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1 # Improving activity of *Lycium barbarum.* polysaccharide on
2 # sub-health mice

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1 **Abstract**

2 Sub-health has been described as a chronic condition of unexplained deteriorated physiological
3 function which falls between health and illness. Fatigue is one of the main manifestations of
4 sub-health. Mitochondrial dysfunctions have been found in fatigue-type sub-health, such as,
5 impaired oxidative phosphorylation and mitochondrial damage. In the present study, we evaluated
6 the effects of *Lycium barbarum.* polysaccharide (LBP-4a), a polysaccharide fraction purified from
7 *Lycium barbarum.*, on anti-fatigue in sub-health mice and relevant mechanisms. Forty mice were
8 divided into the control, model, LBP-4a (L) and LBP-4a (H) groups. Model mice were built
9 through compound factors including forced swim test, sleep deprivation and wrapping restraint
10 stress test. After LBP-4a treatment for 4 w, the gastrocnemius muscles were obtained for
11 morphological observation and detected the activities of SOD, GSH-Px and MDA content.
12 Furthermore, mitochondria membrane potential and Ca^{2+} content were measured in isolated
13 skeletal muscle mitochondria. The results showed that LBP-4a could reduce the damage of the
14 skeletal muscle and MDA level, and enhance the activities of SOD and GSH-Px compared to the
15 model group. The levels of mitochondria membrane potential and Ca^{2+} were increased in skeletal
16 muscle mitochondria treated with LBP-4a, moreover, the high dose group was better than that of
17 low dose group. In conclusion, LBP-4a had anti-fatigue activity on sub-health mice, and the
18 mechanism was closely correlated with reduction of lipid peroxidation level and increase of
19 antioxidant enzymes activities in skeletal muscle tissue, improving the intracellular calcium
20 homeostasis imbalance and increasing mitochondrial membrane potential. These observations
21 provided the background for the further development of LBP-4a as a kind of antifatigue used in
22 sub-health.

23

24 **Keywords:** *Lycium barbarum* polysaccharide; sub-health; fatigue; mitochondria

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2 ## 1. Introduction

3 Sub-health has been described as a chronic condition of unexplained deteriorated
4 physiological function which falls between health and illness. Sub-health is
5 characterised by the presence of lowered energy levels, loss of vitality, altered
6 sleeping patterns and an increased incidence of viral infections [1,2]. The incidence of
7 sub-health is high, therefore, there is an increasing need for scientifically validated
8 therapies which can effectively and simply treat the symptoms of sub-heath including
9 fatigue. The prevention and cure of sub-health are the common concern among
10 traditional Chinese and western medicine [3]. In this study, the sub-health mice were
11 built by compound factors including swimming exercise, sleep deprivation and clamp
12 tail stimulus to mimic the cause and symptoms of sub-health.

13 *Lycium barbarum.* polysaccharide (LBP-4a), the active ingredient extracted from
14 *Lycium barbarum.*, has been found bioactivities such as enhancing the body's immune
15 capacity and lowering blood glucose [4,5]. We have previously shown that LBP-4a is
16 heteropolysaccharides and contained different carbohydrate compositions [6]. In this
17 study, we investigated the effects of LBP-4a on sub-health mice and relevant
18 mechanisms.

19 Skeletal muscle is the main peripheral target organs for fatigue. Fatigue can lead
20 to structure and function obstacle in skeletal muscle and the mechanism is associated
21 with mitochondrial function [7,8]. Previous research has proved the lipid peroxide can
22 destroy the structure and function of mitochondria and affect the process of energy

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1 metabolism, and then, lead to fatigue [9]. Skeletal muscle cells continuously generate
2 reactive oxygen species (ROS), which play a critical role in the modulation of muscle
3 contractility. High levels of ROS promote contractile dysfunction, resulting in muscle
4 weakness and fatigue [10]. Mitochondria have been considered as the main ROS
5 generator during exhaustive exercise, and at the same time, the primary target for
6 oxidative modification [11]. ROS may additionally lead to damage of mitochondrial
7 DNA and membranes thus decreasing membrane fluidity. Lowered levels of
8 antioxidants in fatigue-type sub-health may further aggravate the activated oxidative
9 stress. The antioxidant effect of LBP-4a on skeletal muscle has been scarcely
10 investigated. Given this background, the primary objective of the present study was to
11 examine the therapeutic effects and the possible mechanism of LBP-4 on the
12 improvement of skeletal muscle in sub-health mice. The experimental results may
13 provide comprehensive, scientific evidence for LBP-4a as a suitable dietary natural
14 anti-fatigue agent.

15 2. Materials and Methods

16 *2.1Materials*

17 The fruits of *Lycium barbarum* were collected in the Ningxia Hui Autonomous
18 Region which was the well-known production area of *Lycium barbarum* in China, and
19 were authenticated at the Agricultural college of Northwest A&F University. A
20 specimen (NO.20110609) was deposited in the herbarium of the Botany Department.
21 Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde
22 (MDA) kits were purchased from Nanjing Jiancheng Bioengineering technology

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1 (Nanjing Jiancheng Bioengineering technology Co., LTD, China). Hematoxylin, eosin,
2 rhodamine 123 and Flu3 were purchased from Santa Cruz Biotechnology, Inc. (Santa
3 Cruz, CA). All other reagents and chemicals were of the highest purity grade
4 available.

5 ***2.2 Preparation of LBP-4a from Lycium barbarum.***

6 Isolation, purification and identification of LBP-4a were based on our previous
7 published work[6]. Briefly, the dried fruit samples were refluxed three times to remove
8 lipids with chloroform: methanol solvent (2:1) (v/v). After filtering, the residues were
9 air-dried and then refluxed again with 80% ethanol. The residues were extracted three
10 times in hot water (90℃) and filtered. The combined filtrate was precipitated with
11 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging,
12 the precipitate was collected and vacuum-dried, giving crude polysaccharides (yield
13 was 3.25 %±0.14%, and the purity of LBP was 95.8 %±2.0%). Crude LBP was eluted
14 and isolated on a DEAE cellulose column (ø25 mm×350 mm) with distilled water and
15 0.05-0.5 mol/L NaCl. The collected four fractions were dialyzed, centrifuged, and
16 freeze-dried. For gel permeation chromatography, samples were dissolved in 20 ml of
17 buffer, then applied to a Sephadex G-100 column (ø26 mm×400 mm), and eluted with
18 0.05 mol/L NaCl at a flow rate of 0.5 ml/min. The fractions were collected using an
19 elution pattern and concentrated in an evaporator at 60℃. The concentrate was
20 dialyzed in distilled water for 72 h, and then freeze-dried.

21 ***2.3 Preparation of sub-health model and Treatments***

22 Forty male Kun-ming mice (18-22g) were obtained from medical sciences laboratory

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1 animal center of Changchun University. They were housed in an animal room at
2 22±2℃ and 50±10% relative humidity and had free access to laboratory chow and tap
3 water. The mice were adapted to an inverse 12:12-h light-dark cycle. The animals
4 were treated according to the National Institute of Health Guide for the Care and Use
5 of Laboratory Animals and further approval for their experimentation was obtained
6 from the Animal Ethics Committee of the university.

7 Model mice were built through mimicking the mainly factors of fatigue-type
8 sub-health. The mice were placed in a swimming pool with the diameter of 70 cm and
9 depth of 30 cm, keeping the water temperature at 25℃. The mice were forced to swim
10 for 50 min at 8 a.m. and 4 p.m. every day. The mice were deprived of sleep at the rest
11 of the time until 8 p.m. Since the third week, the mice were put in tubes for 4 h a day,
12 for a total of 7 d. Since the fourth week, the mice were clamped tail using hemostatic
13 forceps wrapped gauze at the cutting-edge. The degree of fatigue in mice was
14 assessed by exhaustive swimming experiments (25℃ water temperature; 7% with
15 lead weights on tail; swimming exhaustion standard: mice nasal tip sank underwater
16 for l0 s). Sub-health mice were randomly assigned to three groups of ten mice each:
17 model group, low dose of LBP-4a (LBP-4a(L)) and high dose of LBP-4a (LBP-4a(H))
18 treated group. In the LBP-4a groups, mice were treated by intragastric administration
19 with LBP-4a (10 mg/kg·d and 20 mg/kg·d) dissolved in normal saline and model
20 group mice received normal saline for four weeks.

21 ***2.4 Skeletal muscle morphological observation***

22 The gastrocnemius of all groups were excised and fixed in 4% formalin, embedded in

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1 paraffin, and cut into 4 μm sections. The sections were stained with H&E staining
2 method and then examined for morphology under light microscope.

3 ***2.5 Measurement of SOD, GSH-Px and MDA of skeletal muscle***

4 1 g gastrocnemius tissues were homogenated. The 10% preparation of tissue
5 homogenate was centrifuged with 3,000 rpm at low temperature for 15 min. The
6 supernatant was collected for determination of SOD, GSH-Px and MDA. The
7 activities of SOD and GSH-Px, and the content of MDA were measured using
8 commercial kit. The manipulation was progressed strictly according to the kit
9 instruction manual. In short, the MDA, formed as an end-product of lipid peroxidation
10 (LPO), was treated with thiobarbituric acid to generate a colored product measured at
11 532 nm. To measure the SOD activity, the sample was diluted with dilution buffer or
12 saline. Sample solution was then aliquoted into the wells of a 96-well plate for each
13 blank or sample, after working solution was added. In addition, an enzyme working
14 solution was added to each sample well and mixed thoroughly. The enzyme reaction
15 was then induced by incubating the mixture plate at 37℃ for 20 min, after which the
16 absorbance was measured using a spectrophotometer at 450 nm.

17 ***2.6 Isolation of skeletal muscle mitochondria***

18 Mice were killed by decapitation under ether anesthesia. The gastrocnemius muscles
19 were quickly excised, trimmed of fat and connective tissue, according to the method
20 [12] with some modifications. In brief, gastrocnemius was shredded and minced with
21 sharp scissors, and placed in extraction medium (120 mmol/L KCl, 20 mmol/L Hepes
22 (pH 7.4), 1 mmol/L EDTA, 5 mmol/L $MgCl_2$). Muscles were centrifuged at 600 rpm

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1 for 10 min. The supernatant was filtered through muslin and centrifuged at 17,000
2 rpm for 10 min. The precipitate was suspended in 20 mL extraction medium and
3 centrifuged at 7, 000 rpm for 10 min. The precipitate was resuspended in 3 mL
4 extraction medium containing 12% Percoll and spreaded in density gradient liquid
5 composed with 3 mL 40% Percoll and 3 mL 19% Percoll, followed by centrifugation
6 at 30,000 rpm for 10 min. Three belts were obtained and collected the homogenate
7 belt accumulated Percoll with 19% and 40%, followed by centrifugation at 16,000
8 rpm for 10 min. And then the precipitate was resuspended in 100 μL extraction
9 medium, which was the purification of mitochondria. All of the above procedures
10 were performed at 4 ℃ as quickly as possible. The protein concentration values of the
11 isolated mitochondria were determined using the Bradford method.

12 ***2.7Measurement of mitochondrial membrane potential（ΔΨm）in skeletal muscle***

13 Mitochondrial membrane potential of skeletal muscle was measured using flow
14 cytometer (excitation: 488 nm; emission: 534 nm), and the procedure in this study
15 was a modification of the method [13]. To get 0.1 mL 1×10^6 cell/mL single cell
16 suspension of skeletal muscle mitochondrial, and then 10% chicken red blood cells
17 were added as internal standard. Rhodamine 123 was added to cell suspension at the
18 final concentration of 25 μmol/L at 37 ℃ in the dark for 30 min. After PBS washing
19 twice, mitochondrial membrane potential was immediately analyzed using flow
20 cytometry instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Data were
21 collected in FSC (forward scatter) and SSC (side scatter) and a total of 10,000 events

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1 were collected for each sample. The level of $\Delta\psi m$ was indicated with fluorescence
2 intensity (I). The computation formula was as follows:

3 $$I=Log\ (x\text{-}mode)\times 340$$

4 ***2.8 Measurement of mitochondrial Ca^{2+} content in skeletal muscle***

5 To get 0.1 mL 1×10^{6} cell/mL single cell suspension of skeletal muscle mitochondrial,
6 and then 10% chicken red blood cells were added as internal standard. 1mL Fluo3 was
7 added in cell suspension at 37 ℃ in the dark for 30 min. After PBS washing twice, the
8 Ca^{2+} content of mitochondrial was immediately analyzed using flow cytometry
9 instrument. Data were also collected in FSC (forward scatter) and SSC (side scatter)
10 and a total of 10,000 events were collected for each sample. The content of Ca^{2+} was
11 indicated with fluorescence intensity (I). The computation formula was as follows:

12 $$I=Log\ (x\text{-}mode)\times 340$$

13 ***2.9 Statistical Analysis***

14 All the results were expressed as the mean ± S.D. *P*-values of less than 0.05 were
15 considered to be significant. Statistical analysis was performed by one-way analysis
16 of variance (ANOVA). All the grouped data were statistically evaluated with SPSS
17 13.0 software. Statistical significance of differences between two groups was
18 determined using the Student's *t*-test.

19 **3. Results**

20 ***3.1 Isolation, purification and characterization of LBP-4a***

21 LBP-4a was identified to be a homogeneous polysaccharide component, which
22 showed a single symmetrical peak following Sephadex G-100 gel chromatography.

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1 The MW of LBP-4a was 33,867 Da and retention time was 8.257 min by HPLC. In
2 addition, the monosaccharide composition of LBP-4a was analyzed by paper
3 chromatography and revealed the presence of six spots, corresponding to galactose,
4 glucose, rhamnose, arabinose, mannose, and xylose respectively. LBP-4a had two
5 absorption peaks at 199 and 260 nm in the UV spectrum, indicating the presence of
6 polysaccharide and protein. According to the IR spectrum, the purified LBP-4a
7 displayed a broadly stretched, intense peak at 3,428 cm^{-1} characteristic of hydroxyl
8 group and a weak C-H peak at around 2,915 cm^{-1}. The relatively strong absorption
9 peak at around 1,710 cm^{-1} indicated the carbonyl group. The absorbance of
10 polysaccharides in the range 1,000-1,200 cm^{-1} was the C-O-C and C-O-H link band
11 positions. The backbone of sugar residues chain in LBP-4a contained 1→6 indican
12 bonds according to periodate oxidation. The results of β-elimination reaction
13 indicated that the chain of polysaccharides and protein were connected by O-linked
14 chemical bond.

15 ***3.2 General situation of sub-health mice***

16 Fatigue-type sub-health mice were established by compound factors. At preliminary
17 stage, model group mice were agitated and irritability. After four weeks, the model
18 group mice were mental burnout, dry hair and eating less. Specifically, the swimming
19 time to exhaustion, the most important index of fatigue, was shortened. The
20 swimming test is often used in anti-fatigue and endurance tests. The maximum
21 swimming time of mice was measured to estimate the effect of LBP-4a on anti-fatigue
22 activity. As shown in Fig. 1, the average swimming time to exhaustion of mice treated

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1 with LBP-4a(L) and LBP-4a(H) was significantly increased ($P<0.05$, $P<0.01$) when
2 compared to that of the model group. However, there was no significant difference in
3 the average swimming time to exhaustion between the LBP-4a (H) group and the
4 normal control group. The result indicated that LBP-4a had an ability to prolong the
5 swimming endurance for sub-health mice.

6 ***3.3 Effects of LBP-4a on the activities of SOD、GSH-Px and the level of MDA in***
7 ***skeletal muscle***

8 Growing evidence indicates that excessive exercise could produce ROS, and muscle
9 fatigue will happen[14,15]. Intracellular antioxidant enzymes could alleviate oxidative
10 stress mediated muscle fatigue. So we investigated the potential effects of a treatment
11 with LBP-4a against oxidative damage induced on mice skeletal muscle. As shown in
12 Table 1, compared with the control group, the activities of SOD and GSH-Px of
13 skeletal muscle were significantly decreased in model group, but the content of MDA
14 was significantly increased ($P<0.01$). LBP-4a treatment for 4 w, compared with the
15 model group, the activities of SOD and GSH-Px of skeletal muscle were increased
16 significantly, and the content of MDA was decreased significantly. Furthermore, the
17 high dose group was better than that of low dose group.

18 ***3.4 Effect of LBP-4a on skeletal muscle***

19 To characterize the internal structure of the muscle fibers, we observed the
20 morphology of the gastrocnemius stained with hematoxylin and eosin (H&E). Under
21 normal conditions, the myofilament was in a state of contraction and the arrangement
22 was orderly and neat. All nuclei were located at the edge of the muscle fibers, whereas

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1 skeletal muscle fibers in the sub-health mice exhibited typical striated muscle
2 degeneration features, such as, blurred or disappeared transverse striation, nucleus
3 pycnosis, hyperchromatic and a mass of fat brown pigment deposition (Fig.2).
4 Furthermore, the structure became loose and the nuclei were distributed in a more
5 disorderly manner. After treatment with LBP-4a for 4 w, especially in high dose group,
6 the anomalies had been improved.

7 ***3.5 Effect of LBP-4a on mitochondrial membrane potential of skeletal muscle***

8 We investigated the effect of LBP-4a on the mitochondrial membrane potential of
9 skeletal muscle. By the end of the experiment on day 28, all animals were executed
10 and the skeletal muscle mitochondrial from control, model and treated mice were
11 harvested. As shown in Fig 3, compared with the control group, mitochondrial
12 membrane potential was significantly reduced in the model group (P<0.01), but, it
13 was significantly increased in LBP-4a (L,H) treatment group (P<0.05 and P<0.01,
14 respectively) compared with model group. Furthermore, the effect of LBP-4a was
15 better at high dose.

16 ***3.6 Effect of LBP-4a on mitochondrial Ca^{2+} of skeletal muscle***

17 To further investigate the mechanism of LBP-4a in sub-health mice, the mitochondrial
18 Ca^{2+} of skeletal muscle, the important regulators in mitochondrial function were
19 evaluated. The result showed that the content of Ca^{2+} in model group was significantly
20 decreased compared with the control group (P<0.01). While after LBP-4a treatment,
21 the contents of Ca^{2+} in treatment groups of LBP-4a(L) and LBP-4a(H) were
22 significantly higher than that in the model group (P<0.05, P<0.01) (Fig 4). The results

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1 indicated that LBP-4a significantly increased mitochondrial Ca^{2+} of skeletal muscle in
2 a dose-dependent manner.

3 **4. Discusstion**

4 To date, many drugs have been developed and applied in clinic for sub-health
5 treatment. However, the effect is not satisfactory. Fatigue, depression and agitation are
6 performance for sub-health. Fatigue is one of the main manifestations of sub-health.
7 In this study, the sub-health model mice were built through compound factors,
8 including forced swim test, sleep deprivation and wrapping restraint stress test. The
9 main symptom in model mice of sub-health is fatigue. Given this, in the present study,
10 we evaluated the effects of LBP-4a for anti-fatigue in sub-health mice and relevant
11 mechanisms. Mitochondrial dysfunction is directly related to excessive fatigue. At the
12 cellular level, fatigue is related to loss of mitochondrial function and diminished
13 production of adenosine triphosphate (ATP). As a result of fatigue, oxidative damage
14 to mitochondrial membranes impairs mitochondrial function[16]. From the perspective
15 of anti-fatigue, this article will discuss LBP-4a, the most promising dietary
16 supplements that were used to treat intractable sub-health and improve mitochondrial
17 function.

18 Excessive exercise can lead to changes in cell structure and function of skeletal
19 muscle [17]. Due to the glucolipid metabolic disorders, the abilities of oxidation and
20 anti-oxidation are imbalance in skeletal muscle, and the non-oxidation way is
21 increased. In our study, skeletal muscle fibers had been damaged in the sub-health
22 mice built by compound factors which led to excessive fatigue, and the structure

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1 became loose and the nuclei were distributed in a more disorderly manner (Fig.2B).
2 After treatment with LBP-4a for 4 w, especially in high dose group, the structure of
3 myofilament was obviously improved (Fig.2D). The exhaustion swimming test, which
4 is a commonly used technique for animal model of fatigue and behavioral despair, has
5 been used extensively for the evaluation of anti-fatigue properties of novel
6 compounds [18]. In this study, the swimming time to exhaustion was shortened in
7 sub-health mice. It indicated that the sub-health mice were fatigue and the survival
8 desire was decreased.

9 Mitochondria have an essential role in energy production through the process of
10 oxidative phosphorylation where nutrients are converted into ATP, which powers
11 many of the cells' activities. In addition to energy production, mitochondria have been
12 implicated in various physiologic processes including the production of ROS and
13 calcium homeostasis and flux. A consequence of the electron transport process is the
14 production of ROS, highly reactive free radicals that are produced as a by-product of
15 oxidative phosphorylation [19]. Increased oxidative stress may inhibit mitochondrial
16 respiration, decrease the activities of the electron transport chain and mitochondrial
17 membrane potential, increase mitochondrial membrane permeability, interfere with
18 ATP production and cause mitochondrial shutdown. Calcium homeostasis imbalance
19 is closely related to cell damage caused by excessive movement. The mitochondria
20 are important respiratory organs and Ca^{2+} repository in cells. Therefore, it plays an
21 important role in maintaining cell function. Too much exercise, mitochondrial
22 oxidative phosphorylation process is suppressed and energy metabolism is disfunction

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1 mainly because Ca^{2+} content in skeletal muscle cytoplasm is increased. At the same
2 time, mitochondrial uptake Ca^{2+} ability and the buffer ability of Ca^{2+} concentration in
3 cytoplasm are decreased, result in lower Ca^{2+} concentration in mitochondria, and
4 eventually cause tissue damage. In our study, the results showed (Fig 3,4), compared
5 with model mice, the levels of mitochondria membrane potential and Ca^{2+} were
6 increased in skeletal muscle mitochondria treated with LBP-4a, moreover, the high
7 dose group was better than that of low dose group.

8 Sub-health is characterized by a significantly decreased antioxidant status.
9 Cellular antioxidant defenses usually maintain ROS levels at concentrations that
10 prevent excess oxidation of cellular molecules [20]. Cellular antioxidant defenses are
11 endogenous and are mediated by GSH-Px, catalase (CAT), SOD, and so on [21]. SOD
12 is one of the most important enzymes in the antioxidant defense system. It quenches
13 the superoxide radical by converting it into O_2 and H_2O_2. H_2O_2 can be reduced to H_2O
14 in the presence of CAT or GSH-Px. GSH is an important biomolecule involve in the
15 antioxidant defense system against toxicants. MDA is one of the final-stage
16 byproducts of lipid peroxidation, and is an indicator of oxidative stress in cells and
17 tissues [22]. A lower MDA level indicates that there is weaker oxidant stress and less
18 lipid peroxidation. Since the antioxidant defense becomes weaker during sub-health,
19 the enhancement of antioxidant enzyme activities can help to fight against sub-health.
20 Also, some dietary plant extracts are reported to have strong antioxidant potential[23-25].
21 *Lycium barbarum.* constituents including polysaccharides found in the plant extracts
22 are effective as radical scavengers and inhibitors of lipid peroxidation[26-28]. A number

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1 of natural supplements have been used to treat nonpsychological fatigue and
2 mitochondrial dysfunction[29]. Enhanced lipid peroxidation and decreased activity of
3 SOD and GSH-Px in skeletal muscle of sub-health mice has been found to be
4 protected following treatment with LBP-4a suggesting protective efficacy of LBP-4a.
5 It was hypothesized that the oxidative stress pathway was the downstream result of an
6 imbalance in skeletal muscle Ca^{2+}, which results in low mitochondrial calcium.
7 LBP-4a treatment decreased lipid peroxidation level and increased antioxidant
8 enzymes activities in skeletal muscle of sub-health mice, and then increased muscle
9 fatigue resistance. The detailed mechanism remains to be further research.

10 5. Conclusion

11 LBP-4a had anti-fatigue activity on sub-health mice, and the mechanism was closely
12 correlated with reduction of lipid peroxidation level and increase of antioxidant
13 enzymes activities in skeletal muscle tissue and improving the intracellular calcium
14 homeostasis imbalance of the skeletal muscle and the increase of mitochondrial
15 membrane potential. These observations provided the background for the further
16 development of LBP-4a as a potential dietary therapeutic agent against sub-health.

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20 Conflict of Interest statement

21 The authors declare that they have no conflict of interest.

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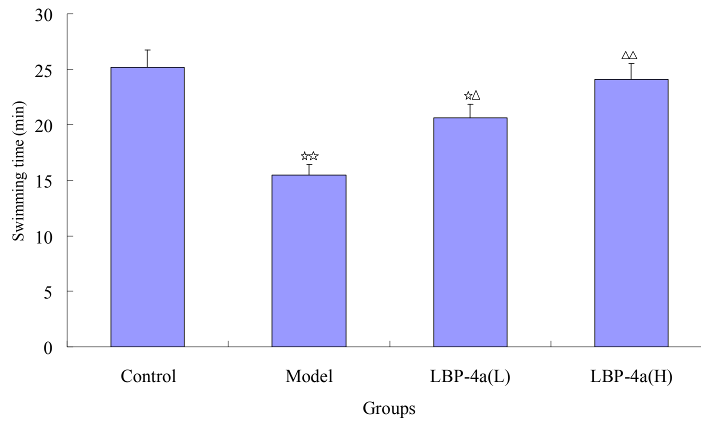

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3 Fig.1. The effect of LBP-4a on the swimming time to exhaustion. Data were expressed as mean ±
4 S.D.(n=10). ☆*P*<0.05, ☆☆*P*<0.01, compared with Control; △*P*<0.05, △△*P*<0.01, compared with
5 Model.

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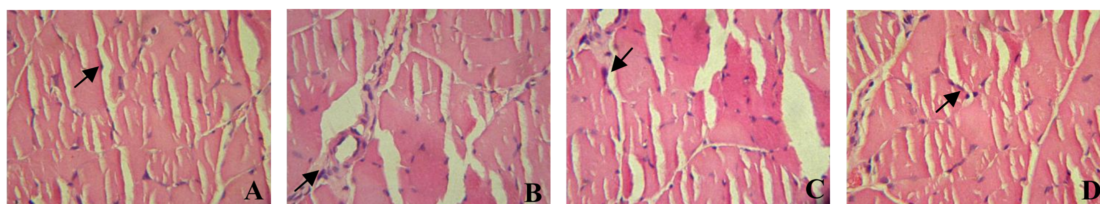

2 Fig.2. Histopathology sections of skeletal muscle tissue. The gastrocnemius were excised and
3 fixed in 4% formalin, embedded in paraffin, and cut in 4 μm sections. The sections were stained
4 with H&E staining method and then examined for morphology under light microscope.
5 A Control group; B Model group; C LBP-4a(L) group; D LBP-4a(H) group (HE×400)
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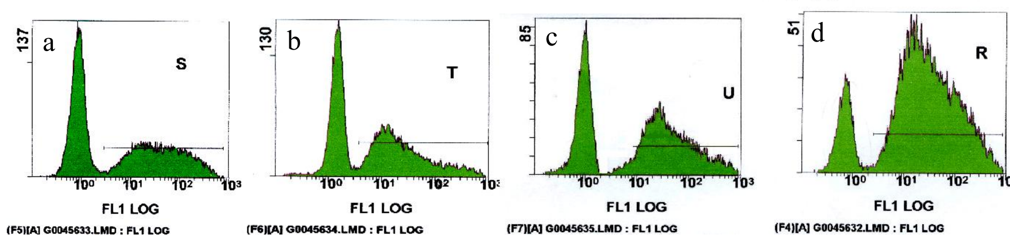

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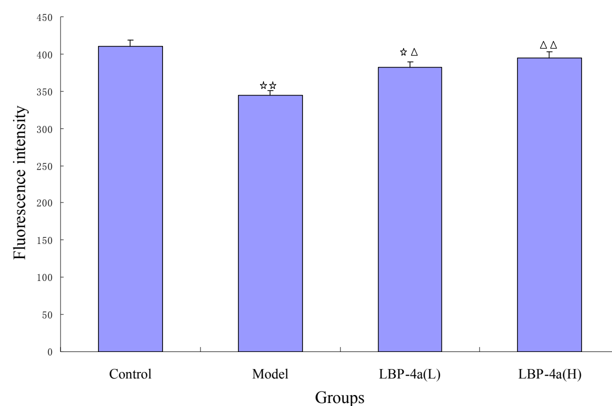

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6 Fig.3. Effect of LBP-4a on mitochondrial membrane potential of skeletal muscle in sub-health
7 mice. (A) Skeletal muscle mitochondrial membrane potential was measured using flow
8 cytometer (excitation: 488 nm; emission: 534 nm). Data were also collected in FSC (forward
9 scatter) and SSC (side scatter) and a total of 10,000 events were collected for each sample. a
10 Control group; b Model group; c LBP-4a(L) group; d LBP-4a(H) group (B) The level of Δψm
11 was indicated with fluorescence intensity (I). The computation formula is as follows: I=Log
12 (x-mode)×340. Data were expressed as mean ± S.D.(n=10). $^{☆}P$<0.05, $^{☆☆}P$<0.01, compared with
13 Control; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01, compared with Model.

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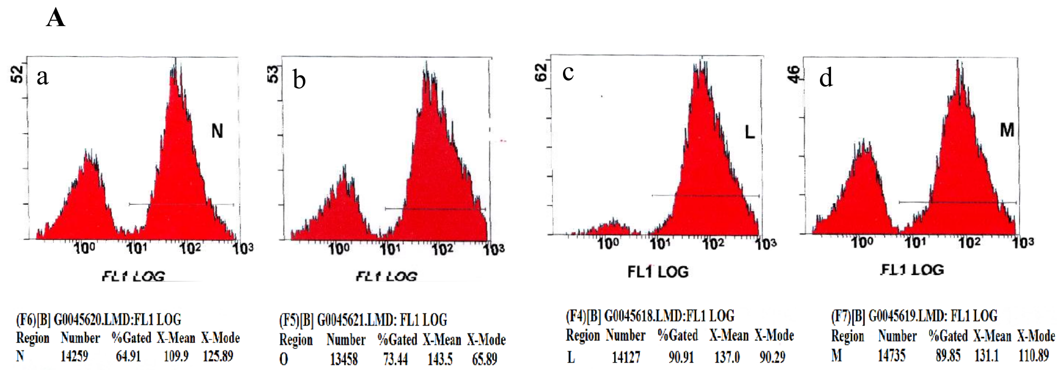

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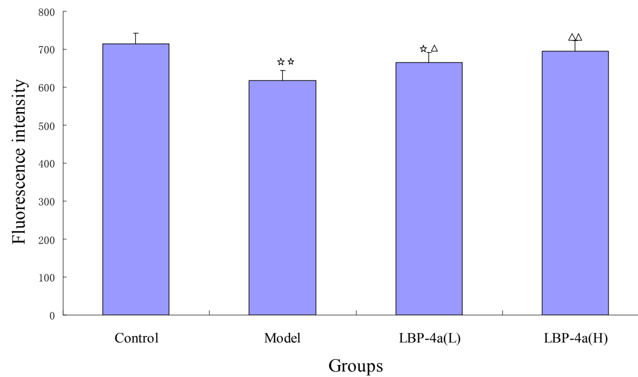

Fig.4. Effect of LBP-4a on [Ca^{2+}] of skeletal muscle mitochondria in sub-health mice. (A) Mitochondrial $Ca2^{+}$ was analyzed using flow cytometry instrument. Data were also collected in FSC (forward scatter) and SSC (side scatter) and a total of 10,000 events were collected for each sample. a Control group; b Model group; c LBP-4a(L) group; d LBP-4a(H) group (B) The level of $Ca2^{+}$ was indicated with fluorescence intensity (I). The computation formula is as follows: I=Log (x-mode)×340. Data were expressed as mean ± S.D.(n=10). $^{☆}P$<0.05, $^{☆☆}P$<0.01, compared with Control; $^{△}P$<0.05, $^{△△}P$<0.01, compared with Model.

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