Interleukin-6, Creatine Kinase, and Antioxidant Enzyme Activities following Platelet-Rich Plasma Treatment on Muscle Injury: A Pilot Study

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Abstract:

The aim of this study was to investigate the effect of autologous platelet-rich plasma (PRP) treatment alongside rehabilitation compared with rehabilitation alone on inflammatory cytokine (interleukin-6, IL-6), creatine kinase muscle type (CKM), and antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT) following hamstring injury. This study was a randomised control trial. Participants diagnosed with grade-2 acute hamstring injury (n=16) were divided into 2 groups of PRP treatment with rehabilitation program (PRP-T) and rehabilitation program (CON). Blood samples were collected at baseline, and 2 fortnightly for the various biochemical assessments. Participants were certified to have recovered upon fulfilling return to play (RTP) criteria. Level of IL-6 and the activities of CKM, SOD, and CAT were measured. PRP-T group benefited from earlier time to RTP with significantly lower IL-6 level and CAT activity compared to CON group. There was no significant difference in CKM and SOD activities between the groups, though a trend of lower values in all variables was observed at week 4 compared to week 0. PRP treatment potentially improves muscle healing process by altering both the inflammatory and oxidative responses, hence hastens time to RTP.

KEY WORDS: Autologous, blood injection, rehabilitation, sports injury, hamstring injury

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Introduction

Musculoskeletal injuries are extremely common in sport which requires rapid acceleration, deceleration, and jumping. An injured athlete could account for significant functional impairment which leads to withdrawal from sport participation [1]. Regardless of the injury mechanisms, skeletal muscle healing progresses through a series of organised overlapping phases, such as haemostasis, inflammation, proliferation, and remodelling [2].

In the early stage of muscle injury, leakage of sarcomeric proteins such as creatine kinase (CK) and myoglobin promote infiltration of neutrophils, leukocytes, and macrophages into the injured area [3]. These responses will subsequently trigger the secretion of cytokines (i.e. interleukin-6, IL-6) which could remain active up to months in order to clear tissue debris and protect the damaged tissues from infection [4]. However, IL-6 does not necessarily represent the presence of muscle injury per se [5], rather it is involved in the latter phase of muscle regeneration mechanism [6].

During the healing process, cell metabolism by-products, such as peroxides are continuously being formed [7]. The increase in metabolic rate causes increase in free radicals which in turn lead to secondary tissue damage [8]. In response to the increased free radicals, the activities of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) are elevated [9].

In order to optimise muscle regeneration, it is essential to limit the formation of muscle fibrosis and secondary tissue damage. Hence, a variety of complementary treatments such as non-steroidal anti-inflammatory drugs (NSAID) and platelet-rich plasma (PRP) have been prescribed alongside common conservative rehabilitation program consisting of rest, activity modification, and physiotherapy [10, 11]. Although NSAID is the most common treatment that attenuates acute muscle inflammation, it has been shown that combined physiotherapy alongside NSAID actually delayed muscle regeneration, with no additive healing effect [12].

Instead, the use of autologous PRP has emerged as an effective and safe treatment due to its healing properties that could ameliorate inflammatory mediators [13], and subsequently enhance muscle recovery [14], alongside rehabilitation program. Earlier laboratory study of Wright-Carpenter et al. [15] found significant increase of satellite cell and size of regenerating myofibril in the autologous conditioned serum treated mice. According to Dimauro et al. [16], PRP could enhance secondary pathways of regeneration process through a balance of proliferative and inhibitory effects. Acceleration of functional restoration was also found in recent human trials injected with ultrasound-guided PRP following muscle injury, where the athletes recovered rapidly and returned to sport without any evidence of excess fibrosis [17, 18].

With the increased popularity of PRP treatment for muscle injury, there is a need for a randomised control trial (RCT) to examine its effect on muscle regeneration factors during the healing process. Thus, the aim of this pilot study was to investigate the changes in the level of a single representative inflammatory cytokine (IL-6), creatine kinase muscle type (CKM), activities of endogenous antioxidant enzymes response to PRP treatment following a hamstring injury, and time to return to play (RTP).

Materials and Methods

Participants

Participants (n=16) were recruited from the Sport Medicine Clinic of University of Malaya
Medical Centre. The inclusion criterion was male and female patients above 18 years old diagnosed with acute (within 7 days from date of injury) grade-2 hamstring strain. Patients who had received other forms of treatment during that time and/or unable to comply with scheduled follow-up assessments were excluded.

**Study design**

The present study was a single-blind (assessor blinded) RCT approved by the University of Malaya Medical Centre Medical Ethics Board (ref no.: 907.25) which complies with the declaration of Helsinki.

**Injury identification**

Patients with injured lower limb were clinically assessed by a physician using palpation, inspection, and functional tests. Those suspected with acute hamstring injury were further diagnosed using ultrasonography (Philips IU 22 ultrasound with 17-5 MHz Probe) by an experienced musculoskeletal radiologist. Identified hamstring injury was graded based on classification as described by Peetron et al. [19]. Patients diagnosed with grade-2 acute hamstring injury were invited to participate in the study.

**Study intervention**

The nature and objective of the study was explained to all participants and written consent was obtained prior to participation. They were randomly allocated into PRP treatment and rehabilitation program (PRP-T) or rehabilitation program (CON) groups through a computer-generated block randomisation list prepared by an independent researcher who had no clinical involvement in this trial. The PRP-T group received a single injection of autologous PRP immediately after randomisation, alongside a rehabilitation program; whereas the CON group prescribed only rehabilitation program. Participants in both groups received the same rehabilitation program, which focused on progressive agility and trunk stabilisation (PATS) exercises (Table 1) as recommended by Sherry et al. [11].

All participants were required to attend a weekly follow-up assessment and rehabilitation session until recovery. A standard clinical examination to assess the participant’s readiness for RTP was performed by a sports physical therapist who was blinded to the study. Throughout the study, the use of non-steroidal medication was prohibited.

Blood samples were collected at baseline (week 0; W0), and 2 fortnightly follow-up visits (week 2; W2 and week 4; W4). Blood was drawn from the antecubital vein into serum separator tubes (SST), allowed to clot, and then centrifuged at 2000 xg for 15 min. The serum was separated and kept at -80°C until assayed. Whole blood was collected in K2 EDTA vacutainer tubes and stored at 4°C for biochemical analysis.
Table 1. The Progressive Agility and Trunk Stabilisation (PATS) exercises

<table>
<thead>
<tr>
<th>Phase 1</th>
<th>Sets</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Low to moderate intensity side-stepping</td>
<td>3</td>
<td>1 min</td>
</tr>
<tr>
<td>2. Low to moderate intensity grapevine stepping, both directions</td>
<td>3</td>
<td>1 min</td>
</tr>
<tr>
<td>3. Low to moderate intensity forward and backward stepping over a tape line while moving sideways</td>
<td>2</td>
<td>1 min</td>
</tr>
<tr>
<td>4. Single-leg stand progressing from eyes open to eyes closed</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>5. Prone abdominal body bridge</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>6. Supine extension body bridge</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>7. Side bridges, left and right side</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>8. Ice therapy in sitting position</td>
<td>-</td>
<td>20 min</td>
</tr>
</tbody>
</table>

*Phase 2

<table>
<thead>
<tr>
<th>Phase 2</th>
<th>Sets</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>9. Moderate to high intensity side-stepping</td>
<td>3</td>
<td>1 min</td>
</tr>
<tr>
<td>10. Moderate to high intensity grapevine stepping, both directions</td>
<td>3</td>
<td>1 min</td>
</tr>
<tr>
<td>11. Moderate to high intensity forward and backward stepping while moving sideways</td>
<td>2</td>
<td>1 min</td>
</tr>
<tr>
<td>12. Single-leg stand windmill touches</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>13. Push-up stabilisation with trunk rotation</td>
<td>2</td>
<td>15 reps</td>
</tr>
<tr>
<td>14. Fast feet in ground</td>
<td>4</td>
<td>20 sec</td>
</tr>
<tr>
<td>15. Proprioceptive neuromuscular facilitation trunk-pull downs (along with Thera-Band), right and left</td>
<td>2</td>
<td>15 reps</td>
</tr>
<tr>
<td>16. Symptoms free practice</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17. Ice therapy if any symptoms of pain</td>
<td>-</td>
<td>20 min</td>
</tr>
</tbody>
</table>

Key: Low intensity, a velocity of movement that is less than or near that of normal walking; moderate intensity, a velocity of movement greater than normal walking but not as great as sport; high intensity, a velocity of movement similar to sport activity.

*Participants were allowed to progress to Phase 2 PATS exercises after the sport physiotherapist has assessed their recovery progression without symptom of pain.
**PRP preparation and injection**

A total of 54 ml blood was drawn from the antecubital vein into a 60 ml syringe primed with anticoagulant citrate dextrose solution, solution A (ACD-A). PRP was harvested using the gravitational platelet separation system GPS™ III (Biomet Biologics, Inc., Warsaw, Ind), based on the manufacturer’s instruction. Approximately 6 ml of PRP was obtained and sodium bicarbonate (8.4%) was added into it. No activating agent was added to the PRP. Using aseptic technique, 3 ml of PRP was infiltrated using an 18G needle into the injured region under US guidance. The injected region was then iced for 15 min.

The amount of platelets and white blood cell (WBC) contained in PRP (1297 x 10³ /µl and 38.3 x 10³ /µl) was significantly higher than the baseline blood (234 x 10³ /µl and 7.3 x 10³ /µl, respectively). Hence, PRP harvested and used in the present study was classified as P4-x-A according to the PAW classification system [20].

**Recovery assessment**

Recovery was determined by the time to RTP, where participants were required to fulfil the RTP criteria recommended by Hamid et al. [17], before they were allowed to recommence their pre-injury level of activity. Hamstring palpation was carried out and those who showed no symptoms of pain was examined with a pain provocation assessment where they performed an isometric hamstring contraction by flexing their knee at 15° in a prone lying position [21]. Participants without any symptoms of tenderness proceeded to the hamstring range of movement (ROM) assessment using the active knee extension test [22]. Participants who were symptom-free and had regained full ROM performed a hamstring muscle strength assessment using an isokinetic dynamometer (System 4 Pro, Biodex Medical System, NY, USA). The test was performed at 3 angular speeds: 60, 180, and 360 °/second with 2 min rest in between. Participants were considered fully recovered when they were symptom-free, had regained full range of motion and ±10% hamstring strength (peak torque) of the uninjured side.

**Determination of biological markers in serum**

Stored serum obtained was thawed and assayed using commercially available enzyme-linked immunosorbent assays (ELISA) kits: IL-6 (Cusabio Biotech Co., Ltd., Wuhan, China), creatine kinase muscle type (CKM) (Cusabio Biotech Co., Ltd., Wuhan, China) and SOD (Northwest Life Science Specialties, LLC, Vancouver, WA), according to the manufacturer’s guidelines. Those plates were pre-coated with a specific monoclonal antibody directed against human IL-6, CKM and SOD, and a polyclonal antibody conjugated to horseradish peroxidase was used for sensitive colorimetric detection at 450 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, Finland). The inter-assay coefficients of variations were <10% for both IL-6 and CKM, and 5.8% for SOD. Samples were analysed in duplicate and the results were expressed as pg/ml, µl/ml, and ng/ml, respectively.

Erythrocyte CAT activity was measured using UV-VIS scanning spectrophotometer (Shimadzu UV-1800, Japan) by measuring the decomposition rate of hydrogen peroxide (H₂O₂) at 240 nm as described by Aebi [23]. The rate constant was determined based on the exponential decay of H₂O₂ and corrected using sodium azide (NaN₃) as CAT inhibitor.

**Statistical Analysis**

Sample size was calculated using the Power and Sample Size software [24], where power
was set at 0.80 with alpha value of 0.05. Delta and sigma values were matched to the earlier pilot study by Wright-Carpenter et al. [14].

All statistical analyses were performed using the Statistical Package for the Social Science version 19.0 (SPSS Inc, Chicago, IL, USA). To analyse the changes over time, data was analysed using two-way repeated measures ANOVA. Bonferroni post-hoc test was used to test for significant differences. Effect size was also determined using the same software. The Shapiro-Wilk test was employed for assessing normality of the distribution of scores (p>0.05). Variables are presented as mean (SD). The significant level was set at p<0.05.

### Results

#### Participants’ characteristics

Sixteen participants diagnosed with a grade-2 hamstring injury participated in present study. The mean age of participants was 24.06 (4.95) years. More than two thirds (68.75%) of the participants were male. Majority of the participants were national athletes (62.5%), while the remaining were state/club/school athletes. The mean time to RTP was 25 (6.87) days and 32.88 (18.43) days for the PRP-T and CON groups, respectively. No significant difference in baseline characteristics and time to RTP between the groups was noted (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Anthropometric description of participants</th>
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<tbody>
<tr>
<td><strong>Groups</strong></td>
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<tr>
<td><strong>Description</strong></td>
</tr>
<tr>
<td>Age, y, mean (SD)</td>
</tr>
<tr>
<td>Weight, kg, mean (SD)</td>
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<tr>
<td>Height, m, mean (SD)</td>
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<tr>
<td><strong>Sports, n (%)</strong></td>
</tr>
<tr>
<td>Athletics</td>
</tr>
<tr>
<td>Soccer</td>
</tr>
<tr>
<td>Others (basketball, hockey, marathon, netball, rugby, tennis)</td>
</tr>
<tr>
<td><strong>Level of participation, n (%)</strong></td>
</tr>
<tr>
<td>National</td>
</tr>
<tr>
<td>Others (state, club, school)</td>
</tr>
<tr>
<td>Time to RTP, days, mean (SD)</td>
</tr>
</tbody>
</table>

**Changes in IL-6 level**

A significant difference of IL-6 level between groups was observed, where the IL-6
level in the PRP-T group was 29.77% lower compared to CON group at W2 ($p=0.032$) (Figure 1). There was no significant change in activity of CKM between PRP-T and CON groups, though there was an apparent (not significant) increase in CON group during W2 (Figure 2).

**Activities of antioxidant enzymes**

The CAT activity was 37.22% lower in the PRP-T group compared to CON group in W2 (Figure 3A). In the PRP-T group, there was a trend of negative progression with time, whereas the CON group showed a significant rise of 31.47% in CAT activity at W2. In contrast, although there was a reduction in SOD activity in both groups, there were no statistically significant differences between groups throughout the assessment time frame (Figure 3B).

![Figure 1](image1.png)

**Figure 1:** The IL-6 level at W0, W2, and W4. *Significant difference between groups.

![Figure 2](image2.png)

**Figure 2:** The CKM activity at W0, W2, and W4.
Discussion

The present study is the first RCT study investigating the effect of PRP treatment on inflammatory cytokine, CKM, and antioxidant enzymes activity in hamstring injury. The main finding showed that PRP treatment alters IL-6 and CAT responses. The PRP-T group also showed an acceleration of recovery by about 8 days compared to CON group.

The IL-6 level was found to be significantly lower in the PRP-T group at W2 compared to CON group. It has been established that IL-6 is produced by muscle cells in response to muscle injury [25], and it is actively involved in the regulation of inflammation during regeneration phase [26]. Therefore, a reduction of IL-6 level in the PRP-T group is in line with past studies which suggest autologous blood product affect anti-inflammatory reactions during the healing process [13]. The healing potential of PRP has been attributed to secretion of bioactive proteins from the concentrated platelets, which enhance pro-stimulatory effect during the cellular proliferation phase, and at the same time, regulates the release of monocyte-mediated pro-inflammatory cytokines [27].

Increased activity of neutrophils and phagocytes due to muscle injury leads to higher productions of oxygen by-products such as H$_2$O$_2$, corresponding with the increase in oxygen consumption [9]. Elevation of H$_2$O$_2$ could have resulted from cascade of activities, where the reduction in superoxide radicals is coupled with the increase in peroxide production, which in turn requires CAT to catalyse the breakdown of H$_2$O$_2$ into water and oxygen [7]. In the present study, CAT activity in PRP-T group was significantly lower compared to the CON group (by 37.22%), which implies that the H$_2$O$_2$ production from inflammatory (IL-6) responses was lower upon PRP treatment. The CAT activity in the PRP-T group is in agreement with an earlier research that showed that autologous blood product could activate antioxidant response elements (ARE) through nerve related growth factor (Nrf) [28]. Nrf1-ARE is an important mechanism that detoxifies ROS, which was mainly produced during the muscle regeneration process [29]. Tohidnejad et al. [28] investigated the effect of Nrf1-ARE pathway by utilizing autologous blood product and they found that ARE

![Figure 3A & 3B: Activities of antioxidant enzymes at W0, W2, and W4.](image_url)

* Significant difference between groups.
† Significant difference from baseline.
pathway is an effective system in regulating the inflammatory responses, and subsequently, enhances muscle regenerative process.

In the present study, although CKM (a muscle type isozyme of CK found exclusively in striated muscle and involved in cellular energetics) was not statistically different between both groups, its activity in CON group tend to be higher than PRP-T group, which indicates that systemic responses towards muscle damage and resolution could have taken place [30]. It has been reported that elevated CKM activity is associated with muscle damage, and is most pronounced between 2 to 7 days following an injury [31]. Although Serrao et al. only examined CKM responses for 7 days, the CKM activity could remain elevated for weeks due to tissue repair mechanisms, hence the CKM activity found in present study should not be just written off as completely invaluable.

Our data showed that CKM activity was relatively lower in the PRP-T group at W2 (difference of 16.75% compared to CON group; \( \eta = 0.24 \) large effect size); clinical practitioners could potentially use this finding when deciding the progression of muscle recovery from initial injury.

The presence of SOD could indicate secondary tissue damage [9], and is reported to increase following a muscle injury [32]. However, the present study revealed no difference in SOD between groups, albeit the SOD activity was still higher compared to normal healthy people (which is typically less than 20 ng/ml) [33]. The pattern of SOD activity change over time was similar in both PRP-T and CON groups, where it showed progressive reduction across time. Thus, PRP treatment does not seem to affect SOD activity.

The present study has some limitations. Although the number of participants enrolled was limited by time, patient availability and suitability, the present study employed an RCT design compared to previous studies which were either case control or non-randomised studies [14, 34], and the minimum sample size of 16 in the present study was estimated based on previous study. A future study with more biological markers and frequent assessments, along with ultrasound illustration of healing dynamic is recommended as this would provide essential information.

Conclusions

In conclusion, PRP treatment in present study showed significant attenuation of both the inflammatory cytokine (IL-6) and antioxidant enzyme activity (CAT) in muscle injury. This is suggestive that ameliorated IL-6 level and CAT activity could minimize secondary muscle damage, thus improved muscle healing process. Although the time to RTP in the present study did not show significant difference between group, time to RTP in PRP-T group was hastened by about 8 days (large effect size; \( \eta = 0.27 \)) compared to CON group, which could be an important decisive information in prescribing this treatment to the injured elite athlete.

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