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Anti-fatigue activities of anthocyanins from *Lycium ruthenicum* Murry

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Abstract

Anthocyanins from *Lycium ruthenicum* Murry (LRAs) were used to explore the anti-fatigue activities of LRAs at different concentrations in a mouse fatigue model. Eight biochemical indexes were determined in liver tissue, muscle tissue and serum. The results showed that the LRAs used in the experiment mainly contained delphinium, petunidin and malvidin. During 21 days of gavage, there was no significant difference in mouse body weight. The exercise time of the LRA-exposed mice increased. LRAs could increase serum glucose (Glu), liver/muscle glycogen and the activity of superoxide dismutase (SOD) and reduce the vitality of lactate dehydrogenase (LDH), the content of malondialdehyde (MDA), the levels of lactic acid (LD) and serum urea nitrogen (BUN). This study indicated that LRAs can enhance exercise endurance, increase sugar reserves, scavenge free radicals and improve metabolism, thereby increasing anti-fatigue activities.

Keywords: *Lycium ruthenicum* Murry; anthocyanins; anti-fatigue.

Practical Application: In China, L. ruthenicum Murry is considered to be a plant with the homology of medicine and food, and it has the function of improving body function, which may be related to its rich anthocyanins. This study took LRAs as the research object, analyzed eight biochemical indicators of liver tissue, muscle tissue and serum in mice, and explored the potential anti-fatigue mechanism of LRAs. It is of great significance for the future research and screening of effective and safe anti-fatigue active ingredients of L. ruthenicum Murry, and also provides reference for the research and development of L. ruthenicum Murry, a Xinjiang characteristic resource product.

1 Introduction

Fatigue is a feeling of weakness caused by many diseases, so it is a nonspecific symptom (Li et al., 2020). There are many diseases related to fatigue, such as inflammation, hypoglycaemia, malnutrition, cardiovascular and cerebrovascular diseases and tumours (Ji et al., 2014; Lee et al., 2006; Xu, 2016). Long-term failure to relieve fatigue will lead to coronary heart disease, cerebral haemorrhage and even karoshi (sudden death caused by overwork) (Wang et al., 2012; Jin et al., 2012). The energy exhaustion theory proposes that the decrease in blood glucose (Glu) and glycogen during exercise leads to the generation of fatigue (Luo et al., 2019), and the oxygen free radical-lipid peroxidation theory proposes that excessive oxygen free radicals will be produced in the body after exercise or under other pressures, resulting in cell metabolism disorder, decreased work efficiency and fatigue (Sun et al., 2014). Although chemical compounds have been reported to significantly enhance physical endurance, delay exercise fatigue and accelerate physical recovery, their application is limited due to some side effects (Liu & Li, 2018). In recent years, extracts of natural active substances with the same origin as traditional medicine and food have attracted extensive attention. Studies have shown that the active ingredients in some plant extracts can achieve anti-fatigue and anti-oxidation effects and have fewer side effects, lower toxicity and better safety than chemical compounds; thus, they have wide application prospects.

L. ruthenicum Murry is a traditional Chinese herbal medicine that is generally distributed in saline desert regions (Jiang et al., 2013), and is rich in anthocyanins, polysaccharides, amino acids, fatty acids and a large number of mineral trace elements. In classical Chinese medicine books such as "*The Four Medical Canon*" and "*Crystal Bead Herbal*", it is said that heart disease can be treated with *L. ruthenicum* Murry, as can menoxenia, eyesight loss and a drop in blood pressure. Modern studies have also confirmed that *L. ruthenicum* Murry can alleviate a series of chronic diseases by regulating intestinal microorganisms (Wang et al., 2018a; Zhu et al., 2020). *L. ruthenicum* Murry is rich in anthocyanins, which reduce oxidative stress to prevent various diseases such as thrombosis, atherosclerosis and other cardiovascular diseases. The content of anthocyanins and the removal of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals were positively correlated. Liu et al. found that the percentages of these radical (DPPH•, •OH and $O_2^{-\bullet}$) scavenging effects had good correlation with the concentration of anthocyanins in *L. ruthenicum* Murry (LRAs) and proposed that *L. ruthenicum*

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Murry had good anti-oxidant activity (Miao et al., 2018). Peng et al. found that the level of anti-oxidant enzymes in the liver of mice taking LRAs for a long time showed an increasing trend, and the levels of oxidases showed a decreasing trend (Peng et al., 2019). Zheng et al. investigated the composition and content of LRAs and found that methanol extracts of these anthocyanins showed potent anti-oxidant activity in terms of DPPH•, $ABTS^{•+}$ and ferric reducing anti-oxidant power (FRAP) assays (Zheng et al., 2011). Tang et al. demonstrated that LRAs promote cell proliferation, regulate the balance between the oxidative system and the anti-oxidative system, and up-regulate anti-oxidant enzyme activity in PC12 cells (Tang et al., 2017). These studies are based on the anti-oxidant function of anthocyanins. However, there are few studies on the anti-fatigue activities of anthocyanins extracts. Hu et al. studied the properties of anthocyanins from purple passion fruit pericarp (PFEA) and evaluated their anti-fatigue effect (Hu et al., 2020).

Because *L. ruthenicum* Murry is rich in anthocyanins, this study took LRAs as the research object, analyzed eight biochemical indicators of liver, muscle and serum in mice, and explored the potential anti-fatigue mechanism of LRAs.

2 Materials and Methods

2.1 Materials and reagents

L. ruthenicum Murry was purchased in Xinjiang, China, and stored at room temperature away from light. The anthocyanin standard product was purchased from German Dr. Ehrenstorfer Company. Assay kits for Glu, liver/muscle glycogen, lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), serum urea nitrogen (BUN), cortisol, and lactic acid (LD) determination were purchased from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). All other reagents used in the present study were of analytical grade.

2.2 Sample preparation and extraction

The extraction and preparation process of LRAs was carried out with reference to the method of Liu et al. with some modifications (Liu et al., 2020). Dried *L. ruthenicum* Murry was crushed and sieved. LRAs were extracted with petroleum ether ultrasonically (KQ-1000 ultrasonic cleaner, Shanghai Baidian Instrument Factory, China). This process was conducted in triplicate, and the samples were soaked overnight, filtered and dried. The defatted *L. ruthenicum* Murry sample was mixed with 80 % acidified ethanol (pH = 3), extracted for 30 min and left to stand overnight. The extracted solution was centrifuged for 30 min (3-30 K high-speed centrifuges, Sigma Laborzentrifugen GmbH, Germany), concentrated by a rotary evaporator (N1000D rotary evaporation, Shanghai Huixi Precision Instrument Co., Shanghai, China), purified with macroporous adsorption resin and lyophilized.

HPLC analysis was performed using a Waters e2695 (Waters Co., USA) equipped with a 2495 UV detector. An Xbridge C18 column (250 mm×4.6 mm, 5 μm, Waters Co., USA) was used for chromatographic separation. The mobile phase consisted of 1 % formic acid (v/v) in water (A) and 1 % formic acid (v/v) in acetonitrile (B). The column flow rate was 1.0 ml/min, the injection volume was 20 μL, the column temperature was 35 °C, and the detection wavelength was set to 530 nm. The gradient elution program was as follows: 0-2 min, 92-88 % A; 2-5 min, 88-82 % A; 5-10 min, 82-80 % A; 10-12 min, 80-75 % A; 12-15 min, 75-70 % A; 15-18 min, 70-55 % A; 18-20 min, 55-20 % A; 20-22 min, 20-92 % A; and 22-30 min, 92 % A.

2.3 Animals and experimental design

A total of 210 male Kunming mice (18 g-22 g) without specific pathogens were purchased from the Animal Center of Xinjiang Medical University. The mice were housed at a constant temperature (25 ± 1 °C) and humidity (50-60 %) under a 12/12-h light-dark cycle with free access to food and water. After one week of acclimatization, the mice were randomly divided into 5 experimental groups (n = 42): the negative control group (N-C, distilled water), positive control group (P-C, 800 mg/kg TAU), low-dose anthocyanin group (L-LRAs, 100 mg/kg LRAs), middle-dose anthocyanin group (M-LRAs, 400 mg/kg LRAs), and high-dose anthocyanin group (H-LRAs, 900 mg/kg LRAs).

According to different exercise methods, each experimental group was further divided into an exhaustive running group and a forced swimming group ($n_1 = 21$, $n_2 = 21$). The exhaustive running group mice were subjected to certain duration of running, and the exhaustive running time was recorded. The forced swimming group was divided into a pre-swimming group (S-1), a post-swimming group (S-2) and a recovery group (S-3). During the 21 days of gavage, the weight changes were recorded every week. The mouse tail was tied to a lead block (weighing approximately 7 % of the body weight). Before the test, blood was collected, and mice from the S-1 group were dissected. After swimming, the time of the S-2 group mice was recorded, blood was collected, and the mice were dissected immediately. After swimming, the S-3 group time was recorded, blood was collected, and the mice were dissected after 30 min.

The animal experiment arrangement was divided into four time periods (Figure 1), and the effect of LRAs on the anti-fatigue activities in mice under three different exercise stages was studied.

2.4 Exhausted running test

The mice in the exhaustive running group were subjected to the exhaustive running test, which was performed according to a previous study described by Shen (Shen et al., 2019). At the beginning of the experiment, the mice were given appropriate exercise training on a treadmill (SANS, Nanjing, China), and each group was subjected to an exhausted running test after the last gavage. The mice were placed on a treadmill, and the exhaustive running time was recorded. The instrument parameters were set as follows: speed, 17 rpm; current, 1 mA; number of channels open, 8; and electric shock duration, 8 s.

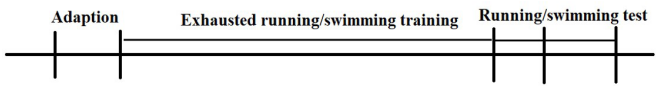

Figure 1. Animal test schedule.

2.5 Forced swimming test

The forced swimming group mice were subjected to the forced swimming test. Some adjustments have been made based on previous research (Yang et al., 2019; Yuan et al., 2019; Ma et al., 2017). After the last gavage, each mouse tail was tied to a lead block (weighing approximately 7 % of the body weight). Mice were individually placed in a swimming pool filled with water (25 ± 1 °C) at a depth of 30 cm until the mice could not surface for 7 s, and the forced swimming time was recorded.

2.6 Biochemical analysis

After the last gavage, the forced swimming group mice were randomly divided into three exercise stages, S-1, S-2, and S-3. First, the eyeballs of S-1 group mice were removed, and blood, liver tissue and muscle tissue were collected. The S-2 group mice and S-3 group mice swam at approximately 25 °C under a heavy load. After exercise, the blood, liver tissue and muscle tissue of the S-2 group mice were collected immediately. After resting for 30 min, the blood, liver tissue and muscle tissue of the S-3 group mice were collected.

The blood of each group of mice was centrifuged for 15 min to prepare serum. The liver and muscle tissue in 0.86 % saline were homogenized for 1 min and then analyzed according to the recommended procedure provided by the test kit. Glu, liver/muscle glycogen, LDH, BUN, cortisol, SOD, MDA and LD were determined.

2.7 Statistical analysis

Each measurement was conducted at least in triplicate, and the data were statistically analyzed by one-way analysis of variance using SPSS 20.0 statistical software. Tukey's multiple comparison tests were performed. The data are expressed as the mean ± standard deviation (SD), and the significance level was set at $p < 0.05$.

3 Results

3.1 Main Ingredients and LRA Content

There are six main anthocyanins in food, namely, pelargonidin, cyanidin, delphinidin, petunidin, peonidin and malvidin, which are found in the cells sap of roots, and plant stems and leaves in nature (Chen et al., 2017). The chromatographic fingerprint of the main LRAs used in the present study is shown in Figure 2. In this study, *L. ruthenicum* Murry mostly contained the anthocyanins delphinium, petunidin and malvidin, and their contents were 0.8 g/100 g, 51.9 g/100 g, and 8.8 g/100 g, respectively.

3.2 Effect of LRAs on Mice Body Weight

The effects of LRAs on mouse body weight were recorded in the initial, intermediate, and terminal stages. With the increase in gavage days (with a maximum of 21 days), the average body weight in each experimental group gradually increased. Moreover, there was no significant difference among the N-C group, P-C group and LRA treatment groups at the same time ($p > 0.05$, Table 1), which indicated that LRA exposure had no significant effects on mouse body weight through gavage administration.

3.3 Effect of LRAs on Exercise Endurance

Exhaustive running time and forced swimming time are usually used to measure the body's exercise endurance (Tan et al., 2012). The present study evaluated the anti-fatigue effect of LRAs by measuring these two indicators. The study determined the exhaustive running time in each group after the last gavage and explored the effect of LRAs on exercise endurance. Compared with that in the N-C group, the running times in the M-LRA group and H-LRA group were longer ($p < 0.01$, $p < 0.05$, Figure 3a), and those in the M-LRA group and H-LRA group increased by 16 min and 34 min, respectively. The effect of LRAs on ameliorating fatigue was explored through a forced swimming test. Compared with that in the N-C group, the swimming times in the M-LRA group and H-LRA group were longer ($p < 0.05$, $p < 0.01$, Figure 3b), and those in the M-LRA group and L-LRA group increased by 6 min and 25 min, respectively. The results showed that a suitable dose of LRAs enhanced exercise endurance by extending the mouse exercise time, indicating that LRAs had good anti-fatigue activity.

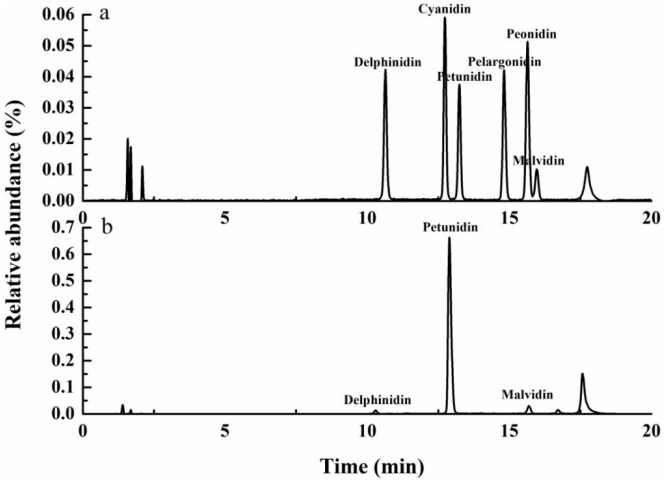

Figure 2. Chromatogram of main components in *L. ruthenicum* Murry anthocyanin (a: Standard, b: *L. ruthenicum* Murry).

Table 1. Effects of LRAs on Mice Body Weight (n = 42).

Group	Initial/g	One week/g	Two week/g	Terminal/g
N-C	20.3 ± 0.7	28.6 ± 1.4	31.0 ± 0.3	32.5 ± 0.8
P-C	19.8 ± 1.4	28.5 ± 0.5	31.5 ± 0.5	33.5 ± 1.8
L-LRA	19.5 ± 0.7	28.6 ± 0.5	29.5 ± 0.7	33.6 ± 1.2
M-LRA	20.9 ± 0.8	28.1 ± 2.1	31.8 ± 0.6	33.3 ± 1.4
H-LRA	20.8 ± 1.4	28.2 ± 1.7	31.5 ± 1.1	32.8 ± 1.6

Data are expressed as mean ± standard deviation (SD), n = 42. *$p < 0.05$, **$p < 0.01$ compared with negative control group (N-C). N-C means negative control group the mice were treated with distilled water. P-C means positive control group the mice were treated with 800 mg/kg taurine. LRA mean that mice were treated with different doses of LRAs as 100 mg/kg, 400 mg/kg, 900 mg/kg.

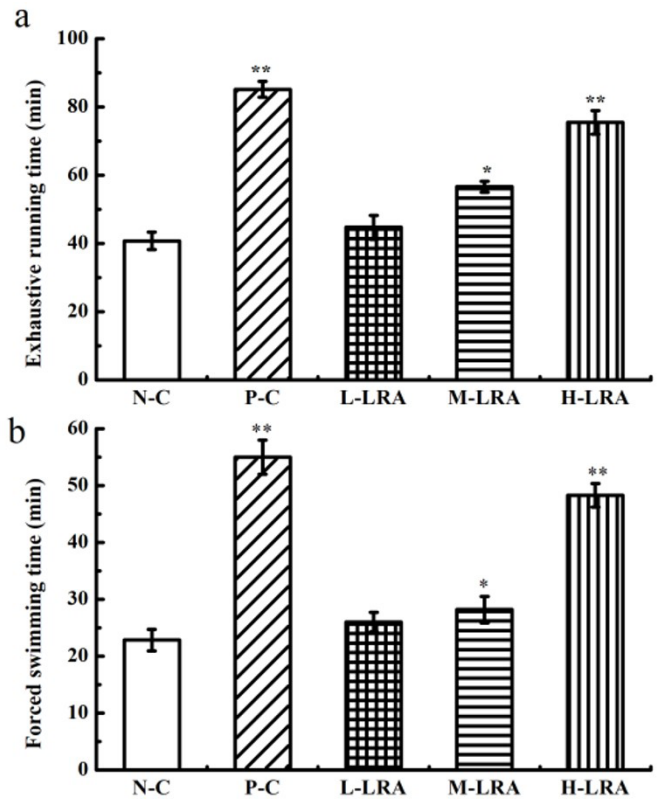

Figure 3. Effect of LRAs on Exercise Endurance. (a) Effect of LRAs on Exhaustive Running Test (n = 21). (b) Effect of LRAs on Forced Swimming Test (n = 21). Data were expressed as mean ± standard deviation (SD), $*p < 0.05$, $**p < 0.01$ compared with negative control group (N-C).

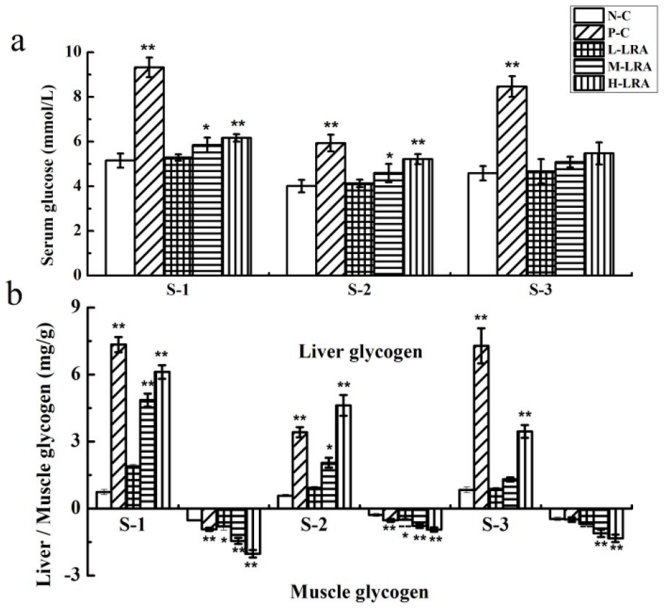

Figure 4. Effect of LRAs on Sugar Reserves (n = 21). (a) Effect on Serum Glucose Content on Mice. (b) Effect on Liver Glycogen and Muscle Glycogen on Mice. Data were expressed as mean ± standard deviation (SD), $*p < 0.05$, $**p < 0.01$ compared with negative control group (N-C).

3.4 Effect of LRAs on Sugar Reserves

Serum Glu, liver glycogen and muscle glycogen are three main sugar reserves in the body; when glycogen is exhausted, exercise endurance will decrease. Therefore, serum Glu and liver/muscle glycogen are important indicators of fatigue (Mani et al., 2018).

The serum Glu levels in the M-LRA and H-LRA groups were significantly higher than those in the N-C group in the S-1 and S-2 stages ($p < 0.05$, $p < 0.01$, Figure 4a). In the S-1 stage of the M-LRA group and L-LRA group, serum Glu levels increased by 0.7 mmol/L and 1.1 mmol/L, respectively; in the S-2 stage, they increased by 0.6 mmol/L and 1.2 mmol/L, respectively. The results indicated that M-LRA and H-LRA can increase the serum Glu reserve, provide more initial energy for exercise, and enhance exercise endurance. The effect of LRAs on increasing serum Glu was not significant in the S-3 stage ($p > 0.05$). This was because the body promotes sugar production through the glucagon secreted by pancreatic B cells after exercise. Decomposition of original Glu and gluconeogenesis levels maintains the stability of serum Glu (Mani et al., 2018), and the mechanism by which LRAs change glucagon levels in the body needs further study.

In the S-2 stage, the glycogen in each group decreased compared to that in the S-1 stage. In the S-3 stage, glycogen increased but did not reach that in the S-1 stage. Compared with the N-C group, the H-LRA group had significantly up-regulated liver glycogen and muscle glycogen in the three exercise stages ($p < 0.05$, $p < 0.01$, Figure 4b). In the S-1 stage, the liver glycogen and muscle glycogen increased by 5.4 mg/g and 1.5 mg/g in the H-LRA group, respectively; in the S-2 stage, they increased by 4.0 mg/g and 0.6 mg/g in the H-LRA group, and in the S-3 stage, they increased by 2.6 mg/g and 0.9 mg/g in the H-LRA group. Supplementation with LRAs can increase liver/muscle glycogen storage and enhance sports endurance. LRAs have a good effect on muscle glycogen.

The results showed that a suitable dose of LRAs can increase the reserve of glucose in serum and regulate liver glycogen and muscle glycogen.

3.5 Effect of LRAs on Free Radical Scavenging

LDH viability can be used to evaluate aerobic metabolism in the body, which is an important indicator to determine exercise load and muscle fatigue damage. When body tissue hypoxia occurs, the body produces a triphenylamine, and the cell membrane is attacked by free radicals, which increases the permeability of the cell membrane and increases LDH vitality in serum (Nam et al., 2018). SOD can scavenge the superoxide free radical O^{2-} through a disproportionation reaction and is an important anti-oxidant enzyme that protects cells. SOD activity is often used to assess the body's oxidative stress level (Bao et al., 2016). MDA is a product of lipid peroxidation degradation and can be used to evaluate cell oxidative stress (Huang et al., 2015). The effect of LRAs on free radical scavenging was explored by determining the changes in LDH, SOD and MDA levels in fatigue model mice.

The changes in LDH vitality in the liver, muscle and serum in three exercise stages were determined. LDH viability in each

group increased in the S-2 stage, and LDH vitality decreased in the S-3 stage. Compared with the N-C group, the LRA groups had reduced LDH vitality, wherein in liver tissue and serum, the effect of the H-LRA group was more significant than that of the L-LRA and M-LRA groups in the three exercise stages ($p < 0.05$, $p < 0.01$, Figure 5a, Figure 5c). In the S-1 stage, the liver LDH decreased by 59.0 U/g pro and the serum LDH decreased by 400.1 U/L; in the S-2 stage, they decreased by 79.2 U/g pro and 361.6 U/L, respectively, and in S-3 stage they decreased by 66.2 U/g pro and 342.3 U/L. The H-LRA group also had a more significant effect on reducing muscle LDH vitality in the S-2 and S-3 stages ($p < 0.05$, $p < 0.01$, Figure 5b). In the S-2 stage, the muscle LDH decreased by 63.3 U/g pro, and in the S-3 stage, the muscle LDH decreased by 62.3 U/g pro.

The results showed that a suitable dose of LRAs can reduce LDH vitality, which means that LRAs eliminate free radicals produced by the body after exercise and reduces the degree of damage to the cell membrane.

In the present study, the changes in SOD activity in the liver, muscle and serum in three exercise stages were determined. Compared with the N-C group, the H-LRA group had more significant effects on liver tissue and muscle tissue at the three different exercise stages ($p < 0.05$, Figure 6a, Figure 6b). In the S-1 stage, liver SOD and muscle SOD increased by 66.6 U/mg pro and 105.7 U/mg pro, respectively; in the S-2 stage, they increased by 63.7 U/mg pro and 92.0 U/mg pro, and in the S-3 stage they increased by 79.1 U/mg pro and 104.1 U/mg pro. The H-LRA group also had a more significant increase in serum SOD activity in the S-1 and S-3 stages than the other LRA groups ($p < 0.05$, Figure 6c). In the S-1 stage, the serum SOD activity increased by 55.2 U/mL, and in the S-3 stage, the serum SOD activity increased by 43.3 U/mL.

The results showed that a suitable dose of LRAs can enhance SOD activity, strengthen the anti-free radical enzyme system, alleviate the damage of free radicals to cells and thus delay fatigue.

The MDA content in liver tissue, muscle tissue and serum in three exercise stages was determined. The MDA content in the S-2 stage was higher than that in the S-1 stage because the body produced a large amount of free radicals after exhaustive swimming. In the S-3 stage, the MDA content decreased. LRAs could reduce the production of MDA in all three exercise stages. Compared with the N-C group, the M-LRA group and H-LRA group had a significant effect on reducing muscle MDA and serum MDA contents at all three different exercise stages ($p < 0.05$, $p < 0.01$, Figure 7b, Figure 7c). In the S-1 stage, muscle MDA and serum MDA contents decreased by 1.3 nmol/mg pro and 1.0 nmol/mL in the M-LRA group, respectively, and they decreased by 1.9 nmol/mg pro and 1.3 nmol/mL in the H-LRA group. In the S-2 stage, muscle MDA and serum MDA contents decreased by 2.5 nmol/mg pro and 1.8 nmol/mL in the M-LRA group, respectively, and they decreased by 3.5 nmol/mg pro and 3.7 nmol/mL in the H-LRA group. In the S-3 stage, muscle MDA and serum MDA decreased by 1.9 nmol/mg pro and 2.6 nmol/mL in the M-LRA group, respectively, and they decreased by 4.6 nmol/mg pro and 3.6 nmol/mL in the H-LRA group. The M-LBA group and H-LBA group also exhibited significant reduced liver MDA contents in the S-2 and S-3 stages ($p < 0.05$, $p < 0.01$, Figure 7a); in the S-2 stage, the liver MDA content decreased by 0.6 nmol/mg pro and 1.0 nmol/mg pro in the M-LRA group and H-LRA group, respectively, and in the S-3 stage, the liver MDA content decreased by 0.5 nmol/mg pro and 0.9 nmol/mg pro in the M-LRA group and H-LRA group.

The results showed that a suitable dose of LRAs reduced the production of MDA, possibly because LRAs can enhance

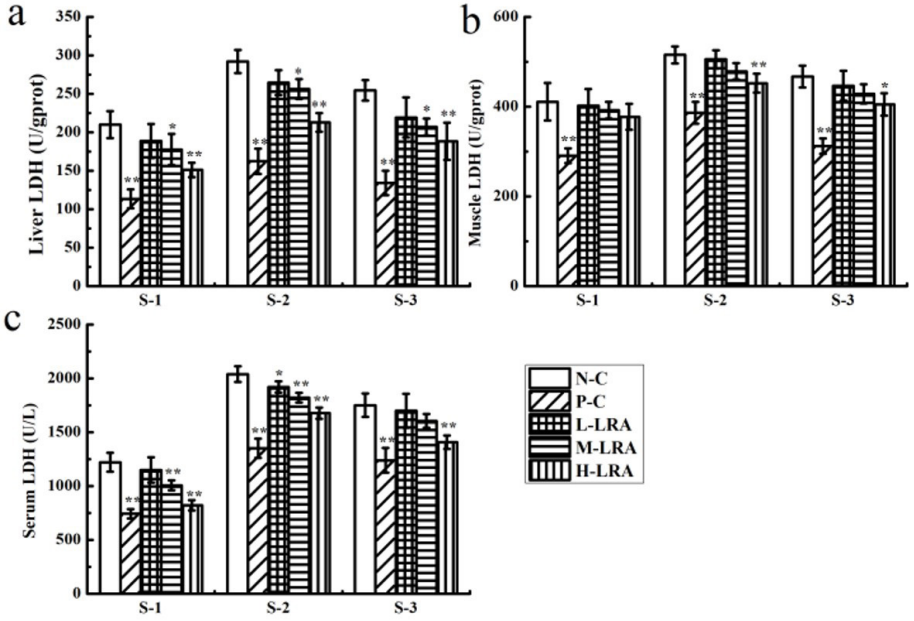

Figure 5. Effect of LRAs on Free Radical Scavenging (n = 21). (a) Effect on LDH Vitality on Mice Liver Tissue. (b) Effect on LDH Vitality on Mice Muscle Tissue. (c) Effect on LDH Vitality on Mice Serum. Data were expressed as mean ± standard deviation (SD), *$p < 0.05$, **$p < 0.01$ compared with negative control group (N-C).

the body's anti-oxidant enzyme activity by reducing oxygen free radicals and protecting cell membranes to enhance anti-fatigue activities.

3.6 Effect of LRAs on Metabolic Levels

BUN is a representative indicator of a strenuous exercise state. This marker is formed in the liver under strenuous exercise and is the end product of protein and amino acid metabolism (Wang et al., 2014). As a sensitive blood biochemical parameter of fatigue, the BUN content is negatively correlated with exercise endurance level and can therefore be used to evaluate the body's ability to withstand high-intensity exercise load (Wu et al., 2014). Cortisol is a steroid hormone closely related to stress and can reflect the body's metabolism and fatigue and maintain life activities by regulating the body's water, salt, sugar and protein metabolism (Viru &Viru, 2004; Ponce et al., 2019). The body needs glycolysis to replenish energy during a short period of

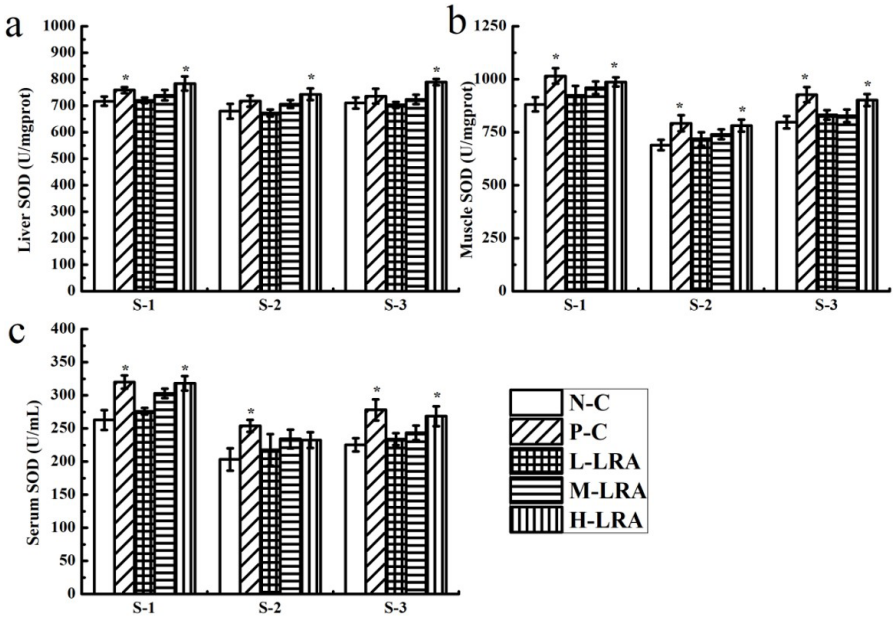

Figure 6. Effect of LRAs on Free Radical Scavenging (n = 21). (a) Effect on SOD Activity on Mice Liver Tissue. (b) Effect on SOD Activity on Mice Muscle Tissue. (c) Effect on SOD Activity on Mice Serum. Data were expressed as mean ± standard deviation (SD), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with negative control group (N-C).

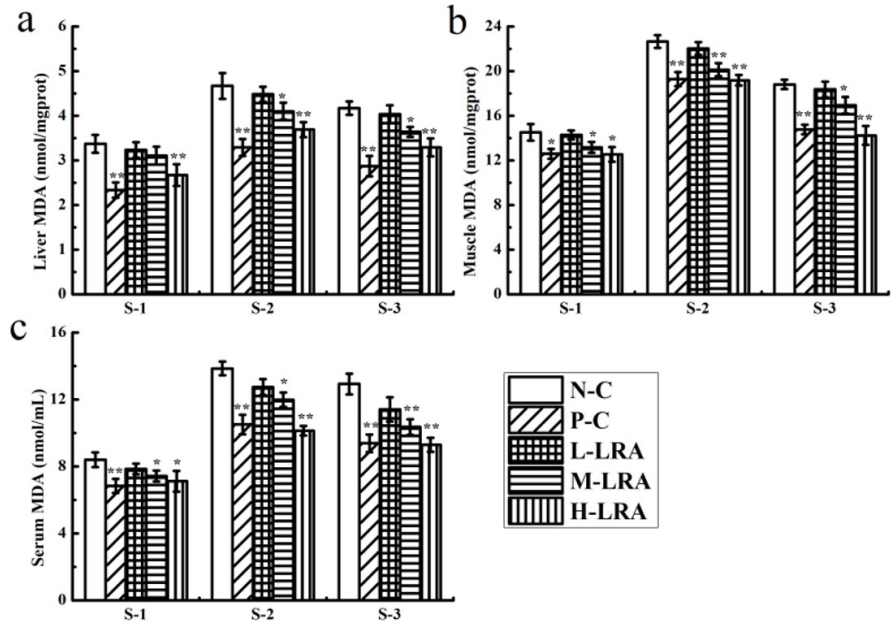

Figure 7. Effect of LRAs on Free Radical Scavenging (n = 21). (a) Effect on MDA Content on Mice Liver Tissue. (b) Effect on MDA Content on Mice Muscle Tissue. (c) Effect on MDA Content on Mice Serum. Data were expressed as mean ± standard deviation (SD), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with negative control group (N-C).

strenuous exercise, and LD is the final product of glycolysis under anaerobic conditions. During long-term exercise, muscles will generate and accumulate excessive acidic metabolites such as LD, and an increase in the LD content causes a decrease in tissue pH, disrupts the balance of the body's environment and leads to a decrease in motion level (Gibson & Edwards, 1985). Therefore, LD is one of the important parameters of exercise intensity or fatigue degree, and it can be used as a physiological index to assess the degree of fatigue (Feng et al., 2009).

BUN was determined in three different exercise stages. Compared with the N-C group, the M-LRA group and H-LRA group had a significant effect on reducing BUN in the three different exercise stages ($p < 0.05$, $p < 0.01$, Figure 8a). In the S-1 stage, the BUN content decreased by 4.5 mmol/L and 5.9 mmol/L in the M-LRA and H-LRA groups, respectively; in the S-2 stage, it decreased by 3.9 mmol/L and 4.7 mmol/L; and in the S-3 stage, it decreased by 2.0 mmol/L and 3.7 mmol/L. The level of BUN in the S-2 stage was higher than that in the S-1 stage because the body breaks down proteins and amino acids to obtain more energy during exhaustive exercise, producing a large number of metabolites such as BUN.

The results showed that a suitable dose of LRAs may increase the body's exercise endurance by increasing the body's glycogen reserve, reducing protein metabolism, relieving the damage of its metabolites to kidney function and reducing the body's fatigue load.

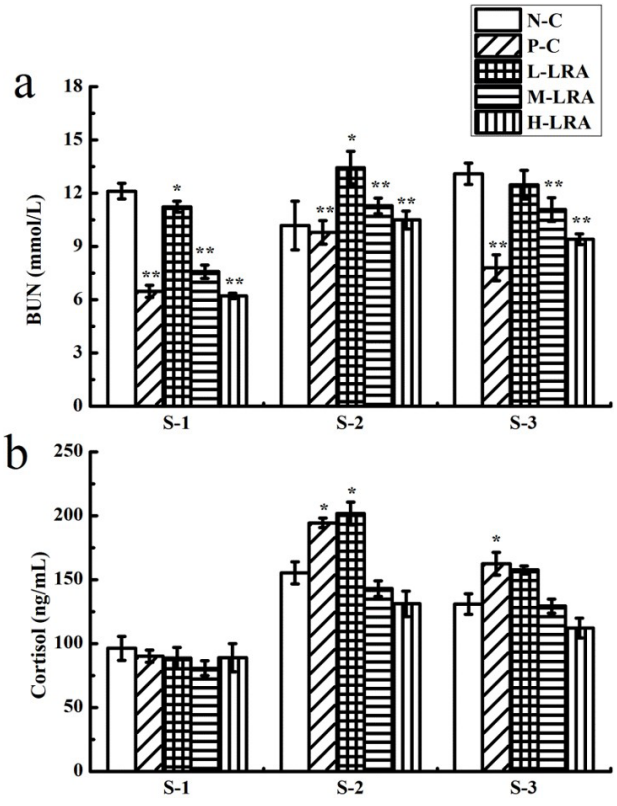

Figure 8. Effect of LRAs on Metabolic Levels (n = 21). (a) Effect on BUN Level on Mice Serum. (b) Effect on Cortisol Level on Mice Serum. Date were expressed as mean ± standard deviation (SD), $*p < 0.05$, $**p < 0.01$ compared with negative control group (N-C).

Cortisol was analyzed in three different exercise stages. Compared with that in the N-C group, the cortisol content in the M-LRA group and H-LRA group was not significantly different ($p > 0.05$, Figure 8b). In the S-2 stage, the cortisol content in the L-LRA group increased significantly ($p < 0.05$), which may be related to the adaptation of the pituitary gland to cortisol concentration feedback during exercise (Taylor et al., 2015). In the S-3 stage, the cortisol concentration gradually decreased, which may be related to exercise intensity, exhaustive swimming time and tissue intake of cortisol. The change in cortisol concentration is affected by the duration and intensity of exercise and by factors such as exercise type, the subject's functional level and mental state (Ponce et al., 2019). Therefore, the mechanism by which LRAs affect cortisol at different exercise stages needs to be further studied and explored.

The changes in LD in liver tissue, muscle tissue and serum in the three different exercise stages were determined. In the S-2 stage, the LD increased, and the body produced a large amount of LD under anaerobic conditions due to insufficient oxygen supply after strenuous exercise. In the S-3 stage, the LD level decreased to a certain extent because of the gradual balance of the production and elimination rates of LD in the body during the rest stage, and the metabolic level gradually recovered. Compared with the N-C group, the H-LRA group had significantly reduced liver LD and muscle LD levels in all three exercise stages ($p < 0.05$, $p < 0.01$, Figure 9a, Figure 9b). In the S-1 stage, the liver LD and muscle LD levels decreased by 0.3 mmol/g pro and 2.2 mmol/g pro, respectively; in the S-2 stage, they decreased by 0.5 mmol/g pro and 3.1 mmol/g pro, respectively; and in the S-3 stage, they decreased by 0.5 mmol/g pro and 3.0 mmol/g pro, respectively. The M-LRA group and H-LRA group had significantly reduced serum LD in the three different exercise stages ($p < 0.05$, $p < 0.01$, Figure 9c). In the S-1 stage, the serum LD levels in the M-LRA group and H-LRA group decreased by 1.0 mmol/L and 1.7 mmol/L, respectively; in the S-2 stage, the serum LD decreased by 1.4 mmol/L and 2.7 mmol/L, respectively; and in the S-3 stage, the serum LD decreased by 1.1 mmol/L and 2.2 mmol/L, respectively.

The results showed that a suitable dose of LRAs can regulate the internal environment and reduce the level of LD to relieve fatigue.

4 Discussions

Lycium ruthenicum Murry is rich in anthocyanins. In the present study, there were three main anthocyanins in *L. ruthenicum* Murry: delphinidin, petunidin and malvidin. Delphinidin has a strong anti-oxidant capacity, and clinical trials have found that delphinidin can soften capillaries and scavenge free radicals in cells (Chen et al., 2017). The anti-oxidant activity of delphinidin was 23 times that of vitamin C and 52 times that of vitamin E (using DPPH as a control). Therefore, moderate intake of delphinidin-rich foods can prevent cancer and reduce inflammation. Many studies have shown that petunidin has certain anti-oxidant and free radical scavenging properties. The anti-oxidation mechanism is mainly manifested as structural changes accompanied by changes in energy (Wang et al., 2018b). Malvidin can reduce the level of cellular oxidative stress and protect vascular endothelial

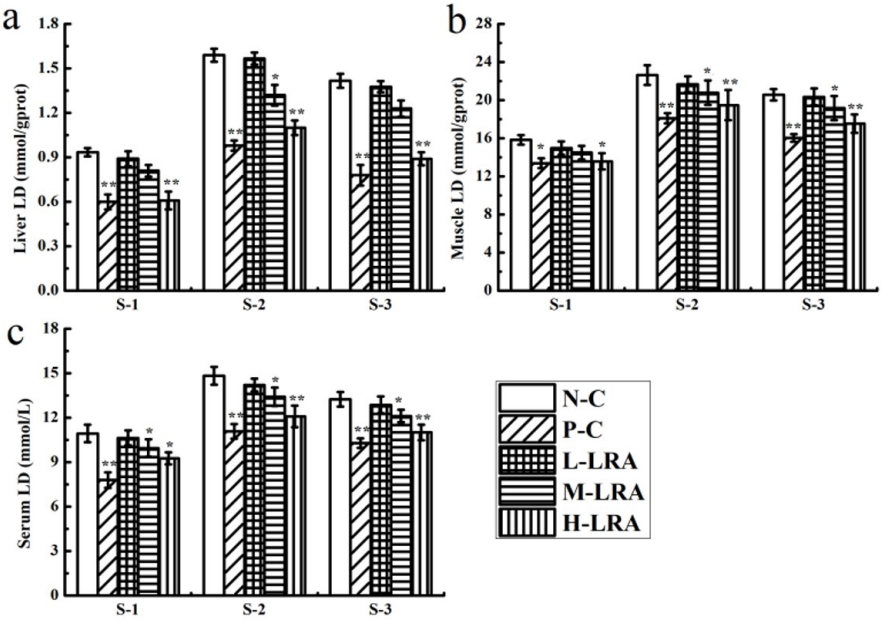

Figure 9. Effect of LRAs on Metabolic Levels (n = 21). (a) Effect on LD Level on Mice Liver Tissue. (b) Effect on LD Level on Mice Muscle Tissue. (c) Effect on LD Level on Mice Serum. Data were expressed as mean ± standard deviation (SD), $^{*}p < 0.05$, $^{**}p < 0.01$ compared with negative control group (N-C).

cells from damage. Therefore, the anti-fatigue function of LRAs is related to scavenging free radicals, inhibiting inflammation and protecting cardiovascular function.

When the body exercises, a series of physiological changes will cause a decrease in muscle contractility and eventually cause fatigue. One of the main characteristics of fatigue is a decrease in exercise endurance (You et al., 2012). The decrease in exercise endurance may be related to factors such as insufficient energy reserves, increased free radicals, accumulation of metabolites and increased inflammatory cytokines related to muscle contraction (Li et al., 2018). Therefore, exercise endurance is one of the main characteristics that is affected by anti-fatigue agents. In the present study, exhaustive running time and forced swimming time were used as indicators to evaluate the anti-fatigue activities of LRAs (You et al., 2012). The mice administered taurine and LRAs had longer running and swimming times than the control mice, especially those in the M-LRA (400 mg/kg) and H-LRA (900 mg/kg) groups. The results indicate that LRAs can improve sports endurance. Some fatigue-related biochemical parameters were determined in this study. Glu and glycogen are indicators related to sugar reserve, Glu plays an important role in resisting fatigue and improving exercise endurance, and glycogen can supplement energy when serum glucose levels are low (Miao et al., 2018). During exercise, the body consumes ATP and Glu in cells, liver glycogen is continuously broken down into Glu for systemic energy supply, and muscle glycogen is used for muscle energy supply. When the decomposition rate of liver glycogen is less than that of muscle glycogen, the serum Glu supply is insufficient, the brain's stress ability decreases, and fatigue occurs. Therefore, Glu and glycogen can help mitigate fatigue and improve exercise endurance. The results showed that LRAs could significantly increase Glu reserves and improve exercise endurance, and their effects may be related to increasing the body's sugar reserves.

LDH is mainly distributed in the liver, muscle and other organs; this enzyme is a marker enzyme of anaerobic oxidation, and it catalyses the mutual conversion of pyruvate and LD under the combined action of NADH and NAD^{+} in the glycolysis pathway (Ye et al., 2017). After strenuous exercise, the cell membrane is attacked by a large number of free radicals, and the damage to cell membrane permeability and serum LDH activity increases. Therefore, LDH can be used to evaluate exercise load, anaerobic metabolism and exercise injury. Strenuous exercise will generate a large amount of oxygen free radicals and oxidative stress, which will lead to fatigue and related diseases (Hong et al., 2015). SOD, as an important endogenous anti-oxidant enzyme, can scavenge superoxide radical O^{2-}, convert it into hydrogen peroxide through a disproportionation reaction and protect cells from damage (Elias et al., 2008). When the cell membrane is attacked by free radicals, the polyunsaturated fatty acids on the membrane are degraded to produce lipid peroxide called MDA, and MDA can reflect the degree of oxidative stress in the body's cells (Ayala et al., 2014). In the present study, treatment with LRAs significantly reduced LDH vitality, strengthened SOD activity and reduced the MDA content in tissues and serum. The results showed that LRAs can enhance the activity of anti-oxidant enzymes, reduce lipid peroxides, protect cell membrane permeability, and maintain the balance between the oxidative system and the anti-oxidant system.

The study also determined indicators related to metabolism. BUN is an important indicator to evaluate the body's ability to withstand high-intensity exercise load and reflects the degree of protein catabolism in the body when carbohydrates and fats cannot meet the energy requirements during exercise. Cortisol is a steroid hormone that can maintain life activities, regulate

body metabolism, promote gluconeogenesis and provide exercise energy. It mainly reflects the body's metabolism and the degree of fatigue. LD is the final product of anaerobic fermentation in muscles and other tissues when the body performs high-intensity exercise. The reduction in the LD content may be related to its conversion to Glu in the liver and storage as liver/muscle glycogen. Therefore, LD is a physiological indicator to evaluate fatigue degree. In the present study, the levels of BUN and LD in mice treated with LRAs were low, indicating that LRAs can reduce the production of metabolites and the burden on the kidney and increase the transformation of Glu and glycogen, thus improving exercise performance. The effect of LRAs may be related to regulating the body's metabolic capacity. The secretion of cortisol is a complicated process that may be related to exercise time, exercise intensity and the dosage of the test substance. The mechanism of LRAs on cortisol requires further study.

The consumption of energy, such as glycogen, the generation of free radicals in cells, and the accumulation of body metabolites, are all potential mechanisms that induce exercise fatigue (Luo et al., 2019). As shown in Figure 10, the potential preliminary anti-fatigue mechanism of LRAs in fatigue model mice can be inferred: (i) LRAs can relieve the insufficient supply of serum glucose and liver/muscle glycogen caused by exhaustive exercise; (ii) LRAs can enhance anti-oxidant enzyme activity (such as that of SOD), reduce the activity of a large number of peroxides and oxidation reaction kinases (such as MDA and LDH), and alleviate the weakening of cell membrane permeability caused by anaerobic metabolism; and (iii) LRAs can adjust the metabolism of proteins during high-strength motion, reduce the production of the metabolite BUN, thereby reducing the burden on the kidney and adjusting internal environmental disorder caused by the LD produced by glycolysis.

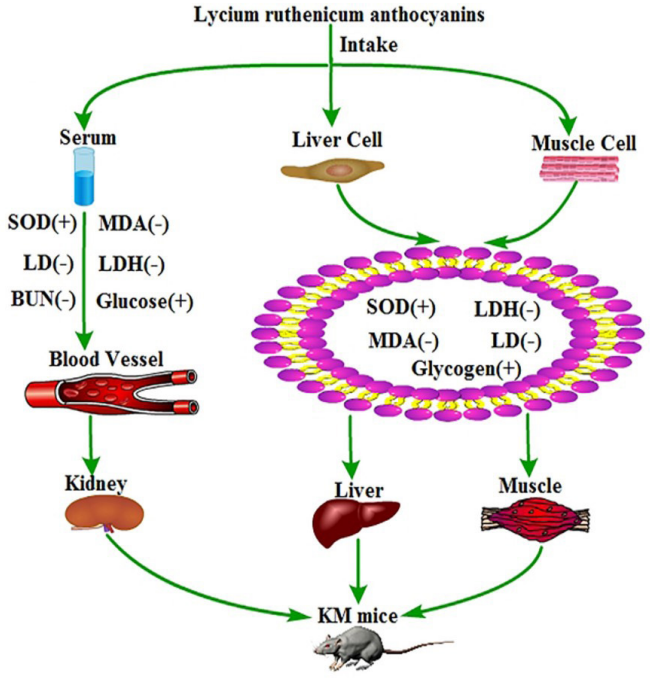

Figure 10. The Potential Anti-fatigue Preliminary Mechanism of LRAs in a Mouse Fatigue Model

5 Conclusions

In summary, the present study demonstrated that LRAs were primarily constituted by three anthocyanins, namely, delphinidin, petunidin and malvidin, as determined by HPLC. After 21 days of gavage, the weight and exercise time were recorded, and the eight indexes related to anti-fatigue properties in liver tissue, muscle tissue and serum in three different exercise stages were determined. The results showed that LRAs had no effect on mouse body weight and that LRAs enhanced mouse exercise endurance, increased sugar reserves, scavenged free radicals and regulated metabolic levels to enhance anti-fatigue activities. LRAs have extensive application prospects in improving sports performance, endurance and eliminating physical fatigue under long-term high-pressure conditions.

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