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Original Article

Potential nutritional strategies to prevent and reverse sarcopenia in aging process: Role of fish oil-derived ω-3 polyunsaturated fatty acids, wheat oligopeptide and their combined intervention

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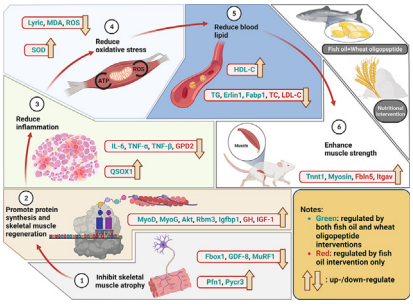
HIGHLIGHTS

- Nutritional support is an essential step to prevent muscle loss in aging process.
- Fish oil and wheat oligopeptide had protective effects against sarcopenia in aging.
- The combined intervention inhibits skeletal muscle atrophy by regulating protein expression.
- The combined intervention promotes protein synthesis and skeletal muscle regeneration.
- The combined intervention also reduces inflammation, oxidative stress and blood lipid.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Nutritional support is potentially considered an essential step to prevent muscle loss and enhance physical function in older adults.
Objectives: This study aimed to assess the role of potential nutritional strategies, i.e., fish oil-derived ω-3 polyunsaturated fatty acids (PUFAs), wheat oligopeptide and their combined intervention, in preventing and reversing sarcopenia in aging process.
Methods: One hundred 25-month-old Sprague-Dawley rats were randomly divided into 10 groups, and 10 newly purchased 6-month-old rats were included in young control group (n = 10). Fish oil (200, 400 or 800 mg/kg body weight), wheat oligopeptide (100, 200 or 400 mg/kg body weight), fish oil + wheat oligopeptide (800 + 100, 400 + 200 or 200 + 400 mg/kg body weight) or the equal volume of solvent were administered daily by gavage for 10 weeks. The effects of these interventions on natural aging rats were evaluated.
Results: All intervention groups had a significant increase in muscle mass and grip strength and reduction in perirenal fat weight when compared to the aged control group ($P < 0.05$). The results of biochemical parameters, magnetic resonance imaging, proteomics and western blot suggested that the combination

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of wheat oligopeptide and fish oil-derived ω-3 PUFA, especially group WFM 2 (400 + 200 mg/kg body weight fish oil + wheat oligopeptide), was found to be more effective against aging-associated muscle loss than single intervention. Additionally, the interventions ameliorated fatty infiltration, muscle atrophy, and congestion in the intercellular matrix, and inflammatory cell infiltration in muscle tissue. The interventions also improved oxidative stress, anabolism, hormone levels, and inflammatory levels of skeletal muscle.

Conclusions: The combination of fish oil-derived ω-3 PUFA and wheat oligopeptide was found to be a promising nutritional support to prevent and reverse sarcopenia. The potential mechanism involved the promotion of protein synthesis and muscle regeneration, as well as the enhancement of muscle strength.

Introduction

The aging of populations, which characterizes the epidemiological trends of our generation, is an incontrovertible trend in both the developing and the developed worlds [1]. Aging causes an imbalance between muscle protein anabolism and catabolism due to a blunted muscle protein synthetic response to protein ingestion [2], thus may contribute to the onset of sarcopenia, an age-related disease characterized by a progressive loss of skeletal muscle mass, strength and function [3]. The pathogenesis of sarcopenia involves various potential mechanisms, including intrinsic factors within skeletal muscle and extrinsic factors in systemic environments (e.g., inflammation, oxidative stress, endocrine, nutrition, immobility) [3–5]. Sarcopenia was recognized as an independent condition with an International Classification of Diseases, 10th revision, Clinical Modification (ICD-10-CM) code M62.84 by the World Health Organization (WHO) in 2016 [6], and has recently attracted attention because the aged population increases and sarcopenia complicates many age-related diseases, reduces quality of life, and increases mortality [7]. Currently, standard diagnostic criteria of sarcopenia have been established by the European Working Group on Sarcopenia in Older People (EWGSOP) and the Asian Working Group for Sarcopenia (AWGS) [8,9]. However, no medications for sarcopenia have been FDA-approved yet [7]. Therefore, it is necessary to develop more effective intervention strategies against sarcopenia by combining nondrug therapies with exercise and nutritional intervention [10].

Nutritional support is potentially considered an initial step to delay the progression of muscle loss and enhance physical performance in older adults, such as a recommended high protein intake [10–12]. However, aging may cause reduced digestive function, declines in mucosal surface area and numerical reduction of the enterocytes in the small intestinal mucosa, resulting in a reduction of nutrients digestibility and utilization [13]. Hence, it is important for older adults to consume adequate protein that is easy to digest and absorb. Wheat oligopeptides are a kind of bioactive oligopeptides hydrolyzed from wheat protein, which have been demonstrated several biological functions including antioxidant, blood lipid reduction and anti-inflammation [14]. The main components include glutamine, leucine, proline, tryptophan and serine, which are essential for muscle protein synthesis and maintenance [15–19]. Our previous study indicated that wheat oligopeptide intervention can enhance the protein absorption and utilization and improve gastrointestinal morphology in rats by promoting the growth of epithelial cells of the gastrointestinal tract and upregulating the activities of aminopeptidase and Na + -K + -ATPase in small intestinal mucosa [20]. As a kind of small-molecule oligopeptide, wheat oligopeptides are easy to digest and absorb, safe to eat for older adults, and were found to enhance the intestinal mucosal barrier and have protective effect on small intestine [21–23]. Therefore, wheat oligopeptide supplementation may have the potential to promote the balance between muscle protein synthesis and breakdown, and can be studied if it could be used in the intervention strategies for sarcopenia prevention.

Fish oil-derived ω-3 polyunsaturated fatty acids (PUFAs), i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are recommended to be used for improving muscle strength and functional capacity as the supplementation with fish oil has demonstrated potential efficacy for enhancing muscle mass and function in older adults [24–27]. Our previous randomized, double-blind, placebo-controlled trial also demonstrated that 6-month supplementation with fish oil-derived ω-3 PUFA beneficially affected the body composition, muscle strength, physical performance and serum lipid profiles in older adults [28]. Thus, fish oil-derived ω-3 PUFA supplementation could be taken into considerations in the endeavor to establish strategies and recommendations to prevent or reverse sarcopenia.

Wheat oligopeptide and fish oil-derived ω-3 PUFA are different kinds of nutritional substances, thus their functional mechanisms may vary and has potential to elicit a joint effect. In this study, we established an animal model of aging to explore the effects of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combined intervention on muscle atrophy and fatty infiltration, so as to find the optimal intervention scheme and investigate the related mechanism by proteomics. Given that sarcopenia is a progressive syndrome characterized by a decline in skeletal muscle mass, strength, and function with aging, we aimed to evaluate the potential of nutritional intervention in reversing sarcopenia by addressing these aspects.

Materials and methods

Animals and study design

One hundred and sixty male Sprague-Dawley (SD) rats aged 6 months were purchased from Animal Core Facility of Nanjing Medical University (SCXK (Su) 2016–0002), raised with a 12/12-h light/dark cycle, an ambient temperature of 22 ± 2 °C and a relative humidity of 50 ± 5% in the Southeast University Laboratory Animal Center (SCXK (Su) 2021–0022). Rats were given free access to food and drinking water. Of the 160 rats, 150 were raised to 25 months old and used as animal model of aging, and 10 were newly purchased and used as young control (YC) group. Based on the previous studies for animal models of sarcopenia, 25-month-old rats are recognized sarcopenia model rats according to Edstrom's Sarcopenia Index [29,30]. Of the 150 25-month-old rats, 50 were excluded because of spontaneous tumors or other diseases. Hence, 100 aged rats were randomly divided into 10 groups including aged control group (AC), fish oil low dose group (FO L), fish oil median dose group (FO M), fish oil high dose group (FO H), wheat oligopeptide low dose group (WP L), wheat oligopeptide median dose group (WP M), wheat oligopeptide high dose group (WP H), wheat

oligopeptide and fish oil mixture group 1 (WFM 1), wheat oligopeptide and fish oil mixture group 2 (WFM 2), and wheat oligopeptide and fish oil mixture group 3 (WFM 3).

Wheat oligopeptide was provided by China National Research Institute of Food & Fermentation Industries (Beijing, China). The preparation, manufacturing process and product properties have been previously described in detail [21,22]. The fish oil was manufactured and provided by the Zhanwang Company (Shanghai, China), and 100 g fish oil contains 70 g EPA + DHA.

According to the 2013 Chinese Dietary Reference Intakes (DRIs), the acceptable macronutrient distribution range (AMDR) of EPA + DHA for adults is 0.25 ~ 2 g/d. The low, median and high dosages were equivalent to 10, 20 and 40 times the recommended dosage for a 60 kg adult. There is no DRIs for wheat oligopeptide intake. Therefore, based on the DRIs, Technical Standards for Testing & Assessment of Health Food (2003 edition) and the dosage of wheat oligopeptide used in the previous study [21,22], FO L, FO M and FO H were administered 200 mg/kg, 400 mg/kg and 800 mg/kg body weight fish oil via gavage; WP L, WP M and WP H were administered 100 mg/kg, 200 mg/kg and 400 mg/kg body weight wheat oligopeptide via gavage; WFM 1, WFM 2 and WFM 3 were administered 800 mg/kg + 100 mg/kg, 400 mg/kg + 200 mg/kg and 200 mg/kg + 400 mg/kg body weight fish oil + wheat oligopeptide via gavage. The solvent was 0.05% sodium carboxymethyl cellulose. YC and AC were administered equal volume of solvent. The intervention time was 10 weeks.

Grip strength test

After 10-week intervention, grip strength of each rat was evaluated using a grip strength meter (Shanghai XinRuan Information Technology Co., Ltd, Shanghai, China). Each rat was placed carefully on the grip strength meter and allowed to grasp a trapeze connected to a force transducer. Then the rat was pulled back gently by holding its tail with rising force until it lost grip. This measurement obtained the peak grip strength force attained. For each rat, the measurement was repeated three times with a two-minute rest between rounds, and the mean value was calculated and recorded.

Magnetic resonance imaging (MRI) measurement

After 10-week intervention, muscle and fat status of rats were examined using a Biospec 7 T/20 USR MRI system (Bruker Biospin, Ettlingen, Germany). Rats were anesthetized with 4 mL/kg intraperitoneal injections of 5% chloral hydrate and then maintained with 1–2% isoflurane anesthesia. Rats were placed prone on the track and fixated by medical adhesive tapes. Each rat was pushed gently into an accurate location of the measuring region. T1-weighted cross-sectional MR images were collected and the maximum cross-sectional area (CSA) of quadriceps femoris was assessed. Measurements and data process including water signal suppression, Fourier transform, Hanning filter, baseline correction, curve fitting, and phase correction of nuclear magnetic resonance spectra, were conducted by ImageJ package.

Sample collection

On the last day of the experiment, rats were fasted for 12 h, but were allowed to drink water freely. All the rats were sacrificed under anesthesia. Their blood samples (via the femoral artery) were centrifuged at 3000 rpm, 4 ℃ for 15 min to obtain separated serum samples. After the rats were euthanized, samples of heart, liver, kidney, perirenal fat, quadriceps femoris, gastrocnemius, tibialis anterior muscle, metatarsal muscle, subcutaneous fat, and perirenal fat were collected and weighed. Small pieces of quadriceps femoris and perirenal fat were cut and fixed in 4% paraformaldehyde solution. The rest of the tissues were immediately frozen in liquid nitrogen, and then stored in −80 ℃ refrigerator.

Histopathological analysis

Quadriceps femoris and perirenal fat fixed in 4% paraformaldehyde solution were dehydrated and embedded in paraffin. Then paraffin sections were cut into slices of 5 μm in thickness and observed using a microscope (Olympus BX41, Tokyo, Japan) after hematoxylin-eosin staining. Myofiber CSA of quadriceps femoris was evaluated by ImageJ. Size and number of adipocytes were quantified at a magnification of 400 times (number/0.57 mm^2/400 ×).

Determination of biochemical parameters in serum and skeletal muscle samples

Serum levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined based on Beckman Coulter UniCel DxC 800 Synchron Clinical Systems (Beckman Coulter, Brea, CA, USA). Muscle-specific F-box protein Atrogin-1 (Fbox-1), myostatin/growth differentiation factor 8 (GDF-8), muscle specific ring finger protein 1 (MuRF-1), myogenic differentiation antigen (MyoD), myogenin (MyoG), growth hormone (GH), insulin-like growth factor 1 (IGF-1), reactive oxygen species (ROS), superoxide dismutase (SOD), malondialdehyde (MDA), interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) in skeletal muscle were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (JRDUN Biotechnology Co., Ltd, Shanghai, China) in strict accordance with manufacturer's instructions.

Proteomic analysis

Proteomic analysis was used to evaluate the differentially expressed proteins in quadriceps femoris samples of FO M, WP M, WFM 2 and AC groups. The process of sample preparation for proteomic analysis included protein extraction, bicinchoninic acid (BCA) assay, acetone precipitation, redissolve, reduction, alkylation, protein digestion, Tandem Mass Tag (TMT) labeling, SDC cleanup, peptide desalting, and high-pH pre-fractionation. Peptides were then collected, separated, and further analyzed by proteomics-based technologies. The detailed information of NanoLC-MS/MS analysis, MS data analysis and bioinformatics analysis has been described previously [31]. The screening criteria for differentially expressed proteins were P-value < 0.05 and fold change < 0.83 or fold change > 1.2.

Western blotting

Based on the results of proteomic analysis, differentially expressed candidate proteins were further selected for verification of the difference between intervention groups and control groups via western blot analysis. The candidate proteins included adhesion protein (Lyric), integrin (Itgav), fibronectin 5 (Fbln5), glycerol-3-phosphate dehydrogenase, mitochondrial (GPD2), RNA-binding motif protein 3 (Rbm3), protein kinase B (Akt), pyrroline-5-carboxylate reductase 3 (Pycr3), fatty acid-binding protein (Fabp1), insulin-like growth factor-binding protein (Igfbp1), myosin, profilin-1 (Pfn1), ER lipid raft-associated 1 (Erlin1), sulfhydryl oxidase 1 (QSOX1), troponin T (Tnnt1). Quadriceps femoris samples were lysed in a RIPA lysis buffer and the concentration of protein was determined via the BCA protein assay. The

western blot procedures have been described previously [22]. The densities of bands were determined by ImageJ.

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (SPSS, Chicago, IL, USA). The data were expressed as mean ± standard deviation. The difference among groups was evaluated by one-way ANOVA. Statistical significance was considered as P value < 0.05. Figures were created by OriginPro 2021 and BioRender (BioRender.com).

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the Ethics Committee on the Care and Use of Laboratory Animals of Southeast University (approval number: SCXK (Su) 2021–0022).

Results

Effects of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination on body weight, grip strength, organ weight and muscle mass of aged rats

Changes of body weight and daily amount of food intake of different groups during the intervention time are shown in Supplementary Figure S1. Before the intervention, there was no significant difference in body weight between groups of 25-month-old rats, whereas the body weight of 6-month-old YC rats was significantly lower than that of other groups. Supplementary Figure S2 shows that after 10-week intervention, the body weight of YC group increased and became significantly higher than that of AC group. The body weight of rats in WP M, WP H, WFM 1, WFM 2 and WFM 3 groups were significantly higher than that of rats in AC group, whereas their body weight was not significantly different from the body weight of YC group. The grip strength of all the intervention groups were significantly elevated when compared with AC group. There was no significant difference in the weight of heart, liver and kidney between intervention groups and AC group. The perirenal fat weight of all the intervention groups were significantly reduced when compared with AC group, whereas the muscle weight of all the intervention groups were significantly elevated when compared with AC group. Furthermore, it is likely that wheat oligopeptide intervention had dose-dependent effect against loss of muscle mass. The combined intervention effect of wheat oligopeptide and fish oil-derived ω-3 PUFA on aging-associated muscle loss may be better than that of single intervention.

Effects of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination on morphology and pathology of skeletal muscle

As shown in the MRI results in Fig. 1A, compared with YC group, fatty infiltration and muscle atrophy could be established in AC group, while the interventions of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination may ameliorate the status to some extent. As shown in Fig. 1B, the muscle cells of YC group were uniform in size and the myofiber CSA of quadriceps femoris was relatively large. However, the result of AC group shows that the myofiber CSA decreased dramatically, many lipid droplets were in the muscle tissue, with congestion in the intercellular matrix and infiltration of a large number of inflammatory cells. After 10-week intervention, the number and size of lipid droplets in muscle tissues of intervention groups were significantly reduced, and the congestion in the intercellular matrix and infiltration of inflammatory cells were alleviated. Fig. 1C and 1D indicate that the maximum CSA and myofiber CSA of quadriceps femoris of AC group were significantly lower than those of YC group. After 10-week intervention, the maximum CSA and myofiber CSA of quadriceps femoris of all the intervention groups apart from FO L group were significantly elevated when compared with AC group. Additionally, there was no difference in both the maximum CSA and the maximum myofiber CSA of quadriceps femoris between WFM 2, WFM 3 and YC group.

Effects of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination on pathology of white adipose tissue and serum lipid levels

As shown in Fig. 2A and 2B, at a magnification of 400 times, the number of white adipocytes decreased and the size of white adipocytes increased in AC group when compared with YC group, with congestion in the intercellular matrix and infiltration of inflammatory cells. The interventions of fish oil-derived ω-3 PUFA, wheat oligopeptide, and especially their combination may alleviate the above status to some extent. Fig. 2C indicates that when compared with AC group, the serum levels of HDL-C and TG of all the intervention groups apart from WP L group were significantly elevated and decreased, respectively. The interventions of fish oil-derived ω-3 PUFA and the combination of fish oil-derived ω-3 PUFA and wheat oligopeptide significantly decreased the serum levels of LDL-C and TC, whereas the intervention of wheat oligopeptide alone may have no impact on them.

Effects of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination on oxidative stress level, anabolism, hormone level and inflammatory level of skeletal muscle

Fig. 3A, 3B, 3C and 3E show that the interventions of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination significantly improved the biomarkers of oxidative stress, anabolism and inflammatory level of skeletal muscle when compared with AC group, and the combined intervention effect of wheat oligopeptide and fish oil-derived ω-3 PUFA especially WFM 2 group on the above biomarkers may be better than that of single intervention. Fig. 3D shows that the interventions of fish oil-derived ω-3 PUFA and the combination of fish oil-derived ω-3 PUFA and wheat oligopeptide significantly increased the levels of GH and IGF-1, whereas the intervention of wheat oligopeptide alone had no significant impact on them.

Proteomic analysis of the differences between FO M group and AC group and validation of proteomic results

Proteomic results of the differences between FO M group and AC group are shown in Fig. 4. We found 81 significant differentially expressed proteins from FO M vs. AC, 54 up-regulated and 27 down-regulated (Fig. 4A). The majority of the significant differentially expressed proteins were found to be either increased or decreased and the differential expression patterns of these proteins were visualized via heatmap (Fig. 4B). To better elucidate the functional diversity of the fish oil-derived ω-3 PUFA intervention, we annotated protein functions with currently available databases, including clusters of orthologous groups of proteins (COG) and KEGG modules. Based on the distribution of COG functions that matched the differentially expressed proteins, the main COG categories with a known function were involved in "Cytoskeleton", "Signal transduction mechanisms", "Posttranslational modification, protein turnover, chaperones", "Lipid transport and metabolism", and "Defense mechanisms" (Fig. 4C). The top 18

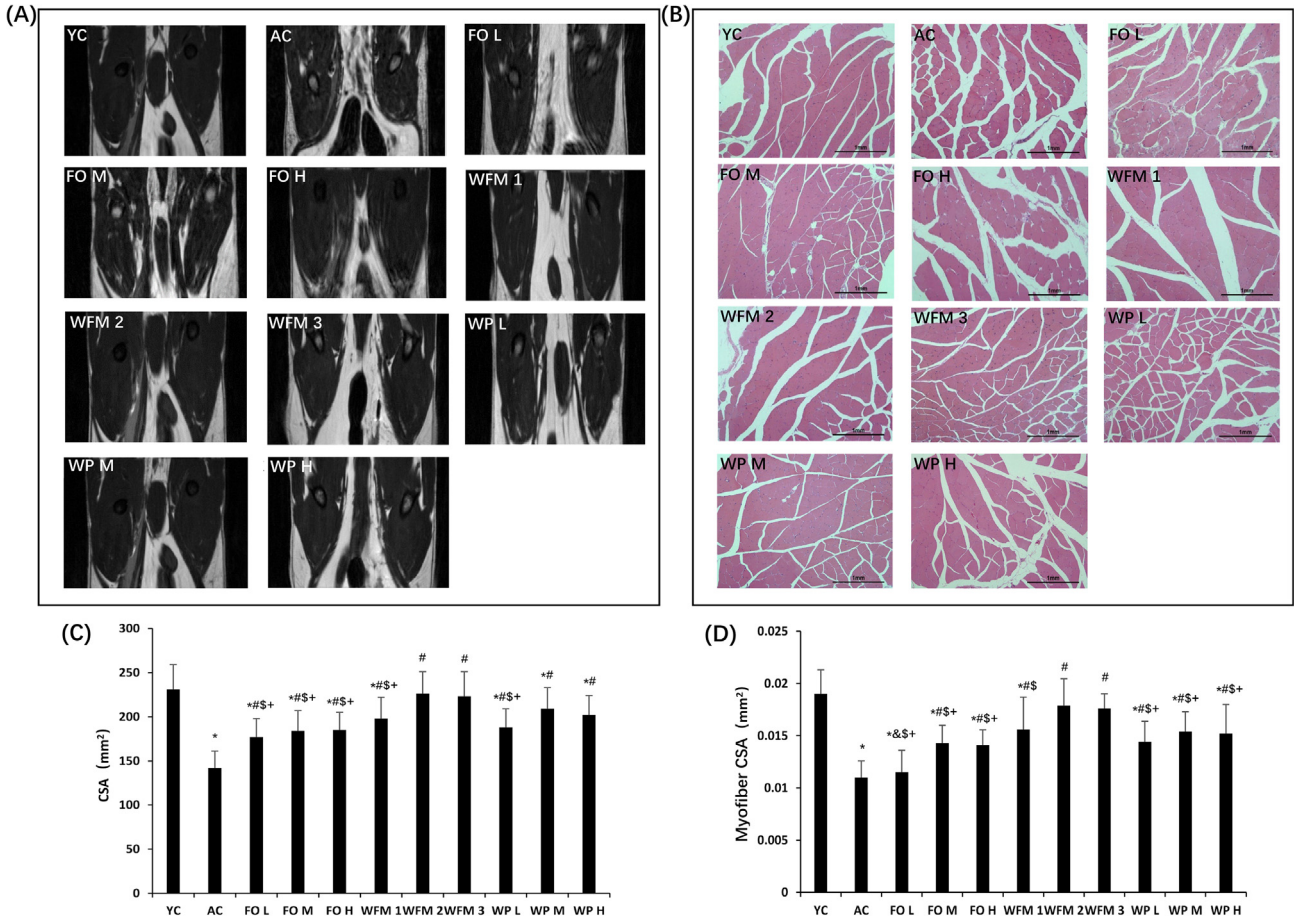

Fig. 1. **(A)** MRI results and **(B)** pathology of skeletal muscle of each group; **(C)** maximum CSA, and **(D)** maximum myofiber CSA of quadriceps femoris of each group after 10-week intervention. *P < 0.05 when compared with YC group; #P < 0.05 when compared with AC group; &P < 0.05 when compared with WFM 1 group; \$$P$ < 0.05 when compared with WFM 2 group; +P < 0.05 when compared with WFM 3 group.

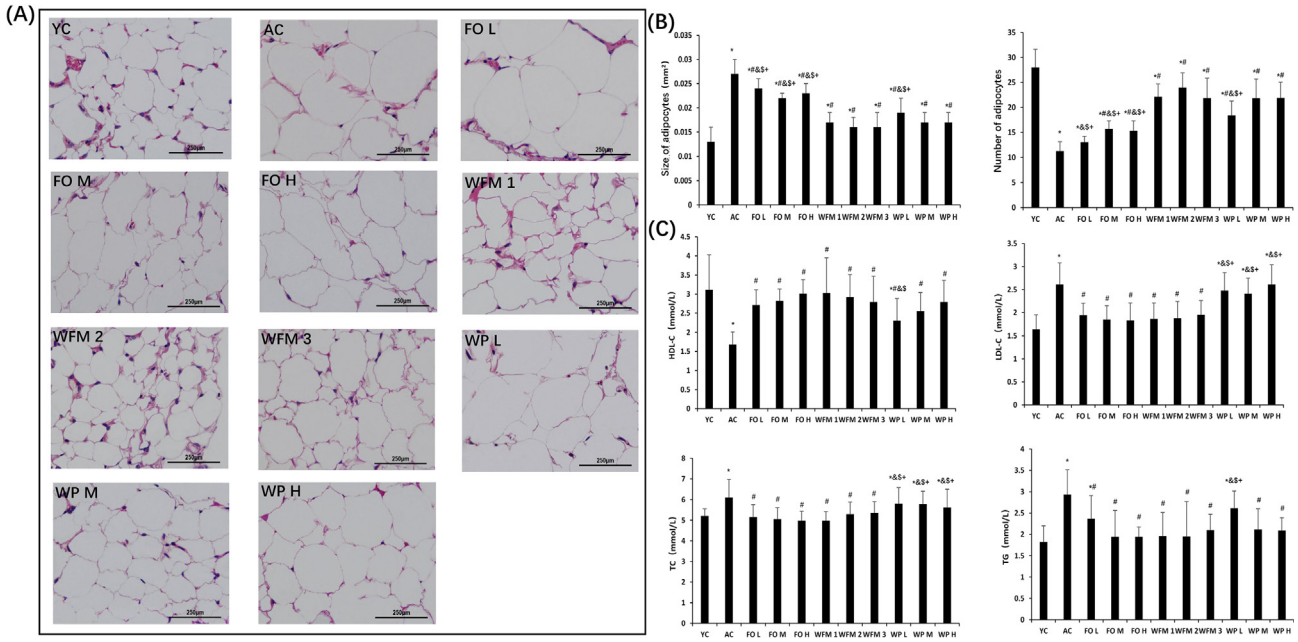

Fig. 2. **(A)** Pathology of white adipose tissue of each group; **(B)** size, and number of adipocytes at a magnification of 400 times; **(C)** serum levels of HDL-C, LDL-C, TC, and TG of each group after 10-week intervention. *P < 0.05 when compared with YC group; #P < 0.05 when compared with AC group; &P < 0.05 when compared with WFM 1 group; \$$P$ < 0.05 when compared with WFM 2 group; +P < 0.05 when compared with WFM 3 group.

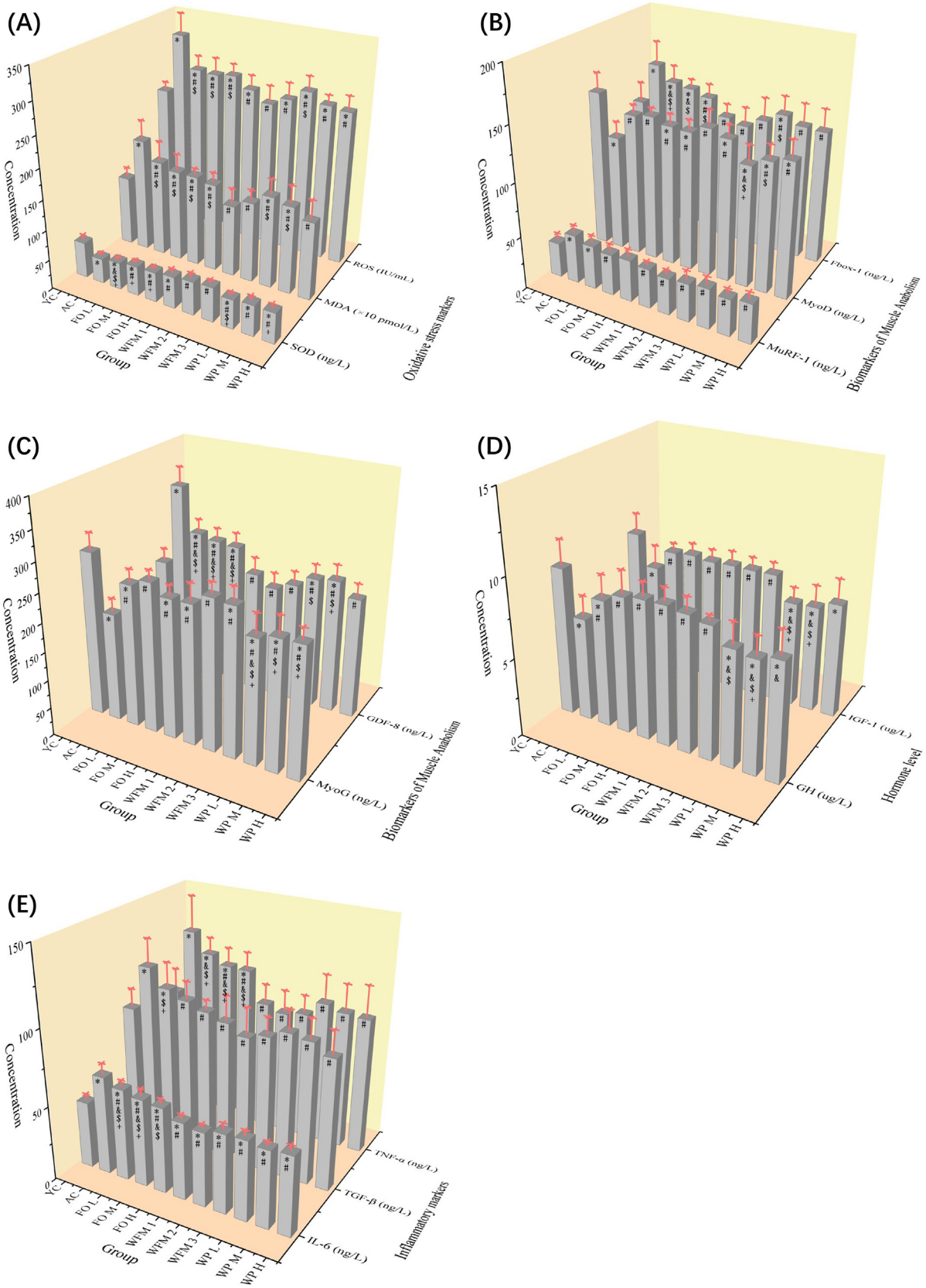

Fig. 3. **(A)** Oxidative stress markers including SOD, MDA and ROS ; **(B)** and **(C)** biomarkers of muscle anabolism including MuRF-1, MyoD , Fbox-1, MyoG and GDF-8; **(D)** levels of hormone including GH and IGF-1; **(E)** inflammatory markers including IL-6, TGF-β and TNF-α in skeletal muscle. *$P < 0.05$ when compared with YC group; #$P < 0.05$ when compared with AC group; &$P < 0.05$ when compared with WFM 1 group; \$$P < 0.05$ when compared with WFM 2 group; +$P < 0.05$ when compared with WFM 3 group.

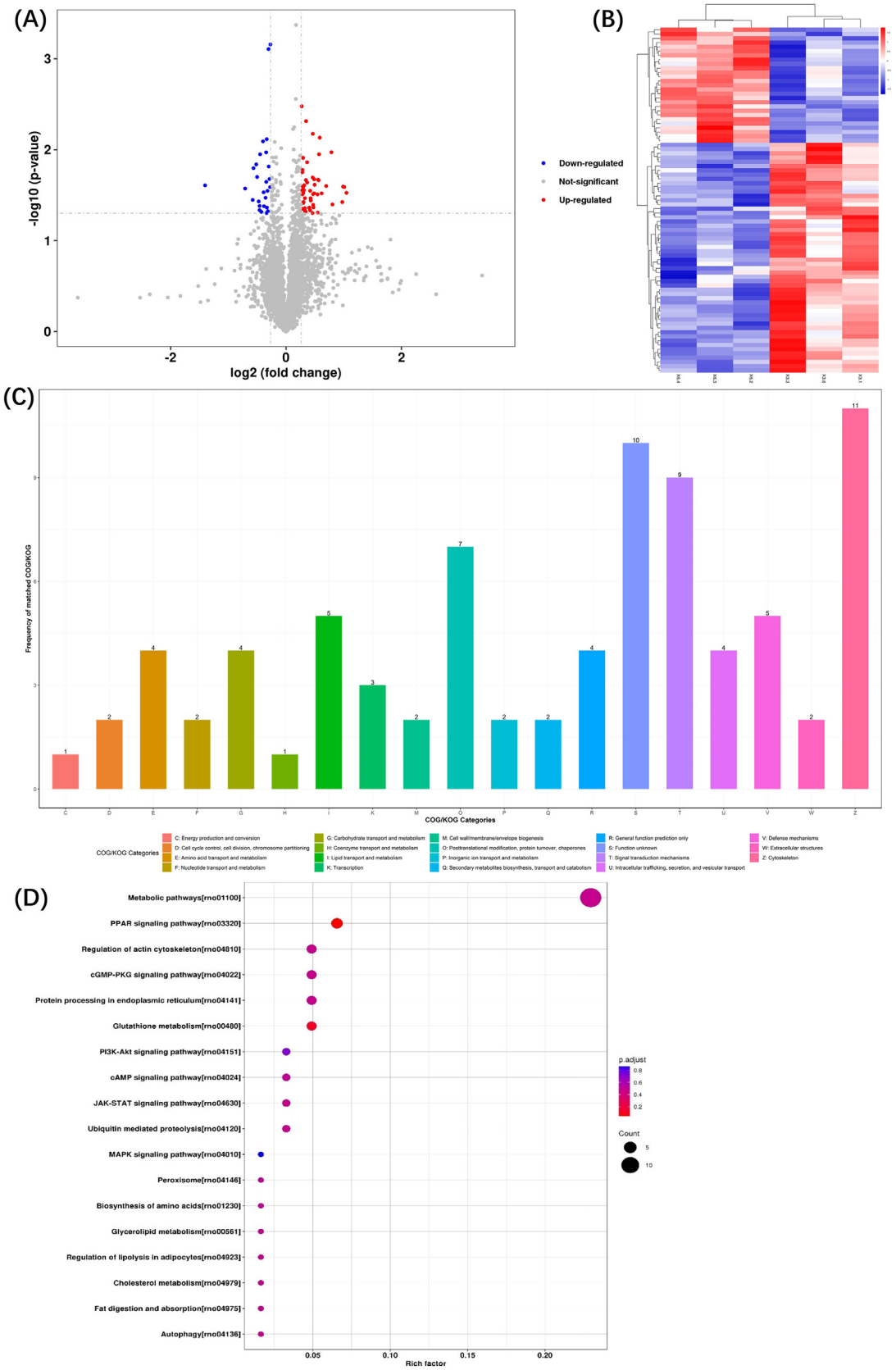

Fig. 4. Proteomic results of the differences between FO M group and AC group. **(A)** Volcano plot for summarizing the results of differential analysis. These points indicate different proteins that display both large magnitude fold-changes (x axis) and high statistical significance (-log10 of *P* values, y axis). Dashed horizontal line shows the *P* values cutoff, and the two vertical dashed lines indicate down/up regulated proteins; **(B)** clustering heatmap of the significant proteins in comparison of AC group; **(C)** COG classification and functional annotation of the differentially expressed proteins in comparison of AC group; **(D)** KEGG enrichment scatter plot. The Rich factor is the ratio of differentially expressed protein numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the Rich factor, the greater the degree of pathway enrichment. A lower adjusted *P* value indicates greater pathway enrichment.

KEGG pathways of differentially expressed proteins in the comparison between FO M group and AC group are shown in Fig. 4D.

Based on the results of proteomic analysis, we further verified 10 differentially expressed candidate proteins via western blot analysis (Fig. 5). The results indicated that compared with YC group, the aging progress significantly up-regulated the expression of Lyric, Erlin1, GPD2 and Fabp1, and down-regulated the expression of Itgav, Fbln5, QSOX1, GAPDH, Igfbp1 and Myosin, while the fish oil-derived ω-3 PUFA intervention alleviated the regulation of protein expression caused by aging. The results were consistent with those of proteomics.

Proteomic analysis of the differences between WP M group and AC group and validation of proteomic results

Proteomic results of the differences between WP M group and AC group are shown in Fig. 6. We found 48 significant differentially expressed proteins from WP M vs. AC, 32 up-regulated and 16 down-regulated (Fig. 6A). The differential expression patterns of these proteins were visualized via heatmap (Fig. 6B). Based on the distribution of COG functions that matched the differentially expressed proteins, the main COG categories with a known function were involved in "General function prediction only", "Signal transduction mechanisms", "Transcription", "Posttranslational modification, protein turnover, chaperones", "Secondary metabolites biosynthesis, transport and catabolism", and "Cytoskeleton" (Fig. 6C). The top 18 KEGG pathways of differentially expressed proteins in the comparison between WP M group and AC group are shown in Fig. 6D.

Based on the results of proteomic analysis, 9 differentially expressed candidate proteins were further verified via western blot analysis (Fig. 7). The results indicated that compared with YC group, the aging progress significantly up-regulated the expression of Lyric and Erlin1, and down-regulated the expression of Rbm3, Pycr3, Pfn1, Akt, p-Akt, QSOX1, Myosin and Tnnt1, while the wheat oligopeptide intervention alleviated the regulation of protein expression caused by aging. Additionally, decreased phosphorylation of Akt protein was found in AC group. Wheat oligopeptide intervention could significantly increase the level of Akt phosphorylation in skeletal muscle of aged rats, and there was no statistical difference between intervention groups and the YC group. The results were consistent with those of proteomics.

Proteomic analysis of the differences between WFM 2 group and AC group and validation of proteomic results

Proteomic results of the differences between WFM 2 group and AC group are shown in Fig. 8. We found 52 significant differentially expressed proteins from WFM 2 vs. AC, 34 up-regulated and 18 down-regulated (Fig. 8A). The differential expression patterns of these proteins were visualized via heatmap (Fig. 8B). Based on the distribution of COG functions that matched the differentially expressed proteins, the main COG categories with a known function were involved in "General function prediction only", "Signal transduction mechanisms", "Cytoskeleton", "Nucleotide transport

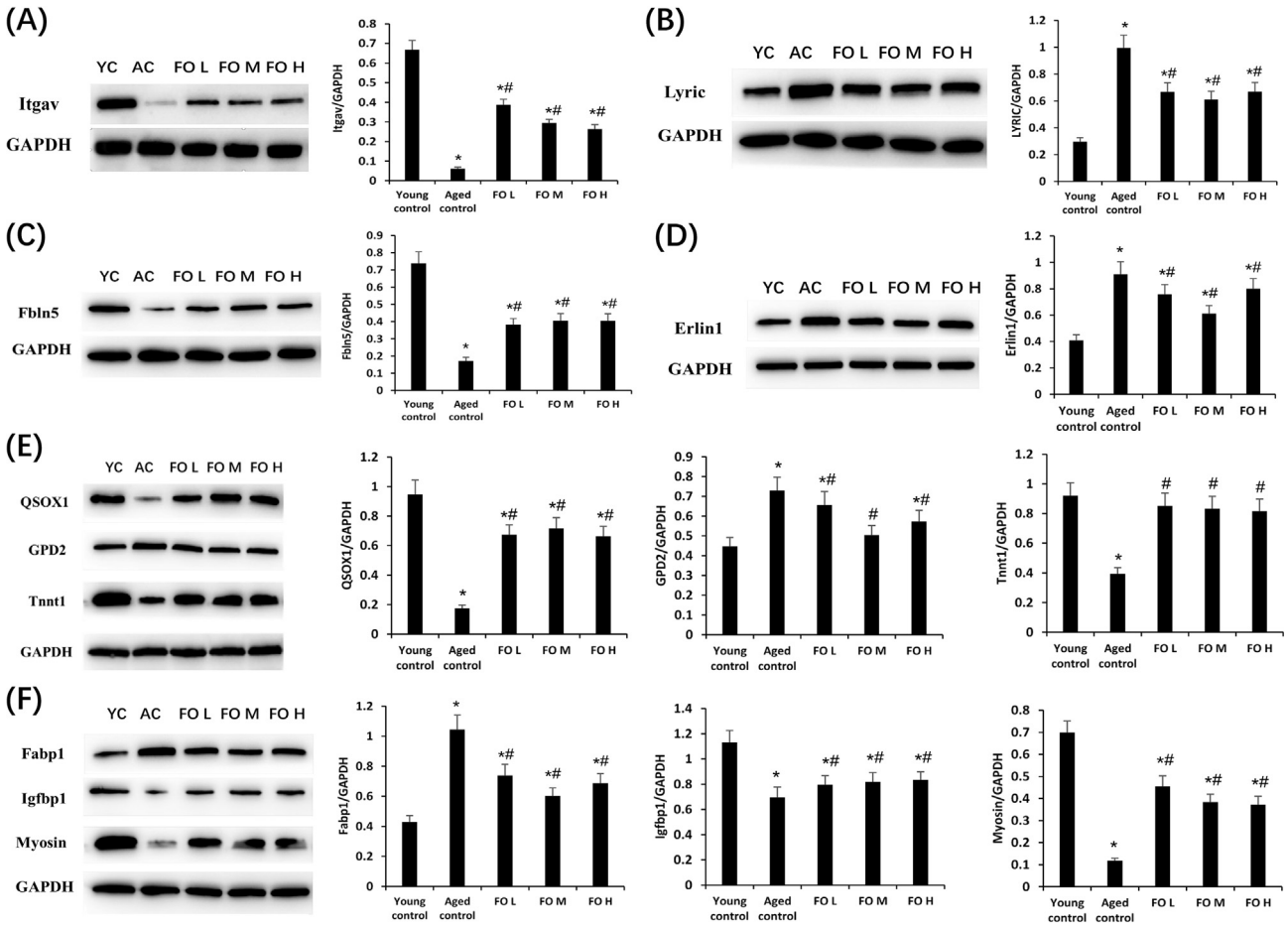

Fig. 5. Effect of fish oil intervention on the protein expression of Itgav, Lyric, Fbln5, Erlin1, QSOX1, GPD2, Tnnt1, Fabp1, Igfbp1, and Myosin in quadriceps femoris. $^{*}P < 0.05$ when compared with YC group; $\#P < 0.05$ when compared with AC group.

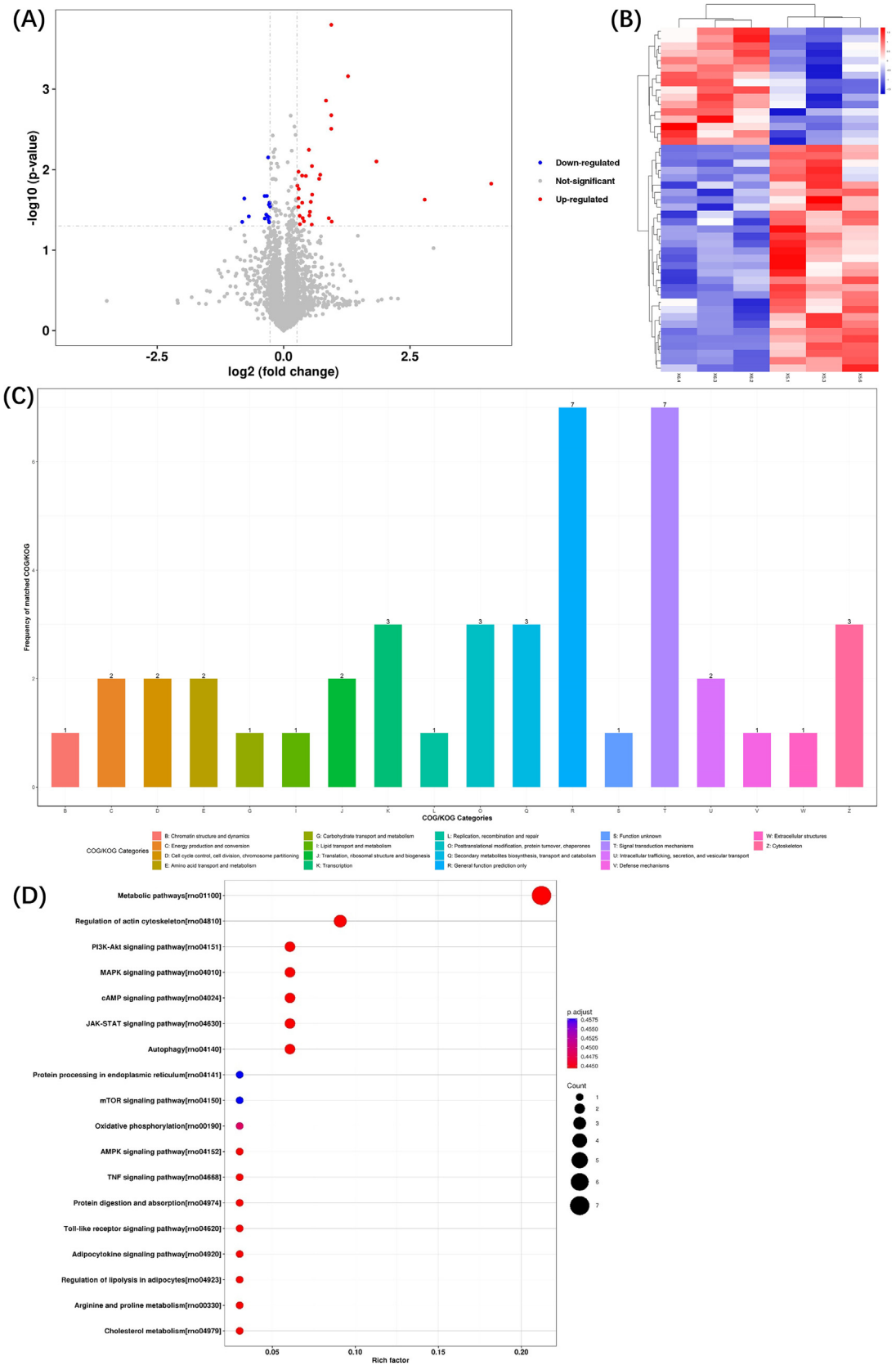

Fig. 6. Proteomic results of the differences between WP M group and AC group. **(A)** Volcano plot for summarizing the results of differential analysis. These points indicate different proteins that display both large magnitude fold-changes (x axis) and high statistical significance (-log10 of *P* values, y axis). Dashed horizontal line shows the *P* values cutoff, and the two vertical dashed lines indicate down/up regulated proteins; **(B)** clustering heatmap of the significant proteins in comparison of AC group; **(C)** COG classification and functional annotation of the differentially expressed proteins in comparison of AC group; **(D)** KEGG enrichment scatter plot. The Rich factor is the ratio of differentially expressed protein numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the Rich factor, the greater the degree of pathway enrichment. A lower adjusted *P* value indicates greater pathway enrichment.

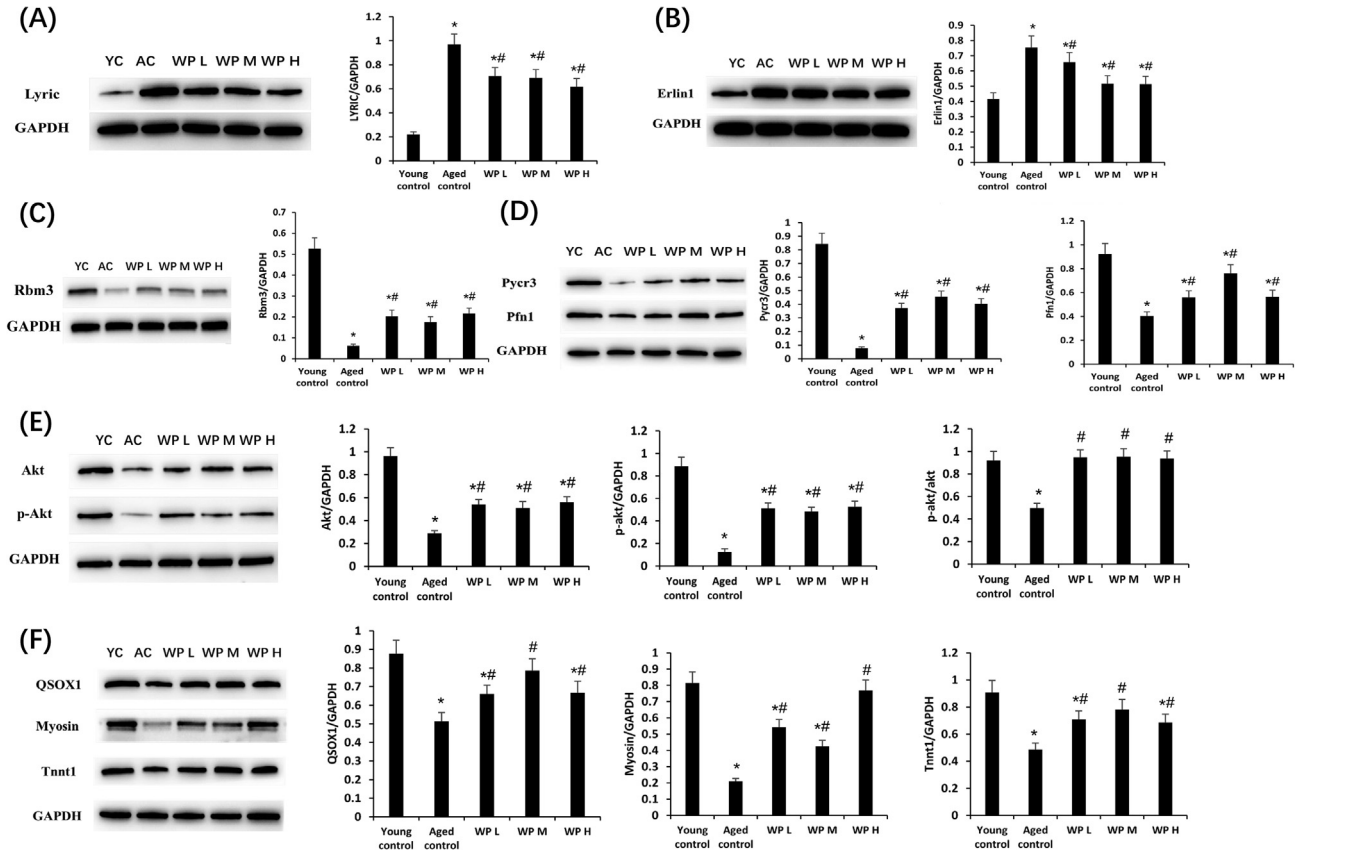

Fig. 7. Effect of wheat oligopeptide intervention on the protein expression of Lyric, Erlin1, Rbm3, Pycr3, Pfn1, Akt, p-Akt, QSOX1, Myosin, and Tnnt1 in quadriceps femoris. *$P < 0.05$ when compared with YC group; #$P < 0.05$ when compared with AC group.

and metabolism", "Translation, ribosomal structure and biogenesis", "Transcription", "Intracellular trafficking, secretion, and vesicular transport", and "Extracellular structures" (Fig. 8C). The top 18 KEGG pathways of differentially expressed proteins in the comparison between WFM 2 group and AC group are shown in Fig. 8D.

Based on the results of proteomic analysis, 7 differentially expressed candidate proteins were further verified via western blot analysis (Fig. 9). The results indicated that the combined intervention of fish oil-derived ω-3 PUFA and wheat oligopeptide, i.e., WFM 1, WFM 2 and WFM 3 groups, significantly alleviated the aging-associated regulation of protein expression of Rbm3, Pycr3, Pfn1, Akt, p-Akt, QSOX1, Fabp1 and Igfbp1. We also found that WFM 2 group had a significant higher expression level of Rbm3, Pycr3, Pfn1 and Akt, and a significant lower expression level of Fabp1 than WP M or FO M group. There was no significant difference between WFM 2 group and YC group in the expression level of Pfn1, Akt, p-Akt and Fabp1. Additionally, the combined intervention of fish oil-derived ω-3 PUFA and wheat oligopeptide could significantly increase the level of Akt phosphorylation in skeletal muscle of aged rats, and there was no statistical difference between combined intervention groups and the YC group, whereas the phosphorylation level of combined intervention groups was significantly higher than that of single intervention groups, i.e., WP M group and FO M group. The results were consistent with those of proteomics.

Discussion

Natural aging rats are closely model human muscle and better suited for assessing preventive and therapeutic strategies [30,32]. Based on the results of muscle mass, grip strength, MRI measurement and histopathological analysis, the animal model of aging was successfully established. Overall, our findings indicated that the interventions involving fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination, to varying degrees, improved the muscle performance and skeletal muscle mass, ameliorated the conditions of fatty infiltration and muscle atrophy, and reduced body fat and blood lipids level in aged rats, highlighting the potential for reversing sarcopenia. Fish oil-derived ω-3 PUFA and wheat oligopeptide had effects on different aspects of some indicators. For example, fish oil-derived ω-3 PUFA may have a greater impact on perirenal fat weight, blood lipids, and hormone level, whereas wheat oligopeptide may have a greater impact on body weight, muscle mass, CSA of quadriceps femoris, size and number of adipocytes, and inflammation. Additionally, among these intervention strategies, the WFM 2 group (400 mg/kg body weight fish oil + 2 00 mg/kg body weight wheat oligopeptide) was observed to potentially have a relatively better efficacy than other groups.

The age-related decline in muscle mass and function is mainly caused by decreased muscle protein anabolism and increased protein catabolism. Proteomic results indicated that consumption of fish oil-derived ω-3 PUFA could promote protein processing in endoplasmic reticulum, biosynthesis of amino acids and glutathione metabolism, and inhibit ubiquitin-mediated proteolysis; consumption of wheat oligopeptide could promote protein digestion and absorption, protein processing in endoplasmic reticulum, and regulation of intracellular protein transport; consumption of their combination could further promote peptide transport and negative regulation of proteolysis.

The western blot analysis of differentially expressed candidate proteins were consistent with the results of proteomics. The proteins regulated by fish oil-derived ω-3 PUFA and wheat oligopep-

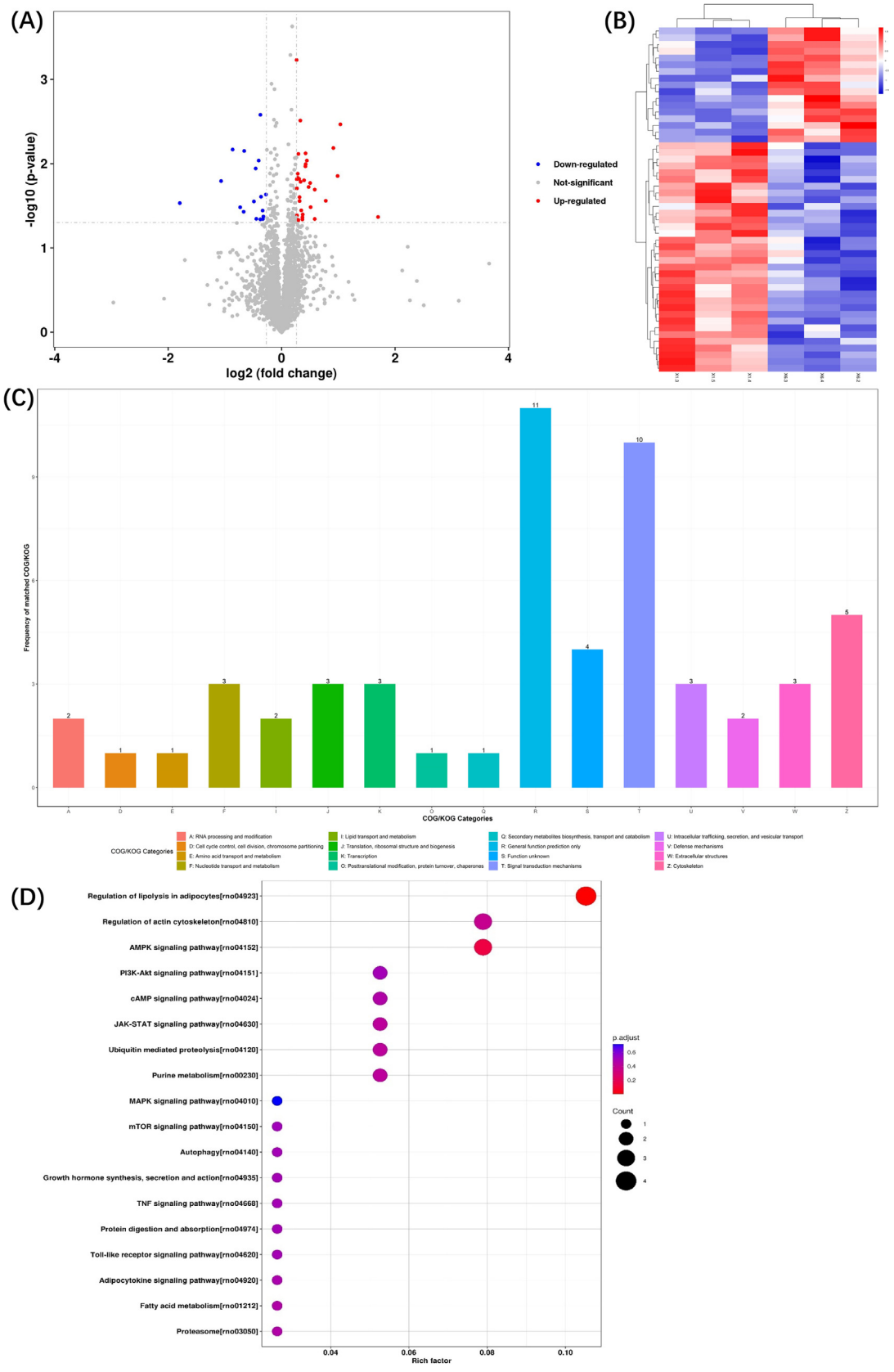

Fig. 8. Proteomic results of the differences between WFM 2 group and AC group. **(A)** Volcano plot for summarizing the results of differential analysis. These points indicate different proteins that display both large magnitude fold-changes (x axis) and high statistical significance (-log10 of *P* values, y axis). Dashed horizontal line shows the *P* values cutoff, and the two vertical dashed lines indicate down/up regulated proteins; **(B)** clustering heatmap of the significant proteins in comparison of AC group; **(C)** COG classification and functional annotation of the differentially expressed proteins in comparison of AC group; **(D)** KEGG enrichment scatter plot. The Rich factor is the ratio of differentially expressed protein numbers annotated in this pathway term to all gene numbers annotated in this pathway term. The greater the Rich factor, the greater the degree of pathway enrichment. A lower adjusted *P* value indicates greater pathway enrichment.

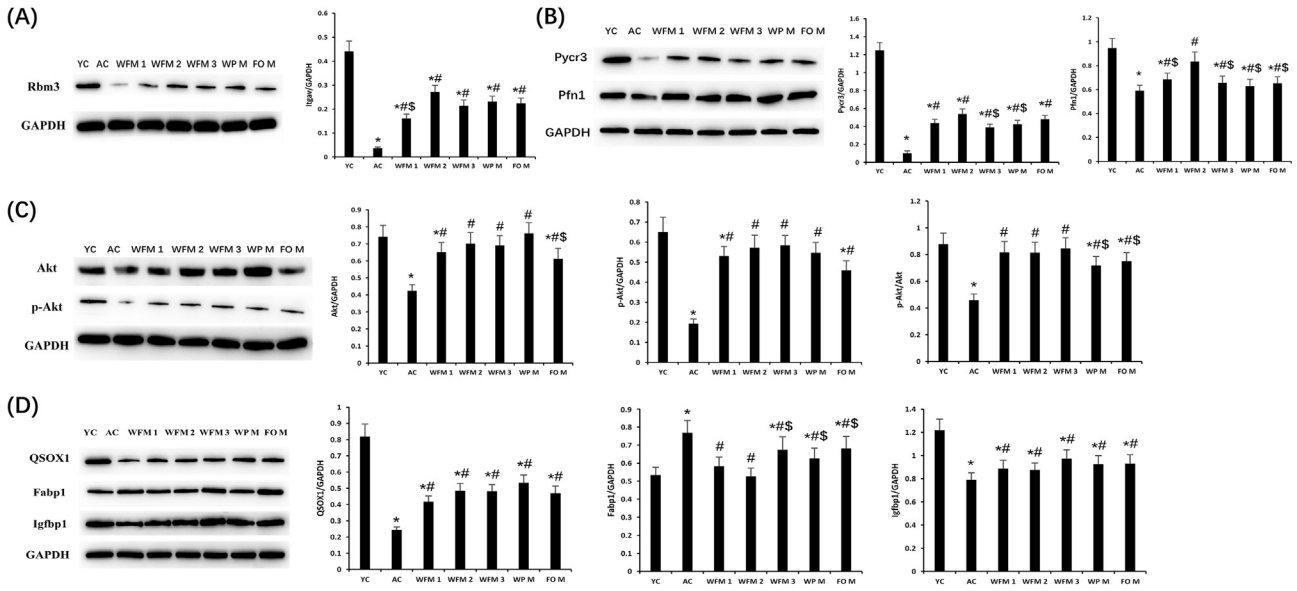

Fig. 9. Effect of combined interventions on the protein expression of Rbm3, Pycr3, Pfn1, Akt, p-Akt, QSOX1, Fabp1 and Igfbp1 in quadriceps femoris. *$P < 0.05$ when compared with YC group; #$P < 0.05$ when compared with AC group; \$$P < 0.05$ when compared with WFM 2 group.

tide and their related functions are illustrated in Fig. 10. Fbox1 and MuRF-1 are atrophy-associated ubiquitin ligases involved in ubiquitin-mediated proteolysis [33], and GDF-8 is the most prominent negative regulator of skeletal muscle mass [34]. Pfn1 and Pycr3 play critical roles in skeletal muscle development. Specifically, Pfn1 functions in the remodeling of cytoskeleton and the

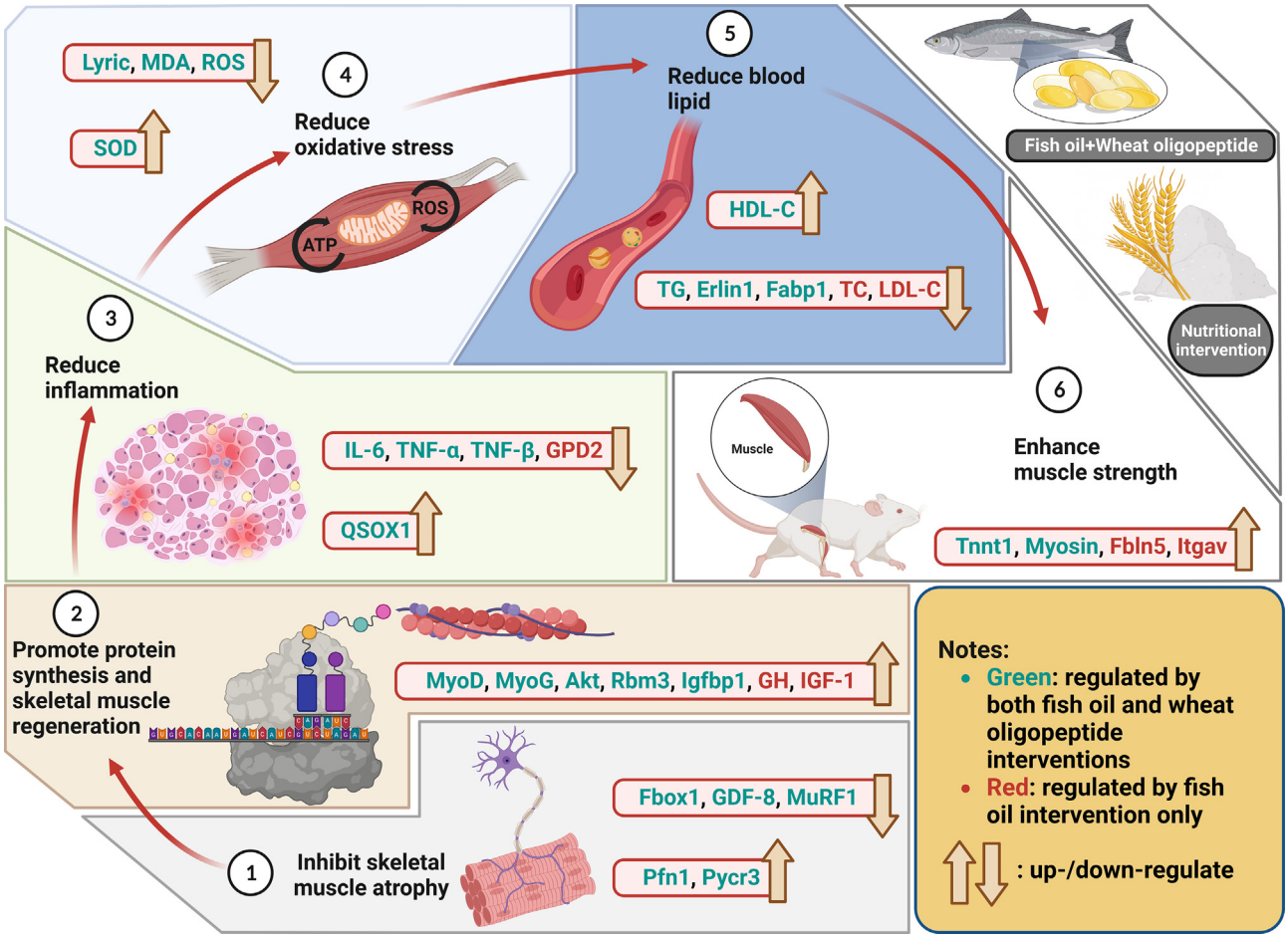

Fig. 10. Diagram of proteins regulated by fish oil and wheat oligopeptide and their related functions.

transmission of intracellular signals to regulate myogenic differentiation [35]; Pycr3 is highly differentially expressed in skeletal muscle tissues with different anatomies [36]. Both fish oil-derived ω-3 PUFA and wheat oligopeptide interventions down-regulated the levels of Fbox1, MuRF-1 and GDF-8, and up-regulated the expression of Pfn1 and Pycr3 to inhibit skeletal muscle atrophy, and activated cAMP signaling pathway, which is essential for controlling and maintaining contractile function and transcriptional homeostasis in skeletal muscle [37]. Previous studies also indicated that animal models deficient in MURF-1 expression exhibit resistance to atrophy [38,39]. It has been reported that muscle size is regulated via a complex interplay of GDF-8 signaling with the IGF-1/PI3K/Akt/mTOR pathway responsible for increase in protein synthesis in muscle [40], which is also proved in the results of western blotting and proteomics in the present study. GH is an anabolic hormone stimulating whole-body protein accretion with protein synthesis in sites including muscle, which is mediated by IGF-1 and modulated by Igfbp1 [41,42]. MyoD and MyoG are transcription factors with the ability to convert various differentiated cell types to myogenesis, as they play important roles in directing satellite cell function to regenerate skeletal muscle via linking the genetic control of developmental and regenerative myogenesis [43]. Rbm3 is an RNA-binding and cold shock protein whose expression in muscle is associated with extended lifespan and maintenance of protein synthesis [44]. Therefore, the interventions of fish oil-derived ω-3 PUFA and wheat oligopeptide may function in the promotion of protein synthesis and skeletal muscle regeneration via increasing the levels of GH, IGF-1, MyoD and MyoG, and up-regulating the expression of Akt, Rbm3 and Igfbp1. Tnnt1 and Myosin are potential quantitative biomarkers of muscle function, mass, strength and quality with aging [45,46]. Specifically, Tnnt1 reflects changes of single fiber muscle force in the absence of significant changes in myofiber CSA in older adults [45]. Fbln5 is essential for elastic fiber formation [47], and Itgav is a transmembrane adhesion protein responsible for connecting laminin in the extracellular matrix with actin in skeletal muscle fibers, thus transmitting longitudinal and lateral forces across the membrane at costameres and myotendious junctions [48]. Hence, combined intervention may enhance skeletal muscle strength by targeting the up-regulation of Tnnt1, Myosin, Fbln5 and Itgav.

In addition, aging also affects the quality and function of skeletal muscle by influencing the structure, function and distribution of adipose tissue. For example, increased ectopic lipid is a consequence of aging. It results in lipid-rich muscle with reduced muscle density and profound negative consequences by increased lipid infiltration within muscle fibers and adipocytes between muscle fibers [49]. The changes in adipose tissue function and distribution with aging affect the secretion of adipose tissue derived hormones and adipokines, and then contribute to a chronic state of low-grade systemic inflammation [50]. Our study found that the interventions of fish oil-derived ω-3 PUFA and wheat oligopeptide ameliorated the state of lipid infiltration in muscle, regulated serum lipid levels and inflammatory markers, and may function in glycerolipid metabolism, regulation of lipolysis in adipocytes, cholesterol metabolism, lipid transport and metabolism, fatty acid metabolism and adipocytokine signaling pathway. Additionally, Erlin1 plays roles in binding cholesterol and regulation of lipid metabolism [51], and Fabp1 is involved in the uptake and transport of lipids and the promotion of lipid accumulation [52]. Both of them were highly expressed in aged rats, and significantly down-regulated by the interventions of fish oil-derived ω-3 PUFA and wheat oligopeptide.

Furthermore, mitochondrial dysfunction and ROS-related oxidative stress are key mechanisms in skeletal muscle atrophy progression [53]. Mitochondrial functions are impaired with advancing age, including decreased mitochondrial protein synthesis and maximal ATP production rate, resulting in reduced muscle performance [54]. Dysfunctional mitochondria produce high amounts of ROS and become more susceptible to ROS damage [55]. The ability of muscle to handle the increased ROS is impaired with advancing age, leading to the accumulation of ROS and disturbed intracellular homeostasis [56]. Fragmentation of the mitochondrial network and increased mitochondrial-derived ROS trigger the activation of the proteasome-ubiquitin system and the autophagy-lysosome axis in myotubes, two key factors contributing to muscle atrophy [57,58]. This study found that the interventions of fish oil-derived ω-3 PUFA and wheat oligopeptide regulated the oxidative stress reaction by reducing ROS and MDA and activating SOD in muscle, and down-regulated the expression of Lyric, which is involved in promoting autophagy [59].

As shown in Supplementary Table S1, previous studies have primarily utilized population-based intervention trials, which have suggested a beneficial effect of fish oil supplementation on sarcopenia; however, the underlying mechanisms have not been fully elucidated. Moreover, animal models have typically employed normal adult animals rather than elderly sarcopenic models, and fish oil has been added to the feed, which made it difficult to control the amount of intake per animal. The present study fills some gaps in the current literature on the mechanisms underlying the beneficial effects of fish oil-derived ω-3 PUFA and wheat oligopeptide on muscle mass and function in the context of sarcopenia and has certain strength and novelty in the field. This study utilized a natural aging rat model of sarcopenia and controlled the amount of fish oil and wheat oligopeptide intake through weight-adjusted gavage. MRI and proteomics were also performed to contribute to the evaluation of muscle tissue and the mechanistic pathways. Furthermore, our study also builds upon the existing body of knowledge and provides new insights into the differences and potential benefits of single and combined interventions. Therefore, by addressing the limitations of previous studies and utilizing a more comprehensive approach, this study provides a more in-depth understanding of the potential benefits of fish oil and wheat oligopeptide in combating sarcopenia. However, the limitation is that whether the results of the animal model can be applied to populations still needs further studies.

Conclusion

Taken together, the interventions of fish oil-derived ω-3 PUFA, wheat oligopeptide and their combination were found to have protective effects against sarcopenia in aged rats. The WFM 2 group (400 mg/kg body weight fish oil + 200 mg/kg body weight wheat oligopeptide) was potential to be the optimal intervention strategy among the intervention groups, thus could be considered a promising nutritional support to delay the progression of muscle loss and improve physical performance in older adults.

Declarations

Compliance with ethics requirements: All experiments involving animals were conducted according to the ethical policies and procedures approved by the Ethics Committee on the Care and Use of Laboratory Animals of Southeast University (approval number: SCXK (Su) 2021–0022).

Consent for publication: Not applicable.

Consent to participate: Not applicable.

Availability of data and material: All data generated or analyzed during this study are included. The technical appendix and statistical procedure are available from the corresponding author.

Declaration of Competing Interest: The authors declare no conflict of interest.

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CRediT authorship contribution statement

Da Pan: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ligang Yang:** Data curation, Investigation, Software, Supervision, Validation. **Xian Yang:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Dengfeng Xu:** Data curation, Investigation. **Shaokang Wang:** Investigation, Project administration, Resources. **Han Gao:** Software, Visualization. **Hechun Liu:** Investigation. **Hui Xia:** Investigation. **Chao Yang:** Investigation. **Yifei Lu:** Investigation. **Jihan Sun:** Investigation. **Yuanyuan Wang:** Investigation. **Guiju Sun:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2023.04.005.

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