



Protective effects of the roots of *Angelica sinensis* on strenuous exercise-induced sports anemia in rats

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ABSTRACT

Ethnopharmacological relevance: Sports anemia is a persistent and severe problem in athletes owing to strenuous exercise-induced oxidative stress and hepcidin upregulation. The roots of *Angelica sinensis* (AS), a familiar traditional Chinese medicine, has been used for replenishing blood since antiquity.

Aim of the study: To evaluate the effects of ethanolic AS extract in a 4-week study on sports anemia in female Wistar rats.

Materials and methods: To induce anemia, a strenuous exercise protocol consisting of running and swimming was employed with increasing intensity. Animals were randomly assigned to the following groups: control group; strenuous exercise group; and strenuous exercise and AS extract-treated group (300 mg kg⁻¹ d⁻¹). After 4 weeks, rats underwent exhaustive swimming and forelimb grip strength test. The blood biochemical markers and hepatic antioxidant activities were determined. Hepatic interleukin-6 and muscle glycogen were observed through immunohistochemical and Periodic acid-Schiff staining, respectively.

Results: AS extract (consisting of ferulic acid, Z-ligustilide, and *n*-butylidenephthalide) treatment improved forelimb grip strength and rescued exercise-induced anemia by significantly elevating the red blood cell counts and hemoglobin concentrations as well as hematocrit levels ($p < 0.05$). AS modulated the iron metabolism through decreasing serum hepcidin-25 concentrations by 33.0% ($p < 0.05$) and increasing serum iron levels by 34.3% ($p < 0.01$). The hepatic injury marker serum alanine aminotransferase concentrations were also reduced, followed by increased antioxidant enzyme catalase expression in the liver ($p < 0.05$). Furthermore, substantial attenuation of hepatic interleukin-6 expression and preservation of muscle glycogen content suggested the additional roles of AS acting on sports anemia and physical performance.

Conclusion: Our findings evidenced a novel and promising therapeutic approach for AS treatment for rescuing the anemic condition induced following 4 weeks of strenuous exercise.

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AS, the roots of *Angelica sinensis*; ASG, strenuous exercise and treated with AS extract group; AST, aspartate aminotransferase; BAT, brown adipose tissue; BUN, blood urea nitrogen; CAT, catalase; CG, control group; CK, creatine kinase; EDTA, ethylenediaminetetraacetic acid; EG, strenuous exercise group; EGTA, ethylene glycol tetraacetic acid; ERK, extracellular signal-regulated kinase pathways; H₂O₂, dihydrogen dioxide; HCT, hematocrit; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hb, hemoglobin; IHC, immunohistochemistry; IL, interleukin; IS, internal standard; JAK, Janus-kinases; LDH, lactic dehydrogenase; LPS, lipopolysaccharide; mTOR, mammalian target of the rapamycin; NF, nuclear factor; RBC, red blood cell; ROS, reactive oxygen species; SEM, standard error of mean; SMAD, son of mother against decapentaplegic; SOD, superoxide dismutase; TAG, triacylglycerol; TCM, traditional Chinese medicine; TNF, tumor necrosis factor; TP, total protein; UA, uric acid; WAT, white adipose tissue; WBC, white blood cell

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1. Introduction

The etiology of sports anemia has been studied for decades (Yoshimura, 1970). Sports anemia can be defined as a condition in which an increased destruction of red blood cells (RBCs) and a decrease in hemoglobin (Hb) levels occur because of exercise (Jenkins, 2005). The prevalence of sports anemia is particularly high in athletes (Fallon, 2004), and is attributed mainly to three factors: (a) hemodilutional effect by the rising plasma volume to adapt to endurance exercises; that is, “sports pseudoanemia” (Weight et al., 1992); (b) mechanical destruction of erythrocytes because of oxidative stress, leading to hemolysis (Bonilla et al., 2005); and (c) upregulation in hepcidin-25 expression, an iron-regulating hormone, resulting in iron deficiency (Kong et al., 2014). Sports anemia is also believed to be associated with hematuria

(McInnis et al., 1998), gastrointestinal blood loss (Babic et al., 2001), and iron loss through profuse sweating (DeRuisseau et al., 2002) during heavy training sessions. Sports anemia has been a persistent and severe problem.

Sports anemia can be associated with several types of exercise such as running (Yusof et al., 2007), swimming, soaking, triathlon, dancing, military training (Bonilla et al., 2005), and possibly rowing (Skarpanska-Stejnborn et al., 2015). Several methods have been proposed for inducing anemia in a rodent model. Liu et al. (2011) adopted a 5-week running protocol on a treadmill. The training started with 30 m min⁻¹ at 0% inclination for 2 min, with 2 min added incrementally per session for 6 d wk⁻¹, and ending at 95 min d⁻¹. The training was performed once a day for the first 2 weeks, and then twice a day for the next 5 weeks. Zhu et al. (2010) established a 5-week swimming protocol involving 5 d wk⁻¹ with no additional load. The durations were as follows: 30 min d⁻¹ for the first week, 60 min d⁻¹ for the second week, 90 min d⁻¹ for the third week, 120 min d⁻¹ for the fourth week, and 150 min d⁻¹ for the fifth week. Both of these exercise protocols have been demonstrated to be extremely effective; however, they had been time-consuming. Because of this limitation, increasing the workload substantially may be an alternative approach to increasing efficiency.

The roots of *Angelica sinensis* (Oliv.) Diels (Apiaceae) (AS) is one of the most well-known traditional Chinese medicines (TCMs) used since antiquity, and is also commonly used in Chinese cuisine (e.g., Angelica chicken soup, Angelica duck, and Angelica thin noodles). Listed in the Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia Commission, 2010), AS is used for replenishing blood, treating abnormal menstruation, and other diseases in women. The most biologically active components of AS are ferulic acid, Z-ligustilide, and *n*-butylidenephthalide (Chao and Lin, 2011). Studies reviewing the effects of TCM on sports anemia are scant; however, studies have reviewed the anticancer, neuro-protective, immunoregulatory, antiinflammatory, and antioxidant effects of AS (Chao and Lin, 2011; Chen et al., 2013). Several recent studies have reported on the effects of AS on hepcidin-25 suppression via the Janus-kinase (JAK)/son of mothers against decapentaplegic (SMAD)/extracellular signal-regulated kinase (ERK) signaling pathways (Zhang et al., 2014) and hematopoiesis and thrombopoiesis promotion (Li et al., 2015; Liu et al., 2010). Moreover, in our previous laboratory experiment, we demonstrated that AS improved exercise performance (Yeh et al., 2014b) by affecting myotube hypertrophy through the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of the rapamycin (mTOR) pathway (Yeh et al., 2014a). These available findings highlight the potential protective role of AS against sports anemia.

The two main methods for ameliorating sports anemia directly involve upregulating the ability of eliminating reactive oxygen species (ROS) and downregulating the hepcidin-25 expression. First, a close relationship exists between oxidative stress and exercise. Exercise-induced oxidative stress can trigger hemolysis because of the structural changes to the erythrocyte membrane. Specifically, spectrin on the inner surface of erythrocytes is most vulnerable to oxidative attacks by ROS (Yusof et al., 2007). Second, iron is an essential trace element for hemoglobin synthesis and oxygen delivery. Hepcidin-25 is responsible for the maintenance of iron homeostasis, which can reduce duodenal iron absorption and trap intracellular iron. Intensive exercise is strongly associated with the elevation of hepcidin-25 (Kong et al., 2014). Hepcidin-25 expression was found to be considerably induced by proinflammatory cytokines (e.g., interleukin (IL)-6) (Banzet et al., 2012). In the present study, a 4-week strenuous exercise protocol consisting of running and swimming with increasing intensity was developed to induce anemia in female Wistar rats. Females may be at a higher risk of sports anemia because of blood loss during

menstruation and hepcidin upregulation caused by higher estrogen levels (Ikeda et al., 2012; McClung, 2012). In this study, the protective effects of AS on physical performance, blood biochemical markers, hepatic antioxidant activities, and inflammation were investigated.

2. Materials and methods

2.1. Chemicals and reagents

The 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylene glycol tetraacetic acid (EGTA), mannitol, sucrose, potassium phosphate, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol [95% (v/v)] was obtained from Echo Chemical (Miaoli, Taiwan). Analytical-grade glacial acetic acid was purchased from PanReac AppliChem (Barcelona, Spain). Analytical-grade acetonitrile was obtained from Avantor Performance Materials (Center Valley, PA, USA). Ferulic acid was purchased from Acros Organics (Geel, Belgium). Z-ligustilide was purchased from Grand Chemical (Bangkok, Thailand). *n*-Butylidenephthalide was purchased from Alfa Aesar (Ward Hill, MA, USA). Baicalein was purchased from Matsuura Yakugyo (Nagoya, Japan). Pure water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Preparation of AS extract

AS (Fig. 1a) was bought from a Chinese medicinal herbs store in Kaohsiung on August 2015, and identified and authenticated as the roots of *Angelica sinensis* (Oliv.) Diels by Prof. Chao-Lin Kuo from China Medical University. A voucher specimen (CMU-AS-105-022) was deposited in School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, China Medical University, Taichung, Taiwan. AS extract was prepared 3 days before the administration to animals. Crude AS pieces (600 g) were pulverized and extracted 3 times with 95% (v/v) ethanol for 8 h. The supernatant was collected and filtered through a filter paper (ADVANTEC, Tokyo, Japan). The filtrate was concentrated through rotary evaporation in vacuum at 40°C for ethanol removal. The residue was lyophilized for water removal. The AS extract (51 g) was obtained and stored in a closed Schott glass bottle (Mainz, Germany) at 4°C for further daily administration.

2.3. Liquid chromatographic analysis of AS extract

The major components of AS extract (ferulic acid, Z-ligustilide, and *n*-butylidenephthalide) were identified and quantified through high-performance liquid chromatography (HPLC). HPLC-ultraviolet (UV) analysis was conducted using an HPLC system (Hitachi, Tokyo, Japan) consisting of a L-2420 UV-Vis detector, L-2200 autosampler, and L-2130 pump. A Luna PFP(2) column (250 × 4.6 mm, i.d., 5 μm, Phenomenex) was used as the analytical column. Baicalein was selected as the internal standard (IS). The mobile phase was composed of solvent A (1% glacial acetic acid) and solvent B (acetonitrile). The elution conditions were as follows: 0–30 min, linear gradient 5–40% B; 30–40 min, linear gradient 40–50%, 40–60 min, and linear gradient 50–80%; finally, the reconditioning step of the column was 5% B isocratic for 10 min. The injection volume was 20 μL, the flow rate was 1 mL min⁻¹, and detection was performed at 284 nm. Linearity was assessed through linear regression with respect to the concentrations and peak area ratios of the standards to those of the IS. The recovery of each component from the AS extract was calculated as follows: recovery (%) = [(measured concentration – basal concentration)/spiked concentration] × 100%. All analyses were performed in triplicate.

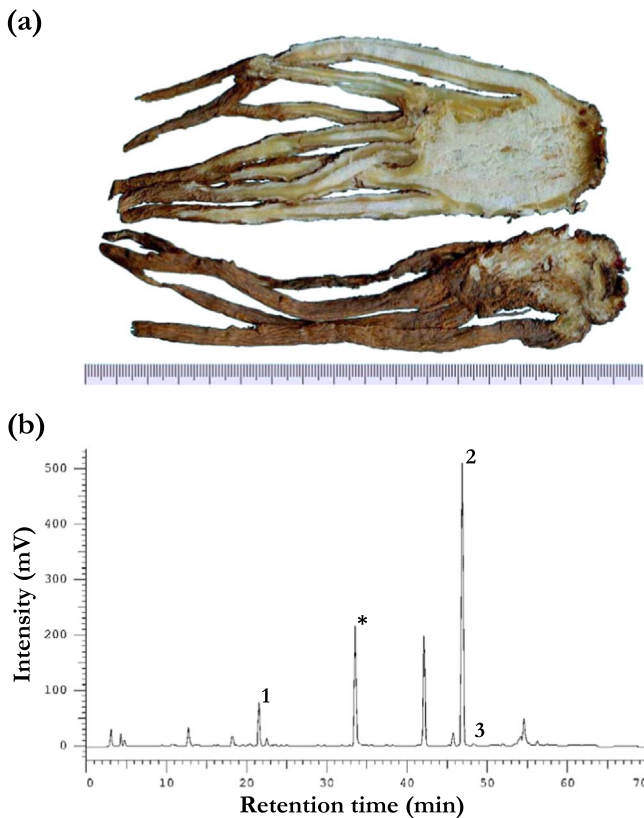


Fig. 1. (a) Crude AS pieces; (b) HPLC chromatogram of AS extract. ¹ ferulic acid, * IS, ² Z-ligustilide, and ³ n-butylidenephthalide.

2.4. Animals and treatment

The animal use protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of National Taiwan Sport University (Taoyuan, Taiwan; approval number: IACUC-10320). Five-week-old female Wistar rats ($n=22$) were purchased from Biolasco (Taipei, Taiwan) and preserved under a 12-h light/dark cycle at $24 \pm 2^\circ\text{C}$ and $65\% \pm 5\%$ humidity with ad libitum access to food (LabDiet 5001 rodent diet, St. Louis, MO, USA) and pure water. After a 1-week acclimation period, the rats were randomly assigned to the following groups: control group (CG; $n=6$); strenuous exercise group (EG; $n=8$); and strenuous exercise and AS extract-treated group (ASG; $n=8$). Daily, $300 \text{ mg kg}^{-1} \text{ d}^{-1}$ of AS extract (prepared in 100 mg mL^{-1} with pure water) was fed once through an oral gavage in the ASG (volume ranged from 0.45 to 0.69 mL, depending on different body

weights), and an equal volume of pure water was provided for the CG and EG. The dose of AS extract used here has been demonstrated to be pharmacologically effective for rats (Circosta et al., 2006; Zhang et al., 2010). The 4-week strenuous exercise training protocol consisted of two exercise sessions: running on a motorized treadmill and swimming in a water container (Table 1). The rats in the EG and ASG were acclimated to performing light exercise on the first 2 days for each session. Thereafter, the exercise duration was progressively increased in main exercise training. The CG rats were placed gently on a treadmill or in the container and kept for an equal time. Bodyweight and food and water intake were recorded semiweekly.

2.5. Physical performance test

2.5.1. Exhaustive swimming test

On the last day prior to sacrifice, the rats in EG and ASG underwent an exhaustive swimming test that involved using constant loads corresponding to 15% of the bodyweight of the rats, as described previously (Huang et al., 2012), with slight modifications. The swimming test was performed in a water container (diameter \times height = $20 \times 65 \text{ cm}$) measuring 30 cm in depth at $28 \pm 2^\circ\text{C}$. The endurance of each rat was recorded as the time (s) from the beginning to exhaustion, which was determined by observing the loss of coordinated movements and failure to return to the surface within 7 s. Times of floating, struggling, and making necessary movements were considered in the swimming duration until exhaustion.

2.5.2. Forelimb grip strength test

On the last day prior to sacrifice, the rats in the EG and ASG underwent a forelimb grip strength test, as described previously (Huang et al., 2012). A low-force testing system (Model-RX-5, Aikoh Engineering, Nagoya, Japan) was used. A force transducer equipped with a metal bar (diameter \times length = $2 \text{ mm} \times 7.5 \text{ cm}$) was used to measure the force of tensile strength of each rat. We grasped each rat at the base of the tail and gently pulled backward as soon as the rat grasped the bar with its forelimb paws; this grasping generated a force of counter pull. The maximal grip strength was recorded in grams.

2.6. Sample collection

At the end of week 4, the rats were anesthetized with 5% isoflurane (0.5 L min^{-1}) and euthanized by exsanguination after 12 h of food deprivation. Blood samples were collected from the abdominal aortas. Whole-blood samples were collected in EDTA tubes, and serum samples were collected in clot activator tubes

Table 1
Four-week strenuous exercise training protocol.

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	AM PM	1 min [#]	2 min [#]	2 min [*]	2 min [*]	3 min [*]	3 min [*]	4 min [*]
Week 2	AM PM	4 min [*]	5 min [*]	5 min [*]	6 min [*]	6 min [*]	7 min [*]	7 min [*]
Week 3	AM PM	10 min [†]	12 min [†]	12 min [‡]	12 min [‡]	14 min [‡]	14 min [‡]	16 min [‡]
Week 4	AM PM	16 min [‡]	18 min [‡]	18 min [‡]	20 min [‡]	20 min [‡]	22 min [‡]	22 min [‡]

[#] Acclimation: running on a motorized treadmill at 9.8 m min^{-1} and an inclination of 16.7%.

^{*} Main exercise: running on a motorized treadmill at 18.6 m min^{-1} and an inclination of 16.7%.

[†] Acclimation: swimming without loads in a water container (30-cm deep) at $28 \pm 2^\circ\text{C}$.

[‡] Main exercise: swimming with loads with additional 10% of bodyweight in a water container (30-cm deep) at $28 \pm 2^\circ\text{C}$.

and isolated by centrifugation at 4000g for 20 min at 4°C. The liver, kidney, spleen, muscle (including gastrocnemius and soleus muscles in the posterior region of the lower legs), brown adipose tissue (BAT), and white adipose tissue (WAT) samples were also collected. The tissue weight (%) relative to individual bodyweight was recorded and calculated. All samples were snap-frozen and stored at –80°C until further analysis. Liver, kidney, spleen, and muscle samples were fixed for histological evaluation in 10% neutral buffered formalin and embedded in paraffin.

2.7. Blood biochemical analysis

All blood biochemical analyses were performed in accordance with the manufacturers' instructions. The levels of whole-blood biochemical parameters, including RBCs, Hb, hematocrit (HCT), and white blood cells (WBCs), were assayed on Sysmex XN-1000 Hematology Analyzer (Kobe, Hyogo, Japan). RBCs and HCT were measured using DC sheath flow detection method. Hb was measured cyanide-free sodium lauryl sulphate method. WBCs were measured using fluorescence flow cytometry. Serum hepcidin-25 levels were measured using a hepcidin-25 bioactive ELISA kit (EIA-5258, DRG, Marburg, Germany). Serum iron, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatine kinase (CK), creatinine, lactic dehydrogenase (LDH), total protein (TP), triacylglycerol (TAG), and uric acid (UA) levels were assayed on Beckman Coulter UniCel Dx C 800 (Brea, CA, USA). AST, CK and LDH were measured using an enzymatic rate method. ALT and ALP were measured using a kinetic rate method. Albumin was measured using a Bromocresol Green method. BUN was measured using enzymatic conductivity rate method. Creatinine was measured using Jaffe's method. TP was measured using rate biuret method. TAG was measured using coupled enzymatic reactions. UA was measured using timed-endpoint method.

2.8. Hepatic antioxidant activity analysis

All hepatic antioxidant activity analyses were performed in accordance with the manufacturer's instructions. Liver tissues were first rinsed with phosphate buffered saline to remove RBCs and clots. For determining superoxide dismutase (SOD) enzyme activity, the liver tissues were homogenized in a cold buffer containing 20 mM HEPES, 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. After centrifugation at 1500g for 5 min at 4°C, the supernatant was collected for assay. A superoxide dismutase assay kit (Item No. 706002, Cayman, Ann Arbor, MI, USA) was used, and absorbance was read at 450 nm. One unit of SOD is defined as the amount of enzymes required to exhibit 50% dismutation of the superoxide radical. The assay measures all three types of SOD (Cu/Zn, Mn, and FeSOD). For determining catalase (CAT) activity, liver tissues were homogenized in a cold buffer containing 50 mM potassium phosphate and 1 mM EDTA. After centrifugation at 10,000g for 15 min at 4°C, the supernatant was collected for assay. Afterward, a catalase assay kit (Item No. 707002, Cayman) was used, and absorbance was read at 540 nm.

2.9. Histological staining of tissues

2.9.1. Immunohistochemical staining of liver tissues

For immunohistochemical (IHC) staining, the Mouse/Rabbit Probe HRP Labeling Kit with DAB Brown (TAHC03D, BioTnA, Kaohsiung, Taiwan) was used. All staining protocols were performed in accordance with the manufacturer's guidelines. The paraffin sections of the liver tissues were deparaffinized in xylene, rehydrated in a graded-alcohol series, and treated with 3% dihydrogen dioxide (H_2O_2) for 10 min. The sections were boiled in a

citrate buffer (pH 6.0) for 30 min, and then blocked with the immunoblock to prevent nonspecific binding for 60 min. Next, the sections were incubated with rabbit anti-IL-6 antibody (diluted 1:200) (bs-0379 R, Bioss, Woburn, MA, USA) for 120 min. Mouse/Rabbit probe HRP labeling was performed for conjugation for 30 min, and peroxidase activity was developed in a 3,3'-diaminobenzidine- H_2O_2 solution for 3 min. The specimens were observed under a light microscope (BX51, Olympus, Tokyo, Japan), and images were captured using a digital CCD camera (DP70, Olympus, Tokyo, Japan).

2.9.2. Periodic acid-Schiff staining of muscle tissues

The deparaffinization, rehydration, and subsequent observation processes were the same as those described previously. Periodic acid-Schiff (PAS) is a staining method to detect polysaccharides, mainly glycogen in muscle tissues. A PAS staining kit (101646, Merck, Darmstadt, Germany) was used. The sections of muscle tissues were oxidized in 0.5% periodic acid solution for 5 min, rinsed in pure water, and placed in Schiff reagent for 15 min. Then, the sections were washed in lukewarm tap water for 5 min, and counterstained in Mayer's hematoxylin for 1 min.

2.9.3. Hematoxylin and eosin staining of spleen, liver, and kidney tissues

The deparaffinization, rehydration, and subsequent observation processes were the same as those described previously. A hematoxylin and eosin (H&E) staining kit (TA01HE, BioTnA, Kaohsiung, Taiwan) was used. The sections of spleen, liver, and kidneys tissues were placed in hematoxylin for 3 min, washed in lukewarm tap water for 5 min, and placed in eosin for 30 s.

2.10. Statistical analysis

Data are expressed as the mean \pm standard error of the mean (SEM). Statistical analyses were conducted using SPSS 20.0. The independent *t*-test was conducted to compare the physical performance in exhaustive swimming and forelimb grip strength test between EG and ASG. One-way ANOVA was performed with Tukey's posttest to compare the differences of all other parameters between groups. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Liquid chromatographic analysis of AS extract

The HPLC chromatogram of the AS extract is shown in Fig. 1b. The retention times of ferulic acid, IS, Z-ligustilide, and *n*-butyridenephthalide were 21.5 min, 33.5 min, 46.9 min, and 48.3 min, respectively. The linearity ranges were 3.96–198 $\mu\text{g mL}^{-1}$, 7.84–392 $\mu\text{g mL}^{-1}$, and 2–100 $\mu\text{g mL}^{-1}$ for ferulic acid ($r^2=0.999$), Z-ligustilide ($r^2=0.999$), and *n*-butyridenephthalide ($r^2=0.998$), respectively. The recovery of each component was within the range of 84.1–108.0% (Supplementary materials S1). The contents of ferulic acid, Z-ligustilide, and *n*-butyridenephthalide in the AS extract were corrected with each recovery, which were 1.57, 17.78, and 0.43 $\mu\text{g mg}^{-1}$, respectively.

3.2. Food intake, water intake, bodyweight, and relative tissue weight

During the experiment, the mean amount of food and water intake was found to be nonstatistically different ($p > 0.05$) (Table 2). At the end of the experiment, the bodyweights of CG and

Table 2
General characteristics of the experimental groups.

Characteristic	CG	EG	ASG
Food intake (g·d ⁻¹)	158.0 ± 7.8	149.9 ± 2.7	156.3 ± 5.8
Water intake (g·d ⁻¹)	289.6 ± 36.3	275.0 ± 24.0	287.1 ± 29.3
Relative weight (%)			
Liver	3.10 ± 0.15	2.95 ± 0.05	2.91 ± 0.09
Kidney	0.76 ± 0.01	0.81 ± 0.03	0.78 ± 0.01
Spleen	0.26 ± 0.01	0.29 ± 0.02	0.26 ± 0.01
Muscle	1.24 ± 0.02	1.20 ± 0.04	1.20 ± 0.02
BAT	0.14 ± 0.02	0.18 ± 0.02	0.19 ± 0.02
WAT	1.17 ± 0.10 [†]	0.79 ± 0.08 [*]	0.79 ± 0.09 [*]

Values are presented as mean ± SEM.

CG; control group (n=6), EG; strenuous exercise group (n=8), ASG; strenuous exercise and AS extract-treated group (n=8), BAT; brown adipose tissue, and WAT; white adipose tissue

^{*} Indicates $p < 0.05$ compared with CG.

[†] Indicates $p < 0.05$ compared with EG.

ASG did not differ ($p > 0.05$); however, the bodyweights of EG rats were significantly lowered by 9.6% ($p < 0.05$ vs. CG) (229.6 ± 4.5 g, 207.4 ± 3.6 g, and 219.5 ± 5.7 g for CG, EG, and ASG, respectively) (Fig. 2a). As listed in Table 2, the relative weights of the liver, kidney, muscle, and BAT samples did not differ among the groups. Compared with the CG, a 15.1% increase in the relative weight of spleen samples was observed in EG; however, the difference was nonsignificant ($p = 0.098$ vs. CG). Strenuous exercise also significantly reduced the relative weights of WAT by 32.5% (both $p < 0.05$ vs. CG) for both the EG and ASG.

3.3. Physical performance

The physical performance tests included exhaustive swimming and a forelimb grip test. The results revealed that the time of exhaustive swimming was prolonged by 95.6% after 4-week AS treatment; however, the value did not differ in both groups ($p = 0.161$ vs. EG) because of the large SEM (450.8 ± 124.5 s and 881.5 ± 262.8 s for EG and ASG, respectively) (Fig. 2b). Grip strength was significantly elevated by 10.2% in ASG ($p < 0.05$ vs. EG; Fig. 2c).

3.4. Blood biochemical parameter assessment

RBC (Fig. 3a), Hb (Fig. 3b), and HCT (Fig. 3c) levels of EG decreased significantly, by 9.2%, 9.0%, and 9.9%, respectively, compared with those of the CG ($p < 0.05$ vs. CG), and AS extract treatment increased these parameters by 13.3%, 10.6%, and 13.0%, respectively, compared with the EG ($p < 0.05$ vs. EG). Compared with the CG, significant increases in WBC levels by 45.2% and 53.3% were observed in EG and ASG, respectively ($p < 0.05$ vs. CG; Fig. 3d). Hepcidin-25 levels (Fig. 4a) were elevated by 33.2% after strenuous exercise in EG; however, no statistical differences were present ($p = 0.088$ vs. CG). The AS extract treatment significantly reduced hepcidin-25 levels by 33.0% ($p < 0.05$ vs. EG), and exhibited no differences in hepcidin-25 levels in CG ($p > 0.05$ vs. CG) (25.4 ± 3.2 ng dL⁻¹, 33.9 ± 2.7 ng dL⁻¹, and 22.7 ± 1.5 ng dL⁻¹ for CG, EG, and ASG, respectively). Iron levels (Fig. 4b) decreased significantly by 27.8% in EG ($p < 0.001$ vs. CG), but increased significantly by 34.3% in ASG ($p < 0.001$ vs. EG). AST levels (Fig. 4c)

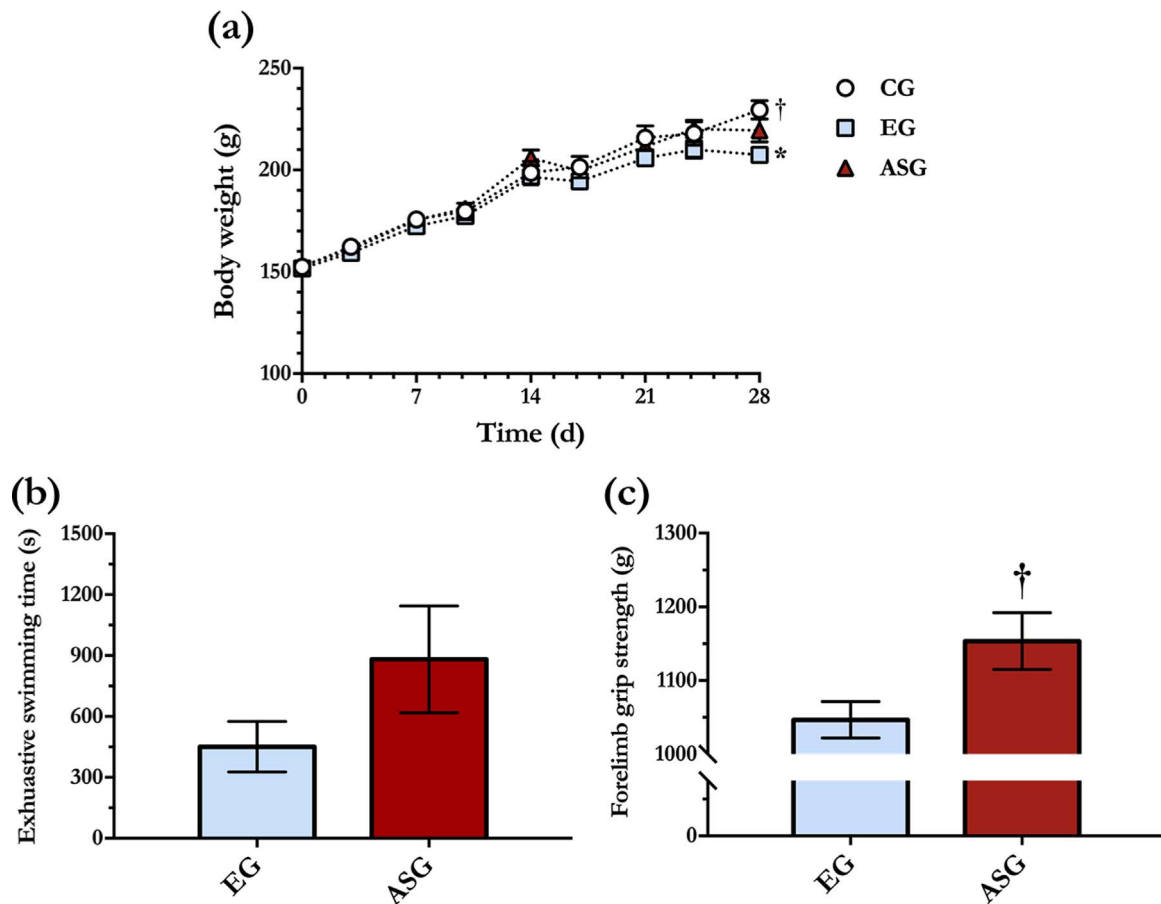


Fig. 2. Effect of strenuous exercise and AS on (a) the bodyweight with time; (b) exhaustive swimming test; and (c) forelimb grip test. Values are presented as mean ± SEM. * Indicates $p < 0.05$ compared with CG. † Indicates $p < 0.05$ compared with EG. CG; control group (n=6), EG; strenuous exercise group (n=8), ASG; strenuous exercise and AS extract-treated group (n=8).

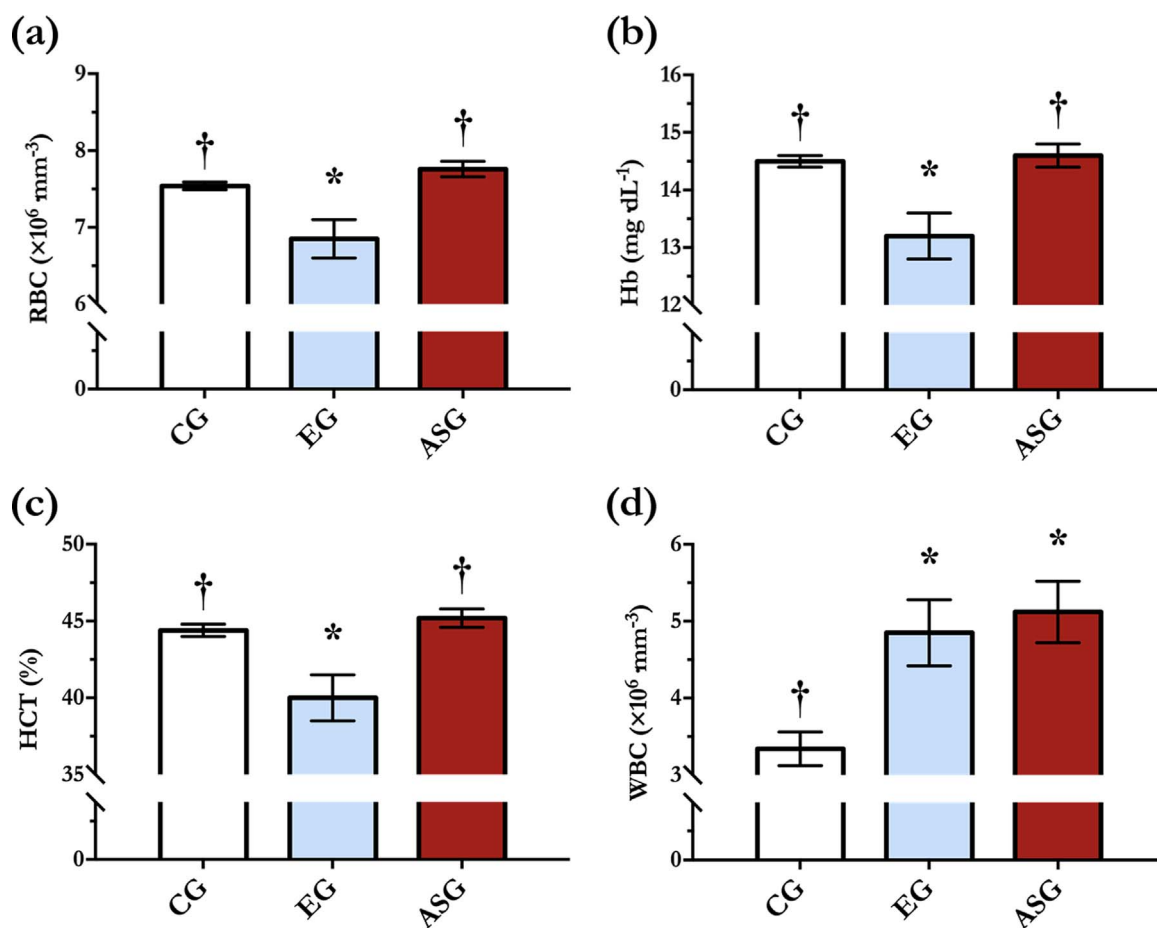


Fig. 3. Effect of strenuous exercise and AS on the levels of (a) RBC; (b) Hb; (c) HCT; and (d) WBC. Values are presented as mean \pm SEM. * Indicates $p < 0.05$ compared with CG. † Indicates $p < 0.05$ compared with EG. CG; control group ($n=6$), EG; strenuous exercise group ($n=8$), ASG; strenuous exercise and AS extract-treated group ($n=8$), RBC; red blood cell, Hb; hemoglobin, HCT; hematocrit, and WBC; white blood cell.

did not differ between the groups ($p > 0.05$). ALT levels (Fig. 4d) increased significantly by 21.3% in EG ($p < 0.05$ vs. CG), but decreased significantly by 14.9% in ASG ($p < 0.05$ vs. EG).

The assessment results of other clinical toxicity-related serum biochemical markers in each group are listed in Table 3. Albumin, ALP, BUN, creatinine, LDH, TP, and UA levels exhibited no differences between the groups ($p > 0.05$). CK levels in the ASG were significantly lower than those in the EG ($p < 0.01$ vs. EG). TAG levels in the EG were significantly lower than those in the CG ($p < 0.05$ vs. CG), as were TAG levels in the ASG ($p < 0.01$ vs. CG).

3.5. Hepatic antioxidant activities

As shown in Fig. 5, hepatic SOD levels exhibited no differences among the groups ($p > 0.05$). Compared with the CG, after strenuous exercise, hepatic CAT levels significantly decreased by 9.4% in the EG ($p < 0.05$ vs. CG), and compared with the EG, AS extract treatment significantly increased hepatic CAT levels by 12.9% in the ASG ($p < 0.01$ vs. EG).

3.6. Histological observation of tissues

Hepatic IL-6 expression was assessed by IHC and presented in brown color (Fig. 6a). The livers in CG exhibited only a weak reaction for IL-6 in the area surrounding the hepatic central vein. However, the livers in the EG exhibited a strongly positive reaction for IL-6, and IL-6 was permeated in the tissues. After AS extract treatment, the livers in the ASG exhibited weak positive staining

for IL-6 in the area surrounding the hepatic central vein.

Muscle glycogen expression was observed by PAS and presented in magenta color (Fig. 6b). Compared with the CG, the glycogen content was almost depleted as energy after strenuous exercise, resulting in weak responses in the EG. In the ASG, however, it preserved a certain amount of glycogen in most of the muscle cells.

Through H&E staining, the red and white pulps in spleen were normal among treatments, no obvious damages were found (Fig. 7a). In liver and kidney tissues, the arrangement of hepatic cords and vascular sinusoids did not differ, nor the structure of renal glomerulus and tubules among treatments (Fig. 7b and c).

4. Discussion

The developed exercise protocol, which included running and swimming, induced anemia effectively. The exercise protocol workload was consistent, and the exercise time was increased incrementally. Our running protocol was set at 18.6 m min^{-1} and an inclination of 16.7% on the motorized treadmill, which was demonstrated to yield more than 80% of maximal oxygen consumption (Bedford et al., 1979). Mechanical trauma by footstrike is documented as a primary factor contributing to exercise-induced hemolysis (Telford et al., 2003); however, we observed that prolonging the volitional training time was difficult, whereas causing injury to the hind limbs of rats was easy. We did not use electricity stimulation because it would have made the rats feel

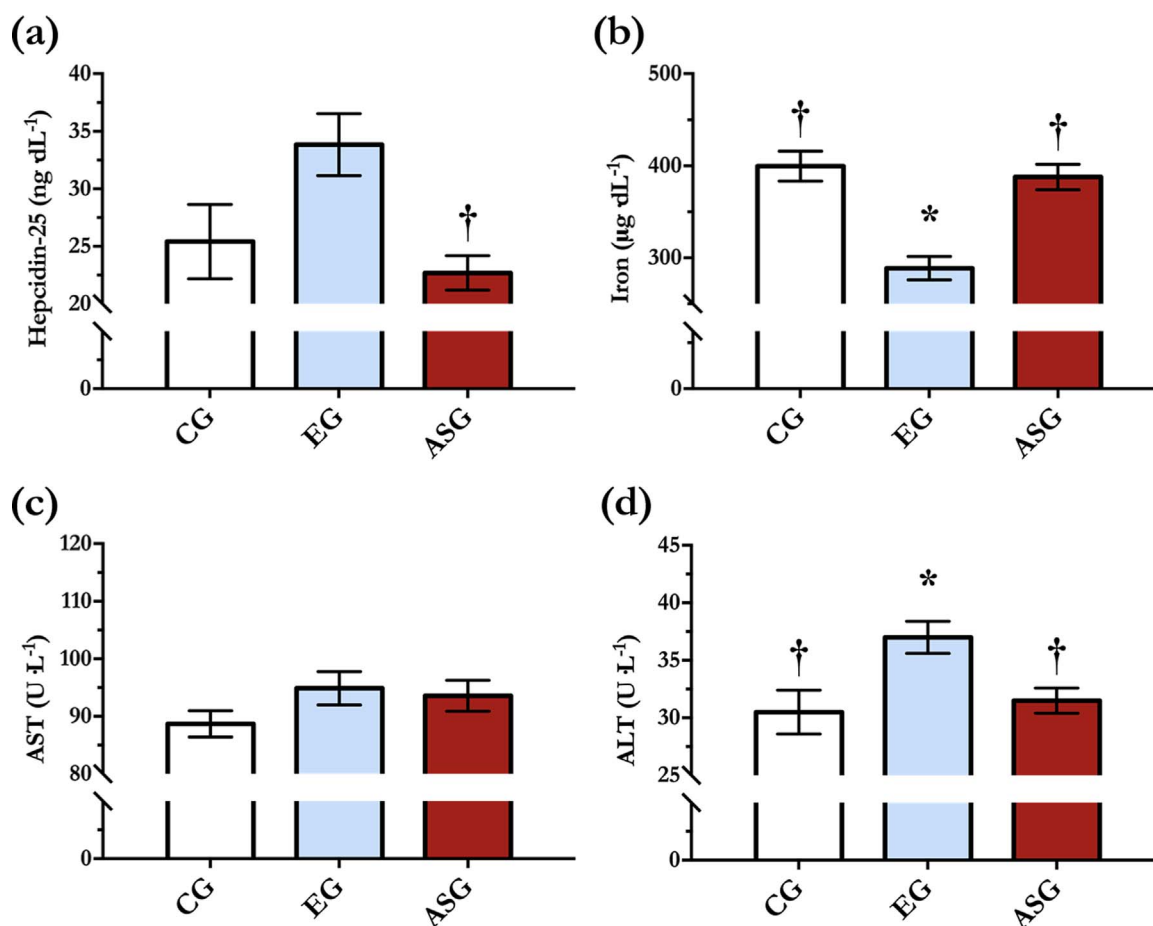


Fig. 4. Effect of strenuous exercise and AS on the serum levels of (a) hepcidin-25; (b) iron; (c) AST; and (d) ALT. Values are presented as mean \pm SEM. * Indicates $p < 0.05$ compared with CG. † Indicates $p < 0.05$ compared with EG. CG; control group ($n=6$), EG; strenuous exercise group ($n=8$), ASG; strenuous exercise and AS extract-treated group ($n=8$), AST; aspartate aminotransferase, and ALT; alanine aminotransferase.

Table 3
Clinical toxicity-related biochemical markers in the serum.

Parameter	CG	EG	ASG
Albumin (g dL ⁻¹)	3.98 \pm 0.06	3.94 \pm 0.04	3.98 \pm 0.06
ALP (U L ⁻¹)	67.5 \pm 3.4	80.0 \pm 3.4	69.4 \pm 6.2
BUN (mg dL ⁻¹)	13.8 \pm 0.7	14.6 \pm 0.4	13.9 \pm 0.5
CK (U L ⁻¹)	550.0 \pm 19.1	590.5 \pm 35.1	451.5 \pm 24.2 [†]
Creatinine (mg dL ⁻¹)	0.42 \pm 0.01	0.43 \pm 0.02	0.40 \pm 0.01
LDH (U L ⁻¹)	674.5 \pm 15.9	707.1 \pm 47.2	628.5 \pm 57.5
TP (g dL ⁻¹)	5.27 \pm 0.07	5.21 \pm 0.04	5.25 \pm 0.07
TAG (mg dL ⁻¹)	40.3 \pm 4.4 [†]	28.5 \pm 2.7 [*]	21.4 \pm 1.9 [*]
UA (mg dL ⁻¹)	1.93 \pm 0.04	1.83 \pm 0.11	1.76 \pm 0.08

Values are presented as mean \pm SEM.

CG; control group, EG; strenuous exercise group, ASG; strenuous exercise and AS extract-treated group, ALP; alkaline phosphatase, BUN; blood urea nitrogen, CK; creatine kinase, LDH; lactic dehydrogenase, TP; total protein, TAG; triacylglycerol, and UA; uric acid

* Indicates $p < 0.05$ compared with CG.

† Indicates $p < 0.05$ compared with EG.

extremely nervous or irritable. Our swimming protocol was set at loading an additional 10% of bodyweight by fastening a soft Velcro strap around the chest of rats instead of on their tail, which may interrupt balance control. Swimming was a relatively difficult training task, and it posed a severe problem because of potential drowning. Overall, we combined two available exercise training tasks for rats with an increasing-intensity approach. Our exercise

protocol exhibited a considerably higher intensity within a shorter exercise duration compared with other studies. However, the results revealed that the anemic condition we triggered was insufficiently severe. RBC and Hb levels decreased by 9.2% and 9.0%, respectively. Liu et al. (Liu et al., 2011) established a 5-week running training program at 30 m min⁻¹ at 0% inclination for 2–95 min d⁻¹, and found that RBC and Hb levels decreased by 32.4% and 32.0%, respectively. Zhu et al. (Zhu et al., 2010) conducted a 5-week swimming training regimen for 30–150 min d⁻¹, and reported that RBC and Hb levels decreased by 36.6% and 11.4%, respectively. Although few studies have investigated the relation between exercise duration and anemia, the available data suggested that our developed protocol was imperfect. Nonetheless, our results revealed significant differences in RBC and Hb levels in the CG and EG. The relative weight of the spleen, an organ that plays a major role in removing old RBCs, reserving blood, and recycling iron in the EG was 15.1% more than that in the CG; however, it did not show obvious differences in histological observation compared with other groups.

We further investigated the protective effects of AS on sports anemia and related responses, such as physical performance, iron status, oxidative enzymes, and inflammation. Iron plays a fundamental role in forming complexes with molecular oxygen in Hb in processes associated with oxygen transport during physical exercise. Hepcidin is the central regulator of systemic iron homeostasis. After a 4-week strenuous exercise period, as expected, AS elevated serum iron levels and reduced serum hepcidin-25 levels. The results suggested that the AS facilitated decrease in hepcidin-

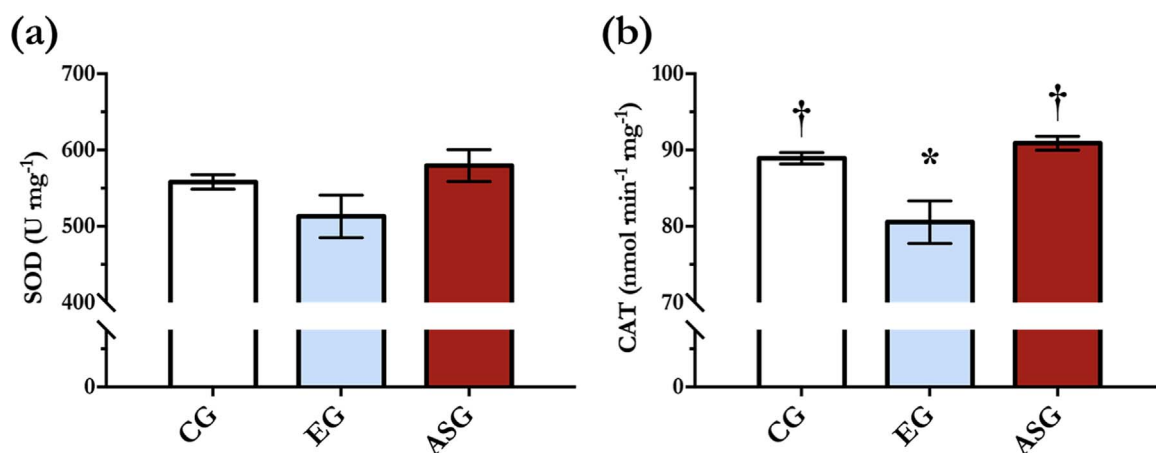


Fig. 5. Effect of strenuous exercise and AS on the hepatic activities of (a) SOD and (b) CAT. Values are presented as mean \pm SEM. * Indicates $p < 0.05$ compared with CG. † Indicates $p < 0.05$ compared with EG. CG; control group ($n=6$), EG; strenuous exercise group ($n=8$), ASG; strenuous exercise and AS extract-treated group ($n=8$), SOD; superoxide dismutase, and CAT; catalase.

25 levels may have induced ferroportin activation, thereby increasing iron release into the serum (mainly from enterocytes, macrophages, hepatocytes, and placental cells) and amending iron depletion (Buratti et al., 2015). The underlying mechanisms of AS acting on hepcidin-25 were examined by suppressing the expression of JAK1/2, phospho-JAK1/2, phospho-SMAD1/5/8, and phospho-ERK1/2, and promoting SMAD7 expression in the liver (Zhang et al., 2014). Furthermore, a novel aspect of iron in muscle physiology was that mTOR is involved in hepcidin inhibition, which may stimulate muscle growth (Mleczko-Sanecka et al., 2014). In our previous study (Yeh et al., 2014a), we demonstrated that AS treatment elevated mTOR phosphorylation levels significantly in myotubes, which strongly supported the potential

attenuation of hepcidin-25 in coordination. In this study, we also observed a significant increase in forelimb grip strength in the ASG. The preservation of glycogen content might explain the elevation of the physical performance. In histological observation, the results suggested that AS prevented the over depletion of muscle glycogen induced by strenuous exercise. Previous study (Wang et al., 2015) has revealed that the AS extract administration increased the glycogen levels in liver and skeletal muscle, whereas our findings are in accordance.

Studies have shown that strenuous exercise can substantially increase the levels of proinflammatory cytokines, particularly IL-6 (Ostrowski et al., 1999; Pedersen et al., 2001). To date, the problem of cytokine-induced hepcidin upregulation remains debatable. The

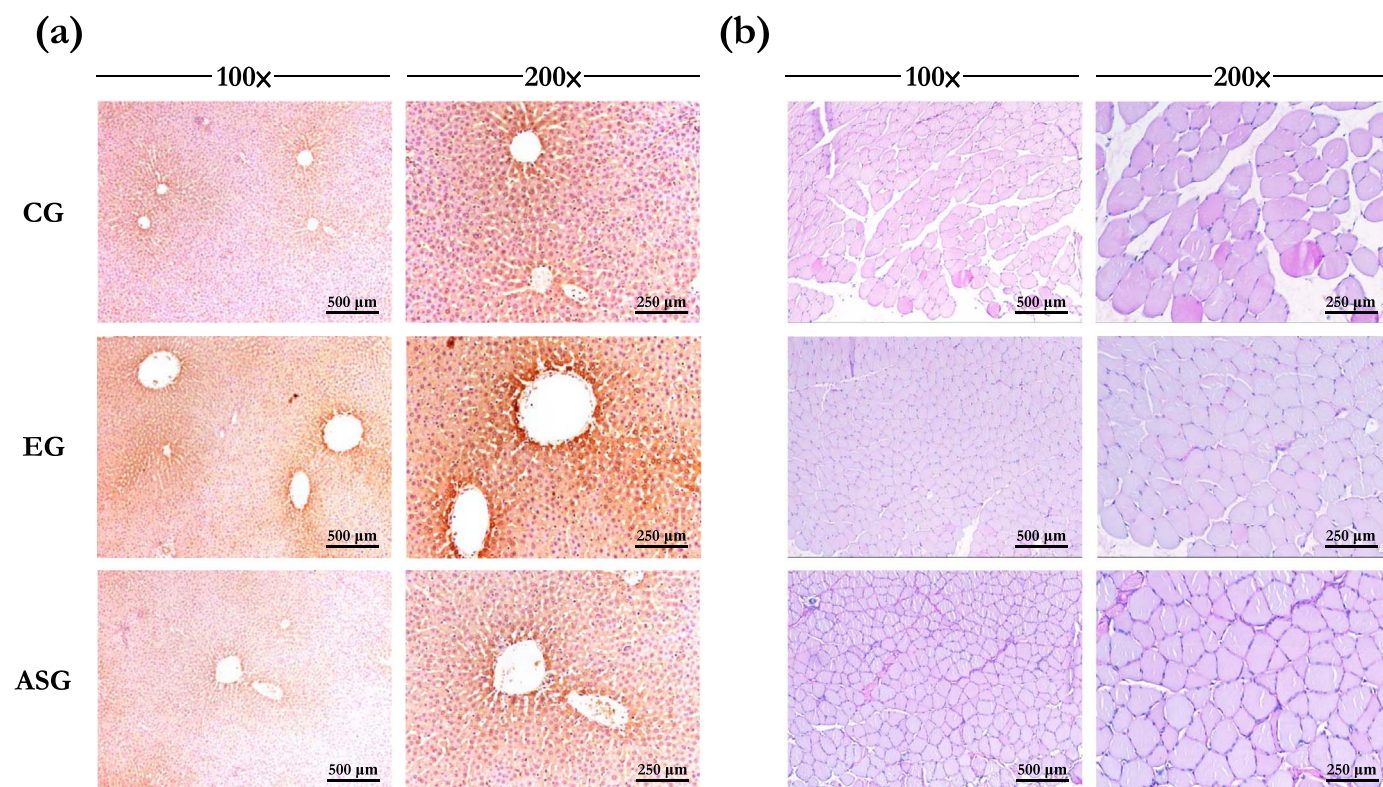


Fig. 6. Effect of strenuous exercise and AS on the histology of (a) liver tissues (IHC staining of IL-6, presented in brown color) and (b) muscle tissues (PAS staining of glycogen, presented in magenta color). CG; control group ($n=6$), EG; strenuous exercise group ($n=8$), ASG; strenuous exercise and AS extract-treated group ($n=8$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

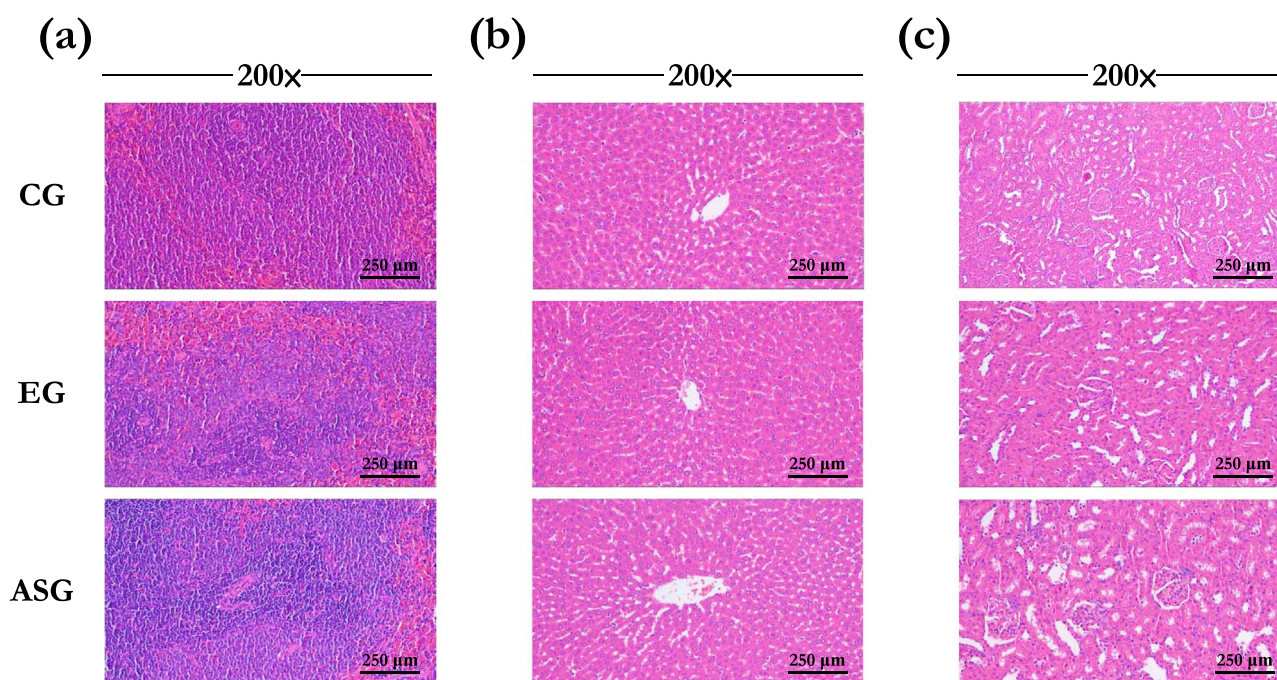


Fig. 7. Effect of strenuous exercise and AS on the histology of (a) spleen tissues; (b) liver tissues; and (c) kidney tissues (H&E staining). CG; control group ($n=6$), EG; strenuous exercise group ($n=8$), ASG; strenuous exercise and AS extract-treated group ($n=8$).

relationship between inflammation and hepcidin has been addressed in several studies (Badenhorst et al., 2015; Banzet et al., 2012; Stoian et al., 2007), but in certain other studies (Burden et al., 2015; Ciniselli et al., 2015), this relationship was found to be independent. Burden et al. (2015) reported that hepcidin is uncorrelated with IL-6. Exercise-induced increases in IL-6 have caused increases in hepcidin levels in athletes with a regular training routine; however, elite athletes may experience less inflammatory response because of adaption. The authors observed a post-exercise increase in hepcidin that was independent of changes in IL-6 after treatment with intravenous iron. Thus, intense exercise contributed to IL-6 production. The liver histological observation in our present study revealed that AS treatment considerably ameliorated the IL-6 production. Studies have reported that all the major constituents of AS potentially contributed to anti-inflammatory activity. Ferulic acid presented an inhibitory activity on the expression of tumor necrosis factor (TNF)- α and IL-1 β cytokine by inhibiting the activation of nuclear factor (NF)- κ B in lipopolysaccharide (LPS)-activated macrophages (Navarrete et al., 2015). Moreover, Z-ligustilide significantly inhibited the upregulation of NF- κ B activation and the production of IL-1 β , TNF- α , inducible nitric oxide synthase, intercellular adhesion molecule-1, and cyclooxygenase-2 in ovariectomized osteopenic rats (Ma and Bai, 2012), and *n*-butylidenephthalide inhibited the LPS-induced production of nitric oxide, TNF- α , and IL-1 β in rat brain microglia (Nam et al., 2013). Most studies have fulfilled the anti-inflammatory properties of AS; however, this might not be the main contribution in alterations to hepcidin levels. Nonetheless, our results evidenced the beneficial effects of AS on attenuating strenuous exercise-induced inflammation.

Oxidative stress is another major cause of sports anemia. Compared with the resting phase, oxygen consumption in muscles increases by 100 times during strenuous exercise. This event induces an electron flow increase through the mitochondrial respiratory chain, resulting in the abundant production of ROS, which are toxic products for the metabolism. ROS could induce lipid peroxidation in erythrocyte membranes, which initiates the hemolytic process (Bonilla et al., 2005). SODs are metalloenzymes

that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide, and CAT is an ubiquitous enzyme involved in the detoxification of hydrogen peroxide (Malstrom et al., 1975). In this study, SOD expression did not undergo changes following exercise or AS treatment. However, exercise intervention diminished CAT expression; AS treatment elevated CAT expression. This suggested that AS treatment played a major role in cellular antioxidant defense. In addition, muscular exercise can release the liver function parameters (e.g., AST and ALT) into the serum, which is indicative of hepatic injury (Pettersson et al., 2008). Our findings revealed that AS treatment reduced ALT levels significantly, and thus, exerted hepato-protective effects. Similar results were reported in (Yu et al., 2013); AS significantly restored carbon tetrachloride-induced hepatotoxicity by normalizing serum AST, ALT, and ALP levels.

We determined clinical toxicity-related biochemical markers in the serum. Our results indicated that albumin, ALP, BUN, creatinine, LDH, TP, and UA levels involved in hepatic, renal, and systemic cell functions did not change after AS treatment. Additionally, it did not differ between groups in histological observation in major organs, liver and kidney. These suggested that the dose of AS used in this study provided physiological benefits without any health risks. We also observed a significant reduction in serum CK levels after AS treatment. CK catalyzes creatine to convert phosphocreatine and adenosine diphosphate by consuming adenosine triphosphate. Clinically, CK is assayed in blood tests as a marker of muscle damage. In addition, compared with CG, serum TAG levels and the relative weights of WAT in EG and ASG exhibited significant decreases, which again suggested that the exercise training was strenuous for the rats.

In conclusion, sports anemia developed in various causes of formation. We first confirmed the protective effects of a TCM, AS, on strenuous exercise-induced sports anemia through several critical perspectives. Our findings evidenced a novel and promising therapeutic approach for AS treatment ($300 \text{ mg kg}^{-1} \text{ d}^{-1}$) for rescuing the anemic condition induced following 4 weeks of strenuous exercise. AS improved grip strength, preserved muscle glycogen after exercise, and significantly elevated RBC, Hb, and

HCT levels. In addition, serum hepcidin-25 levels decreased, and consequently, serum iron levels increased. Moreover, serum ALT as well as hepatic IL-6 levels were attenuated. The antioxidative effect of AS was also verified by increasing hepatic CAT expression. Further research on the effects of AS in humans is warranted.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2016.08.010>.

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