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Original Article

Long-term stability of frozen platelet-rich plasma under −80 °C storage condition

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ABSTRACT

Platelet rich plasma (PRP) is increasingly used in various fields of medicine, aiming to regeneration and repair damaged tissues, cells and organs. High concentration of bioactive molecules including growth factors, cytokines and chemokines are the rationale of using PRP. The aim of this study is to analyze the effect of frozen on the levels of growth factors. In our study, PRP samples were isolated from 50 healthy volunteers using the Trima Accel blood cell separator. The concentration of growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF-1) and platelet factor 4 (PF-4) were assessed in fresh PRP and frozen PRP stored at −80 °C for one to twelve months. The study found that count of platelet in all fresh and frozen PRP samples was significantly increased compared to whole blood baseline. There was no significant difference in the concentrations of PDGF-BB, bFGF, VEGF, and PF-4 between fresh and frozen samples. The concentrations of EGF and IGF in Frozen-PRP group were significantly higher than those in Fresh-PRP group. And the storage condition of −80 °C is suitable for PRP, which will not lead to a decrease in growth factors concentration for at least 6 months.

1. Introduction

Platelet rich plasma (PRP) is an autologous blood derived product that contains platelet concentrations 3- to 7- folds higher than those in whole blood. Platelet play an essential role in hemostasis, and the concept of PRP was first introduced by hematologists in the 1970s as a transfusion product in thrombocytopenia [1]. In the 1980s and 1990s, PRP began to be used in surgical procedures such as maxillofacial surgery and plastic surgery to enhance tissue healing and regeneration [2]. The growth factors were released from the alpha granules of platelet, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF-1), and so on. These notable growth factors produce beneficial effects on healing damaged soft and hard tissues by promoting cell differentiation, proliferation, and regeneration. Recently, with the development of regenerative medicine, scientists focused on the use of growth factors and stem cell-based therapy to elevate the speed and quality of healing, extending the PRP application from the traditional dental and maxillofacial field to the treatment of various injuries. Today, PRP as a biological agent has gained popularity and being used in variety fields of medicine, including orthopedics and traumatology (melasma, skin rejuvenation, periorbital hyperpigmentation, hair growth, soft tissues lesions, nonunion, and loss of bone substance following trauma or excision of cysts), ophthalmology (lesions to the corneal epithelium), diabetic foot ulcers, thin endometrium infertility, musculoskeletal disorders and other specialties (even erectile dysfunction, vaginal atrophy, and so on).

Being an autologous product, PRP is an attractive treatment method that risks and side effects are reduced compared to those

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commonly approved traditional commercial drugs. It was reported that PRP was effective and easy to use, but there was no evidence of standardization of PRP preparation, use and storage [3–7]. Platelet count, methods of preparation, activation methods, leukocytes/red blood cells content, and variable therapeutic protocols are the most argued aspects that may causes different effect on diseases [8]. In order to reduce the protocol-dependent variability, we selected a blood apheresis system for PRP preparation, which running automatically and following standard operation procedures. This system limits the possibility of operator-dependent errors and reduces the risk of microbial contamination.

PRP storage is also a hot topic for clinical application. Since the validity period of fresh platelet is only 5 days [9]. But most patients need multiple PRP treatment sessions [10], each course of blood collection for PRP preparation brings inconvenience to patients and difficulties to medical management. Therefore, it is necessary to explore the storage conditions for PRP to maintain its viability and stability. This study aimed to analyze growth factors concentration in both fresh PRP and frozen PRP to determine the feasibility and shelf life of PRP stored at −80 °C. In addition, we also investigated the possible correlation between the platelet count and growth factors concentration.

2. Materials and methods

2.1. Samples preparation

Fifty volunteers were recruited in this study and obtained informed consents. This study protocol was approved by the Ethic Committee of the General Hospital of Central Theater Command. Donors who had infections and used nonsteroidal anti-inflammatory drug within 5 days prior to blood donation, with serious cardiovascular disease and other diseases that unsuitable for blood cell separation, with hemoglobin values < 120 g/L, or with platelet values $< 120 \times 10^9$/L were excluded.

PRP samples were prepared with the Trima Accel apheresis machine (Terumo BCT, USA), which was reported to be an excellent equipment for platelet apheresis. Oral chewable calcium phosphate was administered to the donor to alleviate any citrate-related side effects. According to the manufacturer's instructions, a single-use apheresis kit was installed in the machine. Acid citrate dextrose (ACD) was used as an anticoagulant. The donor's details, including weight, height, gender, blood group, hematocrit, and platelet count were entered into the software of Trima Accel equipment and the total blood volume of the donor was calculated. Platelet concentration and volume was set as 1500×10^9/L and 80 mL, respectively. After completing the program settings of the machine, venipuncture in the arm was performed for blood apheresis. PRP was yield automatically and collected in the platelet storage bag, and it was a leukocyte-poor product. Fresh PRP was stored at 22 °C, while frozen PRP was stored at −80 °C, after separation.

2.2. Evaluation of platelet count and growth factors of PRP

Platelet count of whole blood and PRP (frozen PRP group tested before freezing) were evaluated using fully automated hematology analyzer (Mindray B-3, China). All PRP samples mixed with activator at a ratio of 10:1, which was composed of 0.05 gm/mL calcium gluconate containing 100 U/L bovine thrombin. The mixture incubated at room temperature for 30 min, and centrifugation of 10,000 g to get release substances for growth factors assessment.

The samples were assayed in duplicate, VEGF, EGF, PDGF-BB, bFGF, IGF and PF-4 were measured using commercially Quantikine ELISA kits (Elabscience, China). According to manufacturer's instructions, the ELISA kits and samples were balanced at room temperature before testing. A dilution series of standard solution and samples were added to the 96 wells plate that coated with human growth factor capture antibodies, in a total volume of 100 μL, and plates were incubated at 37 °C for 90 min. After discarding the solution, add 100 μL of diluted biotinylated antibodies to each well and incubated at 37 °C for 60 min. After five wash steps with washing buffer, 100 μL of HRP-conjugated streptavidin was add to each well and incubated at 37 °C for 30 min. Discard the solution, repeat the washing steps. Add 90 μL of TMB substrate solution and incubated at 37 °C for 15 min. The reaction was terminated by adding 50 μL of stop solution, and OD_{450} was measured immediately. The concentrations of the analytes were expressed in pg/mL.

2.3. Statistical analysis

The data was presented as mean ± standard deviation (SD). And analyzed using SPSS software (SPSS Inc. version 26, Chicago, USA). The normal distribution and homogeneity of variance were measured. When we found that the data did not follow the normal distribution of Shapiro Wilk test or homogeneity of variance test was uneven using Levene test, then the difference between groups were analyzed using the Kruskal–Wallis test (nonparametric one-way analysis of variance). Bonferroni post hoc comparisons were performed when the Kruskal–Wallis was significant. In addition, Spearman correlation analysis was performed between the platelet count and growth factors concentration. The Significant difference was defined as $p < 0.05$.

3. Results

3.1. Preparation of platelet-rich plasma

In this study, all products we prepared with Trima Accel machine were leukocyte-poor PRP. The leukocyte concentration of PRP exceeds the detection range of the hematology analyzer. According to previous study, the mean residual leukocyte content was observed to be 1.3×10^6 per unit by Trima Accel [11]. The mean platelet count of whole blood was $245 \pm 45.5 \times 10^9$/L. The platelet count in fresh PRP and frozen PRP group significantly increased to $865.1 \pm 213.6 \times 10^9$/L and $945 \pm 317.92 \times 10^9$/L, respectively, which was 3.5–3.9 times higher than in whole blood (Table 1).

The concentrations of PDGF-BB, PF-4, bFGF, EGF and VEGF in the Fresh-PRP and Frozen-PRP groups were significantly higher than those in the Whole-blood group (Table 1). The IGF concentration in Frozen-PRP group was significantly higher than that in the Whole blood group, but there was no difference between the Fresh-PRP group and the Whole-blood group (Table 1). There was no significant difference in the concentrations of PDGF-BB, PF-4, bFGF and VEGF between Fresh-PRP group and Frozen-PRP group (Table 1). The concentrations of EGF and IGF in Frozen-PRP group were significantly higher than those in Fresh-PRP group (Table 1).

3.2. The concentration of growth factors at different storage stages

To comparing the variation of growth factors concentration with storage time, we divided 50 PRP samples that from 50 volunteers into 5 groups based on storage periods of 0, 0–2, 2–4, 4–6, 6–12 months. 0 represents the fresh PRP samples that storage at 22 °C for 0–5 days, and >0 represents frozen PRP samples. The results were shown in Table 2. The count of platelet in the 5 groups no significant difference was found (Fig. 1). And there was no significant difference in the concentration of PDGF-BB, PF-4, bFGF and VEGF between the 5 groups (Fig. 1). The concentration of EGF in the 0–2, 2–4, and 6–12 months groups was significantly higher than that in

Table 1
The level of growth factors in whole blood and in fresh and frozen PRP (Mean ± SD).

Growth factors	Whole blood	Fresh PRP	Frozen PRP
PLT ($\times 10^9$/L)	245.00 ± 45.50	865.1 ± 213.56[a]	945.06 ± 317.92[b]
PDGF.BB (pg/mL)	190.24 ± 58.16	400.59 ± 56.67[a]	413.12 ± 54.15[b]
PF-4 (pg/mL)	13,267.88 ± 1697.79	24,314.10 ± 2291.37[a]	23,730.47 ± 2537.39[b]
bFGF (pg/mL)	0.00 ± 0.00	407.99 ± 110.80[a]	338.15 ± 199.69[b]
EGF (pg/mL)	0.00 ± 0.00	163.24 ± 12.98[a]	188.11 ± 33.79[b,c]
VEGF (pg/mL)	0.00 ± 0.00	292.19 ± 176.67[a]	484.74 ± 514.76[b]
IGF (pg/mL)	0.00 ± 0.00	6327.33 ± 6729.83	25,602.1 ± 2800.41[b,c]

[a] There was statistically significant difference between Whole blood and Fresh PRP group.
[b] There was statistically significant difference between Whole Blood and Frozen PRP group.
[c] There was statistically significant difference between Fresh PRP and Frozen PRP group.

Table 2
The concentration of platelet and growth factors at different storage stages (Mean ± SD).

Time (month)	n	PTL ($\times 10^9$/L)	PDGF (pg/mL)	PF4 (pg/mL)	EGF (pg/mL)	VEGF (pg/mL)	bFGF (pg/mL)	IGF (pg/mL)
X = 0	10	865.10 ± 213.55	400.59 ± 56.67	24,314.10 ± 2291.37	163.24 ± 12.98	292.19 ± 176.67	407.99 ± 110.80	6327.33 ± 6729.83
0 < X < 2	10	981.14 ± 281.4	407.26 ± 49.70	22,654.86 ± 1787.40	187.48 ± 27.83	479.63 ± 458.61	394.29 ± 169.67	22,540.64 ± 15,932.48
2 ≤ X < 4	10	762.22 ± 180.65	420.28 ± 69.68	24,660.89 ± 1797.10	193.62 ± 37.36	519.11 ± 544.05	399.32 ± 202.44	31,309.87 ± 19,022.09
4 ≤ X < 6	10	898.25 ± 530.45	388.60 ± 14.80	23,245.25 ± 2571.89	164.32 ± 41.73	128.42 ± 176.51	411.62 ± 239.85	31,369.61 ± 18,685.98
6 ≤ X < 12	10	1134.71 ± 321.13	429.66 ± 57.58	24,962.71 ± 3866.40	195.87 ± 37.09	654.40 ± 686.56	238.20 ± 85.87	21,091.13 ± 11,950.95

the 0-month group (Fig. 1). The concentration of IGF in the 0–2, 2–4, 4–6, and 6–12 months groups were significantly higher than that in the 0-month group (Fig. 1).

3.3. The correlation between platelet count and the concentration of growth factors

The correlation analysis was performed between platelet count and growth factors concentration. The result showed that there was no correlation between the platelet count and the concentrations of PDGF-BB ($r = 0.020$, $P = 0.898$), PF-4 ($r = 0.091$, $P = 0.555$), EGF ($r = -0.035$, $P = 0.822$), VEGF ($r = 0.217$, $P = 0.130$), bFGF ($r = 0.073$, $P = 0.614$), and IGF ($r = 0.006$, $P = 0.967$) growth factors.

4. Discussion

During the last decades, platelet rich plasma (PRP) has been applicated for regenerative purposes in fields such as oral surgery, dermatology, orthopedics, ophthalmology, obstetrics, and so on. The rationale of using PRP is that it contains a lot of bioactive growth factors and molecules, which are released from α-granules of platelet. PRP can be used immediately after preparation in clinical practice, however, increasing number of studies showed multiple application of PRP were required in most diseases [10,12–15]. Therefore, the storage of PRP was needed for the purpose of repeating applications during the treatment cycle. And appropriate storage conditions and duration of PRP are important to optimize its effects on desired clinical outcomes.

This study aims to test whether PRP can be stored as frozen. Thus, we detected the values of PDGF-BB, VEGF, bFGF, EGF, IGF and PF-4 in fresh and frozen PRP. The results showed that, except for IGF, the concentration of growth factors (including PDGF-BB, PF-4, bFGF, EGF, and VEGF) in PRP were significantly higher than that in whole blood. There was no significant difference in the concentrations of PDGF-BB, PF-4, bFGF, and VEGF between the fresh PRP group and the frozen PRP group. Our results are consistent with some previous studies. Roffi's study indicated that no significant difference were detected in VEGF levels between PRP that frozen for 1 h or 7 days and fresh ones [16]. And Hosny's research showed that there was no significant change in VEGF concentration in PRP that frozen at −40 °C for one or three weeks compared to fresh PRP [8]. Interestingly, the concentrations of EGF and IGF in the frozen PRP group were even significantly higher than those in the fresh PRP group. These results are similar to previous studies. Alejandra E. Rodriguez found that PRP can be frozen at −20 °C for 3 months and the concentration of PDGF-BB and EGF in frozen PRP was significantly higher than in fresh one [17]. The phenomenon probably due to the full release of all EGF and IGF growth factors after freezing. And platelet count no significant difference was observed in our study, but other studies found that platelet count decreased significantly in PRP stored in the deep-freezer [8,18]. The reason for this difference may be that we counted the concentration of platelet before freezing, while other studies calculated the platelet concentration after freeze-thaw which may lead to cell lysis during the thawing process.

In order to analyze the effects of −80 °C storage duration on growth factors concentration in PRP. We divided PRP samples to 5 groups: X = 0, 0 < X < 2, 2 ≤ X < 4, 4 ≤ X < 6, 6 ≤ X < 12. X = 0 means that fresh PRP samples were stored at 22 °C for 0–5 days, while the other groups were stored at −80 °C for 0–2, 2–4, 4–6, 6–12 months before detection. Compared with the X = 0 group, there was no significant difference in the concentrations of PDGF-BB, PF-4, bFGF and VEGF among the other groups. This indicated that most of growth factors can maintain stability when PRP was stored at −80 °C even for 6–12 months. The concentration of EGF in 0–2, 2–4, 6–12 months groups were significantly higher than that in 0-month group. The concentration of IGF in 0–2, 2–4, 4–6, 6–12 months groups were significantly higher than 0-month group. These results indicated that the storage condition of −80 °C was suitable for PRP, which would not lead to a decrease in growth factors concentration for at least 6–12 months. And our results are consistent with previous studies. Other authors have found that growth factors exhibits biostability after being stored at −20 °C for 3-months, 6 months and 12 months, respectively [19–21].

In this study, PRP was prepared using Trima Accel apheresis equipment, the platelet count of PRP samples ranged from 407 × 10^9/L to 1665 × 10^9/L. We did not identify a significant correlation between platelet count and growth factors concentration in PRP. Previous studies have also reported similar observations that growth factors concentration did not correlate with variation in platelet count [8,17,22,23]. The result showed that platelet count may not be an appropriate indicator to predict biological activity of PRP, and growth factors concentration may be mainly influenced by

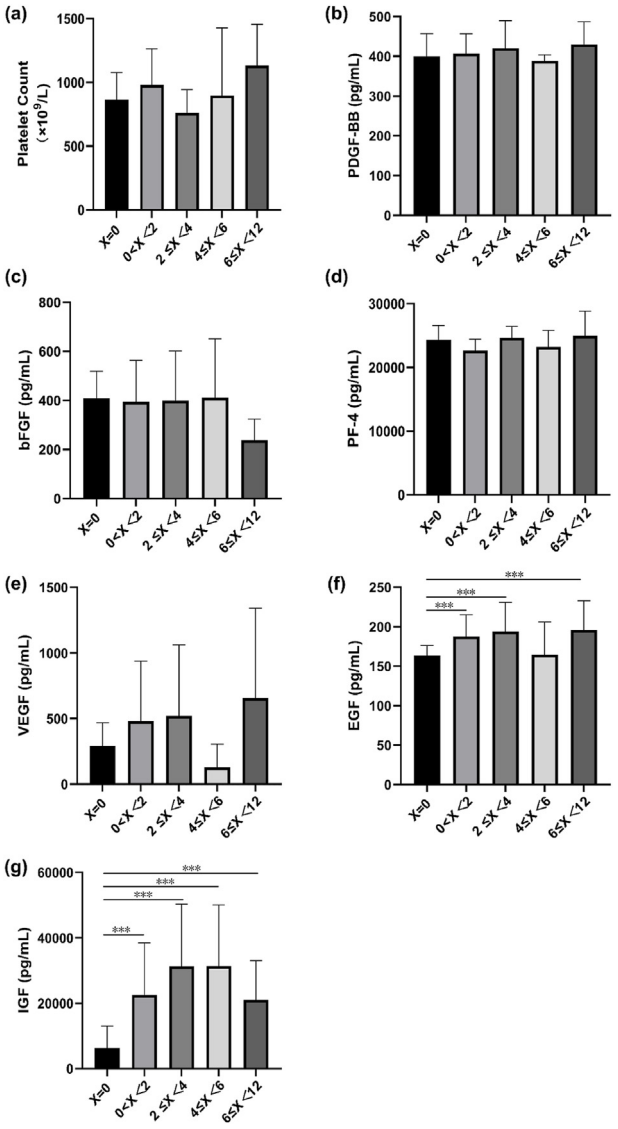

Fig. 1. Analysis of changes in various indicator levels of PRP at different storage stages. (a) Changes in platelet count of platelet-rich plasma samples at different storage stages. (b) Growth factor release profile of PDGF-BB at different storage stages. (c) Growth factor release profile of bFGF at different storage stages. (d) Growth factor release profile of PF-4 at different storage stages. (e) Growth factor release profile of VEGF at different storage stages. (f) Growth factor release profile of EGF at different storage stages. (g) Growth factor release profile of IGF at different storage stages. ***$P < 0.001$.

inter-individual variability. And several limitations should be considered in this study. Firstly, all PRP samples were activated by calcium gluconate and bovine thrombin, which may difference with other studies. Secondly, this is an in vitro study, the clinical significances of growth factors released from fresh and frozen PRP were difficult to confirm. Thirdly, the sample size in this study is small. Therefore, a further study is needed.

5. Conclusions

The concentration of growth factors or bioactive molecules in PRP would not reduce after freezing at −80 °C. And the storage duration can reach up to at least 6 months. This study suggests that frozen can serve as a storage method, thus simplify the management of patients who require multiple PRP injections. Besides, PRP preparation by using a blood apheresis machine can produces 50–200 mL products at once, which can be combined with subsequent packaging and frozen storage, making it more convenient and economical for patients.

Author contributions

G.X. and W.L. conceived the study, designed experimental procedures, and contributed to manuscript writing. Y.L. performed the experimental assays. L.L., T.L., M.D. and J.D. collected and analyzed the date. Y.Q. performed conceptualization and project administration. All authors have reviewed and agreed to the published version of the manuscript.

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Institutional review board statement

This study protocol was approved by the Ethic Committee of the General Hospital of Central Theater Command ([2022]019-01).

Informed consent statement

No image or video has been included in the manuscript. Samples were anonymized before being provided for research. And informed consent was obtained from all subjects involved in the study.

Data availability statement

The data presented in this study are contained within the article. Individual values are available from the corresponding authors upon reasonable request.

Declaration of competing interest

The authors declare that no conflict of interest exists.

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