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Original Research Article

Antifatigue Effect of *Millettiae speciosae* Champ (Leguminosae) Extract in Mice

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Abstract

Purpose: *To evaluate whether Millettiae Speciosae Champ. (Leguminosae) can enhance exercise performance as well as ascertain if it a potential functional food material.*
Methods: *The extract of Millettia speciosa Champ. (MSE) was orally administered to mice in 500, 1000, 2000 mg/kg doses to investigate its anti-fatigue effect in both forced swimming and climbing tests. Glycogen, triglyceride (TG), blood urea nitrogen (BUN) and creatine phosphokinase (CK) levels in plasma which can indicate alterations in energy utilization during exercise performance, were determined to analyze the operating exercise mechanisms.*
Results: *The results showed that swimming time to exhaustion was longer in all treated groups (41.06 ± 1.92, 47.84 ± 1.60, 54.00 ± 2.45 min for 500, 10000 and 2000 mg/kg doses, respectively) than for control (19.45 ± 0.62 min, $p < 0.05$). The middle and high doses of MSE-treated groups significantly prolonged the climbing time compared with control ($p < 0.05$). Furthermore, MSE reduced the content of TG significantly by increasing fat utilization, delayed the accumulation of BUN and decreased the level of CK ($p < 0.05$). In addition, administration of MSE significantly protected the depletion of muscle glycogen when compared with control ($p < 0.05$).*
Conclusion: *The results show for the first time that Millettia speciosa Champ. (Leguminosae) has significant anti-fatigue activity, and also suggest that it is a potential functional food material.*

Keywords: *Radix millettiae speciosae, Anti-fatigue activity, Exercise performance, Serum urea nitrogen, Gastrocnemius muscle glycogen, Triglyceride, Functional food*

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INTRODUCTION

Fatigue is a phenomenon that indicates that health is about to be, or already, subjected to harm, which is often associated with aging, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis and depression [1]. In busy and strained modern societies, fatigue has become a highly prevalent phenomenon, with up to half of the general population reporting fatigue in large surveys [2]. Therefore, it is common in sport medicine to improve athletic ability, postpone fatigue or accelerate the elimination of fatigue with few side effects [3]. However, available therapies for fatigue in modern medicine are still very limited. Thus, increasing number of people tend to take dietary supplements or "tonics" as an alternative [4].

Millettia speciosa Champ. (Leguminosae), commonly called *Niudali*, is grown in tropical and

sutropical regions especially in southeast China, where it is a functional food used traditionally to develop physical strength. However, despite recent studies that reveal that Radix *millettiae speciosae* has multiple bioactivities including liver-protection, expectoration, antitussive, antiasthma and immune boosting [5,6], it remains unclear how it exerts the tonic effect or whether it could enhance exercise performance.

Forced swimming has been employed as a method of assessing the physical work capacity of animals [7]. It is suitable for evaluating the endurance capacity of mice, which induces blood biochemical changes, and gives high reproducibility of results [8]. In this study, therefore, forced swimming and climbing tests were designed to investigate the anti-fatigue property of Radix *millettiae speciosae*.

EXPERIMENTAL

Reagents and chemicals

All chemicals were purchased from Guangzhou Chemical Reagents Co, Ltd (Guangzhou, China) unless otherwise indicated. Commercial diagnostic kits used to determine serum urea nitrogen (BUN), creatine kinase (CK), tissue glycogen and triglyceride (TG) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Plant materials

Millettia speciosa Champ. (Leguminosae) was collected from the Chinese traditional drug market in Guangdong Province, and identified by Professor Ziren Su in Jan. 2013. A voucher specimen (no. 201301290109) was deposited in the herbarium of First Affiliated Hospital of Chinese Medicines, Guangzhou University of Chinese Medicine.

Preparation of extracts

The dried rhizomes of *Millettia speciosa* Champ. was cut, ground in a disintegrator to obtain a coarse powder (particle size < 150 μm). The powder (100 g) was then extracted with boiling water (1:15, w/w) for 3 h. The resulting extract (MSE) was filtered and concentrated under vacuum at 60 ^{o}C to give a semisolid residue, which was then lyophilized to obtain the aqueous extract (13.12 g, yield 13.12 %). MSE was dissolved in distilled water prior to oral administration to mice.

Quantification of total polysaccharide and flavonoids of the extracts

Concentrations of total polysaccharide from MSE were determined by applying sulfuric acid-anthrone using glucose as a standard [9]. The procedure was as follows: anthrone solution (freshly prepared) was added to each tube, vortex-mixed gently, and incubated at 95 ^{o}C in a water bath. After 15 min, the tubes were transferred to an ice bath for 15 min to stop the reaction. Absorbance at 625 nm was read in an ultraviolet spectrophotometer. The content of total flavonoids from MSE was determined as previously reported [10]. MSE and the vanillin reagent were added and kept in boiling water bath for 15 min. The absorbance was read at 360 nm.

Animals

Male Kunming mice were purchased from Medical Experimental Animal Center of Guangdong Province No. 44007200002880, Guangzhou, China). All experiments were carried out according to guidelines of the National Institutes of Health Guide for Care and Use of Laboratory Animals [11], and were approved by the Animal Ethics Committee of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (No. dspf 2014009).

Experimental design

After an adaptation period of 1 week, the mice in the treated groups received three doses of MSE (500, 1000 and 2000 mg/kg for low-MSE group (LMT), medium-MSE group (MMT) and high-MSE group (HMT) respectively) intragastrically and the mice in the control group received distilled water daily for 20 consecutive days. The mice for weight-loaded swimming test and for analyses of biochemical parameters were made to swim for 10 min twice per week without loads as adaptation training before test. The mice for the pole climbing test were made to climb for 5 min twice per week to be accustomed to climbing the pole.

Assessment of animal characteristics

All the animals were observed once daily for morbidity. Changes in the body weight of mice were observed at the initial and terminal stages of the test.

Forced swimming capacity test

The swimming endurance capacity of mice was assessed using an adjustable-current swimming

pool with slight modifications performed from 9:00 am to 15:00 pm [12]. Briefly, an acrylic plastic pool (90 x 45 x 45 cm) was filled with water to a depth of 35 cm. The temperature of the water was maintained at 27±1 °C.

One hour after the final MSE treatment, the mice were subjected to exhaustive swimming with a load (a bundle of lead pieces corresponding to 5 % of their body weight) attached to the tail. Swimming time was recorded immediately from the beginning till exhaustion, determined by observing loss of coordinated movements and failure to return to the surface within 10 s.

Climbing test

Mice were placed on an organic glass bar 60 min after the last MSE administration so that the mouse muscles were in a static tense state. The test was stopped when the mice fell into the water for the third time. The total climbing period was calculated as the summation of all three records [13].

Blood biochemical parameters

One hour later after final administration of MSE, mice were forced to swim for 90 min without lead tied to their tails in the pool. Subsequently, blood was collected from the orbital venous plexus immediately after the forced swimming. Blood samples were centrifuged for 15 min at a speed of 3000 × g and the supernatant plasma was collected.

The levels of plasma BUN, CK and TG were determined using commercial kits by Cosba 8000 automatic biochemistry analyzer (Roche, Germany). In addition, gastrocnemius muscles were quickly dissected out from mice after blood collection, snap frozen in liquid nitrogen, and kept at -70 °C until an analysis for glycogen content. The level of muscle glycogen was determined using commercial diagnostic kit (Nanjing Jiancheng Bioengineering Institute, China).

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). The data were analyzed using Dunnett's t test, and $p < 0.05$ was considered statistically significant.

RESULTS

Contents of polysaccharide and flavonoids of MSE

Consequently, the total polysaccharide and flavonoids in this extract were 4.21 ± 0.13 and 0.33 ± 0.02 %, respectively.

Effect of MSE on body weight change

The body weight of control group increased gradually from day 1 to day 20 as shown in Table 1. Similarly, the body weights of the three MSE groups increased during the whole experiment, but did not show significant difference in comparison with that of control group ($p = 0.735$, 0.876, 0.679 vs control), demonstrating that MSE had no prominent impact on the body weight of mice.

Effect of MSE on swimming endurance capacity

The forced swimming capacity results are shown in Figure 1A. The swimming time to exhaustion of control group was 19.45 ± 0.62 min. In comparison, the LMT, MMT and HMT groups showed significantly prolonged swimming time, of which the swimming endurance was increased by 28, 46 and 77 %, respectively, compared control group ($p < 0.05$). These results evidently demonstrate that MSE elevated the exercise tolerance of mice.

Table1: Effect of MSE on body weight of mice

Group	Treatment (mg/kg)	Body weight (g)	
		Initial	Terminal
Control (C)	---	16.24±0.10	30.14±0.13
Low-dose MSE-treated (LMT)	500	16.36±0.10	29.70±0.18
Medium-dose MSE -treated (MMT)	1000	16.18±0.08	29.89±0.18
High-dose MSE-treated(HMT)	2000	15.52±0.10	29.13±0.20

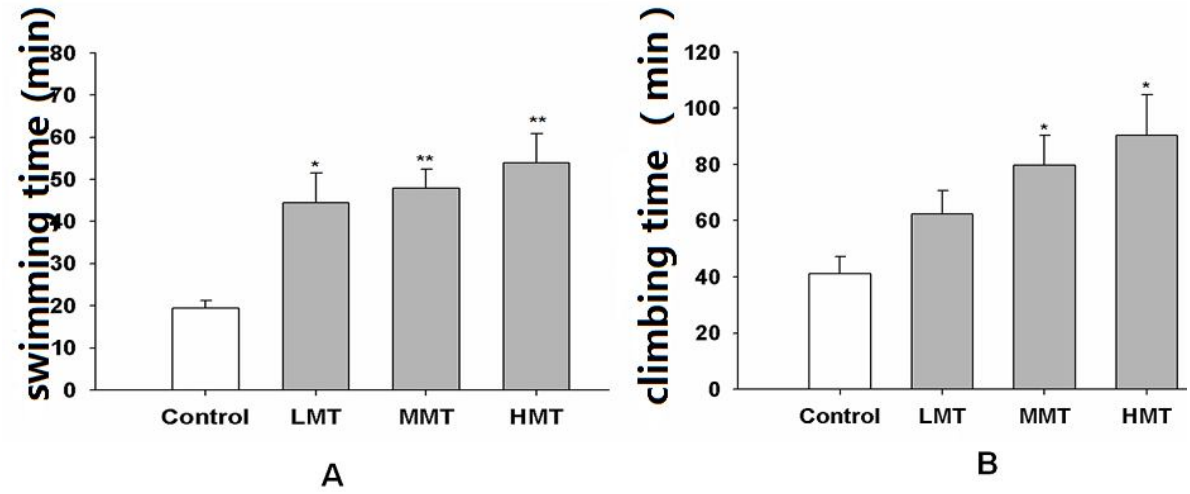

Figure 1: (A) Effect of MSE on physical endurance capacity, by weight-loaded forced swimming test; (B) effect of MSE on physical endurance capacity by climbing test. Control group (open column), MSE-treated group (500, 1000, 2000 mg/kg/d, grey column). Vertical bars represent standard error of mean (SEM, n = 10); Asterisks designate significant differences: **p* < 0.05, ***p* < 0.01, vs control

Effect of MSE on climbing test

As shown in Figure 1B, the climbing time of control group was 41.06 ± 1.92 min. In contrast to the control group, the climbing time in MMT and HMT groups extended by 96.7 and 120.5 %, respectively ($p < 0.05$, vs. control group). This result further confirms that MES may elevate sports endurance.

Effect of MSE on glycogen storage

As shown in Figure 2, after swimming, muscle glycogen contents of the MMT and HMT group were significantly higher than that of the control group, which were increased by 38 and 36 %, respectively. Although the difference between the LMT and control group was not statistically significant, the glycogen content of LMT increased by 21 % when compared to the control group.

Effect of MSE on TG content of plasma

The TG levels in all treated groups were lower than that of the control group (Figure 3A). Significant decline was found in LMT group, which decreased by 17.7 % compared to the control group ($p < 0.05$). Although the MMT and HMT groups didn't show statistical difference compared with the control group, the plasma TG content of these two groups decreased by 7.05 and 8.6 % respectively.

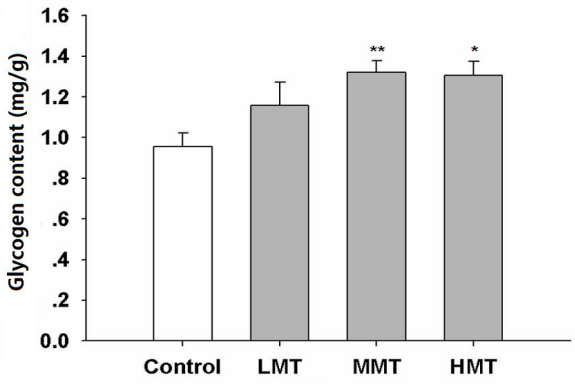

Figure 2: Effect of MSE on glycogen content of the gastrocnemius muscle of mice after forced swimming without load. Control group (open column), MSE-treated group (500, 1000, 2000 mg/kg/d, grey column). Vertical bars represent standard error of mean (SEM, n = 8); Asterisks designate significant differences: **p* < 0.05, ***p* < 0.01, vs control

Effect of MSE on BUN content of plasma

After swimming, the BUN content of LMT, MMT and HMT groups were decreased by 7.43 ± 1.39, 6.81 ± 0.96, and 6.5 ± 0.89 % respectively, which are evidently lower than that of the control group (Figure 3B, $p < 0.05$ vs. control group).

Effect of MSE on CK content of plasma

The plasma CK levels in LMT, MMT and HMT groups were dose-dependently decreased by 39, 49 and 58 %, respectively, when compared to that of control ($p < 0.01$, Figure 4).

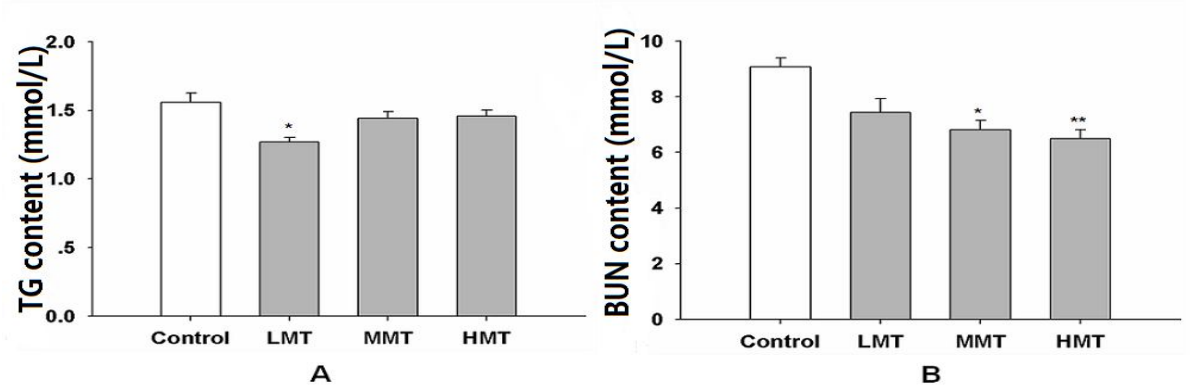

Figure 3: (A) Effect of MSE on TG content of the plasma of mice after forced swimming without load; (B) effect of MSE on BUN content of the plasma of mice after forced swimming without load. Control group (open column), MSE-treated group (500, 1000, 2000 mg/kg/d, grey column). Vertical bars represent standard error of mean (SEM, n = 8); Asterisks designate significant differences: *$p < 0.05$, **$p < 0.01$, vs control

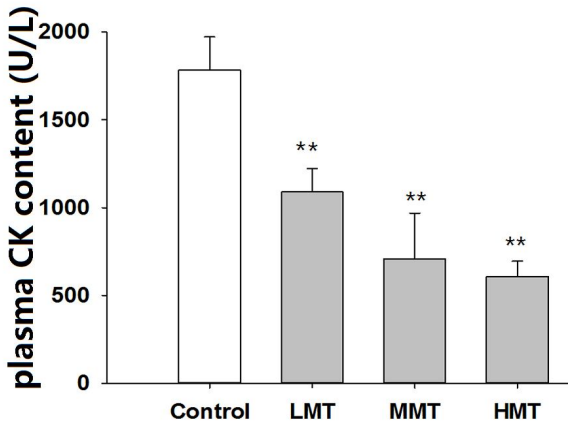

Figure 4: Effect of MSE on CK content of the plasma of mice after forced swimming without load. Control group (open column), MSE-treated group (500, 1000, 2000 mg/kg/d, grey column). Vertical bars represent standard error of mean (SEM, n = 8); Asterisks designate significant differences: **p < 0.01, vs control

DISCUSSION

It is well accepted that the most important physiological inducement of fatigue is limitation in energy metabolism during muscular activity [14]. Thus, improvement of exercise endurance is the most powerful macro representation of anti-fatigue. There into, weight-loaded forced swimming and pole climbing test are widely accepted methods to test exercise endurance, in which, the length of the swimming and climbing time positively related to the static strain, and negatively related to the degree of fatigue in animal movement [15]. In the present study, we examined the anti-fatigue effect of aqueous extracts from MSE on exercise endurance with these two models. The results showed that MSE treatment remarkably prolonged the swimming time to exhaustion and extended the pole climbing time, exhibiting an anti-fatigue effect to elevate the exercise tolerance.

In this study, in order to investigate the energy metabolism after intragastric administration of MSE, we measured the blood levels of TG and BUN in the exhausted mice immediately after the forced swimming test. The results showed that MSE significantly increased the levels of muscle glycogen of mice after swimming, reduced the blood TG and decreased the accumulation of BUN.

It is well established that during prolonged moderate to high intensity exercise, carbohydrate is primarily utilized to support energetic requirements, since it is expeditiously available, and can be directly oxidized to supply ATP in the blood [16]. In humans, the majority of endogenous carbohydrate is stored as glycogen in the muscle and liver. The capacity to sustain muscle contractions at high intensity exercise is highly dependent on the availability of glycogen at these sites [17]. Therefore, muscle glycogen has been well established as the primary metabolic energy substrate during physical exercise and has accordingly been implicated as a limiting factor, when such activity is sustained for a prolonged duration. Coordinately, improving glycogen supply conduces to prolonged endurance capacity and locomotory capacity [15]. Our data showed that MSE markedly increased tissue glycogen contents of mice post exercise, suggesting that MSE plays a positive role to activate energy metabolism and improve metabolic control of exercise, and thus, delay the appearance of fatigue.

Besides glycogen, lipid is also an important substrate during exercise, which was taken up or used for oxidation by muscle, was beneficial to relieve fatigue [18]. Muscular work, performed aerobically in the post-absorptive state, depends mainly on the energy provided by utilization of TG or fatty acids. It is documented that during

prolonged exercises the body increased clearance of plasma TG by skeletal muscle to provide sufficient energy consumption [19]. A lot of folk medicines and functional foods were certified to increase clearance of plasma TG, and thus, make better utilization of fat during swimming [20]. Our results showed that LMT group has significantly lower TG content, which is 17.7 % lower than that of control group. Although MMT and HMT groups didn't show statistical difference when compared with control group, the TG content in these two groups were decreased by 8.7 % and 7.1 %, respectively. This suggests that MSE increased fat utilization during a prolonged swim. This effect of MSE was consistent with herbal medicines previously reported [20]. Another possible explanation for the anti-fatigue effect seen following MSE treatment could involve TG mobilization during exercise, as indicated by the decrease in TG level. Such an effect might become advantageous during prolonged exercise, since better utilization of TG allows the sparing of glycogen and therefore delays fatigue [21]. Further experiments are needed in order to identify the mechanism through which MSE might affect fat mobilization.

Furthermore, when the body fails to obtain enough energy from glycogen and fat catabolic metabolism, for example, in the conditions of fasting and fatigue, protein and amino acids have stronger catabolic metabolism, which help the body adjust to these conditions. However, massive researches demonstrated that accompanied with protein utilization is the accumulation of BUN. BUN is a metabolic product of protein, whose accumulation does harm to the body and seriously lead to azotemia, which is the symptom of preuremic stage [18]. Nowadays, BUN is a well known renal or metabolic biomarker to diagnose some serious disease such as chronic kidney disease [22]. The removal of excess BUN accumulation is of important clinical significance. As we know, urea is formed in the liver as the end product of protein-metabolism. During digestion, protein is broken down into amino acids, part of which is used to produce energy in the tricarboxylic acid cycle [23] and the other of which is reacted with water and carbon dioxide to produce urea in the ornithine cycle. In other words, the worse the body is adapted for exercise tolerance, the more significantly the urea nitrogen level increases [24]. Therefore, BUN is the sensitive indicator to evaluate the body load bearing capacity when human bodies suffer from a physical load. Our results showed that the BUN of MMT and HMT groups were significantly lower than control group. This result demonstrates that MSE could decrease protein metabolism and ameliorate fatigue.

A new explanation of muscle fatigue suggests that it is related to tissue damage and leakage of several cytosolic enzymes, such as CK [25,26]. Fatigue results in the release of reactive oxygen species (ROS) which cause lipid peroxidation of membrane structure and finally damage the muscle [27]. During the process of muscle degeneration, the muscle cells lyse, and CK permeates into the bloodstream. Because most of the CK in the body normally exist in the muscle, an increase of CK in the blood indicates that muscle damage has occurred or is occurring. For example, levels of CK activity are commonly used in clinical pathology to assess the extent of myocardial infarcts [28]. To investigate whether supplementation of MSE decreases muscle fatigue, muscle damage-related biochemical parameters were evaluated. Our results showed that CK level were decreased significantly in the MMT and HMT groups. It can be inferred that MSE inhibited the CK activities and may protect cell against lipid peroxidation, and thus, attenuate subsequent muscle damage induced by exhaustive exercise. Taken together, the results show for the first time that MSE possesses an anti-fatigue activity. The possible mechanism may be attributed to energy metabolism and protection from muscle damage. When the body was suffered from extreme exercise, MSE possibly reduced the level of TG by increasing fat utilization, delayed the accumulation of BUN. Such effects definitely are beneficial for the body to adjust energy metabolism. On the other hand, fatigue may result in muscle damage, since the results revealed that MSE reduces plasma CK level and thus decrease muscle damage induced by exhaustive exercise.

CONCLUSION

The findings of this study corroborate previous results that demonstrate that *Millettia speciosa* Champ. (Leguminosae) is a safe anti-fatigue tonic supplement. However, further studies are required to elucidate the exact underlying mechanisms.

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