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Plantamajoside attenuates exhaustive exercise-induced skeletal muscle injury in rats through oxidative stress- and apoptosis-associated pharmacological modulation

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Abstract

This study investigated the pharmacological protective effects of plantamajoside (PMS), a naturally occurring phenylethanoid glycoside, against exhaustive exercise-induced skeletal muscle injury in rats. Animals were divided into control, model, and low- and high-dose PMS groups. PMS treatment reduced serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities, suggesting attenuation of muscle damage. PMS also increased superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities and decreased malondialdehyde (MDA) levels, indicating an antioxidant-associated response. Histopathological analysis showed that PMS alleviated skeletal muscle fiber disruption and inflammatory infiltration. At the molecular level, PMS treatment was associated with increased PI3K/AKT, Nrf2 and HO-1 expression, increased Bcl-2 expression, and reduced Bax, Caspase-3, Caspase-9 and Keap1 expression. Transcriptome-based screening further suggested that the FBXO31/CDKN1A/CCND1 axis may be involved in exercise-induced muscle injury and PMS-related recovery. Molecular docking was used as an auxiliary in silico analysis and suggested potential interactions between PMS and selected proteins, but these findings should be interpreted as hypothesis-supporting rather than causal mechanistic evidence. Overall, PMS may protect against exercise-induced skeletal muscle injury through pharmacological modulation of oxidative stress-, apoptosis- and cell cycle-associated pathways.

1. Introduction

Moderate, regular exercise is essential for maintaining physical health and follows specific scientific principles. Exercise prescriptions improve muscle strength, bone density, and overall fitness. However, sports injuries, often resulting from muscle fatigue and energy depletion, occur when the body can no longer sustain a certain exercise intensity (Fonseca et al. 2020). The mechanisms underlying these injuries are complex. Studies show that exercise, particularly acute or high-intensity, leads to an increased production of endogenous free radicals. This accumulation of free radicals induces cellular toxicity (Majkić et al. 2020). Skeletal muscle, accounting for 40% of body weight, is highly susceptible to overtraining syndrome, characterized by heightened oxidative stress (Cheng et al. 2020). Exhaustive exercise generates excess reactive oxygen species (ROS), overwhelming the body's antioxidant defenses, weakening enzyme activity, and leading to oxidative stress-induced apoptosis (Kamunde et al. 2023).

The Keap1/Nrf2/HO-1 pathway plays a crucial role in cellular antioxidant defense. Keap1, primarily located in the cytoplasm, regulates Nrf2 by binding to it under normal conditions. During oxidative stress, the interaction between Keap1 and Nrf2 weakens, allowing Nrf2 to translocate to the nucleus and induce the expression of antioxidant genes like HO-1, which is critical in preventing oxidative damage and modulating apoptosis (Zhang et al. 2022a). The PI3K/AKT pathway is a key upstream regulator of Nrf2 (Loboda et al. 2016). It facilitates Nrf2 nuclear translocation, where it activates downstream antioxidant responses (Bellezza et al. 2018; Li et al. 2021a). Exercise exerts a bidirectional influence on PI3K/AKT signaling; moderate exercise supports its activity, while excessive exercise disrupts it, leading to

apoptosis (Lin et al. 2020). This apoptotic process is primarily governed by the balance between Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), where reduced oxidative stress increases Bcl-2 and decreases Bax expression, ultimately reducing apoptosis (Li et al. 2021b). Similar pharmacological studies in Naunyn-Schmiedeberg's Archives of Pharmacology have also used AKT/Nrf2/HO-1 and Nrf2/HO-1 signaling as oxidative stress- and apoptosis-related mechanistic readouts (Abdelnaser et al. 2023; Üremiş et al. 2023).

To prevent and mitigate the adverse effects associated with exhaustive exercise, the field of sports science frequently employs strategies such as balanced nutrition and traditional Chinese medicine. These approaches aim to enhance the body's antioxidant defense system, mitigate the excessive accumulation of reactive oxygen species, and reduce the risk of sports injuries and exercise-induced fatigue (Majkić et al. 2020; Huang et al. 2019). Plantago asiatica, a plant with both medicinal and edible applications, is prevalent across various regions. Traditionally, it has been utilized in folk medicine for its anti-diarrheal and diuretic properties (Komatsu and Hammond 2022). Additionally, Plantago asiatica is frequently incorporated into functional foods and dietary supplements aimed at promoting intestinal health within the healthcare and pharmaceutical industries (Hu et al. 2012). A key component of this plant is plantamajoside (PMS), which has been identified as the primary active ingredient. Research indicates that PMS exhibits a range of biological activities, including antibacterial, anti-inflammatory, antioxidant, anti-apoptotic, tissue repair-promoting, antiviral, and anti-tumor effects (Zhang et al. 2022b; Shang et al. 2019; Lin et al. 2023).

Despite the reported biological activities of plantamajoside (PMS), its pharmacological role in exhaustive exercise-induced skeletal muscle injury remains insufficiently defined. This study used an excessive treadmill training model in rats to evaluate the protective effects of PMS supplementation on skeletal muscle injury and oxidative stress. To explore the underlying mechanisms at a molecular level, transcriptome sequencing was combined with biochemical assays, histopathological assessment, western blot validation and molecular docking. This integrated design aimed to determine whether PMS-mediated skeletal muscle protection is associated with PI3K/AKT-, Keap1/Nrf2/HO-1-, apoptosis- and cell cycle-related signaling changes, thereby providing experimental evidence for PMS as a natural pharmacological candidate for exercise-related skeletal muscle injury.

2. Materials and Methods

2.1 Drugs and reagents

PMS, white powder, 98.56% purity, provided by Chengdu Mansite Biology Science and Technology Co., Ltd. The mammalian protein extraction kit and cytoplasmic protein preparation kit, BCA protein concentration determination Kit (BCA reagent, copper sulfate solution, protein standard, protein standard preparation), pre-staining protein Marker, protease inhibitor and PBS crystals were provided by Fude Biotechnology Co., Ltd. Sodium dodecyl sulfonate (SDS), Twin-20 and Twin-80 were purchased from Beijing Solebo Technology Co., Ltd. SDS-PAGE gel kit (4% and 10% respectively) and the ECL

chemiluminescence solution were obtained from Bio-RAD, USA. Ammonium persulfate (APS) and glycine were obtained from Sigma, Germany. TEMED was provided by Beijing Dingguo Changsheng Biotechnology Co., Ltd. Methanol, anhydrous ethanol and neutral gum were purchased from Sinopharm Chemical Reagent Co., Ltd. Sodium pentobarbital was supplied by Qingdao Jeshikang Co., Ltd. The high protein skim high calcium milk powder was obtained from Yili Group Co., Ltd. HRP labeled goat anti-rabbit secondary antibody, HRP labeled goat anti-mouse secondary antibody, Keap1, Nrf2, HO-1 antibody, PI3K, AKT, FBXO31, CDKN1A, CCND1, Bcl-2, Bax, Caspase-3 and Caspase-9 antibody and GAPDH were purchased from American Abcam company. The HE staining kits were obtained from Qingdao Jieshikang Co., Ltd.

2.2 Experimental animal

Thirty-six male SD rats, SPF grade, weighing (180–220) g, were provided by China Liaoning Changsheng Biotechnology Co., Ltd. (production license number: SCXK (Liao) 2020-0001). The Rats were raised in an environment with a temperature of (24 ± 1)℃ and a humidity of 40%-60%, with 6 rats in each cage and free access to food and water. This experimental research scheme has been approved by the Ethics Committee of Shenyang Medical College, and the approval number is SYYXY2022102101.

2.3 Animal grouping and administration

After adaptive feeding for 1 week, twelve male SD rats were randomly divided into the control group (n = 6) and model group (n = 6) for transcriptome sequencing. Twenty-four male SD rats were randomly divided into the control group (n = 6), the model group (n = 6), PMS low-dose group (7 mg/kg, n = 6), and PMS high-dose group (28 mg/kg, n = 6). The rats were given PMS at 7 mg/kg and 28 mg/kg, respectively. The rats in the model group were given the same amount of normal saline once a day at 8:00–9:00 a.m, and the drugs were now prepared and administered continuously for two weeks.

2.4 Establishment of exhaustive exercise training model in rats

The exhaustive exercise scheme refers to the training model of T G Bedford, etc. An improved exhaustion training program: adaptive treadmill training for one week (speed 10 m/min, 30 min/d). Formal training, the speed of 10 m/min (40%-50%VO_2max) for 10 min, 15 m/min (50%-60%VO_2max) for 10 min, and then 20 m/min (78%VO_2max) exercise until exhaustion, training for 1 week (Zhang et al. 2022a; Bedford et al. 1979). Exhaustion evaluation criteria: during exercise, stimuli such as sound, light, electric current or brush should be used as far as possible to stimulate the animal's vitality, and ensure that the animal can maintain a safe posture before the runway. In grade 3 load exercise, if the rat failed to walk at the prescribed running speed and stayed at the back third of the track for more than 3 consecutive times, and the stimulation was ineffective, it could be judged as physical exhaustion (Zhang et al. 2022a).

2.5 Experimental animal sampling

After the last exhaustive exercise training, every rat was given intraperitoneal injection of pentobarbital sodium solution within 24 h. Blood was collected from abdominal aorta, centrifuged at 3000 r/min for 10 min, and serum was separated and stored in -20℃ refrigerator for testing. The gastrocnemius muscle was immediately taken and placed in an ice bath. The fat and surrounding connective tissue were removed. The blood stains were rinsed with precooled physiological saline, then the filter paper is dried, marked and packed. One part was put in 4% paraformaldehyde solution, and the other part was kept in the refrigerator at -80℃.

2.6 Biochemical index

The activities of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in rats were determined by the kit (Shanghai Jianglai Biotechnology Co., Ltd). The contents of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were determined by the kit of Shanghai Jianglai Biotechnology Co., Ltd, and the instructions were strictly followed.

2.7 Morphological observation of skeletal muscle

The gastrocnemius tissues of rats were fixed with 10% neutral formalin for 24 h, dehydrated with gradient ethanol, transparent with xylene, embedded in paraffin, cooled and solidified into sections with the thickness controlled at 5–8 μm. The sections were then dewaxed, stained with water and hemoxylin eosin, and observed under an inverted microscope.

2.8 Transcriptome sequencing and bioinformatic analysis

The skeletal muscle samples of rats were entrusted to Jin Weizhi Biotechnology Co., Ltd. for library preparation and transcriptome sequencing (3 rats in each group). Transcriptome sequencing procedures included RNA extraction, RNA sample quality detection, library construction, library purification, library detection, library quantification, sequencing cluster generation and computer sequencing. Illumina sequencing was performed by mixing different libraries according to the requirements of effective concentration and target data volume. The process included total RNA quality detection, mRNA capture, mRNA fragmentation, double-stranded cDNA synthesis, terminal repair, adaptor ligation, fragment sorting, PCR amplification, library quality assessment and Illumina sequencing. Differentially expressed genes/transcripts (DEGs) were analyzed and visualized using the R package ggplot2 to generate volcano plots and heat maps. GO enrichment analysis was performed using the GO database (http://geneontology.org/) to classify DEGs into biological process (BP), cellular component (CC) and molecular function (MF) categories. Candidate target genes were further analyzed using the STRING online database (https://cn.string-db.org/) with a medium confidence score of 0.4 for PPI network analysis.

2.9 Western blot

Gastrocnemius tissues were lysed with RIPA buffer supplemented with protease inhibitor, homogenized on ice and centrifuged, and the total protein concentration was determined using the BCA method. Equal amounts of protein from each sample were separated by 10% SDS-PAGE and transferred onto PVDF

membranes. After blocking with 4% skimmed milk for 2 h, the membranes were incubated overnight at 4°C with primary antibodies against PI3K, AKT, Keap1, Nrf2, HO-1, Bcl-2, Bax, Caspase-3, Caspase-9, FBXO31, CDKN1A, CCND1 and GAPDH. After washing three times with 0.1% Tween-PBS for 5 min each, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Protein bands were visualized using ECL chemiluminescence and captured with a gel imaging system under consistent exposure conditions. Band intensities were quantified using Quantity One software, and target protein expression was normalized to GAPDH. Primary and secondary antibodies were used according to the manufacturers' recommended working dilutions, and all antibody sources were reported in the reagent section.

2.10 Molecular docking

The energy of compound PMS was minimized and saved in mol2 format using Chem3D 21.0.0 software. The PI3K (PDB ID: 4UWG), AKT (PDB ID: 2JDO), Keap1 (PDB ID: 6V6Z), Nrf2 (PDB ID: 6T7V), HO-1 (PDB ID: 1N3U), Bcl-2 (PDB ID:1YSW), Bax (PDB ID:5W5Z), FBXO31 (PDB ID:5VZT), CDKN1A (PDB ID:7KYS) and CCND1 (PDB ID:2W96) proteins were downloaded from RSC PDB (https://www.rcsb.org/) database. Then, the protein was dehydrated and hydrogenated by sybyl-X2.0 software, and saved it in PDB format. The interaction between the compound and the target protein was carried out by sybyl-X2.0 software and the molecular docking score was obtained.

2.11 Statistical analysis

All data are presented as mean ± standard deviation (SD). For biochemical indices, histological quantification and western blot data, differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison test. Body-weight data measured longitudinally were analyzed using repeated-measures ANOVA to account for within-subject measurements over time. A two-sided $p < 0.05$ was considered statistically significant. The exact n value for each endpoint is reported in the corresponding figure legend when it differs from the allocated group size.

3. Results

3.1 Effect of PMS on body weight of rats with exercise-induced skeletal muscle injury

As shown in Fig. 1, compared with the control group, the body weight of rats in the model group was decreased ($p < 0.05$). Compared with the model group, the weight of rats in the low dose and high dose PMS groups showed a continuous increasing trend ($p < 0.05$).

3.2 Effect of PMS on CK and LDH in experimental rats

As shown in Fig. 2, compared with the control group, the activities of CK and LDH in serum of rats in the model group were significantly increased ($p < 0.05$). Compared with the model group, the serum LDH activity in the low-dose and high-dose PMS groups was decreased significantly ($p < 0.05$), and the serum CK activity in the low-dose and high-dose PMS groups was also decreased. These results indicated that the activities of CK and LDH were increased after exhaustive exercise, while PMS intervention reduced the activities of CK and LDH, indicating that PMS could repair skeletal muscle damage after exhaustive exercise.

3.3 Effects of PMS on serum SOD, MDA and GSH-Px in experimental rats

As shown in Fig. 3, compared with the control group, the activities of SOD and GSH-Px in serum of rats in the model group were significantly decreased ($p < 0.05$), while the level of MDA was increased ($p < 0.05$). Compared with the model group, the serum SOD activity of rats in the high-dose PMS group was significantly increased ($p < 0.05$), and the SOD activity of rats in the low-dose PMS group was also increased. The activity of serum GSH-Px was also increased in the high-dose and low-dose PMS groups, while the level of MDA was decreased, suggesting that PMS can relieve the oxidative stress of skeletal muscle in exhaustive exercise rats.

3.4 Effect of PMS on skeletal muscle morphological structure in experimental rats

HE staining results were shown in Fig. 4. The muscle tissue of rats in the control group had no obvious malformations, the muscle fiber structure was intact, and the nucleus size and shape were basically normal. In the model group, muscle fibers in skeletal muscle were incomplete, muscle fiber interstitial hyperplasia, nuclear condensation and dissolution, inflammatory cell infiltration and collagen fiber proliferation were obvious. There was only a small amount of inflammatory cell infiltration in the muscle tissue of rats in the low-dose and high-dose PMS groups, and the fibrointerstitial hyperplasia in the muscle tissue of rats was significantly reduced than that in the model group.

3.5 Differentially expressed genes/transcripts in rat gastrocnemius muscle

Differentially expressed genes/transcripts between the control group and model group in rat gastrocnemius muscle were screened using p value < 0.05 as the threshold. A total of 340 differentially expressed genes/transcripts showed significant changes, including 205 up-regulated and 135 down-regulated genes/transcripts in the model group. The volcano plot is shown in Fig. 5A. The heat map showed the expression intensity of these 340 genes/transcripts in the two groups, with up-regulated genes/transcripts shown in red and down-regulated genes/transcripts shown in blue; deeper color indicated higher expression intensity (Fig. 5B).

3.6 Integrated GO enrichment and PPI network analysis of differentially expressed genes/transcripts

As shown in Fig. 6A, a total of 58 GO-enriched entries were obtained ($p < 0.05$), including 10 molecular function (MF)-related entries, 33 biological process (BP)-related entries, and 15 cellular component (CC)-related entries. Among the enriched BP terms, negative regulation of cell growth, negative regulation of cell proliferation, and cellular response to DNA damage stimulus were considered particularly relevant to apoptosis- and cell cycle-associated injury responses.

Negative regulation of cell growth included seven genes, namely BST2, CDKN1A, SERPINE2, SESN1, CTH, OSGIN1, and CDKN2AIP. Negative regulation of cell proliferation included 11 genes, namely TGIF1, NPPC, PTPRU, CDKN1A, TFAP4, SERPINE2, PTPRZ1, CTH, LOC103690190, CYP1B1, and PTGS2. Cellular response to DNA damage stimulus included eight genes, namely CDKN1A, BLM, EGLN3, CCND1, PSME4, AATF, CDKN2AIP, and FBXO31.

To further identify candidate targets, genes involved in these enriched biological processes were imported into the STRING protein interaction database for PPI network analysis. As shown in Fig. 6B, a medium confidence score of 0.4 was used to construct the interaction network. Based on their degree values in the network and their biological relevance to cell cycle regulation and DNA damage-associated responses, FBXO31, CCND1, and CDKN1A were selected as candidate genes for subsequent validation.

3.7 Expression analysis of FBXO31, CCND1 and CDKN1A in skeletal muscle of rats

As shown in Table 1, the results of gene screening showed that compared with control group, the expression level of CCND1 gene in model group was down-regulated, while the expression levels of FBXO31 and CDKN1A gene were up-regulated.

Table 1
Differentially expressed genes in Gastrocnemius muscle of rats in the control group and the model group.

Gene Name	Control group			Model group			LogFC	Changes in the model group
FBXO31	1899	1190	1931	5958	6486	4520	1.757	Up
CDKN1A	210	256	616	838	1098	1701	1.749	Up
CCND1	238	222	224	31	27	21	-3.114	Down

3.8 Effect of PMS on the expression of skeletal muscle protein in experimental rats

As shown in Fig. 7A, the experimental results showed that compared with the control group, the protein levels of PI3K ($p < 0.05$) and AKT in the model group were decreased. Compared with the model group,

the levels of PI3K and AKT protein in skeletal muscle of rats in low-dose and high-dose PMS groups were increased.

As shown in Fig. 7B, the experimental results showed that compared with the control group, the protein levels of Nrf2 and HO-1 in the model group were significantly decreased ($p < 0.05$), and the protein level of Keap1 was significantly increased ($p < 0.05$). The protein levels of Nrf2 and HO-1 in skeletal muscle of rats in low-dose PMS group were significantly higher than those in model group ($p < 0.05$). Compared with the model group, the Keap1 protein level in the PMS low-dose group was reduced. Compared with the model group, the level of HO-1 was dramatically increased ($p < 0.05$), the level of Nrf2 protein was dramatically increased ($p < 0.05$), and the level of Keap1 protein was reduced in the PMS high-dose group. These results indicate that PMS can repair oxidative stress injury of skeletal muscle after exhaustive exercise.

As shown in Fig. 7C, the experimental results demonstrated that compared with the control group, the protein level of Bcl-2 in the model group was signally decreased ($p < 0.05$), and the protein levels of Bax and Caspase-9 were observably increased ($p < 0.05$), the expression of Caspase-3 were increased. Compared with the model group, the skeletal muscle Bcl-2 protein level in PMS low-dose group was significantly up-regulated ($p < 0.05$), the protein levels of Bax and Caspase-9 were significantly down-regulated ($p < 0.05$), and the protein level of Caspase-3 was up-regulated. The protein levels of Bax ($p < 0.05$), caspase-3, and Caspase-9 ($p < 0.05$) in skeletal muscle of rats in the high-dose PMS group were down-regulated, while the protein levels of Bcl-2 were markedly up-regulated compared to the model group ($p < 0.05$). These results indicated that PMS can repair apoptosis damage of skeletal muscle cells after exhaustive exercise.

As shown in Fig. 7D, compared with the control group, the CCND1 protein level in the model group were dramatically down-regulated ($p < 0.05$), while the FBXO31 protein level were up-regulated, and CDKN1A protein level were markedly up-regulated ($p < 0.05$). Compared with the model group, the CCND1 protein level in skeletal muscle of rats in the low-dose and high-dose PMS groups was markedly raised ($p < 0.05$), while the FBXO31 and CDKN1A protein levels were down-regulated.

3.10 Molecular docking analysis

Molecular docking was performed as an auxiliary in silico analysis to explore potential interactions between PMS and selected proteins. In the docking results, total score ≥ 4 and cscore ≥ 3 were considered to indicate acceptable predicted binding, while total score > 7 indicated stronger predicted binding. The scoring results are shown in Table 2. PMS showed predicted binding with PI3K/AKT, Keap1/Nrf2/HO-1, Bcl-2/Bax and FBXO31/CDKN1A/CCND1 proteins. The total scores of PMS with PI3K, AKT, Keap1, Nrf2, HO-1, Bcl-2, Bax, FBXO31, CDKN1A and CCND1 were 8.84, 12.46, 9.63, 7.91, 7.27, 4.29, 8.13, 7.45, 8.22 and 8.44, respectively. These docking data support possible molecular interactions between PMS and the selected proteins, but they do not by themselves demonstrate direct target engagement or pathway causality in vivo. According to the 3D results in Fig. 8, PMS was predicted to occupy cavity pockets of the ten proteins.

Table 2
Docking score of PMS with proteins.

Ligand	Target proteins	Molecular docking score	
		Total score	Cscore
PMS	PI3K	8.84	5
PMS	AKT	12.46	5
PMS	Keap1	9.63	5
PMS	Nrf2	7.91	4
PMS	HO-1	7.27	5
PMS	Bcl-2	4.29	3
PMS	Bax	8.13	4
PMS	FBXO31	7.45	4
PMS	CDKN1A	8.22	4
PMS	CCND1	8.44	3

According to the 2D results, the main types of interaction between PMS and PI3K protein include hydrogen bond, C-H bonds and hydrophobic interaction. For example, PMS formed hydrogen bond with the residues ASP761, LYS636, ASP747 and GLN683. It formed hydrophobic interaction with LEU616, ILE760, TYR670, LEU750; and formed C-H bonds with ASP747. PMS formed hydrogen bonds with AKT protein residues GLU200, ASP293, LYS277, formed C-H bonds with THR292, THR197, ASP293, and formed hydrophobic interactions with VAL166, LYS181, MET229, HJS196. PMS formed hydrogen bonds with Nrf2 protein residues ILE559, VAL604, VAL606, GLY367, VAL512, VAL465, VAL514, VAL561, VAL608 and VAL369, and formed C-H bonds with GLY605, VAL418, CYS513, CYS368 and GLY558, and formed hydrophobic interactions with ALA366. PMS formed hydrogen bonds with HO-1 protein residues GLU62, HIS84, GLU81, GLU62, and formed C-H bonds with GLU81, GLU66, LYS86, and formed hydrophobic interactions with LYS86. The main types of interactions between PMS and Keap1, Bcl-2, Bax, CDKN1A, CCND1 and proteins include hydrogen bond, C-H bonds and hydrophobic interaction. The main types of interaction between PMS and FBXO31 protein also include hydrogen bond and C-H bonds.

4. Discussion

The present study evaluated PMS as a natural pharmacological candidate for exhaustive exercise-induced skeletal muscle injury rather than merely as a sports nutrition supplement. By combining biochemical indicators, histopathology, transcriptome-based screening, western blot validation and docking-based prediction, this study explored whether the protective effect of PMS was associated with oxidative stress-, apoptosis- and cell cycle-related signaling. The sequencing analysis suggested that

skeletal muscle injury was closely related to apoptosis- and cell cycle-associated biological processes. Therefore, subsequent molecular experiments focused on validating selected proteins involved in these injury-response pathways.

Lactate dehydrogenase (LDH) and creatine kinase (CK) are critical enzymes involved in cellular energy metabolism. During exercise, tissue hypoxia and the accumulation of metabolites can lead to increased permeability of skeletal muscle cell membranes or acute muscle cell injury, resulting in elevated serum levels of LDH and CK. Consequently, measuring serum LDH and CK activities provides sensitive biomarkers for assessing muscle tolerance to training loads, as well as for detecting microscopic skeletal muscle injury, adaptation, and recovery. In this study, serum LDH and CK activities were significantly elevated in the model group compared to the control group. Supplementation with plantamajoside (PMS) notably reduced serum LDH and CK levels following exhaustive exercise. Additionally, histological examination revealed that, following exhaustive exercise, the gastrocnemius muscle fibers of rats exhibited swelling, disorganized structure, unclear arrangement, nuclear disappearance, and muscle fiber dissolution (Cheng et al. 2020; Ren et al. 2019; Sikorska et al. 2021). HE staining further confirmed severe skeletal muscle injury in the model group, which was mitigated by PMS supplementation. However, the precise physiological mechanisms by which PMS supplementation alleviates skeletal muscle injury remain incompletely understood.

Research has demonstrated that high-intensity and prolonged exercise can elevate levels of reactive oxygen species (ROS) in skeletal muscles, leading to muscle damage. Exhaustive exercise significantly increases tissue oxygen consumption, resulting in the generation of a large number of free radicals. Concurrently, the demand for antioxidants and trace elements that support antioxidant enzymes rises markedly, causing dramatic changes in the internal environment and hormonal levels, such as body pH and plasma osmotic pressure (Li et al. 2019; Hargreaves and Spriet 2020; Tominaga et al. 2021). Skeletal muscle, as one of the organs most directly involved in exercise, is the largest consumer of energy and oxygen during physical activity and is highly susceptible to damage from free radicals (Henríquez-Olguin et al. 2019; Wu et al. 2022).

Malondialdehyde (MDA) serves as an indicator of ROS production in tissues, while glutathione peroxidase (GSH-Px) helps neutralize ROS and lipid peroxides, thus protecting cell membrane integrity. Superoxide dismutase (SOD) plays a role in blocking peroxidation and eliminating ROS, thereby safeguarding the cell membrane from oxidative stress. In this study, the levels of these three oxidative stress-related factors were measured using ELISA. The results indicated that, compared to the control group, the MDA content in skeletal muscle tissue of the model group was significantly elevated, while SOD and GSH-Px levels were markedly reduced, suggesting that exhaustive exercise induced excessive oxidative stress in skeletal muscle. In contrast, rats in the PMS supplementation groups exhibited reduced MDA levels and increased SOD and GSH-Px contents in their skeletal muscle tissues compared to the model group. These findings suggest that PMS effectively mitigates oxidative stress in skeletal muscle following exhaustive exercise.

To further clarify the antioxidant-associated pharmacological response of plantamajoside (PMS), this study investigated its relationship with the Nrf2/Keap1 signaling pathway. The Nrf2-Keap1 signaling pathway is crucial for mitigating oxidative stress responses. Under normal conditions, Nrf2 and Keap1 interact and inhibit each other. During oxidative stress, this interaction can be disrupted, leading to Nrf2 release and nuclear translocation. In the nucleus, Nrf2 binds to antioxidant response elements (AREs) and promotes the transcription of downstream antioxidant factors such as heme oxygenase-1 (HO-1), which may enhance antioxidant enzyme activities, including SOD and GSH-Px (Maldonado et al. 2021; Kong et al. 2021). In the present study, exhaustive exercise was associated with decreased Nrf2 and HO-1 protein expression and increased Keap1 protein expression in skeletal muscle. PMS supplementation was associated with increased Nrf2 and HO-1 protein expression and reduced Keap1 expression. These findings suggest that modulation of the Keap1/Nrf2/HO-1 pathway may contribute to the antioxidant effect of PMS, although further pathway-blocking experiments are needed to establish direct causality. This interpretation is consistent with recent Naunyn-Schmiedeberg's Archives of Pharmacology studies in which pharmacological protection was linked to oxidative-stress modulation through Nrf2/HO-1 or Keap1/Nrf2/HO-1 signaling (Besong et al. 2023; Zhao et al. 2023).

Oxidative stress is widely recognized as a promoter of apoptosis (Baechler et al. 2019; Le et al. 2020). During intense or exhaustive exercise, the body generates a substantial amount of reactive oxygen species (ROS), which can attack unsaturated fatty acids on cell membranes, leading to lipid peroxidation and direct membrane damage, thereby inducing apoptosis. Tao Jiang's study corroborates this, showing elevated levels of malondialdehyde (MDA) in plasma and skeletal muscle tissues, and a higher apoptosis rate in skeletal muscle tissues in the control group compared to other conditions (Jiang et al. 2021). Additionally, a correlation between apoptosis rate and MDA content has been established (Baechler et al. 2019; Yang et al. 2021). Cell apoptosis and survival hinge on the balance between pro-apoptotic and anti-apoptotic regulatory pathways. The Bcl-2 family plays a central role in apoptosis regulation (Dulloo et al. 2022). Among this family, the expression of the proteins Bcl-2 and Bax are key determinants of apoptosis (Wu et al. 2022; Dulloo et al. 2022; Spitz and Gavathiotis 2021). An increase in Bax expression, coupled with a decrease in Bcl-2 expression, promotes apoptosis (Ahagh et al. 2019). Conversely, increased Bcl-2 expression and decreased Bax levels inhibit apoptosis. Besides the Bcl-2 family, Caspase-3 and Caspase-9 are critical factors in the apoptosis pathway (Duan et al. 2009). In this study, we observed that PMS supplementation led to a significant increase in Bcl-2 protein expression and a marked decrease in Bax and Caspase-3 protein levels compared to the model group. This suggests that exhaustive exercise-induced ROS accumulation in mitochondria leads to oxidative stress, mitochondrial damage, and activation of apoptosis pathways. PMS intervention appears to counteract these effects by modulating apoptosis-related factors, thereby preventing excessive cell apoptosis.

The PI3K/AKT pathway has been reported to act as an upstream regulator of Nrf2, positively modulating Nrf2 gene expression, facilitating its translocation from the cytoplasm to the nucleus and activating downstream antioxidant genes (Bellezza et al. 2018; Ma et al. 2018; Rojo et al. 2018). In this study, PMS supplementation increased PI3K and AKT protein expression in skeletal muscle after exhaustive exercise compared with the model group. These data indicate that PMS-related protection against

skeletal muscle injury may be associated with activation of the PI3K/AKT pathway and subsequent modulation of Keap1/Nrf2/HO-1 signaling and apoptosis-related proteins. However, because no PI3K/AKT inhibitor or genetic intervention was used, this pathway should be interpreted as an associated mechanism rather than as a definitively proven causal target.

Previous studies have suggested that PI3K/AKT signaling may be linked to FBXO31 regulation (Liu et al. 2020; Duan et al. 2021). FBXO31 levels increase following DNA damage and may contribute to activation of CDKN1A, inhibition of CCND1 and disruption of cell cycle progression. In the present study, PMS supplementation decreased FBXO31 and CDKN1A expression and increased CCND1 expression in the skeletal muscle of exercising rats compared with the model group. Molecular docking further suggested potential binding between PMS and proteins in this axis. Together, these results support the hypothesis that PMS-mediated recovery may involve the FBXO31/CDKN1A/CCND1 pathway, but additional functional experiments are required to confirm whether this axis is necessary for the protective effect.

Several limitations should be acknowledged. First, although the current findings support an association between PMS treatment and PI3K/AKT-, Keap1/Nrf2/HO-1-, apoptosis- and FBXO31/CDKN1A/CCND1-related signaling changes, pathway-blocking experiments, gene knockdown/overexpression assays and direct target-engagement analyses were not performed. Therefore, these pathways should be interpreted as mechanistically associated with PMS-mediated protection rather than as definitively proven causal targets. Second, molecular docking was used only as a hypothesis-supporting in silico analysis and does not independently confirm direct protein binding in vivo. These limitations should be addressed in future pharmacological studies. The proposed PMS-associated protective mechanism against exhaustive exercise-induced skeletal muscle injury is schematically presented in Fig. 9, highlighting oxidative stress-, apoptosis-, and cell cycle-associated pharmacological modulation.

5. Conclusion

This study suggests that Plantamajoside (PMS) alleviates skeletal muscle cell apoptosis and ameliorates exhaustive exercise-induced skeletal muscle injury in rats. The protective effect of PMS was associated with PI3K/AKT pathway activation, increased Nrf2 and HO-1 expression, enhanced antioxidant enzyme activities, reduced MDA levels and decreased apoptosis-related protein expression. PMS treatment was also associated with downregulation of FBXO31 and CDKN1A and upregulation of CCND1, suggesting partial restoration of cell cycle-associated recovery responses. These findings support PMS as a promising natural pharmacological candidate for preventing or mitigating exercise-induced skeletal muscle injury. Further studies using pathway inhibitors, gene knockdown/overexpression and direct target-engagement assays are needed to confirm the causal mechanisms underlying PMS-mediated protection.

Abbreviations

Abbreviation	Full term
AKT	Protein kinase B
ANOVA	Analysis of variance
APS	Ammonium persulfate
AREs	Antioxidant response elements
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BLM	Bloom syndrome protein
BP	Biological process
CC	Cellular component
CCND1	Cyclin D1
CDKN1A	Cyclin-dependent kinase inhibitor 1A
cDNA	Complementary DNA
CK	Creatine kinase
DEGs	Differentially expressed genes/transcripts
ECL	Enhanced chemiluminescence
FBXO31	F-box protein 31
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene Ontology
GSH-Px	Glutathione peroxidase
H&E/HE	Hematoxylin and eosin staining
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
MF	Molecular function
mRNA	Messenger RNA

Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate-buffered saline
PDB	Protein Data Bank
PI3K	Phosphoinositide 3-kinase
PMS	Plantamajoside
PPI	Protein-protein interaction
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
SD	Standard deviation; Sprague-Dawley
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
SPF	Specific pathogen-free
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TEMED	N,N,N',N'-Tetramethylethylenediamine
VO_2max	Maximal oxygen uptake

Declarations

Author contributions

Haikun Li: Conceptualization, Methodology, Software, Investigation, Formal Analysis, Writing-Original Draft; Yutong Xie: Visualization, Investigation; Qingchao Qin: Resources, Supervision; Yunen Liu: Conceptualization,Writing-Review & Editing; Zhonghua Luo: Conceptualization, Funding Acquisition, Writing-Review & Editing.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request. At the time of submission, no public accession number has been assigned to the transcriptome dataset; the raw sequencing data can be provided for editorial or peer-review assessment upon reasonable request.

Conflict of interest statement

The authors declare no competing financial interest.

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Figures

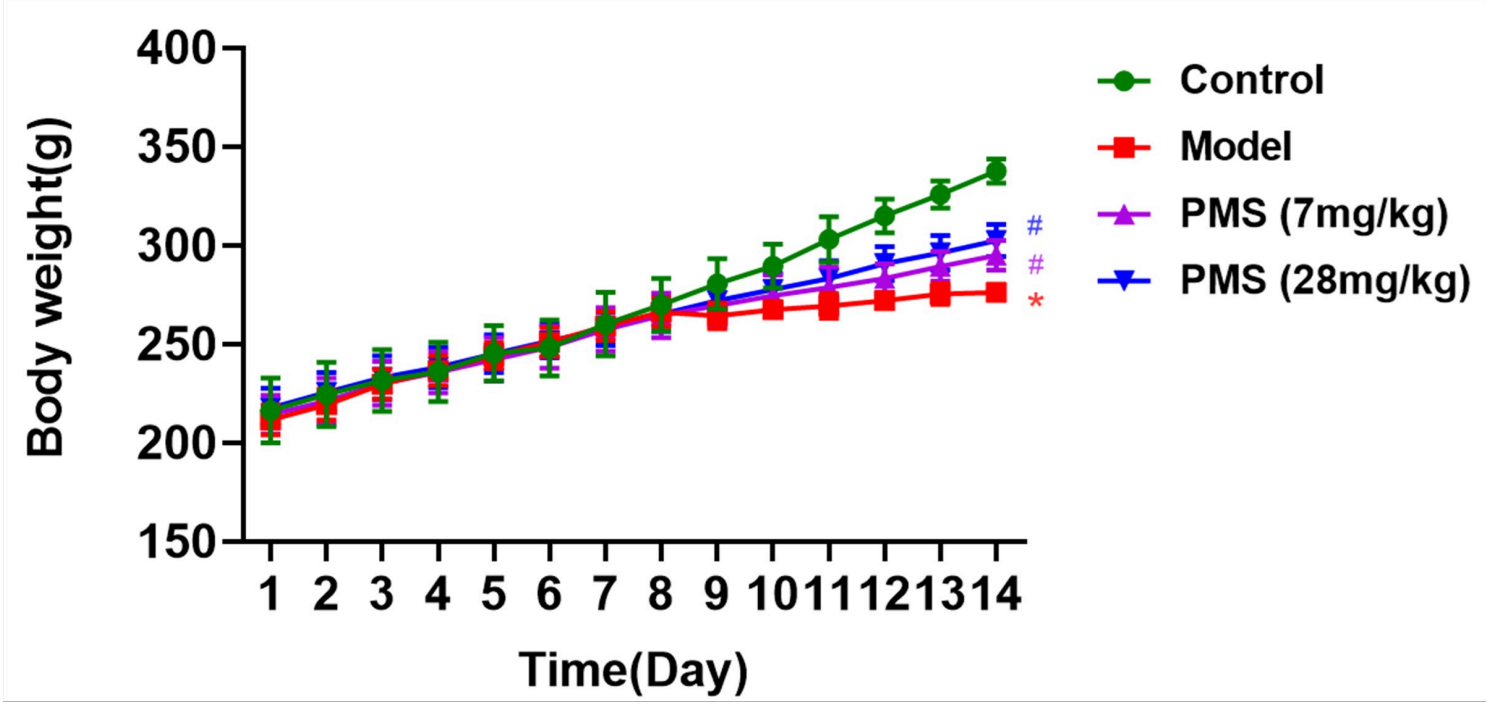


Figure 1

Weight gain of rats in different groups, $^{*}p<0.05$, compared to the control group; $^{\#}p<0.05$, compared to the model group.

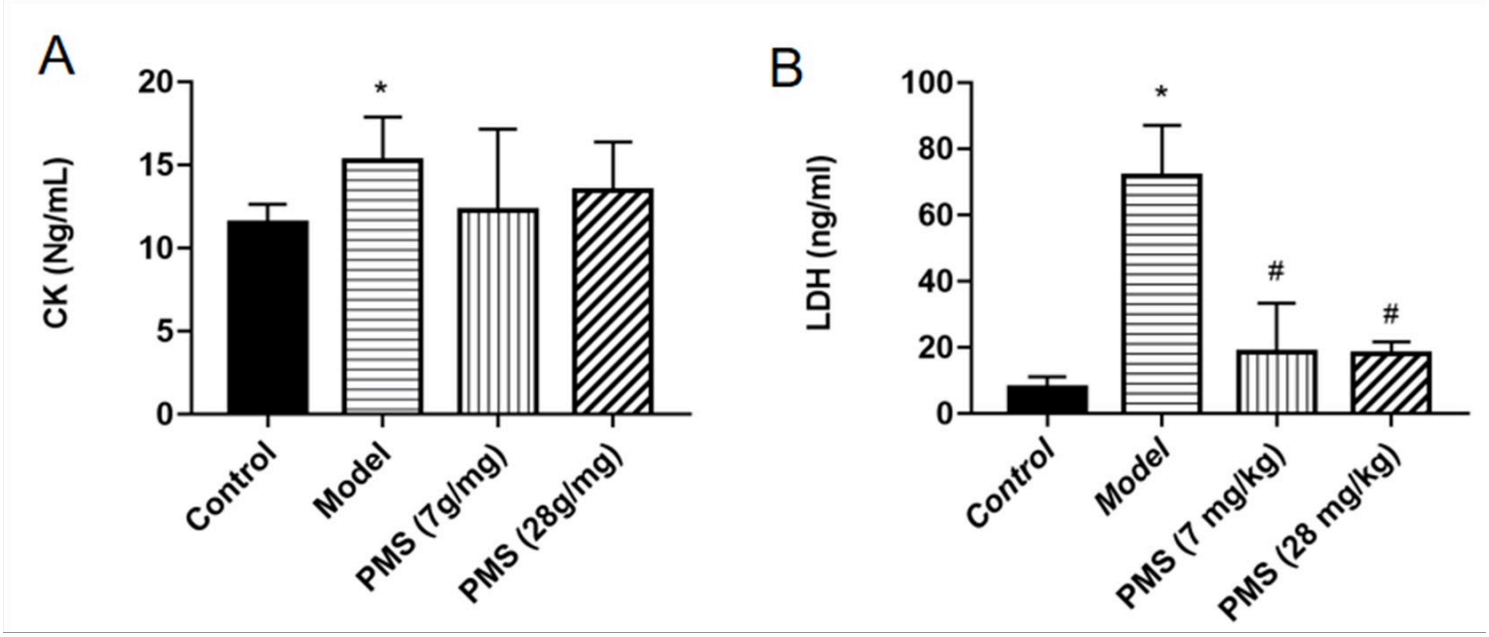


Figure 2

Effects of PMS on the activities of CK and LDH in experimental rats, (A) CK (n=6); (B) LDH (n=6); $^{*}p < 0.05$, compared with the control group; $^{\#}p < 0.05$, compared with the model group.

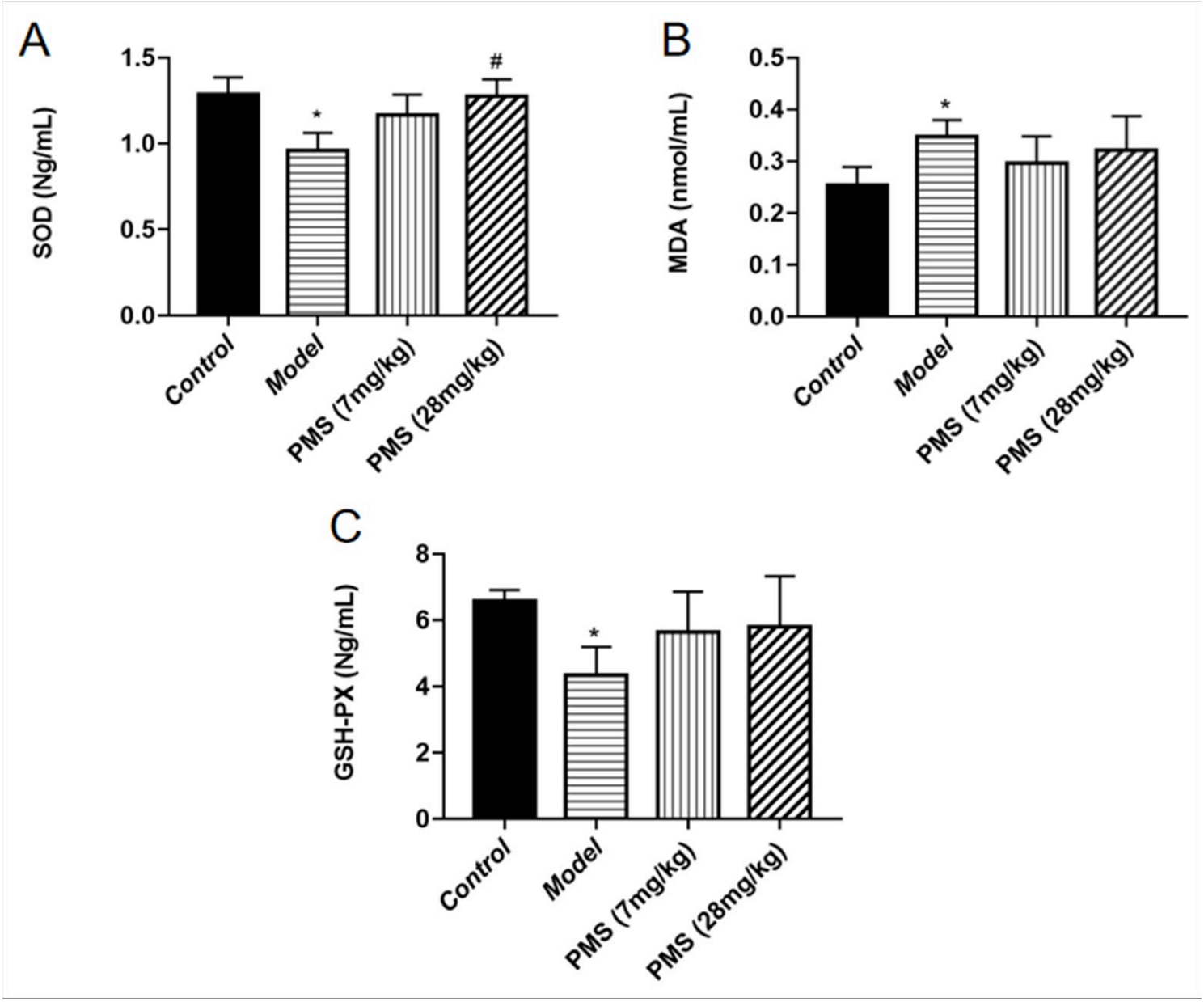


Figure 3

Effects of PMS on SOD, GSH-PX and MDA activities of experimental rats, (A) SOD (n =6), (B) MDA (n =6), (C) GSH-PX (n =6), $^{*}p< 0.05$, compared with the control group; $^{\#}p < 0.05$, compared with the model group.

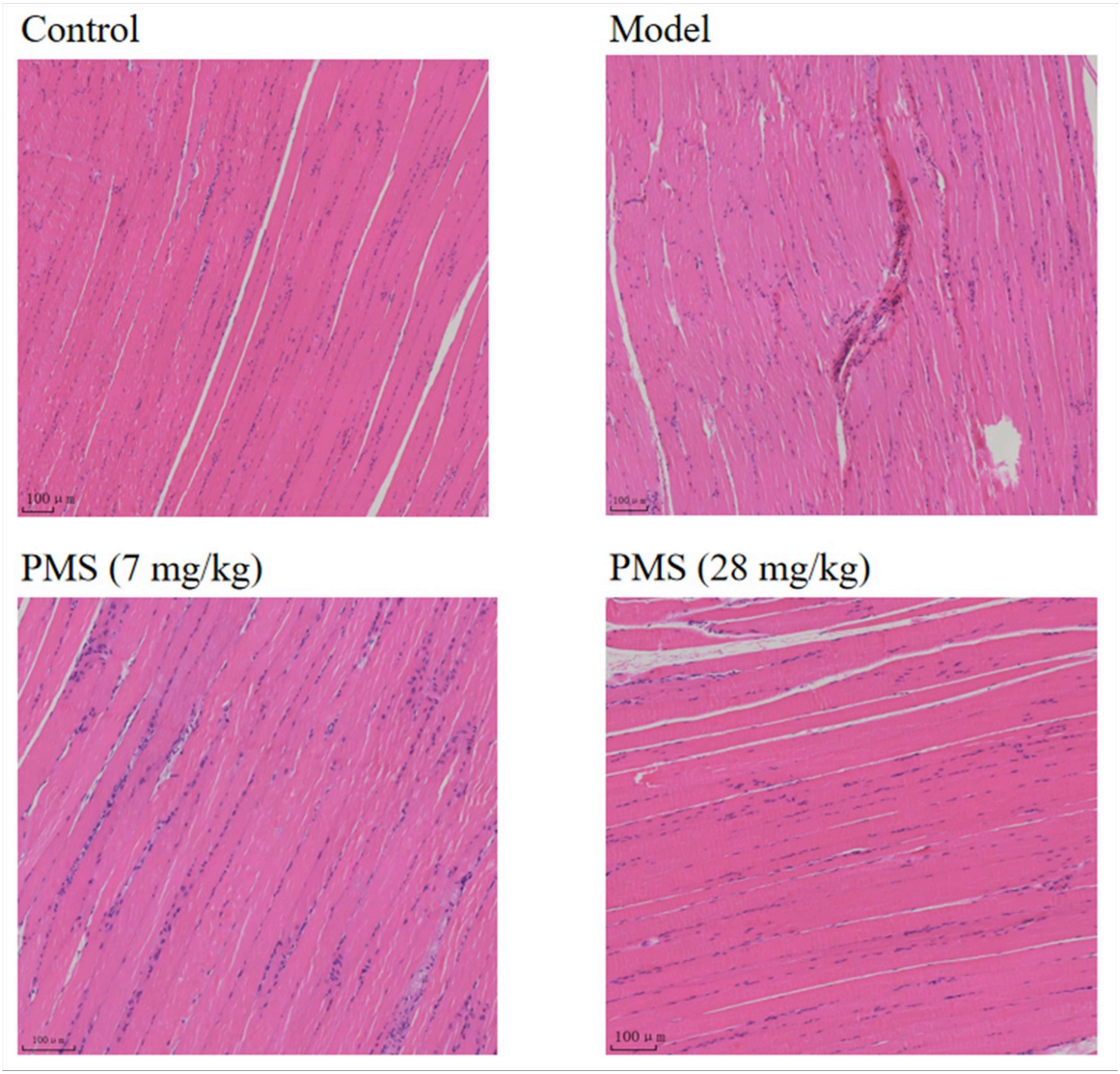


Figure 4

Effects of PMS on histopathology in the experimental rats. The skeletal muscle tissues were stained with H&E, and micrographs were taken at 100× magnification.

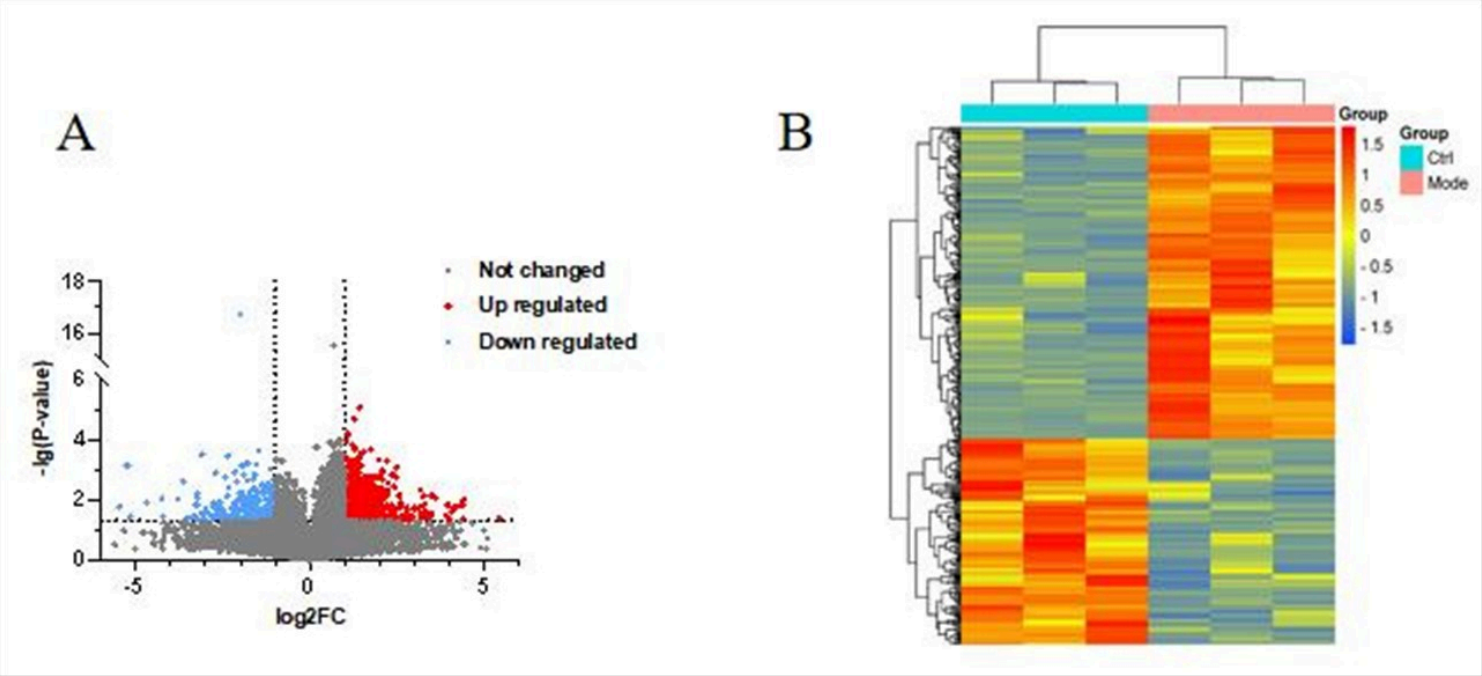


Figure 5

(A) Distribution of differentially expressed genes/transcripts in gastrocnemius muscle between the control group and model group; (B) Cluster analysis of differentially expressed genes/transcripts in gastrocnemius muscle of the control group and model group.

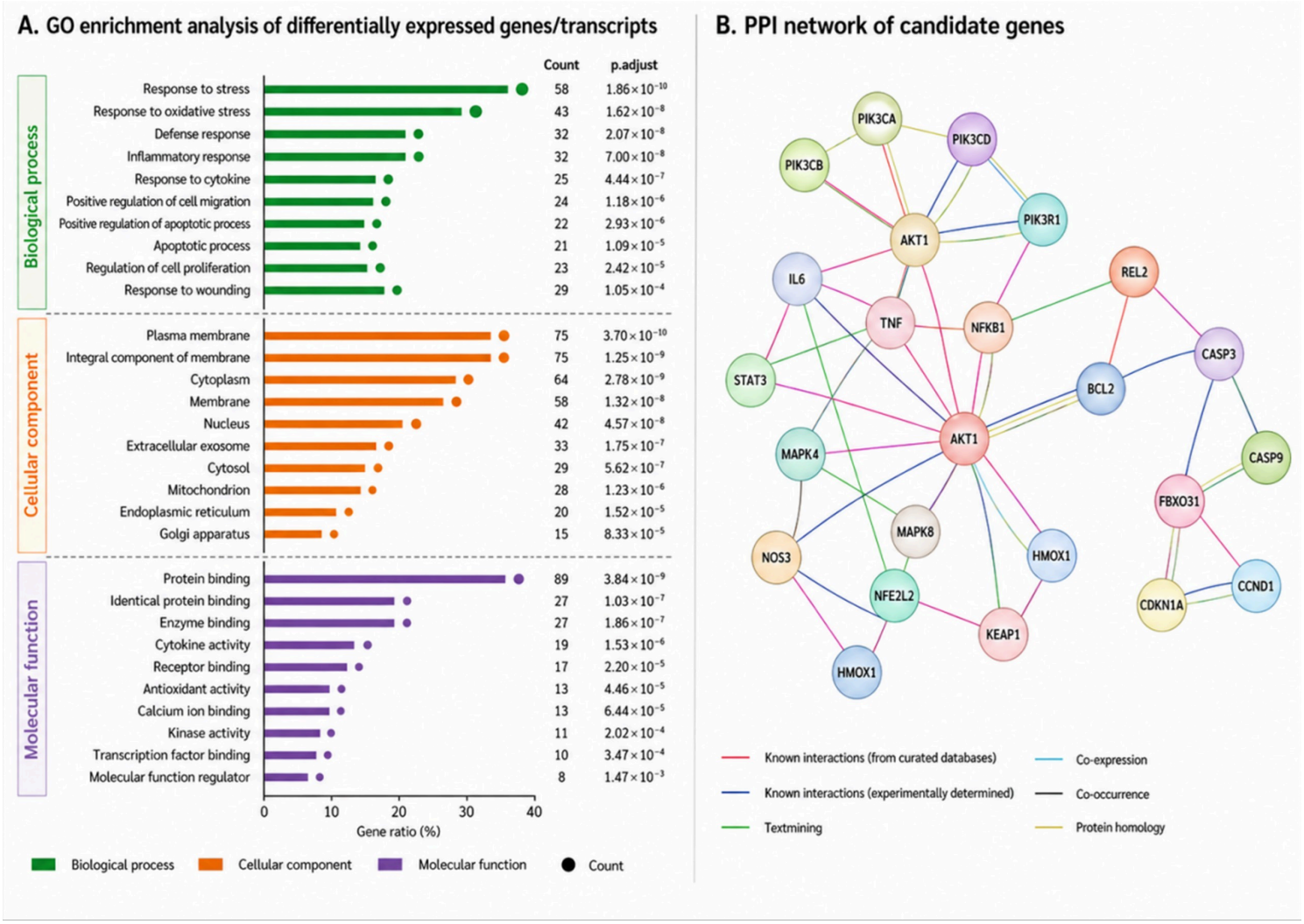


Figure 6

Integrated GO enrichment and PPI network analysis of differentially expressed genes/transcripts in rat gastrocnemius muscle. (A) GO enrichment analysis of differentially expressed genes/transcripts between the control group and model group, showing the major enriched terms in biological process (BP), cellular component (CC), and molecular function (MF).

(B) PPI network analysis of candidate genes/proteins related to apoptosis- and cell cycle-associated responses. The network was generated using the STRING database (confidence score = 0.4), and FBXO31, CDKN1A, and CCND1 were selected for further experimental validation.

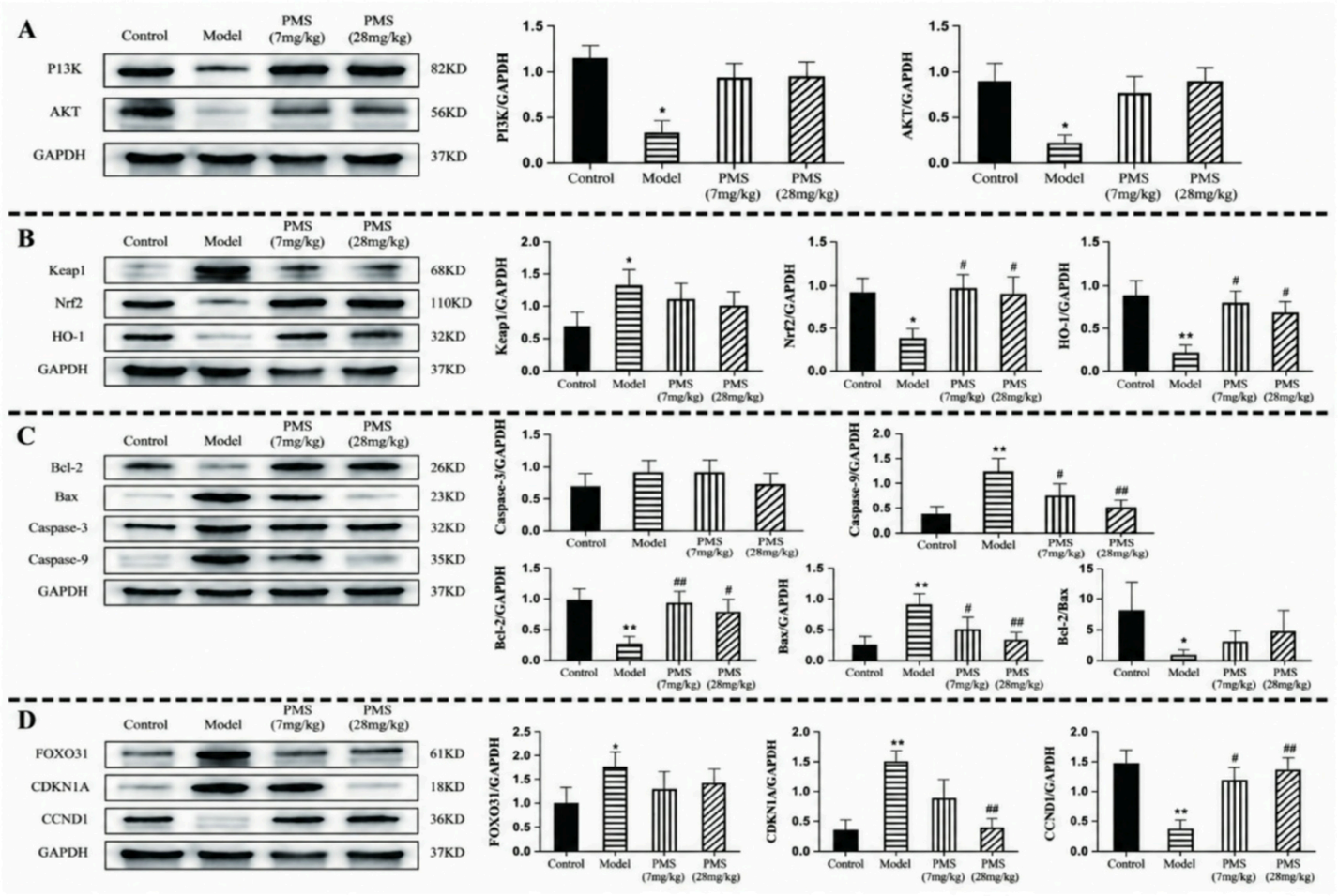


Figure 7

Western blot analysis of PMS-related protein expression in skeletal muscle of experimental rats and quantification of protein expression. Protein levels (PI3K, AKT, HO-1, Nrf2, Keap1, Bcl-2, Bax, Caspase-3, Caspase-9, FBXO31, CDKN1A and CCND1) were normalized to GAPDH. Data are expressed as mean ± SD. $^{*}p< 0.05$ compared with the control group; $^{\#}p<0.05$ compared with the model group.

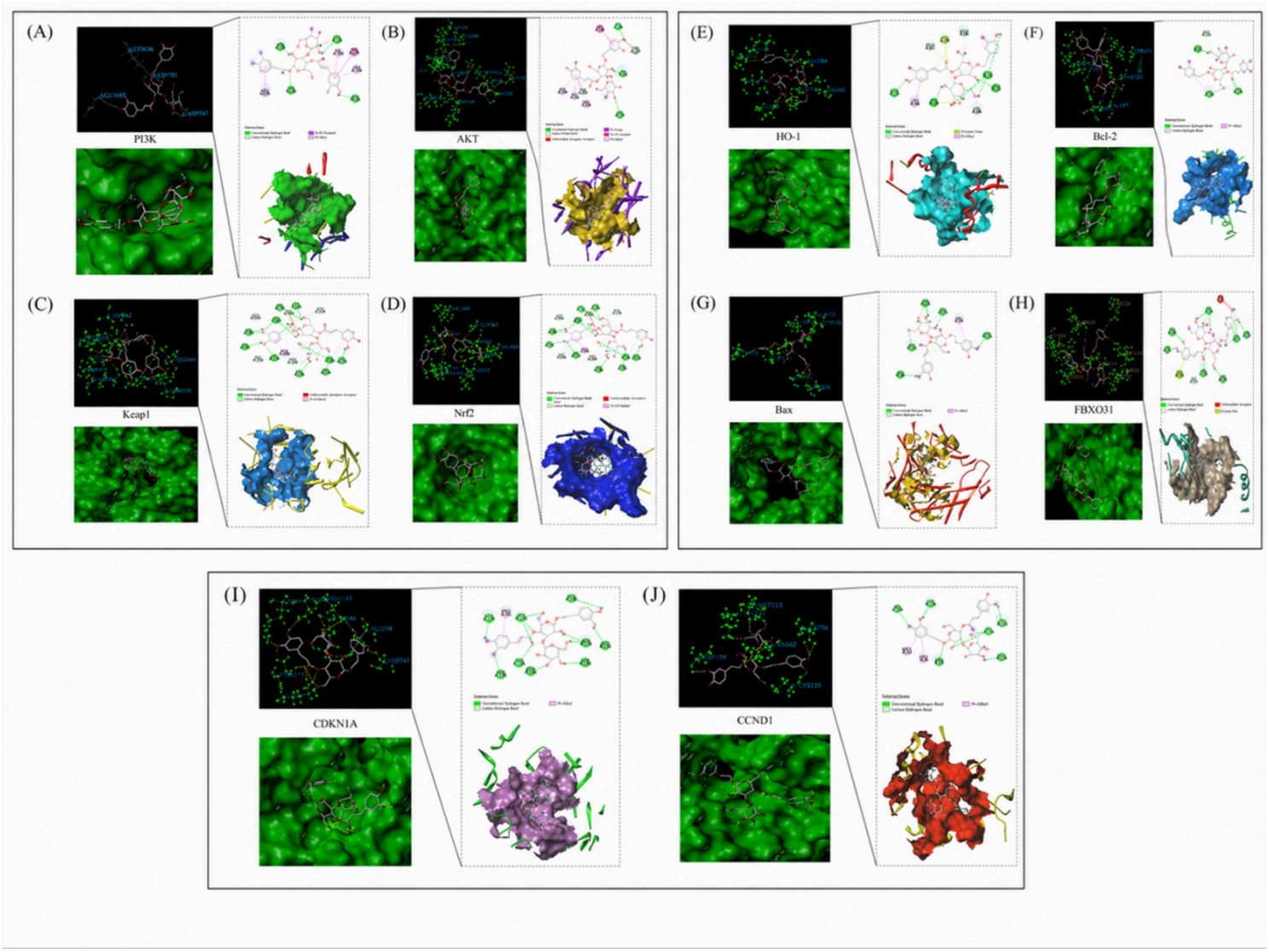


Figure 8

Molecular docking study of PMS binding to PI3K/Akt, Keap1/Nrf2/HO-1, Bcl-2/Bax and FBXO31/CDKN1A/CCND1proteins.

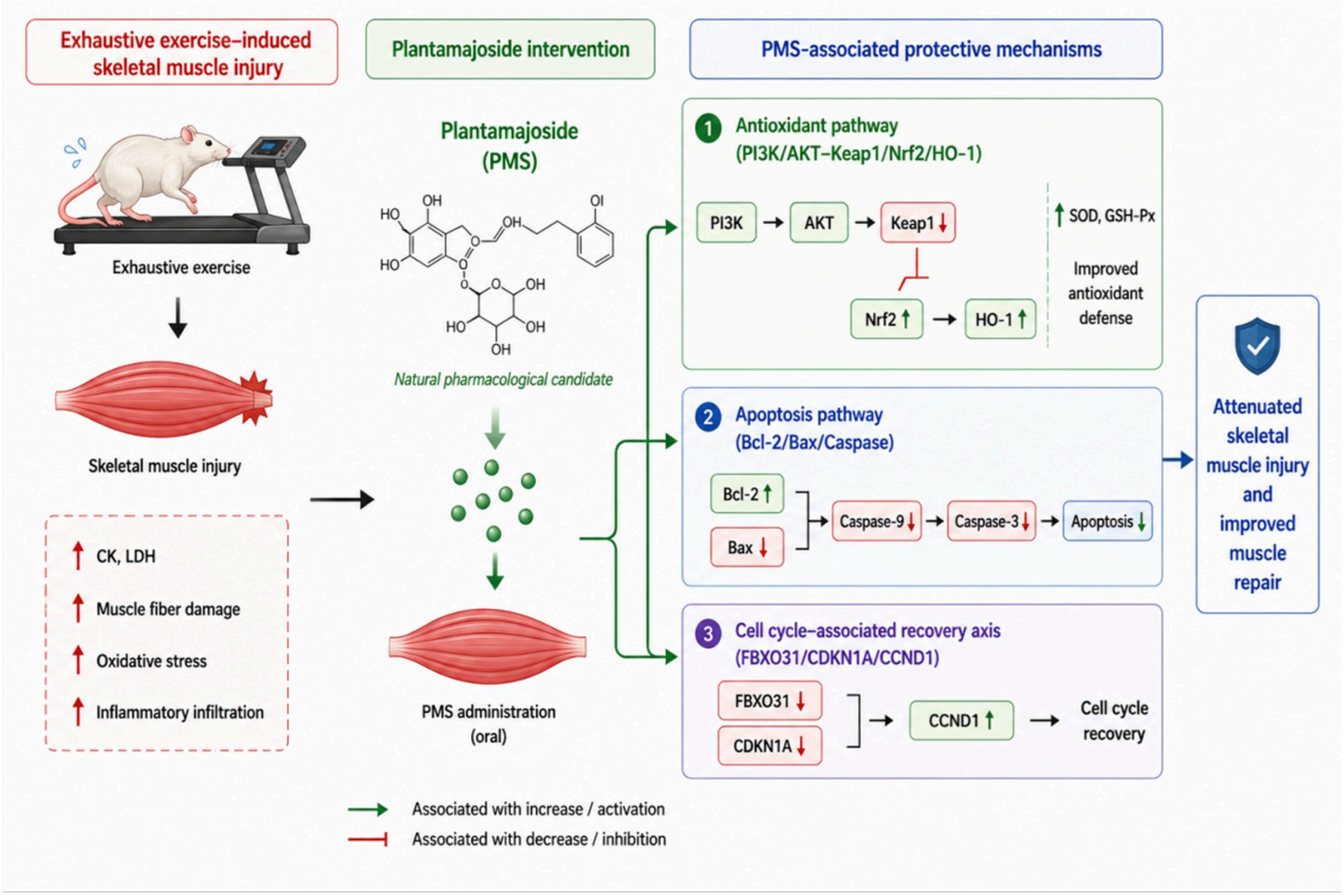


Figure 9

Schematic overview of the mechanisms associated with plantamajoside (PMS)-mediated protection against exhaustive exercise-induced skeletal muscle injury. PMS treatment is associated with modulation of PI3K/AKT–Keap1/Nrf2/HO-1 signaling, apoptosis-related proteins (Bcl-2, Bax, caspase-3 and caspase-9), and cell cycle-related factors (FBXO31, CDKN1A and CCND1). These changes are associated with reduced skeletal muscle injury and improved recovery following exhaustive exercise. The mechanisms shown are based on experimental observations and do not establish direct causality.

Supplementary Files

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