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Efficacy of freeze-dried platelet-rich plasma in bone engineering

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ABSTRACT

Objective: Platelet-rich plasma (PRP) is typically isolated and applied immediately after preparation, making it both a time- and labor-intensive addition to the operative procedure. Thus, it would be convenient if PRP could be preserved. We evaluated the efficacy of freeze-dried PRP (FD-PRP), as compared with freshly isolated PRP (f-PRP) for bone engineering.

Design: FD-PRP was prepared by lyophilization of f-PRP and was subsequently preserved at −20 °C for one month. It was then rehydrated with an equal or 1/3 amount of distilled water (×1FD-PRP, ×3FD-PRP, respectively), and we assessed its gelation properties and the release of growth factors (PDGF-BB, TGF-β1, and VEGF). We also examined the bone forming ability with onlay-grafting on mice calvaria using β-TCP granules as a scaffold.

Results: FD-PRP showed comparable gelation as f-PRP. In terms of growth factor release, ×1FD-PRP released identical concentrations of PDGF-BB and TGF-β1 to f-PRP, while ×3FD-PRP released approximately 3-fold concentrations when compared with f-PRP. *In vivo*, ×1FD-PRP promoted identical levels of the bone formation as f-PRP, and ×3FD-PRP induced more abundant bone formation.

Conclusions: These results suggest that f-PRP can be stored without functional loss by freeze-drying and the concentration of PRP may improve its efficacy in bone engineering.

1. Introduction

PRP is a concentrate of platelets, and is known to promote wound healing for various tissues, including dermis (Kakudo et al., 2012; Marx, 2004), mucosa or connective tissue (Lindeboom et al., 2007; Keceli, Sengun, Berberoğlu, & Karabulut, 2008), tendon (Mishra & Pavelko, 2006; Schnabel et al., 2007) and bone (Marx et al., 1998; Rodriguez, Anastassov, Lee, Buchbinder, & Wettan, 2003; Tajima, Sotome, Marukawa, Omura, & Shinomiya, 2007; Zhong et al., 2012). PRP is generally isolated and applied on site during surgery; however, it is time and labor intensive and it can be difficult to prepare adequate amounts. Thus, it would be useful if PRP could be isolated beforehand and stored until use.

For preservation of PRP, dimethyl sulfoxide (DMSO) (Towell, Levine, Knight, & Anderson, 1986; Guillaumin, Jandrey, Norris, & Tablin, 2008; Tarandovskiy, Artemenko, Panteleev, Sinauridze, & Ataullakhanov, 2013) or sugars such as trehalose (Wolkers, Walker, Tablin, & Crowe, 2001; Crowe et al., 2003; McCarrel & Fortier, 2009) have been added to PRP before freezing or freeze-drying with the intention of preventing platelet structure from being destroyed; however, these additives need to be removed by washing and centrifugation before clinical application. This procedure is also time and labor intensive, and there is a possibility of contamination by these preservation agents. It is also uncertain whether PRP maintains ideal platelet properties. Therefore, we aimed to preserve PRP by freeze-drying without additives in order to quickly use stored PRP by rehydration alone. Furthermore, freeze-dried PRP possesses another advantage, as we can produce super-concentrated platelets by decreasing the amount of water used for rehydration.

We focused on the growth factors and fibrin network in PRP as independent elements of PRP properties, and hypothesized that PRP function would be maintained if these elements are preserved, even if platelet structure is damaged. In this study, we examined these *in vitro* properties, as well as the *in vivo* bone induction, of FD-PRP and super-concentrated FD-PRP for the purpose of assessing their applicability.

2. Materials and methods

Experiments were conducted in accordance with the principles of the Declaration of Helsinki. The Ethics Committee of Nagasaki

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University Graduate School of Biomedical Sciences approved this study using human samples (Approval No. 1180), and blood samples were taken from donor volunteers after obtaining informed verbal consent. All animal experiments in this study were approved by the Biomedical Research Center (BRC) of Nagasaki University (Approval No. 1305131060).

2.1. Preparation of PRP

PRP was prepared from human peripheral blood (PB) collected from 3 healthy donors using a blood phase separator (Medifuge MF 200; Silfradent, Santa Sofia, Italy). Next, 22.5 ml of PB mixed with 2.5 ml of sodium citrate (Citramin; Fuso, Osaka, Japan), divided into four 7 ml plastic tubes (Venoject® II VP-P070K30, Terumo, Tokyo, Japan), was centrifuged for 2 min at 2700 rpm, 4 min at 2400 rpm, 4 min at 2700 rpm, and 3 min at 3000 rpm continuously, using a Medifuge. After centrifugation, 1/10 the volume of whole blood from the border of the plasma and red blood cell layers was marked with a marker, and the upper layer of plasma was discarded, while the lower layer, including platelets, buffy coat and small amounts of red blood cells, was collected with a pipette as PRP (Fig. 1).

2.2. Preparation of autologous serum

After collecting PB from the same donors into glass tubes without sodium citrate, tubes were warmed until rising clot retraction for about 40 min. Samples were then centrifuged at 800 × *g* for 10 min (LC-122; Tomy, Tokyo, Japan). Subsequently, supernatants were collected as autologous serum and were lyophilized, and this was used as the thrombin recombinant substitute for PRP activation.

2.3. Lyophilization of PRP

PRP or serum was pre-frozen at −80 °C for 12 h, and was then lyophilized for 12 h using a freeze dryer (EYELA FD-1000; Tokyo Rikakikai, Tokyo, Japan). After freeze-drying, samples were stored at −20 °C for one month in order to prevent contamination and transformation. We produced different concentrations (equal and 3-folds; ×1FD-PRP, × 3FD-PRP) by resolving FD-PRP to equal or 1/3 amount of distilled water at the evaluation of FD-PRP.

2.4. Blood cell counting

We measured the number of white blood cells (WBC), red blood cells (RBC), and platelets (PLT) in PB and PRP using an automatic hematology analyzer (MEK-6510 Celltac α; Nihon Kohden, Tokyo, Japan). Blood cells were counted 4 times for each of the 3 samples.

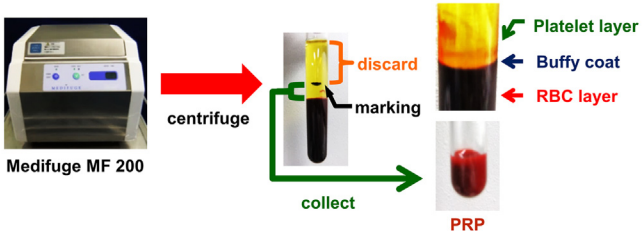

Fig. 1. Preparation of PRP.
(1) Human peripheral blood (PB) was collected and mixed with sodium citrate at a ratio of PB:sodium citrate = 9:1. (2) Blood-sodium citrate mixture was transferred to plastic tubes. (3) Tubes were centrifuged using a blood-phase separator (Medifuge MF 200). (4) Buffy coat was defined as baseline, and a mark was drawn above it. The amount of plasma between the mark and buffy coat was 1/10 the volume of collected whole blood. (5) Platelet-Poor Plasma, which was above the mark, was discarded. (6) The platelet layer and buffy coat were extracted and this plasma fraction was defined as Platelet-Rich Plasma.

The concentration rate for platelets in PRP was calculated by comparing the number of platelets in PRP with that in PB.

2.5. Growth factor assay

PRP mainly contains growth factors derived from platelets, including PDGF, TGF-β, IGF-I, VEGF and EGF. Among these, PDGF and TGF-β are the most abundant in PRP and play significant role in bone engineering (Marx et al., 1998). In addition, VEGF promotes neoangiogenesis, which is an essential element in wound healing and tissue regeneration. Then, the concentration of these three growth factors (GFs; PDGF-BB, TGF-β1 and VEGF) in PRP was measured using an Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Quantikine® DBB00, DB100B, and DVE00; R&D Systems, Minneapolis, MN). To release GFs from platelets in f-PRP, repeated freeze and thaw was performed as described elsewhere (Griffiths, Baraniak, Copland, Nerem, & McDevitt, 2013). GFs in f-PRP, ×1FD-PRP, ×3FD-PRP were assessed in 4 wells for each of the 3 donors, and the ratio of GF concentrations in PRP as compared to f-PRP was calculated.

2.6. Gelation of PRP

We prepared PRP-β-TCP composite gel to assess the fibrin formation properties of FD-PRP. Before preparation of PRP gel, we mixed the same amount of rehydrated FD-serum and 2% $CaCl_2$ (Otsuka Pharmaceutical Factory, Tokushima, Japan) as a PRP activator. Next, 100 μl of f-PRP or rehydrated FD-PRP was mixed with 25 mg of β-TCP granules (OSferion G1; Olympus Terumo Biomaterials Corp., Tokyo, Japan), and this was activated with 20 μl of PRP activator, as described elsewhere (Zhong et al., 2012). We observed the gelation (fibrination) of these mixtures at 10 min after activation.

2.7. Animal experiment

Six-week-old male immunocompromised mice (BALB/c-nu/nu; Clea Japan Inc., Tokyo, Japan) were used for the assessment of *in vivo* bone formation. 0.1 mg/kg of ketamine hydrochloride (Ketalar® 50 mg/ml; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and 0.01 mg/kg of xylazine hydrochloride (Selactar® 20 mg/ml; Bayer Yakuhin, Ltd., Tokyo, Japan), diluted with saline, were used as anesthetic agents. PRPs (f-PRP, ×1FD-PRP or ×3FD-PRP) were mixed with β-TCP granules, and samples were onlay-grafted onto mice calvaria (Figs. 2A and B); 3 mice were used for each PRP from each individual donor, and total 9 mice were used for each PRP group. As a control, a mixture of 100 μl of 10 mg/ml fibrinogen solution (bovine plasma fibrinogen F8630; Sigma-Aldrich, Saint Louis, MO), 10 μl of 100 U/ml thrombin solution (bovine plasma thrombin T9549; Sigma-Aldrich), and 10 μl of 2% $CaCl_2$ with 25 mg of β-TCP granules was onlay-grafted, as described previously (Agata et al., 2012). 4 mice were dead out of 76 mice due to the experimental procedures. We monitored the health and behavior of animals twice a week in a first week and once a week subsequently. Mice were euthanized with the anesthesia and cervical dislocation, and specimens were harvested at 4 and 8 weeks after implantation (Fig. 2C).

2.8. Histological and immunohistochemical assessment of new-bone formation

Harvested samples were fixed in 4% buffered paraformaldehyde for one day, demineralized in 0.5 M EDTA for 10 days, and embedded in paraffin. Samples were sliced parasagittally into 5-μm sections and stained with hematoxylin and eosin (H&E). Slides were de-paraffinized with xylene, and were hydrated in water.

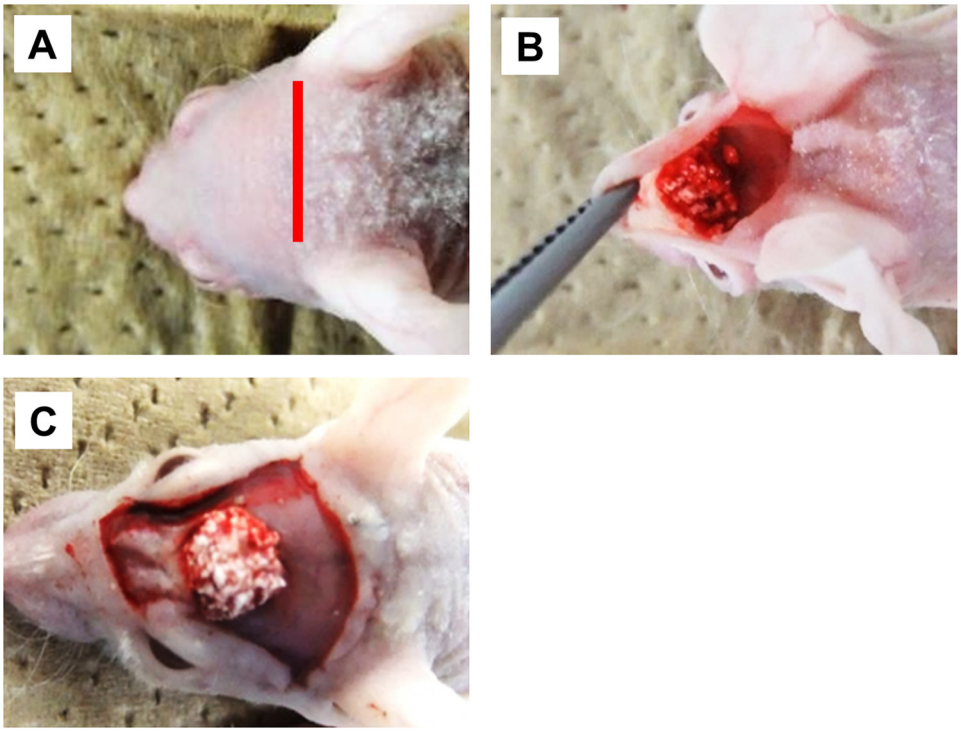

Fig. 2. Transplantation procedure.
(A) Incision line. (B) Periosteum was separated from the calvaria bone, then the transplant was onlay-grafted under the periosteum. (C) Specimens were harvested at 4 and 8 weeks after implantation.

They were stained with Hematoxylin (Mayer's Hematoxylin Solution; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and with Eosin (1% Eosin Y Solution; Wako Pure Chemical Industries). After washing with distilled water, they were dehydrated, and finally, sections were encapsulated by slide glass. Six sectional images were selected every 100 μm through the center of the transplants, and photographs projecting the overall specimens were obtained. The new-bone and transplant area were histomorphometrically measured using NIH Image J (Scion Corp., Frederick, MD). The percentage of new-bone formation and also remaining β-TCP granules (new-bone area, or remaining β-TCP granules area/total transplant area from 6 specimens ×100; %) were then calculated for each group.

In addition, we performed Masson trichrome staining, tartrate resistant acid phosphate (TRAP) staining, and osteocalcin staining to identify new bone formation. We used a staining kit (Trichrome Stain, Connective Tissue Stain, ab150686; Abcam, Tokyo, Japan) for Masson trichrome staining, in accordance with the manufacturer's instructions. For TRAP staining, slides were stained with compound liquid containing phosphatase substrate (naphthol AS-MX phosphate disodium salt N5000; Sigma-Aldrich), *N,N*-dimethylformamide (Wako Pure Chemical Industries), Fast Red Violet LB Salt F3381 (Sigma-Aldrich), sodium (+)-tartrate dihydrate (Wako Pure Chemical Industries) and 0.2 M acetate buffer (pH 5.0). After the initial staining, the slides were stained with methyl green (Wako Pure Chemical Industries). For osteocalcin staining, staining without a primary antibody was performed as a negative control. Sections were de-paraffinized and dehydrated, and peroxidase activity was removed by treatment with methanol and hydrogen peroxide for 30 min. Primary antibody (ab93876; Abcam) at a dilution of 1:100 was then added to each section, followed by incubation at 37 °C for 90 min. ABC complex (VECTASTAIN® ABC HRP kit (peroxidase, rabbit IgG) PK-4001; Vector Laboratories, Burlingame, Ca) was applied to the sections after incubation with biotinylated secondary antibody (VECTASTAIN® ABC HRP kit (peroxidase, rabbit IgG) PK-4001). DAB solution made from a DAB tablet (DAB 4HCl 10 mg/tablet; Wako Pure Chemical Industries) was then used as a chromagen. Finally, the sections were stained with hematoxylin.

2.9. Statistical analysis

Statistical analysis for group comparisons was performed by one-way ANOVA with the Tukey-Kramer test using Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Probability (p) values of less than 0.05 were considered to be significant.

3. Results

3.1. Blood cell counts

Numbers of PLT and WBC were higher in PRP, while the number of RBC was lower. The number of PLT in PRP was enriched from $19.1 \pm 0.9 \times 10^4/\mu l$ to $120.3 \pm 6.6 \times 10^4/\mu l$, from $24.8 \pm 1.9 \times 10^4/\mu l$ to $156.0 \pm 10.8 \times 10^4/\mu l$, and from $20.2 \pm 1.5 \times 10^4/\mu l$ to $131.1 \pm 7.9 \times 10^4/\mu l$, respectively, in the 3 donors. The concentration rate of PLT was similar in each donor, around 6.3 times (Table 1).

3.2. Growth factors assay

3.2.1. PDGF-BB

The ratio of PDGF-BB concentration against that in f-PRP was 1.25 ± 0.10 in ×1FD-PRP, and 2.79 ± 0.22 in ×3FD-PRP. Significant differences were observed between f-PRP and ×1 FD-PRP, and between ×1 FD-PRP and ×3 FD-PRP.

Table 1
Number of blood cells and platelets before and after centrifugation.
PB: Peripheral Blood; PRP: Platelet-Rich Plasma; WBC: White Blood Cells; RBC: Red Blood Cells; PLT: Platelets; PLT ratio: concentration ratio of platelets in PRP.

	Donor A		Donor B		Donor C	
	PB	PRP	PB	PRP	PB	PRP
WBC ($10^2/\mu l$)	62.0 ± 1.0	221.2 ± 9.0	57.7 ± 5.7	200 ± 30.1	44.7 ± 6.1	151.4 ± 19.2
RBC ($10^4/\mu l$)	458.3 ± 2.5	223.4 ± 67.0	441.3 ± 11.6	262.1 ± 55.8	470.0 ± 6.6	271.8 ± 17.4
PLT ($10^4/\mu l$)	19.1 ± 0.9	120.3 ± 6.6	24.8 ± 1.9	156.0 ± 10.8	20.2 ± 1.5	131.1 ± 7.9
PLT ratio (fold)		**6.30 ± 0.11**		**6.29 ± 0.15**		**6.48 ± 0.10**

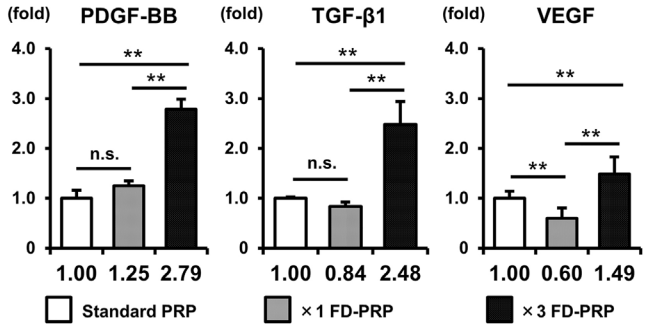

Fig. 3. Comparison of GFs between storage methods.
Concentrations of GFs (PDGF-BB, TGF-β1, and VEGF) were assessed, and the ratio of GF concentration to that in s-PRP among the PRP groups was calculated (×1FD-PRP and ×3FD-PRP). Statistical analysis was performed among s-PRP, ×1FD-PRP, × 3FD-PRP. Data are presented as means ± standard deviation (n = 3). $^{**}p < 0.01$, n.s.: Not significant.

3.2.2. TGF-β1

The ratio of TGF-β1 concentration to that in f-PRP was 0.84 ± 0.10 in × 1FD-PRP, and 2.48 ± 0.52 in ×3FD-PRP. Significant differences were only observed between f-PRP and × 1 FD-PRP, and between × 1 FD-PRP and ×3 FD-PRP.

3.2.3. VEGF

The ratio of VEGF concentration against that in f-PRP was 0.60 ± 0.23 in ×1FD-PRP, and 1.49 ± 0.38 in ×3FD-PRP. VEGF concentrations in ×1FD-PRP were significantly lower than that in f-PRP. However, the concentration in ×3FD-PRP was significantly higher than that in f-PRP and in ×1FD-PRP.

Fig. 3 shows the results of growth factor assay.

3.3. Gelation of PRP and FD-PRP

f-PRP-β-TCP composite gelatinized 10 min after mixture, and the same results were obtained from the composite made from FD-PRP, regardless of preservation term, as shown in Fig. 4.

3.4. Histological and immunohistochemical assessment of new-bone formation

Observations at 4 weeks after transplantation were as follows. The β-TCP group showed bone induction only at the contact with host bone. In the f-PRP and the ×1FD-PRP groups, new-bone was observed around both host bone and β-TCP granules, and these bones connected to the host bone. Furthermore, the ×3FD-PRP group promoted new-bone penetration deeper into β-TCP granules when compared with the other two PRP groups. Masson trichrome staining showed mature bone (red area) surrounding β-TCP granules, and there was abundant immature bone (blue area) (Fig. 5A). At 8 weeks after transplantation, new-bone formation had progressed further than at 4 weeks, although bone formation did not reach the top of the transplant in all groups. Even the β-TCP group showed osteoconduction from host bone, and the formation of bone marrow suggested the maturation of bone in the f-PRP and the × 1FD-PRP groups. The ×3FD-PRP group showed more abundant new-bone formation. Mature bone was extended and there was less immature bone when compared with 4 weeks on Masson trichrome staining (Fig. 5B). The presence of osteoclasts in mature bone surrounding β-TCP granules was confirmed by TRAP staining, and osteoblasts with osteocalcin expression were noted at the surface of new bone (Fig. 5C).

Histomorphometric assessment showed that the average percentage of new-bone formation was 6.6 ± 3.1% in the β-TCP group, 15.3 ± 4.0% in the f-PRP group, 13.0 ± 2.1% in the ×1FD-PRP group, and 19.0 ± 3.8% in the ×3FD-PRP group at 4 weeks, while

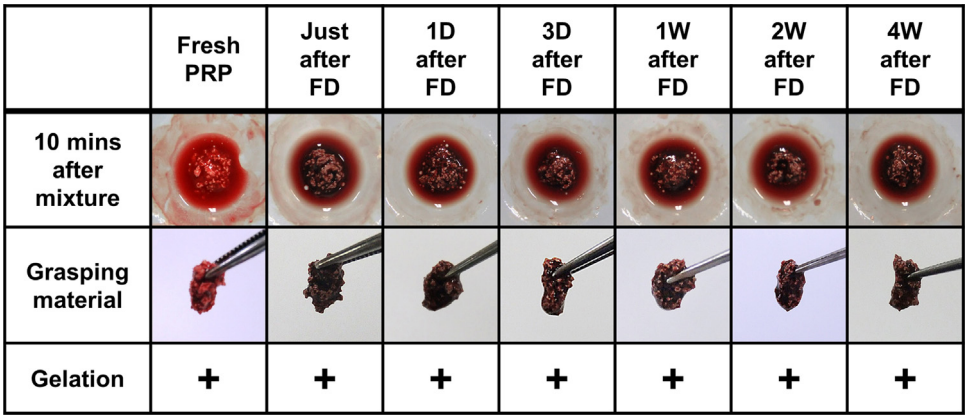

Fig. 4. Gelation of f-PRP gel and FD-PRP.
PRP-β-TCP composite was prepared immediately after producing PRP (f-PRP) and immediately after freeze-drying (FD-PRP), and then at 1 day, 3 days, 1 week, 2 weeks and 4 weeks after freeze-drying. We observed the properties of the composite at 10 min after activation.

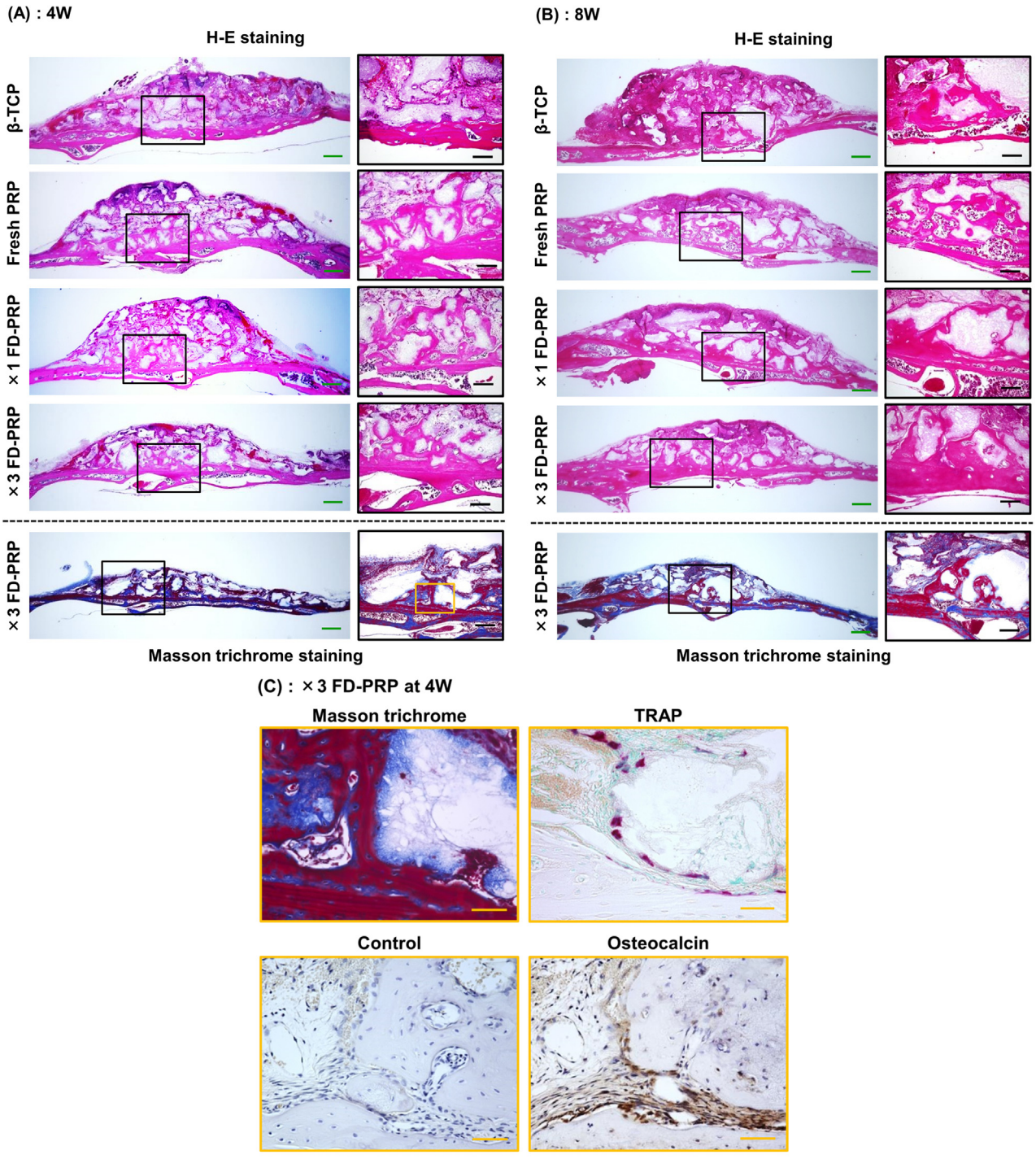

Fig. 5. Histological micrographs of new bone formation at 4 weeks (A) and 8 weeks (B) after transplantation. Immunohistochemical micrographs of new bone formation at 4 weeks after transplantation (C) (For interpretation of the references to color in text, the reader is referred to the web version of this article.).
Sections were stained with hematoxylin and eosin, Masson trichrome, TRAP and Osteocalcin. Scale bars represent 50 μm (yellow), 200 μm (black), 400 μm (green).

that at 8 weeks was 9.7 ± 3.1% in the β-TCP group, 19.4 ± 2.5% in the f-PRP group, 17.4 ± 2.6% in the ×1FD-PRP group, and 22.7 ± 3.3% in the × 3FD-PRP group (Fig. 6A). All PRP groups showed significantly greater new-bone formation than the β-TCP group, and the × 3FD-PRP group was significantly superior to the ×1FD-PRP group at both 4 and 8 weeks. Bone formation at 8 weeks was significantly greater than that at 4 weeks in all groups except the β-TCP group. We also assessed the remaining area of β-TCP granules. As shown in Fig. 6B, the values in each group were around 40% and there were no significant differences among the groups. This indicates that PRP and its derivatives have no effect on the resorption of β-TCP granules.

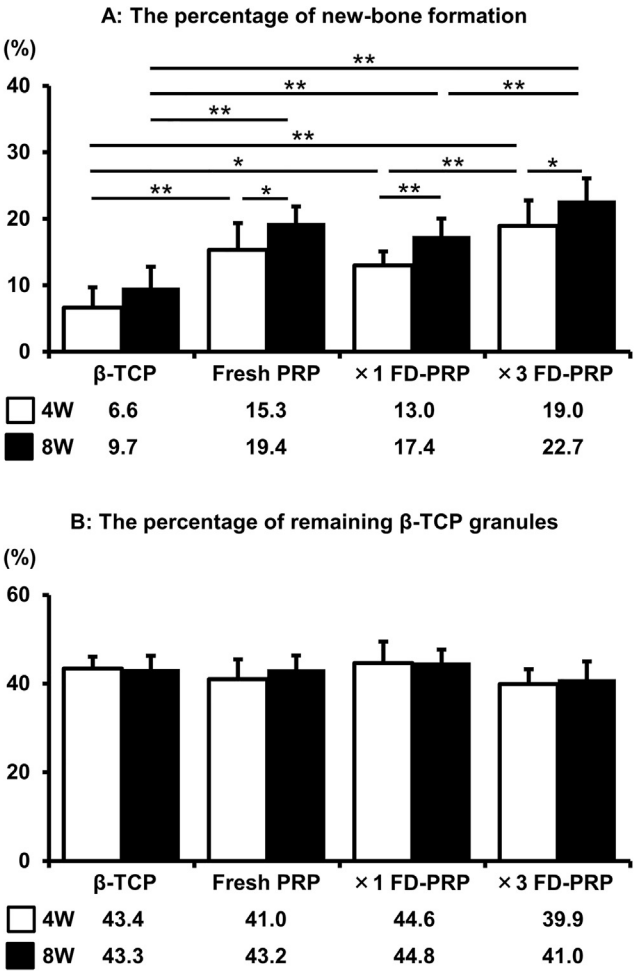

Fig. 6. Percentage of new-bone formation (A). Percentage of remaining β-TCP granules (B).
(A) Rates were calculated by new-bone area and total transplant area. Significant differences were observed between β-TCP and PRPs, ×1FD-PRP and ×3FD-PRP. (B) Rates were calculated by remaining β-TCP granules area and total transplant area. Significant differences were not observed. Data are presented as means ± standard deviation (n = 9). $^{*}p < 0.05$, $^{**}p < 0.01$.

4. Discussion

We demonstrated here that PRP can be preserved by freeze-drying without loss of wound healing properties. Platelets contain various growth factors, such as PDGF, TGF-β and VEGF (Marx, 2001), and these growth factors promote wound healing and tissue regeneration. Concentrated growth factors are an essential element of PRP. Marx suggested that a 4- to 7-fold concentration of platelets in PB, or 100×10^4 platelets/μl, is needed in PRP for tissue healing enhancement (Marx, 2004). In this study, we confirmed that the amount of PDGF-BB and TGF-β1 was preserved after freeze-drying, although VEGF was reduced. PDGF is the fundamental growth factor in tissue regeneration, as it is a mitogen and chemotactic factor for cells of mesenchymal origin (Anitua, Prado, Sánchez, & Orive, 2012). TGF-β1 promotes collagen synthesis in wound tissue (Barnard, Lyons, & Moses, 1990), and also induces osteogenic differentiation of mesenchymal cells in the bone marrow (Zhao, Jiang, & Hantash, 2010). Concentrated PDGF might overcome the influence of VEGF reduction in tissue regeneration, as it is reported that PDGF induces VEGF expression (Carmeliet & Jain, 2011; Xie et al., 2014). We also showed that FD-PRP enhanced *in vivo* bone regeneration similarly to f-PRP. This indicates that the biological activity of growth factors in FD-PRP was maintained.

The fibrin network is also a fundamental element in natural tissue healing response (Anitua et al., 2006), and we demonstrated that gelatinization ability of PRP was maintained after lyophilization. While Marx (2001) reported that platelets secreted approximately 70% of their growth factors within 10 min after activation and almost 100% within an hour, the growth factors in platelets could be released immediately after lyophilization. It is therefore important to prevent growth factors from diffusing. The fibrin network also has a role in sustaining growth factor levels at the injury or surgical site, allowing them to induce the migration and proliferation of tissue cells for early tissue regeneration (Perez et al., 2014). In addition, Pieters, Jerling, and Weisel (2002) demonstrated that there were no differences in fibrin network characteristics between the clots made from freeze-dried and fresh plasma, in accordance with our results.

Storage of PRP with freeze-drying has some advantages in clinical settings, as mentioned above. For example, the surgeon and support staff can avoid confusion at the surgical site, particularly when the staff number is limited, such as in dental office, where the need for PRP is increasing. For patients, repeated use of PRP with only a single blood draw is possible, and chronic pressure ulcer wounds can be readily treated if large amounts of PRP are preserved. Another important advantage of freeze-drying of PRP is that freeze-drying makes it possible to concentrate PRP by reducing the volume of rehydrating fluids. The results of the present study confirmed that higher concentrations of growth factor and greater bone regeneration with three-fold concentrated PRP. This procedure might make it possible to extract the maximum performance of PRP. On the other hand, there are some disadvantages of freeze-drying. Most significantly, the procedure requires a freeze dryer, and the risk of contamination increases with the freeze-drying process. During PRP preparation, technicians should take care to avoid contamination and PRP deterioration via oxidization during storage.

An important limitation of this study is that we assessed the amount of essential growth factors in PRP by ELISA, but we did not directly analyze the biological activity of these growth factors, despite examining *in vivo* activity. We confirmed that the activity of PRP in bone regeneration was maintained, but PRP is known to be effective in healing or regeneration in various types of tissue. Thus, further study is necessary in order to confirm the biological activity of FD-PRP.

In conclusion, the results of this research suggest that f-PRP can be stored without functional loss by freeze-drying and that the concentration of PRP may enhance its efficacy in bone engineering.

Conflict of interest

There is no conflict of interest to declare.

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