【Original article】Freezing procedure without thrombin activation to retain and store growth factors from platelet concentrates

Running title: Growth factors retained after frozen storage of platelet concentrates

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ABSTRACT

Background/purpose: The aim of this study was to examine a new procedure by simply freezing without the need of thrombin to retain and store growth factors from platelet concentrates up to 6 months.

Materials and Methods: After re-suspended with Tyrode's solution, the platelet suspensions were divided into four groups. In negative control group, the platelet supernatants were collected after centrifugation and then frozen at -70℃ until testing. In freezing method group, the platelet suspensions were directly frozen and stored at -70℃; the centrifugation was postponed until prior testing. In conventional method (thrombin) and post-thrombin groups, the procedures were the same as previous two groups, respectively, except thrombin was added prior centrifugations. The concentrations of PDGF-AB and TGF-β1 were assayed by ELISA at the month-0 (the day of starting the experiment), -1, -2, and -4 in first three groups (experiment one), and in all four groups at the month-6 (experiment two).

Results: Similar PDGF-AB and TGF-β1 concentrations were recorded in freezing method and conventional method groups up to 4-month storage. After 6-month storage, similar concentrations were recorded among freezing method, conventional method, and post- thrombin groups.

Conclusion: Because of similar concentrations of growth factors in all groups, except negative control group, after storing up to 6 months, we suggest firstly the concentration of growth factors of platelet concentrates could be storied for at least 6 months, and secondly the growth factors of platelet concentrates can be obtained *via* directly frozen, without the necessity of thrombin activation.

Key words：platelets, growth factors, frozen, thrombin, storage.

INTRODUCTION

The application of autologous platelet concentrates (platelet-rich-plasma) has been introduced in various dental procedures,[1-4] including periodontal regenerations [5, 6] and dental implantations,[7, 8] because of its abundance of growth factors (GFs). The exact effects of platelet concentrates on the bony regeneration are still under investigations. Recently controlled clinical studies have shown that the platelet concentrates may have favorable outcomes in the periodontal infra-bony defect [9, 10] and in the pocket distal to the mandibular second molar after extraction of third molar.[2] Animal studies have demonstrated that greater volume of bone formation around peri-implant when platelet concentrates was applied.[11, 12]

A critical procedure during platelet concentrates preparation is to release the GFs from the activated platelets. A fresh blood sample with thrombin treatment is nowadays commonly recommended to achieve this optimal results.[1, 3, 13, 14] In this study, a more convenient method was tested for storing the GFs from platelet concentrates by frozen up to 6 months without the need of thrombin stimulation. It not only allows in advance and simpler preparation of fresh platelet concentrates to avoid the extra work on the surgery day, but also can save un-used blood for later.

MATERIALS AND METHODS

Preparation and grouping of the platelet concentrates:

The platelet concentrates (25mL/bag) were obtained from 250mL whole blood of healthy volunteer donors in Taipei Blood Donation Center, Taipei, Taiwan. After centrifugation for 10 minutes (2000rpm at 25℃), the platelet suspensions were gently obtained by re-suspended with 25c.c. Tyrode's solution (Sigma® , St. Louis, MO, USA) as our previous studies.[15, 16] The numbers of platelet in the suspensions were measured with the Z1^{TM} Series COULTER COUNTER® Cell and Particle Counter (Beckman Coulter, Inc. Fullerton, CA, USA), and then adjusted to $2x10^5$ and $3x10^5$platelets/μl. Twenty specimens of platelet suspensions with a 1c.c. per eppendorf tube were prepared from each platelets bag/volunteer.

There were four groups of platelet sample prepared in the study ~~(Table 1)~~. The negative control group, which was supposed to have no platelet, was prepared via centrifugation at 7,500prm for 5 minutes and the supernatants were immediately frozen at -70℃ for storage until defrosting for Enzyme-Linked Immunosorbent Assay (ELISA) analysis. The second group, the freezing method group, had the un-centrifuged platelet suspensions being straightway frozen at -70℃ for storage, while the centrifugation was deferred until defrosting was finished before testing. The method in third/conventional method (thrombin) group was the same as negative control group, except a thrombin treatment (5 units/ml, Calbiochem, La Jolla, CA, USA) was received prior centrifugation (300g for 15 minutes) In the fourth group, post-thrombin group, the method was the same as freezing method group; except frozen storage of platelet suspensions was 6 months and a thrombin treatment was received prior centrifugation. The concentrations of GFs in samples of each group were determined.

The present study was composed of two parts. In the experiment part one, the GFs

in negative control, freezing method, and conventional method groups were measured at the examining intervals of month-0(the day of starting the experiment), -1, -2 and -4; while in the experiment part two, the concentrations of GFs in all four groups (negative control, freezing method, conventional method, and post-thrombin groups) were evaluated after 6-month storage.

Determination of growth factors (PDGF and TGF-β1):

Both PDGF-AB and TGF-β1 were assayed using the techniques described in the previous studies.[16, 17] In the PDGF-AB detection, commercially available ELISA kits (R & D System, Minneapolis, MN, USA) and a 1:10 diluted supernatants were used according to the instructions of the manufacturer. The enzyme reaction was measured using an automatic micro-ELISA photometer (DYNEX Technologies, Inc. Chantilly, Virginia, USA) at 450 nm with the minimal detactable dose of 31.25 pg/ml. Following the above procedure, the TGF-β1 ELISA kits (R & D System, Minneapolis, MN, USA) and undiluted supernatants were used to measure the TGF-β1 concentration with the minimal detectable doses for TGF-β1 of 7.0 pg/ml. According to the instructions of manufacturer, the activities of ELISA kits could only be kept for 4 months for the accuracy of the GFs detection. Consequently, a new kit was utilized for the second part of the experiment.

Statistical analysis:

In the experiment part one, the relationships among the concentrations of released GFs, PDGF-AB and TGF-β1 (dependent variables), the method used to prepared platelet concentrates, i.e. by frozen or thrombin activation (between-subjects factor), and the storage durations, i.e. 1, 2 or 4 months (within-subject factor), were evaluated via the repeated measuring analysis of variance. One-way ANOVA was applied to examine the GFs releasing from three different platelet concentrates preparation groups at each

observation interval in the experiment part one and from four different platelet concentrates preparation groups at the end of 6 months in the experiment part two. The Duncan test was applied for post-hoc analysis, and $p < 0.05$ was selected as the significance.

RESULTS

In the experiment part one, there was a significant difference in concentrations of released GFs (PDGF-AB and TGF-β1) among three treatment groups (p = 0.001 and 0.002 for PDGF-AB and TGF-β1, respectively), but not among the examining time-points ~~(Table 2)~~. Significantly less concentrations of PDGF-AB and TGF-β1 were demonstrated in the negative control group than those in the freezing method and conventional method groups at the examining time intervals of 0, 1, 2 and 4 months (Figure 1).

In the experiment part two, in which the post-thrombin group was included, significantly less concentrations of PDGF-AB and TGF-β1 were observed in the negative control group if compared with any of three other groups at the end of 6-month storage (Figure 2); while there were no significant differences in concentration of PDGF-AB or TGF-β1 among freezing method, conventional method, and post-thrombin groups.

DISCUSSION

In the present study, a freezing method depending mainly on freezing was examined for storing the GFs released from platelet concentrates up to 6 months. Our results showed that the concentrations of GFs from the platelets can be preserved after the storage up to 6 months (Figure 2). The data further demonstrated that frozen, a simple physical stimulation could easily activate GFs release from platelets with similar concentration of GFs activated by a thrombin treatment (Figure 1 and 2). Up to date, a thrombin activation to achieve the GFs release from freshly prepared platelet concentrates still commonly recommended. However, the use of thrombin may associate with the formation of antibodies against clotting factors and result in life-threatening coagulopathies.[18] Studies have also shown that thrombin may play a role as a stimulator of bone resorption.[19, 20] Several biomaterials have recently been tested in order to substitute the thrombin.[21, 22] In our laboratory, chitosan (a poly sugar for extra-skeleton of shells) had been tested to replace the thrombin for PRP activation.[16] In this study, the GFs released from platelets were easily achieved by a new method: “frozen”.

A gradual activation of the platelets during the storage of platelet concentrates has been reported in the modern blood bank.[23, 24] To preserve platelet viability and to provide an optimal hemostatic effectiveness, an important drawback of storage of platelet concentrates at low temperature is the risk of spontaneous platelets activation, so called cold-induced activation, has also been observed.[25-27] The cold-induced activation might partly explain the GFs releasing after -70℃ storage of platelets in the present study. Similar procedures in recording the releasing GFs from platelets after frozen storage, without thrombin treatment, have been reported in other studies.[28-30] Nevertheless, this is the first time that the concentrations of GFs released from platelet concentrates up to 6-month storage without the need of thrombin stimulation being

observed.

It has been reported that the platelet counts significantly differed according to donor blood, blood bank platelet concentrates preparation, and self-concentrated platelet concentrates.[31] The efficiency of platelet collection is a main influencing factor, because the end product with a higher platelet count produces higher GF levels.[28, 32] The content of GFs in platelet concentrates is also depending on the system used for the preparation of platelet concentrates. [28] In the present study, the platelets were obtained from a blood bank. Constant number (3×10^5) of platelets from the same individual was used for each group and at each observation interval to avoid having large variations on levels of GFs from the individuals.[31] Although, it was observed that varied levels of GFs were released individually (data not shown), the favorable possibility of successful storage of the released GFs was obviously observed.

Platelet concentrates has been applied in the surgical procedures to deliver GFs in high concentration to the sites requiring osseous regenerations.[1, 11-13] Recent studies have shown that platelet concentrates promotes the cell proliferation of bone cells, osteoblasts, and bone marrow stromal cells;[33-36] the viability and proliferation of alveolar bone cells, however, seems to be suppressed in high platelet concentrates concentrations.[37, 38] The inconsistency of the effect of the platelet count in platelet concentrates on bone regeneration has been demonstrated in rabbits.[39] The healing rate in bony defects on rabbits was higher in combination treatments of platelet concentrates, stromal cells and bone allografts, while lower values were achieved with platelet concentrates treatment alone.[40] No significant increase in bone formation was seen in the addition of platelet concentrates to autogenous bone in cranial bony defects when compared with grafted with autogenous bone or platelet concentrates alone.[41] An *in vitro* study has shown that platelet concentrates produces a number of potent effects on periodontal ligament cells, but its effects do not solely reflect simple combination of

its major known GFs.[42]

In conclusion, the concentrations of GFs released in the PRP with or without thrombin treatment were compared after frozen storage in this study. Our results demonstrated that fewer concentrations in PDGF and TGF were in the negative control group than those in the freezing method, conventional method, and post-thrombin groups after up to 6 months storage, while similar levels in GFs were detected in the later three groups. We, therefore, suggest that firstly the concentration of GFs of platelet concentrates could be retained after storage at -70℃ for at least 6 months, and secondly the GFs of platelet concentrates can be obtained *via* directly frozen preparation, regardless of thrombin activation.

ACKNOWLEDGMENT

This study was supported by the research grant from Tri-Service General Hospital, Taipei, Taiwan, R.O.C. (Grants No. TSGH-96-021).

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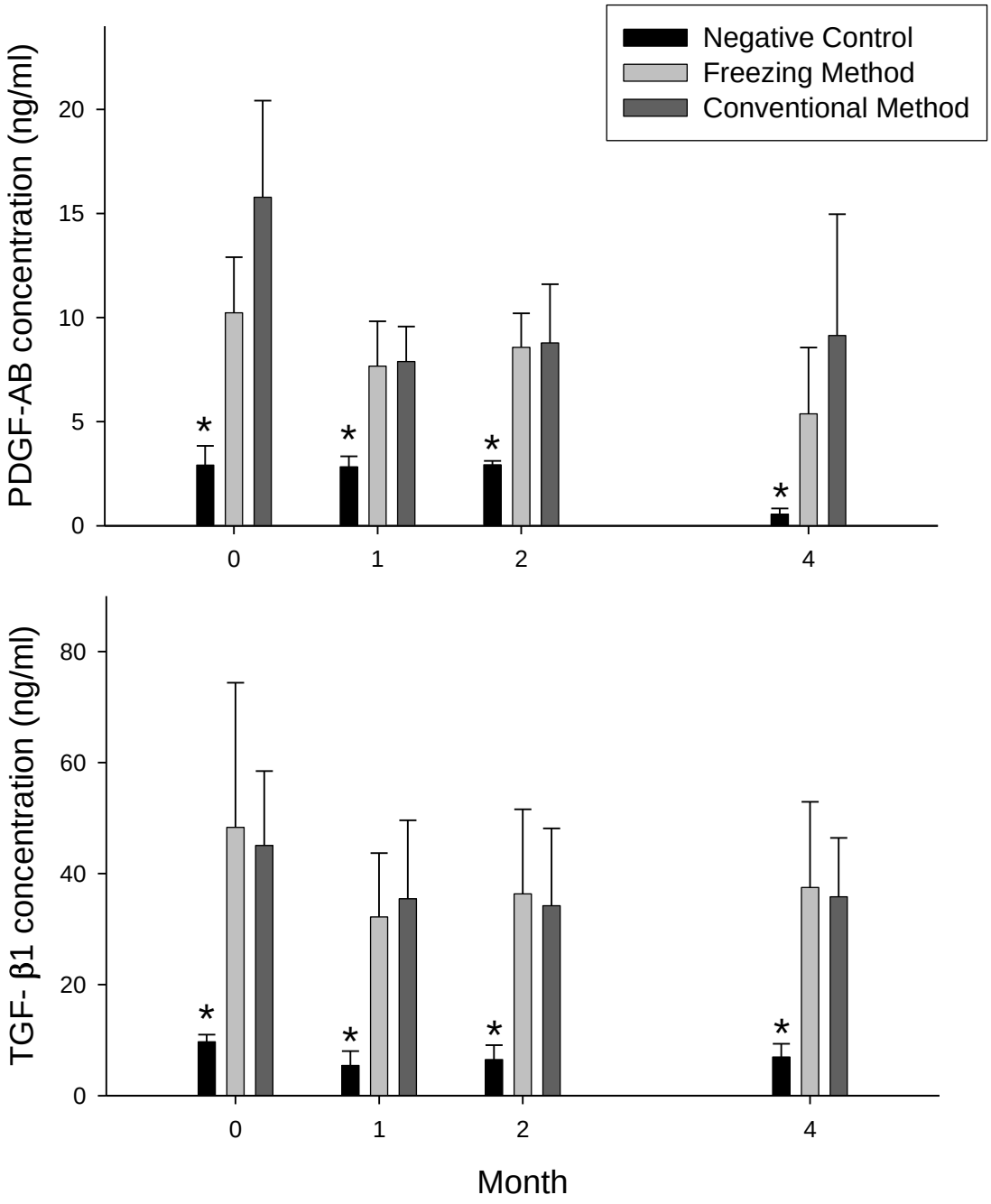
LEGENDS

Figure 1. Comparisons of the concentrations of PDGF-AB and TGF-β1 eleased in the negative control, freezing method and conventional method groups at the observation intervals of 0, 1, 2 and 4 months (means ± SE; *: Significant difference at $p < 0.05$ from the negative control group).

Figure 2. The concentrations of released PDGF-AB and TGF-β1 in the negative control, Freezing method, conventional method, and post-thrombin groups after 6 months storage in the experiment two (means ± SE; *: Significant difference at $p < 0.05$ from the negative control group).



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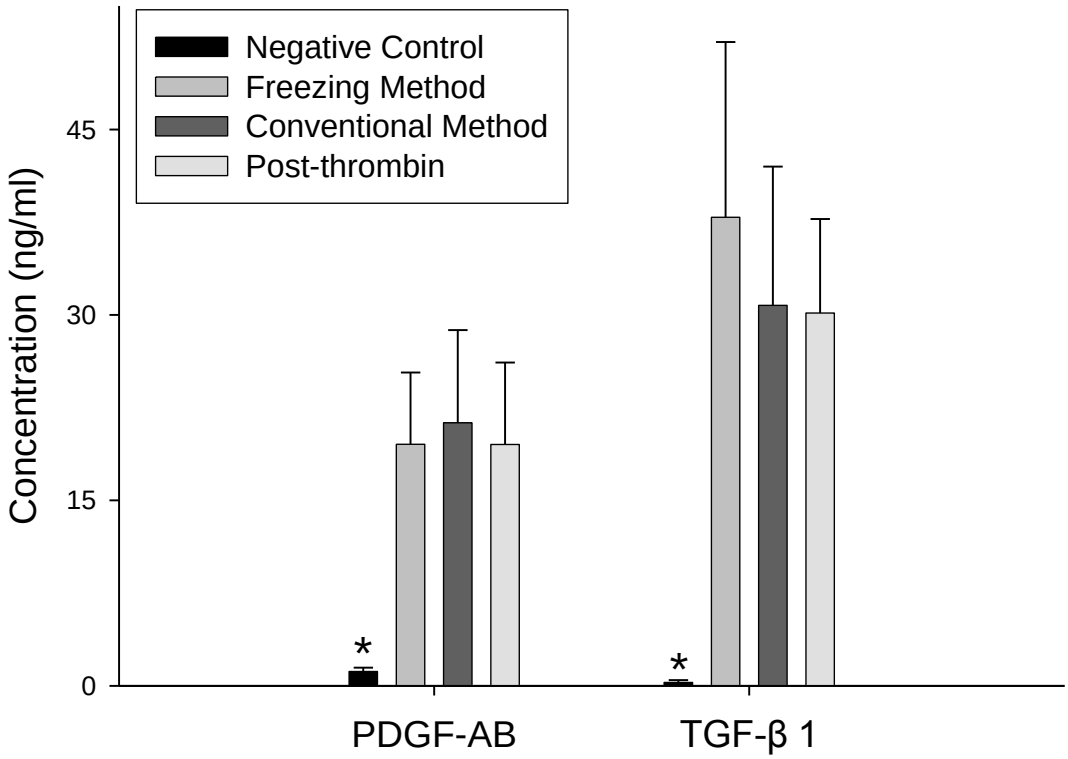

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