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Sustained or higher levels of growth factors in platelet-rich plasma during 7-day storage

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ABSTRACT

Background: The effectiveness of platelet-rich plasma (PRP) for treating soft tissue injuries is still controversial. Most of PRPs were prepared simply by concentrating in volume and were injected right after preparation in physician offices. Neither platelet count nor growth factors were quantitated in advance. We prepared and stored leukocyte and platelet-rich plasma (L-PRP) by regular separation protocols for blood components in the blood bank. And we investigated the dynamic change of growth factors in the L-PRPs over the period of storage.

Methods: The L-PRPs were prepared by 2-step centrifugation and stored agitatedly at 22 °C for 7 days in the platelet incubator of blood bank. Levels of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-basic, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, platelet derived growth factor (PDGF)-AB, endothelial growth factor (EGF), and transforming growth factor (TGF) over the period of storage were evaluated daily after freeze-thawing to release growth factors from platelet.

Results: Compared to original whole blood, platelet concentration, VEGF, FGF-basic, PDGF-AB, EGF, and TGF-beta1 levels of L-PRPs significantly increased after PRP preparation. Both HGF and IGF-1 in L-PRPs remained the original plasma level. Platelet, FGF, and TGF-beta1 concentrations sustained during storage, and concentrations of VEGF, HGF, IGF-1, PDGF-AB, and EGF in L-PRPs increased over the period of storage.

Conclusions: During the storage in blood bank, platelet counts and 7 growth factors sustained or reached higher level than L-PRP obtained on first day. Multiple injections of stored PRPs could become applicable by our protocol.

1. Introduction

Platelet-rich plasma (PRP) opens a vision for potentially restoring the normal function of tissue following injury or degeneration, and it is widely used by the specialist in the fields of physical medicine and rehabilitation, orthopedics and rheumatology. PRP contains various growth factors and cytokines, which were considered to improve tissue repair and regeneration when delivered to target tissue [1,2]. However, the effectiveness of PRP for treating soft tissue injuries is still controversial [3].

The underlying reasons attributing to the controversial therapeutic effectiveness are complicated. Firstly, precise injection of PRP is crucial for delivering growth factors-rich plasma to the culprit. Several procedures such as ultrasound-guidance, arthroscopy-guidance or anatomical guidance were used. Accuracy was proven to be improved with the use of ultrasound-guided injection compared with anatomical guidance [4,5]. Secondly, in literature, the patient parameters were diverse. In studies of PRP treatment in knee osteoarthritis, the outcome was better in younger individuals with a low degree of cartilage degeneration [6,7]. Studies of different populations could hardly be combined together for generating recommendations of high quality and sufficient evidence. Thirdly, treatment efficacy among various PRP preparation protocols and kits was unable to be compared. Most of them were prepared simply by concentrating in volume and were injected right after preparation in physician offices. No consensus dosage protocols could therefore be established due to lack of platelet or

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growth factors quantification in advance [8].

Two types of PRPs, pure PRP (P-PRP, or leukocyte-poor PRP) and leukocyte-and PRP (L-PRP), must be differentiated at least before any comparison [9,10]. PRP method and buffy coat method are two common methods for preparing the platelet concentrate in blood bank. Platelet concentrate belongs to L-PRP. In PRP method, the whole blood is centrifuged by soft spin at first to separate and discard red blood cells, and then the supernatant is centrifuged by high speed to concentrate platelet [8]. Whereas in buffy coat method [8], the whole blood is centrifuged at high speed, and the product is divided into three layers: the bottom layer is red blood cells, the middle part is platelet and white blood cells, and the top layer is platelet poor plasma. The middle-buffy coat layer is retrieved.

Reports on growth factors of PRP mainly focused on vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-basic, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, platelet derived growth factor (PDGF), endothelial growth factor (EGF), and transforming growth factor (TGF) [11]. VEGF, FGF-basic, and PDGF are related with proangiogenic activity and chemotaxis [12]. HGF is a mitogen for endothelial cells [13]; IGF-1 is a mediator in growth and repair of skeletal muscle, chemotaxis and enhance bone formation [14]. EGF could induce tubule formation, endothelial cell proliferation and migration [13]; TGF also involves in anti-angiogenic activity and chemotaxis [12].

Herein, we produced L-PRP by the PRP method for preparing platelet concentrate according to American Association of Blood Banks (AABB) technical manual [15], and evaluated dynamic concentration changes of VEGF, FGF basic, HGF, IGF-1, PDGF-AB, EGF, and TGF-beta1 in L-PRPs through the 7-day storage at 22 °C with agitation in blood bank.

2. Subjects and methods

2.1. Study subjects and blood collection

Peripheral bloods were collected from 6 healthy volunteer donors (3 males and 3 females, 40 to 56 years old) using a close system consisting of a primary blood bag containing anticoagulant citrate-phosphate-dextrose-adenine (CPDA) -1 and two satellite bags (JMS, Corp.; Singapore). The study was approved by the Ethics Committee of the CGMH (IRB no. 201600506A3), and all study subjects signed an informed consent.

2.2. Platelet-rich plasma preparation and storage

Freshly collected whole blood in the primary bag was centrifuged at 515 g for 5 min (J6-MI, Beckman Coulter Inc.; California, USA) to separate and discard red blood cells, and the supernatant plasma containing platelets was transferred into the first satellite bag. The supernatant plasma was centrifuged again at 3427 *g* for 6.5 min to concentrate platelets. Supernatant plasma lacking platelets (known as platelet-poor plasma, PPP) was transferred into the second satellite bag, and 30 ml of PRP remained in the first satellite bag. The final L-PRP was kept in the platelet incubator (Model 628, HOTECH Instruments Corp.; Taiwan) with agitation at 22 °C for 7 days in blood bank.

2.3. Hematological analysis and growth factor quantification

Aliquot of 2.5 mL was drawn out of the L-PRP bag each day during its 7-day storage (Table 1). The plasma of original whole blood specimen (day 0) and all the PRP aliquots (day 1 - day7) were analyzed by a hematological analyzer XE-5000 (Sysmex Corp.; Japan). After hematological analysis, specimens (day 0 - day 7) were frozen and stored at −80 °C for the following growth factors quantification in batch.

Growth factors could be released from platelets by deep freeze-thawing [16]. All frozen stocked specimens were simply thawed in room temperature and then subjected for quantifying growth factors. We used Quantikine ELISA Kits (R&D Diagnostics; Minnesota, USA) to quantify VEGF (Cat. #DVE00), FGF basic (#DFB50), HGF (#DHG00), IGF-1 (#DG100), PDGF-AB (#DHD00C), EGF (#DEG00), and TGF-beta1 (#DB100B). Procedures were carried out following the manufacturer's instructions.

Table 1
Sample collection to the different time points during storage.

	Day 1[a]	Day 2[a]	Day 3[a]	Day 4[a]	Day 5[a]	Day 6[a]	Day 7[a]
L-PRP remained (mL)	30	27.5	25	22.5	20	17.5	15
Aliquot drawn (mL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5

[a] Aliquot of 2.5 mL was drawn from the blood bag immediately after PRP preparation on day 1 and another aliquot of 2.5 mL was drawn from each blood bag every 24 h during the storage period. L-PRP, leukocyte- and platelet-rich plasma.

2.4. Statistical analysis

Paired Student's *t*-test was used to analyze for significant differences of growth factor levels between the original plasma (day 0) and PRP (day 1). Shapiro-Wilk original test was performed for normality of the data. Univariate approach of a repeated measures analysis of variance (rANOVA) with post-hoc Tukey's honestly significant difference (HSD) test was used to determine whether platelet counts and growth factor levels changed significantly throughout the storage period ($p < 0.05$ was considered statistically significant). Mauchly's sphericity test was used to validate the sphericity (an important assumption of rANOVA). If sphericity is violated, Huynh-Feldt correction (when $\varepsilon \geq 0.75$) or Greenhouse-Geisser correction (when $\varepsilon < 0.75$) will be applied. Statistical analysis was performed using Microsoft Office Excel 2007 (Microsoft Inc.; Washington, USA) and SPSS Statistics 18.0 (IBM Corp.; New York, USA).

3. Results

Total 153 to 170 mL whole blood were collected and processed from each of the six healthy volunteers (Table 2). Their platelet concentrations were all above 150,000/μL. Thirty milliliters of PRP were prepared from each donation by the two-step centrifugation. Final concentrations of PRPs became 1.6 to 5.7 folds of their original concentration (Table 2). The WBC reduction rates after PRP preparation were all above 83.0%. Platelet counts of PRPs remained unchanged during storage at 22 °C with agitation for 7 days (Table 3); statistically different platelet concentrations were not observed among storage days (rANOVA, p-value = 0.152).

Plasma VEGF levels of original whole blood specimens (day 0) ranged from 40.3 to 86.0 pg/mL (Table 4A). After centrifuge preparation and deep freezing procedure, the mean VEGF level of PRPs increased significantly from 300.0 pg/mL to 511.9 pg/mL on day 1 (*t*-test, $p < 0.001$). Statistically significant differences (rANOVA, $p < 0.023$) were observed among storage days, and post-hoc Tukey HSD test ($k = 7$, $n = 6$, $\alpha = 0.05$) showed VEGF level was statistically significant increasing between day 1 and day 2, day 2 and day 3, day 4 and day 5, and between day 6 and day 7.

Plasma FGF levels of original whole blood specimens (day 0) ranged from 4.8 to 40.3 pg/mL (Table 4B). After centrifuge preparation and deep freezing procedure, the mean FGF basic level of PRPs increased significantly from 12.3 pg/mL to a plateau level of 192.8 pg/mL on day 1 (*t*-test, $p < 0.001$). No statistically significant difference (rANOVA, $p = 0.146$) was observed among storage days.

Plasma HGF levels of original whole blood specimens (day 0) ranged from 501 to 658 pg/mL (Table 4C). After centrifuge preparation