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The effect of Miya on skeletal muscle changes by regulating gut microbiota in rats with osteoarthritis through AMPK pathway

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Abstract

Background The study aimed to explore whether Miya (MY), a kind of *Clostridium butyricum*, regulated osteoarthritis (OA) progression through adenosine 5′-monophosphate-activated protein kinase (AMPK) pathway.

Methods The OA rats were orally given MY daily for 4 weeks and were intramuscularly injected with AMPK inhibitor once a week for 4 weeks. Hematoxylin eosin (HE) staining was used to observe the histological morphology of the knee joint. The levels of succinate dehydrogenase (SDH) and muscle glycogen (MG) in the tibia muscle of rats were detected by the corresponding kits, as well as the expression of related genes/proteins were assessed by real-time quantitative PCR (RT-qPCR) and western blot.

Results HE staining suggested that MY suppressed the symptoms of OA, which was abolished by AMPK inhibitor. Furthermore, the SDH and MG contents in the OA + MY + AMPK inhibitor group were lower than in the OA + MY group. At last, the levels of *AMPK*, *PI3K*, *AKT1*, *Ldh*, *Myod*, *Chrna1*, and *Chrnd* were notably decreased after AMPK inhibitor treatment, while the levels of *Lcad* and *Mcad* were up-regulated by AMPK inhibitor. Furthermore, their protein expression levels detected by western blot were consistent with those from RT-qPCR.

Conclusion MY may partially regulate skeletal muscle changes and prevente OA development through the AMPK pathway.

Keywords Miya, Osteoarthritis, AMPK, Gut microbiota

Background

Osteoarthritis (OA) is a chronic degenerative disease of bone and joint [1]. The main pathological features of OA are articular cartilage damage, subchondral bone change, osteophyte formation and synovial inflammation of joint [2]. The primary causes of impairment in the aged are joint pain, swelling, stiffness, and loss of function, which are the clinical signs of OA [3]. As life expectancy increases and populations age globally, the prevalence and incidence of OA are increasing [4]. It is estimated that 30.8 million adults in the United States, and 300 million people worldwide suffer from OA [2], creating a significant economic burden in terms of medical costs, lost wages, and lost economic productivity [5]. At present, the main clinical treatment of OA is oral drugs, intra-articular drug injection and surgical treatment [6]. Non-steroidal anti-inflammatory drugs (NSAID), corticosteroids, and opioids can be used to improve joint

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function and delay the progression of OA through anti-inflammatory and pain-relieving effects [7]. However, these drugs are easy to cause gastrointestinal tract, cardiovascular reactions, liver and kidney function damage and other adverse reactions, which have certain limitations on clinical application [8]. Due to the unknown pathophysiology of OA, most treatment methods only relieve symptoms such as pain. Therefore, it is crucial to study the pathophysiology and management of OA.

Gut microbiota (GM) is a complex ecosystem in the body, which affects many physiological processes and is also the pathophysiological basis of many diseases [9]. Recent research has found that the imbalance of GM may be an important environmental factor in the development of OA [10]. The relationship between GM and OA provides a new perspective for further study of the complex pathogenesis of OA. For example, the study of Schott EM et al. provided direct evidence for the association of GM with OA [11], and found that probiotics such as *Bifidobacterium* (*Bifidobacterium pseudolongum*) decreased in the intestines of obesity-related OA mice, and bacteria with pro-inflammatory effects increased [11]. By increasing probiotics such as *Bifidobacterium* (*Bifidobacterium lactis, Bifidobacterium pseudolongum, Bifidobacterium longum, and Bifidobacterium bifidum*) [12] in the intestinal tract of mice, inflammation in the colon and knee joints was reduced, and the development of OA could be inhibited. Probiotics are effective dietary supplements, which are beneficial to maintaining microecological balance, and are the focus of research on microecological therapy for OA [13]. Miya (MY) is a kind of *Clostridium butyricum* Tablets that are reported to be a probiotic for use in humans and livestock, and an anaerobic bacillus with strong acid resistance and corrosion resistance [14]. It can multiply in the intestine to promote the growth of intestinal probiotics such as *Bifidobacterium longum*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, as well as inhibit the reproduction of harmful bacteria. Previous studies have shown that MY promotes mucosal repair, maintains barrier integrity, and restores intestinal function [15, 16]. Besides, MY can be used as an antidiarrheal. It has been reported that MY is beneficial for the neuroprotection of mental health issues, non-alcoholic fatty liver disease, and stomach ulcers [17–19]. In our previous study, we found that MY could enhance the abundance of beneficial bacteria (Lactobacillus, Oscillospira, Clostridium, and Coprococcus), while decline the abundance of pathogenic microorganism (Prevotella, Ruminococcus, Desulfovibrio, and Helicobacter) in gut, as well as regulate energy metabolism-related genes, myogenesis-associated genes, neuromuscular junctions (NMJ)-related genes and interleukin-1β (IL-1β) in muscle, consequently promoting the joint damage repair and protecting OA via the "gut-muscle-joint" axis [20]. However, whether MY is involved in OA progression through other signaling pathways needs to be further explored.

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is a key regulator of energy and metabolic balance, and is reported to participate in regulating cell growth, proliferation, survival, and energy metabolism [21]. Recent studies have demonstrated that AMPK improves the anti-stress ability and survival ability of chondrocytes by regulating the activity of its downstream target molecules in articular chondrocytes, thereby playing an important role in the occurrence and development of OA [22, 23]. When AMPK activity in chondrocytes is reduced the articular cartilage degrades, and leads to OA [24]. A previous study discovered that in human OA, adiponectin could facilitate matrix breakdown of chondrocytes via the AMPK pathway [25]. Another study manifested that AMPK loss in the chondrocytes could accelerate the progression of instability-induced and aging-related osteoarthritis in adult mice, which indicated that AMPK activity in chondrocytes is important in maintaining joint homeostasis and OA development [26]. Thus, AMPK may be a potential therapeutic target for OA. Although there is growing evidence that AMPK activity is involved in the pathogenesis of OA, whether MY can alleviate OA through AMPK pathway, and its molecular pathways are still unclear. Therefore, this study constructed an OA rat model, and then MY (Miyalisan Pharmaceutical Co., Ltd, Japan) and BML275 (dorsomorphin, an AMPK inhibitor, Shanghai yuanye Biotechnology Co., Ltd, China) were used to investigate the roles of AMPK pathways in MY regulating OA progression, which will provide new insights and strategies for drug development in the treatment of OA.

Methods

Animal experiments

A total of 24 of specific pathogen free female Wistar rats (weighing 180–220 g) were purchased from SLAC Laboratory Animal Co., Ltd (Shanghai, China). All the rats were kept in pathogen-free micro-isolated cages in a specific laboratory animal facility at 22–25 ℃ and 20–25% relative humidity, with a 12 h light/dark cycle. During the experiments, all the rats were free access to food and water.

After acclimatization for 7 days, the rats were randomly grouped into 4 groups as follows (n = 6 for each group): control, OA, OA + MY, and OA + MY + AMPK inhibitor groups. The OA rat model was created as previously described [20]. Briefly, the rats were deeply anaesthetized using 2%-4% isoflurane before modeling, and then fixed in lateral position. After that, the knee hair was removed, and the knee incision with a length of about 4 cm was

made. The knee capsule was opened laterally, the anterior cruciate ligament was incised, and muscles, fascia and skin were sutured. After operation, to prevent infections, penicillin (30000U/ time) was administered intramuscularly to the rats once a day for 3 consecutive days, as well as the rats were closely observed in terms of food and water intake, hair color change, incision healing status, suture shedding and infections. However, the rats in the control group did not have their anterior cruciate ligaments dissected.

After 2 weeks of modeling, the rats in the OA + MY, and OA + MY + AMPK inhibitor groups were firstly orally given MY (4×10^5 colony-forming units/mL *C. butyricum*, Miyalisan Pharmaceutical Co., Ltd, Japan) daily for 4 weeks, and then were intramuscularly injected with an equal volume of PBS, and 10 mg/kg BML275 (dorsomorphin, an AMPK inhibitor, cat. no. S31490, Shanghai Yuanye Biotechnology Co., Ltd, China) [27] once a week for 4 weeks, respectively. After that, all the rats were sacrificed by cervical dislocation, and knee joint samples and tibia muscle tissues were collected. All the animal experiments were approved by the Ethics Committee of Shanghai Tenth People's Hospital, Tongji University School of Medicine. And the study is reported in accordance with the relavant ARRIVE guidelines.

Hematoxylin–eosin (HE) staining

The collected knee joint samples were fixed with 4% paraformaldehyde for 24 h, and then, were decalcified, dehydrated, waxed, and embedded in paraffin. After that, the slices were immersed in hematoxylin – eosin (HE, cat. no. C0105S, Beyotime, Shanghai, China) for 5 min. After dewatering and sealing, the images were observed by an inverted optical microscope (IX70, Olympus, Japan).

Determination of succinate dehydrogenase (SDH) and muscle glycogen (MG)

Based on the instructions of the manufacturer, the SDH detection kit (cat. no. A022-1–1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and glycogen detection kit (cat. no. A043-1–1, Nanjing Jiancheng Bioengineering Institute) were respectively used to determine the levels of SDH and MG in the tibia muscle samples of different rats.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from the tibia muscle samples in the OA + MY and OA + MY + AMPK inhibitor groups using RNAiso Plus (Trizol, cat no. 9109,Takara, Tokyo, Japan), and then reverse-transcribed to cDNA using PrimeScript™ RT Master Mix (cat. no. RR036A, Takara). Then, the RT-qPCR was amplified using Power SYBR Green PCR Master Mix (cat. no. 4367659, Thermo Fisher Scientific, USA) in a 7500 RealTime PCR System (ABI, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and the sequences of all primers were shown in Table 1. The relative mRNA levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Western blot

Total protein was isolated from the tibia muscle samples using RIPA (cat. no. P0013B, Beyotime, Shanghai, China), and the concentrations of total protein samples were tested using a BCA assay kit (cat. no. PL212989, Thermo Fisher Scientific). Subsequently, the protein samples (20 μg) were separated via 10% SDS-PAGE using an electrophoresis apparatus (model: EPS300, Tanon Biotech, Shanghai, China), and transferred onto PVDF membranes by a trans-blot transfer (model: VE186,

Table 1 Primer sequences for RT-qPCR assay

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
AMPK	CAGCGATCAACAGGCGAGAC	AGAGATATCCCAGCAAACCTATCCA
AKT	CTTTATTGGCTACAAGGAACGG	CAGTCTGAATGGCGGTGGT
mTOR	AGTGAAGCCGAGAGCAATGAGA	GACAAGGAGATAGAACGGAAGAAGC
p70S6K	CTACAGAGACCTGAAGCCGGAGA	AATGTGTGCGTGACTGTTCCATC
PI3K	ACGGCAATGTGGAGCAGA	GTCGTAGCCAATCAGGGAG
Murf-1	ACCTGCTGGTGGAGAACATC	CTTCGTGTTCCTTGCACATC
MyoD	CGACTGCCTGTCCAGCATAG	GGACACTGAGGGGTGGAGTC
Ldh	GCAGCAGGGTTTCTATGGAG	TGGAGACAGTGGGATTGTCA
Lcad	GCAGTTACTTGGGAAGAGCAA	GGCATGACAATATCTGAATGGA
Mcad	CCACAGTGACCCTTTCTAG	GTGACAGGCTACCTTTCTT
Chrna1	GGCACTTGGACCTATGACGGCTCT	GACGCTGCATGACGAAGTGGTAGG
Chrnd	GCCGCAAGCCGCTCTTCTACATCA	CGTGCTGGGTGTTCGGAAGTGGAT
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

Tanon Biotech). The membranes were incubated with anti-AMPK antibody (cat. no. 2532S, 1: 1000, CST, Danvers, MA USA), anti-cholinergic receptor nicotinic alpha 1 subunit (Chrna1) antibody (cat. no. 10613–1-AP, 1: 1000, Proteintech, Rosemont, IL, USA), anti- lactate dehydrogenase (Ldh) antibody (cat. no. 21799–1-AP, 1: 1000, Abcam, Cambridge, UK), anti-medium-chain acyl-CoA dehydrogenase (Mcad) antibody (cat. no. 55210–1-AP, 1: 1000, Proteintech), anti-myogenic differentiation antigen (Myod) antibody (cat. no. 18943–1-AP, 1: 1000, Proteintech), anti-cholinergic receptor nicotinic delta subunit (Chrnd) antibody (cat. no. ab233758, 1: 1000, Abcam), anti-phosphatidylinositol-3 kinase (PI3K) antibody (cat. no. 20584–1-AP, 1: 1000, Proteintech), anti-long-chain acyl CoA dehydrogenase (Lcad) antibody (cat. no. 17526–1-AP, 1: 1000, Proteintech), and anti-GAPDH antibody (cat. no. 60004–1-lg, 1: 10,000, Proteintech) at 4˚C overnight; and after washing, the secondary antibody (cat. no. 111–035-003 or 115–035-003, 1: 5000, Jackson ImmunoResearch, West Grove, PA, USA) was added, and then incubated at 37˚C for 2 h. Finally, the protein bands were visualized using an enhanced chemiluminescence luminescence (cat. no. P0018AS, Beyotime, Shanghai, China).

Statistical analysis

Statistical analysis was performed using Graphpad prism 5 (GraphPad Software, Inc., San Diego, CA, USA), and data were presented as mean ± standard deviation (SD). Student's t-test was used to compare the differences between two groups; while one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied for the comparison of more than two groups. $p < 0.05$ was considered statistically significant.

Results

Effects of the AMPK inhibitor on OA

The nuclei of chondrocytes were blue-purple and the cytoplasm was pink. In the knee tissue sections of control group, the membrane cells were arranged neatly, the number of chondrocytes was large, the shapes were mostly round or quasi-round, the size was uniform, and the nuclei were visible and the color was uniform. In the knee tissue sections of OA group, synovial tissue cells were hyperplasia and disordered, the number of chondrocytes was reduced, the nuclear staining was shallow, and the four layers of cartilage tissue were indistinct. In the knee tissue sections of the OA + MY group, chondrocytes showed abnormal morphology, disordered distribution and even clustered distribution, while the number of the chondrocytes was higher than that in the OA group, and the arrangement of cartilage tissues was more orderly than that of the OA group. In addition, in the OA + MY + AMPK inhibitor group, the number of the chondrocytes was higher than that in the OA group, whereas was lower than that in the OA + MY group; as well as the arrangement of cartilage tissues was more orderly than that of OA group (Fig. 1).

Effects of the AMPK inhibitor on SDH and MG contents in OA

Skeletal muscle is the body's main organ for absorbing glucose and oxidizing fatty acids, and plays an important role in metabolism. SDH is a respiratory chain enzyme in the TCA cycle, involved in oxidative phosphorylation and respiratory metabolism [28]. MG is involved in the energy supply of muscles, and disturbance of MG levels may lead to loss of muscle strength and function, impairing biological energy metabolism [29]. Therefore, the function of the tibia muscle samples was further examined. The SDH contents in the OA + MY and OA + MY + AMPK inhibitor groups were 7.97 ± 0.21 and 5.93 ± 0.40 U/mg prot, respectively. These indicated that compared to the OA + MY group, the AMPK inhibitor notably decreased the SDH content by 25.6% ($p < 0.05$, Fig. 2A). Additionally, the MG contents in the OA + MY and OA + MY + AMPK inhibitor groups were respectively 5.01 ± 0.18 and 4.40 ± 0.13 mg/g tissue, which displayed that the AMPK inhibitor treatment could significantly reduce the MG content by 12.18% compared to the OA + MY group ($p < 0.05$, Fig. 2B).

RT-qPCR and western blot analysis

AMPK, *PI3K*, *protein kinase B* (*AKT1*), and *mammalian target of rapamycin* (*mTOR*) are closely associated with AMPK pathway; *70-kDa ribosomal protein S6 kinase* (*p70s6k*), *Ldh*, *Lcad*, and *Mcad* are energy metabolism-related genes; *Myod* and *muscle RING finger 1* (*Murf1*) are myogenesis-associated genes; as well as *Chrna1* and *Chrnd* are NMJ- related genes. It is clear that the mRNA expression levels of *AMPK*, *PI3K*, *AKT1*, and *mTOR* were markedly down-regulated in the OA + MY + AMPK inhibitor group compared to the OA + MY group ($p < 0.05$, Fig. 3A-D). However, there was no difference in *p70s6k* mRNA expression between the two groups ($p > 0.05$, Fig. 3E). For *Ldh*, its expression was also significantly decreased after the AMPK inhibitor treatment in comparison with the OA + MY group ($p < 0.05$, Fig. 3F). Relative to the OA + MY group, the mRNA expression levels of *Lcad* and *Mcad* were evidently increased by AMPK inhibitor treatment ($p < 0.05$, Fig. 3G, H). For *Myod*, *Chrna1* and *Chrnd*, the trend of their mRNA expression levels in the two groups were similar with that of the AMPK and *PI3K* mRNA expression ($p < 0.05$, Fig. 3I, K, L). However, no significant difference was found

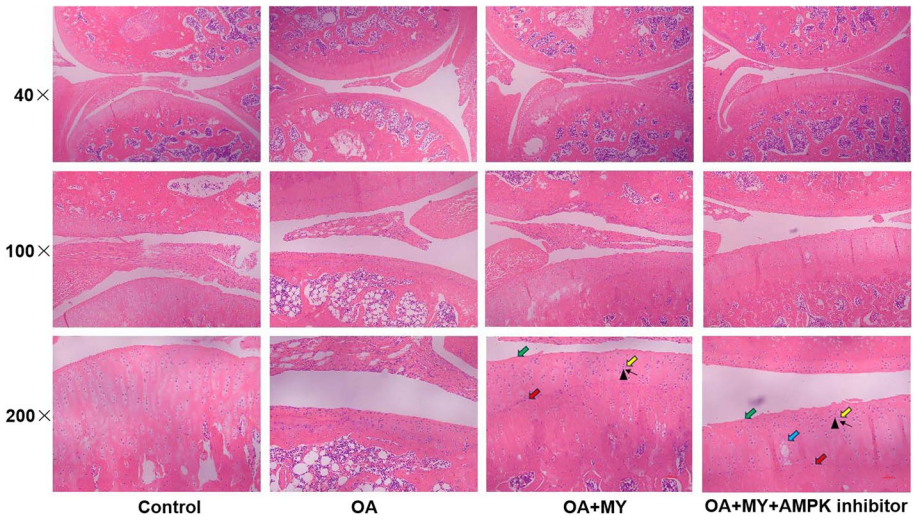

Fig. 1 The histological changes of rats in the different groups observed by hematoxylin–eosin (HE) staining at 40×, 100×and 200×magnification times. The green arrow indicates the surface of the cartilage; the yellow arrow points to cells; the red arrow indicates the tide line; and the blue arrow indicates the absence of cells. The rough triangle represents the nucleus; and the thin arrow represents the cytoplasm. Control: the rats without any treatments; OA: the osteoarthritis (OA) rat model; OA + MY: the OA rats treated with Miya (MY); OA + MY + adenosine 5′-monophosphate-activated protein kinase (AMPK) inhibitor: the OA rats firstly treated with MY, and then treated with BML275 (dorsomorphin, an AMPK inhibitor)

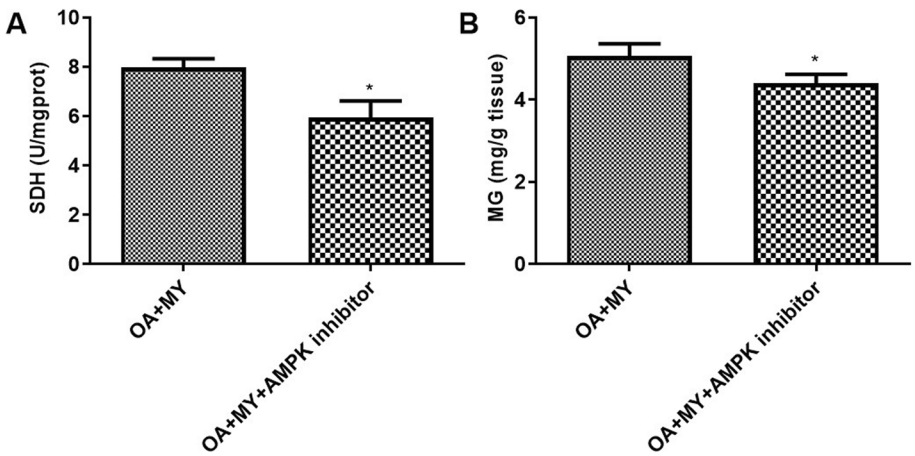

Fig. 2 Effects of the AMPK inhibitor on the succinate dehydrogenase (SDH) and muscle glycogen (MG) in MY-treated OA. **A** The contents of SDH in the tibia muscle of different groups. **B** The contents of MG in the tibia muscle of different groups. * $p < 0.05$, compared with the OA + MY group

in the Murf-1 expression between the OA + MY and OA + MY + AMPK inhibitor groups ($p > 0.05$, Fig. 3J).

Furthermore, western blot was used to determine the protein expression levels of AMPK, PI3K, Ldh, Lcad, Mcad, Myod, Chrna1 and Chrnd. It was found that the tendency of AMPK, PI3K, Ldh, Lcad, Mcad, Myod, Chrna1 and Chrnd protein expression levels in the different groups detected by western blot were consistent with that measured by RT-qPCR ($p < 0.05$, Fig. 4A-I).

Discussion

OA, is a kind of bone and joint disease with multiple factors and pathological changes, has a high morbidity and a disability rate in the elderly population, causing a huge economic burden to society [1]. MY is a probiotic that is used to treat and improve digestive tract diseases [30, 31]. Our previous study found that MY promoted joint injury repair and inhibited OA progression through the "gut-muscle-joint" axis [20]. Then, the research explored the molecular mechanisms by which MY protects against

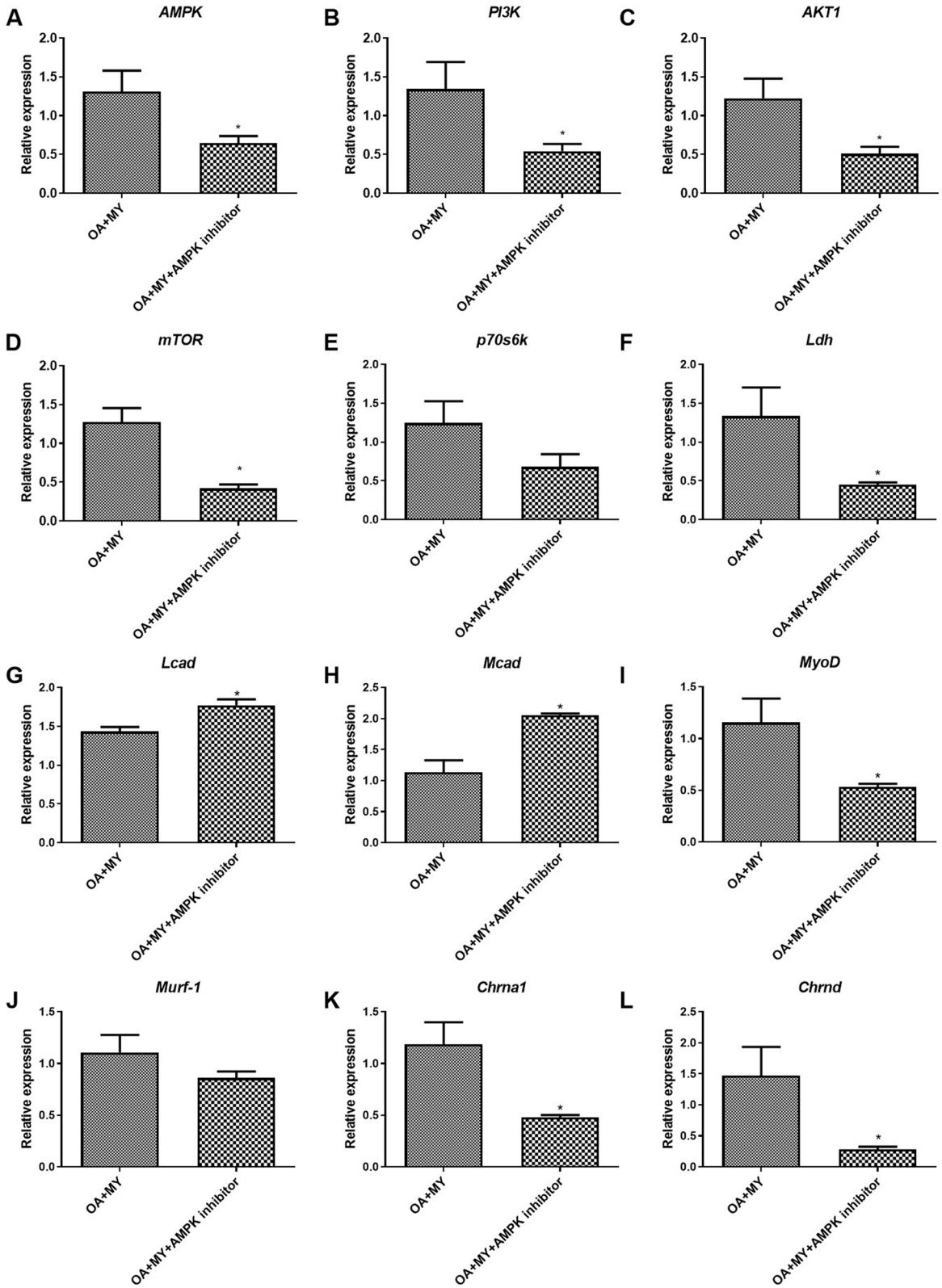

Fig. 3 Effects of the AMPK inhibitor on the mRNA levels of associated genes in the MY-treated OA using real-time quantitative PCR. The mRNA expression levels of (**A**) *AMPK*, (**B**) *PI3K*, (**C**) *AKT1*, (**D**) *mTOR*, (**E**) *p70s6k*, (**F**) *Ldh*, (**G**) *Lcad*, (**H**) *Mcad*, (**I**) *Myod*, (**J**) *Murf-1*, (**K**) *Chrna1*, (**L**) *Chrnd* in the different groups. * $p < 0.05$, compared with the OA + MY group

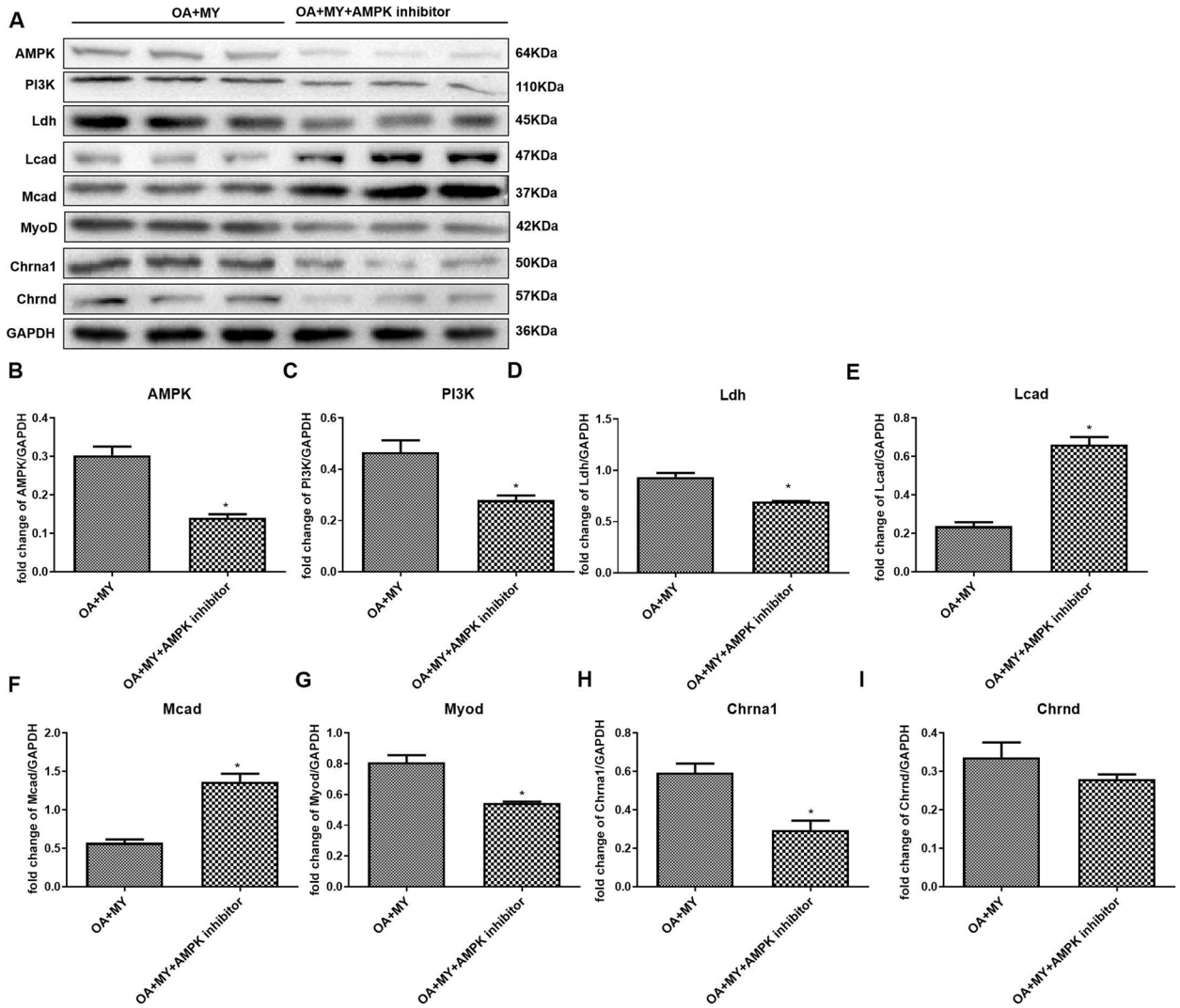

Fig. 4 Effects of the AMPK inhibitor on the protein levels of the associated proteins in the MY-treated OA by western blot. **A** The protein bands of all the associated proteins visualized by western blot analysis. The protein expression levels of (**B**) AMPK, (**C**) PI3K, (**D**) Ldh, (**E**) Lcad, (**F**) Mcad, (**G**) Myod, (**H**) Chrna1, (**I**) Chrnd in the different groups. * $p < 0.05$, compared with the OA + MY group

OA. AMPK has been extensively investigated and has attracted a lot of attention due to its critical function in preserving the body's energy balance, which was found to be significantly up-regulated after MY administration. However, the roles of AMPK in MY-treated OA remains unclear. Therefore, a rat OA model was constructed, and treated with MY and an AMPK inhibitor to study the roles of the AMPK inhibitor in MY-mediated OA. This study found that MY could regulate skeletal muscle changes, alleviate cartilage injury, and prevent OA development, while the AMPK inhibitor could partially reverse the actions of MY in OA.

AMPK not only plays the role of intracellular energy sensor, but also participates in maintaining cellular homeostasis under various stress conditions [32]. It has been reported that when the AMPK activity in chondrocytes was declined, the articular cartilage would become degenerative, thus triggering OA occurrence and progression [33]. A previous study demonstrated that metformin (an AMPK agonist) could inhibit the development and progression of OA by activating AMPK signaling [23]. Another study in a randomized controlled trial showed that methotrexate, a kind of AMPK agonists, could significantly relieve pain in patients with knee OA and synovitis [34]. In vitro and in vivo experiments showed that chitosan oligosaccharides could suppress the expression of inducible nitric oxide synthase and cyclooxygenase-2 in TNF-α-induced synovial cells

through an AMPK-dependent mechanism, thereby alleviating inflammation in vivo through activating AMPK signaling pathway [35]. These reports indicate that AMPK agonists can delay the development of experimental OA. In our previous study, MY treatment significantly increased AMPK mRNA and protein expression in the OA rat models [20]. Considering the roles of AMPK in OA, an AMPK inhibitor (BML275, dorsomorphin) was used to investigate whether MY inhibited OA progression through the AMPK signaling pathway in a rat model of OA, and we found that the AMPK inhibitor could reverse the effects of MY on OA partially, further indicating the roles of AMPK in MY-treated OA.

Skeletal muscle is critical in the evolution of knee structural destruction and functional changes in OA patients [36, 37]. Skeletal muscle components of OA patients are changed, which release myogenic cytokines involved in the pathogenesis of OA [37]. Muscle strength training helps delay OA while improving joint function and relieving pain [38]. Myogenic cytokines are a kind of cytokines or proteins secreted by muscles that regulates muscle development and growth, and plays an important role in the formation of bone and fat [39]. Our previous study found that SDH and MG contents were decreased in the OA model rats; while MY could significantly elevated the contents caused by OA [20]. However, in this study, the SDH and MG contents were notably decreased after AMPK inhibitor treatment. SDH is widely present in mitochondria, and is tightly bound to the inner membrane of mitochondria [40].

Mitochondria produce ATP through tricarboxylic acid cycling and oxidative phosphorylation, affecting reactive oxygen species production, inflammation regulation, chondrocyte senescence, stromal catabolism, apoptosis induction, and calcium homeostasis in the pathological process of OA [41, 42]. Succinic acid is an intermediate product of the tricarboxylic acid cycle, and its accumulation ultimately promotes the increase of inflammatory factor IL-1β, leading to inflammatory response [43]. SDH is one of the important enzymes in the electron transport chain and oxidative phosphorylation in cellular glucose metabolism [44]. The decrease in SDH activity may cause the cell energy metabolism to slow down or stop, and the decrease of adenosine triphosphate production, contributing to the disturbance of glucose metabolism [45]. Glycogen is an important exercise energy substance that is mainly stored in the liver and muscle tissue. MG is considered an important energy source for high-intensity and endurance exercise, and is an important factor in sustained muscle movement [46]. The germ-free mice were reported to have lower MG levels than individuals with normal GM components [47]. Taken together, it can be inferred that MY could increase the contents of SDH and MG in muscles, while is abolished by the AMPK inhibitor, which indicated that MY may affect the levels of SDH and MG via AMPK pathway.

In addition, we observed that the AMPK inhibitor could significantly down-regulate the expression levels of AMPK, PI3K, *AKT1*, and *mTOR*. It has been reported that AMPK can be abnormally activated in articular chondrocytes, and phosphorylated activation of AMPK can promote phosphoinositide 3 kinase (PI3K) to reduce apoptosis [48]. PI3K is believed to be closely related to articular cartilage injury, and has been paid close attention by researchers [49, 50]. AKT is a direct downstream target of P13K [51], which included three subtypes of AKT1, AKT2 and AKT3. Among them, AKT1 and AKT2 are involved in the balance regulation of bone cell synthesis and metabolism. PI3K/AKT is an important intracellular signal transduction pathway [52]. In recent years, due to its involvement in the regulation of chondrocyte proliferation, differentiation and survival, PI3K/AKT pathway, as a core pathway in the pathogenesis of osteoarthritis, has received extensive attention [53, 54]. A previous study of Chen et al. showed that Fuzi decoction could increase the cell viability and wound healing ability of chondrocytes, and reduce the PI3K/AKT signaling pathway, thus relieving OA [53]. Another research demonstrated that overexpression of circFOXO3 could alleviate chondrocyte apoptosis and promote the anabolism of extracellular matrix by activating FOXO3, PI3K/AKT pathway, and autophagy [54]. It is well known that activation of PI3K/AKT pathway can promote chondrocyte autophagy and prevent cartilage injury [55]. mTOR is a kind of serine/threonine kinases, and a key regulator of cell growth, which regulates autophagy and inflammation [56]. Ji et al. [57] reported that apigenin could reduce the protein levels of p-mTOR, apoptosis regulator BAX and caspase 3, while increase B-cell lymphoma-2 in macrophages, as well as down-regulate the levels of IL-1, IL-6, and TNF-α in chondrocytes, thereby inhibiting the chondrocyte inflammation and alleviating OA. Additionally, PI3K/AKT and mTOR can jointly constitute the PI3K/AKT/mTOR signaling pathway, which participates in a variety of biological regulatory processes, such as regulating cell growth, differentiation and metabolism [58]. Among them, AKT receives upstream PI3K signal, and transmits the signal to mTOR, the downstream target of AKT, playing a "connecting role". Zhang et al. [59] showed that daurisoline could alleviate H2O2-induced chondrocyte autophagy via activating the PI3K/AKT/mTOR signaling pathway, thus protecting OA. Another study manifested that inhibition of recombinant chemokine C–C-motif receptor (CCR10) could alleviate IL-1β-induced chondrocyte damage by inhibiting the PI3K/Akt/mTOR pathway, suggesting that CCR10

may be a promising target for OA treatment [60]. Combined with our results, we speculate that AMPK may participate in MY-treated OA through regulating the PI3K/AKT/mTOR pathway. However, the specific actions of PI3K/AKT/mTOR pathway in MY regulating OA need to be investigate in the future.

Further, the effects of the AMPK inhibitor on the levels of energy metabolism-associate genes (*Ldh*, *Lcad*, and *Mcad*), myogenesis-associated genes (*Myod* and *Murf-1*), and NMJ- related genes (*Chrna1* and *Chrnd*) in the tibia muscle tissues were further explored. In this study, we found that the AMPK inhibitor treatment could significantly down-regulate *Ldh*, *MyoD*, *Chrna1* and *Chrnd* expression, and up-regulate *Lcad* and *Mcad* expression. LDH is a key enzyme in anaerobic glycolysis of glucose, and an indicator of muscle damage [61]. The main function of LDH is to catalyze the reaction between lactic acid and pyruvate, and lactic acid is produced by anaerobic glycolysis of glucose catalyzed by LDH. Under pathological conditions, LDH activity is enhanced and catalyzes lactic acid oxidation, which is a protective compensatory mechanism of automatic mechanical regulation in the body. It has been reported that AMPK can regulate lipid metabolism by regulating key enzymes of fatty acid synthesis and autophagy function of cells, thus delaying the development of OA [62]. Lcad and Mcad are two key enzymes that regulate fatty acid synthesis and oxygenation [63]. Vitamin D could activate AMPK, and elevate the levels of Lcad and Mcad in skeletal muscle, boosting muscle fat formation and mitochondrial activity [64]. MyoD is the first discovered myogenic regulator, and can regulate myoblast differentiation during muscle regeneration [65]. Furthermore, MyoD can also transform many other types of cells, such as fibroblasts, pigment cells, nerve cells, fat cells, liver cells, etc. into skeletal muscle cells [65]. NMJ plays an important role in maintaining the conduction of nerve impulses and the transport of nutrients, and its disruption can lead to muscle atrophy [66]. A previous study showed that knee OA contributed to muscle atrophy and NMJ remodeling of the tibial anterior muscle [67]. Choline is a substrate derived from the neurotransmitter acetylcholine, and is essential for membrane integrity [68]. Chrna1 and Chrnd encoding different acetylcholine subunits and acetylcholine receptors have been reported to be closely related to the development and function of NMJ [69]. These reports, together with our findings, we can hypothesize that the AMPK inhibitor may regulate energy metabolism-associate genes (*Ldh*, *Lcad*, and *Mcad*), myogenesis-associated genes (*Myod*), and NMJ- related genes (*Chrna1* and *Chrnd*) to influence MY-treated OA, thus reducing the effect of MY on OA.

Conclusion

In conclusion, MY may partially regulate skeletal muscle changes and prevent OA development through the AMPK pathway. These findings provide a new idea for studying the role of MY in bone and joint disease, and also provide a reference and basis for better clinical treatment of bone and joint diseases with AMPK as the novel potential target.

Abbreviations

MY	Miya
OA	Osteoarthritis
GM	Gut microbiota
NMJ	Neuromuscular junction
HE	Hematoxylin-eosin
SDH	Succinate dehydrogenase
MG	Muscle glycogen
RT-qPCR	Real-time quantitative PCR
LDH	Lactate dehydrogenase

Supplementary Information

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Supplementary Material 1.

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None.

Authors' contributions

Conception and design of the research: T.Y.X. and F.Q.; acquisition of data: Y.W.Z., Y.S.F. and Y.H.G.; analysis and interpretation of data: Z.H.L., H.L.L. and Y.W.Z.; Statistical analysis: Y.W.Z.; Obtaining funding: G.D.L.; Drafting the manuscript: S.W., Z.W.D. and Z.H.L.; revision of manuscript for important intellectual content: T.Y.X. and D.Y. All authors read and approved the final manuscript.

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Data availability

The data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate

All animal studies were conducted in compliance with guidelines and regulations. All the animal experiments were approved by the Ethics Committee of Shanghai Tenth People's Hospital, Tongji University School of Medicine. And the study is reported in accordance with the relevant ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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