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ORIGINAL ARTICLE

Attenuation of diabetes-mediated muscle atrophy in rats by fish oil enriched omega-3 polyunsaturated fatty acids supplementation

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Abstract

Diabetes is associated with an increased risk of muscle wasting/atrophy, which adversely affects quality of life. We hypothesized that long term supplementation of fish oil may have protective effects against sarcopenia or muscle atrophy in streptozotocin (STZ) and high-fat (HF) diet-induced diabetic rat model. Wistar rats at age of 7 weeks were injected with saline or STZ to induce hyperglycemia. After one week, they were fed on a normal control diet or HF diet with/without supplementation of fish oil for 18 weeks. Feeding diabetic rats with a fish oil-enriched diet alleviated body weight loss and the impaired glucose tolerance using OGTT test. Although fish oil did not improve the decreased muscle mass, the muscle atrophy induced by diabetes was attenuated by fish oil in gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus muscles. Fish oil supplementation reversed the decreased expression of phospho (p)-AKT, p-mTOR, and p-p70s6k, which are molecules related to protein synthesis. Besides, protein degradation-related signaling pathways were inhibited by fish oil, such as increasing p-FoxO1 and decreasing Atrogin-1 and MURF1 protein expression. Fish oil down-regulated the expression of autophagy-related molecules including ATG5, p62, and LC3B II/I ratio, which may result in less muscle atrophy. Inflammation-related signaling regulators including TNF-α, NF-κB, AGEs, and RAGE were suppressed by fish oil supplementation as well. Moreover, the down-regulated p-AMPKα, SIRT1, and PGC-1 in diabetic rats were counteracted by fish oil, which may improve mitochondrial function and further block FoxO action. These data suggest that long-term fish oil supplementation exerts protective effects against diabetes-induced muscle atrophy, which may in turn ameliorate insulin resistance and impaired glucose tolerance.

Keywords: Diabetes mellitus, Fish oil, Muscle atrophy, Omega-3 polyunsaturated fatty acids, Sarcopenia

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia, originated from defects in the secretion and/or action of insulin, and appears one of the most common health conditions for the elderly, influencing approximately one-quarter of adults at ages of 65 and older [1]. Patients with type 2 DM (T2DM) are at an increased risk of developing sarcopenia compared with euglycemic subjects, and those patients with poor glycemic control are particularly more susceptible to muscle atrophy and loss [2,3]. Sarcopenia, which corresponds to a progressive and generalized loss of muscle mass and function occurred commonly during aging, has recently been recognized as one of the comorbidities of diabetes [4,5]. T2DM contributes to the sarcopenia-mediated muscle atrophy through various mechanisms, including impaired insulin-regulated signaling pathways, accumulation of advanced glycation end-products (AGEs), peripheral neuropathy, vascular complications, oxidative stress, and chronic inflammation [6–8]. Accounting for approximately 40% of total body weight and 50–75% of total proteins [9], skeletal muscle is the largest tissue in the body and

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plays a fundamental role in the regulation of glucose and protein metabolism; therefore, the loss of muscle mass and function by sarcopenia may display a negative feedback on glycemic control and further lead to metabolic disturbance, which doubtlessly forms a vicious cycle [7]. Despite the risk of sarcopenia can be reduced by appropriate lifestyle management, such as resistance-exercise training and nutritional strategies, not much attention has been paid to the diabetes-induced muscle atrophy, sarcopenia, or muscle dysfunction, which can have deleterious consequences on physical and psychosocial health of diabetic patients and may progressively lead to poor quality of life and increased mortality [10,11].

Dietary interventions using natural bioactive food compounds have emerged as promising therapeutic tools in the management of chronic metabolic disorders, with limited deleterious side effects. Omega-3 polyunsaturated fatty acids (n-3 PUFAs), notably α-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), have been reported to have a wide spectrum of biological roles in human health [12,13]. Convincing evidence shows that n-3 PUFAs possess anti-inflammatory and hypolipidemic potential and may have benefits on myriad health conditions and diseases, such as cancer, mental illness, cardiovascular disease, obesity, and diabetes [14]. Several studies have shown that n-3 PUFAs or fish oil, which enriches with long-chain n-3 PUFA (EPA and DHA), exerts beneficial effects on the regulation of glucose homeostasis, insulin sensitivity, and the prevention of T2DM in human subjects and in animal models [15–18]. The potential mechanisms underlying the impact of n-3 PUFAs on glucose metabolism or insulin sensitivity include reducing adiposity and altering adipokine secretion [19], modulating adipose tissues features [20,21], dampening the inflammatory process [16], stimulating GLUT4 up-regulation [19], and promoting mitochondrial function and fatty acid oxidation [16,22].

Interestingly, recent studies have highlighted a positive influence of n-3 PUFAs or fish oil on skeletal muscle and sarcopenia [23–26]. The n-3 PUFAs are believed to be capable of directly incorporating into the membrane phospholipids after absorption, and the enrichment of these membrane with EPA and DHA may modulate various cellular functions, through signaling pathways and gene expressions [27] and then regulate muscle protein turnover by enhancing protein synthesis pathways or decreasing factors that promote protein degradation [28,29]. This raised the possibility that fish oil may have benefits on the development of diabetes-induced muscle atrophy. As such, the streptozotocin (STZ) and high-fat (HF) diet-induced diabetic rat model was undertaken in this study, and fish oil was incorporated into the daily diet for 18 weeks to evaluate the potential effects of fish oil on glucose homeostasis and diabetes-related muscle atrophy with emphasis on the involved mechanisms related to protein synthesis and degradation.

2. Materials and methods

2.1. Animal model and experimental design

All animal procedures in the current study were approved by the Animal House Management Committee of the National Taiwan Ocean University (IACUC No.:110019) and carried out in strict accordance with the guidelines for care and use of laboratory animals. All the studies were performed in male Wistar rats, which were obtained from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan), and maintained under controlled temperature (22–24 °C), with relative humidity of 40–60%, and on a 12-h on-off lighting cycle. We decided on a total sample size of 24, with 8 rats in each group, based on our previous studies and experiences [30–32], and also adhered to the 3Rs principles of minimizing animal usage. To confirm that this sample size was appropriate, we performed a post hoc power calculation using G*Power software (ver. 3.1.9.7; Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) [33]. The analysis revealed that our study design, with a total sample size of 24, had 81.9% power to detect significant differences when measuring blood glucose levels at a 5% significance level, which Indicates our sample size was appropriate and provided adequate power to show meaningful results. Following the schedule shown in Fig. 1, rats at age of 6 weeks were ad libitum given rodent chow (LabDiet 5001, PMI Nutrition International, St. Louis, MO, USA) and water for one week, and then randomly divided into two groups. One is subcutaneous (SC) injection of saline (n = 8), and the other is SC injection of 45 mg/kg streptozotocin (STZ) in 0.1 M citrate (n = 16). After one week, rats that administered STZ via SC injection, following by a high fat diet for 18 weeks were regarded as DM group (n = 8). In DM + Fish oil (DF) group, we substituted 5% lard with 5% fish oil in the high fat diet (n = 8). Control rats were administered with saline injection and continued on a normal diet (NC group). The STZ-injected, high fat-fed rat model, with lower mortality rates compared to a single high dose of STZ injection model, provides a good animal model of T2DM that mimics insulin resistance

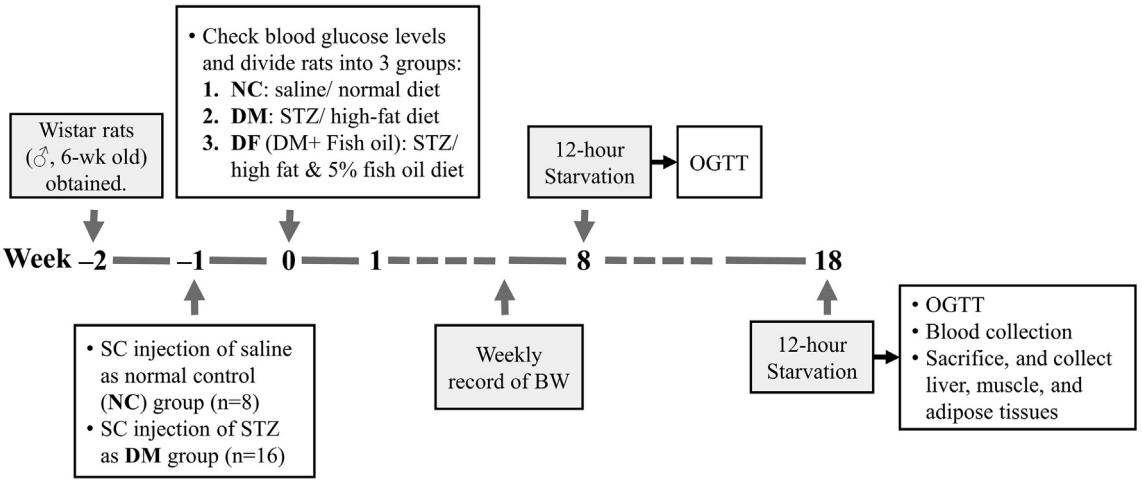

Fig. 1. Experimental design and timeline.

syndrome and is suitable for evaluating the antidiabetic activity of test compound [31,34]. The composition of these diets and the fatty acid composition of fish oil were performed as previously described [35]. In brief, the normal diet contained soybean oil 2% and lard 3%, the high fat diet contained soybean oil 2% and lard 13%, and the fish oil diet contained soybean oil 2%, lard 8%, and fish oil 5%. Proximately 30.6% EPA and 19.2% DHA were included in the fish oil composition. Body weights were monitored weekly.

One week after STZ injection, blood samples were obtained from the retro-orbital sinus using heparin-coated capillary tubes to measure blood glucose levels. Eight and eighteen weeks after feeding different experimental diets, rats were subjected to the oral glucose tolerance tests (OGTT). On the last day of experiment after 12-h fasting, animals were euthanized under carbon dioxide (CO_2: O_2 = 7:3) and the blood samples were withdrawn from the abdominal aorta into heparin-coated tubes. Skeletal muscles, adipose and livers were collected, weighted and stored at −80 °C for western blotting or in 10% formaldehyde for histological staining.

2.2. Determination of fasting plasma glucose and oral glucose tolerance test (OGTT)

Blood sampling from retro-orbital sinus was used for measuring the fasting glucose levels, which were detected by a Glucose Enzymatic Kit (Randox, Crumlin, UK). The OGTT was performed by oral gavage of a glucose load (1.5 g/kg BW) to rats, and the plasma glucose levels were measured at 0 (before glucose challenge), 30, 60, 90, and 120 min post administration using Accucheck glucometer (Roche diagnostics). The area under the curve (AUC) of the OGTT was calculated using Prism 6 (GraphPad Software, La Jolla, CA, USA) as a measure of glucose tolerance.

2.3. Measurement of insulin and homeostatic model assessment for insulin resistance (HOMA-IR)

The plasma samples collected from rats kept on 12-h fasting were used for measuring insulin levels, which were detected using a rat Insulin ELISA kit (Mercodia AB Inc., Uppsala, Sweden). The fasting glucose and insulin data were then used to generate the HOMA-IR with the following formula. HOMA-IR = [Fasting Blood Glucose (mmol/L) x Fasting Insulin (mU/L)]/22.5.

2.4. Measurement of blood biochemical parameters

After centrifuging, plasma was separated and stored at −80 °C for use. Concentrations of triglyceride (TG) were determined using colorimetric-enzymatic kits (Randox Laboratories Ltd., Crumlin, UK). Insulin level was detected using a rat insulin ELISA kit (Mercodia AB Inc., Uppsala, Sweden). Plasma level of TNF-α was analyzed using a rat TNF-α enzyme immunometric kit (R & D systems Inc., Minnesota, USA). AGEs content was measured using an OxiSelect™ AGE ELISA kit (Cell Biolabs, San Diego, CA, USA). All the tests were conducted following the instructions of the manufacturers.

2.5. Histological analysis of skeletal muscle

Fresh muscle samples were fixed in 10% formalin, embedded in paraffin, and prepared in 5-μm sections, which were then stained with hematoxylin

and eosin (H&E) as described previously [36]. Nuclei were stained in blue or purple, and muscle cells were in pink. Then, five images were taken randomly for each sample at 200× magnification, and the cross-sectional area (CSA) for individual myofibers was quantified using the image J software (National Institutes of Health, Bethesda, MD, USA).

2.6. Western blot analysis

Protein from gastrocnemius muscle samples were extracted using radioimmunoprecipitation lysis buffer, and protein concentration was estimated using a Pierce BCA protein assay kit. Protein samples (50 μg per lane) were loaded and run on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transblotted onto a polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). Afterwards, membranes were blocked with 5% skim milk in 1x tris-buffered saline with Tween (TBST) at room temperature for 1 h and then incubated with primary antibodies (1:1000 v/v dilution) for phosphorylated FoxO1a (Ser256), FoxO1a, phosphorylated AKT (Ser473), AKT, phosphorylated NF-κB-p65 (Ser536), NF-κB-p65, Atrogin-1, PGC-1α (Abcam, Cambridge, MA, USA), phosphorylated AMPKα (Thr172), AMPKα (Cell Signaling Technology, Danvers, MA, USA), MURF1, phosphorylated mTOR (Ser2481), mTOR, and ATG5, p62, LC3BI, LC3BII, phosphorylated p70s6k (Ser434), p70s6k, RAGE, SIRT1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Following primary antibody incubation, membranes were washed with 1x TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 v/v dilution, Cell Signaling Technology) for 1 h at room temperature. Finally, membranes were exposed to enhanced chemiluminescence reagent (BioRad Laboratories, Redmond, WA) and the signal was detected using X-ray films (Fujifilm, Tokyo, Japan). Densitometric analysis was performed using ImageJ software (National Institutes of Health, USA) on scanned films and data were from at least three independent experiments. All protein band intensities were normalized to GAPDH and expressed as arbitrary units.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6 software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was used for multiple comparisons. Result values are presented as mean ± standard deviation (SD) of the mean, and considered significant if p values are <0.05.

3. Results

3.1. Fish oil supplementation alleviates diabetes-induced impaired glucose tolerance

Initially, impaired glucose homeostasis and insulin resistance were successfully established in single dose STZ-injected rats fed a high fat (HF) diet for 18 weeks (Table 1). As expected, the DM rats exhibited higher plasma levels of glucose and insulin, as well as a higher HOMA-IR value, compared with the normal control (NC) diet-fed rats. Supplementation of fish oil significantly reduced the plasma levels of glucose, but had no effects on insulin levels. The HOMA-IR value was 23.8% lower in DF rats (DM rats supplemented with fish oil) compared with that in DM rats; however, the difference between these two groups was not statistically significant due to the big error bars (Table 1). Next, OGTT was then undertaken to further evaluate the insulin secretion and action. The DM rats both after 8 and 18 weeks of the dietary intervention showed the highest glucose levels throughout the measuring points compared to the other two groups, indicating a typical phenotype of insulin resistance (Fig. 2A for 8-week; Fig. 2C for 18-week). DF rats, after 8-week diet intervention, showed a significant decline of postprandial blood glucose levels at all the measuring time points, as compared to those in DM rats; whereas the blood glucose levels decreased only at 30 min post glucose challenge in DF rats after 18-week diet intervention (Fig. 2A and C). However, the AUC calculated from OGTT was reduced in DF rats both after 8- and 18-week diet intervention (Fig. 2B for 8-week; 2D for 18-week). Moreover, the plasma levels of total cholesterol were elevated in DM rats (Table 1), which was reversed by fish oil

Table 1. Plasma glucose, insulin, total cholesterol and insulin resistance index (HOMA-IR) in rats fed with different experimental diets for 18 weeks.

Diet	NC	DM	DF
Glucose (mg/dL)	226.4 ± 23.0^{b}	313.5 ± 102.3^{a}	237.8 ± 26.8^{b}
Insulin (μg/L)	0.45 ± 0.16^{b}	0.84 ± 0.49^{a}	0.78 ± 0.25 ^{ab}
Total cholesterol (mg/dL)	85.4 ± 15.8^{b}	140.1 ± 76.3^{a}	83.6 ± 20.0^{b}
HOMA-IR	6.34 ± 2.62^{b}	14.7 ± 5.41^{a}	11.2 ± 3.38^{a}

Results are expressed as mean ± S.D. for each group (n = 8). Different letter indicates statistical significance (p < 0.05). NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil; HOMA-IR, homeostatic model assessment for insulin resistance.

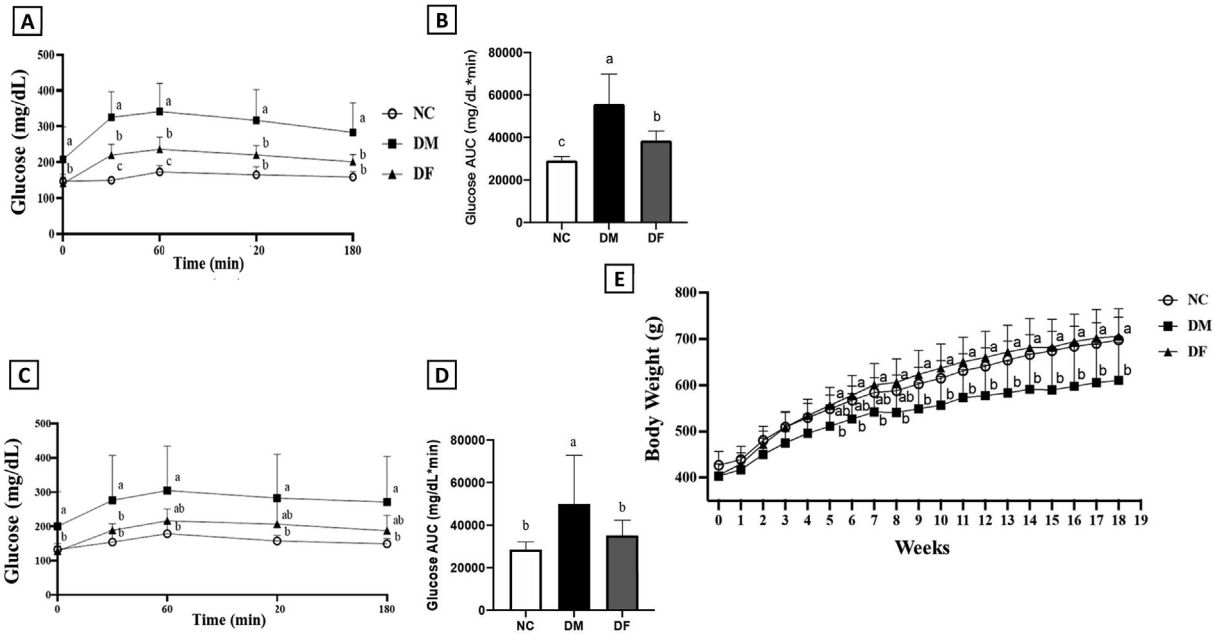

*Fig. 2. The changes of plasma glucose concentration and area under curve (AUC) after oral glucose tolerance test (OGTT) and body weight in rats fed with different experimental diets for 8 weeks and 18 weeks during the experimental period. Rats were subjected to OGTT test and blood was sampled at 0 (baseline), 30, 60, 90, and 120 min for glucose levels after 8-week (A) and 18-week (C) dietary intervention. AUC was calculated and expressed as mg/dL*min for 8-week group (B) and 18-week group (D). The body weight was recorded weekly (E). Results are expressed as mean ± S.D. for each group (n = 8). Different letters indicate statistical significance ($p < 0.05$) by one-way ANOVA post hoc Duncan's multiple range test. NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.*

supplementation. These observations collectively indicate that the DM rats displayed the typical characteristics of T2DM, with evidence of insulin resistance, hyperglycemia, hyperinsulinemia and dyslipidemia, and fish oil supplementation may improve insulin-regulated glucose disposal and alleviated glucose intolerance.

3.2. Effects of fish oil supplementation on the diabetes-induced body weight and muscle weight loss

The body weight decreased significantly in DM rats compared with NC rats since week 5 (Fig. 2E). The food intake in NC, DM and DF rats was 28.3 ± 1.76, 29.8 ± 8.13, and 24.9 ± 1.77 g/day, respectively, which was no significant difference between groups. After 18-week intervention, the weights of gastrocnemius and tibialis anterior muscles was significantly reduced in DM rats, although soleus and extensor digitorum longus muscle showed a tendency to decrease but no statistical difference compared to that in NC rats (Table 2). Fish oil administration to DM rats significantly restored the body weight to normal range since week 9 (Fig. 2E), suggesting that fish oil attenuated body weight loss following diabetes development. However, the reduced muscle mass of gastrocnemius and tibialis anterior muscle was not alleviated by fish oil supplementation (Table 2). Notably, the increase of triglyceride (TG) content in gastrocnemius muscle induced by diabetes was reduced by fish oil supplementation, demonstrating that fish oil improves skeletal muscle adiposity.

Table 2. Muscle weight and TG content in rats fed with different experimental diets for 18 weeks.

	Diet		
	NC	DM	DF
Muscle weight			
Gastrocnemius muscle (g)	7.06 ± 0.32[a]	6.27 ± 1.02[b]	6.36 ± 0.35[b]
Soleus muscle (g)	0.53 ± 0.09	0.46 ± 0.05	0.52 ± 0.04
Tibialis anterior muscle (g)	2.24 ± 0.39[a]	1.90 ± 0.27[b]	1.84 ± 0.11[b]
Extensor digitorum longus muscle (g)	0.52 ± 0.06	0.47 ± 0.09	0.45 ± 0.05
Muscle adiposity			
TG (mg/g muscle)	3.21 ± 1.10[b]	4.56 ± 1.13[a]	3.39 ± 0.83[b]

Results are expressed as mean ± S.D. for each group (n = 8). Different letter indicates statistical significance ($p < 0.05$) by one-way ANOVA post hoc Duncan's multiple range test. NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil; TG, triglyceride.

3.3. Fish oil supplementation improves diabetes-altered myofiber morphology and distribution of myofiber size

To observe the muscle fiber alteration, H & E staining was performed to measure the cross-sectional area (CSA) of muscle fibers. The morphology and distribution of myofiber size in gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus muscles was presented in Fig. 3A and B, respectively. DM mice showed a leftward shift in the distribution of myofiber sizes of all the muscles compared to NC rats, whereas fish oil supplementation partially, but significantly, reversed the leftward trend (Fig. 3B). It indicated that fish oil improved the diabetes-altered myofiber morphology and distribution of myofiber size.

3.4. Fish oil supplementation suppresses ubiquitin-proteasome system (UPS) and autophagy-lysosome system (ALS)

Upregulation of muscle protein breakdown by stimulating UPS and ALS, which are two major protein degradation pathways, is involved in muscle atrophy or loss of fiber size [37,38]. To investigate the molecular mechanism underlying the effect of fish oil on diabetes-induced muscle atrophy, we first checked UPS-related molecules forkhead box protein O1 (FoxO1), Atrogin-1 and muscle RING-finger protein 1 (MURF1) in gastrocnemius muscles [39]. The Western blotting results showed that the decrease of phosphorylated expression of FoxO1a and the increase of protein expression of Atrogen-1 and MuRF1 in DM rats was reversed by fish oil supplementation (Fig. 4A–C). The phosphorylated FoxO1a in DF rats was even higher than that in NC rats (Fig. 4A), and the Atrogen-1 protein expression in DF rats was even lower than that in NC rats (Fig. 4B). Next, we evaluated the inhibitory effect of fish oil on ALS markers, autophagy protein 5 (ATG5), p62/sequestosome 1 (p62) and the ratio of microtubule-associated protein light chain 3 β II (LC3B II) to LC3B I in gastrocnemius muscle. Fish oil supplementation decreased protein expression of ATG5 and p62 compared to NC and DM rats, although diabetes did not increase their expressions (Fig. 5A and B). Additionally, the ratio of LC3B II to LC3B I increased by diabetes was reversed by fish oil supplementation (Fig. 5C). Collectively, these results suggest that the improvement of diabetic muscle atrophy by fish oil can be attributed to the reversal of muscle protein degradation through inhibiting both UPS and ALS.

3.5. Fish oil supplementation activates the AKT/mTOR/p70s6k pathway

Given that the AKT/mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (p70s6k)

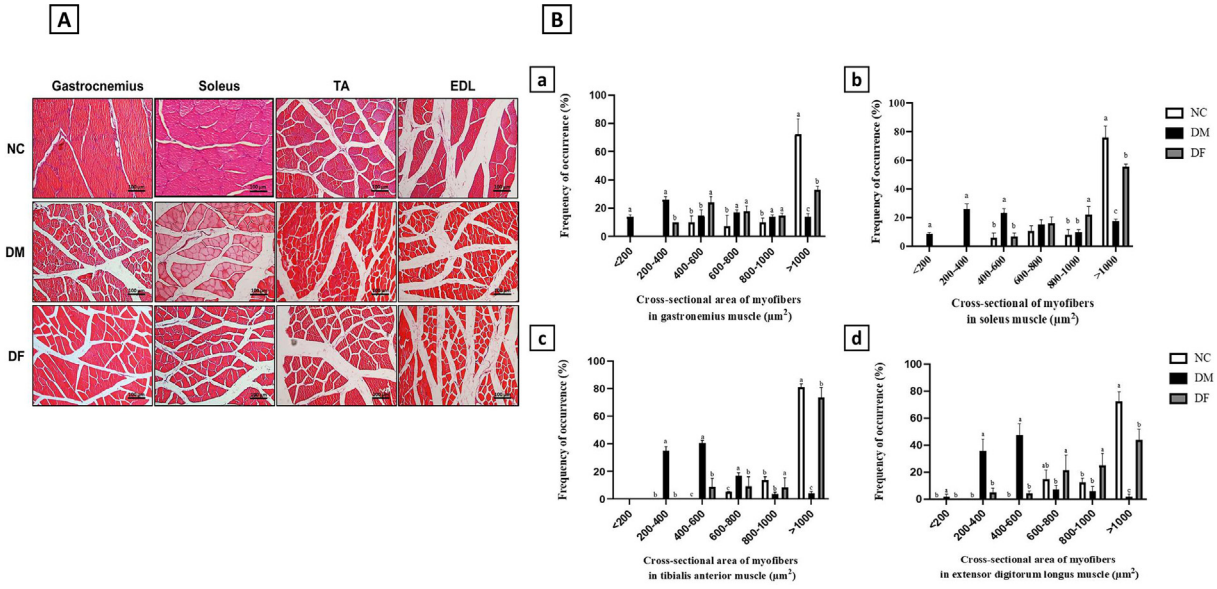

Fig. 3. Histological analysis of skeletal muscles. The gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus muscles dissected from rats fed with different experimental diets were determined by hematoxylin and eosin (H & E) staining. (A) The representative H & E stained images were shown in left panel. (B) The frequency of distribution of myofiber CSA in gastrocnemius (a), soleus (b), tibialis anterior (c) and extensor digitorum longus (d) was calculated and shown in right panel. Results are expressed as mean ± S.D. for each group (n = 8). Different letters indicate statistical significance (p < 0.05). NC, normal control diet. DM, DM in high fat diet with 13% lard. DF, DM in high fat diet with 8% lard and 5% fish oil.

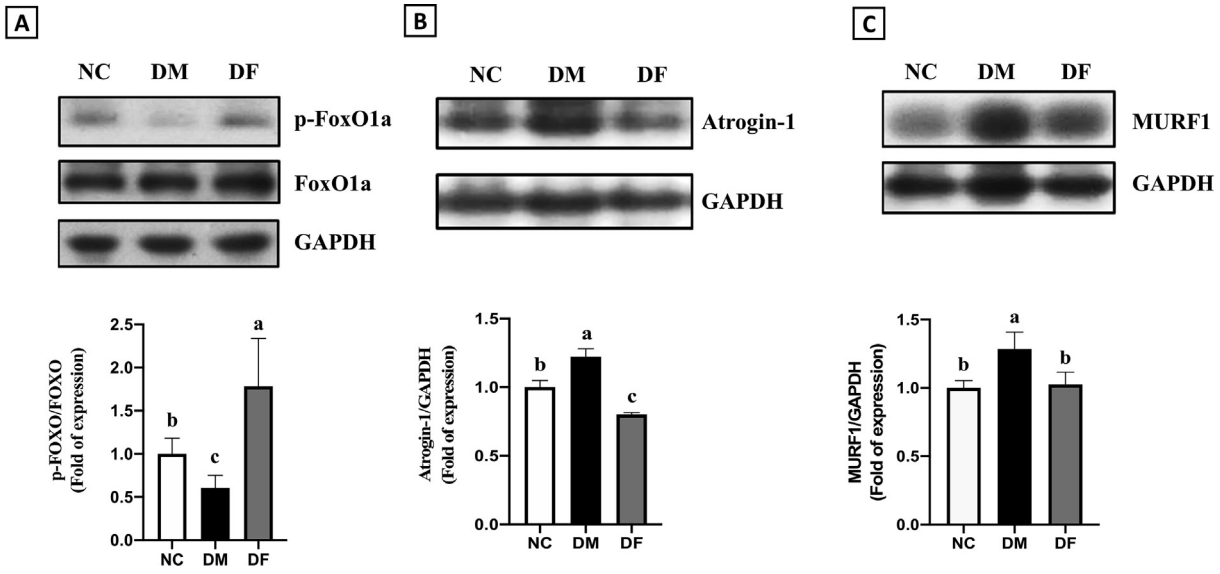

Fig. 4. Effects of fish oil on ubiquitin-proteasome system-related three major proteins in rats fed with different diets for 18 weeks. At the end of experiment, protein expression of phosphorylated FoxO1a (p-FoxO1a), atrogin-1 and MURF1 were measured in gastrocnemius muscle by Western blotting. Representative immunoblots and relative optical density of p-FoxO1a/FoxO1a (A), Atrogin-1/GAPDH (B), and MURF1/GAPDH (C) were shown. GAPDH was used as an internal control. Results are expressed as mean ± S.D. for each group (n = 4). Graph bars with different superscript letters are significantly different (p < 0.05). NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.

pathway is a key signaling pathway of protein synthesis [40], we further studied whether the improved muscle atrophy by fish oil is through stimulating protein synthesis. The expression of phosphorylated mTOR (p-mTOR) and phosphorylated p70s6k (p-p70s6k) in gastrocnemius muscle decreased in DM rats; however, the phosphorylated AKT (p-AKT) was no difference between NC and DM group (Fig. 6A–C). Fish oil supplementation was found to reverse the decreased p-mTOR and p-p70s6k protein expression of DM group. Moreover, p-AKT was up-regulated by fish oil compared to DM group. These findings suggest that increased protein synthesis by enhancing AKT/mTOR/p70s6k pathway is participated in the benefits of fish oil on diabetes-induced muscle atrophy.

3.6. Fish oil supplementation blocks TNF-α/NF-κB-mediated inflammatory signaling and AGEs/RAGE signaling

Inflammation plays a role in inducing muscle atrophy in diabetes, and TNF-α is known to be one of immune modulator to trigger muscle atrophy through activation of NF-κB [8] We therefore evaluated the effect of fish oil on the changes of TNF-α and NF-κB in diabetes-mediated muscle atrophy. Our data showed a significant increase of plasma TNF-α levels in DM rats as compared with that in NC rats, which could be reduced by fish oil supplementation (Fig. 7A). Besides, the phosphorylated protein expression of NF-κB-p65 in gastrocnemius muscle was highly induced in DM rats, and fish oil markedly reduced the induction to the level even lower than that in NC rats (Fig. 7C). Next, advanced glycation end products (AGEs)/receptor for AGE (RAGE) signaling modulates the inflammatory response and oxidative stress in injured muscles, contributing to sarcopenia [41]. We then investigated the plasma levels of AGEs and muscle protein expression of RAGE. As shown in Fig. 7B, DM rats showed a 4.7-fold increase of AGEs levels, which could be reduced by fish oil supplementation from 34.0 to 19.1 μg/mL. Although muscle RAGE protein expression did not differ between NC and DM rats, supplementation of fish oil significantly decreased RAGE expression as compared to both NC and DM groups (Fig. 7D).

3.7. Fish oil supplementation stimulates the AMPK/SIRT1/PGC-1α pathway

Mitochondrial dysfunction-associated abnormal energy metabolism and oxidative stress in skeletal muscle can lead to muscle atrophy or sarcopenia [42,43]. The signaling cascade mediated by adenosine monophosphate-activated protein kinase (AMPK), sirtuin1 (SIRT1) and peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α) has been reported to act as an energy-

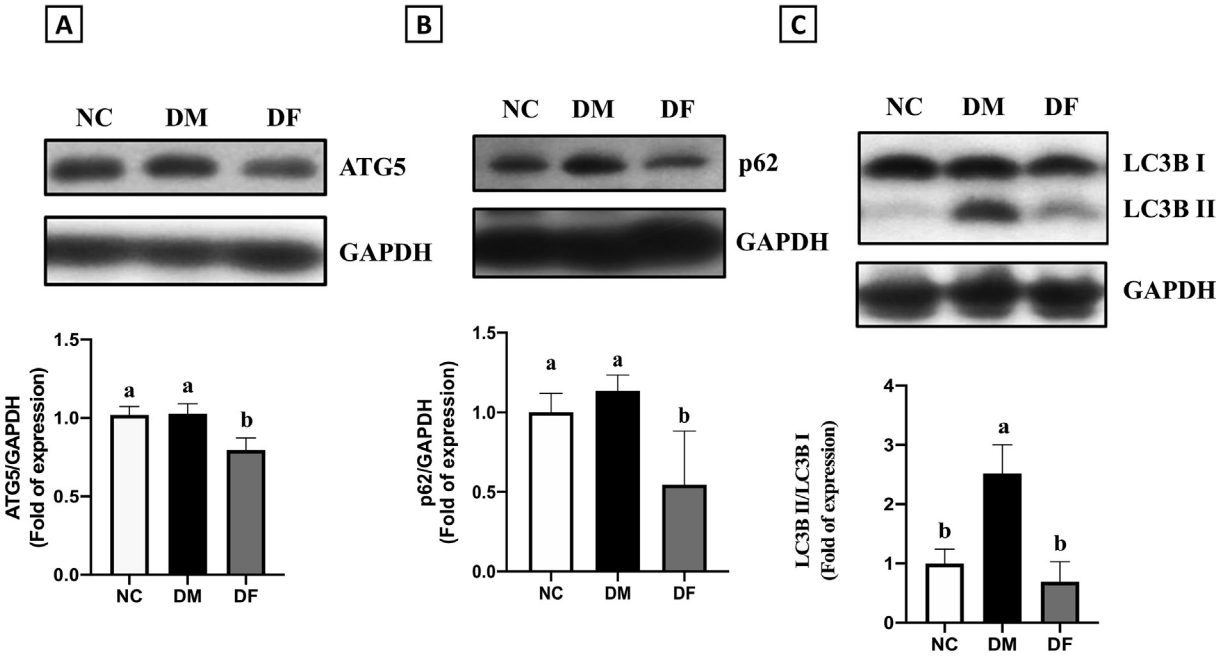

Fig. 5. Effects of fish oil on three protein markers involved in autophagy-lysosome system in rats fed with different diets for 18 weeks. At the end of experiment, the protein expression of ATG5, p62 and LC3B II/LC3B I was measured in the gastrocnemius muscle by Western blotting. GAPDH was used as an internal control. Densitometric analysis for protein levels corrected to each internal control was shown. Results are expressed as mean ± S.D. for each group (n = 4). Different letter indicates statistical significance (p < 0.05). NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.

sensing network that plays a pivotal role in the regulation of mitochondrial biogenesis, energy metabolism, and oxidative stress [44,45]. Hence, the activation of AMPK/SIRT1/PGC-1α pathway might be a possible mechanism of action by which fish oil improves muscle atrophy. The protein expression of the AMPKα, SIRT1 and PGC-1α was then analyzed. We found that all the expression of phosphorylated

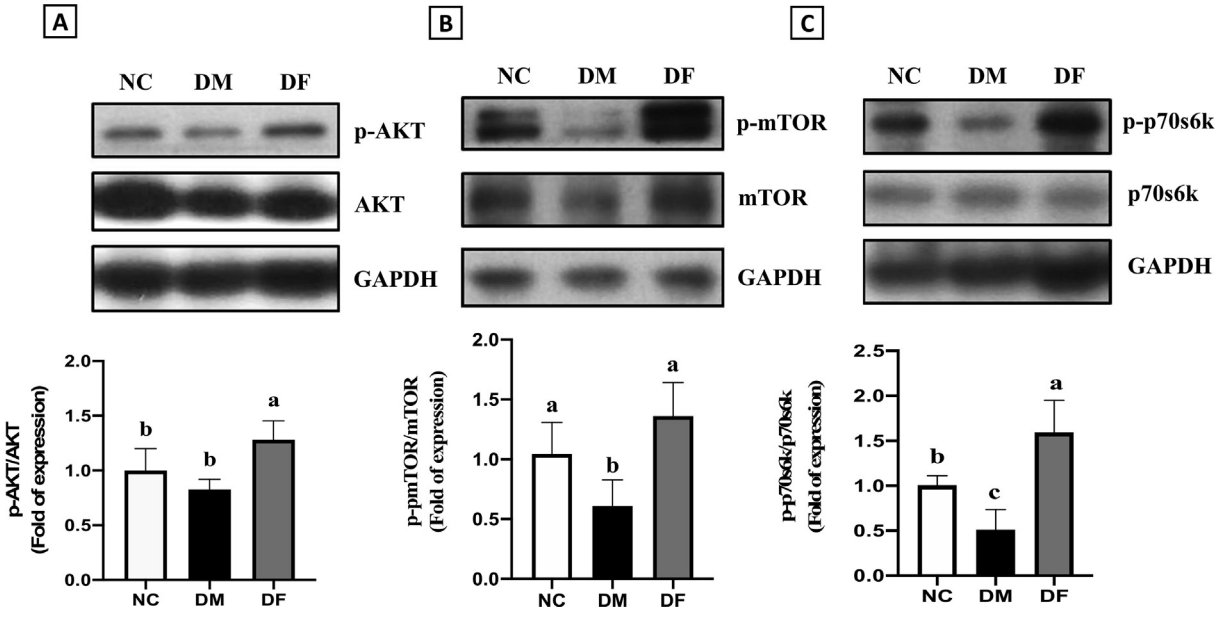

Fig. 6. Effects of fish oil on AKT/mTOR/p70s6k phosphorylation in gastrocnemius muscle from rats fed with different diets for 18 weeks. At the end of experiment, the protein expression of phosphorylated AKT (p-AKT)/AKT (A), phosphorylated mTOR (p-mTOR)/mTOR (B) and phosphorylated p70s6k (p-p70s6k)/p70s6k were measured by Western blotting. GAPDH was used as an internal control. Densitometric analysis for protein levels corrected to each internal control was shown. Results are expressed as mean ± S.D. for each group (n = 4). Different letter indicates statistical significance (p < 0.05). NC, normal control diet; DM, DM in high fat diet with13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.

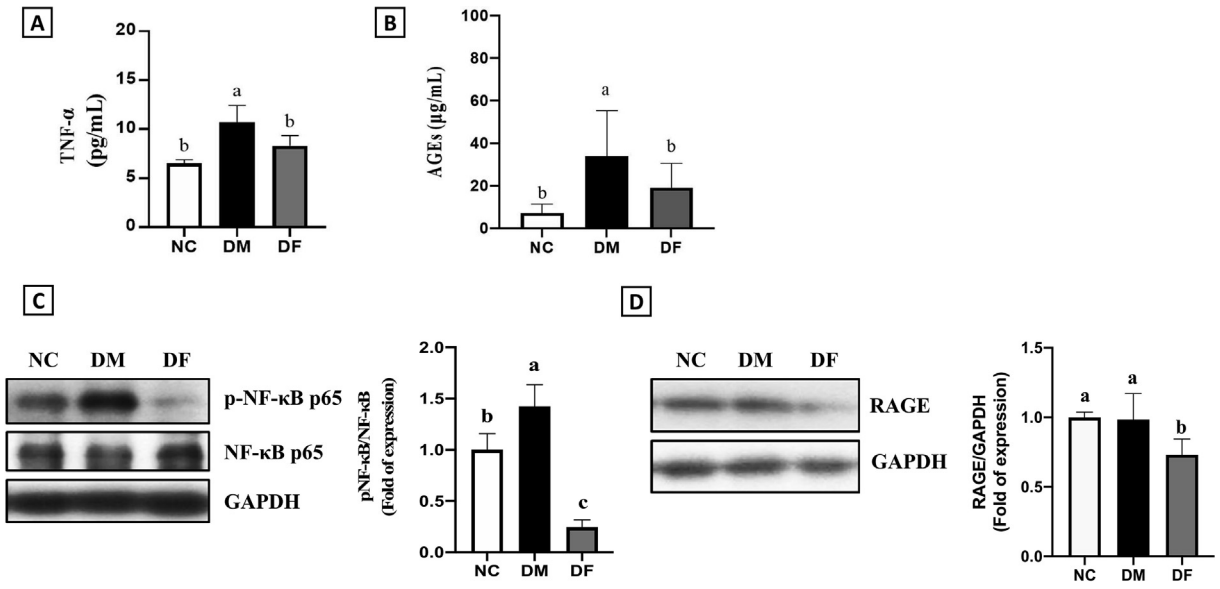

Fig. 7. Plasma TNF-α and advanced glycation end products (AGEs) levels and muscle NF-κB and RAGE protein expression in rats fed with different experimental diets for 18 weeks. ELISA was performed to determine the plasma levels of TNF-α (A) and AGEs (B) (n = 8). Protein expressions of NF-κB and RAGE were measured by Western blotting (n = 4). Densitometric analysis for protein levels corrected to each internal control was shown. Results are expressed as mean ± S.D. for each group. Different letter indicates statistical significance (p < 0.05). NC, Normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.

AMPK α (p-AMPKα), SIRT1 and PGC-1α in gastrocnemius muscle of DM rats decreased as compared to that of NC rats (Fig. 8A–C). The decline of p-AMPKα, SIRT1 and PGC-1α expression in DM rats was significantly recovered by fish oil supplementation, suggesting that improvement of mitochondrial function by regulating AMPKα/SIRT1/PGC-1α signaling pathway might be involved in the effects of fish oil on the muscle atrophy triggered by diabetes.

4. Discussion

This study was designed to test the hypothesis that the positive effect of dietary n-3 PUFAs on diabetes-induced muscle atrophy is associated with improving the balance between protein synthesis and degradation in skeletal muscle. After 18 weeks of high fat diet, the body weight gain was reduced and glucose tolerance was impaired in STZ/HF diet-induced diabetic rats, and these alterations could be prevented by supplementation with fish oil. Although the diabetes-induced loss of skeletal muscle mass was not significantly reversed by fish oil, the skeletal muscle adiposity was improved by fish oil. In addition, diabetic rats showed muscle atrophy with an increase in skeletal muscle protein degradation, as demonstrated by upregulating the UPS- and ALS-related signaling molecules, and a decrease in skeletal muscle protein synthesis by evidence of inhibiting AKT/mTOR/p70s6k signaling. Fish oil supplementation abolished these deleterious signaling pathways and achieved better balance between protein synthesis and degradation. Moreover, diabetes-induced inflammatory signals, such as TNF-α/NF-κB and AGEs/RAGE pathways, were significantly counteracted in diabetic rats supplemented with fish oil. The down-regulation of AMPKα/SIRT1/PGC-1α pathway by diabetes was ameliorated by fish oil as well, suggesting the impaired mitochondrial function was improved by fish oil. It therefore suggests that the beneficial effects of fish oil on diabetes-induced muscle atrophy could be related to its beneficial effects on inflammation and mitochondrial function.

In this study, the fasting glucose levels in NC rats was 226.4 mg/dL, which seems a little bit too high in contrast to existing literature data. Nonetheless, the observation is consistent with our prior studies that the fasting glucose levels of control rats typically fell within 170~220 mg/dL range [30,31,46]. Moreover, the HOMA-IR value in DF group was slightly lower than in the DM group, however, there was no significant difference between the two groups, which was likely due to the fact that the fasting insulin levels did no differ between DM and DF groups, as HOMA-IR is calculated based on the fasting blood glucose and insulin levels. It is known that fasting insulin-based indices reflect not only insulin sensitivity, but also insulin secretion and metabolic clearance [47]. Thus, the evaluation of insulin sensitivity solely based on plasma measurement will

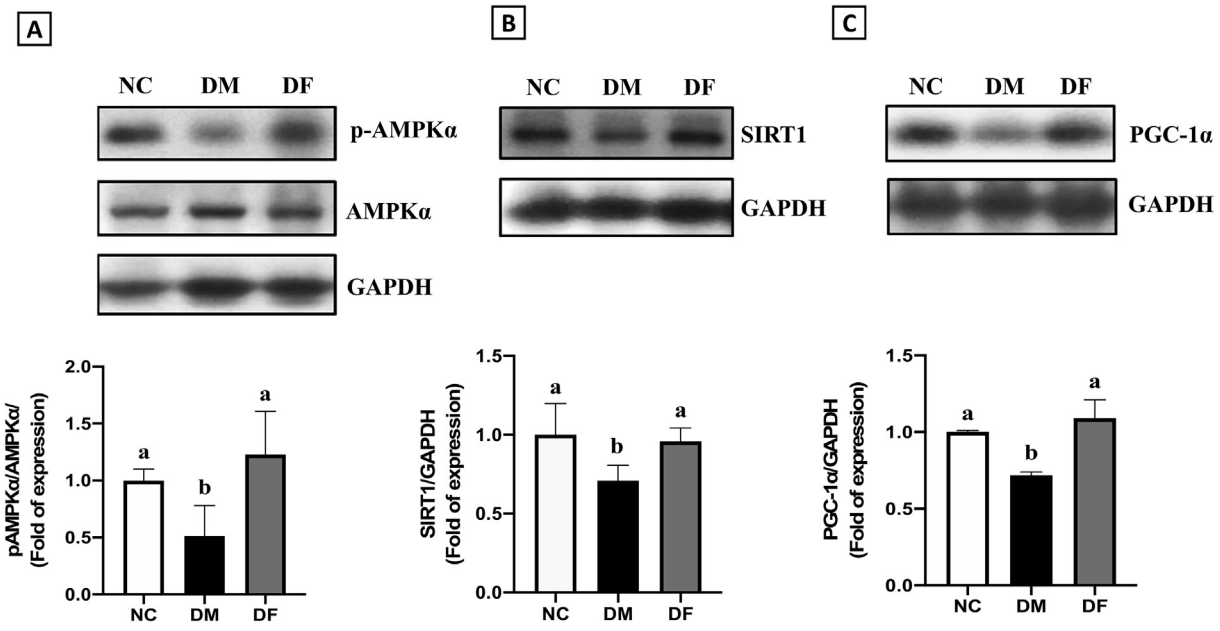

Fig. 8. Effects of fish oil on AMPKα/SIRT1/PGC-1α signaling in gastrocnemius muscle from rats fed with different diets for 18 weeks. The protein expression of phosphorylated AMPKα (p-AMPKα)/AMPKα (A), SIRT1 (B) and PGC-1α (C) were measured by Western blotting. GAPDH was used as an internal control. Densitometric analysis for protein levels corrected to each internal control was shown. Results are expressed as mean ± S.D. for each group (n = 4). Different letter indicates statistical significance (p < 0.05). NC, normal control diet; DM, DM in high fat diet with 13% lard; DF, DM in high fat diet with 8% lard and 5% fish oil.

not provide accurate reflection of β-cell dysfunction in diabetes. OGTT is utilized to assess glucose tolerance in clinical practice and animal research, as well as being a surrogate measure of insulin sensitivity, and applied in evaluating β-cell dysfunction in obesity, prediabetes, and T2DM [48]. Our data showed that diabetes-induced elevation of glucose levels and OGTT parameters were reduced by fish oil supplementation, demonstrating the HF diet substituted with 5% fish oil has beneficial effects on diabetes in our setting. These results are in agreements with observations by others in different rodent models and in human studies that n-3 PUFAs have a positive effect on insulin resistance and glucose homeostasis. In spontaneous diabetes models, such as Otsuka Long-Evans Tokushima Fatty rats and WBN/Kob rats, n-3 PUFAs- EPA had favorable effects on blood glucose and insulin resistance [49,50]. In a high-fat diet-induced obese mice model, EPA prevented and reversed insulin resistance and hyperglycemia via modulating adipose tissue inflammation [51]. In an obese, diabetic KK-Ay mice, long-term colonic delivery of n-3 PUFAs- DHA improved impaired glucose tolerance via GLP-1 secretion [52]. In a sucrose-fed, insulin-resistant rat model, dietary fish oil normalized insulin resistance and adiposity via positively regulating plasma leptin and adiponectin levels [53]. Oh et al. demonstrated that administration of fish oil led to improved insulin sensitivity, enhanced muscle and hepatic insulin sensitivity, and decreased hepatic steatosis in HF diet-fed mice through G protein-coupled receptor 120-mediated pathways [54]. Zhuang et al. also reported that EPA and DHA differentially reversed hyperglycemia and insulin resistance in HF diet-induced obese mice by altering gut microbiome [55]. In overweight T2DM patients, EPA supplementation at doses of 2 g/day for 3 months improved insulin sensitivity and blood glucose [56]. Chen et al. indicated that consumption of fish oil or constant use of fish oil supplements was associated with a lower risk of T2DM [18]. Despite the role of n-3 PUFAs for the prevention and treatment of diabetes in human subjects are inconclusive, most animal studies support a beneficial effect of n-3 PUFAs on insulin sensitivity and glucose metabolism [16,57].

In our STZ/HF diet-induced diabetes model, we did not see the body weight gain during the experiment period. Instead, the diabetic rats exhibited body weight loss since week 5. Notably, body weight change may not be a good indicator of T2DM model, as both T2DM and T1DM will face a significant weight loss with time [58]. Indeed, studies in the literature reported that the body weights of HF diet/STZ-induced diabetic rats may be increased, no change, or decreased following the development of diabetes, as compared to that of

control rats [59–61]. Likewise, normalizing muscle weight by body weight is not appropriate in order to compare the difference of muscle mass between NC and DM groups in this diabetic animal model because the body weight of DM rats was significantly reduced, which is supported by Geng et al. [62]. On the other hand, the analysis of cross-sectional area (CSA) of muscle fibers, including the morphology and distribution of myofiber size in gastrocnemius, soleus, tibialis anterior, and extensor digitorum longus muscles, indicated that fish oil improved the diabetes-altered myofiber morphology and distribution of myofiber size.

Skeletal muscle atrophy is defined as a decrease in muscle mass and fiber size [63]; therefore, improvement of muscle atrophy might be accompanied with gain of muscle mass. In our present study, fish oil supplementation to DM rats showed visibly larger CSA of muscle fibers, as compared with that of DM rats. However, fish oil had no effect on reversing diabetes-induced decline of muscle mass. Notably, the increase of TG content in gastrocnemius muscle of DM rats was reduced by fish oil supplementation, suggesting that fish oil-mediated decrease of muscle adiposity may mask fish oil-mediated increase of muscle protein mass, which resulted in no statistical difference in muscle mass between DM and DF groups. Further studies need to be conducted to clarify this hypothesis.

Maintenance of skeletal muscle mass and function depends on the balance of complicated signaling networks regulating muscle protein synthesis and degradation [9]. In muscle atrophy, the balance shifts towards a catabolic state leading to a net loss of myofibrillar proteins, organelle and cytoplasm causing shrinkage of muscle size, which can be due to activation of the two major intracellular proteolytic systems, such as UPS and ALS, or decrease of protein synthesis [38,64]. Coordinated induction of ubiquitin ligases, such as atrogin-1 and MURF1, by FoxO1 transcription factors are markedly involved in developing UPS-mediated muscle atrophy. Consequently, atrogin-1 and MURF1 are now widely used as markers of UPS-mediated proteolysis. During autophagy, LC3B-I is converted to LC3B-II through lipidation mediated by ATG family, and LC3B-II are incorporated into the expanding membrane of autophagosome. By recurring Atg5/Atg12/Atg16 complex, the mature autophagosome is formed where LC3B-II may function as binding proteins for adapters such as p62, that recruits cellular components for degradation and recycle. Therefore, it is well accepted LC3B-II/LC3B-I ratio, ATG, and p62 could be good biomarkers for evaluating the activation of autophagy. The major anabolic pathway regulating protein synthesis in skeletal muscle is Akt/mTOR/p70s6k signaling, which is also known to further promote protein accumulation by suppressing FoxO transcription factors [64,65]. Here, we showed that not only ubiquitin ligases Astrogen-1 and MuRF1 of the protein degradation pathway but also autophagy markers ATG5, p62, and LC3B-II were activated in diabetic rats. Inhibition of AKT/mTOR/p70s6k signaling was observed in diabetic rats as well. Fish oil was capable of interfering these signaling pathways, and ameliorating muscle protein synthesis/degradation imbalance in diabetic rats. Besides, insulin signaling plays a pivotal role in controlling muscle size, as it potently stimulates protein synthesis by activating PI3K/AKT/mTOR signaling [8], it is conceivable that fish oil supplementation might alleviate muscle protein synthesis in diabetic rats by improving insulin resistance. The anabolic effects of n-3 PUFAs on muscle protein turnover were previously described in many animal models, such as dexamethasone, fasting, and muscle disuse (hindlimb suspension)-induced muscle atrophy, and in pathophysiological conditions including aging, sepsis and cancer cachexia [66–70]. In the present study, we confirm that diabetes-induced muscle atrophy was also improved by fish oil supplementation, and supports the idea that dietary n-3 PUFA ingestion increases the anabolic sensitivity of skeletal muscle, and alleviates the anabolic resistance associated with insulin resistance [26,42].

Supplementation with n-3 PUFAs affects muscle not only directly by regulating muscle protein turnover, but also indirectly by decreasing systemic inflammation, which thus improves the skeletal muscle function and insulin resistance [19,28]. Modulation of inflammation and its related mediators, such as TNF-α, IL-6, AGEs and RAGE, may represent another potential explanation to address the benefits of fish oil on diabetes-mediated muscle atrophy, in addition to controlling protein turnover and insulin signaling. According to previous studies, TNF-α is associated with diabetes-mediated muscle atrophy via NF-κB activation, which in turn transcriptionally increases the expression of MURF1 and astrogen-1 [8,39]. In our study, TNF-α/NF-κB signaling induced by diabetes was blocked in response to fish oil supplementation, indicating the inhibition of TNF-α/NF-κB inflammatory signaling pathway might be one of the mechanisms responsible for the effects of fish oil on muscle atrophy in diabetes. Recently, AGEs and its receptor RAGE signaling have been proposed to be involved in aging- and diabetes-related disease such as sarcopenia, as they induce inflammation and oxidative

stress by intracellular signal transduction such as activation of NF-κB [71–73]. One of the mechanisms underlying the AGE/RAGE axis in muscle atrophy might be through down-regulating AKT signaling, which is mediated by AMPK [74]. AGEs/RAGE axis has also been reported to promote mitochondria dysfunctions which further lead to vicious circle of age-related inflammation and sarcopenia [75]. Our data presented here suggests that the blockade of AGEs/RAGE signaling might be also participated in the benefit of fish oil on diabetes-mediated muscle atrophy, by which the inflammatory response and oxidative stress of skeletal muscle are improved.

Diabetes-induced renal damage and hyperglycemia can be improved by activation of AMPK/SIRT1/PGC-1 signaling pathway [76,77]. Here, we found that, in response to fish oil supplementation, diabetes-induced muscle atrophy might be alleviated by this signaling cascade as well. In skeletal muscle, diabetes causes mitochondrial dysfunction, including decreased mitochondrial biogenesis, impaired quality control, reduced oxidative capacity, inactivated satellite cells, and increased oxidative stress, which may lead to muscle atrophy [78,79]. Martins et al. showed that supplementation with fish oil increased the expression of PGC1-α and AMPK, which are associated with mitochondrial biogenesis and function and resulted in attenuation of obesity and insulin resistance in a HF-diet-fed mouse model [22]. Liu et al. demonstrated that fish oil prevents obesity-induced muscle wasting by activating AMPK/PGC-1α signaling [36]. PGC-1α is also considered to protect skeletal muscle atrophy by suppressing FoxO action [80]. Consistent with our findings, we observed AMPK/SIRT1/PGC-1α signaling was stimulated by fish oil supplementation in diabetic rats, which suggests that improvement of defective mitochondrial function, abnormal energy metabolism and excessive oxidative stress might contribute to the anabolic effects of fish oil on preserving muscle mass. Additional studies are required to better elucidate the role of mitochondria and oxidative stress in the benefits of fish oil on the regulation of diabetes-mediated muscle atrophy.

Although the exact mechanisms by which fish oil or n-3 PUFAs regulates skeletal muscle metabolism during diabetes are not clear, several signaling pathways are proposed to explain the anabolic effects of fish oil on diabetes-induced muscle atrophy. As shown in Fig. 9, both UPS and ALS proteolysis pathways are down-regulated, whereas protein synthesis via AKT/mTOR/p70s6k signaling

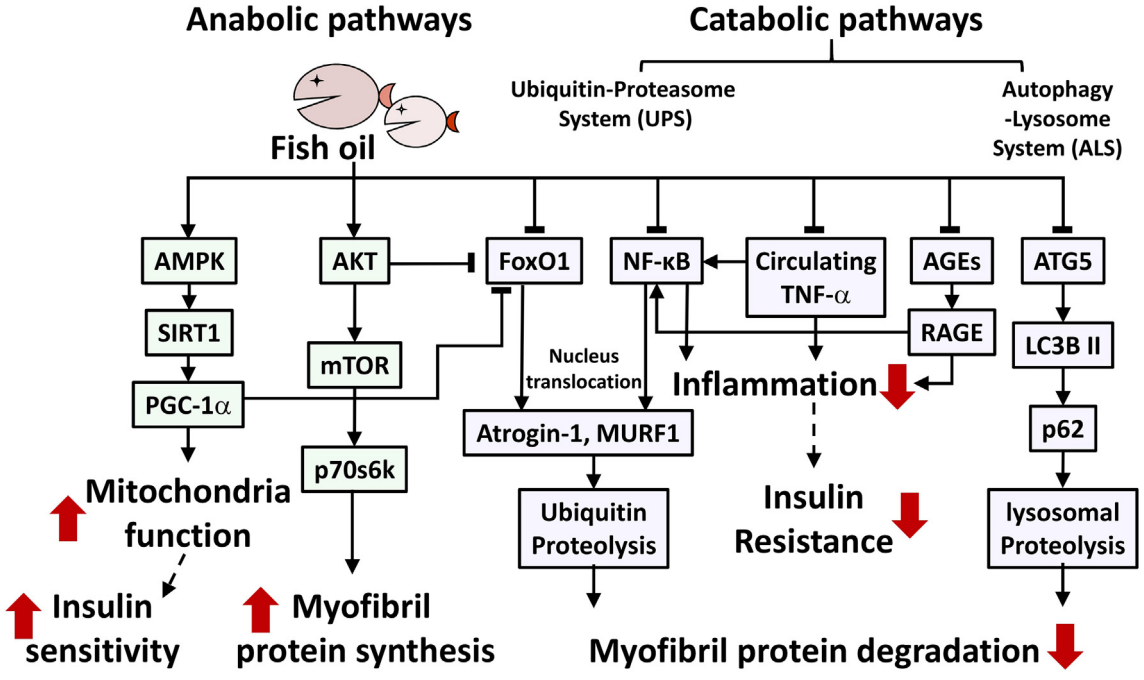

Fig. 9. Proposed molecular mechanisms for the benefits of fish oil on muscle atrophy triggered by diabetes. Fish oil improves skeletal muscle health through enhancing myofibril protein synthesis, reducing myofibril protein degradation (UPS and ALS), relieving inflammation, and improving mitochondria function, which may lead to net attenuate muscle atrophy along with improved insulin sensitivity. Abbreviations: AGEs, advanced glycation end products; AMPK, adenosine monophosphate-activated protein kinase; ATG5, autophagy protein 5; FoxO1, forkhead box O 1 transcription factor; LC3B II, microtubule-associated protein light chain 3 β II; mTOR, mammalian target of rapamycin; MURF1, muscle RING-finger protein 1; p62, p62/sequestosome 1; p70s6k, p70 ribosomal S6 kinase; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 α; RAGE, receptor for AGEs; SIRT1, sirtuin1.

is up-regulated by fish oil, which results in net protein accumulation in skeletal muscle and may compensate the muscle loss induce by diabetes. Activated AKT may deactivate FoxO1 to further mitigate proteolysis. Improvement of inflammatory environment by inhibiting TNF-α/NF-κB and AGEs/RAGE signaling is also participated in the positive effects of fish oil on diabetes-induced muscle atrophy. Additionally, some effects of fish oil appear to be mediated by amelioration of mitochondria-associated energy metabolism and oxidative stress via activating AMPK/SIRT1/PGC-1α pathway. PGC-1α may further block FoxO-induced atrophy program. Insulin resistance can thus be improved by the reduced inflammatory state and the enhanced mitochondrial function of skeletal muscle.

The study has potential limitations. The experimental period seems not long enough to see muscle mass change by fish oil; hence, increasing the dosage or extending the feeding duration of fish oil may be a good way to see whether the absolute muscle mass can be considerably enhanced by fish oil. Besides, utilizing different diabetic model might be an alternative approach to further examine the anabolic effects of fish oil. Overall, our study offers some insight which may inspire researchers to further explore the potential of fish oil on improving muscle health in individuals with diabetes.

5. Conclusion

Diabetes, sarcopenia and diabetes-associated sarcopenia are huge challenges to the health and economy worldwide. In order to maintain and improve their quality of life, it is important to delay or decrease the extent of muscle loss and weakness in the elderly, especially those with diabetes. It is well accepted that maintaining or improving muscle mass is an effective way to manage blood glucose, as skeletal muscle is the protein reservoir in our body playing a pivotal role in glucose and lipid homeostasis. In the study, we demonstrate that the deleterious effects induced by diabetes on impaired glucose tolerance and skeletal muscle atrophy are attenuated, at least partially, in response to fish oil supplementation. The beneficial effects of fish oil on diabetes-mediated muscle atrophy are mainly achieved by ameliorating the imbalance between catabolic and anabolic pathways. Intake of n-3 PUFAs-enriched fish oil may be a good supplementation option for elderly patients with diabetes to help maintain muscle health, and delay the development of sarcopenia.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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