Studies on the anti-fatigue activities of *Irpex lacteus* polysaccharide-enriched extract in mouse model

Juan Wang, Chenliang Li, Wenji Hu, Lanzhou Li, Guangsheng Cai, Yang Liu and Di Wang*
School of Life Sciences, Jilin University, Changchun, China

Abstract: *Irpex lacteus* is a white rot basidiomycete proposed for a wide spectrum of biotechnological applications. However, few studies examined its effects on exercise performance and physical fatigue. The present study evaluated the potential beneficial effects of *I. lacteus* extract (ILE) on physical fatigue in mice. Anti-fatigue activities of ILE were evaluated in Kunming mice using the forced swim test, rotating rod and forced running test. Serum and liver biochemical parameters were determined by an autoanalyzer and commercially available kits. Seven-day ILE administration at doses of 0.04, 0.2 and 1.0g/kg failed to influence mouse horizontal and vertical movement indicating its safety on the central nervous system. Compared with normal mice, ILE significantly increased persistent period during rotating rod and swimming tests, and reduced shock times in forced running test. Additionally, ILE resulted in 23.4% and 64.5% increments on adrenocorticotropic hormone and cortisol levels in serum. Compared with normal mice, an 209.0% increment on adenosine triphosphate level in liver (up to 2.5 mmol/gHb) were noted in ILE-treated mice. Moreover, ILE increased the level of super oxide dismutase and reduced the level of malondialdehyde in the liver suggesting its antioxidant activity. Data obtained from western bolt suggests that ILE-improved endurance capacity is mainly acquired through activating 5′-AMP-activated protein kinase (AMPK). ILE enhanced the endurance capacity of mouse by an elevation of antioxidant at least partially associated with AMPK pathway. Our data highlight the potential of *I. lacteus* as an antioxidant in the treatment of fatigue-related diseases.

Keywords: *Irpex lacteus*, anti-fatigue, anti-oxidant, AMPK.

INTRODUCTION

Fatigue is a feeling of excessive physical or mental tiredness, which attacks more than 20% of people (Nozaki *et al*., 2009, Tanaka *et al*., 2008)**.** The symptoms of chronic fatigue including headaches, sleep disturbances, difficulties with concentration, and muscle pain are commonly observed in patients. During exercise, excessive free radicals are produced in body tissues leading to the disruption of muscle contractile function (Anand *et al*., 2014). Antioxidants supplementation successfully prolongs exercise performance and reduces metabolites and physical fatigue (Su *et al*., 2014, Wu *et al*., 2013).

In molecular level, 5′-AMP-activated protein kinase (AMPK) is considered as a key regulator of cellular and whole-body energy balance (Nakagawasai *et al*., 2013). AMPK activation protects cells from overproducing and accumulating reactive oxygen species (ROS) in mitochondria (Shin and Kim, 2009). *Cordyceps sinensis* improves the exercise endurance capacity of rats via modulation AMPK phosphorylation (Kumar *et al*., 2011). In skeletal muscle and liver, AMPK displays an important role in glycogen breakdown, glucose uptake, and fatty acid oxidation (Hardie and Sakamoto, 2006). As reported, the AMPK agonist AICAR significantly enhances the endurance performance in a mouse model (Kobilo *et al*., 2014). AMPK is considered to be a biomarker of physical fatigue (Nakagawasai *et al*., 2013). In the other hand, stress-induced hyper-activation on hypothalamo-pituitary-adrenal (HPA) axis is closely related to chronic fatigue (Hong *et al*., 2015). An alteration in cortisol secretion occurs in the process of fatigue development (Kumari *et al*., 2009).

Herbs show significant effect on alleviating symptoms of fatigue (Chen *et al*., 2015), such as *Rhodiola rosea*, a commonly used anti-hypoxia traditional Chinese medicine, displays fatigue recovery ability (Huang *et al*., 2009). Rhodioloside and salidroside, active components of *R. Rosea* extract were found to protect against oxidative damage in physical, emotional, and mental exhaustion (Panossian *et al*., 2010). *Irpex lacteus*, one of the white-rot basidiomycetes, displays a remarkable therapeutic effect on chronic glomerulonephritis, and suppresses erythropoiesis and urinary protein content in chronic nephritis patients (Zhang *et al*., 2012). However, scanty information is available on its potential anti-fatigue activity.

In this study, several mice models were applied to observe the anti-fatigue effect of *I. lacteus* extract (ILE) and biochemical parameters related to fatigue including adenosine-triphosphate (ATP), malondialdehyde (MDA), super oxide dismutase (SOD), adreno-cortico-tropic-hormone (ACTH) and corticosterone (CORT) were determined. Relevant signaling including protein kinase B (AKT) and AMPK in liver were measured to analyze its

**Corresponding author:* e-mail: jluwangdi@outlook.com

underlying mechanism. The results will be helpful in the development of novel anti-fatigue dietary supplements for humans.

MATERIALS AND METHODS

Strain culture

I. lacteus (obtained from China Center for Type Culture Collection, China) was grown in a defined liquid medium containing: 20g/L lactose, 10g/L peptone, 10g/L yeast extract powder, 0.5g/L $MgSO_4 \cdot 7H_2O$, 0.5g/L KH_2PO_4, and 0.1g/L Vitamin B1. The mycelium was filtered and lyophilized for further using. All the chemical reagents used in the submerged fermentation were obtained from Sigma-Aldrich, USA.

I. lacteus extract preparation

I. lacteus was extracted twice at 80°C for 4h by double distilled (D.D.) water. After centrifugation at 8,000rpm for 10 min, the supernatant was sequentially concentrated in an evaporator under reduced pressure, following with freeze-drying. Our preliminary experiments revealed that polysaccharides-enriching *I. lacteus* extract (ILE) contains 33.7% polysaccharides, 5.5% mannitol, and 0.4% adenosine.

Animal care

The experimental animal protocol was approved by the Animal Ethics Committee of Jilin University. Kunming mice (6 weeks, 18-22g, equal numbers of male and female; purchased from Norman Bethune University of Medical Science, Jilin University, Jilin, China) (SCXK(JI)-2014-0003) were maintained on a 12-h light/ dark cycle (lights on 07:00-19:00) at 23±1°C with water and food available *ad libitum*. Eight hours before the experiment, the animals were deprived of food with free access to water. All the following experiments were performed in a quiet room and each animal was used only once.

KM mice are the most commonly used outbred mouse line in China. It is believed that the original colony of KM mice in China was started from Swiss mice brought to Kunming, China. Due to their high disease resistance, good adaptive capacity, high breeding coefficient and good survival rate, KM mice has been widely utilized in pharmacological, toxicological, medicinal and biological research (Shang, Wei *et al*., 2009).

Measurement of anti-fatigue capacity

After a week adaptation, KM mice were randomly divided into five groups (n=20/group; equal numbers of male and female), and orally treated with double distilled (D.D.) water (Serving as control group), 0.6g/kg of *R. Rosea* (Serving as positive group) and ILE at doses of 0.04, 0.2 and 1.0g/kg once a day for seven days. At the end of drug administration, following experiments were performed.

Autonomic activity test: Mice were placed in a multi channel activity box (ZZ-6, Chengdu Taimeng Software Co. LTD, China) and allowed locomotor activities for 5 min. Due to its infrared sensor with multiple Fresnel lens, vertical movements such as jumping, as well as horizontal movements such as walking and running, were recorded. The experiments were performed between 12:00 and 16:00.

Weight-loaded forced swimming test: After seven-day ILE treatment, a weight-loaded forced swimming test was applied to evaluate the endurance and performance of each mouse. Mice loaded with 10% of their bodyweights swam in water with 22±1°C temperature and 30 cm depth. Exhaustion time was recorded from the beginning of swimming to the point at which mouse failed to return to the water surface within 15 s.

Forced running test: A treadmill (FT-200, Chengdu Taimeng Software Co. Ltd., China) was performed to test the endurance of mouse. Before the formal experiment, mice were allowed to run at a set speed of 20 mph for 1 min for practice and repeat for three times. And then, mice were putting on the treadmill with a set speed of 20 mph. During the experiment, once the mouse stopped running, they would be shocked by the electrode. The number of mice shocked within 5 min was used to evaluate their running performance.

Rotating rod test: A fatigue turning device (ZB-200, Chengdu Taimeng Software Co. Ltd., China) was used to test mouse performance after seven-day ILE administration. Before the formal test, three times exercise training at a speed of 20 rpm for 1 min was applied. During the fatigue analysis, mice were putting on the turning device at a speed of 20 rpm. The total duration on the rod was recorded.

Sample collection

KM mice (n=24/group; equal numbers of male and female) were orally treated with D.D. water (Serving as control group), 0.6g/kg of *R. Rosea* (Serving as positive control group) and ILE at doses of 0.04, 0.2 and 1.0g/kg once a day for seven days. Sixty min after last treatment, 12 mice (equal numbers of male and female) in each group were forced to swim for 30 min in water with 22±1°C temperature and 30cm depth. After 10-min recess, blood samples were collected from the caudal vein in swimming and non-swimming mice. At the end of the experiment, all mice were sacrificed. Liver tissues were quickly dissected, washed in the ice-cold physiological saline solution, and homogenated in D.D. water.

Parameters determination

The levels of ATP, SOD, and MDA in liver were determined according to the procedures provided by the related assay kits (Nanjing Biotechnology Co. Ltd., Nanjing, China). The levels of ACTH and CORT in serum

were measured using enzyme-linked immunosorbent assay (ELISA) according to the procedures provided by the related assay kits (Calbiotech, USA).

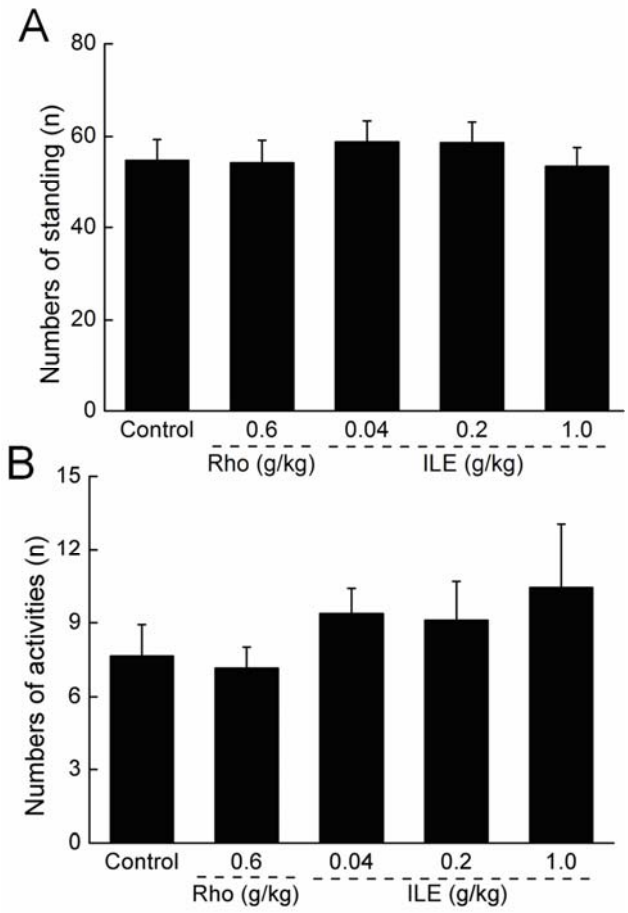

Fig. 1: The effects of ILE (0.04, 0.2 and 1.0g/kg) and *R. Rosea* (0.6 g/kg) on mouse spontaneous standing (A) and locomotor activities (B) were detected, respectively. Data are expressed as mean ± S.D. (n = 20) and analyzed using a one-way ANOVA followed by Dunn's test.

Western blot analysis

Liver tissue samples were homogenized with lysis buffer (Sigma-Aldrich, USA) containing 1mM of phenylmethanesulfonyl fluoride (PMSF) and 1X protease inhibitor cocktail (Sigma-Aldrich, USA). The homogenate was centrifuged at 12,000 rpm for 10 min and supernatants were used as the whole protein extract. Total protein was estimated using Bradford method. About 30 µg of protein was separated by 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (0.45µm, Bio Basic, Inc. USA). After blocking with 5% bovine serum albumin (BSA)/tris-buffered saline (TBS) at room temperature for 3 h, The membranes were then incubated overnight with the primary antibodies against phosphor (p)-AKT, total (t)-AKT, p-AMPK, t-AMPK, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Cell Signaling Technology, Beverly, MA) and subsequently incubated with peroxidase-conjugated secondary antibodies (1:2000, Santa Cruz, USA). Immunoreactive bands were visualized with an electrochemiluminescence (ECL) detection kits (GE Healthcare, UK). The intensity of the bands was quantified by scanning densitometry using software Image J (National Institutes of Health, USA).

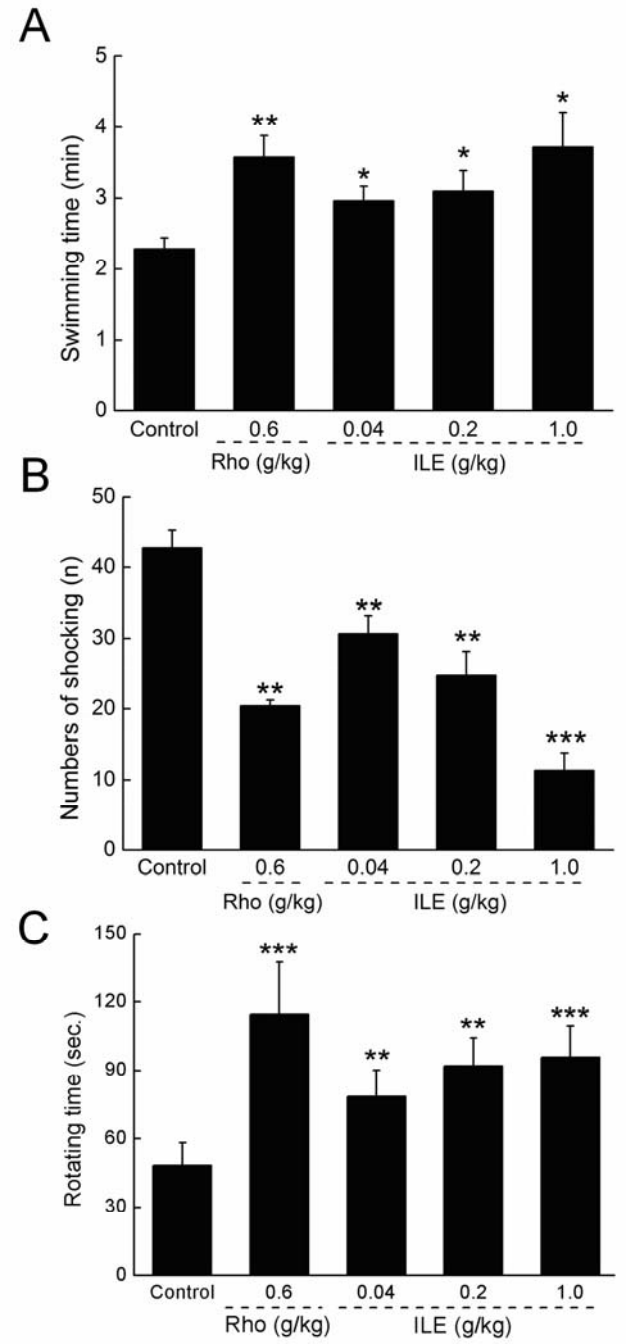

Fig. 2: The anti-fatigue activities of ILE (0.04, 0.2 and 1.0 g/kg) and *R. Rosea* (0.6 g/kg) were analyzed via forced swimming test (A), forced running test (B) and rotating rod test (C). Data are expressed as mean ± S.D. (n = 20) and analyzed using a one-way ANOVA followed by Dunn's test. **P*<0.05, ***P*<0.01 and ****P*<0.001 versus control group.

STATISTICAL ANALYSIS

All of the values were expressed as mean ± S.D. A one-way analysis of variance (ANOVA) was used to detect statistical significance followed by post-hoc multiple comparisons (Dunn's test) using SPSS 16.0 software (IBM Corporation, Armonk, USA). A *P*-value of less than 0.05 was considered to be statistically significant. Graphs were plotted with the OriginPro 8.5 software (OriginLab Corporation, USA).

RESULTS

The effects of ILE on autonomic activity

ILE showed no significant effects on mouse autonomic activities indicating that ILE is a safe agent for further experiment (*P*>0.05; fig. 1).

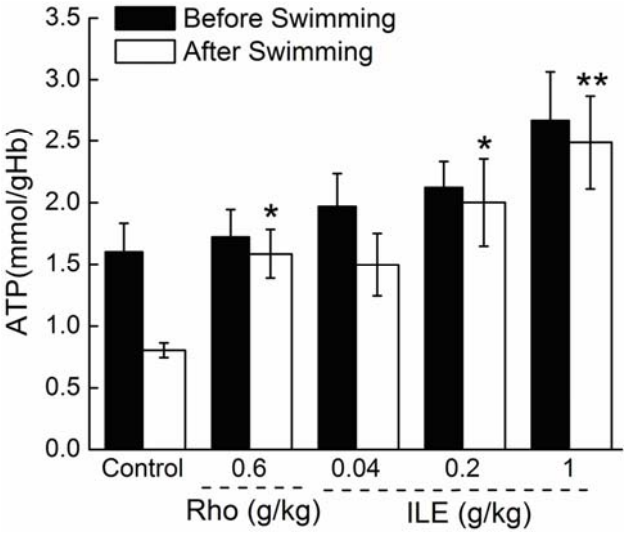

Fig. 3: After seven-day ILE (0.04, 0.2 and 1.0 g/kg) and *R. Rosea* (0.6g/kg) treatment, the effects on ATP concentration in liver was analyzed before and after 30-min swimming. Data are expressed as mean ± S.D. (n = 12) and analyzed using a one-way ANOVA followed by Dunn's test. **P*<0.05 and ***P*<0.01 versus control group.

The anti-fatigue activities of ILE

Behavior tests were always applied to evaluate the anti-fatigue performance of drugs (Kang *et al*., 2015, Mercer *et al*., 2003). Similar to *R. Rosea,* compared with non-treated group, ILE dose-dependently improved the residence time in forced swimming test (*P*<0.05; fig 2A). In forced running test, compared with non-treated mice, 0.04, 0.2 and 1.0 g/kg of ILE decreased nearly 28.2%, 42.2% and 73.4% shock times (*P*<0.01; fig. 2B). ILE at 1.0g/kg significantly enhanced rotating time with a maximum record of 95.8 s versus 48.5 s in control group (*P*<0.001, fig. 2C).

The effects of ILE on ATP, SOD and MDA level in the liver

ATP is a direct energy source for normal physiological activity. The shortage of ATP leads to a variety of physiological dysfunction, especially in skeletal muscle performance (Tan *et al*., 2014). Seven-day ILE treatment at doses of 0.2 and 1.0g/kg approximately increased 148.6% and 209.0% ATP concentration in the liver compared with that of control group after 30-min swimming (*P*<0.05, fig. 3). However, no significant enhancement of ATP level was observed before exercise (fig. 3). MDA is one of the end products result from degradation of cell membrane by radicals (Nallamuthu *et al*., 2014). 1.0g/kg of ILE resulted in 46.1% and 42.8% reduction on MDA concentration in the liver before and after swimming, respectively (*P*<0.05, fig. 4A). SOD protects cells via catalyzing the conversion of super oxide radicals to O_2 and H_2O_2. Compared with non-treated mice, 61.4% and 86.5% increment of SOD level in liver were noted in ILE-treated mice at doses of 0.2 and 1.0g/kg before exercise (*P*<0.001, fig. 4B). Moreover, 0.2 and 1.0g/kg of ILE enhanced 130.5% and 171.3% SOD level after exercise (*P*<0.001; fig. 4B). Compared between before and after swimming, among all groups, no significant changes were found on ATP, SOD, and MDA concentration in liver.

The effects of ILE on ATCH and CORT concentration in serum

HPA axis activates circulating ACTH and stimulates the release of CORT, helping the body to compete stress (Mariappan *et al*., 2013). Seven-day ILE treatment at 1.0g/kg resulted in 18.2% (before swimming) and 23.4% (after swimming) increment of serum ACTH concentration compared with control group (*P*<0.05; fig. 5A). Moreover, 0.2 and 1.0 g/kg of ILE enhanced serum CORT level significantly before and after exercise (*P*<0.01; fig. 5B).

The effects of ILE on the activation of p-AKT and p-AMPK

AMPK is a key cellular sensor of energy supply which is activated by the increased ratio of AMP/ATP (King *et al*., 2006). *R. Rosea* displayed no significant effects on the expressions of p-AKT and p-AMPK (fig. 6). ILE at a dose of 1.0g/kg enhanced nearly 85.6% and 45.9% phosphorylation of AKT and AMPK in liver compared with non-treated mice (*P*<0.05, fig. 6).

DISCUSSION

Toxicity assessment is the most important data for a new drug registration (Gao *et al*., 2013). In our separated experiment, an acute toxicity test in KM mouse was performed to exam the safety of ILE. After treatment with ILE at doses range from 0.5 to 5.0g/kg, no significant changes on general behavior, defecation, postural abnormalities, dietary amount and drinking amount in both male and female mice were noted during two-week monitor (data not shown).

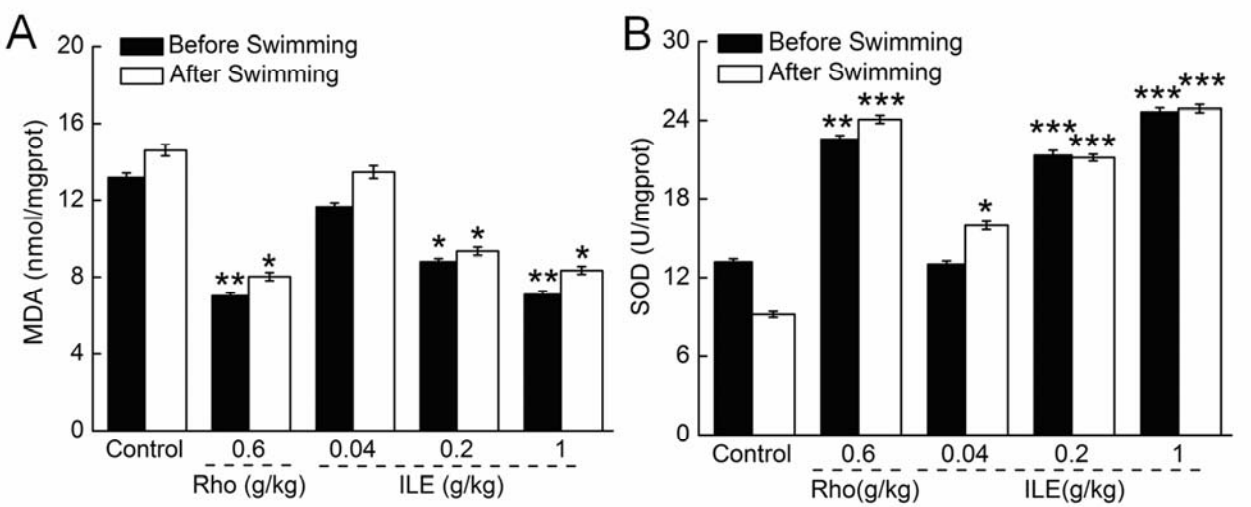

Fig. 4: Mice were treated with ILE (0.04, 0.2 and 1.0 g/kg) and *R. Rosea* (0.6 g/kg) for seven days, before and after 30-min swimming, the MDA (A) and SOD (B) levels in liver were determined respectively. Data are expressed as mean ± S.D. (n = 12) and analyzed using a one-way ANOVA followed by Dunn's test. * P<0.05, ** P<0.01 and ***P<0.001 versus control group.

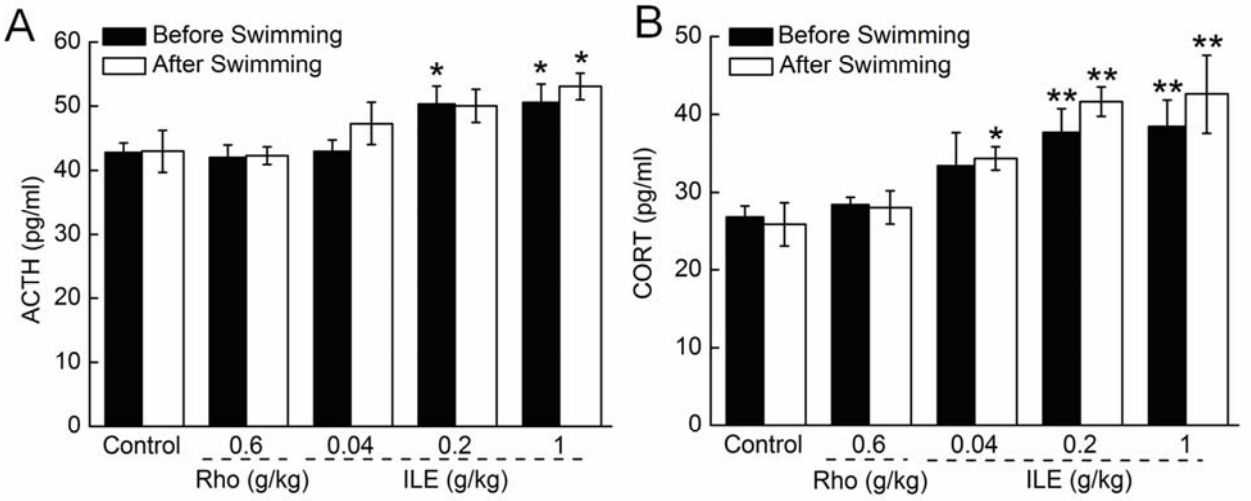

Fig. 5: Mice were treated with ILE (0.04, 0.2 and 1.0 g/kg) and *R. Rosea* (0.6 g/kg) for seven days, before and after 30-min swimming, the ACTH (A) and CORT (B) levels in serum were determined respectively. Data are expressed as mean ± S.D. (n = 12) and analyzed using a one-way ANOVA followed by Dunn's test. * P<0.05 and **P<0.01 versus control group.

Our present study successfully confirmed the anti-fatigue activity of polysaccharides-enriching *I. lacteus* water extract. The enhanced levels of ATP and SOD, and the reduced level of MDA in live were observed in ILE-treated mice after 30-min exercise. As reported, antioxidant enzymes play important roles in preventing oxidative injury in mouse (Virmani *et al*., 2002). Intense exercise causes an imbalance between the body's oxidation and anti-oxidation systems, thereby over-producing ROS, which contributes to the development chronic disease and aging (Ma, 2014)**.** Enzymatic antioxidant system, such as GPx and SOD, is important in scavenging free radicals (Qi *et al*., 2014). Oral treatment with antioxidants successfully prevent or reduce oxidative stress, decrease muscle damage, and improve exercise performance (Peternelj and Coombes, 2011). Due to suppressing skeletal muscle oxidative stress, GRb1 efficiently enhanced mice anti-fatigue endurance (Lortz *et al*., 2013). Polysaccharides separated from *Panax ginseng* improve behavioural alterations associated with chronic fatigue via inhibition oxidative damage (Huang *et al*., 2014)**.** *I. lacteus* produces simultaneously lignin peroxidase, Mn-dependent per oxidase, nonspecific per oxidase, and laccase; therefore, displays significant anti-oxidative effects (Novotny *et al*., 2009). The results obtained here indicated that the anti-fatigue effects of ILE probably occurs through preventing lipid oxidation and further protecting cells from oxidative injury via modifying activities of the oxidative system and related factors.

ACTH is produced in response to biological stress, which influences CORT production and release (Ehlert *et al*., 2001). The high stress hormone levels are characteristic of patients with chronic fatigue disorders (Gottschalk *et al*., 2005). Recently, *Hovenia Dulcis* extract is reported to show a significant anti-fatigue activity via the inhibition of stress hormone levels (Na *et al*., 2013). Additionally, via activating AMPK, adiponectin stimulates ACTH secretion in pituitary corticotroph cells (Chen *et al*., 2014). Although the enhancement effects of ILE on the serum levels of ATCH and CORT were monitored during the experiments, we failed to conclude the relationship between them and antifatigue activities.

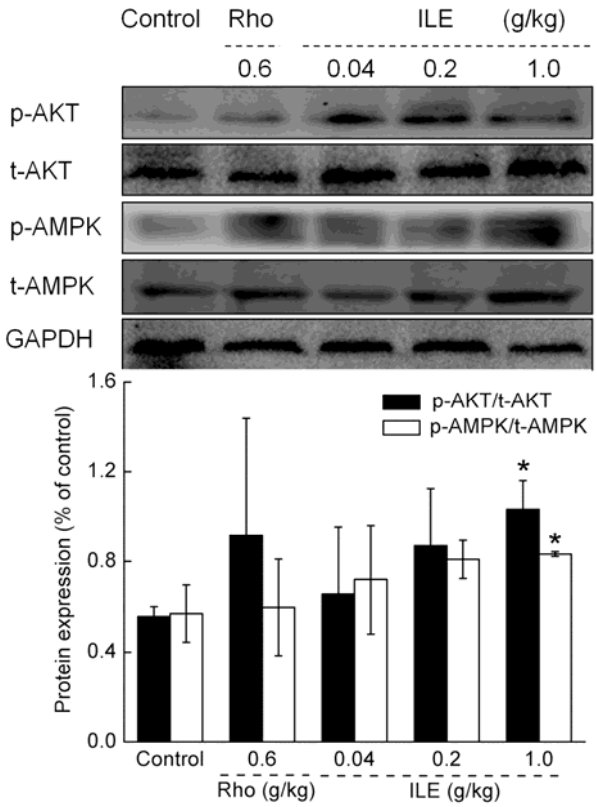

Fig. 6: Mice were treated with ILE (0.04, 0.2 and 1.0 g/kg) and *R. Rosea* (0.6g/kg) for seven days, after 30-min swimming, the levels of p-AKT and p-AMPK in liver tissue were analyzed via western blot. Quantification data of the expressions of p-AKT and p-AMPK were normalized by corresponding t-AKT and t-AMPK. Data are expressed as mean ± S.D. (n=6) and analyzed using a one-way ANOVA followed by Dunn's test. **P*<0.05 versus control group.

After 30-min swimming, the improved activities of AMPK in the liver were observed in ILE-treated mice. AMPK is a metabolic survival factor which can be effectively antagonized by high concentration of ATP (Hardie and Sakamoto, 2006). AMPK activation helps to maintain ATP level under conditions of shortage of energy sources (Ceddia, 2013). During exercises, AMPK activation prevents cells from overproducing and accumulating ROS in mitochondria (Shin and Kim, 2009) which further leads to fatigue. In skeletal muscle and liver, the activation of AMPK increases glycogen breakdown, glucose uptake, and fatty acid oxidation (Hardie and Sakamoto, 2006). Altogether, the activation of AMPK plays a central role in ILE-mediate anti-fatigue effect.

Additionally, the enhanced phosphorylation of AKT was noted in ILE-treated mice. AKT is sensitive to regulate growth factors and energy metabolism (Wullschleger *et al*., 2006). During exercise, to promote protein synthesis and translation, AKT signaling is activated (Bolster *et al*., 2003). However, as reported, drugs widely used to activate AMPK always caused dephosphorylation of AKT (King *et al*., 2006). Based on our present data, whether AKT signaling is involved in ILE's anti-fatigue activities is unascertainable. Since a large number of complex mechanisms are involved in the exercise-induced fatigue, the molecular pharmacology mechanisms of ILE-enhanced fatigue endurance still needs further investigation.

CONCLUSION

Altogether, our results indicate that ILE elevates the endurance capacity at least partially via activating AMPK-linked antioxidant pathway, which provides experimental evidence in supporting the clinical use of ILE as an effective agent against fatigue. Further pharmacological research and identification of the active constituents in ILE will be required.

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