

# A Cycle of Freezing and Thawing as a Modified Method for Activating Platelets in Platelet-rich Plasma to Use in Regenerative Medicine

İbrahim Eker<sup>1</sup>, Soner Yılmaz<sup>2</sup>, Rıza A Çetinkaya<sup>3</sup>, Aytekin Ünlü<sup>4</sup>, Aysel Pekel<sup>5</sup>, Raşan I Sağkan<sup>6</sup>, Zerrin Ertaş<sup>7</sup>, Orhan Gürsel<sup>8</sup>, Hacı U Muşabak<sup>9</sup>, Ertuğrul Yazıcı<sup>10</sup>, Sebahattin Yılmaz<sup>11</sup>, Cengizhan Açikel<sup>12</sup>, İsmail Y Avcı<sup>13</sup>, Ferit Avcu<sup>14</sup>, Emin Kürekçi<sup>15</sup>, Patrizio Petrone<sup>16</sup>

## ABSTRACT

**Background:** Platelet-rich plasma (PRP) has been widely used to improve wound healing and tissue repair. The objective of this study was to assess a cycle of freezing/thawing and its effect in the amount of growth factors in PRP.

**Materials and methods:** After the preparation of PRP using standard methods, the samples were equally divided into two groups: The first group was activated by adding 10% calcium gluconate, while the second group was cryopreserved at  $-80^{\circ}\text{C}$  for 24 hours. Enzyme-linked immunosorbent assay was used for aliquots of PRP to measure concentrations of insulin-like growth factor-1, platelet-derived growth factor, and basic fibroblast growth factor.

**Results:** 15 mL of whole blood was obtained from 20 volunteers and collected into citrated tubes. The mean platelet count of the donors and the autologous PRP were  $238.5 \pm 44.7 \times 10^3/\mu\text{L}$  and  $544.7 \pm 161.5 \times 10^3/\mu\text{L}$ , respectively. There were no significant differences between the growth factor levels of freeze-thawed and calcium-activated PRP. A cycle of freezing/thawing was the only independent factor associated with the growth factor yield in the multivariate model.

**Conclusion:** With its features of being simple, inexpensive, and easy for standardization, a cycle of freezing/thawing may be the method of choice for PRP activation procedure, without inducing fibrin matrix. This study was performed in a laboratory setting; therefore, future clinical trials are recommended.

**Keywords:** Basic fibroblast growth factor, Freezing, Insulin-like growth factor-1, Platelet-rich plasma, Platelet-derived growth factor, Thawing.

## RESUMEN

**Antecedentes:** El plasma rico en plaquetas (PRP) se ha utilizado ampliamente para mejorar la cicatrización de heridas y la reparación de tejidos. El objetivo de este estudio fue evaluar un ciclo de congelación/descongelación y su efecto en la cantidad de factores de crecimiento en PRP.

**Métodos:** Después de la preparación de PRP utilizando métodos estándar, las muestras se dividieron por igual en dos grupos: el primer grupo se activó agregando gluconato de calcio al 10%, mientras que el segundo grupo se crioconservó a  $-80^{\circ}\text{C}$  durante 24 horas. El ELISA se usó para partes alícuotas de PRP para medir el factor de crecimiento-1 similar a la insulina, el factor de crecimiento derivado de plaquetas y las concentraciones básicas del factor de crecimiento de fibroblastos.

**Resultados:** Se obtuvieron 15 mL de sangre completa de 20 voluntarios que fueron colocados en tubos citratados. El recuento medio de plaquetas de los donantes y el PRP autólogo fue de  $238.5 \pm 44.7 \times 10^3/\mu\text{L}$  y  $544.7 \pm 161.5 \times 10^3/\mu\text{L}$ , respectivamente. No hubo diferencias significativas entre los niveles de factor de crecimiento de PRP congelado-descongelado y activado por calcio. Un ciclo de congelación/descongelación fue el único factor independiente asociado con el rendimiento del factor de crecimiento en el modelo multivariante.

**Conclusión:** Con sus características de ser simple, económico y fácil de estandarizar, un ciclo de congelación/descongelación puede ser el método de elección para el procedimiento de activación de PRP, sin inducir la matriz de fibrina. Este estudio se realizó en un laboratorio, por lo tanto, se recomiendan ensayos clínicos futuros.

**Palabras clave:** Plasma rico en plaquetas, Congelar, Descongelar, Factor de crecimiento-1 similar a la insulina, Factor de crecimiento derivado de plaquetas, Factor básico de crecimiento de fibroblastos.

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## INTRODUCTION

Platelet-rich plasma (PRP) preparations are safe, reliable, and cost-effective products that are used for accelerating healing after a surgical procedure or injury. Platelet-rich plasma comprises an increased concentration of platelets compared to whole blood. As a growth factor pool for improving tissue regeneration, PRP has been widely used in different clinical scenarios, such as in orthopedics and ophthalmology. Depending on the purpose, PRP can be used with or without previous platelet activation. Upon activation under physiological and pathological circumstances,

<sup>1</sup>Department of Pediatric Hematology, Afyonkarahisar Health Sciences University, Afyonkarahisar, Turkey

<sup>2,11</sup>Regional Blood Center, Health Sciences University, Gulhane Training and Research Hospital, Ankara, Turkey

<sup>3</sup>Department of Infectious Disease, Health Sciences University, Sultan Abdulhamid Han Training and Research Hospital, Istanbul, Turkey

<sup>4</sup>Department of General Surgery, Health Sciences University, Gulhane Training and Research Hospital, Ankara, Turkey

<sup>5</sup>Deren Laboratories, Ankara, Turkey

platelets release a large amount of bioactive molecules that possess regenerative features.<sup>1-4</sup> Platelet-rich plasma and other platelet-derived products seem to be promising alternative tools besides the treatment options for infected wounds.<sup>5,6</sup> Platelets can be activated by adding collagen, thrombin, and/or calcium in contact with glass. Cryopreservation process has also been recently shown to activate platelets and activation associated markers such as P-selectins.<sup>7</sup> The purpose of this study is to evaluate a cycle of freezing/thawing and its effect on the amount of growth factor in PRP.

## MATERIALS AND METHODS

### Study Design

Approval from the Gulhane Military Medical Academy Ethical Committee was granted (Approval # 37). Samples of whole blood were obtained from voluntary donors who have similar demographic characteristics and have not associated risks, medications, or comorbidities by venipuncture and collected into two citrated tubes (BD-Plymouth, PL6 7BP; UK). The centrifugation process involved two phases: During the first phase, the red blood cells (RBC) and white blood cells (WBC) were eliminated with minimal platelets loss (RCF: 300×g, centrifugation time: 5 minutes; soft spin). Next, the whole plasma above the buffy coat was transferred into a sterile tube without an anticoagulant (PRP1). During the second phase, tubes were centrifuged at a higher speed (RCF: 700×g, centrifugation time: 17 minutes; hard spin) to obtain the best platelet yield. PRP and platelet-poor plasma (PPP) were, respectively, located on the lower and upper thirds of the centrifuged solution. Platelet pellets were formed at the bottom of the tube. Platelet-rich plasma (PRP2) was obtained by removing the PPP. PRP2 was divided into two groups. Activation of the first group was obtained by adding 10% calcium gluconate to finalize PRP with a volume ratio of 1:10 [calcium piken amp. 10% 10 mL (Adeka Pharm. Ind.; Turkey)]. The second group was cryopreserved at 80°C for 24 hours and thawed at room temperature. Centrifugation at 10,000g for 10 minutes was performed to remove the platelets, and to measure the growth factors in both groups. Aliquots of 150 µL from the obtained PRP were used to measure insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF-BB), and basic fibroblast growth factor (bFGF) concentrations by using the ELISA kit (RayBiotech; Norcross, Georgia, USA).

### Statistical Analysis

Statistical analysis was performed by using SPSS v.22 (IBM Inc.; Armonk, New York, USA). The amount of the released GF was expressed as mean value in pg/mL ± the standard deviation. Normality analysis was performed by One-Sample Kolmogorov-Smirnov tests. Continuous variables between autologous PRP, post-freeze PRP, and calcium-activated PRP groups were compared

<sup>6</sup>Department of Medical Biology, Uşak University, Uşak, Turkey

<sup>7</sup>Department of Hematology, Health Sciences University, Gulhane Training and Research Hospital, Ankara, Turkey

<sup>8</sup>Department of Pediatric Hematology, Health Sciences University, Gulhane Training and Research Hospital, Ankara, Turkey

<sup>9</sup>Department of Allergy and Immunology, Baskent University, Ankara Hospital, Ankara, Turkey

<sup>10,13</sup>Department of Infectious Disease and Clinical Microbiology, Health Sciences University, Gulhane Training and Research Hospital, Ankara, Turkey

<sup>12</sup>Clinical Reaearch International Ltd., Genter Str. Cologne, Germany

<sup>14</sup>Department of Hematology, Memorial Hospital, Ankara, Turkey

<sup>15</sup>Department of Pediatric Hematology, Lösante Children's and Adult Hospital, Ankara, Turkey

<sup>16</sup>Department of Surgery, NYU Langone Health - NYU Winthrop Hospital, NYU Long Island School of Medicine, Mineola, New York, USA

**Corresponding Author:** Patrizio Petrone, Department of Surgery, NYU Langone Health - NYU Winthrop Hospital, NYU Long Island School of Medicine, Mineola, New York, USA, Phone: +1 (516) 663-9571, e-mail: patrizio.petrone@nyulangone.org

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by one-way analysis of variance tests. A multivariate analysis of covariance was performed to evaluate the effect of the platelet activation variables after adjusting for possible covariates factors, such as donors' age, weight, height, body mass index (BMI), and baseline platelet counts that affected the growth factor yield and were analyzed by multivariate linear regression, using a backward elimination procedure. Statistical significance was set at *p* value < 0.005.

## RESULTS

Twenty donors were included in the study, from whom 5 mL of whole blood was obtained. Their mean age was 36.3 ± 8.4 years, and the mean weight was 83 ± 10 kg. The mean platelet count and the autologous PRP were 238.5 ± 44.7 × 10<sup>3</sup>/µL and 544.7 ± 161.5 × 10<sup>3</sup>/µL, respectively. Overall, there was an increase in the concentration of the three growth factors (Table 1), but only PDGF was statistically significant. There were not any significant differences between the growth factor levels of freeze/thawed and calcium-activated PRP. A cycle of freezing/thawing was the only independent factor associated with growth factor yield in the multivariate model.

**Table 1:** Comparison of mean growth factor concentrations in the fresh autologous PRP, post-freeze PRP and calcium-activated PRP

Growth factor	Fresh autologous PRP (pg/mL)	Post-freeze PRP (pg/mL)	Calcium-activated PRP (pg/mL)	<i>p</i> value*
IGF-1	0.71 ± 0.12	0.66 ± 0.13	0.80 ± 0.17	0.683
PDGF-BB	78.95 ± 49.42	218.90 ± 30	365.97 ± 101	0.0001
bFGF	47.74 ± 18.15	63.09 ± 18.15	70.14 ± 25.75	0.324

\*Statistically significant *p* < 0.05

IGF-1, insulin-like growth factor-1; PDGF-BB, platelet-derived growth factor-BB; bFGF, basic fibroblast growth factor; PRP, platelet-rich plasma

## DISCUSSION

The rationale behind the widespread use of platelet-derived products is the abundance of growth factors and other signal transmitting molecules along with their ease to acquire them.<sup>8</sup> Under normal circumstances, growth factors and bioactive peptides are secreted in response to injury, acting in a well-organized and controlled medium for contributing to the healing process. Their contribution in the healing process occurs sequentially in the inflammation, repair, and remodeling phases. In general, platelets remain in the inactivated, quiescent state and require a trigger signal to actively participate in hemostasis and wound healing.<sup>9</sup> During the latter, activated platelets secrete both dense and alpha granules. Dense granules contain ADP, ATP, serotonin and calcium, and alpha granules contain clotting factors and platelet-derived growth factors. The contents of these granules play a critical role in normal platelet activity.<sup>10</sup> Platelet-derived growth factors have specific cellular target receptors. Upon binding their targets, they show morphogenic and mitogenic effects, induce cell migration, and support cell proliferation, differentiation and chemotaxis. Additionally, they support mesenchymal cells in bone marrow, adipose tissue and cord blood, increase the effective proliferating number of cells, and cell differentiation, thus speeding up the wound healing process. The secreted platelet-derived growth factors bind to the tyrosine kinase receptors and induce the propagation of signal transduction toward the nucleus by the explicit pathways and signaling cascades and activate target expression.<sup>11</sup>

Platelet-rich plasma is obtained by transferring the blood sample into the acid/citrate/dextrose tubes and centrifuged them. In order to gain the supernatant, consisting of PRP, platelet pellet, and PPP, a high-speed centrifugation is required. Besides the protocol for obtaining PRP, platelet activation is required for the secretion and enmeshment of growth factors. However, the preferred method of activation may exert a negative impact on matrix development, growth factor content, and wound healing.<sup>12</sup> The most preferred method of activating the platelets in PRP is the addition of pig thrombin and calcium, which results in the formation of platelet rich gel. When these reagents are added, gel formation occurs quickly, being these reagents frequently applied to the wound site. Most protocols only use calcium to activate platelets due to the risk of developing antibodies against pig thrombin, and clotting factors V and IX, with the resultant development of severe coagulopathy. Currently, a standard method to obtain platelet gel does not exist.

As mentioned earlier, addition of pig thrombin and calcium for activating the platelets in PRP results in the formation of platelet-rich gel. Under some circumstances, it may be preferred that the activated and degranulated platelets remains in liquid form, such as PRP drops used for the treatment of dry eye syndrome and corneal ulcers.

Use of PRP gel form have been previously described. Cetinkaya et al.<sup>5</sup> reported that antimicrobial properties of PRP have led to the suggestion that PRP may be a viable treatment option for the treatment of surgical site infections. *In vitro* studies on the antimicrobial efficacy of PRP have shown that PRP inhibits the growth of methicillin-sensitive and -resistant *Staphylococcus aureus* (MRSA), Group A Streptococci, *E. coli*, and *N. gonorrhoeae*, while other authors have shown that PRP used in conjunction with vancomycin is effective against MRSA growth. The conclusion of this animal study was that PRP gel seems to enhance MRSA-infected

surgical wound healing, by decreasing chronic suppurative inflammation and MRSA cell counts in wounds.

Later, Çetinkaya and associates<sup>6</sup> revealed in their *in vitro* study that PRP and PPP had antimicrobial effect on three resistant microorganisms such as MRSA, ESBL-positive *K. pneumoniae*, and carbapenem-resistant *P. aeruginosa*, while they had no significant effect on VRE. When these results were considered as a whole, antimicrobial effect of PRP was more effective than PPP; however, it was statistically significant only in MRSA and *P. aeruginosa* group and only in the first hours of the study. This group stated that considering the trend on diabetes and antibiotic resistance rates, treating chronic infected wounds is going to continue to be one of the most important and the most difficult fields in medicine in the future. The conclusion of that study was that emerging PRP and other platelet-derived products seem to be a promising alternative tool besides antibiotic treatment, debridement, negative pressure wound therapy, hyperbaric oxygen therapy, and other treatment options for treating such infections.

Other authors describe the implications of the cycle of freezing and thawing effects over platelet-derived growth factor functions. Sekido et al.<sup>13</sup> studied cell cultures and showed that activities of growth factors released from cryopreserved platelets were similarly effective and adequate as the growth factors released from fresh platelets. Similarly, Roffi et al.<sup>14</sup> demonstrated that a cycle of freezing and thawing did not change the secretion of platelet-derived growth factors and their effects on chondrocytes and sinoviocytes. Ronci et al.<sup>15</sup> showed that the use of PRP activated after a cycle of freezing and thawing, efficiently treated persistent ocular epithelial defects.

The concept of lyses of platelets by freezing was also addressed in a publication by Weed et al.<sup>16</sup> There are differences between their study and our own regarding the method of PRP product preparation. In their study, an average of 240 mL of leukocyte-reduced platelets and a similar amount of plasma were collected using the Spectra blood cell separator. Aliquots of platelets and plasma were placed into 12 vials and frozen at less than or equal to  $-18^{\circ}\text{C}$  within 12 hours of collection. In our study, samples of whole blood were obtained from voluntary donors by venipuncture and collected into citrated tubes, and PRP was obtained by using centrifuge as detailed in the methods section, not by apheresis as described in the Weed' study. While the PDGF increase obtained by our method was approximately three times higher than the basal levels, in their study, they obtained approximately forty times more compared to the basal levels. But as their method needs, apheresis is neither practical nor easy to apply. Also, our samples were cryopreserved and ready immediately at  $-80^{\circ}\text{C}$  for 24 hours, not at  $-18^{\circ}\text{C}$  within 12 hours of collection. Lastly, their PRP product did not lead to statistically significant accelerated wound healing. They stated that there could be many reasons for this, but did not discuss timing and degree of freezing.

The concept of lyses of platelets by freezing was addressed by Sonker et al.<sup>17</sup> In their study they aimed to compare different preparation methods, concentration techniques, and storage durations in order to optimize the yield of growth factors. Concentration of growth factors was performed by using double freeze thaw technique or  $\text{CaCl}_2$ -induced degranulation technique. Double freeze thaw is a different concentration technique compared to the single freeze thaw concentration technique we used in our study. With theirs the PRP sample is frozen at  $-80^{\circ}\text{C}$ , it is thawed at room temperature after 48 hours, and refrozen

immediately thereafter. Just before analyzing it was again thawed and centrifuged for 10 minutes at 10,000 rpm in a microcentrifuge. They did not explain or give any reference about why they used this method, but they probably aimed to lead a complete release of all growth factors from intracellular granules into the surrounding liquid. Further studies are needed to compare the levels of growth factors in the lysate and the lysate activity between both single and double freeze thaw concentration techniques.

Our study showed that growth factors were released at higher concentrations from the platelets after activation with a cycle of freezing/thawing compared to the basal values in the autologous PRP without formation of gel. Although it is thought that the platelet levels present in the blood-derived products play a key role in the regenerative process rather than the fibrin matrix or the methods chosen for platelets activation, our study established that a cycle of freezing/thawing was the only independent factor affecting the growth factors content of the PRP. In the freezing/thawing activated PRP, platelets activation has been obtained without inducing fibrin matrix or coagulation, but there was different amounts of fibrin matrix or coagulation in the calcium activated PRP.

It is important to recognize that standardization of PRP preparations is one of the major concerns in the field of regenerative medicine, and although biological studies like ours give important indications for the development of treatments, the results do not always directly translate into clinical findings.

## CONCLUSION

With its features of being simple, inexpensive and easy for standardization, a cycle of freezing/thawing may be the method of choice for PRP activation procedure, especially for obtaining PRP rich in PDGF, without inducing fibrin matrix, like the eye drops rich in GF used for the treatment of persistent ocular epithelial defects. Further clinical studies are recommended to obtain a better understanding of the effects of a cycle of freezing/thawing for activation of platelets in PRP on patients' symptoms and functional improvement.

## INFORMED CONSENT

The study was approved by the Institutional Ethics Committee of Gulhane Military Medical Academy. Informed consent was obtained from all study participants.

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