Milk fat globule epidermal growth factor 8 (MFG-E8) and whey protein have emerged as promising bionutrient supplements for enhancing skeletal muscle mass and function. In the present study, aging-related sarcopenia rat model was employed to elucidate the effects of the combined administration of MFG-E8 and whey protein on the catabolism and anabolism of gastrocnemius protein. Combined intervention led to notable enhancements in the antioxidative stress status and mitochondrial biogenesis capacity of gastrocnemius muscle fibers in the aging rats, concomitant with a significant inhibition of lipid accumulation. Moreover, the synergistic effect of MFG-E8 and whey protein was found to exert modulatory effects on key signaling pathways, including PI3K/Akt/PGC-1α pathway and MAPK/ERK signaling pathways in the gastrocnemius muscle of the aging rats. Specifically, this combined intervention was observed to promote mitochondrial biogenesis and regulate the expression of protein anabolism and catabolism-related regulators, thereby facilitating the alleviation of mitochondrial oxidative stress and enhancing biogenesis in gastrocnemius tissues. The findings of our study provide compelling evidence for the potential of MFG-E8 as a promising dietary supplement with antisarcopenic properties to ameliorate muscle protein metabolism disorders and mitigate mitochondrial-mediated myoblast apoptosis induced by oxidative stress.

**Key words:** sarcopenia, MFG-E8, whey protein, PI3K/Akt/PGC-1α signal pathway, mitochondria function

**INTRODUCTION**

Sarcopenia, a condition commonly observed in the elderly population, is characterized by progressive neuromuscular changes, alterations in anabolic hormone levels, and metabolic diseases (Daily and Park, 2022; Li et al., 2023). The aging process often leads to the loss of skeletal muscle mass, bone density, strength, and function, which significantly affects physical performance and quality of life (Ham et al., 2020). During the age-related process of muscle loss, the rate of protein synthesis was generally declined, whereas the rate of protein breakdown increased. This leads to a net loss of protein in muscle tissue and a slowdown in the protein anabolism pathway (Nishikawa et al., 2021). Meanwhile, the increased risk of oxidative stress associated with aging causes a decline in mitochondrial function, resulting in insufficient energy supply to mitochondrial biogenesis and function, thereby affecting normal muscle function and protein synthesis (Alizadeh Pahlavani et al., 2022). Although drug treatments for sarcopenia exist, they often come with noticeable side effects. As a result, nutritional interventions have gained global attention as a promising approach to improving muscle function in elderly individuals with reduced exercise capacity (Daily and Park, 2022; Nunes et al., 2022). In particular, protein nutrition and its effect on elderly health have emerged as a key area of interest in recent years (Wu et al., 2022).

High-quality protein supplementation has been recognized as an effective strategy for mitigating conditions such as obesity, sarcopenia, and sarcopenic obesity without necessitating significant dietary changes (Daily and Park, 2022; Nunes et al., 2022). Among various protein sources, whey protein has been identified as particularly beneficial in reducing body fat and promoting muscle protein synthesis, owing to its high caloric value (Wu et al., 2022). Whey protein has been found to improve muscle mass and address glycolipid metabolism disorders (Mitchell et al., 2015; Gilmartin et al., 2020). Mechanistically, it is believed that whey protein exerts its effects on sarcopenia through the activation of the insulin-like growth factor 1 (IGF-1)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and adenosine 5′-monophosphate-activated protein kinase (AMPK)/sterol regulatory element
binding proteins-1c (SREBP-1c) signaling pathways, which help regulate skeletal muscle lipid metabolism and promote muscle protein anabolism (Shin et al., 2020; Wu et al., 2022). This, in turn, inhibits skeletal muscle lipid accumulation (Castro et al., 2017; Corrochano et al., 2018). As a functional protein, whey protein is often used as a dietary supplement in combination with other drugs or nutrients to investigate potential synergistic effects on age-related degenerative diseases, such as obesity and sarcopenia (Bauer et al., 2015; Hassanzadeh-Rostami et al., 2020).

The milk fat globule membrane (MFGM) is a unique 3-layered structure composed of phospholipids, sphingolipids, and membrane proteins (Watanabe et al., 2020; Li et al., 2023). Clinical studies have revealed that MFGM protein contains numerous active components, some of which have shown potential in alleviating skeletal muscle mass loss and dysfunction even at low doses (Soga et al., 2015; Wu et al., 2021). Therefore, ongoing research is continuously uncovering the potential of MFGM protein components in addressing sarcopenia (Raza et al., 2021; Li et al., 2023). One such component of MFGM is milk fat globule epidermal growth factor 8 (MFG-E8), a highly abundant protein secreted from mammary epithelial cells during milk production (Li et al., 2022a). MFG-E8 is also expressed in various tissues by different types of cells, including macrophages, fibroblasts, dendritic cells, and epithelial cells (Li et al., 2022a). Current research on the role of MFG-E8 in sarcopenia has primarily focused on the cellular level, with evidence suggesting that MFG-E8 exerts anti-inflammatory and antioxidative stress effects on myoblasts through activation of signaling pathways such as PI3K, AMPK, mitogen-activated protein kinase (MAPK), and nuclear factor kappa-B (NF-κB), which in turn promotes myoblast proliferation and differentiation, as well as muscle protein synthesis (Bu et al., 2021). In comparison to other high-quality animal and plant proteins, MFG-E8 has been shown to exert higher promotion effect on myoblast proliferation and differentiation, making it valuable protein resource within MFGM (Li et al., 2019). However, the precise effects of MFG-E8, particularly in combination with whey protein, on regulating oxidative stress and mitochondrial biosynthesis in the aging gastrocnemius muscle and cells, as well as the underlying mechanisms in alleviating sarcopenia, remain to be fully elucidated.

The signaling cascades of PI3K/Akt/peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1α) and MAPK/extracellular regulated protein kinase (ERK) are crucial in the development of skeletal muscle satellite cells and myoblasts (Li et al., 2021b). PGC-1α, a transcriptional coregulator that regulates energy metabolism and cellular respiration, has been shown to be upregulated by activating the PI3K/Akt signaling pathway, further to alleviate sarcopenia through regulating skeletal muscle mitochondrial biogenesis and protein synthesis (Kunkel et al., 2011; Liu et al., 2019). Additionally, reducing the phosphorylation expression of MAPK/ERK has been found to be beneficial in maintaining muscle protein homeostasis and muscle contraction, thereby preventing aging-related muscle mass and function (Li et al., 2022b). Studies showed that whey protein supplementation combined with resistance training can enhance muscle mass, strength, and function of aging population (Shen et al., 2019). Ensslin and Shur (2007) revealed that the absence of MFG-E8 led to significantly reduced levels of activated MAPK and decreased cell proliferation. Our previous in vitro cell experiments have provided evidence for the role of MFG-E8 in improving sarcopenia and its potential mechanisms, which can promote protein synthesis, cell cycle, and cell proliferation and inhibit cell apoptosis by mediating the PI3K/Akt and MAPK/ERK signaling pathways in muscle cells (Li et al., 2019).

The objective of this study was to assess the potential synergistic effect of MFG-E8 in combination with whey protein on oxidative stress and mitochondrial biosynthesis, and to elucidate the underlying mechanisms involved in regulating sarcopenia. To evaluate the in vivo effects of exogenous supplementation of MFG-E8 combined with whey protein, a d-galactose (d-gal)-induced aging-related sarcopenic rat model was employed. Parameters such as BW, lipid profiles, liver function, and the expression of regulators related to protein anabolism and catabolism were examined.

**MATERIALS AND METHODS**

**Materials**

The MFG-E8 protein was isolated and purified from MFGM using cellulose DE-52 (Solarbio, China) ion exchange chromatography. The whey protein concentrate used for the experiment was obtained from Sachsenmilch Leppersdorf GmbH Inc. (Sachsen, Germany). The purity of MFG-E8 was identified as 98.33% by LC-MS/MS and the purity of whey protein concentrate is 85%. Sprague Dawley rats were procured from the animal facility of the Second Affiliated Hospital of Harbin Medical University (Harbin, Heilongjiang, China) under permission number SCXK 2013-001.

**Animals, MFG-E8 Intervention, and Experimental Design**

Forty male Sprague Dawley rats was kept in an isolated cage with free access to water and food and...
fed with a normal specific-pathogen-free (SPF) diet (research diet D12450B, containing bovine casein, corn starch, maltodextrin, sucrose, cellulose, soybean oil, l-cystine, bitartrate, vitamins, minerals, approximately 20 kcal% protein, 70 kcal% carbohydrate, and 10 kcal% fat). Each rat, approximately 2 mo old, was induced to develop sarcopenia following a modified protocol from our previous studies (Li et al., 2021b). Rats were administered 10% d-gal daily over a successive 6-wk period (200 mg/kg per day) through subcutaneous injection on the nape back. The rats were then divided into 4 groups and orally administered MFG-E8 (16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk (Table 1). Dosages were calculated using clinically targeted drug dosage and drug equivalent dose ratios between humans and rats (Li et al., 2021b). After slaughter, biochemical assays were performed on blood, organs, and tissues. Gastrocnemius was located on the dorsal side of the hind leg, trimmed the fur of the gastrocnemius collection area, exposed the muscle tissue and cleaned with alcohol. Separated gastrocnemius from surrounding tissue, carefully removed the blood vessels and nerves connected to the gastrocnemius muscle, cut the connection between the gastrocnemius muscle and the Achilles tendon. Then, separated the gastrocnemius and took a suitably sized midsection of gastrocnemius muscle for hematoxylin and eosin (H&E) staining experiments. All animal protocols were approved by the animal ethics committee of Harbin Institute of Technology, School of Life Science and Technology, and efforts were made to minimize suffering. The calculation of rat organ coefficients was shown as follows:

\[
\text{Organ coefficients (\%)} = \frac{m_{\text{organ weight}}}{m_{\text{body weight}}} \times 100\%,
\]

where \(m\) = mass.

**Biochemical Analysis**

The blood was collected from anesthetized rat heart under nonfasted conditions via chloral hydrate vein. The collected blood was kept overnight at 4°C and centrifuged at 3,000 × g for 15 min to obtain serum. Serum levels of triglyceride (TG) and nonesterified fatty acid (NEFA) were measured using a TG and NEFA testing kit (Lengton Bioscience, Shanghai, China). The enzymatic activities of superoxide dismutase (SOD), glutamic-oxaloacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT), as well as the levels of malondialdehyde (MDA), were measured using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute, China. Insulin-like growth factor 1 was measured using a rat IGF-1 Immunoassay kit (Lengton Bioscience, Shanghai, China). The profiles of biochemical analytes were shown in Supplemental Table S1 (https://doi.org/10.17632/cywmmx3hy3.1, Guan et al., 2023).

**Histopathological Analysis of Gastrocnemius**

Histopathological evaluation was conducted by a pathologist at the hospital of Harbin Institute of Technology. Gastrocnemius samples were pre-fixed in 10% formalin solution, processed for paraffin embedding, 3- to 5-μm-thick sections of gastrocnemius were used and stained with H&E. Six images of different locations in one muscle H&E section were captured at 100× and 400× magnification. Based on H&E staining, histological alterations such as muscle fiber spacing, cell nucleus changes, and cell edema were evaluated under a microscope.

**Isolation of L6 Cells from Gastrocnemius**

The L6 cells were isolated following our previous protocol (Li et al., 2021b). The large hind limb gastrocnemius of rats in the control, MFG-E8, whey protein, and MFG-E8 + whey protein groups were isolated, respectively. The gastrocnemius without adipose tissue was subjected to enzymatic dissociation with 0.2% collagenase Type II (Solarbio) for 60 min, then with 0.04 U/mL dispase II (Solarbio) for 45 min. The cell suspension was filtered through a cell strainer. The resulting cell suspension was identified as L6 cells.

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**Table 1.** Detailed information on animal grouping, feeding conditions, and gastric dosing

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Basic feed</th>
<th>Amount/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>SPF animal feed</td>
<td>0.01 mol/L PBS</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>10</td>
<td>SPF animal feed</td>
<td>16 mg MFG-E8</td>
</tr>
<tr>
<td>Whey protein</td>
<td>10</td>
<td>SPF animal feed</td>
<td>1.26 g whey protein</td>
</tr>
<tr>
<td>MFG-E8 + whey protein</td>
<td>10</td>
<td>SPF animal feed</td>
<td>16 mg MFG-E8 + 1.26 g whey protein</td>
</tr>
</tbody>
</table>

1Nutritional intervention for 4 wk. MFG-E8 = milk fat globule epidermal growth factor 8.

2SPF = specific pathogen free.
Intracellular Mitochondrial Morphology

The structure of mitochondria in L6 cells was observed by electron microscopy following the previously described method (Guan et al., 2021; Li et al., 2022b). In brief, L6 cells were collected and subsequently centrifuged. The cells were then pre-fixed with 2.5% glutaraldehyde at 4°C overnight. The cell pellets were postfixed with 2.5% glutaraldehyde and 1% osmium tetroxide for 2 h. After dehydration in graded ethanol, the cells were embedded in Epon 812. Serial ultrathin sections were stained with both uranyl acetate and lead citrate. The images were observed and analyzed using a Zeiss 900 transmission electron microscope (TEM) and Image J (1.8.0 version) software.

Intracellular Mitochondrial and Lysosome Immunofluorescence Label

The nucleus, mitochondria, and lysosomes were marked and analyzed by Hoechst33342 blue (excitation 360 nm/emission 485 nm), mito-tracker red (excitation 530 nm/emission 590 nm), and lyso-tracker green (excitation 488 nm/emission 530 nm), respectively, following the previously described method (Li et al., 2022b). Mito-tracker red, lyso-tracker green, and Hoechst33342 blue working solutions were prepared and added to cells washing with PBS, respectively. Then the images of the nucleus, mitochondria, and lysosomes were observed on a fluorescence microscope.

Immunocytochemistry Analysis of PGC-1α

L6 cells were fixed and incubated with PGC-1α antibody (1:500, Proteintech) overnight at 4°C. After washing with PBS, the cells were further incubated with fluorescein-conjugated affinity pure goat anti-mouse IgG secondary antibody (1:1,500 dilution) for 1 h. Nuclei were visualized by counterstaining with 4',6-diamidino-2-phenylindole (DAPI). The images of PGC-1α and nuclei were acquired using a Zeiss confocal scanning laser microscope.

Quantitative Real-Time PCR

Total RNA was isolated from gastrocnemius using a RNaprep pure animal tissue kit (Tiangen Biotech, Beijing). The cDNA was synthesized using 3 μg of RNA with a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Ishiyama, Japan) following the manufacturer's protocol. The assay was carried out on a CFX96 Real-Time PCR system (Bio-Rad Laboratories) with iTaqTM Universal SYBR Green Supermix (Bio-Rad) and gene-specific primers for IGF-1, mechanistic target of rapamycin (mTOR), myogenic differentiation (MyoD), myoglobin (MyoG), MuRF, and atrogin-1, or GAPDH (Table 2). The PCR conditions were as follows: 95°C for 5 s followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. The quality of RNA was assessed by OD260/OD280, RNA used in the experiment had an OD260/OD280 ratio between 1.8 and 2.0. Relative expression was first quantified using a standard curve and calculated using the \(2^{-\Delta\Delta Ct}} \) data were normalized to GAPDH mRNA. The primer efficiency was determined based on cycle threshold (Ct) value and melting curve. The Ct value of each mRNA was kept ≤30, and melting curve was controlled single peak. The mRNA and protein expressions were represented by relative expression; the formula used for relative expression was as follows:

\[
\Delta Ct \ (test) = Ct \ (target, test) - Ct \ (ref, test);
\]

\[
\Delta Ct \ (calibrator) = Ct \ (target, calibrator) - Ct \ (ref, calibrator);
\]

\[
\Delta \Delta Ct = \Delta Ct \ (test) - \Delta Ct \ (calibrator);
\]

\[
mRNA \ relative \ expression = 2^{-\Delta \Delta Ct};
\]

\[
Relative \ expression = \left(2^{-\Delta \Delta Ct} \ \text{experiment \ group} - 2^{-\Delta \Delta Ct} \ \text{control \ group}\right)/2^{-\Delta \Delta Ct} \ \text{control \ group} \times 100%.
\]

Western Blot

Western blot analysis was carried out to detect proteins including GAPDH, β-actin (1:800), PGC-1α (1:400), P3K, phosphorylated (p)-P3K, Akt1/2/3, p-AktSer473, ERK, p-ERK, JNK, p-JNK, p38 and p-p38 Tyr182, utilizing specific antibodies as previously described (Li et al., 2019). Gastrocnemius homogenate was prepared and proteins were extracted by radio-immunoprecipitation assay buffer. Subsequently, 100 μg of protein was separated by SDS-PAGE electrophoresis and then electrophoretically transferred to a nitrocellulose membrane for 4 h at 200 mA. The nitrocellulose membranes were then blocked using 5% nonfat milk in Tris-buffered saline with Tween 20 buffer for 1 h, followed by overnight incubation with primary antibodies (Santa Cruz Biotechnology Inc.) at 4°C. After incubation with anti-rat, anti-goat, or anti-rabbit secondary antibody (1:2,000; Santa Cruz Biotechnology Inc.) at 37°C for 1 h, the protein bands were visualized by alkaline phosphatase chromogenic assay. The relative expression level of the western blot band is based on the analysis of the grayscale (fluorescence intensity) of each protein band, the results are obtained by using Image J software for relative quantification calculation.
### Table 2. Primer sequences

<table>
<thead>
<tr>
<th>Protein</th>
<th>NCBI number</th>
<th>Forward primer (5’ to &gt;3’)</th>
<th>Reverse primer (5’ to &gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>24383</td>
<td>GACACCCCGTCCTTTTGATCGTGCCTCT</td>
<td>AAGGCAAGACCCGGAGAAAGTGGAGAGAAG</td>
</tr>
<tr>
<td>mTOR</td>
<td>56718</td>
<td>TCGCCGCACAAATTTCAAC</td>
<td>TCGCTGTTGCGTGGATGAGTC</td>
</tr>
<tr>
<td>MyoD</td>
<td>337868</td>
<td>TCAAGGGACTTTTGAGAGATCGA</td>
<td>CGAAAGCAGTTGGAGAGTT</td>
</tr>
<tr>
<td>MyoG</td>
<td>29148</td>
<td>AGAGGAGCCACCTGCTCAAC</td>
<td>TGATCTCCCTTGGTGAGACC</td>
</tr>
<tr>
<td>MuRF</td>
<td>140393</td>
<td>GGAAAGAAGCACCCTGCTCAAC</td>
<td>AACGAACCCAGACATGGG</td>
</tr>
<tr>
<td>Atrogin-1</td>
<td>17043</td>
<td>ATCCCCTGAGTGCTTCCATCGC</td>
<td>CTCCCTACAGTTAGCGGT</td>
</tr>
</tbody>
</table>

1mTOR = mechanistic target of rapamycin; MyoD = myogenic differentiation; MyoG = myoglobin; MuRF = muscle specific RING finger.

### Statistical Analysis

Statistical analysis was conducted using SPSS 24.0 software (IBM Corp.). Data are presented as mean ± standard error of the mean. One-way ANOVA was used for statistical comparison, followed by Dunnett’s test for pairwise comparison. Significance was considered at $P < 0.05$ for all tests.

### RESULTS

Skeletal muscle is a versatile tissue involved in critical biological processes, such as movement, metabolism, and alterations in muscle mass and composition, that can have a profound effect on health and disease (Ham et al., 2020). To replicate sarcopenia in vivo, excessive intake of d-gal has been widely used due to its ability to generate a large number of high-reactivity molecules or ions with unpaired electrons (free radicals), which induced oxidative stress, mitochondrial dysfunction (decrease in mitochondria mass and decline in mitochondrial biogenesis and oxidative phosphorylation) and lipid peroxidation (Wei et al., 2005; Gao et al., 2016). Our previous research demonstrated that MFG-E8 promotes cell proliferation and differentiation via the PI3K/Akt signaling pathway in vitro myoblasts (Li et al., 2021b). However, the synergistic mechanism and relative activity of MFG-E8 combined with whey protein in promoting muscle protein synthesis compared with whey protein alone remains unknown. Therefore, we conducted an in vivo rat experiment to investigate the potential synergistic effect of MFG-E8 and whey protein in attenuating aging-related sarcopenia. The workflow of the study is illustrated in Figure 1.

#### Synergistic Effects of MFG-E8 and Whey Protein on Sarcopenic Rats

This study used d-gal-induced aging rats, a well-established and representative model of aging-associated sarcopenia. The final weight of rats in d-gal was significantly lower than normal group (Supplemental Table S2, https://doi.org/10.17632/cywmmx3hy3.1, Guan et al., 2023, $P < 0.05$). Gastrocnemius and soleus coefficients of d-gal group were reduced by 11.3% and 12.5%, respectively ($P < 0.05$). The SOD activity was reduced by 22.6% and the content of MDA in the d-gal group was increased by 38.6% ($P < 0.05$, compared with normal group). Moreover, compared with normal group, the number of fibers and nuclei decreased in the gastrocnemius of the d-gal-treated rats ($P < 0.05$), edema of the cells was more serious, and there was significant difference in the total cross-sectional area, cells arrangement irregularity, various forms of muscle fibers, and nuclei of the gastrocnemius between the control and d-gal group. Based on the above results, d-gal induces impairment of skeletal muscle mass in rat, which is consistent with the symptoms of sarcopenia.

To explore the in vivo function of MFG-E8 (Wei et al., 2005; Zhang et al., 2018). We evaluated the efficacy of MFG-E8 intervention by weekly measuring the rats’ BW and observed that the statistical differences of MFG-E8 and MFG-E8 + whey began to emerge at the end of 2 wk supplementation ($P < 0.05$), MFG-E8, and MFG-E8 + whey protein intervention maintained normal growth status (strong physical abilities, rapid weight gain and reaction speed, slightly yellow and glossy fur, mild hair loss, and good muscle endurance) while facilitating significant weight gain compared with control group ($P < 0.05$). After 4 wk of MFG-E8 or whey protein supplementation, the coefficients of gastrocnemius and soleus showed significant enhancement with the highest increase in the MFG-E8 + whey protein group ($P < 0.05$). Moreover, the coefficients of liver and kidney indicated a significant increasing trend ($P < 0.05$). Whey protein treatment did not affect BW significantly but minimized the mass of epididymal fat, which was inversely associated with the gastrocnemius and soleus coefficients ($P < 0.05$; Figure 2 and Table 3).

#### Combination of MFG-E8 and Whey Protein Ameliorated Pathological Changes of Gastrocnemius

Oxidative stress has been shown to play a key role in the development of lipid peroxidation and cellular damage, resulting in decreased antioxidant enzyme.
activity and increased nitrite concentration (Sharma et al., 2020). Our study has provided evidence for a synergistic effect of MFG-E8 and whey protein in promoting muscle health in aged rats (Figure 3). Specifically, we found that treatment with either MFG-E8 or whey protein alone significantly increased the cross-sectional area and nucleus of gastrocnemius fibers in aged rats compared with the control group (Figure 3A). However, the combination of MFG-E8 and whey protein was even more effective in improving muscle quality and function, as shown by the reduction in edema and fat content. Moreover, we observed a significant decrease in TG and NEFA contents after combined intervention with MFG-E8 and whey protein (P < 0.05, compared with control, MFG-E8 or whey protein; Figure 3B and 3C). These findings suggest that MFG-E8 in combination with whey protein may offer a promising strategy for combating muscle atrophy and improving overall muscle health in aging individuals.

**Combination of MFG-E8 and Whey Protein Ameliorates d-Gal-Induced Oxidative Stress Injury**

To evaluate the effect of MFG-E8 and whey protein on aging-related indicators, several measurements related to antioxidant activity and liver function were performed on aged rats (Figure 4). As shown in Figure 4A, 4B, and 4C, the SOD activity was significantly increased by 42.79%, 32.31%, and 44.71% in the MFG-E8, whey protein, and MFG-E8 + whey protein groups, respectively, compared with the control group (P < 0.05, Figure 4A). MDA contents were significantly reduced by 41.89%, 24.85%, and 49.63%, respectively (P < 0.05, Figure 4B). The combination of MFG-E8 and whey protein resulted in increased levels of SOD/MDA antioxidant activity by 26.20% and 51.17%, respectively, compared with the MFG-E8 or whey protein group (P < 0.05). Furthermore, IGF-1 levels in the MFG-E8 + whey protein group were significantly increased by 4.63% and 22.34% (P < 0.05, compared with the MFG-E8 and whey protein group, Figure 4C).

Ketones are small, water-soluble circulating lipids produced by the liver. After intervention with MFG-E8 or whey protein, especially MFG-E8 + whey protein, the ketone contents decreased significantly by 17.04% (P < 0.05, compared with control group, Figure 4D). Moreover, the levels of GOT and GPT, which are indices for evaluating liver health, were significantly reduced in the MFG-E8 (28.31% and 18.60%, respectively) and whey protein (25.96% and 10.57%) groups, compared with the control group (P < 0.05, Figure 4E and 4F). The MFG-E8 + whey protein group showed
the most significant reduction in GOT and GPT levels ($P < 0.05$, vs. MFG-E8 or whey protein group), indicating an excellent capability in repairing liver function associated with MFG-E8 and whey protein.

To evaluate mitochondrial function, TEM, mito-tracker staining, and immunofluorescence were conducted. The TEM showed that the number of mitochondria in cells increased, whereas mitochondrial autophagy and vacuolation decreased in the MFG-E8 + whey protein group (vs. MFG-E8 or whey protein group, Figure 4G). Mito-tracker staining demonstrated that MFG-E8, especially in combination with whey protein, significantly alleviated d-gal-induced $\Delta\Psi_m$ depolarization and optimized $\Delta\Psi_m$ status ($P < 0.05$,

**Figure 2.** Milk fat globule epidermal growth factor 8 (MFG-E8) ameliorated skeletal muscle atrophy in aged rats. (A) Representative gastrocnemius and soleus images of different groups; (B) BW of rat recorded in the 4 wk; (C) coefficients of gastrocnemius and soleus; (D) coefficients of liver, kidney, spleen, testis, epididymis adipose, and perirenal fat. Different letters (a–d) in graphs indicate significant differences between groups ($n = 3$, $P < 0.05$). Data are presented as mean ± SD. Treatment groups: MFG-E8 (16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk.
compared with control, Figure 4H). Immunofluorescence analysis revealed that PGC-1α was remarkably increased in the MFG-E8 and whey protein groups \((P < 0.05,\) compared with control, Figure 4I). These results suggest that the protective role of MFG-E8 or whey protein, especially in combination, may be related to the alleviation of mitochondrial-mediated apoptosis induced by oxidative stress.

**Combination of MFG-E8 and Whey Protein Inhibits Muscle Protein Degradation via Activating PI3K/AKT and MAPK/ERK Pathway**

As detailed above, we sought to investigate the mechanism underlying the antimuscle protein degradation effect of MFG-E8 in combination with whey protein. To this end, we conducted quantitative real-time PCR analysis to determine the mRNA levels of various genes involved in muscle protein metabolism (Figure 5A). Our results demonstrate that both MFG-E8 and whey protein treatment led to increased relative expression of IGF-1, mTOR, MyoD, and MyoG mRNA. In contrast, the expression of atrogin-1 and MURF mRNA was significantly reduced by 36.20% and 72.5%, and 55.2% and 70.60% for the MFG-E8 and whey protein treatment, respectively \((P < 0.05,\) compared with the control group). Notably, the combined treatment of MFG-E8 and whey protein resulted in a significant increase in IGF-1 and MyoG mRNA levels by 6.41% and 8.13%, respectively. Moreover, the levels of MyoG mRNA were decreased by 11.34% and 35.50% \((P < 0.05,\) compared with the MFG-E8 or whey protein group), which is in

**Table 3. Body weight changes in rats during nutrition supplementation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial (g)</th>
<th>1 wk (g)</th>
<th>2 wk (g)</th>
<th>3 wk (g)</th>
<th>4 wk (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>403.4 ± 9.31(^a)</td>
<td>408.2 ± 11.27(^a)</td>
<td>436.5 ± 7.90(^b)</td>
<td>446.5 ± 7.90(^b)</td>
<td>453.3 ± 10.34(^b)</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>402.1 ± 8.52(^a)</td>
<td>415.1 ± 10.42(^a)</td>
<td>459.7 ± 10.31(^b)</td>
<td>478.67 ± 10.31(^b)</td>
<td>481.3 ± 9.43(^b)</td>
</tr>
<tr>
<td>Whey</td>
<td>403.5 ± 9.26(^a)</td>
<td>414.7 ± 9.94(^a)</td>
<td>444.9 ± 6.23(^a)</td>
<td>457.9 ± 6.23(^a)</td>
<td>462.7 ± 6.47(^a)</td>
</tr>
<tr>
<td>MFG-E8 + whey</td>
<td>402.8 ± 10.71(^a)</td>
<td>415.9 ± 6.45(^a)</td>
<td>458.7 ± 9.49(^b)</td>
<td>476.7 ± 9.50(^b)</td>
<td>484.8 ± 10.96(^b)</td>
</tr>
</tbody>
</table>

\(^a,b\)Different superscript letters represent significant differences among groups \((P < 0.05)\).

\(^1\)The rats were divided into 4 groups and orally administered with milk fat globule epidermal growth factor 8 (MFG-E8; 16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk.

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*Figure 3. Milk fat globule epidermal growth factor 8 (MFG-E8) alleviates the pathological changes of skeletal muscle lipid deposition. (A) Histological analysis for gastrocnemius; 6 images in each group were observed, and representative images were exhibited. Blue arrows indicate fat accumulation and increasing spacing between muscle fibers. (B) Triglyceride (TG) levels of gastrocnemius; (C) nonesterified fatty acid (NEFA) levels of gastrocnemius. Different letters in graphs indicate significant differences between groups \((n = 6, P < 0.05)\). Data are presented as mean ± SD. Treatment groups: MFG-E8 (16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk.*

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Figure 4. Combination of milk fat globule epidermal growth factor 8 (MFG-E8) and whey protein on antioxidant activity and mitochondrial function. (A) Levels of superoxide dismutase (SOD); (B) levels of malondialdehyde (MDA); (C) levels of insulin-like growth factor 1 (IGF-1); (D) levels of ketone; (E) levels of glutamic-oxaloacetic transaminase (GOT); (F) levels of glutamic-pyruvic transaminase (GPT); (G) effect of MFG-E8 or whey protein on mitochondria observed by transmission electron microscopy (magnification 5,000 to 30,000×); (H) mitochondrial membrane potential (ΔΨm) detected by mito-tracker and lyso-tracker; (I) analysis of peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) expression by confocal laser. Different letters in graphs indicate significant differences between groups (n = 3, P < 0.05). Data are presented as mean ± SD. Treatment groups: MFG-E8 (16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk.
line with our previous in vitro myoblast experiment. These findings suggest that MFG-E8 and whey protein, particularly when used in combination, can promote the formation of multinucleated myotubes by upregulating the phosphorylation expression of mTOR via activation of the PI3K/Akt signaling pathway, upregulating the expression of MyoD, and entering the metaphase of differentiation. The upregulated expression of MyoG mRNA shortens the cell differentiation cycle, facilitates muscle fiber formation, and promotes fusion.

Additionally, we investigated the role of PI3K and MAPK signaling pathways in the balance of skeletal muscle protein metabolism both in vitro and in vivo. We aimed to determine how MFG-E8 mediates these pathways to regulate protein anabolism and catabolism. We determined the expression of PI3K, ERK, JNK, and p38 protein, as well as the levels of phosphorylation, in the gastrocnemius muscle (Figure 5B). Our results revealed that MFG-E8 significantly increased the expression of phosphorylated PI3K and ERK. Furthermore, the combined treatment of MFG-E8 and whey protein led to an obvious increase in the expression of phosphorylated PI3K and ERK ($P < 0.05$, compared with the control group). The transcriptional activities of phosphorylated JNK and phosphorylated p38 were decreased. Interestingly, the levels of p-PI3K/PI3K (2.25-fold) were significantly higher than those of p-ERK/ERK (1.35-fold), p-JNK/JNK (0.80-fold), and p-p38/p38 (0.68-fold), respectively ($P < 0.05$). These findings are consistent with our previous in vitro myoblast experiment and suggest that the mechanism by which MFG-E8 regulates the balance of muscle protein metabolism mainly involves the activation of PI3K/Akt and MAPK/ERK multisignal pathway cascades. Overall, these results provide valuable insight into the underlying mechanism of MFG-E8 in mitigating sarcopenia.

**Effect of MFG-E8 on Mitochondrial Biogenesis via PI3K/AKT/PGC-1α**

Akt, a key downstream regulator of PI3K, is subject to control by a variety of factors, including hormones, cytokines, and growth factors. In skeletal muscle, Akt has been identified as a critical regulator of mitochondrial bioenergetics and PGC-1α-driven mitochondrial biogenesis. As illustrated in Figure 6, MFG-E8 or whey protein can stimulate the phosphorylation of Akt at Ser473 and positively regulate Akt activation through the PI3K signaling pathway, mimicking the response pattern of IGF-1. The p-Akt/Akt ratio was significantly increased in the MFG-E8 (25.90%) and whey protein (8.99%) groups ($P < 0.05$, compared with the control group), particularly with combined treatment of MFG-E8 and whey protein ($P < 0.05$, compared with the MFG-E8 and whey protein group; Figure 6A and 6B). PGC-1α is a crucial regulator of oxidative phosphorylation, which governs mitochondrial biogenesis and respiration in myoblasts. PGC-1α expression is associated with insulin-resistant states and inversely correlated with plasma free fatty acids and aging. MFG-E8 (58.53%) and whey protein (67.75%) can significantly increase the expression of PGC-1α ($P < 0.05$, compared with the control group; Figure 6C), and the co-effect of MFG-E8 and whey protein significantly enhances PGC-1α expression by 20.81% and 13.98%, respectively ($P < 0.05$, compared with the MFG-E8 and whey protein group; Figure 6C), indicating the repairment of aging-related gastrocnemius mitochondria damage. Therefore, the results suggest that the synergistic effect of MFG-E8 and whey protein upregulates the phosphorylation expression of Akt and participates in PGC-1α-regulated mitochondrial biogenesis (Figure 6D).

Our findings suggested a potential synergistic effect of MFG-E8 and whey protein combination in maintaining skeletal muscle mass and function. MFG-E8 in synergy with whey protein exhibited a significant increase in gastrocnemius and soleus coefficient. The serum content and muscle RNA expression of IGF-1 showed a significant increase and serum content of MDA, ketone and GOT showed significant decrease. The protein expression demonstrated that combination of MFG-E8 and whey protein can mitigate d-gal-induced sarcopenia through PI3K/Akt/PGC-1α and MAPK/ERK signaling pathways. These results indicated that the synergistic effect of combining MFG-E8 and whey protein is superior to the individual effects of MFG-E8 and whey protein alone in alleviating inflammatory response, oxidative stress, muscle loss, and promoting mitochondrial function in the body. Thus, these findings provided support for the potential use of MFG-E8 and whey protein as interventions for sarcopenia associated with aging.

**DISCUSSION**

Skeletal muscle is a crucial component of overall body health, serving as an important factor in promoting joint protection and regulating normal contraction and relaxation (Daily and Park, 2022; Li et al., 2023). Gastrocnemius and soleus muscles are particularly significant contributors to knee joint function, functioning as both flexors and stabilizers of the joint (Barber et al., 2017). Our study found that the coefficients of gastrocnemius and soleus muscles in the MFG-E8 and whey protein treatment groups were significantly increased compared with the control group ($P < 0.05$).
Figure 5. Effect of MFG-E8 combined with whey protein on the phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase (MAPK) signaling related mRNA and protein expression in the gastrocnemius of aged rats. (A) The mRNA levels of insulin-like growth factor 1 (IGF-1), mechanistic target of rapamycin (mTOR), atrogin-1, muscle specific RING finger (MURF), myogenic differentiation (MyoD), and myoglobin (MyoG) in gastrocnemius; (B) western blot and quantitative analysis of the expression of PI3K, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 in gastrocnemius. The mRNA and protein expression was represented by relative expression. Different letters in graphs indicate significant differences between groups (n = 3, *P* < 0.05). Data are presented as mean ± SD. Treatment groups: MFG-E8 (16 mg), whey protein (1.26 g), MFG-E8 + whey protein (16 mg + 1.26 g, respectively), or 0.01 mol/L PBS (control) daily for 4 wk. p-PI3K, p-ERK, p-JNK, and p-p38 represent phosphorylated-PI3K, phosphorylated-ERK, phosphorylated-JNK, and phosphorylated-p38.
Although whey protein has been recognized as a superior source of protein for improving skeletal muscle mass and function, our study found that a micro-dose of MFG-E8 (<78.8-fold, compared with whey protein) can produce a similar effect to a full dose of whey protein. Thus, MFG-E8 stands out as the most valuable resource among MFGM proteins. Our results suggested that MFG-E8 could be used as a potential nutrient to promote muscle protein synthesis and improve muscle function in individuals with muscle-related disorders.

Biomarkers SOD and MDA are associated with oxidative stress. Studies have shown that during muscle atrophy, there was an increase in oxidative stress levels within the muscles, leading to decreased SOD and increased MDA levels (Cheng et al., 2020). Our study observed that the supplementation of MFG-E8 or whey protein, especially when taken together, led to an increase in SOD activity and a reduction in MDA levels. This resulted in a significantly higher SOD/MDA ratio ($P < 0.05$), indicating that MFG-E8 and...
whey protein have a synergistic effect in promoting antioxidation. Moreover, ketones are incomplete oxidation products of fatty acids that can provide energy to various organs and inhibit muscle protein breakdown (Garcia-Fernandez et al., 2011). However, abnormal liver function can lead to ketone accumulation in the blood (Ponziani and Gasbarrini, 2018). Studies have shown that MFG-E8 and whey protein supplements, particularly MFG-E8, can promote liver health by facilitating the increase of liver coefficients, GPT and GOT activity, and a decrease in ketone levels. Such nutrient supplements can decrease aging-induced oxidative stress and improve gastrocnemius repair by protecting the liver (Ponziani and Gasbarrini, 2018). Previous research also demonstrated that MFG-E8 can promote oxidative phosphorylation, enhance liver cell activity, repair mitochondrial dysfunction, and alleviate the aging process (Li et al., 2021b).

Inflammation is a key contributor to muscle protein degradation in cancer cachexia, and the pro-inflammatory transcription factor NF-κB plays a significant role in inducing cytokine expression (Wu et al., 2022). Whey protein has been shown to have anti-inflammatory effects by reducing serum levels of TNF-α, IL-1β, and MCP-1, improving intestinal flora distribution, and reducing liver damage caused by inflammation, which can increase liver coefficient (Wu et al., 2022). However, dyslipidemia, a common age-related metabolic disorder characterized by abnormal lipid metabolism, can contribute to the development of sarcopenia (Daily and Park, 2022; Nunes et al., 2022). The accumulation of free fatty acids in tissues such as the liver and muscle can result in the production of toxic lipid metabolites ceramide and diglycerides, causing systemic inflammation in skeletal muscles (Daily and Park, 2022). This inflammation can further impair skeletal muscle function and promote the loss of muscle mass. Whey protein and MFG-E8 have both been found to have beneficial effects on lipid metabolism. The MFG-E8 activates the PI3K/Akt signaling pathway to inhibit the activation of inflammatory factor signaling pathways mediated by NF-κB and TNF-α, reducing serum levels of NEFA and preventing sarcopenia caused by systemic inflammation. Meanwhile, whey protein activates the AMPK signaling pathway to upregulate the expression of lipid-decomposing genes (e.g., PPARα and ATGL) and enhance the β-oxidation process, ultimately reducing circulating TG and NEFA levels and regulating lipid metabolism. Thus, MFG-E8 and whey protein may have a synergistic effect in attenuating dyslipidemia and improving lipid metabolism in liver tissue.

Increasing IGF-1 expression is crucial for maintaining skeletal muscle structure and function. Muscle protein degradation is regulated by ubiquitin-proteasome system, which involves the attachment of multiple ubiquitin molecules to a protein substrate by ubiquitin E3 ligases such as muscle RING-finger protein (MuRF) and atrophy-related gene-1 (atrogin-1; Park et al., 2020). Expression of MuRF and atrogin-1 is regulated by various signaling pathways, including the IGF-1 and NF-κB pathways (Glass, 2005; Ji et al., 2020). Activation of the PI3K pathway promotes muscle protein anabolism, while inhibition of the NF-κB/MuRF and Akt/FOXO/atrogin-1/MuRF signaling pathway reverses muscle protein degradation (Jang et al., 2021). In this study, MFG-E8 and whey protein upregulated IGF-1 mRNA expression in gastrocnemius tissue, regulated the PI3K/Akt signaling pathway, and reduced gene transcription activity of atrogin-1 and MuRF, ultimately suppressing aging-induced muscle protein catabolism (Hassanzadeh-Rostami et al., 2020). The findings suggested that MFG-E8 and whey protein regulate interrelated networks of ubiquitin E3 ligases and PI3K signaling, promoting the upregulation of MyoD and MyoG (Park et al., 2020). The whey protein group had the lowest MuRF mRNA expression due to its promotion of insulin secretion, immune modulation, anti-inflammatory effects, and inhibitory effects on protein degradation pathways caused by apoptosis.

The PI3K and MAPK signaling cascades are crucial for transducing extracellular signals in various cellular processes, including survival, migration, transcription, and metabolism (Prokopidis et al., 2021). The MAPK/ERK signaling pathway is necessary for the progression of mitosis in myoblasts, while the JNK and p38 signaling pathways are activated by pro-inflammatory cytokines and inhibit myoblast activity (Guan et al., 2021). In this study, the combination of MFG-E8 and whey protein was found to promote mitochondrial biogenesis and muscle protein metabolism homeostasis through the PI3K and MAPK pathway cascades. The expression of PGC-1α, which is responsible for promoting mitochondrial biogenesis and regulating muscle growth and differentiation, is negatively regulated by d-gal-induced reactive oxygen species production and activation of the NF-κB signaling pathway. Whey protein and MFG-E8 can stimulate insulin secretion by augmenting the PI3K/Akt signaling pathway, inhibiting the activation of NF-κB and TNF-α-mediated inflammatory factor signaling pathways, reducing tissue inflammation caused by aging, and repairing damaged gastrocnemius fibers. The reparative and regenerative effects of MFG-E8 and MFG-E8 + whey protein on rat gastrocnemius were found to be superior to those of the whey protein group, as evidenced by histopathological sections, cell spacing analysis, number of nuclei, and cross-sectional area of muscle fibers.
CONCLUSIONS

The present study aimed to investigate the synergistic effects of MFG-E8 and whey protein in mitigating sarcopenia in aged rats and to identify the underlying mechanisms. Our findings demonstrated that the combined supplementation of MFG-E8 and whey protein effectively reduced oxidative stress damage, mitochondrial dysfunction, dyslipidemia, gastrocnemius ceroidosis, and lipid deposition induced by d-gal. Moreover, the activation of PI3K/Akt/PGC-1α and MAPK/ERK signaling cascades was identified as a fundamental mechanism underlying the alleviation of aging-related sarcopenia. These results provided novel insights into the potential mechanisms of MFG-E8 and whey protein in mitigating sarcopenia and provide compelling evidence for their rational use in promoting skeletal muscle health. The nutrient formula developed in this study has the potential to serve as a novel intervention strategy for sarcopenia. Overall, these findings have important implications for the development of effective interventions to combat sarcopenia and improve skeletal muscle health in aging populations.

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**ORCIDs**

He Li ORCID: https://orcid.org/0000-0001-9345-042X
Kaifang Guan ORCID: https://orcid.org/0000-0003-0438-7356
Hongchun Wang ORCID: https://orcid.org/0000-0002-3747-7553
Ying Ma ORCID: https://orcid.org/0000-0002-2641-7926