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Standardized Extract (HemoHIM) Ameliorated High Intensity Exercise Induced Fatigue in Mice

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Abstract – HemoHIM was used as a Korean traditional medicine for anti-inflammatory and antioxidant effects. However, there is no study on the effect of HemoHIM on fatigue. We examined the potential use of HemoHIM to determine whether it can induce anti-fatigue effects. Mice were administered with HemoHIM and VEH for 14 days. On the last day of treatment, mice were subjected to behavioral tests. Subsequently, their plasma and muscle were collected after the treadmill test to measure lactate, lactate dehydrogenase (LDH), ammonia, corticosterone, glycogen, and creatine kinase (CK). We found that HemoHIM moderately increased the running time (s) in the treadmill and mobility duration in the cold swimming tests. In addition, the VEH group showed a significant increase in lactate, LDH, and corticosterone levels in the plasma compared to the group that did not perform the test. However, this was moderately reduced in HemoHIM treatment. Moreover, the HemoHIM-treated group showed significant differences in LDH and glycogen levels, and showed significantly different CK levels in the muscle. HemoHIM is considered to be effective in improving fatigue, given the duration of cold swimming or running time on a treadmill. Also, HemoHIM treatment resulted in reduced concentrations of blood and muscle parameter analysis.
Keywords – HemoHIM, Anti-fatigue, Treadmill, Cold swimming test, Mice

Introduction

Fatigue is difficult to define academically, but it generally leads to incapable work output and impairment in physical activity.[1] Approximately 10-17 million people visit clinics or offices annually in the USA.[2] Fatigue often causes anxiety along with depression and is linked to cognitive impairment, sleep quality, dysfunction, and energy balance.[3] Fatigue is also associated with autoimmune diseases,[4] cancer,[5] Parkinson's disease,[6] and heart disease.[7] In addition, it can induce diverse disorders associated with biological regulation and the immune system.[8] Overall fatigue is considered an important issue that threatens human health.[9] Fatigue is a warning for body abnormalities and is important to avoid fatigue to prevent the occurrence of secondary diseases. It would be helpful to recover from fatigue through adequate sleep, exercise, lifestyle changes (moderation in drink, quitting smoke), and intake of nutrients such as protein, vitamins, minerals, and healthy functional foods.[10]

HemoHIM is a herb mixture consisting of three kinds of traditional Korean medicinal plants mixtures: Angelica Radix (the root of *Angelica gigas* Nakai) used for invigorating blood circulation for over 2000 years in China,[11] Cnidium Rhizoma (rhizome of *Cnidium officinale*

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Makino) known to have a regulatory property for blood circulation and inflammatory diseases,[12] and Paeonia Radix (the root of *Paeonia japonica* Miyabe) to treat various illnesses including gastralgia, gynopathy, enterorrhagia, and headache.[13,14] It has been reported to improve inflammatory activity, Th1-like immune responses fractionated γ-irradiation, and airway.[14-16] In addition, it has been reported to inhibit various activities of human mast cells,[17,18] enhances antitumor effects during chemotherapy and radiotherapy,[19] improve immune function,[18,20] and ameliorate oxidative stress induced by irradiation.[21] There are reports that inflammatory and oxidative stress are associated with fatigue syndrome.[22–24] HemoHIM has anti-inflammatory and antioxidant effects, which might improve fatigue. However, there is no detailed study on the effect of HemoHIM on fatigue.

Therefore, in this study, we confirmed the anti-fatigue effects of HemoHIM *in vivo*. We conducted a cold swimming test (CST) and treadmill test to induce fatigue in an animal model for behavioral evaluation. In addition, biomarkers, including lactate, corticosterone, lactate dehydrogenase (LDH), and ammonia level changes in blood were checked. Moreover, glycogen, LDH, and creatine kinase (CK) levels were evaluated in the skeletal muscle. This study is an additional work to Kwon *et.al* (2021) that adds treadmill and cold swimming test.

Experimental

Animals – Male ICR mice (20–25 g) used in this study were obtained from Hanlim Laboratory Animals Co. (Hwaseong, Korea). Animals were housed in groups in an animal room with controlled temperature and humidity ($22 \pm 2^{o}C$ and $55 \pm 5\%$, respectively). Food and water were provided ad libitum, except at night before the experiment. The animal room was kept on a 12 h/12 h light/dark cycle (7 AM to 7 PM). All animals were acclimatized to the housing conditions for 7 days prior to the experiment. The experiments were conducted after 14 days of treatment. Different dosages (0, 125, 250, and 500 mg/kg) of test substances were orally administered. Animal treatment and maintenance were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85 - 23 revised 1985) and the Animal Care and Use Guidelines of Sahmyook University, Korea (SYUIACUC 2020-003).

Preparation of HemoHIM – HemoHIM was prepared with three herbs used in traditional Korean medicine, *Angelica gigas*, *Cnidium officinale*, and *Paeonia japonica.* Nodakenin (50-150 mg/100 g), chlorogenic acid (25-60 mg/100 g), and paeoniflorin (200-400 mg/100 g) containing in standardized HemoHIM (Batch no: HHH09) were produced by Kolmar BNH (Sejong-si, Republic of Korea). HPLC data was provided in the previous report.[25] *Angelica gigas*, *Cnidium officinale*, and *Paeonia japonica* were extracted for 4 h in boiling water to obtain a total extract of HIM-1. We added 4 volumes of 100% ethanol to obtain an ethanol-insoluble polysaccharide fraction and half of the extract precipitated as an insoluble polysaccharide fraction. HemoHIM was prepared by adding the ethanol-insoluble polysaccharide fraction to the other half of HIM-1. Test substances were dissolved in distilled water (DW) before the experiment.

Treadmill Test – Thirty minutes after administration of the test substances in mice, high-intensity exercise was performed on a treadmill (5-lane treadmill, DJ-344, Daejonglab, Korea). All the experimental mice followed a nine-stage protocol, of which, the first three stages were habituating stages. Mice were running for 9 min at the speed of 15, 20, and 25 m/min without an incline or electrical stimulation for habituation. After habituation, in every stage, we increased the incline and applied electrical stimulation every four min from stages 4 to 9. The inclination increases by 1° and electrical stimulation increases by 2 mA every two stages. In stage 9, the experiment was terminated when the mice stopped moving after physical or electrical stimulation.

Cold swimming test – The cold swimming test is used to evaluate an animal's ability to resist stress, maintain exercise, and control its body temperature. Mice were administered with the experimental substance 30 min before conducting the experiment. The cold swimming test was conducted at a water temperature of 15ºC. The temperature was maintained to ensure that the animal was moving enough. The experiment ended when the mouse completely stopped moving, even when physically stimulated.

Open field test (OFT) / Rota-rod test – The OFT was conducted to evaluate general behaviors such as movement failure, sedation, excitement, and increased impulsivity. Mouse locomotor activity was assessed in a square black plexiglass container with an open field measuring 42 × 42 × 42 cm. Thirty minutes after administering the experimental substance into the mouse, the mouse was placed at the center of the box and its movement was observed for 12 min. We measured the locomotor activity for the remaining 10 min except for the first 2 min of habituation using the EthoVision system (Noldus IT b.v., Netherlands), a behavioral observation device and program.

The rota-rod test was used to assess the balance and

motor coordination of animal using a rota-rod, rpm-adjustable experiment device (UgoBasile, Varese, Italy). The rota-rod was fixed at 36 rpm. All the animals were trained for 3 min on a rotating rod for 2 d prior to the rota-rod test. Latency time (i.e., time of the first fall in second) and falling frequency were recorded for 20 min.

Blood parameter analysis – After the end of the treadmill test, blood from the mouse was collected through heart puncture, and the plasma was used to evaluate the levels of lactate, corticosterone, lactate dehydrogenase (LDH), and ammonia. The collected blood was centrifuged at 4,000 rpm and 4°C for 15 min to separate the plasma and stored at -80°C. A column switching method and an UV detector are used to detect corticosterone. All equipment used semi-microbore HPLC nanospace si-2 series (Shiseido, Tokyo, Japan) equipment and consisted of two pumps, column thermostat, UV detector, 3012 switching valve, 3023 auto sampler, and 3009 mobile purge system. Lactate was measured using Accutrend® Plus. We used general ammonia assay kit (Mybiosource, Catalog No. MBS8305402), and lactate dehydrogenase activity assay kit (Sigma, Cat no. MAK06) to measure ammonia and LDH levels, respectively, as per the manufacturer's instructions.

Muscle parameter analysis – The gastrocnemius muscle of the mice was collected and stored at -80°C. Muscle tissue (80 mg) was added to a tube containing 400 μL of cold phosphate buffered saline and homogenized using a homogenizer (T25 digital homogenizer, IKA, Germany). The resulting tissue was centrifuged at 13,000 rpm at 4°C. The levels of creatine kinase (CK), glycogen, and LDH in the muscle were determined using a creatine kinase activity assay kit (ab155901), glycogen assay kit (ab65620), and an LDH assay kit (ab102526, Abcam, Cambridge, UK), respectively, according to the manufacturer's instructions.

Data and statistical analysis – All data are presented as mean ± standard error of the mean (S.E.M.) and were analyzed using GraphPad Prism 7.0 software (San Diego, CA, USA). Data were analyzed using the *t*-test.

Result and Discussion

The results of the treadmill test after 14 days of HemoHIM administration are shown in Fig. 1. This behavioral test measured the running time until the mice were completely exhausted, which was done by gradually increasing their incline and speed after habituation. HemoHIM treatment significantly increased the running time of mice at all dosages.

The results of the cold swimming test after 14 days of HemoHIM administration are shown in Fig. 2. In this test, as the time spent moving at low temperatures increased, there was an effect against stress fatigue at cold temperatures. The HemoHIM-administered group showed a tendency to increase mobility duration compared to the VEH group, although this lacked statistical significance.

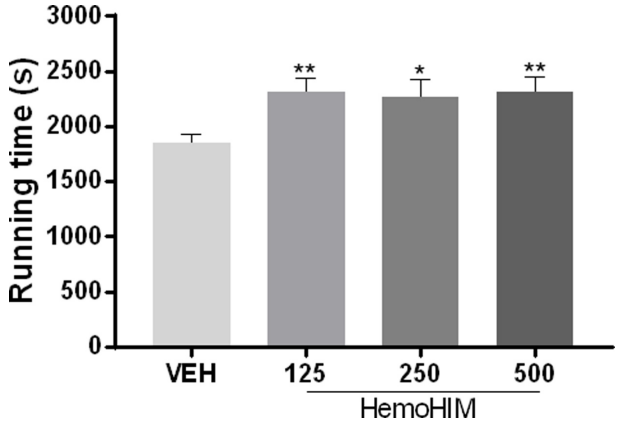

Fig. 1. Running time of VEH, HemoHIM treated mice in the treadmill test. Treadmill test in ICR mice after treatment of VEH, HemoHIM [125, 250, 500 mg/kg showing the running time. n = 10 animals per group. Values are expressed as mean ± SEM. *p < 0.05, **p < 0.01, significantly different from the VEH (t-test).

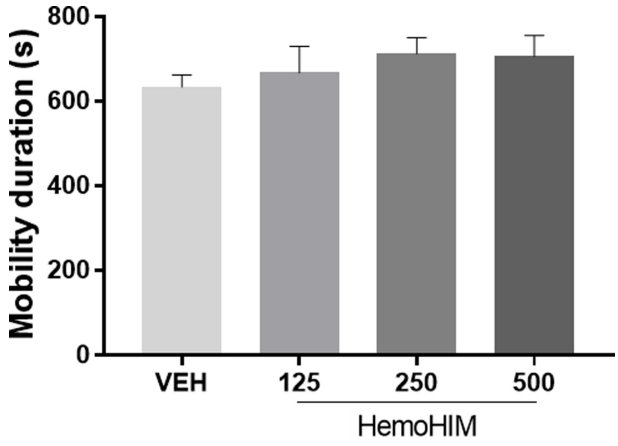

Fig. 2. Mobility duration of VEH, HemoHIM treated mice in the cold swimming test. Cold swimming test in ICR mice after treatment of VEH, HemoHIM [125, 250, 500 mg/kg] mobility duration. n = 10 animals per group. Values are expressed as mean ± SEM.

The results of the OFT and rota-rod test are shown in Fig. 3. The OFT and rota-rod were checked to observe changes in locomotor activity and motor balance and coordination. The HemoHIM-administered group did not show any significant difference in distance moved and movement duration. In the rota-rod test, the HemoHIM-administered group did not show any significant difference

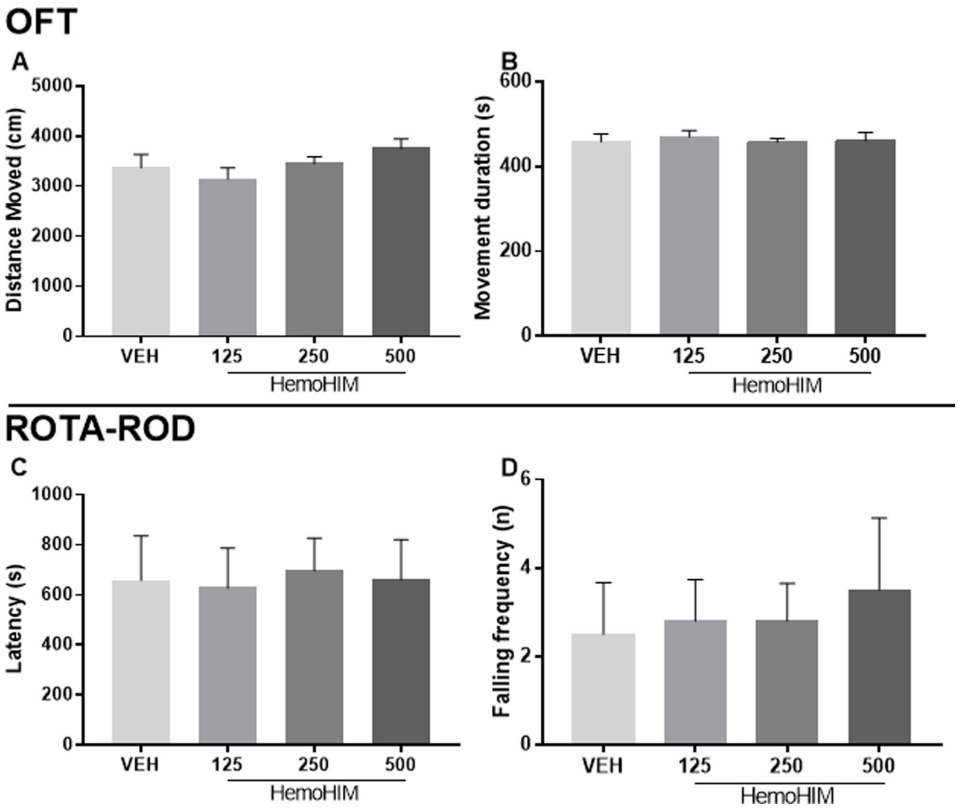

Fig. 3. Evaluation of open field and rota-rod test. OFT and rota-rod test in ICR mice after treatment of VEH, HemoHIM [125, 250, 500 mg/kg] showing the (A) distance moved, (B) movement duration, (C) latency to first fall, (D) falling frequency. n = 10 animals per group. Values are expressed as mean ± SEM.

in latency time and falling frequency.

HemoHIM was administered for 14 d, and blood was collected after the treadmill test. The results of various parameters from the plasma are shown in Fig. 4. As shown in Fig. 4A, lactate levels were significantly increased in the VEH group compared to the CON group (group that did not perform the experiment). HemoHIM treatment of 250 and 500 mg/kg significantly decreased lactate level compared to the VEH group. Corticosterone level is shown in Fig. 4B. The VEH group also showed a signifi- cant increase in corticosterone level compared to the CON group, which was significantly decreased by HemoHIM treatment at 500 mg/kg in a dose-dependent manner. As shown in Fig. 4C, the VEH group showed a significant increase in LDH level compared to the CON group. The HemoHIM-treated group (125 and 500 mg/kg) showed a significant decrease compared to the VEH group. In addition, 250 mg/kg HimoHim reduced LDH levels, but did not show any significant difference. Moreover, as shown in Fig. 4D, the VEH group tended to have higher ammonia level than the CON group, but the difference was insignificant. The HemoHIM-treated group tended to have lower ammonia level than the VEH group; 250 mg/kg of HemoHIM showed a significant decrease in ammonia level.

LDH, glycogen, and CK levels after the treadmill test are shown in Fig. 5. As shown in Fig. 5A, only 500 mg/kg HemoHIM dosage group showed a significant decrease in LDH level after the treadmill test. As shown in Fig. 5B, 500 mg/kg HemoHIM showed a significant increase in glycogen level and other HemoHIM administered groups tended to show an insignificant increase compared to the VEH group. As shown in Fig. 5C, 125 mg/kg HemoHIM significantly reduced the CK activity after the treadmill test. However, the other groups did not show any difference.

This study was conducted to determine the anti-fatigue function of HemoHIM. The effect of the test substance on the general and fatigue behaviors was evaluated. Fatigue biomarker changes due to continuous exercise were also evaluated. HemoHIM showed a tendency to increase the duration and running time in the cold swimming and treadmill tests. There were no significant changes in the open field and rota-rod tests. This shows the possibility

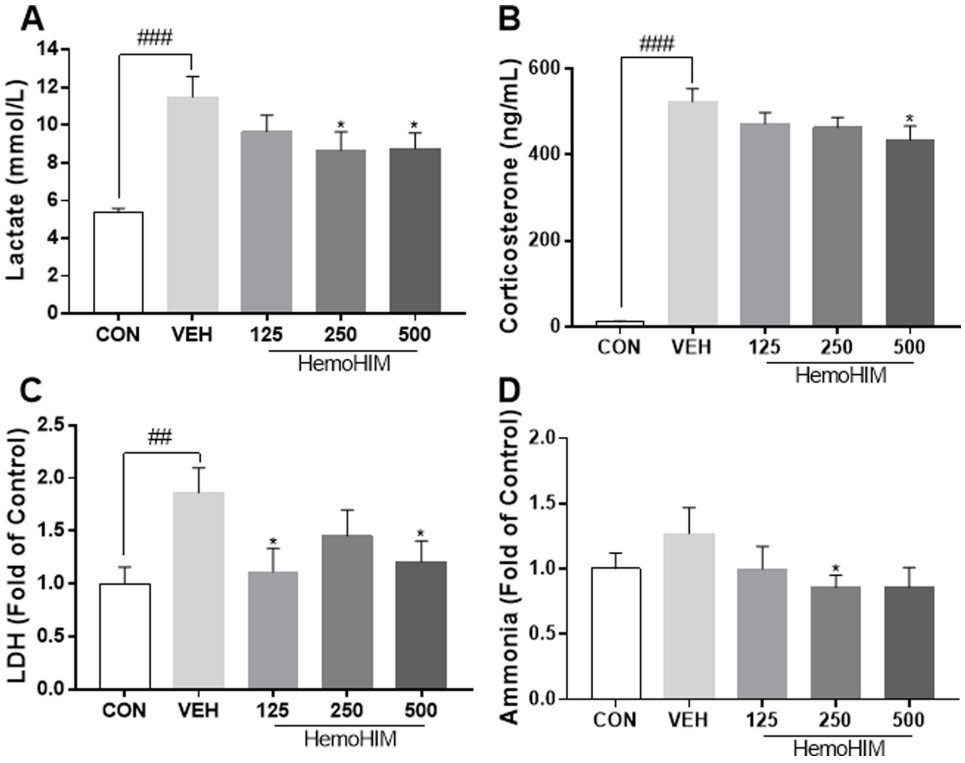

Fig. 4. Evaluation of blood parameters in mice. Blood parameter in ICR mice after treatment of VEH, HemoHIM [125, 250, 500 mg/kg], CON (mice did not conduct the test) showing the (A) lactate, (B) corticosterone, (C) Lactate dehydrogenase (LDH) and (D) ammonia levels in plasma. n = 10 animals per group. Values are expressed as mean ± SEM. ##$p < 0.01$, ###$p < 0.001$ significantly different from the CON (t-test). *$p < 0.05$, significantly different from the VEH (t-test)

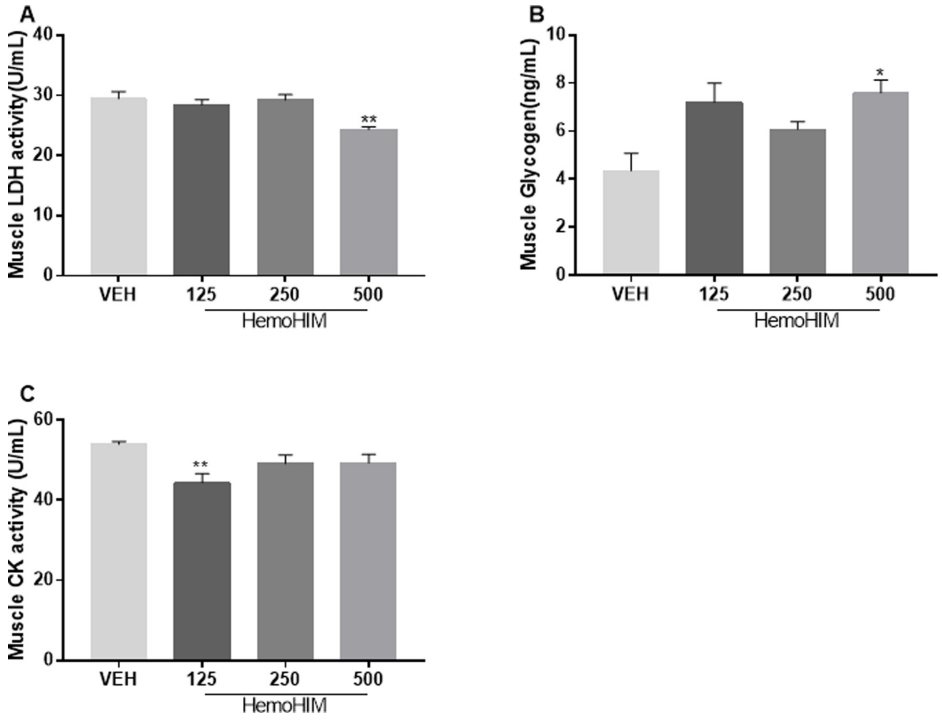

Fig. 5. **Evaluation of muscle parameter in mice.** Muscle parameter in ICR mice after treatment of VEH, HemoHIM [125, 250, 500 mg/kg], showing the (A) Lactate dehydrogenase (LDH), (B) glycogen, (C) creatine kinase (CK) activity in muscle. n = 10 animals per group. Values are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$ significantly different from the VEH (t-test).

that HemoHIM is resistant to the fatigue behavior of cold swimming and running. There were no significant effects on general behavior, which could suggest that the possibility of side effects from HemoHIM administration may be considered low.

Excessive exercise can cause the production and accumulation of metabolic products, such as lactic acid, in the body.[26] Lactate is an acidic substance produced as a result of the anaerobic metabolism of glucose, which causes exercise fatigue by acidifying tissues and blood.[27] In the case of intense aerobic exercise, lactate production increases due to a lack of oxygen supply. In this study, we found that treadmill tests under harsh conditions increased the production of lactic acid through high-intensity aerobic exercise. The concentration of lactate in the plasma increased more in the VEH group than in the CON group, which did not participate in the experiment. In addition, the increase in lactate levels tended to be lower in the HemoHIM-treated group in a dose-dependent manner. LDH is released into the blood by heavy physical exercise and could be a muscle damage indicator related to fatigue.[28] In our study, LDH levels showed a decreasing trend in the HemoHIM-treated group. In a previous study, an increase in ammonia concentration in blood could be related to fatigue.[29] Additionally, ammonia released from muscle may direct access to brain tissue and cause a toxic effect on the central nervous system (CNS).[30] The ammonia concentration was also decreased by HemoHIM treatment. Stress can activate the hypothalamic-pituitary-adrenal (HPA) axis and alter the corticosterone level.[31] In our study, the treadmill can act as a stressor because of forced exercise. We observed corticosterone levels and checked the changes in its level. Mice subjected to the treadmill test showed a high level of corticosterone. Glycogen is a complex glucose polymer that serves as a storage form for glucose found in many tissues, mainly in the skeletal muscles and liver. In addition, the ability of the muscle to exercise is damaged when glycogen stores are reduced to low levels.[32] After the treadmill test, muscle glycogen levels significantly increased in the HemoHIM 500 mg/kg group, and other groups tended to show an increase. Moreover, the LDH level in the muscle was significantly reduced in the 500 mg/kg group. One of the causes of fatigue is the change in high-energy phosphates (i.e., ATP and ADP), and CK is the key enzyme for maintaining a constant ATP/ADP ratio during rapid energy turnover.[33] In this study, the HemoHIM 125 mg/kg group showed a significant decrease in CK activity in the muscle. Therefore, HemoHIM is considered to be effective in improving fatigue, given the duration of cold swimming or running time on a treadmill. HemoHIM treatment resulted in reduced concentrations of lactate, LDH, and ammonia in the plasma. Moreover, it increased glycogen levels, reduced LDH levels, and CK activity in the muscle. We suggest that HemoHIM could be a potential anti-fatigue agent.

Competing interests

The authors declare that there are no conflicts of interest

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