doi.org/10.36721/PJPS.2024.37.2.REG.321-326.1

Experimental study on the antifatigue effect of icariin

Mosi Chen[1], Qi Li[2], Chenglan Su[1], Yuping Chen[1], Li Dai[1] and Genlin Chen[1]*

[1]School of Basic Medical Sciences, Jiangsu Vocational College of Medicine, Yancheng, Jiangsu, China
[2]Department of Neurosurgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

Abstract: Fatigue is a serious disturbance to human health, especially in people who have a severe disease such as cancer, or have been infected with COVID-19. Our research objective is to evaluate the anti-fatigue effect and mechanism of icariin through a mouse experimental model. Mice were treated with icariin for 30 days and anti-fatigue effects were evaluated by the weight-bearing swimming test, serum urea nitrogen test, lactic acid accumulation and clearance test in blood and the amount of liver glycogen. The protein expression levels of adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC1-α) in the skeletal muscle of mice in each group were measured by western blotting. Results showed that icariin prolonged the weight-bearing swimming time of animals, reduced the serum urea nitrogen level after exercise, decreased the blood lactic acid concentration after exercise and increased the liver glycogen content observably. Compared to that in the control group, icariin upregulated AMPK and PGC1-α expression in skeletal muscle. Icariin can improve fatigue resistance in mice and its mechanism may be through improving the AMPK/PGC-1α pathway in skeletal muscle to enhance energy synthesis, decreasing the accumulation of metabolites and slowing glycogen consumption and decomposition.

Keywords: Icariin, antifatigue, AMPK, PGC1-α.

INTRODUCTION

Fatigue, which can be objectively observed as a decrease in physical strength or muscle endurance, commonly occurs following intense physical activity or overexertion. Additionally, certain illnesses may also cause fatigue and chronic fatigue syndrome is an enigmatic condition that can result in feelings of exhaustion and other related symptoms. Notably, not all individuals who experience unexplained fatigue necessarily suffer from chronic fatigue syndrome. Fatigue is considered to be one of the most common long-term complaints in individuals who have previously been infected with COVID-19 and recent research (Joli *et al.*, 2022) has demonstrated that COVID-19 patients may experience persistent fatigue for weeks or even months, a condition known as "long COVID" or "chronic course COVID", which shares similarities with chronic fatigue syndrome. Some clinically used herbal formulations have been shown to improve breathlessness and fatigue resulting frompost-COVID-19 fatigue (Pang *et al.*, 2022), suggesting that we can search for anti-fatigue functional active substances from natural products. Therefore, the development of safe and effective anti-fatigue medications holds significant potential for widespread application.

Epimedium is the dry leaves of Berberis [*Epimedium brevicornu* Maxim.], it is a traditional Chinese medicine with anti-fatigue effects (Yu *et al.*, 2023), but the specific mechanismis still unclear. Icariin is one of the effective components of Epimedium (shown in fig. 1). The objective of present research was to verify the anti-fatigue efficacy of icariin and investigate the mechanism by which icariin improves physical fatigue in mouse experimental models.

The body primarily relies on glycolysis to generate energy during exercise. Prolonged physical activity can lead to fatigue, which may result from a depletion of liver glycogen stores and a reduction in the body's capacity for movement. The accumulation of metabolite lactic acid also aggravates the exercise fatigue in the process of glycolysis. As exercise intensity increases, protein catabolism and metabolic waste production increase, leading to elevated blood urea levels during fatigue. Depletion of energy substrates (liver glycogen), buildup of metabolites and excessive protein consumption are key mechanisms underlying fatigue. The external manifestation of fatigue can be revealed by exhaustion time and the internal manifestation can be measured by the content of liver glycogen, blood lactic acid and urea nitrogen.

Adenosine monophosphate-activated protein kinase (AMPK) in skeletal muscle cells is hypersensitive to energy change and conversion and AMPK can activate the synthesis effect of mitochondria through phosphorylation (Morales-Alamo *et al.*, 2016). Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC1-α) plays a vital role in mitochondrial biosynthesis, lipid and glucose metabolism (Kang *et al.*, 2016). Studies have found that the transcriptional genes regulated by AMPK and PGC1-α are

*Corresponding author: e-mail: jsmc_cgl@163.com

mostly the same. As the upstream regulator of PGC1-α, AMPK can directly phosphorylate PGC1-α, which enhances the transcriptional activity of PGC1-α, promotes the synthesis of mitochondria and provides energy for tissue cells (Yu *et al.*, 2018, Kim *et al.*, 2018, Zhang *et al.*, 2019). Relevant studies have shown that AMPK can be used as a target center to explain the potential molecular mechanism of anti-exercise fatigue through signaling pathways correlated with the regulation of mitochondrial biosynthesis, cellular oxidative stress adaptation, metabolic substrate utilization and changes in muscle fiber types.

MATERIALS AND METHODS

Experimental materials

Icariin was prepared in the laboratory *and* its control product was purchased from the China Institute for Food and Drug Control. Assay kits for liver glycogen, serum urea nitrogen and lactic acid were provided by Nanjing Jiancheng Bioengineering Institute. A total of 160 SPF BALB/C adult male mice were used, weighing 18-23 g *and* were provided by Hubei Experimental Animal Research Center. The animal experiments fully met ethical requirements.

Preparation and administration of Icariin

Epimedium glycoside extraction technology conditions: Epimedium dry leaves at 15 times the amount of 60% ethanol solution reflux extraction twice every 2 hours, the epimedium extract on macroporous adsorption resin column type (DM130 macroporous adsorption resin), with purified water, 20% ethanol elution in turn after impurities, with 50% ethanol elution epimedium glycoside. After purification by macroporous resin, the elution rate of icariin was above 90%. The yellow-brown powder was obtained by recrystallization with ethyl acetate and 60% ethanol. The content of icariin was over 92%, as determined by HPLC.

Icariin was dissolved in 0.5% sodium carboxymethyl cellulose. Experimental animals were randomly divided into 4 groups by weight: a control group and icariin low/medium/high-dose groups (30, 100 and 300 mg/kg), dosages for animal were converted from the clinical dosage for human (Yong *et al.*, 2021). Test substances were given by oral gavage. Each group was given oral gavage with 0.2 ml/10 g bw for 30 days.

Weight-bearing swimming test

Forty adult male BALB/C mice were preliminarily examined and divided into 4 groups as described above, with 10 mice in each group. Mice in the control group were given normal saline and the experimental groups were given icariin in different dosages. The weight of the mice was measured every 5 days before and after administration. Thirty minutes after the last intragastric injection, the tail root of the mouse was loaded with 5% of the body weight of lead skin and the animals were carefully put into the box for swimming (water depth of 40 cm and temperature of 25±1°C). The time from swimming to death, due to exhaustion, was recorded.

Serum urea nitrogen test

Mice were continuously given icariin, except for the control group (a total of 40 mice, grouped as above) for 30 days. Thirty minutes after the last intragastric administration, the orbital blood of each group of mice was taken after swimming in a 30°C water tank for 20 minutes. The serum was separated and urea nitrogen was determined by an automatic biochemical analyzer according to the kit instructions.

Blood lactic acid accumulation and clearance test

Mice were continuously given icariin, except for the control group, (a total of 40 mice, grouped as above) for 30 days and orbital blood was collected for the first time 30 min after the last oral gavage. After blood collection, mice were put into a 30±1°C water tank swam for 10 min and were immediately removed and wiped dry for the second blood collection. After swimming, the mice were placed in a quiet state and rested for 20 min and the orbital blood was collected again for the third time. Blood lactic acid levels of mice were measured before swimming, 10 min after swimming and 20 min after rest precisely according to the kit instructions.

Detection of liver glycogen and expression of AMPK/PGC1-α protein

Mice were continuously given icariin, except for the control group, (a total of 40 mice, grouped as above) for 30 days and then sacrificed 30 min after the last administering oral medications. The liver was rinsed with normal saline and dried with filter papers, 75 mg was accurately weighed and the content of liver glycogen was determined by anthracone colorimetry according to the kit instructions.

The expression of AMPK/PGC1-α in skeletal muscle tissue was detected by western blot: approximately 50 mg of muscle tissue was taken from the quadriceps muscle area and placed in a centrifuge tube and 1000 μL of RIPA fission buffer and 10 μL of benzoyl sulfonyl fluoride (PMSF) were added to extract total protein from skeletal muscle tissue of mice in each group. After the total protein concentration was determined by the BCA method, the sample amount was calculated. Target proteins were transferred to PVDF membranes after electrophoresis and five percent skim milk powder solution was incubated at room temperature for one hour. After washing with PBS, AMPK (1:5000) and PGC1-α (1:5000) were added. The β-actin (1:5000) phase corresponding to the primary antibody solution was incubated overnight at 4℃. After removal, TBST was

used to wash the membrane three times for 15 min each time and the corresponding secondary antibody (1:5000) was added. The film was shaken for 1 h and TBST was used to wash the film three times for 15 min each time. An ECL chemical luminous solution was added, the appropriate strip exposure was selected and an imaging system was used to develop read-out bands for analysis.

Ethical approval

This study was allowed by the Laboratory Animal Ethics Committee of Huazhong University of Science and Technology (approval No. 2021459/TJ).

STATISTICAL ANALYSIS

The results of western blot were quantified by Image-Pro Plus 6.0 software, and data were analysed by IBM SPSS Statistics 26. Statistical comparison of data was performed by one-way analysis of variance (ANOVA) when the data conformed to a normal distribution *and* the Wilcoxon rank sum test was used when data did not comply with a normal distribution. The results of data were presented as the mean±SEM, $p<0.05$ was considered significant.

RESULTS

The effects of drugs on weight

Each group of mice gained weight over the course of the experiment, but no significant difference was observed in body weight of animals between groups during the same period (fig. 2), indicating that the treatment had no significant effect on the growth of mice.

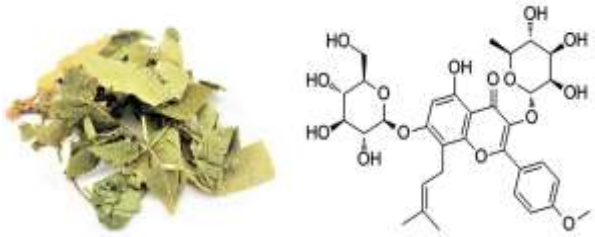

Fig. 1: Epimedium and the structure of Icariin.

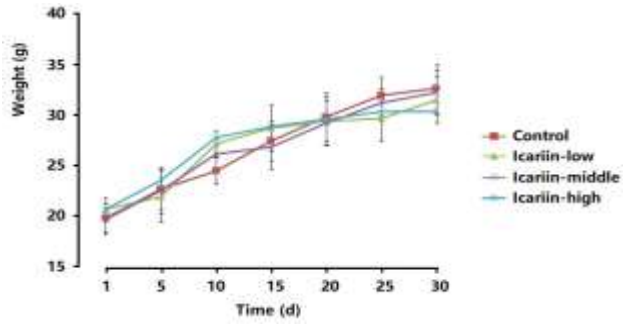

Fig. 2: The effect of icariin on the growth and weight of mice ($\overline{X}$ ±SEM).

Weight-bearing swimming test

After the experiment, results of the mouse weight-bearing swimming test showed that compared with the control group, the swimming time of mice in icariin groups were significantly increased *and* proportional to the dose, indicating the test object could enhance the ability to keep moving and delay motor exhaustion, as shown in fig. 3.

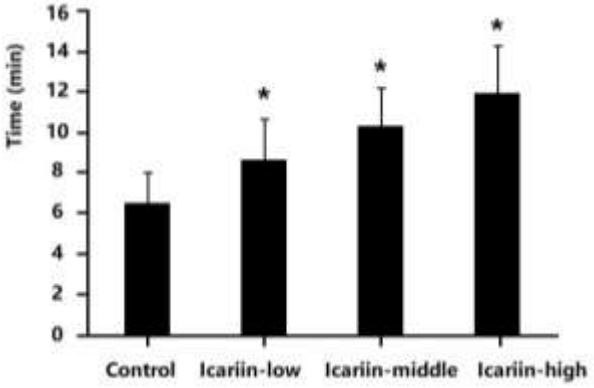

Fig. 3: The effects of icariin on the weight-bearing swimming test ($\overline{X}$ ±SEM), n=10, *p<0.05 compared with the control group.

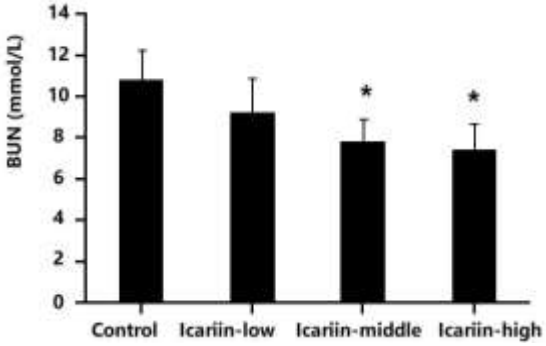

Fig. 4: Results of the serum urea nitrogen test ($\overline{X}$ ±SEM), n=10, *p<0.05 compared with the control group.

Serum urea nitrogen test

The serum urea nitrogen (BUN) of mice after swimming exercise showed that BUN levels of mice in the middle-dose and high-dose icariin groups were lower than that in the normal control group *and* the difference in results was significant. The results indicated that icariin could reduce the BUN level of mice after exercise, as shown in fig. 4.

Blood lactic acid accumulation and clearance test

The results of the blood lactic acid accumulation and clearance test showed that the lactic acid level in blood increased significantly after exercise *and* decreased rapidly after 20 min of rest. The area under the blood lactic acid curve of mice in the medium- and high-dose icariin groups after exercise was significantly lower than that in the control group, indicating icariin could reduce the production of lactic acid, a product of hypoxia after exercise. Datas were shown in fig. 5.

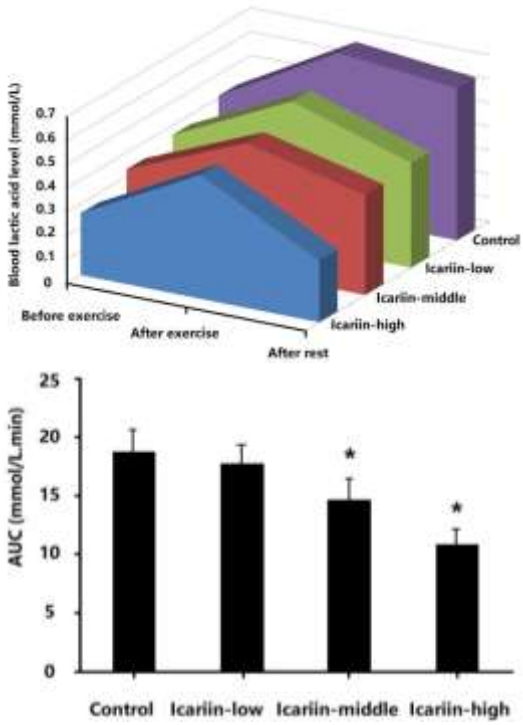

Fig. 5: Effect of icariin on blood lactic acid levels in mice before and after exercise ($\overline{X}$ ±SEM, mmol/L), n=10, *p<0.05 compared with the control group.

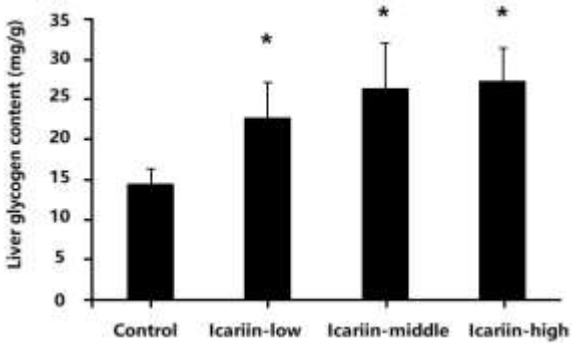

Fig. 6: Effect of icariin on the liver glycogen content of mice ($\overline{X}$ ±SEM), n=10, *p<0.05 compared with the control group.

Detection of liver glycogen

The data results of the liver glycogen test showed contents of liver glycogen were significantly increased in icariin groups at all doses compared with that in the control group, indicating icariin had the ability to increase the content of liver glycogen in mice, as shown in fig. 6.

Expression of AMPK/PGC1-α protein

Compared with mice in the control group, the expression of AMPK and PGC1-α in the skeletal muscle of mice in the icariin groups were significantly increased, as shown in fig. 7.

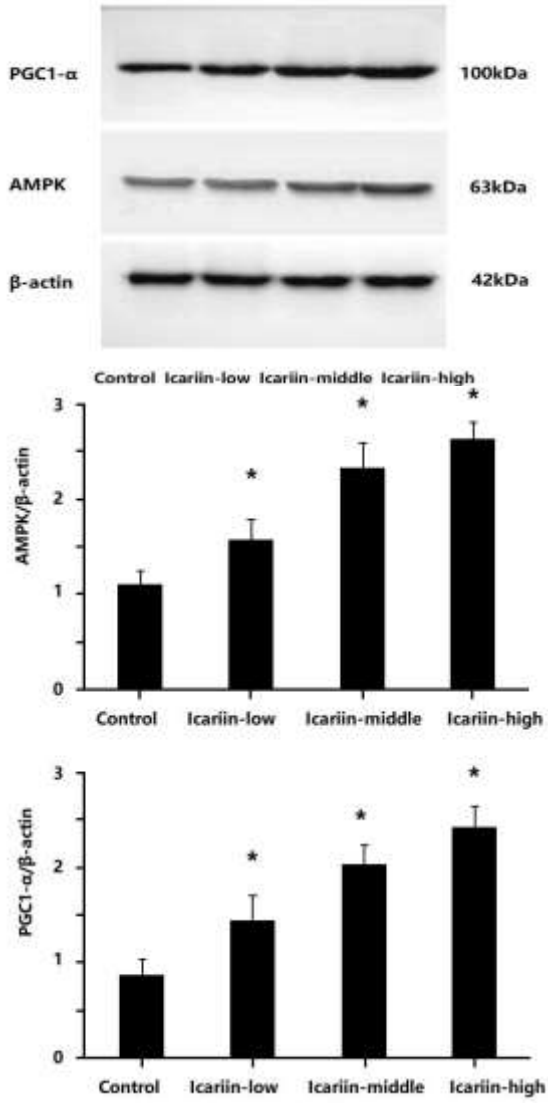

Fig. 7: Effect of icariin on skeletal muscle AMPK and PGC1-α protein expression in mice with exercise-induced fatigue ($\overline{X}$ ±SEM, n=10), *p<0.05 compared with the control group.

DISCUSSION

Although fatigue is common with excessive continuous exercise or certain disease states, there is currently no clear mechanism and lack of regulated treatment at present, but some anti-fatigue health supplements are popular, due to multi-pathway characteristics of active ingredients in natural product, which show unique advantages in the treatment of fatigue. Delaying the occurrence of exercise fatigue can be reflected in increased exercise endurance and better adaptability to energy metabolic stress and oxidative stress. AMPK is an important molecular target for regulating energy metabolism, involved in a variety of energy substrate metabolic pathways and associated with energy overconsumption causing fatigue, including glucose

utilization, fatty acid oxidation promotion, mitochondrial biosynthesis and even muscle fiber type conversion. AMPK participates in the regulation of cellular oxidative stress pathways, thus it also plays a significant role in the regulation of exercise fatigue.

The molecular mechanism underlying anti-exercise fatigue may be that AMPK phosphorylates GTPase, activates Tre-2/BUB2/cdc1 domain family 1(TBC1D1), promotes the fusion of protein glucose transporter type 4 (GLUT4) vesicles with the cell membrane, transports extracellular glucose molecules into the cell *and* further oxidizes metabolic capacity or glycogen synthesis in the cell (Hardie *et al.*, 2012). It was found that (Lee-Yong *et al.*, 2007) the oxygen utilization capacity and exercise capacity of overexpressed dominant inactivated mutant AMPK mice were significantly weakened compared with those of wild-type mice. AMPKα2 knockout mice showed a 27% reduction in the maximum allowable running speed and decreased fatty acid oxidation capacity.

The activation of AMPKα2 increased the expression of pyruvate dehydrogenase kinase 4 (PDK4) and inhibited the activity of pyruvate dehydrogenase (PDH). Therefore, the glucose oxidation rate is limited, while the increased intake of fatty acids by mitochondria and the oxidation and elimination capacity of fatty acids are conducive to glycogen synthesis and speed up the elimination of fatigue (Fritzen *et al.*, 2015).

It has been shown that increased expression of PGC-1α in skeletal muscle can increase muscle weight and promote lactic acid metabolism. To improve exercise ability, the molecular mechanisms involved include the CaMKK-AMPK-PGC1α and NO-CREB-PGC-1α signaling pathways (Matsukawa *et al.*, 2015, Villareal *et al.*, 2018). Under the same exercise intensity, intermittent exercise-induced recurrent metabolic fluctuations were more conducive to enhancing the phosphorylation of AMPK, CaMKII and p38 mitogen-activated protein kinases (P38-MapK) than sustained exercise. Thus, the expression of PGC-1α in skeletal muscle is enhanced, the biosynthesis of mitochondria is promoted *and* the adaptive response of cells to oxidative stress is regulated (Combes *et al.*, 2015).

It was confirmed (Zimmermann *et al.*, 2015) that AMPK activation enhances the Nrf2/heme oxygenase 1 (HO-1) signaling pathway in mouse embryonic fibroblasts by xanthohumol, an activator of AMPK and Nrf2. This indicates that there is a close cooperative relationship between the cellular redox signaling pathway and the energy metabolism signaling pathway. Furthermore, AMPK drives expression of antioxidant enzyme-encoding genes directly and indirectly by regulating the combination of Nrf2 and antioxidant response element (ARE). Then, the cellular oxidative stress balance is regulated (Joo *et al.*, 2016).

In addition, the proportion of muscle fibers in the muscles has a great effect on athletic endurance. AMPK regulates the activity of activated PGC-1α, which can promote the transformation of muscle fibers into type I and type IIa fibers beneficial to improving exercise tolerance (Wen *et al.*, 2020). Therefore, AMPK can be used as a target center to elucidate the potential molecular mechanisms of anti-exercise fatigue from above aspects. The experimental results showed administration of icariin could significantly increase the expression of AMPK and PGC1-α in mice compared with control group, indicating that icariin could improve the function of skeletal muscle mitochondria, promote energy generation in skeletal muscle and delay fatigue.

Fatigue has become a symptom that cannot be ignored especially in people who have a severe disease, such as cancer, or have been infected with COVID-19 (Fox *et al.*, 2020, Thong *et al.*, 2020), but there is a lack of clinical medication recommendations or definitive guides for various fatigue classifications. Many natural products, including herbs, have been used to relieve fatigue as nutritional supplements or clinical prescriptions, which may be attributed to the complex chemical ingredients and action mechanisms (Yu *et al.*, 2023). It is very promising to study the antifatigue effect and related mechanisms to develop safe and effective new drugs. Icariin may have potential efficacy in the treatment of COVID-19 (Khezri *et al.*, 2022), so it is worth further development.

CONCLUSION

In summary, icariin can enhance the fatigue resistance of mice through multiple mechanisms. First, icariin increases liver glycogen content and thus provides more energy reserves prior to exercise. Second, it enhances mitochondrial oxidative phosphorylation by activating the skeletal muscle AMPK/PGC1-α pathway, thereby promoting energy synthesis during exercise. Third, icariin improves metabolism and reduces the accumulation of metabolites such as urea nitrogen and lactic acid. Therefore, it has the potential to be further developed into new drugs to relieve fatigue symptoms, such as COVID-19 infection.

REFERENCES

Combes A, Dekerle J, Webborn N, Watt P, Bougault V and Daussin FN (2015). Exercise-induced metabolic fluctuations influence AMPK, p38-MAPK and CaMKII phosphorylation in human skeletal muscle. *Physiol. Rep*., **3**(9): e12462.

Fox RS, Ancoli-Israel S, Roesch SC, Merz EL, Mills SD, Wells KJ, Sadler GR and Malcarne VL (2020). Sleep disturbance and cancer-related fatigue symptom cluster in breast cancer patients undergoing chemotherapy. *Support Care Cancer*, **28**(2): 845-855.

Fritzen AM, Lundsgaard AM, Jeppesen J, Christiansen ML, Biensø R, Dyck JR, Pilegaard H and Kiens B (2015). 5'-AMP activated protein kinase α2 controls substrate metabolism during post-exercise recovery via regulation of pyruvate dehydrogenase kinase 4. *J. Physiol*., **593(**21): 4765-4780.

Hardie DG, Ross FA and Hawley SA (2012). AMPK: A nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol*., **13**(4): 251-262.

Joli J, Buck P, Zipfel S and Stengel A (2022). Post-COVID-19 fatigue: A systematic review. *Front Psychiatry*, **11**(8): 947973.

Joo MS, Kim WD, Lee KY, Kim JH, Koo JH and Kim SG (2016). AMPK Facilitates Nuclear Accumulation of Nrf2 by Phosphorylating at Serine 550. *Mol. Cell Biol*., **36**(14): 1931-1942.

Kang C and Ji LL (2016). PGC-1α overexpression via local transfection attenuates mitophagy pathway in muscle disuse atrophy. *Free Radic. Biol. Med*., **93**(4): 32-40.

Khezri MR, Nazari-Khanamiri F, Mohammadi T, Moloodsouri D and Ghasemnejad-Berenji M (2022). Potential effects of icariin, the Epimedium-derived bioactive compound in the treatment of COVID-19: A hypothesis. *NaunynSchmiedebergs Arch. Pharmacol*., **395**(9): 1019-1027.

Kim MB, Kim T, Kim C and Hwang JK (2018). Standardized kaempferia parviflora extract enhances exercise performance through activation of mitochondrial biogenesis. *J. Med. Food*, **21**(1): 30-38.

Lee-Yong RS, Ayala JE and Birnbaum MJ (2007). Coupling of oxidative metabolism to muscular work in healthy and insulin resistant conscious mice: Role of AMPK signaling. *DiabetesPro*., **56**(Suppl 1): A63.

Matsukawa T, Motojima H, Sato Y, Takahashi S, Villareal MO and Isoda H (2017). Upregulation of skeletal muscle PGC-1α through the elevation of cyclic AMP levels by Cyanidin-3-glucoside enhances exercise performance. *Sci. Rep*., **20**(7): 44799.

Morales-Alamo D and Calbet JAL (2016). AMPK signaling in skeletal muscle during exercise: Role of reactive oxygen and nitrogen species. *Free Radic. Biol. Med*., **98**(9): 68-77.

Pang W, Yang F, Zhao Y, Dai E, Feng J, Huang Y, Guo Y, Zhou S, Huang M, Zheng W, Ma J, Li H, Li Q, Hou L, Zhang S, Wang H, Liu Q, Zhang B and Zhang J (2022). QingjinYiqi granules for post-COVID-19 condition: A randomized clinical trial. *J. Evid. Based Med*., **15**(1): 30-38.

Thong MSY, van Noorden CJF, Steindorf K and Arndt V (2020). Cancer-related fatigue: Causes and current treatment options. *Curr. Treat Options Oncol*., **21**(2): 17.

Villareal MO, Matsukawa T and Isoda H (2018). L-citrulline supplementation-increased skeletal muscle PGC-1α expression is associated with exercise performance and increased skeletal muscle weight. *Mol. Nutr. Food Res*., **62**(14): e1701043.

Wen W, Chen X, Huang Z, Chen D, Chen H, Luo Y, He J, Zheng P, Yu J and Yu B (2020). Resveratrol regulates muscle fiber type conversion via miR-22-3p and AMPK/SIRT1/PGC-1α pathway. *J. NutrBiochem*., **77**(3): 108297.

Yong EL, Cheong WF, Huang Z, Thu WPP, Cazenave-Gassiot A, Seng KY and Logan S (2021). Randomized, double-blind, placebo-controlled trial to examine the safety, pharmacokinetics and effects of epimedium prenylflavonoids, on bone specific alkaline phosphatase and the osteoclast adaptor protein TRAF6 in post-menopausal women. *Phytomedicine,* **91**(10): 153680.

Yu W, Song C, Lei Z, Li Y, He X, Yu J and Yang X (2023). Anti-fatigue effect of traditional Chinese medicines: A review. *Saudi Pharm. J*., **31**(4): 597-604.

Yu Y, Zhao Y, Teng F, Li J, Guan Y, Xu J, Lv X, Guan F, Zhang M and Chen L (2018). Berberine improves cognitive deficiency and muscular dysfunction via activation of the AMPK/SIRT1/PGC-1a pathway in skeletal muscle from naturally aging rats. *J. Nutr. Health Aging,* **22**(6): 710-717.

Zhang Q, Liang XC (2019). Effects of mitochondrial dysfunction via AMPK/PGC-1 α signal pathway on pathogenic mechanism of diabetic peripheral neuropathy and the protective effects of Chinese medicine. *Chin. J. Integr. Med*., **25**(5): 386-394.

Zimmermann K, Baldinger J, Mayerhofer B, Atanasov AG, Dirsch VM and Heiss EH (2015). Activated AMPK boosts the Nrf2/HO-1 signaling axis – A role for the unfolded protein response. *Free Radic. Biol. Med*., **88**(Pt B): 417-426.