Regenerative Therapy 27 (2024) 200–206

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Comparison of bioactive substances in novel-developed freeze-dried platelet-rich plasma (PRP) and activated normal PRP, and investigation of bioactive substance levels after long-term storage

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ARTICLE INFO

Article history:
Received 8 February 2024
Received in revised form
15 March 2024
Accepted 20 March 2024

Keywords:
Orthobiologics
Platelet rich plasma
Growth factor
Preservation

ABSTRACT

Introduction: In recent years, biotherapy in orthopedics has become widespread, and platelet-rich plasma (PRP) has been readily used to treat sports injuries and osteoarthritis. Production of freeze-dried PRP (PRP-FD) results in PRP that is in powder form, allowing it to be stored for long periods at room temperature. Using this technology, we have developed Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD). However, whether VFD contains sufficient levels of bioactive substances (BS) remains unknown and retains the same levels of BS during long-term storage. In this study, we examined whether VFD contains sufficient amounts of BS and whether they retain these BS levels during long-term storage.

Methods: Peripheral blood was collected from 10 healthy men (mean ± SD: 46.5 ± 15 years old) and various BS, including transforming growth factor β (TGF-β), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), tissue inhibitors of metalloproteinases-1 (TIMP-1), interleukin-1 receptor antagonist (IL-1ra), matrix metallopeptidase-9 (MMP-9), and interleukin-6 (IL-6), were compared between VFD and normal PRP samples, including both leukocyte-rich PRP (LR-PRP) and leukocyte-poor PRP (LP-PRP). VFD was prepared using two rounds of centrifugation. LP-PRP and LR-PRP were activated by freezing and thawing before measurement. To evaluate the effects of long-term storage, the BS of VFD purified from five professional football players was compared between baseline and 1 year after storage.

Results: In terms of the growth factors, the TGF-β and EGF levels were higher in LR-PRP than in VFD and LP-PRP ($p < 0.05$), while the bFGF levels were higher in VFD than in the LR-PRP and LP-PRP groups ($p < 0.01$). In terms of anti-inflammatory cytokines, the TIMP-1 level was lower in VFD than that in the other groups ($p < 0.01$), whereas the IL-1ra levels were higher in VFD than those in LP-PRP ($p < 0.05$) and lower than those in LR-PRP ($p < 0.01$). In terms of inflammatory enzymes and cytokines, the IL-1ra level was higher in VFD than that in LP-PRP ($p < 0.05$) and lower than that in LR-PRP ($p < 0.01$), whereas the IL-6 levels did not differ among the groups. Furthermore, the TGF-β, bFGF, TIMP-1, and IL-1ra levels were 5.61 → 3.38 ($x10^3$ pg/μL), 61.0 → 63.0 (pg/μL), 3.4 → 2.7 ($x10^5$ pg/μL), and 14.9 → 14.5 ($x10^3$ pg/μL) at baseline and 1 year later, respectively. No significant differences in the BS levels were observed between baseline and 1 year after storage.

Conclusions: The VFD samples prepared in this study exhibited higher levels of anti-inflammatory cytokines than LP-PRP and contained growth factor levels similar to LP-PRP and LR-PRP. In addition, the BS levels in VFD samples were maintained after one year of storage. These results suggest that VFD can be

Abbreviations: PRP, platelet-rich plasma; PRP-FD, platelet-rich plasma-freeze-dried; VFD, Valuable Platelet-Derived Factor Concentrate Freeze Dry; LR-PRP, leukocyte-rich platelet-rich plasma; LP-PRP, leukocyte-poor platelet-rich plasma; TGF-β, transforming growth factor-beta; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; TIMP-1, tissue inhibitors of metalloproteinases-1; IL-1ra, interleukin-1 receptor antagonist; MMP-9, matrix metallopeptidase-9; IL-6, interleukin-6; ESSKA, European Society of Sports Traumatology, Knee Surgery and Arthroscopy; ELISA, enzyme-linked immunosorbent assay.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2024.03.021

prepared and stored and may serve as a novel treatment strategy for sports injuries in high-risk groups, such as athletes.

1. Introduction

In recent years, biotherapy has become increasingly widespread in the field of orthopedics. Among these therapies, platelet-rich plasma (PRP) therapy has been applied clinically. When platelets are destroyed or activated, various bioactive substances, such as transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF), are released from platelet α-granules into the plasma, and these cytokines have anabolic and catabolic effects on the tissue healing process. Therefore, PRP therapy has the potential to be a simple, safe (autologous), and minimally invasive technique, and it is currently used to treat sports injuries and osteoarthritis [1–3]. The clinical progress of PRP therapy is currently hampered because PRP formulations, dosing, and treatment protocols are not standardized for clinical use [4]. Furthermore, from an operational perspective, PRP cannot be provided without qualified staff for blood manipulation and medical facilities that comply with regulatory requirements to ensure sterility and safety.

To address some of these challenges, freeze-dried PRP (PRP-FD) has been developed in recent years: PRP is activated, bioactive substances (BS) are released, and the liquid component is processed into powder form. Because PRP-FD is cell-free, it can be stored at room temperature for long periods. Furthermore, PRP-FD is prepared in a central laboratory uniformly, which should theoretically provide more consistent product quality than the PRP processing methods used in individual clinics.

One key disadvantage of PRP-FD is the time required for its preparation, with PRP-FD typically taking several weeks to produce. In other words, PRP-FD cannot be immediately used to treat sports-related injuries, such as ligament injuries or muscle strains. However, preparing PRP-FD in advance for high-risk athletes prone to injury could be performed to mitigate the time constraints associated with PRP-FD preparation. Thus, assessing whether the bioactive substances (BS) in PRP-FD are maintained over an extended period has become necessary. Using this technology, we have developed Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD®) in collaboration with Cell Source Corporation.

At present, it remains unclear whether VFD contains adequate levels of BS and sustains consistent BS levels during prolonged storage. Therefore, this study aimed to investigate whether VFD possesses BS levels comparable to those of active PRP and whether it maintains these amounts during extended storage.

2. Methods

The procedures conducted in this study were approved by the Institutional Review Board of Juntendo University Hospital. Informed consent was obtained from participants. Peripheral blood samples were collected from 10 healthy men who are not particularly sports enthusiasts and do not undergo regular pharmacological treatments for disease (mean ± SD: 46.5 ± 15 years old), and the levels of various BS, including TGF-β, basic fibroblast growth factor (bFGF), EGF, tissue inhibitors of metalloproteinases-1 (TIMP-1), interleukin-1 receptor antagonist (IL-1ra), matrix metallopeptidase-9 (MMP-9), and interleukin-6 (IL-6), were compared between VFD and normal PRP, including both leukocyte-rich PRP (LR-PRP) and leukocyte-poor PRP (LP-PRP). LP-PRP and LR-PRP were activated by freezing and thawing before measurement. The platelet counts of the LP-PRP, LR-PRP, and VFD samples were 3.0-, 3.0-, and 4.1-fold higher than that of the peripheral blood, respectively, and the leukocyte counts of the LP-PRP, LR-PRP, and VFD samples were 0.4-, 2.6-, and 1.1-fold higher, respectively, than that of the peripheral blood (Table 1). To evaluate the effects of long-term storage on the BS (TGF-β, bFGF, TIMP-1, and IL-1ra) levels, VFD was produced using blood samples from another five professional football players, and the BS levels at baseline were compared to those that had been stored for 1 year. VFD powder was packed in opaque bottles and stored in a blackout environment at room temperature setting of 1–30 °C.

2.1. PRP and VFD preparation

To obtain PRP, 40 mL of whole blood was drawn from each volunteer. To obtain LP-PRP, peripheral blood samples were collected using a MyCells® PRP preparation kit and transferred into four sets of MyCells® PRP harvesting kits (11 mL each). After centrifugation at 2000×*g* for 7 min at 21–25 °C, the supernatant was discarded, and the remaining 1.5 mL of plasma from each kit was collected as LP-PRP (6 mL in total) after pipetting out the buffy-coat layer. To obtain LR-PRP, 44 mL of blood was collected in a 50 mL syringe (BD Biosciences, Bedford, MA, USA) pre-filled with 4 mL of 3.8% sodium citrate solution, which served as an anticoagulant. The blood mixture was then equally distributed (11 mL per tube) into four 15 mL centrifuge tubes and centrifuged at 400×*g* for 10 min. After the initial centrifugation, the contents were carefully drawn off 4–5 mm below the buffy-coat layer and subjected to a second centrifugation at 2000×*g* for 3 min. The supernatant was removed after the second centrifugation, and 1.5 mL of plasma from each tube was collected to obtain a total volume of 6 mL LR-PRP. To prepare VFDs, 40 mL of whole blood was extracted from the same cohort of volunteers and shipped to a cell processing facility (CellSource, Tokyo, Japan). The samples were centrifuged at 300×*g* for 10 min. The supernatant plasma, including the buffy coat, was isolated and centrifuged at 1400×*g* for 10 min at room temperature. After the second centrifugation, the supernatant was removed, leaving 0.5 mL of plasma from each tube (total PRP volume of 1.0 mL). The isolated PRP was subjected to a freeze-thaw cycle; initially frozen at −60 °C for 10 min and subsequently thawed at 37 °C for 15 min. The PRP was then diluted with 6.5 mL of

Table 1
Number of white blood cells and platelets in each platelet-rich plasma (PRP) sample.

(Ave±SD)	WBC (x10^3/μL)	Platelet (x10^5/μL)
Whole Blood	3.5 ± 0.7	2.22 ± 4.0
LP-PRP	1.73 ± 0.9	6.68 ± 2.0
LR-PRP	10.9 ± 3.5	6.65 ± 2.0
VFD	4.4 ± 1.6	9.10 ± 1.8

*White Blood Cells (WBC).
*Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD).

Table 2
Methods used to prepare each PRP sample.

	Blood collection	PRP	Centrifugation method
VFD	40 mL	6 mL	Double spin 300×*g* 10 min 1400×*g* 10 min
LP-PRP	44 mL	6 mL	2000×*g* 7 min (Mycells ®)
LR-PRP	44 mL	6 mL	Double spin 400×*g* 10 min 2000×*g* 3 min

*Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD).

phosphate-buffered saline and filtered through a 0.45 μm membrane to eliminate cellular debris. Finally, the product was lyophilized (Table 2). Since LP-PRP, LR-PRP, and VFD were prepared from each of the 10 volunteers, each volunteer underwent a blood collection of 124 mL.

2.2. Measurement of bioactive substance levels

The cytokine and growth factor concentrations present in VFD and normal PRP were assessed using an enzyme-linked immunosorbent assay (ELISA). Quantikine ELISA kits (R&D systems, Minneapolis, MN, USA) were used to measure the concentrations of growth factors and cytokines: TGF-β (DB100B), bFGF (DFB50), EGF (DEG00), TIMP-1 (DTM100), IL-1ra (DRA00B), IL-6 (D6050), and MMP-9 (DMP900). All procedures were performed according to the manufacturer's instructions. Prior to the ELISA, the VFD solution was mixed with 5 ml of sterile saline. LP-PRP and LR-PRP were activated by freezing and thawing before measurement.

2.3. Statistical analysis

Graph Pad Prism (version 9.3.1) software was used to perform analysis of variance (one-way ANOVA), followed by the Tukey-Kramer post-hoc test to compare the differences between three groups. The Wilcoxon matched-pairs signed rank test was used to compare differences between the two groups, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Comparison of growth factors in VFD and normal PRP samples

First, we compared the bioactive substances present in the VFD and normal PRP samples. The normal PRP samples were activated using the freeze-thaw method before being used for measurements. In terms of growth factors, the TGF-β levels were 4.76, 12.6, and 6.10 (x10^4 pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 1). The TGF-β level was significantly higher in LR-PRP than that in VFD and LP-PRP. No significant difference was observed between VFD and LP-PRP. The bFGF levels were 60.5, 37.7, and 80.8 (pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 1). bFGF was significantly higher in VFD than that in LR-PRP, whereas no significant difference was observed between LR-PRP and LP-PRP. The EGF levels were 15.5, 23.8, and 16.7 (x10^2 pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 1). The EGF levels were significantly higher in LR-PRP than that in VFD or LP-PRP, with no significant difference observed between VFD and LP-PRP. These results suggest that the VFD purified in our study contained growth factor levels comparable to those of LR-PRP and LP-PRP.

3.2. Comparison of anti-inflammatory cytokines in VFD and normal PRP samples

The levels of anti-inflammatory cytokines, specifically TIMP-1, were 11.9, 12.4, and 7.9 (x10^4 pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 2). The TIMP-1 levels were significantly lower in the VFD group than that in the LP-PRP and LR-PRP groups, with no significant difference observed between LR-PRP and LP-PRP. The IL-1ra levels were 0.22, 14.4, and 4.59 (x10^3 pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 2). The IL-1ra level of VFD was significantly higher than that in LP-PRP and significantly lower than that

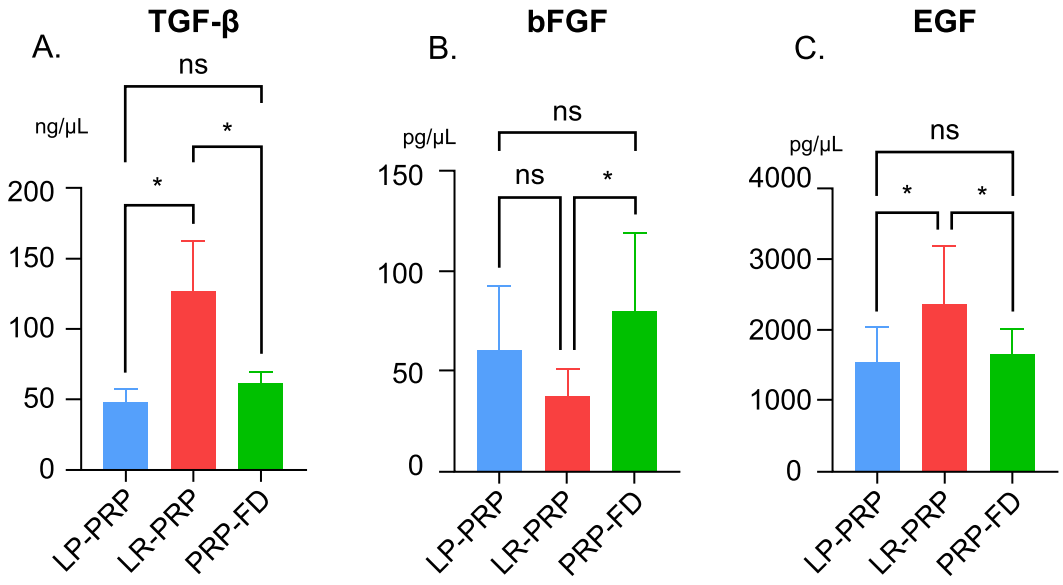

Fig. 1. The level of growth factors in each PRP. Growth factor in leukocyte-poor platelet-rich plasma (LP-PRP), leukocyte-rich platelet-rich plasma (LR-PRP), Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD). (A) Transforming growth factor-beta (TGF-β), (B) basic fibroblast growth factor (bFGF), (C) epidermal growth factor (EGF). Data are presented as the mean ± SD (*$p < 0.05$).

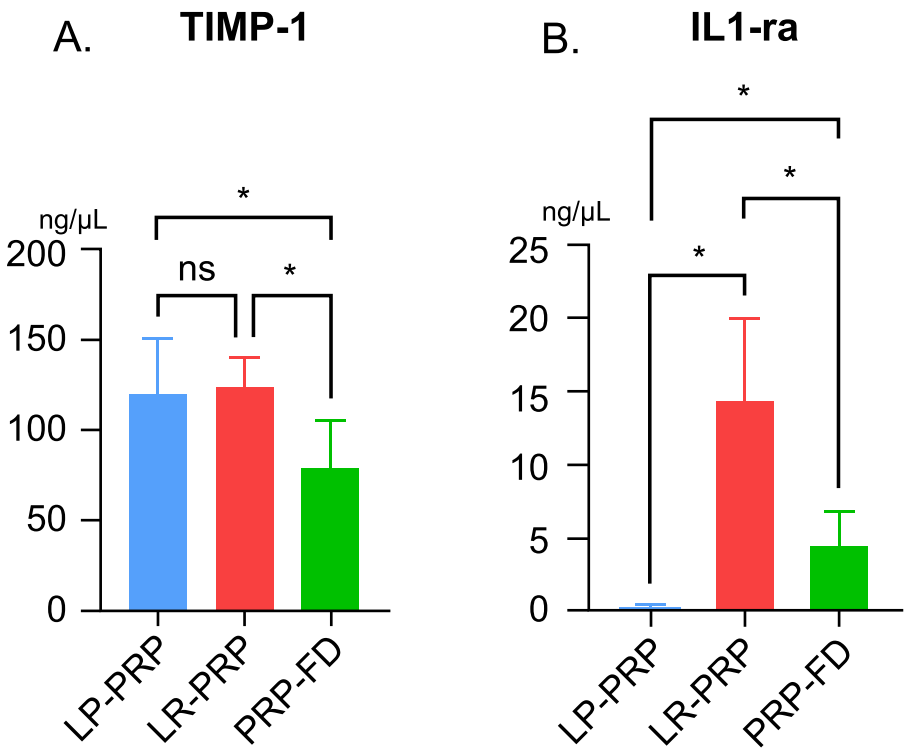

Fig. 2. The level of anti-inflammatory cytokines in each PRP. Anti-inflammatory cytokines in leukocyte-poor platelet-rich plasma (LP-PRP), leukocyte-rich platelet-rich plasma (LR-PRP), Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD). (A) Tissue inhibitors of metalloproteinases-1 (TIMP-1), (B) interleukin-1 receptor antagonist (IL1-ra). Data are presented as the mean ± SD (*p < 0.05, **p < 0.01).

in LR-PRP. These results indicate that VFD contains intermediate quantities of anti-inflammatory cytokines compared with LR-PRP and LP-PRP.

3.3. Comparison of proteolytic enzymes and inflammatory cytokines in VFD and normal PRP samples

In terms of catabolic enzymes, the MMP-9 levels were 0.3, 3.3, and 1.4 (x10^5 pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 3). The MMP-9 level in VFD was significantly higher than that in LP-PRP and significantly lower than that in LR-PRP. In terms of the inflammatory cytokines, the IL-6 level was 6.5, 5.2, and 5.6 (pg/μL) for LP-PRP, LR-PRP, and VFD, respectively (Fig. 3). There were no significant differences in the IL-6 levels among the three groups. These results indicate that VFD contains an intermediate level of catabolic enzymes compared with that in LR-PRP and LP-PRP, and there is no significant difference in the levels of inflammatory cytokines among the groups.

3.4. Evaluation of the maintenance of bioactive substances after long-term VFD storage

Finally, we investigated whether VFD maintains its bioactive substance levels after long-term storage. To evaluate the effects of long-term storage, bioactive substances (TGF-β, bFGF, TIMP-1, IL-1ra) purified from another five professional football players were compared at baseline and after storage for 1 year. The levels of TGF-β, bFGF, TIMP-1, and IL-1ra were 5.61 → 3.38 (x10^4 pg/μL), 61.0 → 63.0 (pg/μL), 3.4 → 2.7 (x10^5 pg/μL), and 14.9 → 14.5 (x10^3 pg/μL) at baseline and 1 year later, respectively (Fig. 4). No significant differences were observed between baseline and following 1 year of storage. These results indicate that VFD retains its bioactive substance levels, even after storage at room temperature for 1 year.

4. Discussion

In this study, we investigated the levels of bioactive substances in the newly developed VFD and compared them with those in normal PRP samples. To the best of our knowledge, we are the first to demonstrate that VFD contains bioactive substance levels that are equivalent to those in “activated” PRP samples. Additionally, we demonstrated that VFD contains more anti-inflammatory cytokines than LP-PRP. This suggests that VFD not only exhibits tissue repair-promoting effects but also anti-inflammatory effects. Furthermore, this study demonstrated that the bioactive substances in VFD samples remain stable and can be preserved for one year. Based on these findings, VFD could be considered a novel, PRP treatment option that requires fewer blood collection procedures and may contribute to the advancement of biotherapy.

PRP therapy is clinically applied as a new, minimally invasive treatment for musculoskeletal disorders. However, several challenges have been identified, including the lack of a standardized method for preparing PRP, the need to maintain cell cultures and processing facilities for preparation, the need for skilled technicians, and the time-consuming nature of blood sampling and processing [5]. To address these limitations, VFD can be prepared at a centralized facility, allowing standardization of the preparation procedure and reducing the burden on individual medical facilities.

Araki et al., reported that lyophilized PRP contains significantly more growth factors than “non-activated” PRP [6]. Generally, PRP is believed to exert its effects through the gradual activation of platelets and the release of bioactive substances after administration into the body, whereas VFD acts as a cytokine therapy that is

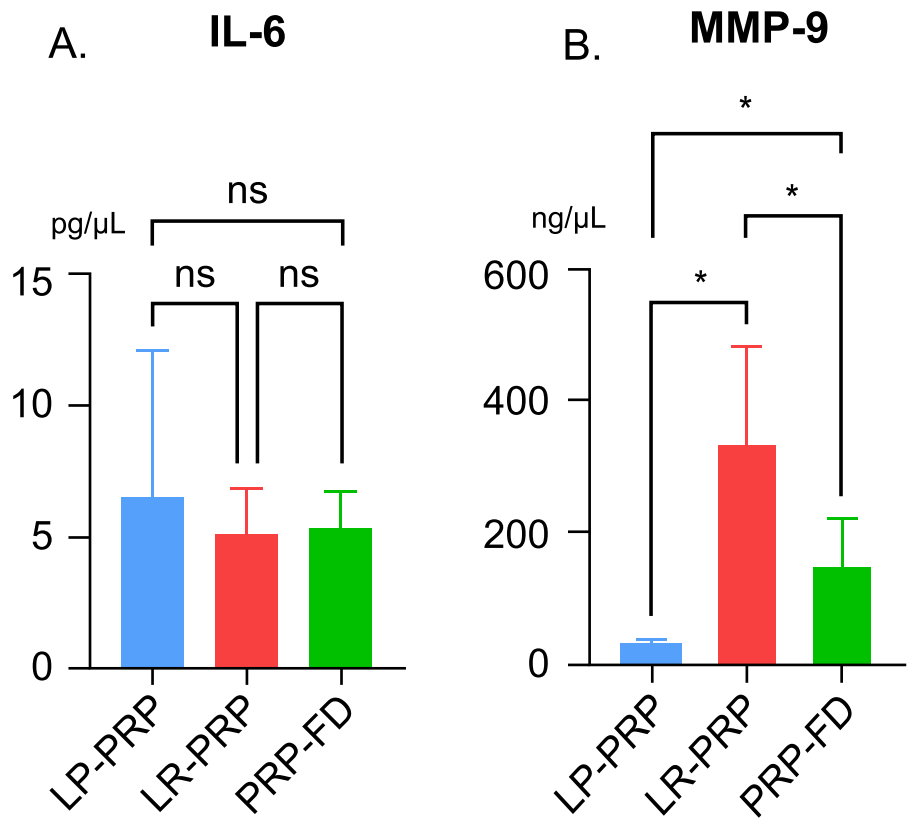

Fig. 3. The level of inflammatory enzymes and cytokines in each PRP. Inflammatory enzyme and cytokine in leukocyte-poor platelet-rich plasma (LP-PRP), leukocyte-rich platelet-rich plasma (LR-PRP), Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD). (A) Matrix metallopeptidase-9 (MMP-9), (B) interleukin-6 (IL-6). Data are presented as the mean ± SD (*p < 0.05).

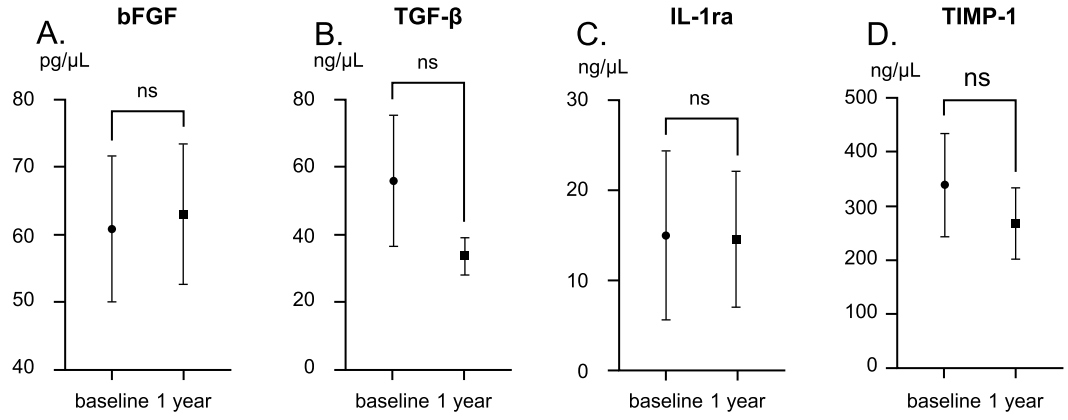

Fig. 4. Comparison of bioactive substances in Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD) at the time of purification and one year after storage. Comparison of bioactive substances immediately after purification and one year after purification in Valuable Platelet-Derived Factor Concentrate Freeze Dry (VFD). (A) Transforming growth factor-beta (TGF-β), (B) basic fibroblast growth factor (bFGF), (C) tissue inhibitors of metalloproteinases-1 (TIMP-1), (D) interleukin-1 receptor antagonist (IL-1ra). Data are presented as the mean ± SD (*p < 0.05).

expected to be effective immediately after administration. In other words, PRP and VFD may differ in terms of the time of onset and duration of the effects. The results of this study demonstrated that VFD contained similar levels of bioactive substances compared with activated PRP. This suggests that VFD may have the same degree of efficacy as normal PRP when used clinically. Based on these findings, the preparation and storage of VFD prior to injury may be a novel treatment option for sports injuries.

In our study, VFD contained higher levels of bFGF compared with normal PRP and similar levels of TGF-β and EGF as LP-PRP. Growth factors, such as bFGF and TGF-β, in PRP are recognized for their crucial roles in cell proliferation, chemotaxis, cell differentiation, and angiogenesis [3]. FGF plays an important role in the proliferation and differentiation of a wide variety of cells and tissues and mediates angiogenesis during the healing process in normal tissue [7]. EGF is also known to play a role in cell

proliferation, endothelial chemotaxis, and angiogenesis [8]. TGF-b, present in humans as three subtypes (TGFb1, TGF-b2, and TGF-b3), stimulates the proliferation of undifferentiated mesenchymal stem cells, chemotaxis of endothelial cells, and angiogenesis [8].

One characteristic of PRP is its dual effect in promoting tissue repair and possessing anti-inflammatory properties. We observed lower levels of TIMP-1 in PRP-FD than the other PRPs, whereas the IL1-ra levels were comparable between LR-PRP and LP-PRP. IL-1ra can suppress IL-1b, a key cytokine involved in inflammation and matrix degradation [9]. These findings suggest that freeze-drying may be advantageous in preserving the stability of proteins in some cases but not in others, suggesting that these possibilities vary for each protein. The VFD samples used in this study were prepared using LR-PRP. Therefore, they may contain anti-inflammatory agents and inflammatory cytokines. The IL-6 levels in our study did not differ significantly between the groups. Furthermore, many other inflammatory cytokines were not measured in this study; therefore, the results obtained may not definitively conclude that VFD has lower levels of inflammatory cytokines. Similar to normal PRP, there are a number of different preparation methods for VFD. Future investigations in this field should focus on comparing the efficacy of VFD prepared using different methods.

PRP is expected to have anabolic effects; however, it also contains catabolic enzymes. In cases where tissue metabolism is expected, PRP with a higher catabolic enzyme content may be preferred. However, PRP containing a large concentration of MMP is considered unsuitable for intra-articular administration because it promotes articular cartilage matrix degradation [10,11]. In this study, the MMP-9 level in VFD was higher than that in LR-PRP and lower than that in LP-PRP (Fig. 3). Considering the low levels of TIMP-1 (Fig. 2), which is the counterpart of MMP [12] in VFD, it is suggested that VFD has a moderate catabolic effect compared with normal PRP. The selection of PRP based on the pathology is crucial; therefore, the characterization of PRP properties, as conducted in this study, is essential [13].

The findings of this study demonstrate that VFD can retain its level of BSs at room temperature for a minimum of one year. Additionally, there are several advantages associated with the clinical application of VFD, including the preservation of PRP bioactivity, facilitation of standardization of platelet count and growth factor levels, ability to adjust doses, avoidance of inter-donor variability, ease of handling and compatibility with other biomaterials, rapid reconstitution through rehydration at the point of care, elimination of the need for venipuncture at the point of care, timely utilization in emergency situations, and convenience in terms of shipping and transport [5]. In cases where multiple administrations are required, the advantage of requiring only a single blood collection is less invasive for patients. However, the blood volume collected, at 50 mL, is not significantly lower compared to other PRP therapies. Therefore, while the freeze-drying procedure reduces the invasiveness in terms of blood collection times, it does not address the issue of blood drawing volume. Moreover, considering the prevalence of sports injuries and similar incidents, there is a concern that individuals at high risk, despite undergoing pre-procedure blood draws, may experience heightened invasiveness if the treatment remains unused for over a year. From these perspectives, it is believed that, similar to previous PRP therapies, tailored utilization according to the specific characteristics of each product will be necessary.

This study has several limitations. First, blood samples were obtained from healthy individuals rather than from patients, which could potentially yield different results when extrapolated to older individuals with chronic conditions, such as knee osteoarthritis. Second, all volunteers were male, and the sex of the patients may have influenced the levels of BSs. Third, long-term storage was only examined in professional football players, making it less applicable to a broader population. Finally, the number of bioactive substances measured was limited, and other factors, such as PDGF, that might contribute to clinical outcomes should be evaluated.

In conclusion, the newly developed VFD contained growth factors equivalent to those of LR-PRP and LP-PRP and more anti-inflammatory cytokines than LP-PRP. These findings suggest that VFD not only possesses tissue repair-promoting effects but also anti-inflammatory effects. Additionally, the bioactive substances in VFD were maintained, even after one year of storage at room temperature. The advantage of creating multiple doses from a single blood sample is apparent, particularly in patients requiring multiple treatments. Moreover, preparing and stocking VFD for high-risk groups, such as athletes prone to injuries, allows for the immediate implementation of PRP therapy in the event of trauma. We believe that these results will contribute to the advancement of biotherapies for musculoskeletal disorders, and plan to further validate the clinical efficacy of VFD in future studies.

Funding

This study was funded by CellSource Co., Ltd. (Tokyo, Japan).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The corresponding author is affiliated with the Department of Sports and Regenerative Medicine, Juntendo University Graduate School of Medicine, which is sponsored by CellSource Co. Ltd., Tokyo, Japan.

Acknowledgments

We thank Yui Miyabayashi for her valuable assistance in this project.

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