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Biological features of freeze-dried platelet-rich plasma for bony union in spinal fusion surgery

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Abstract

Platelet-rich plasma (PRP) has been reported to accelerate bone union in spinal fusion surgery in a clinical situation as well as a rat experimental model. However, one of the major issues with fresh PRP is its short half-life, of only several hours. To overcome this problem, the use of freeze-dried PRP (FD-PRP) and its efficacy in bone union *in vivo* and *in vitro* have been investigated. Using a rat model of spinal posterolateral fusion (PLF), we showed that the ability of FD-PRP to accelerate bone union is comparable to that of fresh PRP and BMP (human recombinant bone morphogenetic protein 2). FD-PRP has the ability to remodel the bone with thinner and tangled trabecular bone with confirmed rigidity. In conclusion, FD-PRP has a bone union ability comparable to that of fresh PRP and BMP and thus, together with its safety and more affordable price, could be an adequate alternative to fresh PRP and BMP.

Key words: Platelet-rich plasma (PRP), freeze-drying, spinal fusion, bone union, platelet-derived growth factor (PDGF)

I . Introduction

During orthopedic surgery for conditions such as fracture or unstable spinal pathologies, stabilization of unstable segments is one the most important aspects of the treatment strategy. Arthrodesis surgery is one of the most viable options to achieve this stability[1-3].

Postoperative bone union can take from a few weeks to 1 year and, occasionally, delayed or inadequate bone union can cause pseudarthrosis, which in turn can lead to adverse outcomes such as chronic instability and pain, resulting in a poor quality of life. Thus, acceleration of postoperative bone union is required for an effective

arthrodesis surgery.

Platelet-rich plasma (PRP) is known to accelerate tissue healing and has already been introduced in the field of plastic surgery, dentistry, and orthopedics [4-8]. Additionally, we have reported on its clinical usefulness in spinal fusion surgery with a 2-3 month earlier bone fusion[9-12]. Basic studies also show that PRP administration in rat models of spinal fusion led to a significantly faster bone union without any complications[9,10]. However, one of the possible barriers to the clinical applications of fresh PRP is its short half-life of merely several hours. To overcome such issues, the use of freeze-dried PRP (FD-PRP) has been suggested and developed[13]. In the current article, we reviewed the biological features of FD-PRP, confirmed by *in vivo* and *in vitro* animal experiments by introducing our associated basic studies.

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Basic experimental conditions and Ethics issues in the related researches

Basic experiments included in the current review article used male Sprague-Dawley rats or partly human blood samples. All of the protocols for experimental procedures were approved by the ethics committees of our institution and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (2010 revision).

Fresh- and FD-PRP preparation and biological properties [14-16]

PRP has been reported to contain a large number of growth factors and to have a role in promoting tissue regeneration [17-19]. In our previous studies, we evaluated and confirmed the biological properties of fresh- and FD-PRP. Fig. 1 shows how fresh PRP and FD-PRP are prepared using human autologous whole blood [20].

The procedure for PRP preparation using animals was as follows: after the donor rats were anesthetized, fresh whole blood was obtained transcardially and centrifuged at 1500 rpm for 10 minutes. Subsequently,

the plasma fraction was separated from the red blood cells and further centrifuged at 3000 rpm for 10 minutes to pellet the platelets as previously described. Pelleted platelets were collected and separated from the supernatant platelet-poor plasma (PPP). PRP was generated by mixing the platelets with 2.5-mL PPP. Test tubes containing aliquoted PRP were then rotated in an ethanol bath at -60°C for membranous freezing (preliminary freezing) and then immediately frozen at -30°C for 4 h. Tubes were then attached to a vacuum freeze-dryer to complete the process and stored for 8 weeks at 4°C to maintain FD-PRP in a powdery state [16]. Prior to the experiments, we resuspended FD-PRP powder in distilled water. To avoid any changes in component concentrations, the weight after dissolving was matched to the weight before freeze-drying. FD-PRP was activated by adding calcium chloride solution and thrombin solution to the sample.

One of the studies showed that FD-PRP contained a significant number of platelets, in fact 4.9 times that of whole blood [16,20]. Furthermore, enzyme-linked immunosorbent assay (ELISA) showed that concentrations of platelet-derived growth factor BB (PDGF-BB) and transforming growth factor $\beta 1$ (TGF- $\beta 1$) were significantly higher in fresh PRP and FD-PRP than in whole blood [16].

II. In vivo evaluation of the efficacy of FD-PRP in bone fusion in animal lumbar posterolateral fusion models [16]

The efficacy in bone fusion was confirmed using animal spinal fusion models [16]. Posterolateral fusion (PLF) was performed in rats (Fig. 2) and then animals were divided into five experimental groups: one sham group and four experimental groups identified by the graft materials: artificial bone group (artificial bone – alone), autologous bone group, FD-PRP (artificial bone + FD-PRP) group, and human recombinant bone morphogenetic protein 2 (BMP) + artificial bone group as a positive control [16]. Refit[®] (Hoya Corporation, Tokyo, Japan) was used as the artificial bone graft substitute, which is also used in clinical spine surgery.

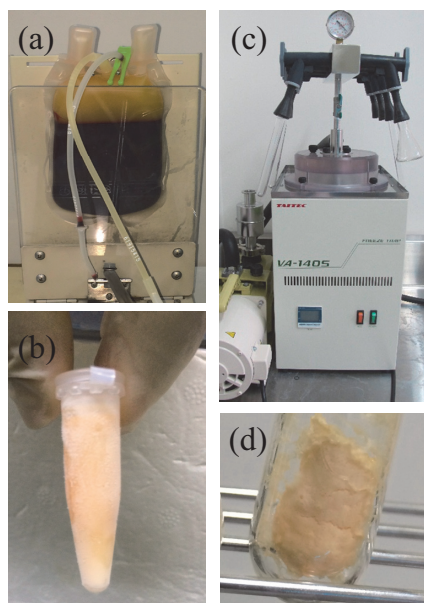


Fig. 1 PRP preparation using human blood [20].

- (a) PRP was sequestered from the supernatant of the centrifuged whole blood.
- (b) PRP frozen at -80°C
- (c, d) Freeze-dried PRP was made into powder form using a freeze dryer

The FD-PRP group used a mixture of 0.5 mL Refit and 0.5 mL gelled-activated FD-PRP (Figs. 2 (b) and 2 (c)). In the BMP group, 0.5 mL Refit and 5 μ g of BMP (Sigma-Aldrich Corporation, St. Louis, MO, USA) were mixed and transplanted [21,22]. In the autologous bone group, rats were implanted with ground spinous

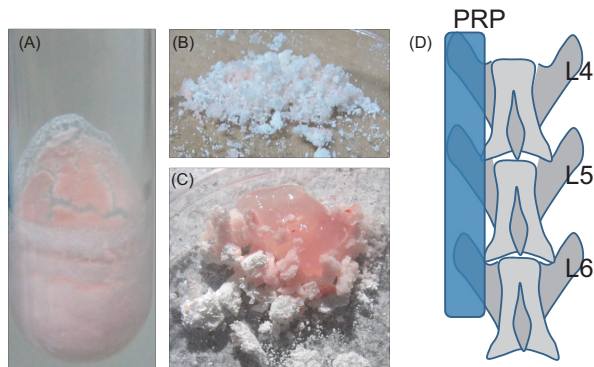


Fig. 2

- FD-PRP preparation [16]. FD-PRP is in powder form and can be dissolved in distilled water to the same concentration of fresh PRP.
- Artificial bone is crushed into powder.
- FD-PRP is mixed with the powdered artificial bone, followed by activation using thrombin and calcium chloride before use.
- Schema of the spine (transplantation site). The graft material was implanted over the transverse processes of L4-L6.

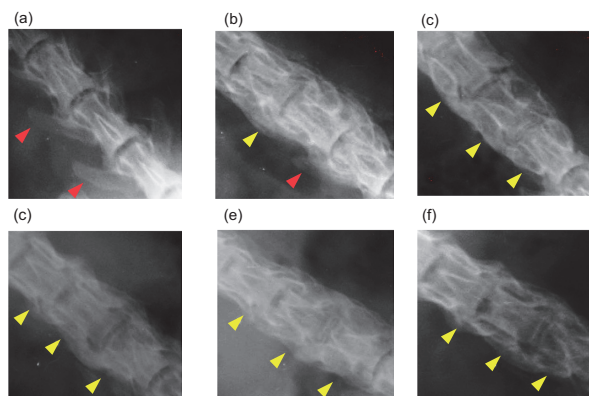


Fig. 3 Anteroposterior radiographs of the spinal segment of the platelet-rich plasma group 4 weeks after surgery [16].

- Sham group
- Artificial bone - alone group
- Autologous bone group
- Artificial bone + FD-PRP group
- Artificial bone + BMP group

FD-PRP and BMP groups showed greater bone formation compared with the autologous bone group.

processes of T10-L2. The posterolateral lumbar spine in each rat was exposed through a midline skin incision, followed by two paramedian fascial incisions to expose the lamina and the transverse processes of L4-L6 (Fig. 2 (d)).

Radiological evaluation showed that FD-PRP combined with artificial bone significantly accelerated bone fusion comparable to BMP (Fig. 3). Histological evaluation showed trabecular bone formation with a more tangled bony structure and many thin branches at the early stage of 4 weeks after surgery with comparable rigidity to autologous bone, indicating that bone fusion induced using FD-PRP showed different structure in bone fusion (Fig. 4). Biomechanical tests showed that the harvested spinal column from the FD-PRP group showed significantly stronger rigidity compared to that of the artificial bone-alone group and slightly inferior, although not significantly, compared to the autologous bone group (Fig. 5). Thus, FD-PRP enables earlier bone union with an increased amount of bone formation and trabecular bone remodeling with considerable rigidity.

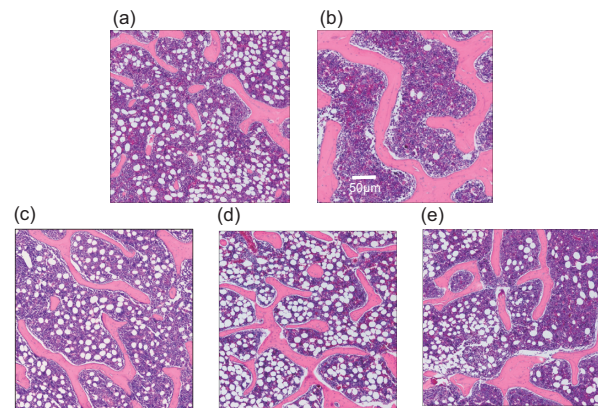


Fig. 4 Histological images of trabecular bone [16].

- Artificial bone - alone group
- Autologous bone group
- Artificial bone + fresh PRP group
- Artificial bone + FD-PRP group
- Artificial bone + BMP group

Trabecular bone formation of the FD-PRP group consisted of a tangled structure with more thin branches, compared to the autologous bone group. Trabecular bone formation is similar to that of the BMP group.

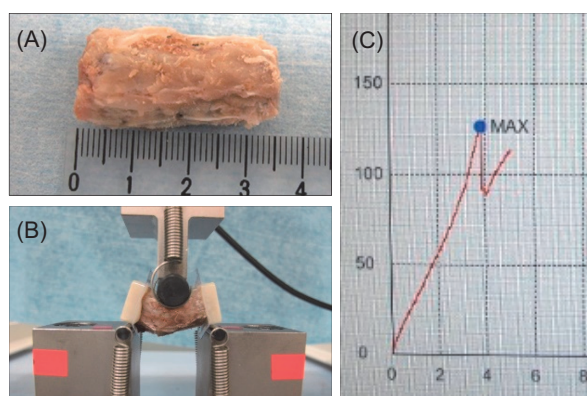


Fig. 5 Mechanical strength evaluation: three-point bending test[16].

- (a) Harvested lumbar spine (L4-L6)
- (b) Three-point bending
- (c) Representative plotting for initial peak pressure measurement.

III. Biological activity of long-term-stored FD-PRP [20]

Previous work shows that the activity of FD-PRP is maintained after an 8-week long-term-storage[20]. PRP prepared from human whole blood was tested to assess its platelet activation features as well as growth factor content after 8-week preparation[20]. Assessment of activated platelets using flow cytometry showed a platelet activation of about 80%, which did not differ between the freeze-drying and pre-activation conditions (Fig. 6) [20]. An antibody array detected growth factors such as PDGF (AA, AB, BB), IGFBP-2, VEGF, TGF- β , EGF and M-CSFR in fresh PRP (Fig. 7 (a)). If stored at room temperature for 8 weeks, almost none of the growth factors were detected (Fig. 7 (b)). However, thinner but significant spots were detected in the PRP frozen after preparation (Fig. 7 (c)). Results showed that in FD-PRP, almost all growth factors were preserved to a considerable level compared with those in fresh PRP (Fig. 7 (d)). These assays showed that storage of PRP at room temperature led to a significant decrease in platelet count while frozen/freeze-drying maintained it for about 8 weeks. Platelet activation properties were also maintained when the activated PRP was stored in frozen or free-dried states. Compared with the frozen PRP, the FD-PRP showed a more significant preservation of growth factors, comparable to that of the fresh one.

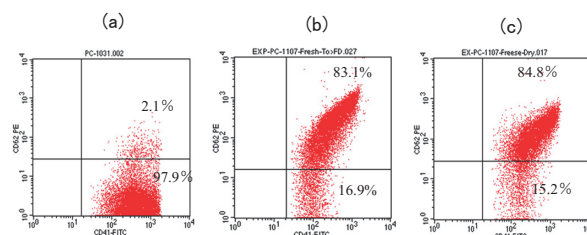


Fig. 6 Assessment of activated platelets using flow cytometry. X axis represents anti-CD41a antibody (platelet marker) and the Y axis represents anti-CD62P antibody (marker for activated platelets) [20].

- (a) Fresh PRP immediately after preparation (before activation).
- (b) Activated fresh PRP
- (a, c) Activated freeze dried PRP (8 weeks after preparation)

The percentages of activated platelets in freeze dried samples after 8 weeks were almost the same as in fresh PRP.

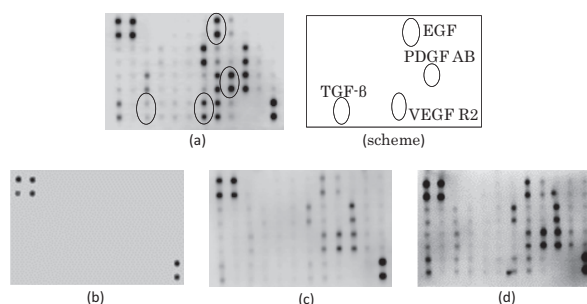


Fig. 7 Growth factor detection using antibody arrays [20].

- (a) Fresh PRP
Growth factors such as PDGF AB, VEGF R2, TGF- β , and EGF were detected as spots. Each detailed location is shown in the scheme.
- (b) PRP in RT stored for 8 weeks
Almost all growth factors disappeared.
- (c) Frozen PRP stored for 8 weeks
Thinner but significant spots were detected.
- (d) Freeze dried PRP stored for 8 weeks
Almost all growth factors were observed as robust spots.

In vitro evaluation using osteoblasts

We also reported that PDGFs in FD-PRP retain their activity, promoting the proliferation of osteoblasts via the PDGFR-mediated ERK signaling pathway. This activity is preserved even after freeze drying and 4-week storage, resulting in two-fold proliferation of osteoblasts [15].

IV. Safety and availability of PRP

The fact that FD-PRP showed comparable bone union ability to that of BMP is important, particularly given that BMP is expensive, and thus unavailable in some countries, as well as the fact that it has associated risks such as tumor formation and excessive inflammation [23-25]. Compared to BMP, PRP is safer because it is made from autologous blood and its clinical use has few significant complications [6]. Considering that growth factors are still preserved in the long-term stored FD-PRP, it should be useful if it is prepared before the surgery, or even if it is made from other origins such as induced pluripotent stem cells.

In conclusion, FD-PRP shows potential for the purpose of accelerating bone union, derived from a preserved and enhanced concentration of bone union-related growth factors by achieving increased and enhanced structure of the remodeled bone. FD-PRP could be of great use in our ageing society, which leads to an increased number of spinal pathology patients requiring spinal fusion surgery.

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Conflict of interest

None.

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