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# Reduction of hemoglobin, not iron, inhibited maturation of red blood cells in male rats exposed to high intensity endurance exercises

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ABSTRACT

The existence of sports anemia, induced by strenuous or long-term exercise and characterized by decreases of red blood cells (RBCs), hemoglobin and iron content, remains to be doubtful. To observe the effects of endurance exercise on RBCs and explain the underlying reason, we designed this study by observing RBCs parameters and iron metabolism in 8-weeks training rats and effects of iron supplement or protein supplement on RBCs. Results showed that erythrocyte counts, hematocrit, mean corpuscular volume and hemoglobin content decreased while RBC distribution width increased in exercised rats at later stage during 8 weeks training. But the contents of serum iron and ferritin decreased only at 1-week and 2-week and returned to normal at 4-week and 8-week. Same as iron content, apparent iron absorption rate was declined at early stage but restored to normal level at 8-week, as well as serum adrenaline, cortisol and insulin levels. Instead, the contents of total protein and albumin in serum were decreased at later stage during 8-weeks training. Furthermore, we observed that protein supplement ameliorated RBCs parameters in rats exposed to 8 weeks swimming exercise, but iron supplement had no effects on RBCs, though it obviously increased iron content of serum and the liver. Based on these results, we drew a conclusion that transient changes of iron metabolism, which may be induced by stress hormone changes, was not the reason for RBCs decrease in endurance exercises but hemoglobin reduction, induced by defects in protein supplement, impeded development of RBCs.

## 1. Introduction

Sports or manual labor capacity depends on sufficient ATP supplied by aerobic metabolism [1,2], which needs enough oxygen transported by blood. Hemoglobin is the carrier of oxygen in RBCs and tightly connected with sports capacity while iron is an indispensable trace element for composite of hemoglobin and maturation of RBCs [3]. It is reported that strenuous or long-term sports decreased the quantities of red blood cells (RBCs), hemoglobin and iron in blood [4], which is called sports anemia [5]. So far, a number of causes inducing sports anemia have been proposed, including stress hormones alterations, hemolysis, dehydration, disturbances of iron metabolism and so on [6,7]. Among these, iron dyshomeostasis is the spotlight for researchers. Dr. Kong and colleagues review that iron metabolism in sports anemia is regulated by hepcidin [7], which is changed after intensive exercises resulted from hypoxia, inflammation, erythropoietin and insulin as we reported before [8]. Besides, iron supplement has been used to improve iron status and exercise performance of female athlete [9].

But other researchers raised some controversial opinions about sports anemia and iron supplement. It was reported that hemoglobin and serum iron levels did not differ significantly between two periods involving different training regimens in female runners [10]. Nabatov and colleagues also found that iron content did not differ between non-athletic female group and female athletes groups [11]. Moreover, it was even increased in both men and women after exercise in Fragala's research [12]. Meanwhile, oral iron supplement has been criticized because of hazard effects of iron overload [9], which is proved to be capable of inducing hepatic inflammation [13]. In addition, not all iron supplement trails are successful to ameliorate iron status and exercise performance in athletes, as Dr. Tsalis and colleagues reported [14]. The contradictory results from different studies lead to unconsensuses on the existence of sports anemia, the induction of iron deficiency by physical activities, and the requirement of iron supplement for athletes

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or labor workers.

The kind, intensity, duration of sports and the physique, sex-difference and diet of individuals may be reasons for opposite conclusions obtained by researchers. In order to verify the existence of sports anemia and effects of iron supplement or protein supplement, in this study, we used male Sprague Dawley rats with identical genetic background, age, and diet and exposed to running or swimming with same duration and intensity for detection of RBCs parameters and iron content. Further, we evaluated protein nutrition status and iron absorption rate of rats during long-term endurance exercises to explore underlying reasons for hemoglobin and iron content alterations. We also conducted experiments of iron supplement or protein supplement to identify the key factor influencing RBCs parameters in rats during long-term endurance exercise.

## 2. Methods

### 2.1. Animals

We used 8-weeks aged, 160 ± 10 g weighted, male Sprague Dawley rats (purchased from Slac Laboratory Animal, Shanghai, China) and conducted all animal experiments according to the "Guide for the Care and Use of Laboratory Animals" with the approval of Second Military Medical University Institutional Animal Care and Use Committees. In intensive exercises experiment, 96 rats were randomly divided into 3 groups: Control, Running and Swimming. Each group contained 4 sub-groups: 1-week, 2-week, 4-week and 8-week (n = 8 in each sub-group). Rats in Running group received 2 h running per day on a JD-PT running platform at the speed of 25 m/min and rats in Swimming group were forced to swim for 2 h per day in a 32 ± 2 ℃ water pool. Running or swimming were performed continuously for 8 weeks, 5 days per week. All rats received formula diet ad libitum, containing 45 ppm iron and 18% protein according to AIN-93. In iron supplement and protein supplement experiment, 24 rats were divided into 4 groups: Control, Swimming, Swimming + Iron and Swimming + Protein (n = 6 in each group). Rats in swimming groups received 8 weeks exercise as above and were fed by control diet (45 ppm iron and 18% protein), iron supplement diet (adding iron-dextran, containing 500 ppm iron) and protein supplement diet (containing 40% protein) respectively. All rats received supplemented diet ad libitum as well. Rats were anaesthetized at each time-point for collection of blood and tissues.

### 2.2. Determination of red blood cells parameters

Rat blood was taken from heart into BD Vacutainer (Cat. 367843, containing EDTA-K2). The blood samples were loaded to automatic hematology analyzer (KX-21, Sysmex, Japan) for detection of counts of RBCs, hemoglobin (Hb) concentrations, hematocrit (HCT), mean corpuscular volume (MCV), mean cellular hemoglobin contents (MCH) and RBCs distribution width (RDW) based on principles of electric pulse method and colorimetry.

### 2.3. Determination of serum hormone content

We used radioimmunoassay kits to quantitate the content of adrenaline, cortisol, insulin and erythropoietin in serum samples following the manual protocols. In brief, $I^{125}$ labeled antigen-antibody compound was supplied by the kit, then we incubated our serum samples with these compounds to compete with $I^{125}$ labeled antigen and set standard samples to obtain standard curve. At last, we detected the signals from samples and calculated for concentrations of target antigen in serum. All kits were purchased from North Institute of Biological Technology Co (Beijing, China).

### 2.4. Determination of Iron content of the liver and serum

The liver iron content was determined by a flame atomic absorption spectrophotometer (FAAS, Z-8100, Hitachi, Tokyo, Japan) and normalized by the dry tissue weight for each sample [13]. The dried liver tissues were firstly needed to be digested by mixed acid (nitric acid: perchloric acid = 4: 1) into transparent solution in a heater. Then the digested liquid was added to 10 ml and determined absorbance by FAAS with iron standard-samples together. The iron standard-samples were diluted from iron standard solution (100 μg/mL, 99% purity, Cat.GBW (E)080624, Shanghai Institute of Measurement and Testing Technology) and divided into 6 standard-samples at concentration of 10 μg/mL, 8 μg/mL, 6 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL by deionized water. All standard-samples and test-samples were prepared in the same way and detected the absorbances at the same time. After determining the absorbances of standard-samples and test-samples, we established standard curve based on absorbances and concentrations of standard-samples ($R2 > 0.99$), then we calculated iron concentration in test-samples according to their absorbances and therefore calculated iron content in the liver. Serum iron was separated from transferrin in acidic medium, then reduced to ferrous bivalent by reductant, and a purple-red compound was formed with ferrozine. There is an absorption peak at 562 nm of this compound. The content of serum iron can be calculated by standard curve obtained from iron standard solution treated in the same way.

### 2.5. Determination H2 of protein content in serum

Total protein content in serum was determined by a BCA kit (Cat. 23225, Thermo Scientific). And we used commercial ELISA kits for detection of serum content of albumin (Cat. ml028495, Enzyme-linked Biotechnology, Shanghai, China), ferritin (Immunology Consultants Laboratory, Inc.) and soluble transferrin receptor (Cat. JL21128, JianglaiBio, Shanghai, China).

### 2.6. Determination of diet intake and iron apparent absorption rate

We used comprehensive laboratory animal monitoring system (CLAMS, Coulumbus Instruments, USA) to record the weight of consuming feed and the feces for 24 h at 1-, 2-, 4-, 8-week time point. After determination of the iron content in feed and feces, we calculated the iron apparent absorption rate by the following formula. Apparent absorption rate = (feed iron content – feces iron content) / feed iron content × 100%.

### 2.7. Statistics

All data were represented as Mean ± SD. One-way ANOVA was used for multigroup comparison, if the data obeyed the normal distribution and homogeneity of variance. If not, Kruskal-Wallis test was used for multigroup comparisons. We set $p < 0.05$ as statistically significant level, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs. Control.

## 3. Results

### 3.1. Long-term endurance exercise induced immaturities of RBCs

To identify the appearance of anemia after long-term exercises, we detected RBCs parameters of male SD rats with identical genetic background and same exercises at different time points during 8 weeks training (Table 1). The results showed that the counts of RBCs did not change at 1-, 2-, 4-week time points, but slightly decreased at 8-week time point in both running and swimming groups with statistical significances. The contents of hemoglobin, HCT and MCH decreased while RDW increased after 8 weeks exercises, in which MCH decrease meant that the hemoglobin content in each RBC was decreased while RDW

**Table 1**
RBCs parameters of rats during 8 weeks endurance exercises. All data were represented as Mean ± SD. *: $p < 0.05$, vs. Control (n = 8).

| | Time (week) | Control | Running | Swimming |
|---|---|---|---|---|
| RBC counts (10^12) | 1 | 6.82 ± 0.21 | 6.78 ± 0.22 | 6.67 ± 0.37 |
| | 2 | 7.38 ± 0.5 | 7.18 ± 0.30 | 7.14 ± 0.46 |
| | 4 | 7.48 ± 0.17 | 7.42 ± 0.22 | 7.41 ± 0.16 |
| | 8 | 7.93 ± 0.32 | 7.35 ± 0.40* | 7.23 ± 0.19* |
| Hemoglobin (g/L) | 1 | 136.61 ± 3.71 | 135.01 ± 3.61 | 134.72 ± 8.72 |
| | 2 | 145.51 ± 9.80 | 133.32 ± 4.51 | 135.60 ± 10.41 |
| | 4 | 143.80 ± 3.01 | 138.01 ± 3.60 | 137.21 ± 6.20 |
| | 8 | 145.11 ± 4.81 | 137.01 ± 3.82* | 133.50 ± 3.25* |
| HCT (%) | 1 | 41.52 ± 1.21 | 40.91 ± 1.21 | 42.82 ± 3.22 |
| | 2 | 42.8 ± 3.01 | 41.70 ± 1.12 | 41.52 ± 2.71 |
| | 4 | 41.82 ± 0.81 | 40.91 ± 1.22 | 39.33 ± 2.00 |
| | 8 | 43.02 ± 1.12 | 39.20 ± 0.31* | 38.91 ± 0.78* |
| MCV (fL) | 1 | 60.81 ± 1.62 | 59.23 ± 1.50 | 59.32 ± 2.52 |
| | 2 | 59.72 ± 1.7 | 52.71 ± 1.75* | 51.91 ± 1.60* |
| | 4 | 59.51 ± 1.23 | 51.21 ± 1.50* | 50.10 ± 2.30* |
| | 8 | 54.30 ± 1.31 | 53.31 ± 2.32 | 52.02 ± 1.03 |
| MCH (pg) | 1 | 20.12 ± 0.58 | 19.20 ± 0.51 | 19.31 ± 0.69 |
| | 2 | 19.70 ± 0.86 | 19.61 ± 0.62 | 19.61 ± 0.62 |
| | 4 | 18.82 ± 0.30 | 17.61 ± 0.50* | 17.21 ± 0.71* |
| | 8 | 18.30 ± 0.41 | 16.21 ± 0.52* | 16.80 ± 0.41* |
| RDW (%) | 1 | 13.42 ± 0.67 | 13.50 ± 0.41 | 13.50 ± 0.62 |
| | 2 | 13.21 ± 0.63 | 14.33 ± 0.31* | 14.80 ± 0.71* |
| | 4 | 13.50 ± 0.64 | 14.43 ± 0.42* | 14.82 ± 0.51* |
| | 8 | 13.62 ± 0.40 | 14.90 ± 0.93* | 15.61 ± 0.92* |

increase meant that the differences of RBCs volume increased. These results suggested immature RBCs were increased because the volumes of erythrocyte precursors were bigger. Obviously, the above parameters did not change dramatically and were still at the range of normal reference values of hematological parameters [15,16] in rats after 8 weeks exercises, which did not meet the criteria of anemia [17,18], indicating that long-term endurance exercises would not induce sports anemia in rats.

### 3.2. Iron metabolism was disrupted at early stage but restored to normal at later stage during long-term endurance exercise

Considering that iron deficiency might be the reason for changes of RBCs parameters, we observed iron metabolism during 8 weeks running and swimming programs (Fig. 1). Surprisingly, the indicators related to iron metabolism, including serum iron content (Fig. 1A), serum ferritin content (Fig. 1B), serum soluble transferrin receptor content (Fig. 1C), liver iron content (Fig. 1D) and apparent iron absorption rate (Fig. 1E) were completely unaffected at 8-week time point during long-term endurance exercises, which were inconsistent with the above changes of hemoglobin and RBCs. In addition, iron storage in the liver and soluble transferrin receptor in the serum were not affected at early stage of endurance exercises (1- and 2-week) and RBCs parameters were maintained at normal level, though apparent iron absorption rate decreased and serum iron and ferritin content decreased. These opposite results implied that disruption of iron metabolism at early stage of long-term endurance exercises was temporary and might be not the reason for changed RBCs parameters at later stage.

### 3.3. Long-term endurance exercise induced malnutrition of protein

In addition to iron, nutritional status of protein is another factor affecting hemoglobin content and RBCs development. Hence, we evaluated protein nutritional status in long-term endurance exercised rats to find the reason for the decreases of hemoglobin and immaturities of RBCs. Our results showed that body weights of rats in both running and swimming groups were significantly lower than rats in control group and the increasing rates of body weight were also slower than control rats (Fig. 2A), but there was no difference of the weight of intake diet among these three groups (Fig. 2B). By determining the contents of serum total protein and albumin, we found that total protein and albumin declined in exercised rats at 8-week time point (Fig. 2C & D), which were consistent with alterations of hemoglobin and RBCs during 8 weeks exercise (Table 1). These results indicated that long-term endurance exercise led to body weight loss and protein malnutrition, which could be the cause of hemoglobin decrease and RBCs immaturation.

### 3.4. Long-term endurance exercise induced acute stress response at early stage but was adapted at later stage

It was reported that stress inducing changes of hormones levels could modulate homeostasis of iron metabolism [19]. For explanation of changes of iron metabolism at early stage during long-term endurance exercise and considering that the high intensity of running and swimming in this study were stressful stimulations, we detected levels of several hormones including adrenaline, cortisol, insulin and erythropoietin in serum (Fig. 3). At 1- and 2-week, serum adrenaline content (Fig. 3A) and serum cortisol content (Fig. 3B) of rats in running and swimming groups were significantly higher than rats in control group, while serum insulin content (Fig. 3C) of exercised rats were significantly lower. But later at 4- or 8-week, serum content of adrenaline, cortisol and insulin were returned to normal level. These results meant that rats received running or swimming exercises showed acute stress responses, which might induce disruption of iron metabolism. The adaption of long-term endurance exercise brought levels of serum hormones back to normal, with the recovery of iron absorption and relevant indicators at later stage. For the content of erythropoietin in serum (Fig. 3D), rats in running group showed enhanced expression level at 2-, 4- and 8-week, while there were no changes in swimming group.

### 3.5. Instead of iron supplement, protein supplement restored the changed RBCs parameters in rats exposed to intensive exercise

To ensure roles of iron deficiency and protein malnutrition in changes of RBCs, we used iron-rich diet and protein-rich diet to supply iron or protein for rats and observed their effects on RBCs parameters during long-term exercise. In rats exposed to 8 weeks swimming, RBC counts, hemoglobin content, MCV, MCH and RDW were significantly altered (Table 2). Most importantly, we observed that iron supplement had no improving effects on RBCs parameters but protein supplement effectively ameliorated the decreases of RBC counts, hemoglobin content, HCT and the increase of RDW (Table 2). By detecting serum iron content (Fig. 4A), serum transferring saturation (Fig. 4B), serum ferritin content (Fig. 4C) and liver iron content (Fig. 4D), we confirmed that iron was accumulated in rats fed by iron-rich diet, which further identified that iron deficiency was not the reason for changed RBCs parameters. However, protein nutritional status was elevated in rats fed by protein-rich diet as serum total protein and serum albumin were increased in protein supplement group (Figure 4E & 4 F). These results showed that block of protein malnutrition could correct RBCs abnormities, which conversely proved that protein malnutrition was the cause of hemoglobin decrease and RBCs immaturation.

## 4. Discussion

In this study, we observed RBCs parameters, iron metabolism, protein nutrition status and hormones levels in male rats received 8 weeks running or swimming exercise. The RBCs parameters altered slightly only at later stage of 8 weeks exercises and did not meet the criteria of anemia, though with statistical significance. MCV was decreased and RDW was increased in both running and swimming rats at later stage, implying that long-term endurance exercises could induce

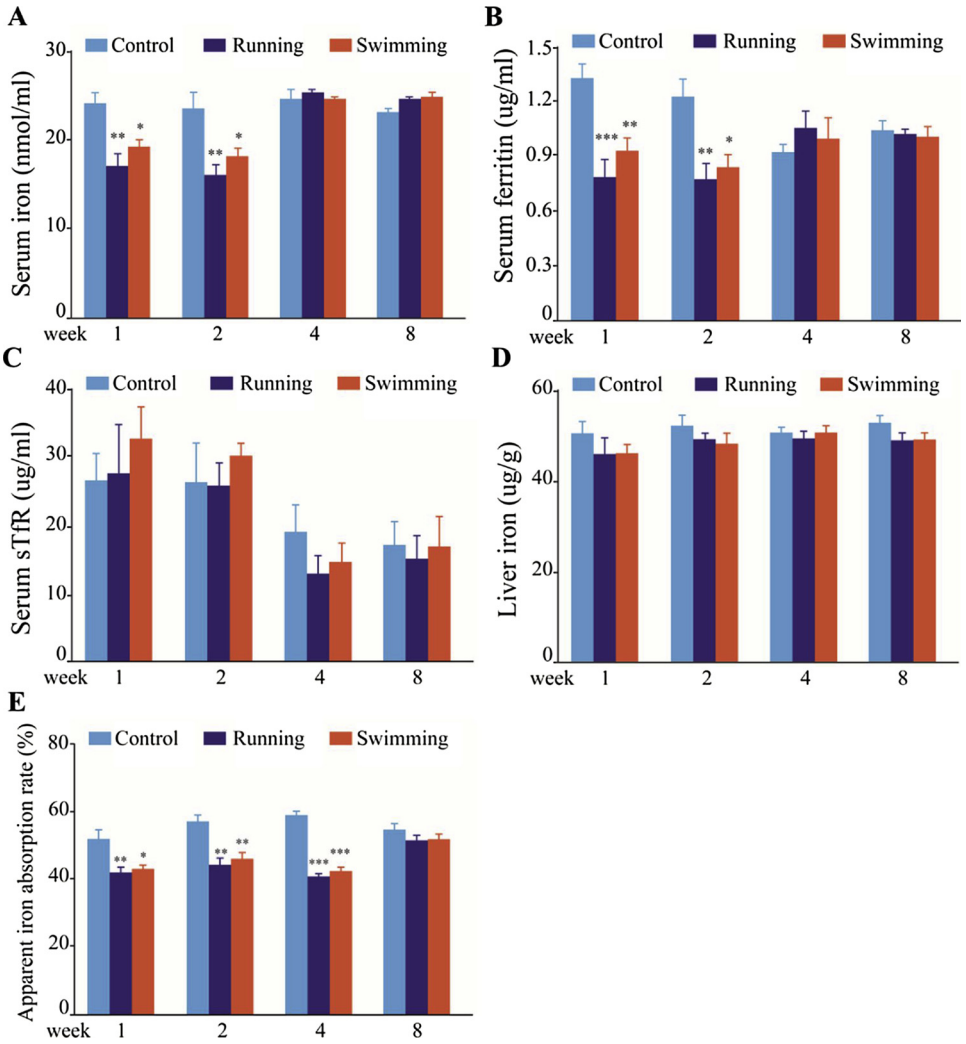


**Fig. 1.** Iron metabolism in rats during 8 weeks endurance exercises. Rats in running or swimming group were respectively received running or swimming for 2 h a day, 5 days per week for 8 weeks. At time point of 1, 2, 4 and 8 week, rats were anaesthetized for detections of serum iron content (A), serum ferritin content (B), serum soluble transferrin receptor (sTfR) content (C) and liver iron content (D). Apparent iron absorption rate was calculated by (feed iron content – feces iron content)/feed iron content × 100% (E). Data were represented as Mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs. Control (n = 8).

immaturation of RBCs. Though the parameters of RBCs were still in normal range, some researchers proposed that it brought disadvantages for individuals suffered from heavy physical activities [20,21]. Indeed, rats exposed to endurance exercises needed more RBCs to carry oxygen for energy production. Therefore, even a slight weakness of RBCs could impair the performance in long-term endurance exercise, which meant that appropriate interventions were required to ameliorate abnormalities of RBCs for improvement of sports performances or work capacities.

It was reported that iron metabolism was dysfunctional in dramatic or long-term physical activities [20,22,23]. As an essential element for hemoglobin, iron is the active site binding with oxygen. Anemia caused by iron deficiency is characterized by RDW increase, MCV decrease and hemoglobin decrease [24], which were same as changes of RBCs parameters after high intensity physical activities. Consequently, disruption of iron metabolism was deemed to be an initial factor of abnormal RBCs development and iron supplement was accordingly used for athletes and laborers undertaking high levels of physical activities [25–27]. But not all iron supplement shows beneficial effects for individuals and iron supplement by orally taking iron reagent incurs side effects and iron toxicities. Another question is that it is hard to distinguish the protective effects from iron supplement or protein supplement in positive experiments or trails of iron supplement by iron-rich food, which usually contains plenty of protein. Besides, the paradox results in this study aroused our attention: exercised rats showed iron reduction in serum at early stage in long-term exercise, which probably was induced by decrease of apparent iron absorption rate. But there was no change of storage iron in the liver and serum soluble transferrin receptor, which meant that supply of iron in blood circulation was not insufficient. In addition, content of hemoglobin was not decreased at early stage but at later stage of 8 weeks exercise when the iron content in serum and apparent iron absorption rate were returned to normal level. These contradictory results suggested that dysfunctional iron metabolism at beginning of 8 weeks endurance exercise might not be the reason for abnormal RBCs at the end. Considering that both acute or chronic iron overload were reported to be adverse for health [13,28], we thought it needed more discussion about whether iron supplement was necessary for the people or not.

Including our previous studies, many researchers reported that stress was capable of disrupting iron metabolism [19,29–31]. A typical response of stress was the activation of hypothalamic-pituitary-adrenal axis (HPA axis), characterized by elevated levels of serum adrenocorticotropic hormone and adrenocortical hormone [32,33]. Due to levels of adrenaline and cortisol in serum were significantly altered in exercised rats at early stage of high intensity endurance exercises, we thought that the strenuous exercises could cause acute stress response

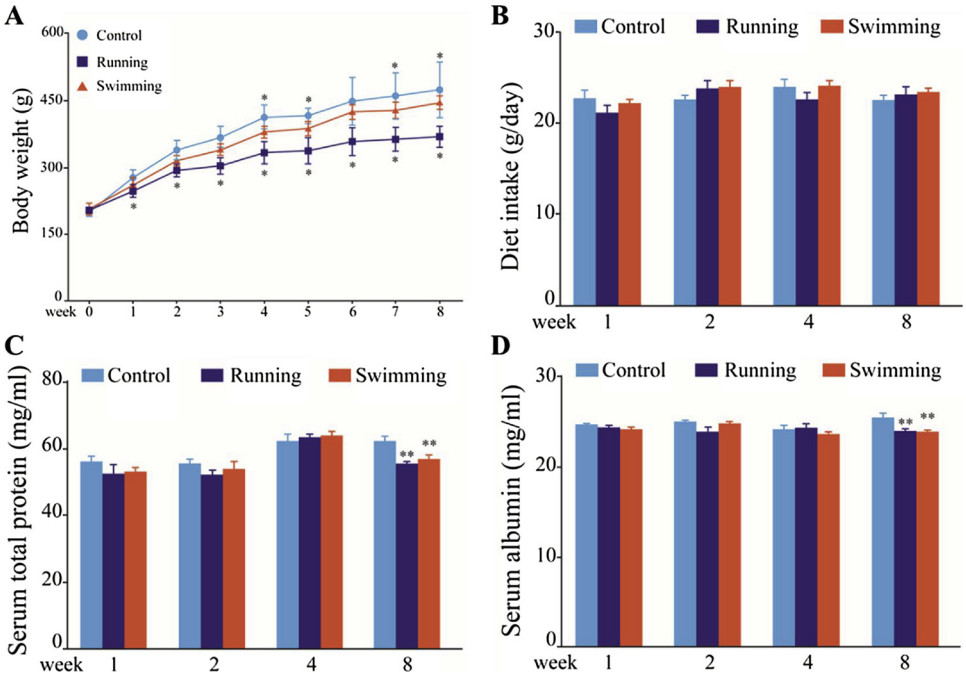


**Fig. 2.** Protein nutritional status in rats during 8 weeks endurance exercises. A: body weights of rats in Control, Running and Swimming groups at every week of 8 weeks. B: diet intake of rats at 1-, 2-, 4- and 8- week. C: serum total protein content of rats at 1-, 2-, 4- and 8- week. D: serum albumin content of rats at 1-, 2-, 4- and 8- week. Data were represented as Mean ± SD. *: $p < 0.05$; **: $p < 0.01$, vs. Control (n = 8).

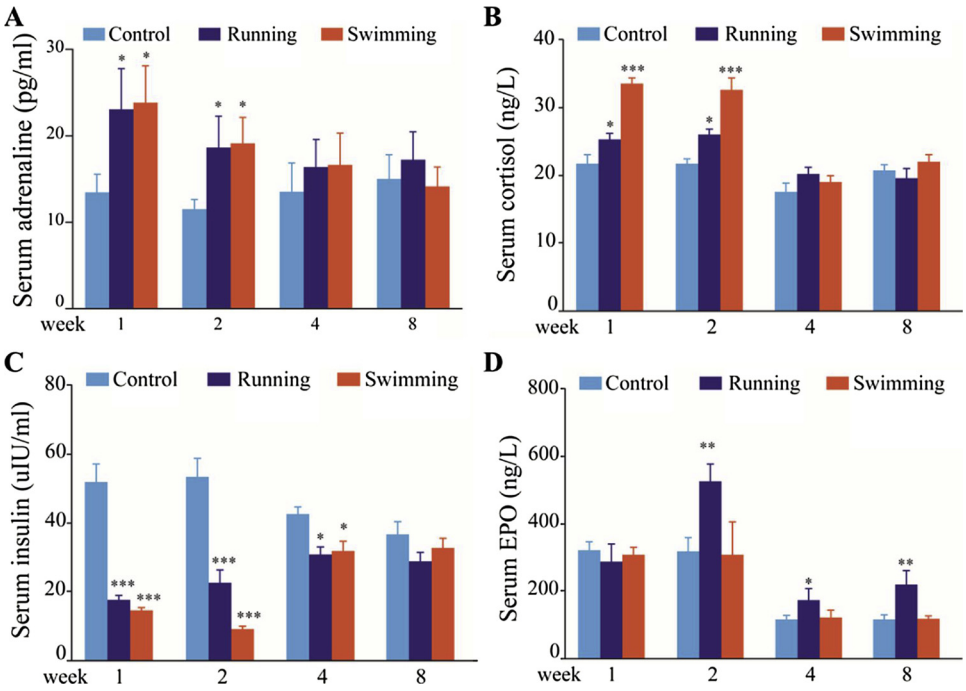


**Fig. 3.** Serum hormones levels of rats during 8 weeks endurance exercises. A: serum adrenaline content of rats in Control, Running and Swimming groups at 1-, 2-, 4- and 8- week. B: serum cortisol content of rats at 1-, 2-, 4- and 8- week. C: serum insulin content of rats at 1-, 2-, 4- and 8- week. D: serum erythropoietin (EPO) content of rats at 1-, 2-, 4- and 8- week. Data were represented as Mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ vs. Control (n = 8).

**Table 2**
Effects of iron supplement or protein supplement on RBCs parameters of rats exposed to 8 weeks swimming exercises. All data were represented as Mean ± SD. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$, vs. Control. #: $p < 0.05$; ##: $p < 0.01$; vs. Swimming (n = 6).

| | Control | Swimming | Swimming +Iron | Swimming +Protein |
|---|---|---|---|---|
| **RBC counts (10^12)** | 8.51 ± 0.44 | 6.88 ± 0.18** | 6.74 ± 0.38* | 7.62 ± 0.62# |
| **Hemoglobin (g/L)** | 142.0 ± 2.15 | 125.8 ± 4.01** | 126.8 ± 4.46* | 140.3 ± 1.56## |
| **HCT (%)** | 43.80 ± 1.51 | 36.55 ± 1.56** | 35.03 ± 1.58*** | 44.55 ± 1.39### |
| **MCV (fL)** | 61.80 ± 0.50 | 59.13 ± 0.94 | 59.47 ± 1.69 | 60.50 ± 1.04 |
| **MCH (pg)** | 19.43 ± 0.11 | 17.63 ± 0.40** | 16.77 ± 0.81** | 18.52 ± 0.36* |
| **RDW (%)** | 12.10 ± 0.13 | 16.07 ± 0.58*** | 17.13 ± 0.81*** | 14.23 ± 0.37***# |

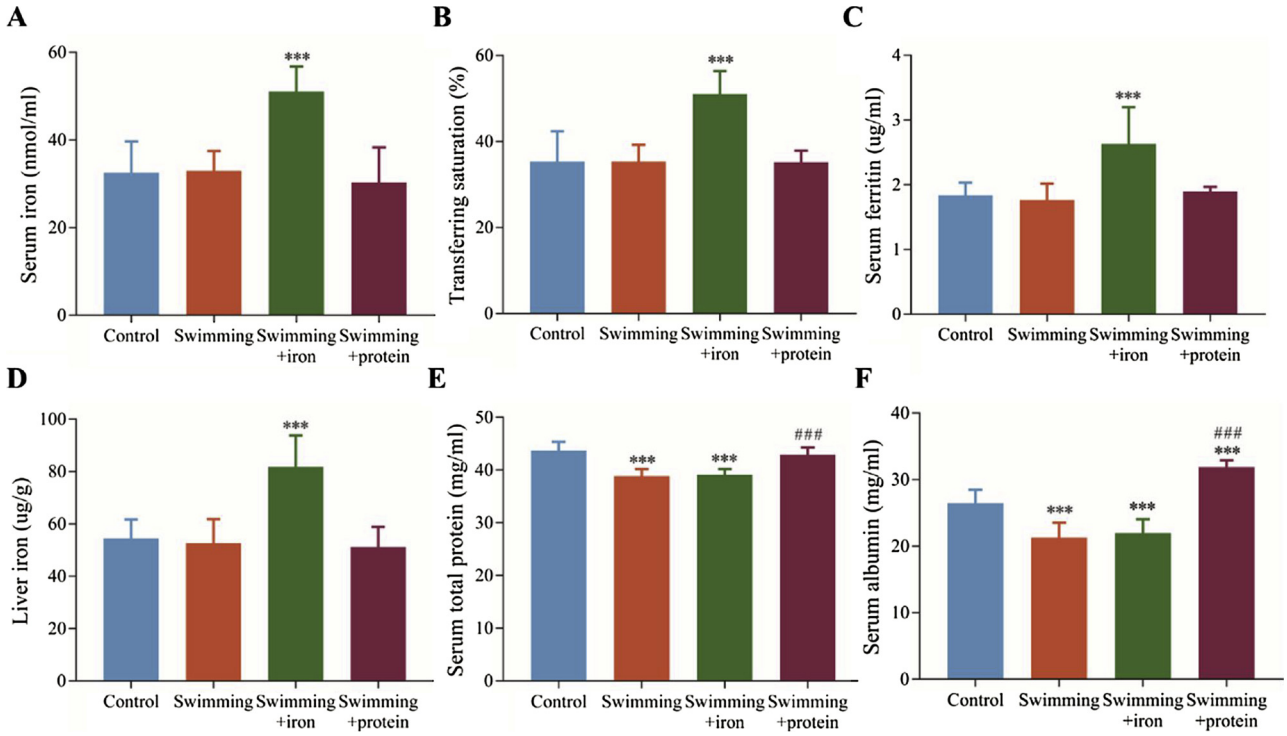


**Fig. 4.** Nutritional status of iron and protein in rats exposed to 8 weeks swimming exercise with supplement of iron or protein. 24 rats were randomly divided into 4 groups (n = 6): Control group, Swimming group (Swimming), Swimming group with supplement of iron (Swimming + iron) and Swimming group with supplement of protein (Swimming + protein). Exercise protocol of swimming and intervention of diet were described in Methods. A: serum iron content in 4 groups; B: serum transferring saturation in 4 groups; C: serum ferritin content in 4 groups; D: liver iron content in 4 groups; E: serum total protein content in 4 groups; F: serum albumin content in 4 groups. Data were represented as Mean ± SD. ***: $p < 0.001$, vs. Control. ###: $p < 0.001$, vs. Swimming.

in rats. Besides, insulin, a hormone with the mighty of modulating iron metabolism [8], was decreased at early stage. At later stage, the rats adapted to the exercise routine as hormones levels were restored to normal. Meanwhile, iron metabolism, including iron absorption rate, serum iron content and serum ferritin content, changed following the identical pattern of hormones during 8 weeks exercises. To this extent, it was reasonable to regard disruption of iron metabolism as a result of stress induced alterations of hormones. We also noticed that serum erythropoietin contents increased in running rats but unchanged in swimming rats. It was possibly induced by acute RBCs destructions resulted from running, which producing more impacts on body than swimming [34–36].

In the case that iron was not the cause of hemoglobin reduction and RBCs immaturation, we evaluated status of protein nutrition because protein was another part of hemoglobin except iron. Meaningfully, with high intensity of physical activities, rats in experimental group had no more intake diet compared to rats in control group. In addition, body weights of exercised rats were significantly lower than control rats, with the decrease of serum total protein contents and serum albumin contents. It was no doubt that protein malnutrition led to developmental retardation, including immaturation of RBCs [37,38]. In experiment of iron or protein supplement, we observed that iron supplement did not improve RBCs abnormities, which confirmed that iron deficiency was not the underlying reason. However, protein supplement effectively ameliorated decreases of RBCs and hemoglobin content by increasing protein content. Our results showed that normal formula diet was not enough for large amounts of physical activities in male rats. A supplement of protein abundant diet was needed instead of iron supplementation.

In conclusion, our study proposed that, at least in male rats, long-term endurance exercise led to abnormal development of RBCs but did not reach the threshold of anemia. Though iron metabolism was disrupted in acute stress responses induced by high intensity of exercise, it was not the cause of hemoglobin reduction and RBCs immaturation. Instead, malnutrition of protein, a fundamental factor easy to neglect, might be the key point causing abnormities of RBCs and impairments of physical performance. Next, a direct link between protein malnutrition and RBCs immaturation need to be created. More profoundly and meaningfully, a well-designed and compelling trial on human beings was urgently needed to elucidate relationships among high intensity of activities, iron metabolism, protein nutritional status and physical capacity.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgment

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