# Exercise and the platelet activator calcium chloride both influence the growth factor content of platelet-rich plasma (PRP): overlooked biochemical factors that could influence PRP treatment

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## ABSTRACT

**Background** There is strong evidence that exercise affects platelet haemostasis factors, but this potential effect on growth factor concentrations in platelet-rich plasma (PRP) has never been studied. In addition, there is a paucity of studies focusing on the effects of activating agents used in conjunction with PRP. The first aim of this study was to evaluate the effect of exercise on platelet and platelet-derived growth factors (PDGF)-AB, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) concentrations in PRP. The second aim was to study the effect of the activating agent calcium chloride ($CaCl_2$) on growth factor concentration in relation to different exercise states.

**Methods** Controlled laboratory study. Ten healthy participants performed 1 h of submaximal exercise with blood being withdrawn immediately pre, post and 18 h following. PRP was prepared in each condition in both an activated $CaCl_2$ and non-activated form. Concentrations of PDGF-AB, HGF, IGF-1 and VEGF were evaluated using standard ELISA systems.

**Results** Exercise had no significant effect on platelet concentration, but significantly suppressed both VEGF and PDGF-AB concentrations. Exercise state had no significant effect on IGF-1 or HGF concentration. Activation with $CaCl_2$ resulted in a significant increase in PDGF-AB and IGF-1 concentrations, unchanged VEGF and significantly reduced HGF concentrations.

**Conclusions** Exercise significantly impacts on PDGFs in PRP with significantly reduced concentrations of VEGF and PDFG-AB. Furthermore, the activation of PRP with $CaCl_2$ results in a differentiated GF release from platelets. These relevant factors can potentially influence outcome in daily clinical practice and are recommended to be accounted for in future study design.

## INTRODUCTION

A recent meta-analysis on the efficacy of the widely used autologous platelet-rich plasma (PRP) for 14 musculoskeletal indications showed conflicting evidence for its use, predominantly because of the shortcomings in standardisation of study protocols and confounding factors affecting the platelet application.[1] The authors strongly recommended basic science studies focusing on the optimal preparation, dosage, effects of activating agents and timing of injecting autologous blood products.

Despite its apparent clinical popularity and a steadily increasing number of clinical studies,[2–6] there is still only limited understanding of the role of the cellular and extracellular elements, optimal concentrations of platelets, leucocytes and released growth factor (GF) dose, timing and activation.[7–9] Platelet-derived GF (PDGF) are stored in α-granules found within platelets and are released in a selective manner upon activation.[10 11] Platelet activation is dependent on specific platelet membrane glycoproteins binding to ligands, kinase activation[12] and cytoplasmic calcium influx from both the dense tubular system and extracellular milieu,[13 14] and may be initiated in vivo by a range of factors including thrombin,[15] calcium, collagen[9 14] and shear stress.[16] Each platelet contains about 80 α-granules, which, in addition to GF, contain adhesive proteins, chemokines, fibrinolytic proteins and pro-coagulant molecules. In vitro, calcium and thrombin are routinely utilised to induce GF release from PRP; in clinical practice, preactivation of PRP is widely used.[17–19] However, evidence and consensus on the therapeutic requirement for preinjection activation is lacking.[20–24]

GF released from the α-granules of platelets are assumed to provide the regenerative benefits of PRP. Haematological studies have shown that exercise may affect platelet function with an increased release of platelet-derived pro-coagulant microparticles following exercise.[16 25] Subsequently, it is possible that exercise may also influence GF release from platelets, and thereby affect the clinical efficacy of the PRP.

The first aim of this study was to evaluate the effect of exercise on platelet and PDGFs PDGF-AB, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) concentrations in PRP. The second aim was to study the effect of the activating agent calcium chloride ($CaCl_2$) on GF concentration in relation to different exercise states.

## METHODOLOGY

### Participants

Eleven Caucasian male patients were recruited for the study after a thorough explanation of all the risks and benefits of participation in the study. The patients were subsequently excluded from analysis if they were suffering from any type of injury or were taking any medications that would impact on the platelet function. The study protocol was approved by our Institutional Medical Ethics Committee. All patients provided written informed consent.

### Experimental overview

Each participant was asked to report to the exercise laboratory having abstained from exercise for 36 h and following a 12 h fast (with the exception of water). Upon arrival at the controlled environmental laboratory (~21°C, 40–60% RH, 760–770 mm Hg), body mass and sports participation were recorded. Resting blood pressure was measured and venous blood (10 ml) sampled from an antecubital vein. Participants then undertook a modified[26] submaximal cycling test[27] on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands) to predict maximal aerobic capacity and peak power output (PPO). A single researcher supervised all cycling tests.

After 7 days, the initial laboratory procedure was repeated with the identical fasting and exercise abstention protocol. Prior to exercising, participants had 54 ml of blood withdrawn, which was immediately prepared as described below. The participants were then required to exercise at 50% of their estimated PPO (based upon the previous test) for 1 h. Immediately upon the cessation of exercise, a further 54 ml of blood was sampled. The participants were advised to abstain from exercise; 18 h following the exercise bout, each subject had a further 54 ml of blood withdrawn for analysis.

### Haematological analysis

The blood was sampled from the antecubital vein with 10 ml drawn into an EDTA-coated tube for immediate analysis of the whole blood platelet count. All platelet analysis was completed using the CELL-DYN 3700 SL analyser (Coulter Count; Abbot Diagnostics, Chicago, USA).

PRP was prepared using the manufacturer's instructions from the GPS III centrifuge separation system (Biomet Recover, GPS III Platelet Separation System). In a 60 ml syringe, 54 ml of whole blood was added to 6 ml of ACD-A anticoagulant, and immediately centrifuged for 15 min at 3200 rpm. The extraction of PRP and platelet-poor plasma (PPP) was completed based on the method outlined in the commercially available separation system.

The volumes of PRP and PPP were recorded and each sample was separated into 500 μl aliquots with a portion utilised for platelet concentration (PC) analysis as shown above. The remaining samples were either immediately stored at −80°C or underwent activation with $CaCl_2$ (25 mM) for 1 h at 37°C. Following the activation, the samples were centrifuged at 4000 rpm, 4°C for 10 min and the fibrin clot was separated from the supernatant. The supernatant was aliquoted and frozen at −80°C until analysis. GF levels of PDGF-AB, HGF, IGF-1 and VEGF in the PRP, both the non-activated and following in vitro activation, were evaluated by commercially available ELISA (R&D systems, Oxon, UK). All inter- and intra-assay coefficients of variation were <10%.

### Statistical analysis

All statistical analyses were performed using SPSS (V.19.0). Data were screened for outliers and deviation from normality, and hence no transformations were applied. In addition, different GFs (HGF, IGF, PDGF-AB and VEGF) in PPP and PRP, respectively, were analysed using a linear mixed model, including fixed effect terms for activation status (activated or not activated) and time group (0 h (resting), immediately postexercise and 18 h postexercise) and random effect terms for participants and residuals. The effect size (ES) was calculated as partial $\eta^2$. ES of 0.01, 0.06 and 0.14 indicate small, medium and large association, respectively. Where a significant effect was found, post

hoc pair wise comparisons were performed. Statistical significance was accepted at p<0.05.

## RESULTS

One participant was excluded from the study due to concurrently utilising a platelet-inhibiting medication (clopidogrel), and subsequently the data from 10 participants (mean age 34.3±3.2 years; mean body mass index 26.1±4.28 kg/m²) were analysed. All participants were physically fit and regularly engaged in moderate-to-intense physical exercise.

Mean PC in whole blood was within expected ranges (271.9±54.0×10³ μl), increased significantly in PRP (1065.3±4144.8×10³ μl) and decreased significantly in PPP (43.2±16.3×10³ μl). Mean white blood cell concentration (WBC) increased significantly from baseline whole blood levels (7.34±1.72×10³ μl) in PRP (34.06±11.06 × 10³ μl) and significantly decreased in PPP (0.04±0.04 × 10³ μl). In the PRP, PC was not associated with HGF, VEGF or IGF-1 concentrations. PC was positively associated with PDGF-AB (p<0.001); with every one unit increase in PC, a 25.2 unit increase is associated with PDGF.

### Effect of exercise state on PRP

There was no significant change in PRP PC from resting to the postexercise conditions (figure 1). The analysis showed that there was an overall effect of exercise state on PDGF-AB (F (2,41.9)=4.0; p=0.025; ES=0.311) and VEGF (F (2,42.3)=3.7; p=0.034; ES=0.456), with significant suppression of both VEGF (resting vs 1 h p=0.024; and vs 18 h p=0.001) and PDGF-AB (resting vs 1 h p=0.021; and vs 18 h p=0.003) concentration (figure 2). Exercise state had no significant effect on IGF-1 and HGF concentrations.
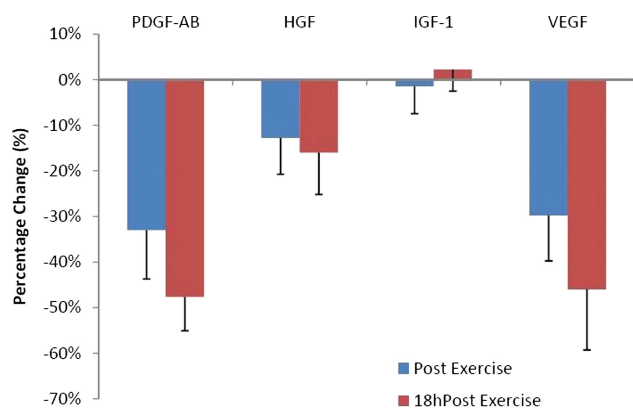
### Effect of activation state on PRP

There was an overall effect of activation state on PDGF-AB (F (1,41.5)=63.6; p<0.001, ES=0.886), HGF (F (1,42.1)=69.9; p<0.001, ES=0.752) and IGF (F (1,42.2)=11.3; p=0.002, ES=0.584), but not VEGF. The activation resulted in a significant increase in PDGF-AB (resting p=0.012; 1 h p<0.001; 18 h p<0.001) and IGF-1 (1 h p=0.022; 18 h p=0.010) concentrations. HGF concentrations were significantly reduced after activation (resting p<0.001; 1 h p<0.001; 18 h p<0.001). Postactivation, no significant differences were found for VEGF concentrations (figure 3).
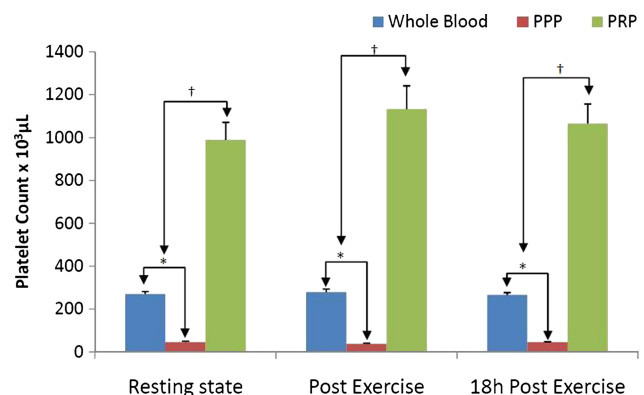


* Whole blood PC significantly higher than PPP (p<0.001)
† PRP PC significantly higher than Whole blood PC and PPP PC (P<0.001)

**Figure 1** Platelet concentration (mean±SE) of platelet-rich plasma (PRP) at resting state, immediately postexercise and 18 h postexercise.

**Figure 2** Effect of exercise state on platelet-rich plasma. Change in concentration of platelet-derived growth factors (PDGF)-AB, hepatocyte growth factor, insulin-like growth factor-1 and vascular endothelial growth factor (without preactivation) from resting state, immediately postexercise and 18 h postexercise (mean±SE). Compared with the resting state, percentage increase or decrease was calculated from average means for the 10 participants.

There was a significant interaction effect between activation status and time on PDGF-AB (F $(1,41.5)$=63.6; $p<0.001$; ES=0.312) and VEGF (F $(2,42.1)$=4.1; $p=0.023$; ES=0.398).

## DISCUSSION

This investigation suggests that exercise may influence GF concentration in PRP, and that the activation of PRP with $CaCl_2$ results in a differentiated GF release. These are important observations since the assumption is that the GF present in PRP provides the regenerative stimulus to tissues. However, we still have only a limited understanding of the multitude of factors, which may impact on GF concentrations, and subsequently the outcomes of treatment with PRP.[8][9][28]

The platelet counts, WBC and factor of increase of platelets observed in this study are comparable with earlier reports where the same separation system was used.[28][29] Levels of 600 000–1 000 000 platelets/μl are considered as being appropriate for PRP, but there is little clinical evidence as to what level is optimal.[17][30–33] In contrast to the previous studies,[9] our finding

that platelet count in PRP was not associated with three of four GF concentrations, casts doubt on the clinical relevance of absolute platelet count. Of note, the WBC was observed to increase in this preparation of PRP. The merits of either elevated or reduced WBC in PRP remain clinically indeterminant, and may ultimately depend on the clinical indication.[28]

While previous authors have reported a 25% increase in PC in whole blood immediately following strenuous exercise, no significant change was found in either whole blood or PRP PC following our exercise protocol.[25] We did, however, observe a significant effect of exercise on GF concentrations within PRP. It is well recognised that platelet α-granules are heterogeneous in their content, and that different stimuli may result in differential granule and factor release.[10][11][34] Furthermore, it has been previously illustrated that in response to the exercise-induced shear stress, there will be an increase in platelet-derived factors involved in haemostasis.[16][25] Our finding that exercise significantly reduced the circulating concentrations of PDGFs, VEGF and PDFG-AB, but had no effect on HGF concentrations, supports this differential release of contents from α-granules. IGF-1, which is not a primary constituent of platelet α-granules, was also unaffected by strenuous exercise. Based on this finding, it would appear that exercise may be a confounder to α-granule release and exercise proximity should to be considered when preparing PRP and reporting clinical outcomes. It remains to be determined if a change in GF concentration of the magnitude demonstrated will be clinically relevant. Although quality evidence for the clinical use of PRP for chronic degenerative tendon indications is lacking,[1] the potency of GFs in acute muscle injuries has not been studied yet and remains unknown. Factors such as the timing, dose and relative proportions of GF may be relevant;[32][35][36] anything that modifies the GF content of PRP may have a confounding effect on outcomes.

Both the requirement and rationale for preinfiltration activation of PRP in the clinical setting remain controversial. One school of thought suggests that activation prior to the application ensures optimal levels of GF release,[37] while the contrasting argument suggests that the platelets will respond to the in vivo environment and release appropriate GF. While each of these arguments has a theoretical basis, there remains no clinical evidence to delineate an answer. Our data imply that the
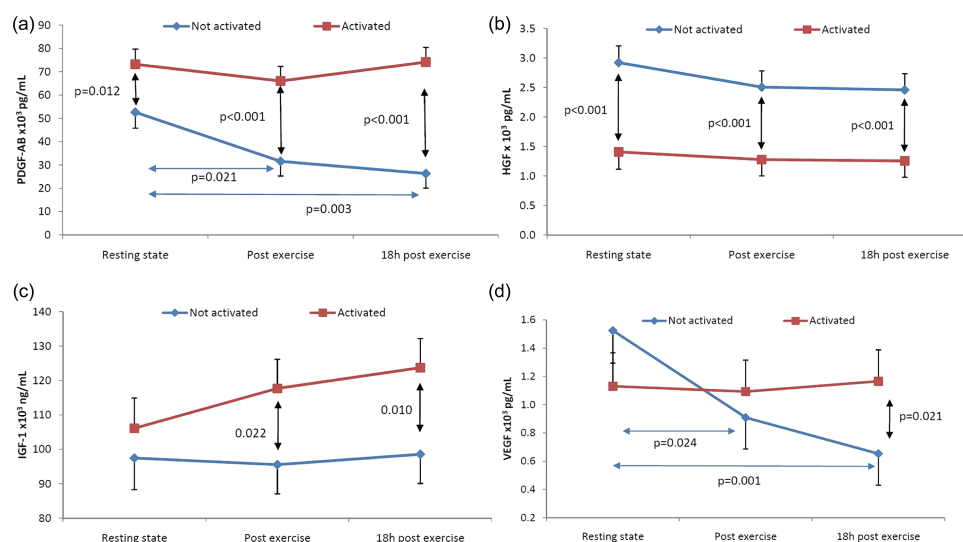


**Figure 3** Effect of activation state. Platelet-derived growth factor (PDGF) concentrations PDGF-AB (A), hepatocyte growth factor (B), insulin-like growth factor-1 (C) and vascular endothelial growth factor (D) for the inactivated and activated state (mean±SE).

activation of PRP with $CaCl_2$ results in a specific cellular response, with increased plasma concentrations of PDGF-AB and IGF-1, but paradoxically reduced concentrations of HGF and had no effect on VEGF concentrations. The cause of the paradoxical reduction in HGF concentration remains unknown. Although it cannot be excluded that HGF containing α-granule release was inhibited by $CaCl_2$, this would seem unlikely. We cannot rule out a technical error and further studies are required to test the reproducibility of the presented data. Similarly, IGF-1 is typically not considered a constituent of α-granules. The mechanism by which activation with $CaCl_2$ increased the IGF-1 concentration remains to be determined, but may be considered an extraplatelet effect. From a clinical perspective, the differential release of GF has no relationship to the clinical milieu in which the PRP will subsequently be infiltrated. A further study to delineate the results of preinfiltration activation with respect to the in vivo demands of the tissue is required.

An obvious limitation of this study is that only four GFs in 10 male participants were evaluated. Nonetheless, these GFs are among the most extensively studied[9][37] and thought to play a prominent role in the regeneration process. With the chosen study design, it cannot be excluded that the fasting state, which was part of the resting state protocol, might have influenced GF concentrations. A control group without fasting is needed to clarify its potential confounding influence. A further confounder in this study may be that some GFs remained in the activated clot and were therefore not evaluated in the supernatant, thereby resulting in an underestimate of the actual GF release. The differential GF concentrations and a wide range of concentrations in response to both exercise and activation with $CaCl_2$ suggest that further research in this area is required. As platelet function and effectiveness might be influenced by hormonal factors, validity in a female population remains unknown. We would recommend that in any future study design, exercise state be clearly defined.

## CONCLUSION

This study illustrates that exercise may impact upon GF concentrations in PRP, with significantly reduced concentrations of VEGF and PDFG-AB. Furthermore, the activation of PRP with $CaCl_2$ results in a differentiated GF release from platelets. These potentially confounding factors may influence clinical outcomes and should be accounted for both in future study design and when assessing clinical efficacy.

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### What are the new findings?

► This study illustrates that exercise may suppress both vascular endothelial growth factor and platelet-derived growth factor-AB concentrations in PRP, thereby illustrating the importance of knowing the exercise state of subjects and patients when observing and reporting clinical outcomes.

► Furthermore, activation with $CaCl_2$ resulted in a differential, non-uniform alteration in GF concentration. This latter finding is an important consideration for those researchers trying to establish the mechanisms behind any PRP efficacy, and highlights the complexity of this biological tool.

► This novel study adds important information for clinicians and researchers utilising PRP, and highlights the need for further detailed in vivo and in vitro research in a larger population.

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### How might this study impact on clinical practice?

In the future, the application of GF in the form of autologous blood products should be targeted to specific GFs at precise times in the healing process. This study highlights that exercise may affect the GF concentration, and therefore efficacy of PRP, and this may need to be considered in the future.

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