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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.
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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.

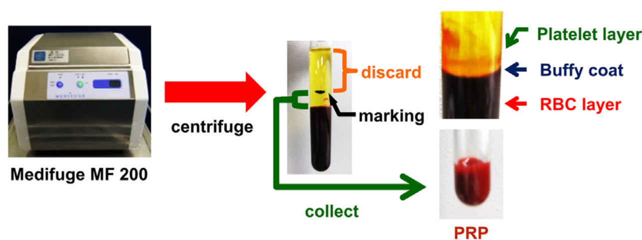

Fig. 1. Preparation of PRP.
(1) Human peripheral blood (PB) was collected and mixed with sodium citrate at a ratio of PB:sodium citrate = 9:1. (2) Blood-sodium citrate mixture was transferred to plastic tubes. (3) Tubes were centrifuged using a blood-phase separator (Medifuge MF 200). (4) Buffy coat 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.

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