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Original Research Article

SINGLE DOSE OF INTRA-MUSCULAR PLATELET RICH PLASMA SUPPRESS THE INCREASE IN PLASMA HEPCIDIN LEVEL: PROTECT ROLE IN EXERCISE INDUCED IRON LOSS?

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Abstract: Background: In general, platelet rich plasma (PRP) injection treatment is used for skeletal muscle injury. PRP is regulated tissue regeneration by controlling autocrine and paracrine biomolecules including growth factors. We recently have reported that PRP administration reverses in plasma iron levels response to post-exercise recovery days. As a continuation of this study that PRP can be affected on iron regulated hormone plasma hepcidin levels during the post-exercise inflammation process. The exercise-induced muscle damage exercise (EIMDE) used as an acute model for muscle inflammation. The purpose of this investigation the effects of intramuscular delivery of PRP on hematologic and hepcidin responses and recovery strategy muscle inflammation induced by exhaustive muscle damage exercise.

Methods: Volunteers were assigned to a control (n =6) and PRP application (PRP, n=6) and they performed exhaustive exercise with maximal voluntary contraction of the elbow. Then, saline or PRP injections was applied on painfully arm of the subjects. Blood samples were obtained in the morning to establish a baseline value and also following the injections 1., 2.,3. and 4 days post-exercise.

Results: The baseline levels in white blood cells (WBC), neutrophils (Neut), lymphocytes (Lymph), red blood cells (RBC), hemoglobin (Hb), and hepcidin levels were similar in both saline and PRP injections group. However, 24-hour following exercise a significant increase in Hb, WBC, Neut, hepcidin were observed in control during the recovery days. Interestingly, PRP administration inhibited effects on these parameters.

Conclusion: PRP administration improved inflammation by suppressing the increase hepcidin level in the post-exercise and it may favourable effect in the exercise induced iron losses.

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Introduction: Exhaustive physical exercise results in a number of unfavourable changes, such as a prooxidant shift in the prooxidant-oxidant balance, hyperthermia, metabolic acidosis, hypoglycemia, and hemoconcentration [1,2]. These processes contribute to a decrease in the osmotic resistance of erythrocytes

resulting in greater susceptibility to haemolysis. This results in an increase in the free iron level, which not only induces free radical-mediated processes, but also enhances inflammation [3,4]. Increases in plasma and/or urinary hepcidin levels have been consistently reported in response to a single bout of prolonged and intense exercise in humans [5-8], an increase is noted at the beginning of the exercise and a peak is reached during the early recovery period [6]. Hence, it has been suggested that exercise enhances hepcidin synthesis and inhibits iron absorption. A major advance in recent years has been the identification of hepcidin, as an acute-phase reactant, and a key regulator of whole-body iron homeostasis and it is likely that hepcidin acts beyond the acute phase [9]. However, only a few studies have investigated the acute post-exercise kinetics of hepcidin and its association with iron metabolism in athletes [4, 6, 7]. Most studies have shown an elevated hepcidin level 24 h post-exercise, preceded by acute increase of serum iron and inflammation parameters [4,6,7].

Exercise-induced muscle damage and inflammation in both human and animal studies is well-described in the literature [10-13]. It has also been shown that damaged muscle will induce an inflammatory response [14]. The effects of acute exercise on WBC trafficking and function have been extensively studies and it is accepted that acute exercise increases the number of circulating WBC which causes a temporary depression on several aspects of immune cell function [13]. Moreover, the symptoms of EIMD include muscle weakness and delayed- onset muscle soreness (DOMS) and this may have a negative impact on performance [15, 16]. Nevertheless, among athletes, it is usual practice to search for strategies, including pharmacologic ones, to alleviate muscle discomfort and impairment of performance quality. However, the effectiveness of most of these resources is controversial, with some being potentially unfavourable to adaptive responses to training [17, 18]. However, the studies have shown that the inflammations and oxidation-reduction (redox) homeostasis play a role in muscle repair and regeneration [15,16,19].

Generally, platelets are not active when it becoming active players in haemostasis and wound healing [20,21]. In regard to, the therapeutic effect of PRP is consisting of various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor β, fibroblast growth factor, insulin-like growth factor-1, 2 (IGF-1, IGF-2), vascular endothelial growth factor, epidermal growth factor, and also some cytokines [22] primarily stored in alpha granules. Accordingly, PRP is widely applied to help treat injuries to tendons, ligaments and muscle. The physiological mechanism of the PRP is a rich source of growth factors and cytokines, which should aid the healing process and reduce inflammation. Evidently, we reported that post-exercise PRP administration improves inflammation by reversing the increase in free iron levels and suppressing the plasma ferritin levels during the recovery phase compared to control values [23]. However, information regarding the physiological effects of locally administered intramuscular PRP has not yet been elucidated in exercise induced muscle damage and inflammation. Therefore, as a continuation of this study, we hypothesized that PRP may affect the iron regulated hormone plasma hepcidin levels during the post-exercise inflammation process. Thus in this study the effects of intramuscular delivery of PRP on hematologic and hepcidin responses on muscle inflammation induced by high intensity muscle damage exercise is reported.

Materials and Methods

Participants

Twelve moderately active male volunteers participated in this randomized double-blind placebo-controlled trial to verify the effects of the intramuscular PRP administration on hematologic and hepcidin level on muscle recovery after an eccentric/concentric exercise. Subjects were randomly divided into two groups: PRP (n=6, mean age 23±3 year, body weight 94 ± 6.9 kg, height 183.6 ± 3.2 cm) and control (n=6, mean age 22±2 year, body weight

86 ± 7.8 kg, height 177.6 ± 8.7 cm). The subjects informed that they have no any injury of the arm and shoulder area and also have not been participate in any regular weight-training program. The possible risks of the study procedures were explained to the subjects, and signed informed consent to participate in this study was collected. This study Ethical approval was established from The University Medical Faculty Ethics Committee (2013/14) and each subject assigned written informed consent prior to the study.

Muscle damage exercise protocol: The exercise-induced muscle damage test was performed as previously described by Punduk et al. [23], the subjects were seated on a dumbbell bench, their arm was positioned in front of their body and rested on a padded support, such that their shoulder was secured at a flexion angle of 0.79 rad (45^0) and their forearm was maintained in the supinated position throughout the exercise. Subjects were repeatedly weight-loaded upon dumbbell lowering to achieve an 80% of maximum voluntary contraction (MVC), 2-min rest between the sets of elbow extension from the flexed position at 90^0 to fully extended position slowly over 5 s, until exhaustion was experienced. The control group performed a mean average number of repetitions and number of set (47±6 and 13±3) respectively whilst the PRP group performed a mean average number of repetitions and number of set (44±3 and12±2) respectively. The subjects were also given verbal encouragement by the investigator to maintain constant speed throughout the procedure. The volunteers were instructed to continue with their normal activities and to abstain from any strenuous exercise at least 2 weeks prior to the commencement of the study. Moreover, they were asked to continue with their usual food intake, not to change the amount or frequency of dietary meat and not to use any dietary supplements, anti-inflammatory drugs, or anything else that could affect muscle soreness and damage until the end of the study.

Platelet-rich plasma and placebo

Each participant was allocated to either receive a control (saline) or PRP group, injection in the non-dominant arms with post-24 h DOMS exercise based on computerized randomization. PRP preparation method was used as previously described by Punduk et al. [23] For PRP blood taken from the dominant arm (8 mL). Then, the blood samples were centrifuged for 9 min at 3500 revolutions per minute (H-19F, RegenCentrigel, Regen ACR-C; Regen Lab, Switzerland) according to manufacturers recommendation. Subsequently, 4 mL of PRP was obtained and this plasma injected using a 20-gauge needle into the pain full region of the non-dominant arm under sterile aseptic conditions.

Biochemical analysis: The blood samples were put into tubes containing K3-EDTA and were measured to flow-cytometry for count of the RBC, WBC, Neut, Lymph, Platelets and Hb concentration using fully automated Blood Cell Counter Gen-S (Beckman Coulter, Coulter Corporation, USA). Serum was separated by centrifuging at 825 g for 10 min and stored at -80°C for analyses of hepcidin. Serum level of hepcidin was determined by enzyme-linked immuno-sorbent assay (ELISA) using commercially available kits (Shanghai Sunred Biological Technology Company,) on a diagnostic instrument (Thermo Scientific – Varioskan Flash Multimode Reader, Finland).

Statistical analysis: All values of the calculations were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The values of serum hepcidin level were presented as raw values as area under the curve (AUC) during the experimental period. The AUC was calculated as the sum of four or five trapezoid areas separated by each supplement time point Two-way mixed model analyses of variance (2groups X 5 times) by repeated measures. Differences in continuous variables between groups were assessed using independent *t* test. Data is expressed as means ± SD and the level of significance was set at $p < 0.05$.

Results: There was no significant difference in body weight, height, age and exercise performance between the PRP and CONTROL group. Previous published study has shown that PRP administration decreased free iron levels (

p= 0.002) compared to the control group but this was only observed on the day 2 and 3 post-exercise [23]. Also, exercise induced increased plasma ferritin levels; however PRP had no effect on ferritin [23]. As a continuation of this study result of the white blood cells (WBC), neutrophils (Neut), lymphocytes (Lymph), red blood cells (RBC), hemoglobin (Hb) and hepcidin levels were similar in both the control and PRP group (p <0.05, Table 1). However, 24 h post-exercise a significant decrease in the level of the WBC on the first and second day (p> 0.05, Table 1) was observed during the recovery period when compared to the baseline values in the control group. In control group, Neut levels were decreased on the first day of the post-exercise level while Hgb levels were increased during the recovery days (p> 0.05, Table 1). Moreover, exercise induced up-regulated the level of the hepcidin in both control and PRP group (p=0.001, Fig. 1) during the recovery days. However, PRP administration suppressed the exercise induced increase in hepcidin level, especially on the third day of the recovery period (P=0.001, Fig. 1) and similar pattern of WBC, Neut and Hb levels was observed.

Table 1. Effect of intramuscular PRP injection on haematological parameters.

			Post-exercise days			
Measure	***Group***	***Baseline***	***Day 1***	***Day 2***	***Day 3***	***Day 4***
RBC $10^3/\mu L$	Control	5.30 ± .18	5.1 ±.21	5.2 ±.17	5.4 ± .26	5.4 ± .22
	PRP	5.18 ± .14	5.1 ± .13	5.2 ± .19	5.0 ± .13	5.0 ±.12
Hb *g/dL*	Control	14.5 ± .70	14.9 ± .85[a]	14.8 ± .69[a]	14.9±.60[a]	15.0 ± 1.25[a]
	PRP	15.4 ± .28	15.4 ± .30	15.6 ±.29	15.0 ± .66	14.7 ± .33
WBC $10^3/\mu L$	Control	6.4 ±.55[a]	5.6 ± .41[a]	5.6 ± 1.13[a]	5.5 ± .54	5.5 ±.79
	PRP	7.3 ± .92	6.5 ± .58	6.6 ± .56	6.0 ± .71	5.6 ±.61
Neut	Control	4.34 ±. 61[a]	3.66 ± .48[a]	3.52 ±.47	3.44 ±.42	3.20 ±.49
	PRP	4.56 ±.63	4.12 ±.39	4.0 ±.42	3.84 ±.49	3.44 ±.46
Lymph	Control	1.7 ±.16	1.70 ±.17	1.54 ±.16	1.74 ± .16	2.0 ±.34
	PRP	1.86 ±.20	1.86 ±.19	2.0±.24	1.78 ±.28	1.7 ±.18
Platelets $10^3/\mu L$	Control	252 ± 11	226 ± 7.7	238 ± 12	240 ± 16	240 ± 19.6
	PRP	232 ± 14	235 ± 14	230 ± 14	238 ± 17	229 ± 16.3

Table 1. Haematological parameters were analysed on post-exercise days induced by exercise (Mean ± SD). [a]p< 0.05 compared to baseline analysed by repeated measures by ANOVA. No significant differences between control and PRP, by independent-samples *t* test observed between control and PRP. Control, platelet rich plasma (PRP). RBC: red blood cells: Hb: haemoglobin; WBC: white blood cells; Neut: neutrophils; Lymph: lymphocytes.

Figure 1. Post-exercise serum hepcidin level in PRP and Control group.

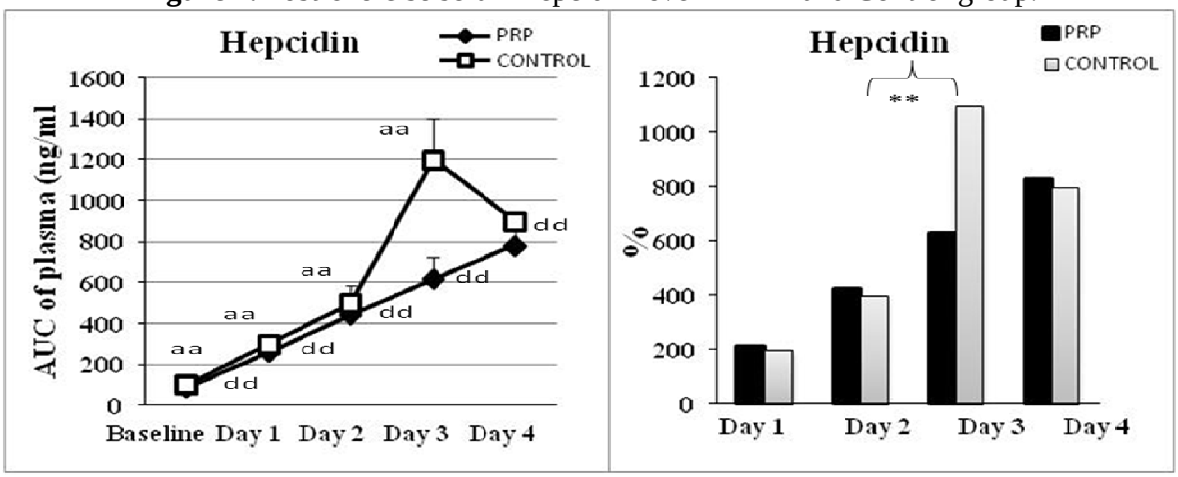

Figure 1. AUC of plasma of hepcidin was calculated at baseline and on days 1, 2, 3 and 4 post-exercise (mean ± SD). $^{aa}p< 0.001$, $^{dd}p< 0.001$, compared with baseline analyzed by repeated measures by ANOVA. $*p< 0.001$ (A). Percentage change (%) was calculated by comparison to baseline levels on days 1, 2, 3 and 4 post-exercise in groups, significant differences between control and PRP, analyzed by independent-samples *t* test (B).

Discussion: The present has shown that during acute exhaustive exercise there is an increase in muscle damage markers, including serum hepcidin. This result is consistent with previous findings reported in the literature that elevated serum hepcidin levels have been observed after different exercise regimens [24, 25]. The results of the present study suggest that exhaustive exercise induce hepcidin levels and it can be an important possible inflammatory marker as a result of exercise induced iron loss. Recent studies have reported on the effects of exercise on hepcidin-dependent control of iron status. Inflammation-induced hepcidin up-regulation may represent a new mechanism behind iron deficiency (possibly leading to anemia) in athletes and may have important practical implications [26]. It can be speculated that hepcidin is not up-regulated if exercise intensity is not sufficient to trigger inflammation. Indeed, hepcidin was decreased after 3 wk of endurance running that did not induce C-reactive protein [27] and low intensity cycling did not up-regulate hepcidin, in moderately active human subject [28]. Iron needs for higher hemoglobin synthesis are met by inhibiting hepcidin expression, thereby increasing ferroportin activity and iron availability to the erythropoietic compartment. Interestingly, recently one study reported a dramatic decrease in hepcidin expression in the livers of tg6 mice with excessive erythropoiesis caused by constitutive over expression of Epo [29]. Moreover, serum and urinary hepcidin levels are decreased in healthy humans treated with Epo [30, 31, 32]. Additionally, our previous study demonstrated that intramuscular PRP injection plays a key role as an anti inflammatory marker by suppressing the effect of increased free iron in plasma during the muscle damage recovery [23]. Moreover, the present study provides additional evidence that the intramuscular PRP administration depresses the increase in hepcidin levels in the post- exercise recovery days. The treatment of the muscle damage of the inflammatory conditions have used non-steroidal anti-inflammatory drugs (NSAIDs) although they are ineffective in reducing muscle pain and do not increase muscle performance during muscle recovery period [33-35]. As an alternative to conventional treatments, platelet-rich therapy has been applied due to its potential in protecting iron stores and it may play a protective role in exercise-induced anemia. However, the effect of the intramuscular injection PRP on iron related parameters and hepcidin has not been defined. Evidently, one related study showed that hepcidin was

suppressed by mTOR signalling [36] or growth factors [37]. This is novel in muscle physiology, particularly, the role of the mTOR pathway as muscle growth may not just be only related to the activation of protein synthesis [38]. In fact, increased iron availability triggered by low hepcidin levels may provide iron that contributes to muscle growth. Understanding the role of this inhibitory pathway will be important because it could contrast the effect of exercise/inflammation-mediated hepcidin induction on hepcidin levels and have consequences on the possible development of iron deficiency and anaemia in athletes.

An exhaustive and strenuous exercise causes muscle damage that defines clinically as muscular pain and involves proteins impair and ultrastructural alters (delayed-onset muscle damage). Studies have reported that especially eccentric muscle contraction delayed-onset muscle damage is mainly induced by mechanical stress, [39] and disturbances of calcium homeostasis [40]. Phagocytes such as macrophages/monocytes and neutrophils are the major subsets of leukocytes that infiltrate the muscle tissue [41-43]. The effects of acute exercise on leukocytes trafficking and function have been investigated extensively. It is approved that acute exhaustive exercise increases the number of circulating leukocytes; it causes a temporary depression in several aspects of immune cell function. These effects are related to both the duration and intensity of exertion, with strenuous exercise (prolonged and/or high-intensity) yielding the most profound effects [44]. In this study we observed that the levels of WBC and Neut were slightly suppressed and Hb was increased after exhaustive acute muscle exercise in control. In general, exercise induced hemolysis leads to the release of Hb and iron in to the circulation. The studies evidence suggests that the alters in circulating Neut after eccentric exercise are dependent on the muscle groups used, or the amount of muscle mass during eccentric exercise. Furthermore, WBC, Neut and Hb level were not altered by intra-muscular PRP administration in this study. Therefore, it seems that PRP administration may exert a systemic effect when healing processes are concerned, at least during the very first stages of inflammation. Considering the short half-life of growth factors which is in the order of minutes [45], it is difficult to consider that growth factors released locally by platelets may be acted on injured muscle. This result agrees with the recent studies in which the systemic levels of some growth factors, such as vascular endothelial growth fact and epidermal growth factor, were modified within 24 hours after PRP injection [46]. Further studies are needed to clarify this issue, with particular regard to physiological mechanism of intramuscular PRP injection.

Conclusion: Our results indicate that exhaustive exercise induced hepcidin level and this can be important as a possible inflammatory marker in the mechanisms of exercise induced iron loss. Inflammation-induced hepcidin up-regulation may represent a new mechanism behind iron deficiency in athletes and may have important practical implications. PRP administration improved the inflammatory response by suppressing serum iron and hepcidin levels and it may be provide a protective role in exercise induced iron loss.

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