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Research Article

Mangiferin Alleviates Exercise-Induced Fatigue through Inhibition of NFκB-Mediated Inflammation and Oxidative Stress Based on Network Pharmacology

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Background. Muscle fatigue is defined as a decrease in maximal force or power production in response to contractile activity, which can cause muscle damage and limit athletic performance. A mass of phytochemicals were certified feasibly in relieving exercise-induced fatigue. Mangiferin, a multifactorial pharmacological phytochemical, was a promising candidate in theory; nonetheless, the potential molecular mechanisms of mangiferin on exercise-induced fatigue need to be elucidated. *Methods*. In this research, a mouse fatigue model was induced by forced swimming 4 weeks, and mangiferin was supplied in 100 mg/kg. The protective effects of mangiferin were illustrated by rotating rod test, serum biochemical indicators (LA, CK, BUN, ALT, and AST), and gastrocnemius morphology. Then, the targets of mangiferin against exercise-induced fatigue were predicted using network pharmacology through a comprehensive search and analysis with STITCH, PharmMapper Serve, STRING, and AutoDock Vina. Furthermore, predicted target genes were verified in gastrocnemius by immunohistochemistry. *Results*. Mangiferin has a significant antifatigue effect by prolonging the rotating time of the rod test. Serum biochemical indicators were mitigated (LA, CK, BUN, ALT, and AST), and gastrocnemius injury was easing. Network pharmacology was used to find that mangiferin might alleviate exercise-induced fatigue through regulating NFκB, PTGS2, TNF-α, and CCL2, and those aberrant expressions were verified in gastrocnemius. Molecular docking indicated that NFκB may be the most important molecule locking with mangiferin. Furthermore, the levels of IL-1β, TNF-α, IL-6, and MDA were significantly upregulated, while SOD and GSH-PX were downregulated in serum of fatigue mice, which were reversed with mangiferin treatment. *Conclusion*. Mangiferin is a feasible treatment to mitigate exercise-induced fatigue by anti-inflammation and antioxidative stress, through regulating the expression of NFκB and its downstream molecules.

1. Introduction

Physiological exercise induces muscle fatigue in intense and prolonged exercise both in athletes and individuals. Exercise-induced fatigue causes a decline or loss of power generation and endurance ability and even contributes to muscle damage that limits athletic performance [1]. Furthermore, the negative effect of exercise-induced fatigue engages not only single limbs but also the whole body, which is involved in the development of many diseases, including neurological, muscular, and cardiovascular disorders, as well as ageing and frailty, which threatens public health [2–5]. The pathogenesis of exercise-included fatigue is closely correlative with increased energy demands to satisfy

contraction in a vigorous metabolic state. Additionally, multiple mechanisms were involved in the progression of exercise-induced fatigue, including the output of neurotransmitters, the release of calcium, the supply of energy, the volume of blood flow, the balance of oxidation, and the response of inflammation [1].

Apart from energy exhaustion and metabolite accumulation, oxidative stress and inflammation are critical to the pathogenesis of exercise-induced fatigue [6]. Substantial investigations demonstrated that rigorous and/or prolonged exercise increased the production of free radicals and reactive oxygen species (ROS) both in serum and skeletal muscle fibres, thus leading to oxidative damage [7, 8]. Excessive ROS aggravated muscle fatigue through (1) attacking biomacromolecules (causing protein oxidation and lipid peroxidation), (2) accelerated muscle weakness (influencing intramyofibrillar Ca^{2+} turnover and sensitivity, Na^+/K^+-ATPase activity, and actin-myosin kinetics), and (3) activating biochemical signalling pathways that contribute to exercise-induced adaptation in the contracting muscle fibres [9–10]. Repetitive contraction gives rise to microtrauma and oxidative stress in the weak muscle, leading to inflammation. Smith's cytokine hypothesis considers that overtraining with insufficient recovery induces musculoskeletal trauma and enhances the production of proinflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) [6, 12]. The accumulated proinflammatory cytokines communicate with multiple organs and dysregulate mitochondrial function, contributing to a decline in physical performance [13]. Finding exercise-induced fatigue relief drugs with definite efficacy and fewer side effects is a hot topic in sports medicine, and phytochemicals are one of the most promising and feasible candidates attributed to their cost-effectiveness, superior bioavailability, and lower toxicity. It is well known that various nutrients from food sources can relieve fatigue, and increasing research has demonstrated that many natural plant-derived chemicals also have good antifatigue effects, such as ginsenosides [14, 15], resveratrol [16], and curcumin [17].

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-β-D glucoside) is a bioactive phytochemical isolated from the mango plant *Manfigera indica* L. and exhibited significant therapeutic effects in various chronic diseases including neurodegenerative disorders, cardiovascular diseases, renal and pulmonary diseases, diabetes, and obesity [18]. It has already exhibited favourable properties, presenting itself as a promising candidate for improving muscle function in several population-based experiments. Previous clinical research verified that the combination of mangiferin and luteolin counteract fatigue and improve exercise performance in sprint exercise [19, 20]. In addition, supplementation with a mango leaf extract (rich in the natural polyphenol mangiferin) combined with quercetin attenuates muscle damage and accelerates recovery after strenuous exercise [21, 22]. However, the potential molecular mechanisms of mangiferin have not been further elucidated. Studies *in vitro and in vivo* of mangiferin revealed that it has multifactorial pharmacological properties through different mechanisms of activity. With advantages in the prediction of pharmacology and molecular biology, network pharmacology is a unique way to research the multifactorial pharmacological function and complex disease and supply critical gene networks for further research and development. Here, we use network pharmacology to investigate potential molecular mechanisms of mangiferin in exercise-induced fatigue.

In this research, we investigated that mangiferin, a phytochemical, alleviates exercise-induced fatigue through inhibition of nuclear factor-κB (NFκB)-mediated inflammation and oxidative stress based on network pharmacology (Figure 1). First, we demonstrated the protective effects of mangiferin on muscle damage and the enhancement of exercise performance. Second, prostaglandin-endoperoxide synthase 2 (PTGS2), NFκB, and C-C motif chemokine ligand 2 (CCL2) were the critical genes in antioxidative stress and anti-inflammation of mangiferin through network pharmacology and experimental verification. Therefore, this study provides further insights into the mechanism linking mangiferin and exercise-induced fatigue. Mangiferin is a promising candidate for further research and development in antifatigue.

2. Materials and Methods

2.1. Experimental Animals. Eight-week-old male C57BL/6 mice weighing 21–24 g ($n = 28$; Shanghai SLAC Lab Animal Co., Ltd.) (Shanghai, China) were housed in a temperature- and humidity-controlled environment ($22.5 \pm 0.5^{\circ}$C, $50 \pm 5\%$ humidity) with a 12 h light-dark cycle. All mice had free access to food and water. All animal studies were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Animal Treatment. Our animal experiments were approved by the committee on ethics of the Naval Medical University, PLA. The mice were randomly assigned into four groups: control group (Ctrl; $n = 7$), exercise-induced fatigue group (EF; $n = 7$), mangiferin group (MF; $n = 7$), and mangiferin-treated exercise-induced fatigue group (EM; $n = 7$). The mice in the EF and EM groups were challenged by forced swimming over a 4-week test to build an exercise-induced fatigue mouse model: mice were individually forced to swim for 1 h with weight-bearing (5% of weight) attached to the proximal end of the tail per day in a water tank (30 cm high × 30 cm in diameter) filled 2/3 full of water at room temperature (20–25°C). The mice were determined to be exhausted when they sunk into the water and could not rise to the surface of water within a 10 s period. The day's training would be interrupted. Mangiferin was dissolved in a saline solution at a concentration of 12.5 mg/mL. The mice in the MF and EM groups were gavaged with mangiferin at a dose of 100 mg/kg body weight, and the Ctrl and EF groups were treated with the same volume of saline.

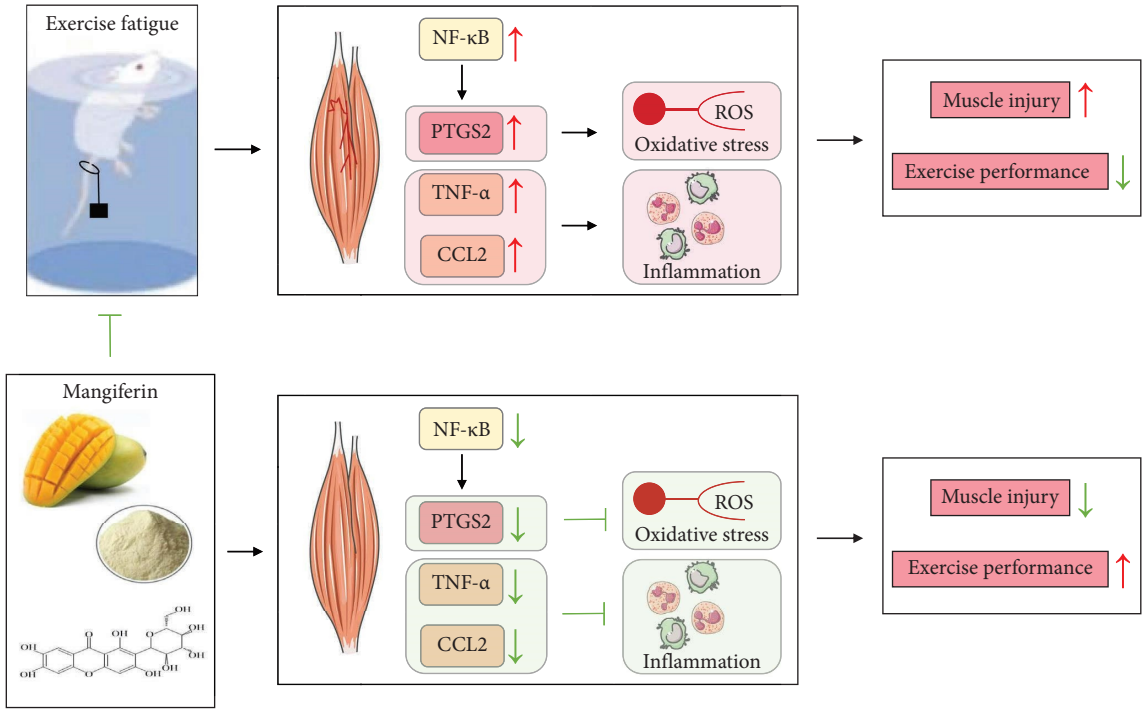

Figure 1: Diagram of the effects of mangiferin on exercise-induced fatigue.

2.3. Rotating Rod Test. In the rota-rod test, the mice in four groups were placed on an accelerating rota-rod cylinder (Shanghai Jiliang Software Technology Co. Limited, China). The test was divided into two steps: a training period and a test period. The mice were trained three times on the first day and tested on the second day. In the training period, the rota rod was accelerated from 7 to 20 r·min^{-1} in 10 min (intertrial interval = 60 min). In the test period, the rota rod was accelerated from 5 to 35 r·min^{-1} in 5 min. The duration was measured.

2.4. Serum Biochemical Assay. At the end of the experiment, the mice were sacrificed, and blood was collected from the eyeballs and centrifuged at 3500 rpm for 15 min at room temperature. Serum was obtained, and serum lactic acid, creatine kinase (CK), blood urea nitrogen (BUN), alanine transaminase (ALT), and aspartate transaminase (AST) were determined by a spectrophotometric detection method using commercially available kits (Jiancheng Biotech. Sci. Inc., China). The levels of these parameters were determined by the manufacturer's protocols.

2.5. Network Pharmacology. Relevant data on exercise fatigue were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The target proteins of mangiferin were searched in the Search Tool for Interactions of Chemicals (STITCH) database (https://stitch.embl.de) and PharmMapper Serve database (https://www.lilab-ecust.cn/pharmmapper/). Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses were performed using the clusterProfiler package. The protein-protein interaction (PPI) for the intersection targets was assessed by the Search Tool for Retrieval of Interacting Genes (STRING) database (https://string-db.org/), and the PPI network was constructed using Cytoscape (https://cytoscape.org) software. Molecular docking was carried out using AutoDock Vina (version 1.1.2) software. Ligplus (version 2.24) software and PyMoL (version 4.5.0) software were used to study protein-ligand interaction and visualize the results as two-dimensional and three-dimensional figures, respectively.

2.6. Tissue Section Staining and Immunohistochemistry. Gastrocnemius tissues were separated and kept in 4% formaldehyde for 24 h. Hematoxylin and eosin (HE) staining was used to evaluate the gastrocnemius pathology of mice in the four groups. The gastrocnemius was removed, dehydrated by gradient ethanol, embedded in paraffin, and sectioned at 4 μm thickness. Paraffin sections of gastrocnemius were stained with HE by standard procedures. For immunohistochemistry (IHC) analysis, paraffin sections were stained with PTGS2 antibodies (1 : 500, Cat. GB11077-1, Servicebio, China), TNF-α antibodies (1 : 200, Cat. GB11188, Servicebio, China), NFκB antibodies (1 : 500, Cat. GB11997, Servicebio, China), and CCL2 antibodies (1 : 500, Cat. GB11199, Servicebio, China). Finally, observations and photos were taken with an optical microscope.

2.7. Biomarker Measurement in Serum. Serum ELISA IL-1β ELISA kit (Cat. GM1152, Servicebio, China), TNF-α ELISA kit (Cat. GM1150, Servicebio, China), IL-6 ELISA kit (Cat. GM1154, Servicebio, China), malondialdehyde (MDA) kit (Cat. GM1134, Servicebio, China), superoxide dismutase (SOD) kit (Cat. GM1133, Servicebio, China), and

glutathione peroxidase (GSH-PX) kit (Cat. GM1135, Servicebio, China) were used to detect the corresponding protein levels in the serum. The ELISA experiments were performed according to the manufacturer's instructions.

2.8. Statistics. Experimental data were expressed as the mean ± standard error of the mean (SEM) and were analyzed with SPSS 22.0 statistical software (SPSS Inc., USA). Student's *t*-test was used to compare results between two groups. One-way ANOVA followed by Tukey's post-test was used for comparisons among multiple groups. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Mangiferin Mitigates Exercise-Induced Fatigue Effectively. Gastrocnemius tissue sections stained with HE were examined under a light microscope for histopathological analysis to determine the effect of mangiferin on exercise-induced fatigue. The damage to muscle fibres and the consumption of muscle glycogen and liver glycogen increased in EF group mice, and mangiferin alleviated the damage and consumption (Figure 2(a)). Likewise, mangiferin improved the decline in motor function, including the rotating stick time of the rota-rod test (Figure 2(b)), and reduced serum biochemical indices, including lactic acid (LA), CK, and BUN (Figures 2(c)–2(e)). In addition, the increased levels of ALT and AST in serum of fatigue mice were improved by mangiferin (Figures 2(f) and 2(g)), suggesting that mangiferin also alleviated liver damage.

3.2. Related Targets and Pathways of Mangiferin in Exercise-Induced Fatigue. Relevant data on exercise-induced fatigue were downloaded from the dataset (GSE209706, Supplementary Table S1), and 1495 targets of exercise-induced fatigue were screened. The STITCH database and PharmMapper Serve were used together to screen the targets of mangiferin. Two hundred (Supplementary Table S2) and 298 (Supplementary Table S3) potential targets were retrieved from the above two databases. In total, 475 mangiferin targets were obtained after removing the repetitive targets. Next, the intersection of exercise-induced fatigue targets and mangiferin targets was examined, and 42 intersection targets were obtained (Figure 3(c)). The top 20 KEGG pathways (Figure 3(a), Supplementary Table S4) and the top 10 GO terms (Figure 3(b), Supplementary Table S5) of cell composition (CC), biological processes (BP), and molecular functions (MF) were performed. After enrichment analysis, the top two oxidative- and inflammatory-related signalling pathways were obtained: the IL-17 signalling pathway and the TNF signalling pathway. PTGS2, TNF-α, NFκB, and CCL2 were the hub targets identified by the PPI network using the degree method of Cytoscape software (Figure 3(d)).

To further explore the changing trend of the four hub targets, IHC analysis of PTGS2, TNF-α, NFκB, and CCL2 was carried out in the Ctrl group, EF group, EM group, and MF group of muscle tissue. As compared with the Ctrl and MF groups, the expressions of PTGS2, TNF-α, NFκB, and CCL2 in muscle tissue in the EF group were significantly upregulated. With the treatment of mangiferin, the expressions of the four proteins were significantly decreased (Figure 4(a)). After docking, the binding energies with PTGS2, TNF-α, NFκB, and CCL2 were −3.5 kcal/mol, −7.8 kcal/mol, −9.9 kcal/mol, and −8.3 kcal/mol, respectively (Table 1). According to the binding energy and the number of hydrogen bonds, NFκB has the maximum absolute values of binding energy (Figure 4(b)), suggesting that the molecular docking effect is ideal.

3.3. Mangiferin Influences the Expression of the Oxidative- and Inflammatory-Related Proteins in Exercise-Induced Fatigue. To further explore the mechanism of mangiferin against exercise-induced fatigue, the indicators of inflammation and oxidative stress were detected in the four groups. As shown in Figure 5, the levels of IL-1β, TNF-α, IL-6, and MDA in serum of mice were significantly upregulated, while SOD and GSH-PX were significantly reduced in the EF group compared to the Ctrl group. After treatment with mangiferin, the levels of these indicators were reversed, indicating that mangiferin might mitigate exercise-induced fatigue by suppressing inflammation and oxidative stress.

4. Discussion

Physical exercise is an extensive protective factor against chronic diseases and thus has been promoted and adopted in our daily life [23–25]. However, prolonged and intense exercise induces muscle fatigue. Skeletal muscle is confronted with a high need for ATP and accumulated metabolites. Meanwhile, excessive free radicals and inflammatory factors were generated. If resolved unfavourably, accumulated oxidative stress and inflammation cascade aggravate fatigue and even muscle damage. Many phytochemicals that act as antioxidants have been proven effective in relieving exercise-induced fatigue [26–28]. In this study, we demonstrated that mangiferin could alleviate exercise-induced fatigue by mitigating oxidative stress and inflammation via regulating NFkB. Overall, mangiferin is an effective antifatigue phytochemical, which might have a promising prospect in clinical use.

Mangiferin has been demonstrated to possess multiple properties, including antioxidant, antidiabetic, antihyperuricemic, antiviral, anticancer, and anti-inflammatory activities [29]. Mangiferin acts as a modulator in exercise-induced fatigue in theory due to its potential capability of scavenging free radicals, which is derived from its C-glucosyl linkage and polyhydroxy groups [30]. In our research, the decreased levels of serum SOD and GSH-PX in mice induced by exercise were reversed by mangiferin. Along with antioxidant properties, we predict that potential auxiliary effects of mangiferin could reduce defects of excessive ROS and thus interrupt oxidative stress cascade effects in exercise-induced muscle damage. It has been reported that the cardioprotection of mangiferin in doxorubicin (DOX) cardiotoxicity relied on upregulating calcium regulatory

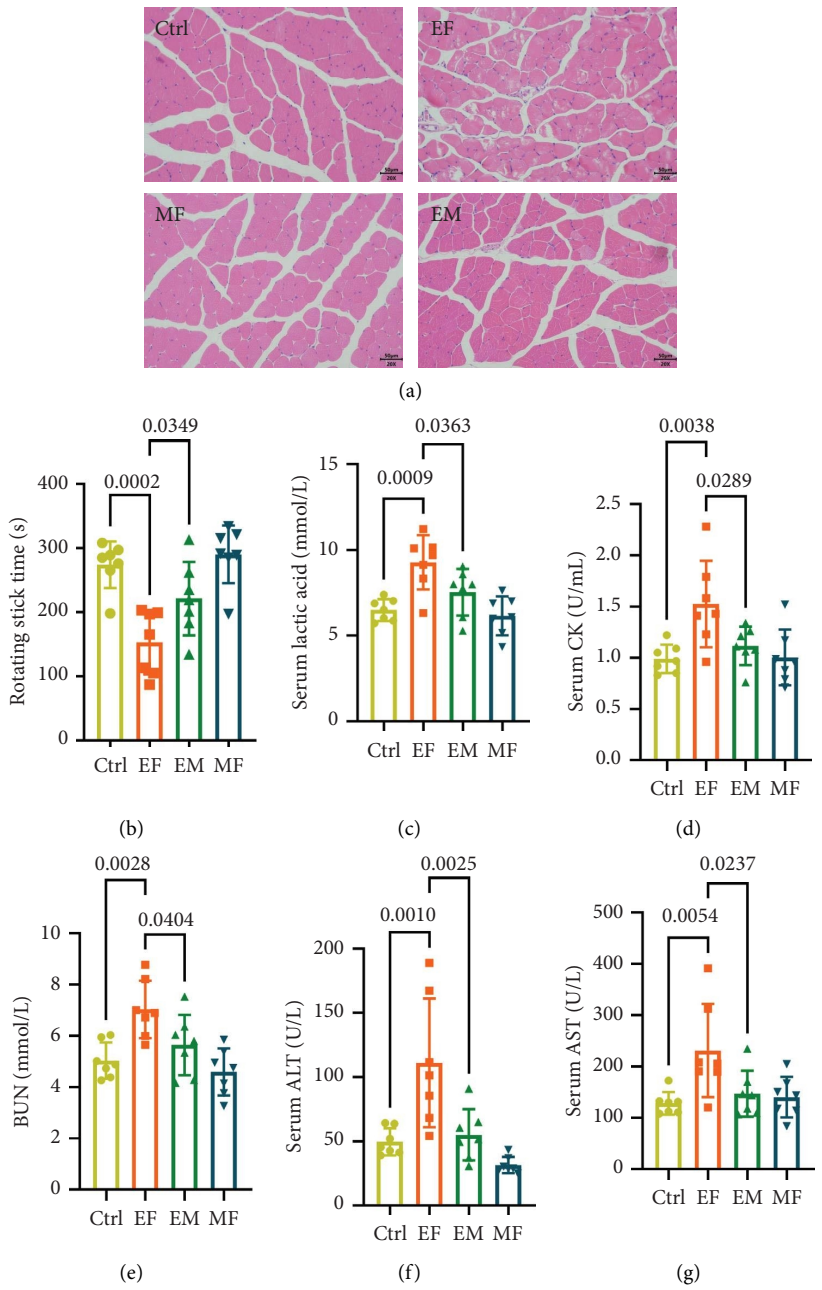

FIGURE 2: Effect of mangiferin on exercise-induced fatigue in mice. (a) HE staining of muscle tissue sections in the ctrl, EF, MF, and EM groups ($n = 7$). (b) The rotating stick time of the rota-rod test in the ctrl, EF, MF, and EM groups state ($n = 7$). (c–g) Serum biochemical levels of lactic acid, CK, BUN, ALT and AST in the Ctrl, EF, MF, and EM groups ($n = 7$).

gene (SERCA2a) and restoring intracellular Ca^{2+} concentration [31]. Another research declared that aqueous extract of *Mangifera indica* L. leaves significantly increased the Na^+-K^+ ATPase activity in the small intestine [32]. These two independent research studies give us some bases that mangiferin alleviates exercise-induced fatigue through antioxidant properties and cascade effects, like restoring intracellular Ca^{2+} and increasing the Na^+-K^+ ATPase activity. Interestingly, mangiferin also possesses iron chelation property, cutting off the generation of hydroxyl radicals in Fenton's reaction and corresponding lipid peroxidation reactions [33–36]. In our research, the level of serum MDA was downregulated with mangiferin treatment, suggesting that mangiferin might rescue lipid peroxidation reactions due to its iron chelation property. Apart from its capacity to scavenge free radicals, mangiferin also possesses the ability to modulate the expression of different genes involved in several signalling cascades, like NFκB and Nrf2 [18].

The NFκB signalling pathway is sensitive to oxidative stress and activated by excess ROS [37]. Oxidative stress

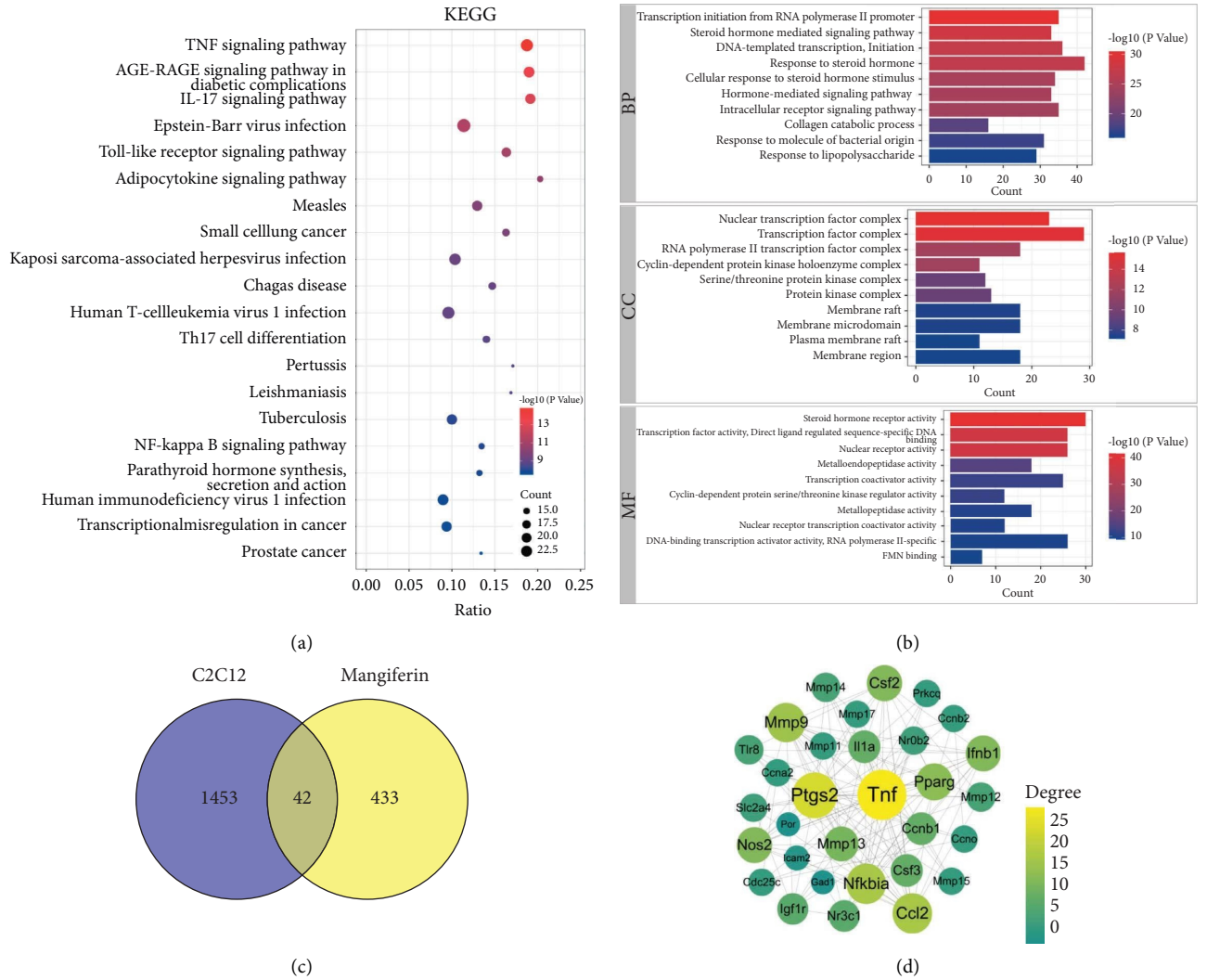

Figure 3: Screening the targets and pathways of mangiferin against exercise-induced fatigue. (a) The top 20 significantly enriched KEGG pathways of predicted targets of mangiferin against exercise-induced fatigue. (b) The top 10 GO terms (including biological processes, cellular components, and molecular function) of predicted targets of mangiferin against exercise-induced fatigue. (c) Venn diagram showing the overlapping targets of mangiferin and exercise-induced fatigue. (d) Visualization of PPI network based on predicted targets of mangiferin against exercise-induced fatigue.

increases the production of cytokines, including TNF-α [38]. Oxygen-containing derivatives act as the second messenger to activate transcription factor NFκB and activate protein 1 (AP-1). The oxidation of reduced Coenzyme H (NADPH) oxidase complex on the cell membrane produces ROS to amplify oxidative stress. It can further stimulate the activation of inflammatory cells, thus forming a vicious spiral between oxidative stress and inflammatory response. In addition, increased ROS excites tyrosine kinase and induces the phosphorylation of Raf-1 and its downstream enzymes, which further activates the intracellular ribosome S6 kinase. The phosphorylation of the Tyr4 residue of IκBα promotes its degradation, which induces activation of NFκB. It was reported that NFκB selectively binds to DNA response elements and regulates the transcription of antioxidative enzymes and inflammatory cascade in skeletal muscle [39]. Prolonged or high-intensity exercise is also linked with substantial inflammatory responses that could augment mitochondrial dysfunction and ROS production, as well as precede muscle damage. Thus, there is elegant evidence which shows NFκB activation both in serum and muscle in terms of exercise-induced damage [39–43]. Consistently, the increased expressions of NFκB, PTGS2, TNF-α, and CCL2 were detected in our exercise-induced fatigue model of mice. Furthermore, molecular docking analysis implied that NFκB might be the key molecule locking with mangiferin. Among these four molecules, mangiferin shows the best research evidence for NFκB [18]. On the basis of the anti-inflammatory and antioxidant effects of mangiferin on exercise-induced fatigue, we analyzed key inflammatory factors and oxidative stress indicators in serum of mice, and the results were also in line with our expectations. The regulation of mangiferin in NFκB has been demonstrated in various inflammatory-related diseases [44]. It was predicted

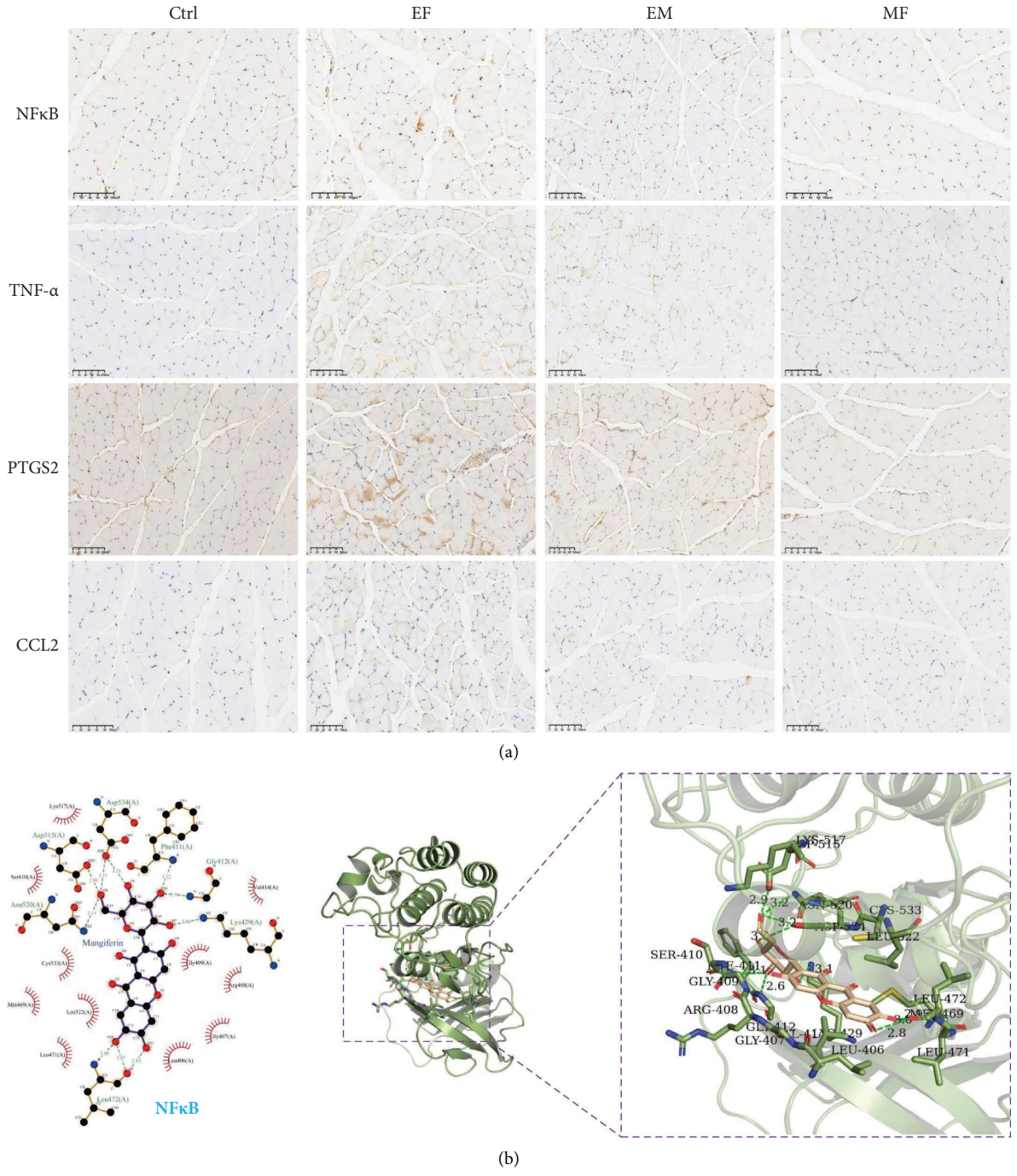

Figure 4: The hub targets were validated by IHC and molecular docking. (a) The expressions of PTGS2, TNF-α, NFκB, and CCL2 in muscle tissue were displayed by IHC analysis in the ctrl group, EF group, EM group, and MF group. (b) The structural diagram of the simulated docking of mangiferin with NFκB.

Table 1: The binding energies of mangiferin docking with NFκB, PTGS2, TNF-α, and CCL2.

Phytochemical	Protein	PDB ID	Ligand	Affinity (kcal/mol)
Mangiferin	NFκB	4DN5	AGS	−9.9
	CCL2	4DN4	GOL	−8.3
	TNF-α	2AZ5	307	−7.8
	PTGS2	1CX2	S58	−3.5

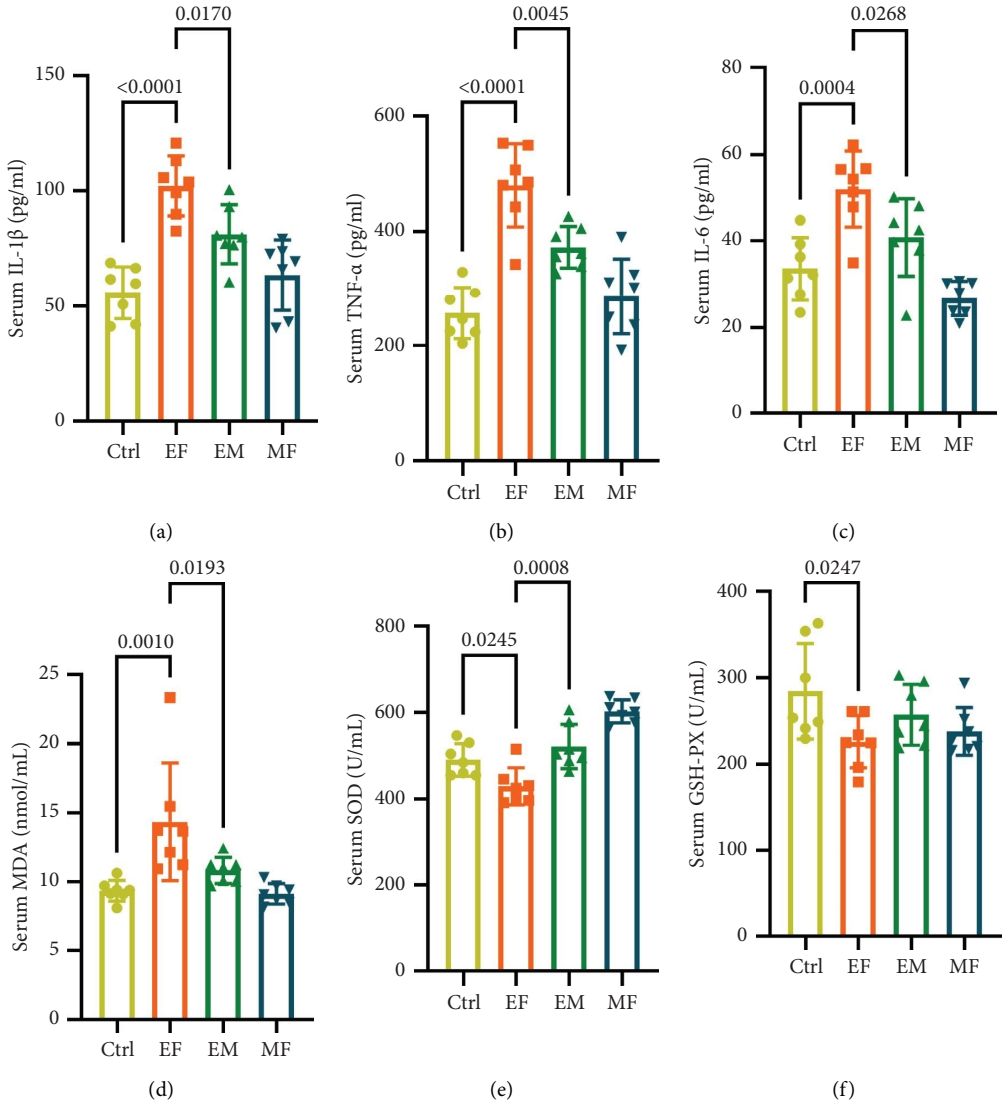

Figure 5: Mangiferin alleviated inflammation and oxidative stress in fatigue mice. (a–f) The levels of IL-1β (a), TNF-α (b), IL-6 (c), MDA (d), SOD (e), and GSH-PX (f) in serum were determined by corresponding kits in the ctrl group, EF group, EM group, and MF group ($n = 7$).

that mangiferin blocked the p50-p65/DNA interaction of NFκB, resulting in the loss of the functions of this heterodimeric member through molecular dynamic simulations [45]. In addition, NFκB is a pleiotropic nuclear transcription factor, which regulates the expression of a vast array of genes, including PTGS2 and CCL2. A single bout of exercise accelerates NFκB activation as well as PTGS2 expression. As exercise intensity increased, both PTGS2 expression and NFκB DNA-binding activity were enhanced in an intensity-dependent manner [39]. In addition, electrically stimulated C2C12 mimics muscle contraction in an *in vitro* system and releases CCL2 in an NFκB-dependent manner to induce monocyte chemoattraction in exercise-induced muscle inflammation [46]. A complex regulatory network is formed among them, and NFκB is most likely the initiating molecule of exercise-induced inflammation and oxidative stress damage [47].

Collectively, this research suggested that mangiferin could improve exercise-induced fatigue by suppressing inflammation and oxidative stress via NFκB regulation. The most prominent innovation of our research is the first to prove that mangiferin has an antisports fatigue effect and to analyze possible potential targets using network pharmacology methods. Concurrently, there are some shortcomings in this study. For example, this study did not conduct in-depth research on the anti-inflammatory and antioxidant mechanisms, nor did it prove whether mangiferin has direct or indirect effects on PTGS2, TNF-α, CCL2, and NFκB. Regardless, this study is a valid attempt to screen for potential antifatigue phytochemicals. The concern of pharmacological activities and therapeutic molecular mechanisms of mangiferin were explored in this research, providing a reference for further drug research and development in exercise-induced fatigue.

5. Conclusion

In conclusion, our study suggested that mangiferin alleviates exercise-induced fatigue through inhibition of NFκB-mediated inflammation and oxidative stress. Therefore, we propose mangiferin as a potential phytochemical for the prevention and treatment of exercise-induced fatigue.

Data Availability

All data generated or analyzed during this study are included within the article (and its supplementary information files).

Additional Points

Research Highlights. (1) Mangiferin has a protective effect against exercise-induced fatigue. (2) Mangiferin has anti-inflammatory, antioxidant, and antifatigue effects. (3) Mangiferin alleviates exercise-induced fatigue by regulating the NFκB pathway.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

CL, ML, SX, and HL completed the experimental analysis and wrote the manuscript. YZ organized the results. YS analyzed the data. WC bred the mice. HL, JC, DG, and XG were responsible for formal analysis, proposed the methodology, and used the software. HS and HL designed, analyzed, and edited the manuscript. All authors contributed to the article and approved the submitted version. CL, ML, YZ, and SX have contributed equally to this work and share first authorship.

Acknowledgments

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Supplementary Materials

Relevant data on exercise-induced fatigue were downloaded from the dataset (GSE209706, Supplementary Table S1). Two hundred (Supplementary Table S2) and 298 (Supplementary Table S3) potential targets were retrieved from the STITCH database and PharmMapper Serve databases to screen the targets of mangiferin. After the intersection of exercise-induced fatigue targets and mangiferin targets, 42 overlapping targets were obtained. The first 20 KEGG pathways (Supplementary Table S4) and the top 10 GO terms (Supplementary Table S5) of cell composition (CC), biological processes (BP), and molecular functions (MF) were performed with clusterProfiler package. (*Supplementary Materials*)

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