Pentosan polysulfate regulates hepcidin expression in native Mongolian horses

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ABSTRACT. The aim of this study was to investigate the anti-hepcidin effect of pentosan polysulfate (PPS) in Mongolian horses. Twenty-six healthy horses were randomly allocated to two groups; one group was treated with a PPS once a week for 4 weeks while another group was kept as a placebo. Blood samples at day 0 (D0), before race (BR; day 28) and after race (AR; day 28) were analyzed for serum biochemistry, hepcidin and iron concentrations. Significant reduction of hepcidin was observed at AR in PPS group when compared with BR placebo (P<0.05) and AR placebo (P<0.01). Mean hepcidin concentration difference of D0-BR and BR-AR in PPS was greater than the placebo whereas the iron concentration difference is reduced compared to placebo. Results indicate a novel therapeutic application of PPS as an anti-hepcidin compound to control hepcidin in horses while emphasizing further molecular studies.

KEYWORDS: hepcidin, horse, iron, pentosan polysulfate

Hepcidin, synthesized in the liver has been found to be the key regulator of iron homeostasis and leads to hypoferremia during inflammation [7, 22, 23, 25]. Hepatocytes synthesized hepcidin secreted into the bloodstream binds to the iron exporter ferroportin (FPN) in target cells, such as macrophages, and enterocytes and to some extent in hepatocytes (Fig. 1). Physical exercises and stress factors have significant effect on animal metabolism and physiological processes proceeding lots of changes of the concentrations of several blood variables including iron. Iron is a crucial element in the metabolic structures involved in oxygen transport and deficiency can be frequently observed among athletes [5, 15, 20, 29, 32]. Over the past decade, an increasing amount of attention has been focused on the influence of exercise-induced hepcidin elevation on reduction of iron absorption, which may compromise an athlete’s iron availability [17, 19]. Expression of hepcidin is influenced by iron and variety of stimuli, including anemia, hypoxia, and inflammation (interleukin-6 (IL-6)), and erythropoiesis [1, 6, 8, 26, 28]. Among those several physiological factors, exercise-induced IL-6 is suggested to be an important stimulus for hepcidin production [21].

Pentosan polysulfate (PPS) has been used to treat horses with mild or early-stage osteoarthritis (OA) with its ability to reduced articular cartilage fibrillation with no adverse effects [18, 27]. Pentosan is a semi-synthetic polysulfated poly-saccharide derived from beech wood hemicellulose that has been reported to modulate the progression of OA as a disease modifying osteoarthritis drug (DMOADs) [9]. Further, extensive in vitro and animal model studies using PPS have shown that this compound is effective at reducing joint inflammation, promoting fibrinolysis, stimulating hyaluronan synthesis by synovial fibroblasts and stimulating proteoglycan synthesis by chondrocytes [9, 10, 12]. The findings of our in vitro study showed that PPS possesses promising anti-arthritic effects on type II collagen-induced arthritis in rats by ameliorating clinical and functional outcome [30]. However, PPS has acquired more attention as a source of potential long-term safe pharmaceutical compound due to its pharmacologic properties [4, 11].

The present work was carried to verify if PPS has an anti-hepcidin activity in horses reared in field condition. The study on hepatoma cell line and in mice proved the possibility to use PPS as an efficient anti-hepcidin molecule, with marginal side effects [2]. Through the in vitro study, we have identified that PPS is able to control the formation and function of hepcidin 1 treated osteoclast derived from canine bone marrow and thereby intracellular iron concentration through the in vitro study [31]. To the best of our knowledge, present study is the first attempt to identify the effect of PPS on hepcidin in Mongolian horses. We hypothesized that PPS with its anti-hepcidin effect would ameliorate availability of iron which is a fundamental element in the metabolic systems involved in oxygen transport.
Twenty-six (2 years old) native Mongolian horses (mean body weight 256 ± 7 kg; mean ± SD) were randomly selected at a feeder in one plain in Khentii province, Mongolia. Every horses had free access to grass and water daily in the same pasture condition. Before being assigned to this study, horses were subjected to a general physical examination, serum biochemistry profiles including hepatic and renal functional measures and orthopedic fitness being normal and healthy (Table 1). All procedures were done by registered veterinary professionals in Mongolia under the approval and ethical standard guidelines for animal of School of Veterinary Medicine, Mongolian University of Life Science.

The horses were randomly allocated into placebo and PPS (OJI-200EI, Oji Holdings Co., Tokyo, Japan) subgroups which consists of 13 horses in placebo while 13 horses in PPS. Four doses of PPS at 3.0 mg/kg were injected intramuscularly once a week for 13 horses among 26. The selected concentrations of PPS are within the previously proved non-cytotoxic range [13]. Placebo groups were treated with phosphate-buffered saline solutions at the same course/same time as PPS treatment.

Horse blood samples were collected by venipuncture in the morning for routine hematological and biochemical analyses at the day 0 (D0), before race (BR) and after race (AR). For hematological analysis, the blood was collected in tubes with anticoagulant ethylenediamine tetraacetic acid (EDTA). For serological and biochemical analyses, collection tubes with clot activator gel were used to extract the serum. Except for the samples collected in EDTA tubes, the others were centrifuged at 700 × g for 10 min, aliquoted into microtubes, and stored in a freezer at a temperature of −80°C until analysis.

Evaluation of horses general physical and orthopedic conditions was done every week when PPS was injected. Serum biochemistry profiles, including total alanine aminotransferase (ALT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN) and creatinine (Cr) were measured (Fuji Dry Chem NX500, Fujifilm, Tokyo, Japan) after serum being separated and prepared. Serum hepcidin concentration of all horses was measured according to the manufacture’s guideline of horse hepcidin (HEPC) Enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource.com, San Diego, CA, USA). Iron assay kit was used to measure the serum iron levels by ferrozine chromogenic method (MG Metallogenics.com, Chiba, Japan) for all horses. All assays were performed in duplicate.

Data were analyzed using IBM SPSS version 23 (SPSS inc., Chicago, IL, USA). ELISA data was analyzed using Graph Pad Prism v.7.03 software (San Diego, CA, USA). Non-normally distributed data were analyzed using the Mann Whitney U Test to compare two groups. The results were considered significant at a 95% confidence level (P < 0.05).

At the beginning of the study, mean value of the serum hepcidin of 26 horses was 110.13 ± 40.15 ng/mL (mean ± SD) which was within the normal range [16]. After 4 weeks (BR) of PPS treatment, mean serum hepcidin level of PPS group was reduced comparatively to placebo group (Fig. 2A). However, significant reduction of hepcidin was observed at AR in PPS group when
compared with BR placebo ($P<0.05$) and AR placebo ($P<0.01$) (Table 2). At the beginning of the study, mean value of the serum iron of 26 horses was $150.81 \pm 22.52$ μg/dL (mean ± SD) which lies in the range of serum iron in healthy individual (50–195 μg/dL) [16]. This study data showed that hepcidin negatively correlated (correlation coefficient; $R^2=0.3704$) with serum iron in horses (Fig. 2B).

Concentration differences between D0-BR and BR-AR were calculated in placebo and PPS groups to detect the degree of changes of serum hepcidin and iron levels. The hepcidin concentration difference among D0 and BR (D0 hepcidin–BR hepcidin) was comparatively higher in PPS group than placebo when compared the mean values (Fig. 2C). The same phenomenon was found at the BR and AR

![Graph](image)

**Table 2.** Serum hepcidin and iron concentration of horses

| Compound | Groups    | N  | Mean   | ± SD | ± SEM | $P$ value |
|----------|-----------|----|--------|------|-------|-----------|
| Hepcidin | Placebo-BR| 13 | 111.49 | 38.52| 10.68 |           |
|          | PPS-BR    | 13 | 86.69  | 25.41| 7.05  |           |
|          | Placebo-AR| 13 | 141.18 | 47.72| 13.23 |           |
|          | PPS-AR    | 13 | 72.27  | 23.32| 6.47  | 0.039 (a) 0.001 (b) |
| Iron     | Placebo-BR| 13 | 100.78 | 28.52| 7.68  |           |
|          | PPS-BR    | 13 | 111.54 | 37.79| 5.05  |           |
|          | Placebo-AR| 13 | 79.98  | 20.41| 6.23  |           |
|          | PPS-AR    | 13 | 100.84 | 33.32| 5.47  |           |

BR: Before race; AR: after race; SD: Standard deviation; SE: Standard error; Hepcidin and iron concentration in μg/dL. Significance vs Placebo-BR- (a): $P<0.05$; Significance vs Placebo-AR- (b): $P<0.01$. "Fig. 2. Serum hepcidin concentration during study period. Serum hepcidin concentrations of all horses in placebo (n=13) and pentosan polysulfate (PPS) (n=13) groups at Day 0, before race (BR) and after race (AR) were measured by horse hepcidin (HEPC) Enzyme-linked immunosorbent assay (ELISA) kit. (A) After 4 weeks (BR) of PPS treatment, mean serum hepcidin level of PPS group was reduced comparatively to placebo group. However, significant reduction of hepcidin was observed at AR in PPS group when compared with BR placebo and AR placebo. Data are expressed as mean ± SD. **$P<0.01$; *$P<0.05$, compared with placebo group. (B) This study data showed that hepcidin negatively correlated with serum iron in horses. (C) The hepcidin concentration difference among D0 and BR (D0 hepcidin–BR hepcidin) was comparatively higher in PPS group than placebo when compared the mean values. (D) The hepcidin concentration difference among BR and AR (BR hepcidin–AR hepcidin) comparatively higher in PPS group than placebo when compared the mean values."
(BR hepcidin–AR hepcidin) for hepcidin concentration difference where we detected the higher concentration changes in PPS groups than placebo. Even though, the concentration difference of hepcidin among BR and AR placebo and PPS groups showed favorable response, statistically significant was achieved in mean concentration only AR in PPS group when compared with BR placebo ($P<0.05$) and AR placebo ($P<0.01$). Intriguingly, five horses have revealed negative values for concentration deference (BR-AR), showing increase of AR hepcidin concentration in placebo group (Fig. 2D).

Iron concentration was decreased in both placebo and PPS groups towards AR while standing it in normal range (Fig. 3A). However, changes between D0-BR and BR-AR were comparatively low in PPS group reveling the surge of serum iron level with response to declining of hepcidin level (Fig. 3B, 3C). Further, statistically significant different was not observed in concentration difference between placebo and PPS (Table 2).

Previous studies on athletic horses showed that physical exercise influences iron homeostasis and affect athletic performance through low hemoglobin concentration resulting in reduction in the oxygen transport capacity and decreasing the maximal oxygen uptake to muscles [16, 24, 29]. On the other hand, heparin derivatives were found as potent inhibitors of hepcidin through in vitro and in vivo studies. Fascinatingly, PPS (a semi-synthetic sulfated polysaccharide with a molecular weight of about 4,000–6,000 Da) seems to be a promising compound to control hepcidin as was proved in a previous study conducted in mice [3]. In light of this evidence, PPS could be a promising compound to control hepcidin expression. On the basis of this knowledge, the present study demonstrates for the first time that PPS acts as an inhibitor of hepcidin expression in native Mongolian horses.

In this study, PPS was injected in 3.0 mg/kg dose in once a week for 4 weeks as an intramuscular injection and that was able to ameliorate serum iron concentration while inhibiting hepcidin expression. The previous studies on experimental induced OA and healthy horses have shown the beneficial effect of PPS as a novel DMOADs without any adverse reactions. Further, it has demonstrated that PPS-treated horses have higher chondroitin sulfate concentrations than placebo group, suggesting a systemic upregulation of aggrecan synthesis [18]. It has been already proven that PPS positively correlates to values of procollagen II C-propeptide, an anabolic marker of cartilage metabolism while showing negative correlation to values of cartilage catabolic marker of oligomeric matrix protein [27]. Moreover, previous data explained that anti-hepcidin activity of PPS is due to its greater negative charge density distributed on the polysaccharide chain, that induce a high affinity for the basic clusters of the protein [3] and inveterate the necessity of further studies to understand the molecular interaction between PPS and hepcidin. Previous studies have shown that anti-hepcidin activity of heparins is achieved through its high sulfation level [2]. Pentosan Polysulfate which is categorized under heparinoid class carries high sulfation [14] and that would be the rationale of possible anti-hepcidin effect of PPS. The finding of this study shows that PPS inhibits hepcidin expression facilitating consequent systemic iron distribution in order to maintain the constant oxygen supply to the body tissues without causing evident side effects.

CONFLICTS OF INTEREST. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
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