
VEGF	ACTTTCTGCTGTCTTGGGTG	TTCGTGATGATTCTGCCCTC
TNFα	ACCTCTCTCTAATCAGCCCTC	GTTATCTCTCAGCTCCACGCCA

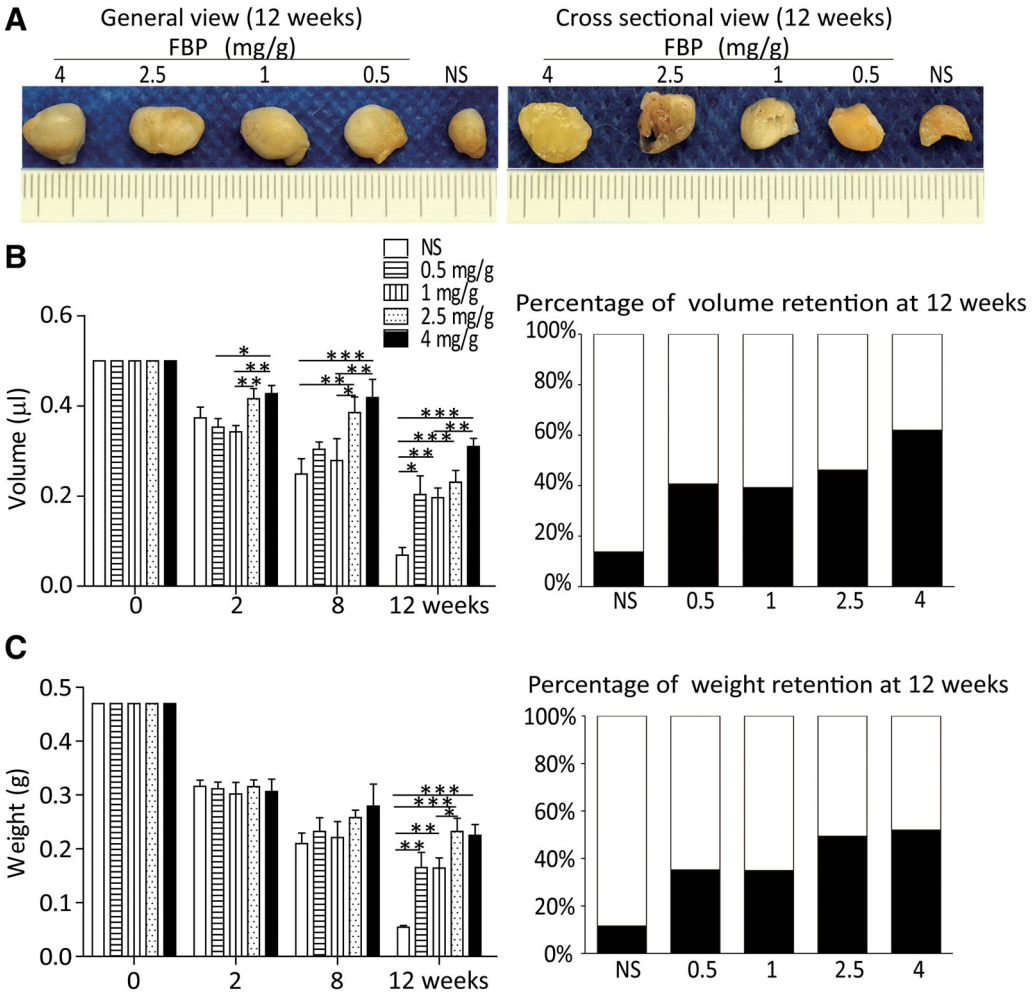

Figure 1. Macroscopic views of grafted tissue and volume/weight assessment. **(A):** Representative images of general and cross-sectional views after 12 weeks implantation; **(B, C):** the left panels illustrate the average volume and wet weight of the grafts in all groups at 2, 8, and 12 weeks, whereas the right panels indicate the percent retention of original volume and wet weight at 12 weeks. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: FBP, fructose 1,6-bisphosphate.

the initial volume and weight, respectively (Fig. 1B). Moreover, FBP at 0.5 and 1 mg/g provided limited protection against retention, whereas 2.5 and 4 mg/g FBP demonstrated effects that were stronger and that occurred earlier. There was no significant difference between the 4 and 2.5 mg/g groups. In summary, these data suggested that FBP obviously improved volume and weight retention and mitigated the resorption of the graft post-implantation, especially at doses of 2.5 and 4 mg/g.

Histological Examination of Fat Graft

Panoramic scanning of HE staining was used to determine a view of the fat grafts in the NS, 1, 2.5, and 4 mg/g groups after 2 and 12 weeks postimplantation. Different degrees of tissue necrosis were observed in the center areas of every sample in all groups as early as 2 weeks after implantation (Fig. 2A). H&E-stained slides that were imaged using high-magnification fields from both surviving and necrosis zones further showed that adipocytes in the peripheral areas and newly regenerated adipocytes at the center regions were both better preserved in the 2.5 and 4 mg/g groups (Fig. 2A). Quantification of the number of adipocytes showed that 2.5 and 4 mg/g FBP caused an increase in both central and peripheral areas (Fig. 2B, 2C) from 2 weeks up to 12 weeks.

Cell Viability and Revascularization of Fat Graft

To further analyze the viability of adipocytes after grafting, we used Bodipy 558, a class of lipophilic fluorescent dyes, to label

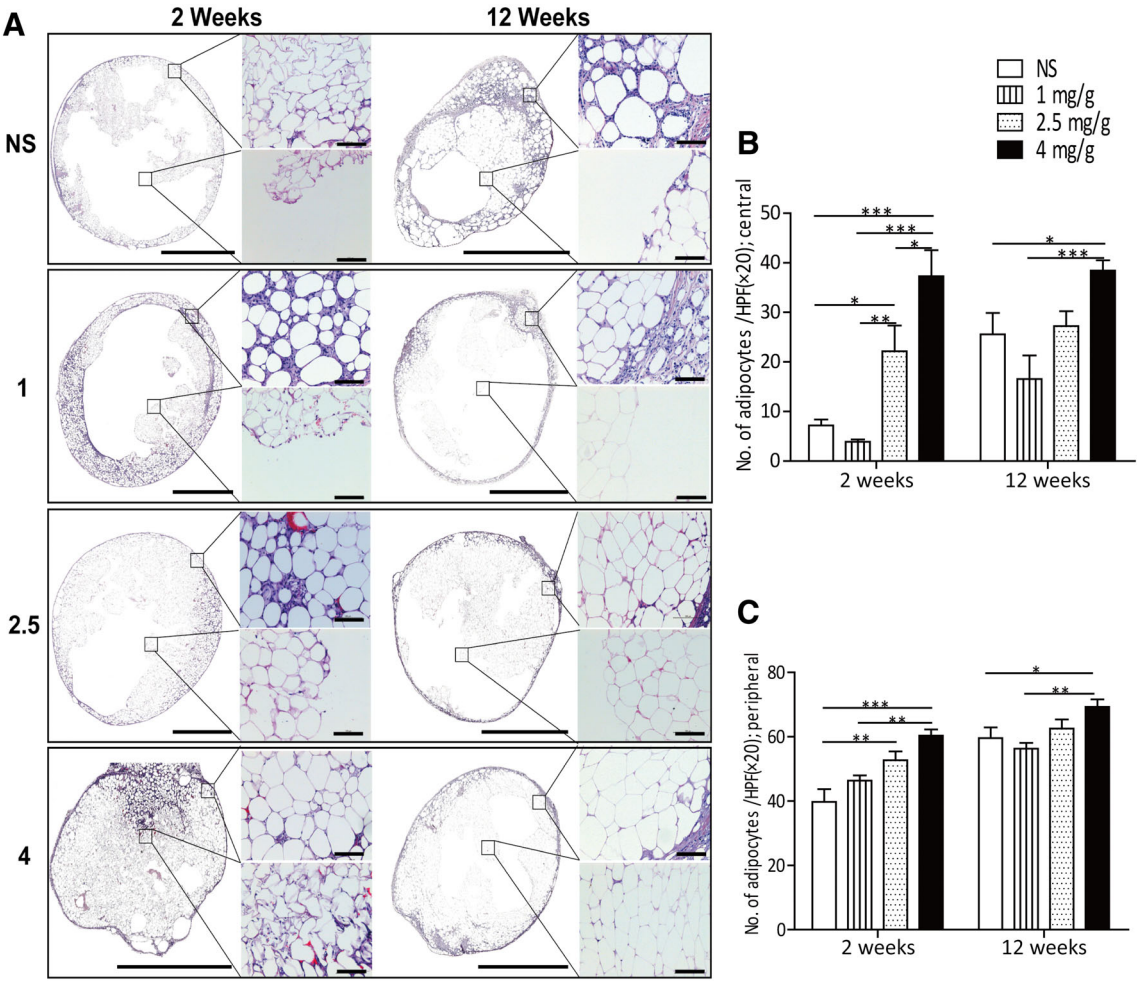

Figure 2. H&E staining of the fat graft sections at 2 and 12 weeks. **(A):** Representative panoramic view of the cross section of fat grafts in all groups, a higher magnification image of the boxed area (original magnification, ×20). Adipocytes in the peripheral area are shown in the upper right side; adipocytes in the central area are shown in the bottom right side; **(B)** number of adipocytes at the center areas; **(C)** number of adipocytes at the peripheral areas. Data are expressed as the mean ± SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviation: HPF, high power field.

lipid droplets. At 2 and 12 weeks postimplantation, the Bodipy-positive lipid droplets in the 4 mg/g FBP group were higher in number and exhibited a stronger fluorescence intensity (Fig. 3A). To provide a more stringent quantitative measurement of the metabolic activity of the fat grafts, the activity of the adipogenic enzyme GPDH, a cytosolic enzyme that is activated in functional adipocytes, was measured. The results showed that the GPDH activity of fat grafts in the 2.5 and 4 mg/g groups was higher, but not statistically higher, than that in the NS groups at 2 weeks postimplantation; this high level was maintained until 12 weeks after implantation (Fig. 3B).

Immunofluorescence staining of vWF was applied to evaluate the revascularization status in the fat grafts. The results showed that a positive staining for vWF was barely observed in the NS group; however, it was obvious in the 4 mg/g FBP group at 2 weeks. As quantified, the capillary numbers were significantly higher in the 4 mg/g group compared with that in the other two groups at 2 weeks, which indicated that the vascularity increased the most in the 4 mg/g group early postimplantation. However, by 12 weeks after transplantation, the vessel networks were all well-rebuilt in the three groups, there was no significant difference among them (Fig. 3C).

FBP Effects on Fat Graft and Infiltrated Macrophages at an Early Stage Postimplantation

Above results indicated that FBP exerted a significant effect on fat-graft survival in the 4 mg/g group at 2 weeks after transplantation, we further examined adipocytes viability, vascularization, and inflammation of fat graft at an early stage. Round-shaped cells that were strongly positive for perilipin were defined as viable adipocytes [17]. As early as the fifth day, a significantly higher number of perilipin-positive adipocytes was confirmed in the 4 mg/g FBP group and continuing till 2 weeks (Fig. 4A). Consistent with the Bodipy and vWF staining, the mRNA expressions of PPAR-γ and VEGF in the 4 mg/g group were significantly higher than that in control group at 2 weeks (Fig. 4B, 4C), although there was no significant difference at the fifth day. These data corroborate that FBP aids adipocyte activity and revascularization.

To further define the differences in fat inflammation, we investigated the recruitment and distribution of specific immune cells as assessed using CD68 immunostaining, a marker for pan macrophages, and performed real-time PCR for inflammatory cytokine genes. CD68 staining showed that significantly less macrophages infiltrated into the graft in the 4 mg/g group both at 5 days and

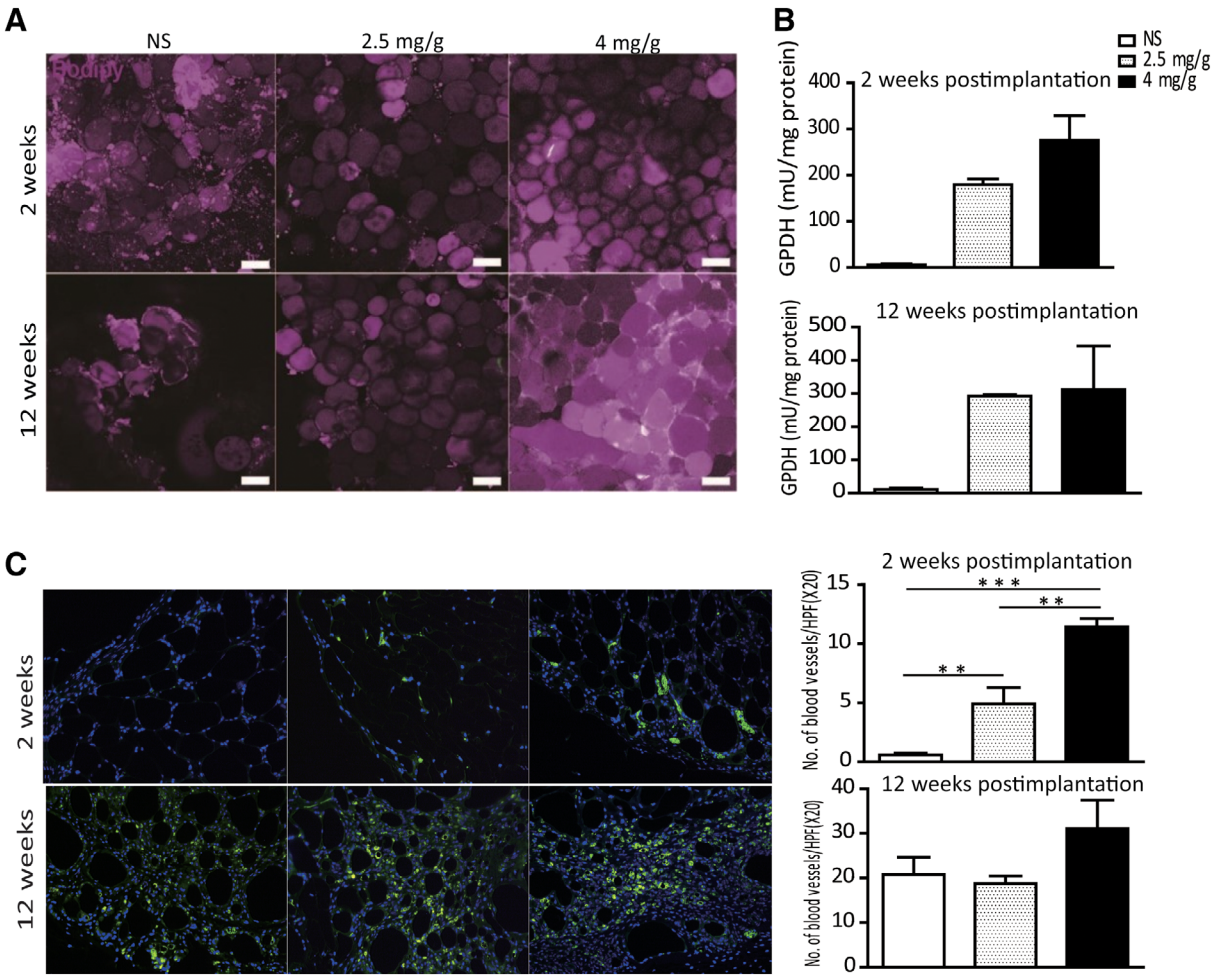

Figure 3. Cell viability and revascularization examination at 2 and 12 weeks. **(A):** Example images of Bodipy 558 staining of the fat grafts; **(B)** GPDH activity of the fat grafts at 2 and 12 weeks; **(C)** immunofluorescent staining for Von Willebrand factor (vWF) and quantification of vWF-positive staining at 2 and 12 weeks. **, $p < .01$; ***, $p < .001$. Abbreviation: GPDH, glycerol 3-phosphate dehydrogenase.

2 weeks (Fig. 5A). Additionally, the tumor necrosis factor-α (TNF-α) expression levels decreased significantly in the FBP group as early as 5 days postimplantation, which indicated that FBP reduced inflammation of the fat graft at an early stage (Fig. 5B).

FBP Effects on Human ASCs in vitro Cultured under Ischemia-Mimicking Conditions

To analyze the cellular effects of FBP in ischemia condition (hypoxia and low nutrients), human ASCs were cultured under ischemia-mimicking conditions (hypoxia without FBS) with or without FBP treatment for 12 hours and examined. The number of viable cells was significantly decreased under ischemia-mimicking conditions; in contrast, the 1 mmol/l FBP group exhibited more viable cells compared with the ischemia group, indicating the protective effects of FBP (Fig. 6AI, 6BI). The results also showed that hypoxia or an absence of FBS alone also led to a decrease in cell viability, whereas the administration of 1 mmol/l FBP significantly increased cell viability (Fig. 6AII,III, 6BII,III). FBP at 0.5 mmol/l exhibited no effect on cell viability under any culture condition. Moreover, exogenous 1 mmol/l FBP could improve the cell-proliferation potential under an absence of FBS (Fig. 6CIII), but not under hypoxia or ischemia-mimicking conditions (Fig. 6CI,II). FBP could significantly increase the VEGF expression of ASCs under ischemia-mimic condition (Fig. 6DI). Moreover, gene expression of VEGF increased in response to hypoxia (Fig. 6DII), whereas the absence of FBS decreased VEGF expression of ASCs (Fig. 6DIII). For adipogenic differentiation, under hypoxia alone condition, the expression of the adipogenic marker PPAR-γ significantly increased, indicating that hypoxia effectively induced adipogenesis (Fig. 6EII). In line, studies have reported that hypoxia induces adipogenic differentiation of MSCs [18, 19]. However, the expression of PPAR-γ displayed only a marginal increase in the FBP group under all conditions (Fig. 6EI–III), which suggested that FBP did not affect the adipogenic capacity of ASCs.

Discussion

Fat-grafting procedure has been considered to be a regenerative, cell-directed therapy [20] and successfully used in many different clinical situations; however, there are ongoing concerns regarding the survival and longevity of fat grafts after implantation and the unpredictability of a long-term outcome. Improvement in therapy efficiency and preventing ischemia should be a primary goal in fat grafting to optimize early and long-term results. For years, FBP has been used as a protective agent both

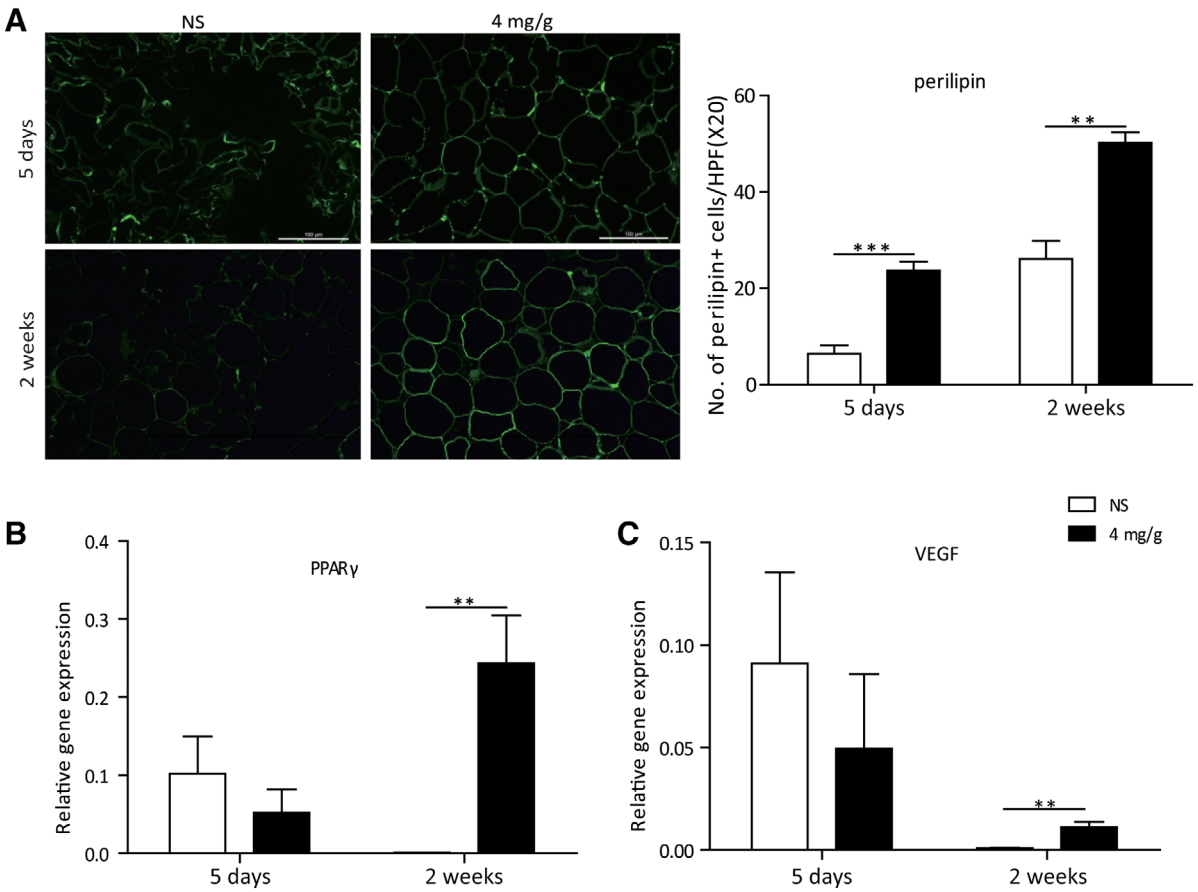

Figure 4. Cell survival analysis at the early stage. **(A):** Representative images of perilipin immunostaining and quantitative analysis of the perilipin-positive staining at 5 days and 2 weeks; scale bars represent 100 μm **(B)**; mRNA expression levels of PPAR-γ were determined via quantitative reverse transcription polymerase chain reaction (qRT-PCR); **(C)** mRNA expression levels of VEGF were determined via qRT-PCR. The expression of each gene was normalized to the amount of β-actin RNA to calculate the relative amount of mRNA. All tests were performed in triplicate, and the data are expressed as the mean ± SEM. **, $p < .01$; ***, $p < .001$. Abbreviations: PPAR-γ, peroxisome proliferator activated receptor.

clinically and experimentally during ischemia environment, and the primary benefits of FBP are attributed to its ability to sustain glycolysis and increase ATP production. There are no studies that have evaluated the potential use of FBP as a supplement in autologous fat-grafting surgery, in which a region of fat grafts is subjected to a period of ischemia. In the current study, exogenous FBP administration was found to attenuate the volume and mass reduction of fat grafts postimplantation. Our results also demonstrated that FBP enhanced adipocyte viability and function, increased blood-vessel formation, and decreased inflammation. Finally, in vitro cell experiments showed that FBP could protect ASC viability from hypoxia and an absence of FBS. Current hurdles to the clinical translation of substitute materials, such as autologous tissues, allogeneic tissues, or synthetic biomaterials, include protection of the cells and graft survival and stability both pre transplantation and post-transplantation. Our study may provide an efficient protective strategy for the application of biomedical implants in regenerative therapies.

In animal models, an aqueous solution of FBP can be dispensed exogenously via different routes, including oral, i.v., and intraperitoneal injections; the dosage will vary depending on the route used. Xu and Stringer verified that after intraperitoneal administration of a single 0.5 g/kg dose of FBP, after 1 hour, FBP levels could be measured in tissues including fat [21]. According to other literatures, FBP-induced protection will vary depending on the tissue type, and it often appears to be dose dependent [10]. For instance, a concentration range between 5 and 10 mM FBP was the most effective for increasing metabolism at least for cardiac cells [22]. In our study, we used 0.5, 1, 2.5, and 4 g/kg doses intraperitoneally and found that FBP at 2.5 and 4 g/kg doses are more effective for fat grafts.

The cellular survival theory that was introduced by Peer [23] described that the grafted adipocytes passed through an ischemic phase, in which the mature adipocytes were very fragile and exhibited a low level of resistance to hypoxia and ischemia. A common phenomenon that occurs in hypoxic or ischemic cells is the lethal cell injury by oxidative stress. The modulation mechanisms of FBP in decreasing oxidative stress include a reduction in ROS production and counteracting the decrease of NADPH due to hypoxia [24]. Additionally, Seaman et al. observed that exposure to collagenase exhibited an adverse effect on human adipocytes and interstitial cell viability [25]. The study about the fate of adipocytes after free fat grafting showed that viable adipocytes decreased in number sharply on day 1, followed by a further decrease over time until day 5 [26]. With the addition of FBP in our study, the number of adipocytes in both peripheral and central areas increased significantly and more functional viable adipocytes

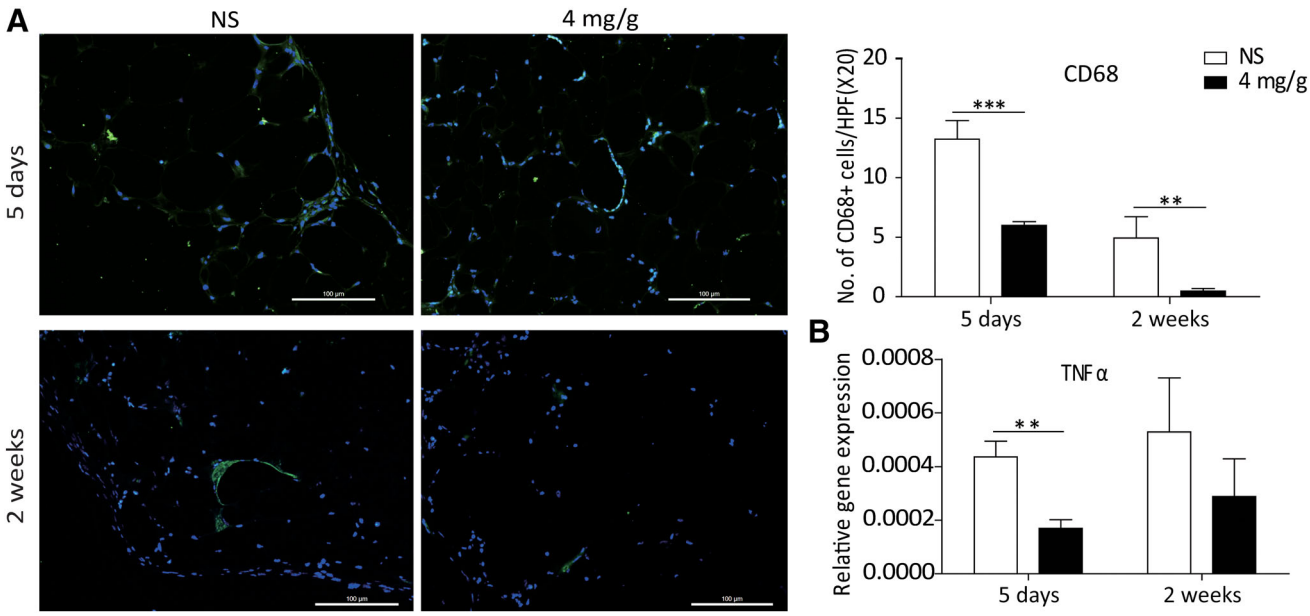

Figure 5. Effect of fructose 1,6-bisphosphate on macrophage infiltration and inflammatory cytokine gene expression of the fat grafts at 5 days and 2 weeks. **(A):** Representative images of CD68 staining and quantitative analysis of the CD68-positive staining cells. The presence of cell nuclei was indicated by 4′,6-diamidino-2-phenylindole staining. **(B):** mRNA expression level of inflammatory cytokine genes TNF-α was determined via quantitative reverse transcription polymerase chain reaction. The expression of each gene was normalized to the amount of β-actin RNA to calculate the relative amount of mRNA. All tests were performed in triplicate, and the data are expressed as the mean ± SEM. **, $p < .01$; ***, $p < .001$.

were preserved as early as 5 days postimplantation. These findings suggested that FBP could prevent mature adipocytes dying under ischemic conditions at the early time, which is essential for survival of grafts. Exogenous FBP could enter cells, where it acts as a glycolytic substrate, and stimulate the glycolytic pathway. The advantage of FBP as the initial substrate is that, whereas the net yield of the anaerobic metabolism of 1 mol of glucose is two ATP moles, 1 mol of FBP metabolized in the same conditions would result in a net gain of four ATP moles since FBP does not require phosphorylation [27]. These features may explain, at least in part, how FBP helps cells save energy and survive under hypoxic or ischemic challenges. The preventive administration of FBP could attenuated ischemic injury and act as an alternative energy source.

It is noteworthy that administration of FBP reduced inflammatory infiltration in fat grafts. It has been proposed that, among the different cellular and molecular mechanism, the tissue inflammation due to hypoxia may enhance oxidative stress and affect fat tissue graft survival [28]. Macrophages have attracted considerable recent attention in adipose tissue biology following the discovery that they play a substantial role in the inflammatory process [29]. TNF-α is a pivotal cytokine produced by macrophages in inflammation and ischemia injury tissue. It has been found that TNF-α could induce apoptosis through a series of signaling pathways and blocking its expression by anti-TNF-α antibody could significantly attenuate cell apoptosis and preserve the quality of the transplanted fat tissue [30]. We found that less macrophages infiltrated into the graft and the expression levels of TNF-α appeared to be reduced in the FBP administration group. The anti-inflammatory effect of FBP has been documented [31, 32]. The addition of FBP to the culture medium inhibited the release of TNF-α and interleukin-6 production in lipopolysaccharide-exposed alveolar macrophages in the early phase of the inflammatory response [33], which suggest that FBP acts as a cytokine inhibitor and provides anti-inflammatory effects.

The quantity of fat-graft vascularization is among the long-term viability determinates. Chung showed a significantly greater graft sustainment and vascularization in the VEGF-injected group [34]. Assessment of fat transplants in our mouse model showed that FBP increased the vascularization of transplanted adipose tissue, manifested as more positive-vWF staining, and upregulated VEGF mRNA expression in 2 weeks. When reviewing the literature, there is no direct evidence that FBP can promote vascularization. It has been proven in preclinical studies that ASCs secrete multiple angiogenic growth factors and cytokines, which contribute to enhanced graft viability [35, 36]. In our study, we demonstrated that FBP promoted ASC viability, which may result in much more angiogenic growth factors secreted to promote vascularization.

There is a substantial amount of evidence that supports the therapeutic use of ASCs to improve long-term graft survival and retention. The overall volume of a fat graft retained has been reported depending on the degree of survival of the regenerating zone, which contains ASCs with the potential for differentiation and replacement of adipocytes that are lost in the necrotic zone [26]. ASCs have also been implicated in encouraging graft revascularization via paracrine effects. Oxygen plays pivotal roles in cell viability and angiogenesis, and in our experiment, hypoxia induced significant increase in VEGF expression, which is consistent with other studies [37]. Our in vitro findings demonstrated that FBP exhibited to promote cell viability in both hypoxia and low-nutrient conditions with a marginal adipogenic induction potential, whereas FBP increased VEGF expression only in hypoxia together with low-nutrients conditions. The results suggested that FBP induced VEGF increasing through promoting

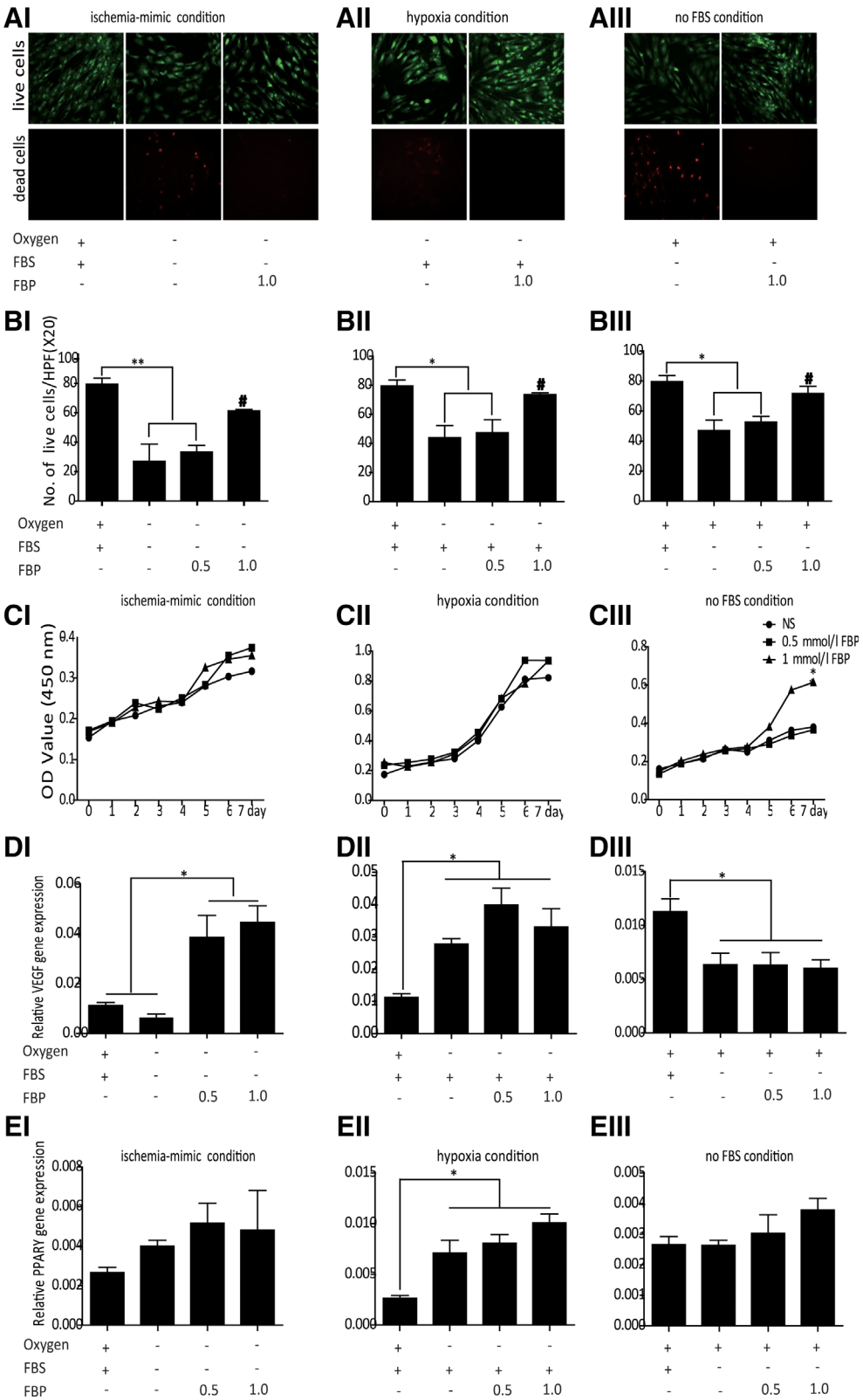

Figure 6. Effect of FBP on human adipose-derived mesenchymal stem cell viability and proliferation in vitro. **(AI–III):** Representative images of live and dead cells stained with a live/dead cell-viability assay kit. **(BI–III):** Number of live cells under different conditions; **(CI–III)** cell proliferation analysis via MTT assay. **(DI–III):** The gene expression of VEGF in human adipose-derived stem cells (hASCs) was measured 12 hours after culturing under different conditions. **(EI–III):** The expression of the adipogenic differentiation gene PPAR-γ in hASCs was measured 12 hours after culturing under different conditions following adipogenic induction for 7 days via quantitative reverse transcription polymerase chain reaction; *, $p < .05$; **, $p < .01$; #, $p < .05$ versus without FBP under the same culture conditions. Abbreviations: FBP, fructose 1,6-bisphosphate; FBS, fetal bovine serum; PPAR-γ, peroxisome proliferator activated receptor.

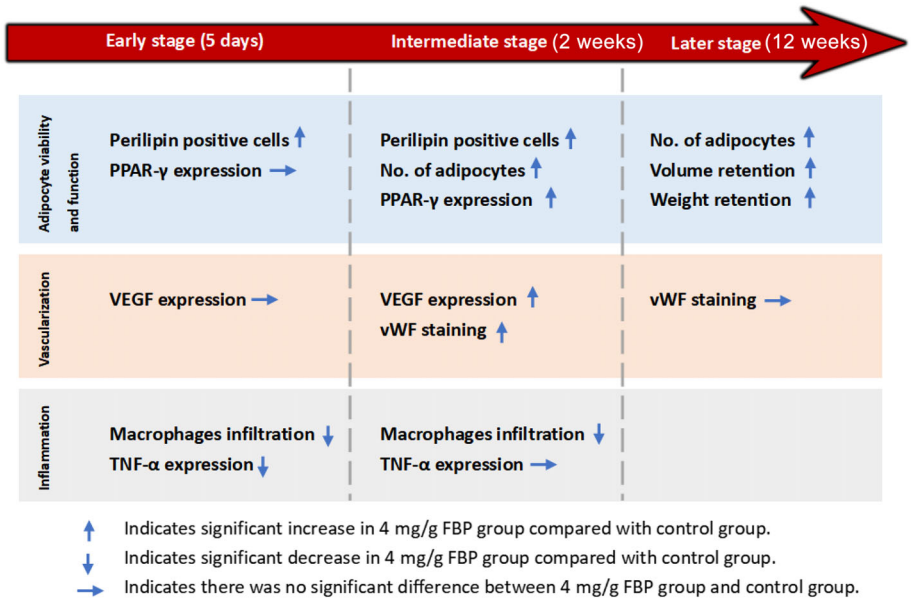

Figure 7. Timeline of cell and molecular changes related to FBP effects. Abbreviations: FBP, fructose 1,6-bisphosphate; PPAR-γ, peroxisome proliferator activated receptor; vWF, Von Willebrand factor.

cell viability, which may explain its role in promoting angiogenesis in vivo. Qi et al synthesized FBP dicalcium (Ca_2 FBP) porous microspheres and found that 10 μg/ml Ca_2 FBP porous microspheres could promote human bone marrow MSCs osteogenic differentiation without obviously influencing proliferation [38]. The authors analyzed that FBP-induced calcium-chelating ability and Ca_2 release are the possible reasons. Future studies to investigate the effect and mechanism of FBP on proliferation and differentiation of stem cell could provide additional insights regarding the use of FBP in stem-cell-based regenerative medicine.

We used a timeline-based classification to provide a summary of the molecular changes related to FBP effects (Fig. 7), which is helpful to understand the effects and time sequence of FBP. In the early stage of transplantation, more perilipin-positive cells suggested that FBP protected adipocytes intact, and the decreased macrophages infiltration and TNF-αexpression in fat grafts implied decreased inflammation. In the intermediate stage, the protective effect of FBP on adipocytes and the inhibition of macrophages infiltration were still observed. The increased expression of PPAR-γhinted increased number of ASCs at this stage, which expressed more VEGF and promoted vascularization showing higher vWF staining in 4 mg/g FBP group. Through protecting cell viability and function, inhibiting inflammation and promoting vascularization in the first 2 weeks, FBP eventually promoted larger volume and weight retention of fat grafts in the long time.

Our data confirmed the role of FBP in promoting the survival of fat grafts; however, there were still many problems to be solved in translation to clinical application. Vexler et al. [39] demonstrated the toxicity of FBP in neonatal rats, which receiving 8,000 mg/kg and 27% of those receiving 6,000 mg/kg of FBP intraperitoneally died, because of the chelation of the ionic calcium, creating a reduction in plasmatic ionic calcium, and resulting in convulsions and cardiac fibrillations. Additionally, according to Li et al. study [40], the consumption of FBP by the intestinal epithelium cells could be a major barrier for FBP absorption during oral administration, and a higher dosage may be used when FBP was given orally to achieve the desired clinical therapeutic effect. In our experiment, the administration of FBP in mice is 4 mg/g intraperitoneally, whereas the recommended dose of FBP for patients is 1 g, tid i.v., that is, 42.8 mg/kg per day. Therefore, high-dose FBP intraperitoneal administration appeared to indicate a systemic effect; whether safe dose of FBP is efficient for fat tissue transplantation need to be further studied. FBP mixed with fat grafts during implantation could be an alternative strategy, which may reduce the systemic side effects of FBP and increase its local concentration, but it is difficult to maintain the effective concentration as a short half-life of 10–15 minutes. In a word, it is important to continue to refine our knowledge on the complex roles of FBP and to explore the optimal application dosage and time in order to facilitate the improvement of clinical outcomes.

Conclusion

In this study, we report the application feasibility of FBP for free fat transplantation. Our results demonstrate that FBP could improve long-term volume and weight retention of fat grafts. We also provide evidences that FBP effectively increases adipocyte viability and function promotes the vascularization of fat grafts, and exerts anti-inflammatory properties as well. Another important finding is that FBP effectively promotes ASC viability under ischemia conditions. Therefore, FBP may represent a new therapeutic strategy for free fat grafting. Moreover, because of the advantages of FBP under ischemia conditions, it may be used in other substitute biomaterials such as engineered tissue transplantation and stem-cell-based regenerative medicine.

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Author Contributions

T.L.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Y.G.: provision of study material or patients; J.B., N.K.: collection and/or assembly of data, data analysis and interpretation; Z.Y., X.F.: data analysis and interpretation, manuscript revision for intellectual content; Q.W., Y.L.: administrative support, data analysis and interpretation; X.L., Y.C., R.X.: conception and/or design, data analysis and interpretation, manuscript writing, final approval of manuscript, financial support.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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