



Brief Report

A novel method for the preparation and frozen storage of growth factors and cytokines obtained from platelet-rich plasma

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ABSTRACT

Platelet-rich plasma (PRP) therapy is employed to treat damaged connective tissues and osteoarthritis. PRP is collected in the presence of an anticoagulant to avoid premature activation. The PRP is then activated by various activation methods that all have regulatory or cost drawbacks. Additionally, activated PRP can only be stored for a limited time. The purpose of this study was to assess the biological stability of a PRP composition obtained from platelets of healthy volunteers using a mechanical activation by passing PRP through a 0.22 μm filter and stored for up to 9 months. The PRP fraction was isolated and then activated using either mechanical or thrombin techniques: 9 samples were evaluated in each experiment. The concentrations of interleukin (IL)-4, IL-10, IL-13, platelet-derived growth factor, transforming growth factor beta 1, insulin-like growth factor 1, and vascular endothelial growth factor were compared in samples that were freshly collected and samples that were previously stored at -80°C for 9 months. Protein concentration analysis showed no statistically significant differences in the composition of the PRP when the platelets were activated by thrombin or mechanical activation. There was also no statistically significant difference in the concentration of cytokines and growth factors in the PRP autologous composition after storage for 9 months at -80°C . Mechanical activation is an efficient method to activate PRP, and the PRP-derived autologous composition is capable of being stored for up to 9 months without affecting the concentration of the analyzed proteins.

Introduction

Autologous platelet-rich plasma (PRP) treatment appears to provide a safe method for the treatment of osteoarthritis and other musculoskeletal conditions.^{1–3} Inflammatory cytokines, including interleukin (IL)–1, IL-6, IL-8, and tumor necrosis factor- α (TNF- α), play a role in the pathogenesis of osteoarthritis by exerting catabolic effects on the surrounding joint tissues, such as cartilage and bone.⁴ This catabolic effect can be successfully blocked by platelet-derived inhibitory cytokines, such as IL-4, IL-10, and IL-13.⁴ It has been reported that PRP is also a source of various bioactive regenerative proteins, including growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor, and transforming growth factor beta 1 (TGF β -1).^{5,6,7} PRP is a heterogeneous term encompassing different preparations of platelets due to the varied production protocols. Although PRP treatment has been proven effective in various medical conditions,^{1,2,8–10} it remains controversial in the broader medical community due to a lack of standardization and optimization with respect to its preparation methods, detailed cytokine analysis, and treatment protocols. Platelet activation—the release of bioactive molecules secreted by platelet α -granules—can be triggered by the production protocols; therefore, there may be varying concentrations of growth factors and cytokines in the PRP dependent on the production protocol utilized.^{11,12} PRP fractions are collected in the presence of an anticoagulant, such as sodium citrate, to avoid premature activation

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of the platelets.¹³ Platelet activation can then be achieved through several methods, such as addition of calcium chloride (CaCl) or thrombin, freezing and thawing of platelets, and the use of ultrasonic waves;^{11–17} however, each of these activation methods has drawbacks. Thus, the addition of CaCl changes the chemical composition of the final PRP product. Thrombin is a key regulator of robust platelet activation in response to vascular injury and is perhaps the most effective activator of platelets *ex vivo*¹⁴; however, homologues or xenoproteins of thrombin can lead to an allergic reaction or be a source of pathogens. Additionally, repeated freeze-thaw cycles can lead to the degradation of biologically active proteins, such as PDGF and TGF β -1.¹⁵ Finally, there are regulatory issues with the use of ultrasonic waves since this technique does not meet the minimum manipulation regulatory requirements applicable in Canada and the United States for PRP treatment approval. Here we compare a mechanical PRP activation method using a small pore cell filter with effective *ex vivo* platelet activator thrombin¹⁴ to create an efficient protocol for activating platelets.

Activated PRP can typically only be stored for about 6 hours at room temperature before losing biological activity¹⁸; however, some PRP therapy methods require multiple blood draws (at each treatment visit), which is invasive, time-consuming, and costly. To mitigate these concerns, mainly to enhance treatment safety by significantly reducing the risk of insertion-site infection and potential contamination of collected blood, we propose a new PRP preparation method allowing storage (up to 9 months) of mechanically-activated PRP at $-80\text{ }^{\circ}\text{C}$ for future use. Previous research has compared growth factor and cytokine concentrations in fresh and frozen PRP stored for an extended period of time and reported a significant decrease in insulin-like growth factor 1 (IGF1), PDGF and TGF β -1 concentrations in frozen storage conditions.^{19,20} The aim of this study is to evaluate the effect of prolonged storage at $-80\text{ }^{\circ}\text{C}$ of mechanically-activated PRP protein concentrations compared to baseline concentrations.

Material and methods

Ethics statement

This study was approved by the Chesapeake International Review Board (CR00082114). The study population included healthy males or females between the ages of 21 and 60 with a body weight of at least 110 pounds. Twelve patients were recruited voluntarily, and informed consents form were signed.

PRP sample collection and preparation

Venous blood samples (9.5 cc) were collected in polystyrene tubes (BD Biosciences) containing 0.5 cc 4% sodium citrate (Baxter, JB7747) (Fig. 1A) and centrifuged at 7500 rpm for 30 s (Fig. 1B). A standard PRP activation method includes the use of a single dose of thrombin or CaCl, stimulating a rapid release of cytokines and growth factors. Normal platelets are 1.5 to 3 μm in diameter; therefore, applying mechanical force by passing the platelet concentrate through a 0.22 μm pore size polyethersulfone membrane will physically damage the platelet membranes initiating the release of the granule content. Harvested PRP was passed through a 0.22 μm filter (Millipore, SLGP033RS) (Fig. 1C). Bovine thrombin (Cedarline, 528–50-EA) was added as a control to inactivated samples: 100 units per 1 cc PRP. Samples were aliquoted as 2.5 mL portions under sterile, laminar flow conditions and stored at $-80\text{ }^{\circ}\text{C}$ for 9 months. Nine samples were used for each assay. All samples were analyzed in triplicate. The effect of storing the mechanically-activated PRP for 9 months at $-80\text{ }^{\circ}\text{C}$ on the concentrations of IL-4, IL-10, IL-13, vascular endothelial growth factor (VEGF), PDGF, basic fibroblast growth factor (bFGF), TGF β -1 and IGF1 levels was analyzed by comparing frozen samples to fresh mechanically-activated PRP.

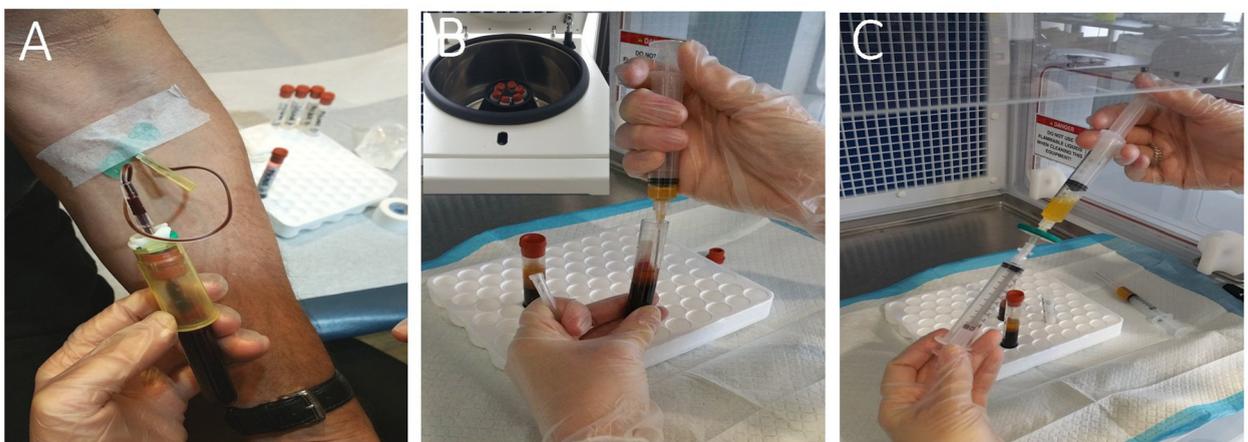


Fig. 1. Platelet-rich plasma sample collection and preparation method. **A,** Blood draw. **B,** Centrifugation and collection. **C,** Mechanical activation by passing through a 0.22 μm filter.

Cytokines quantification

Concentrations of IL-4, IL-10, IL-13, VEGF, PDGF, IGF1, bFGF, and TGF β -1 were quantified in experimental and control samples using an optimized multiplex test (BIO-PLEX Assay, Bio-Rad) in a Multiplex reader (Bio-Plex MAGPIX, Bio-Rad) according to the supplier's protocol. The results were analyzed using the xPONENT™ software. The absolute concentrations of the samples were determined by the construction of a standard curve for each analyte.

Statistical analysis

Twelve samples were analyzed for each experiment. Three separate measurements were conducted for 3 sets of samples (triplicates). A power analysis was performed using GPower 3.0 software. The observed power results varied from 0.68 to 0.99 for statistically significant effects ($P < .05$) and from 0.08 to 0.7 for $P > .05$ results. Two-tailed unpaired t tests were performed. A P value less than .05 indicated significance. All statistical tests were performed using GraphPad Prism™ version 5.01. Data are expressed as the mean \pm standard error of the mean.

Results

Comparative analysis of cytokine and growth factor concentrations in PRP fractions obtained by human thrombin or mechanical activation

Previously published data has shown that the release of cytokines and growth factors from platelets could also be achieved by disruption of the platelet cell membrane.¹⁶ To test this hypothesis, we compared the concentrations of IL-4, IL-10, IL-13, VEGF, PDGF, and bFGF in PRP samples activated by the 2 different methods: thrombin activation or by applying mechanical force via a 0.22 μ m filter. As shown in Figure 2 no statistical differences were observed in any of the cytokine or growth factor concentrations between the samples, confirming the efficacy of using mechanical activation as part of the final PRP preparation.

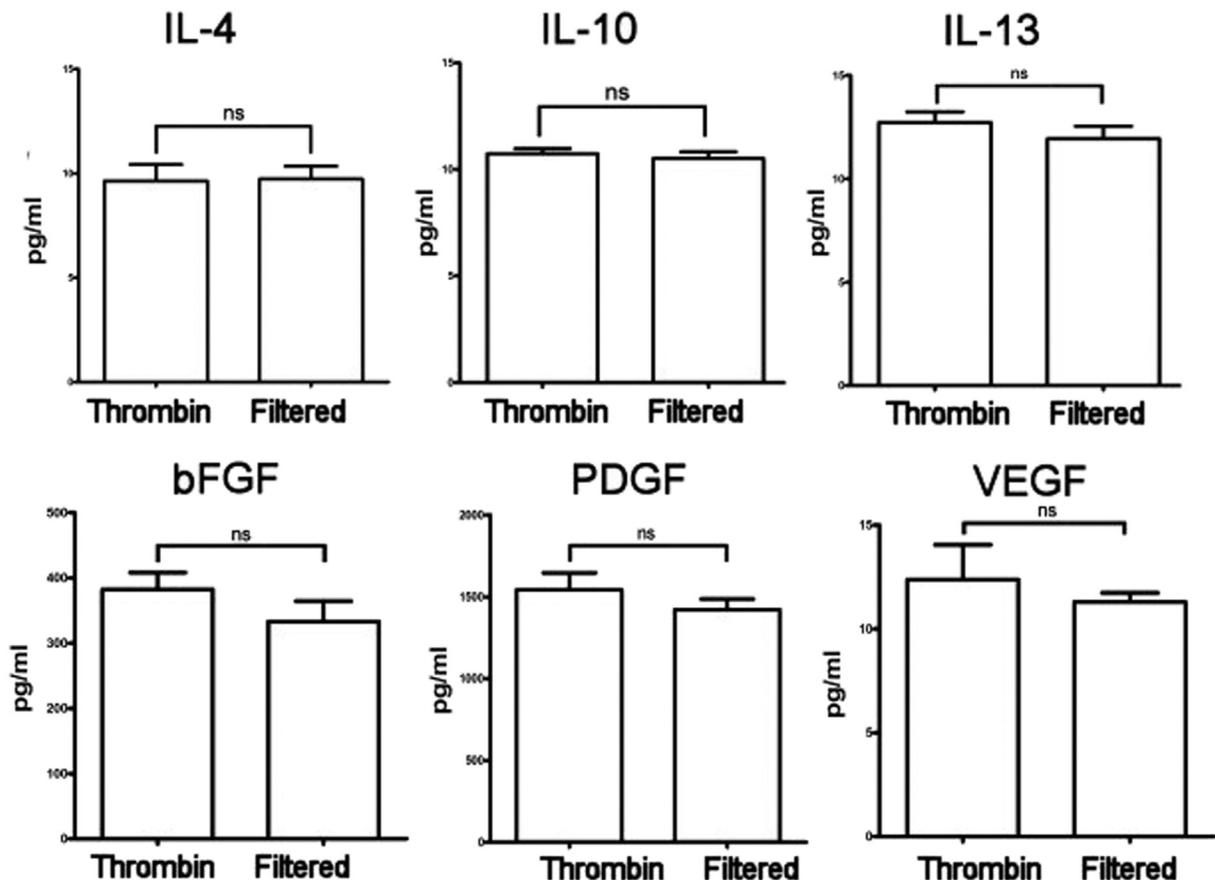


Fig. 2. A comparison of the cytokine and growth factor concentrations in thrombin or mechanically-activated human PRP samples. Concentrations of interleukin (IL)-4, IL-10, IL-13, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) were compared in PRP samples activated by thrombin (thrombin) or by applying mechanical force through a 0.22 μ m filter (filtered). Data are expressed as the mean \pm standard error of the mean. Two-tailed unpaired t tests showed no significant difference between the methods. ns, not significant; PRP, platelet-rich plasma.

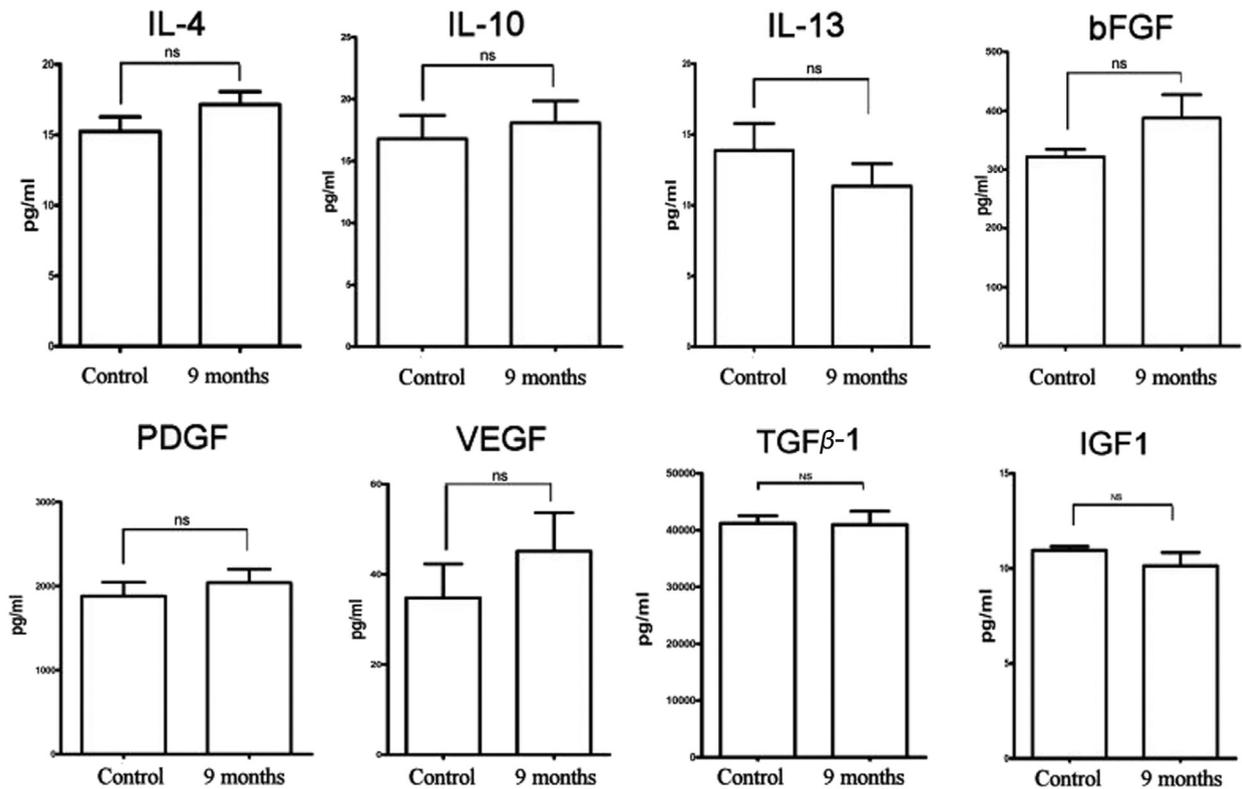


Fig. 3. A comparison of the cytokine and growth factor concentrations in the mechanically-activated human PRP samples freshly collected or stored for 9 months at -80°C . Concentrations of interleukin (IL)-4, IL-10, IL-13, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF β -1), and insulin-like growth factor (IGF1) were compared in mechanically-activated human PRP samples freshly collected or stored at -80°C for 9 months previously. Data are expressed as the mean \pm standard error of the mean. Two-tailed unpaired *t* tests showed no significant difference between the methods. ns, not significant; PRP, platelet-rich plasma.

The effect of prolonged storage of mechanically-activated PRP without preservation agents on the concentration of anti-inflammatory cytokines and growth factors

Previous studies have pointed out differences in the protein profiles of fresh or frozen stored PRP.^{19,20} Figure 3 shows that there was no change in any of the cytokine and growth factor concentrations between the fresh or previously frozen samples of mechanically-activated PRP. TGF β -1 and IGF1 are 2 potentially strong therapeutic agents due to their ability to suppress a chronic inflammation pathway and promote collagen synthesis.^{21,22} Unfortunately, previously described freeze-thawing PRP protocols were found to lead to a significant decrease in TGF β -1 and IGF1 protein levels. Intriguingly, as shown in Figure 3, no significant differences were observed, suggesting that mechanical activation of PRP samples can provide a novel method for allowing longer-term storage of PRP.

Discussion

The first goal of this study was to investigate a new PRP activation protocol utilizing mechanical activation. We approached this using a $0.22\ \mu\text{m}$ pore filter to activate PRP since applying a mechanical force physically damages the platelet membranes initiating the release of the granule content. Previously, researchers have successfully used freeze-thaw cycles as an activation method for PRP by rupturing the platelet membrane.¹⁸ However, the application of this approach in clinical medicine can cause certain complications due to the presence of membrane fragments in the final product that can stimulate the inflammatory process. We show data confirming that filtered PRP comprises the same active protein concentrations as PRP activated by thrombin, presenting the advantage of being potentially clinically safer due to its acellular nature. Platelet activation is a step that might affect the PRP biological activity in healing processes; however, there are inconsistent reports in the literature describing the therapeutic potential of PRP endogenous (surrounded tissue) vs exogenous (thrombin, CaCl, etc) activation.^{23,24} Although some published studies suggest that PRP does not need to be activated prior to the treatment,^{25,26} define concurrent data highlight positive effect of activation protocol employment.²⁴

Future in vivo studies should aim at further investigating the effect of the mechanical activation strategy to analyze the clinical outcomes.

The effect of PRP storage conditions, for example, the optimal duration and temperature of freezing, on the PRP protein concentrations compared to fresh PRP has been previously investigated.^{19,22,27,28} The authors reported that PDGF levels were no different in activated human PRP after 4 weeks of storage in -80°C but that its concentration decreased after that; additionally, the concentration of TGF β -1 decreased by 8 weeks.²¹ Another study analyzed the concentrations of IGF1, PDGF, and TGF β -1 in human CaCl activated PRP stored for up to 14 days at -20°C ²⁸; data showed a significant decrease in all growth factors under these storage conditions.²² It has previously been shown that the clot formation that accompanies thrombin and CaCl PRP activation might retain substantial concentrations of growth factors,²⁹ which are lost upon freezing. This present study demonstrated, for the first time to our knowledge, that mechanically-activated human PRP could be stored at -80°C for up to 9 months without diminishing the PRP-derived protein concentrations.

Our study has some limitations. We report the result of sample analysis from 12 subjects. The eligibility criteria included healthy subjects and were age-restricted, which may limit generalizability: there are high variations in serum cytokine levels among individuals depending on age, sex, and health status.^{30,31} Other limitations may include the variations in commercially-available PRP preparation methods and the storage time. Further studies to evaluate PRP cytokine concentration according to various commercially-available methods of preparation in the heterogeneous subject populations are required for the proper PRP analysis. Changes to the protein conformation, such as the partial unfolding of protein molecules during freeze-thawing, which might affect the therapeutic benefit of frozen PRP, have been previously described.³² Therefore, further work will be needed to assess whether this issue affects mechanically-activated PRP stored at -80°C . Future clinical studies are required to explore the clinical benefits of the presented PRP activation and storage method.

Author contributions

I.B. designed and performed the in vitro experiments, interpreted the data, and wrote the manuscript; A.M.G. designed the experiments and interpreted data.

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Ethics approval

The Chesapeake International Review Board approved the experimental procedure and all participants signed informed consent forms. International review board approval number: CR00082114.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This research was funded by the Antnor LTD Company. Antnor LTD holds a product patent and A.M. Galea is a stockholder.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.jcjp.2022.100089](https://doi.org/10.1016/j.jcjp.2022.100089).

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