High glucose induces apoptosis, glycogen accumulation and suppresses protein synthesis in muscle cells of olive flounder Paralichthys olivaceus

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Abstract
The effect and the mechanism of high glucose on fish muscle cells are not fully understood. In the present study, muscle cells of olive flounder (Paralichthys olivaceus) were treated with high glucose (33 mM) in vitro. Cells were incubated in three kinds of medium containing 5 mM glucose, 5 mM glucose and 28 mM mannitol (as an isotonic contrast) or 33 mM glucose named the Control group, the Mannitol group and the high glucose (HG) group, respectively. Results showed that high glucose increased the ADP:ATP ratio and the reactive oxygen species (ROS) level, decreased mitochondrial membrane potential (MMP), induced the release of cytochrome C (CytC) and cell apoptosis. High glucose also led to cell glycogen accumulation by increasing the glucose uptake ability and affecting the mRNA expressions of glycogen synthase and glycogen phosphorylase. Meanwhile, it activated AMP-activated protein kinase (AMPK), inhibited the activity of mammalian target of rapamycin (mTOR) signalling pathway and the expressions of myogenic regulatory factors (MRF). The expressions of myostatin-1 (mstn-1) and E3 ubiquitin ligases including muscle RING-finger protein 1 (mrf-1) and muscle atrophy F-box protein (mafb) were also increased by the high glucose treatment. No difference was found between the Mannitol group and the Control group. These results demonstrate that high glucose has the effects of inducing apoptosis, increasing glycogen accumulation and inhibiting protein synthesis on muscle cells of olive flounder. The mitochondria-mediated apoptotic signalling pathway, AMPK and mTOR pathways participated in these biological effects.

Key words: Glucose: Olive flounder: Mitochondria: Muscle: Cell

Glucose is an important energy and carbon source. Owing to the protein-saving effect of carbohydrate, the addition of carbohydrate can reduce the use of protein raw materials in fish feed. Appropriate dietary carbohydrate can both promote fish growth and reduce feed cost(1). However, excessive dietary carbohydrate can exert negative impacts on the growth performance and physiological functions(2).

Moreover, fish muscle quality can also be changed by excessive dietary carbohydrate(3,4). The research in dentex (Dentex dentex) found that higher dietary carbohydrate level (28 % v. 4 %) decreased the muscle hardness and caused higher glycogen content in muscle(5). In post-mortem fish muscles, glycogen produces lactic acid by anaerobic respiration, which leads to a decrease of muscle pH and meat softening. Glycogen level was proved to be the principal determinant of post-mortem pH in fish(6). Meanwhile, the massive intracellular glycogen accumulation in muscle was proved to be associated with the soft texture in another study of Atlantic salmon (Salmo salar)(7).

In our previous study, excessive dietary carbohydrate (24 %) reduced the muscle hardness of olive flounder (Paralichthys olivaceus). Meanwhile, it increased the muscle glycogen content(3). These results suggested that in fish, the dietary carbohydrate may influence the meat quality by affecting the glycogen content in muscle.

At the same time, research found that excessive dietary carbohydrate content can suppress the immune functions and the antioxidative capacities as well as reduce the growth performance in some fish species(2,7). In the study of mammalian cell, high glucose was proved to stimulate reactive oxygen species (ROS) production and suppress mammalian target of rapamycin (mTOR) signalling pathway(8). High glucose leads to mitochondrial dysfunction and promotes the release of cytochrome C (CytC) form mitochondria, which results in oxidative stress and cell apoptosis(9). ROS was proved to impair flesh quality. It can lead to the oxidation of subcellular membranes and structural proteins, which affect both the meat texture and the muscle water holding

Abbreviations: CytC, cytochrome C; HG, high glucose; MMP, mitochondrial membrane potential; MRF, myogenic regulatory factors; MTOR, mammalian target of rapamycin; ROS, reactive oxygen species.
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The phosphorylation level of mTOR, which negatively affects ROS in fish and thereby affect the fish muscle quality. Tor, glucose may also play a role in promoting the production of oxidative stress in meat. As an important nutritional factor, glucose may also play a role in promoting the production of ROS in fish and thereby affect the fish muscle quality.

High glucose-induced oxidative stress was found to inhibit the phosphorylation level of mTOR, which negatively affects the protein synthesis and cell growth. Researchers found that the oxidative stress can disrupt the balance between degradation and resynthesis of skeletal muscle proteins. The application of H2O2 inhibited the proliferation accompanied by decreasing the activity of mTOR and p70 ribosomal S6 kinase (p70s6k). At the same time, ROS can also promote proteolysis.

Study performed in C2C12 cells found H2O2 treatment increased the mRNA expressions of the two E3 ubiquitin ligases (MAFbx and MuRF-1), which mediate in skeletal muscle catabolism. In some research with fish cells, the uptake and metabolism of glucose was studied. However, there is little research on the effect of high glucose on fish cells, the mechanism of high glucose on retarded growth and oxidative stress is not yet entirely clear in fish species.

Olive flounder (P. olivaceus) is an economically important marine carnivorous fish species in East Asia. Previous studies showed that excessive dietary carbohydrate level negatively regulated the growth performance and muscle quality of olive flounder. Further research is needed to understand how the high glucose condition affect the muscle of olive flounder. In the present study, the primary cultured muscle cells of olive flounder were used to focus on the accumulation of glycogen, apoptosis and muscle growth under high glucose condition. The aim of the present study is to explore the effect of high glucose on fish muscle cells and its potential mechanism.

Materials and methods

Ethical statement

The present study was performed in strict accordance with the recommendations in the Guide for the Use of Experimental Animals of Ocean University of China. The protocols for animal care and handling used in this study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Animals

Olive flounder with weight ranging from 8 to 10 g were purchased from a commercial fish farm in Haiyang city, Shandong, China. After disinfection, fish were kept in 0.4 m³ tanks with a re-circulating water system at 23°C.

Primary cell cultures

The muscle cells of olive flounder were isolated according to the protocols described by Magnoni et al with some modifications. The detail steps of cell culture and verification were according to the previous study. Briefly, fish was euthanised by immersion in eugenol (Sigma, St. Louis, MO, USA) (80 mg/l). White epaxial muscle was excised under sterile conditions and minced. The Dulbecco’s Modified Eagle Medium (without glucose) (Sangon Biotech and Ham’s F12 medium (F12) (HyClone) were mixed 1:1 to make the basal medium (5 mM D-glucose). The basal medium was supplemented with 10% fetal bovine serum (FBS) (Bioind) and antibiotics (Penicillin-Streptomycin, 100 U/ml) (HyClone, Logan, UT, USA) to make the complete medium. The fragments were centrifuged and digested by Type II collagenase (0-2%) (MP Biomedicals, Solon, OH, USA) and trypsin (0-1 % final concentration in the basal medium) (HyClone). The suspension was centrifugated, and the supernatant was collected in cold basal medium containing fetal bovine serum (FBS) (Bioind) and antibiotics (Penicillin-Streptomycin, 100 U/ml) (HyClone). After centrifugation, the resulting pellet was resuspended in the complete medium and filtered through a 40-μm nylon cell strainer. Cells were incubated at 23°C without CO₂.

Glucose concentration in medium of high glucose condition

As there is little research on effects of high glucose concentration on fish cells in vitro, the present study referred the literatures from other species. The treatment of high glucose on cells usually used the medium containing 20 mM, 25 mM, 33 mM or 50 mM of glucose. To choose a suitable ‘high’ glucose level, a series of medium with graded levels of glucose were designed. The cell viability was tested by Cell-Counting Kit-8 (CCK-8) reagents (Sigma) after the 24-h incubation of the medium containing 5 mM, 20 mM, 25 mM, 33 mM or 50 mM of D-glucose (Sigma). Using the decrease of cell viability as indicator, 33 mM and 50 mM of glucose were considered as high glucose level for olive flounder muscle cells (Fig. 1(a)). However, 55 mM might be excessive high compared with 33 mM. In order to avoid the potential effects unrelated to glucose, the 33 mM of glucose was chosen as the high glucose condition in the present study to investigate the effect of high glucose on olive flounder muscle cells.

Analysis of reactive oxygen species

The levels of intracellular ROS were determined by 2, 7-dichloro-5.8-dichlorofluorescein diacetate (DCFH-DA) (Sigma) assay. Briefly, olive flounder muscle cells were plated in 96-well plates (Corning) (5 × 10^3 cells/well) or in 6-well culture plates (Corning) (1 × 10^5 cells/well) in the complete medium. After 24-h incubation, cells were treated with the complete medium containing 5 mM D-glucose, 5 mM D-glucose and 28 mM mannitol (Merck KGaA, Darmstadt, Germany) (as an isotonic contrast) or 33 mM D-glucose. These three groups were named as the Control group, the Mannitol group and the HG (high glucose)
group, respectively. After 24 h, the cells were incubated with 10 μM DCFH-DA for 15 min at 23°C. For the cells in 96-well plates, intracellular fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (Spectra Max i3x, Molecular Devices). The values were normalised on cell proliferation by MTT assay (C0009, Beyotime Institute of Biotechnology). Three independent experiments were performed for each assay condition. Meanwhile, the ROS levels in cells in 6-well plates were determined using a fluorescence microscope (Echo Laboratories).

**Apoptosis assay**

The apoptosis of cells was detected by an annexin V-fluorescence image in the fluorescein isothiocyanate and propidium iodide kit (Vazyme, A21101). The cells were incubated as stated above. After staining, the cells were analysed on a flow cytometer (Beckman, CA, USA) to determine the rate of apoptosis. Briefly, cells were trypsinised, centrifuged, washed twice with ice-cold PBS (HyClone). The cells were then resuspended in binding buffer containing annexin-fluorescence image in the fluorescein isothiocyanate and propidium iodide.
Cells were incubated at room temperature for 10 min and then analysed by flow cytometry within 1 h.

**Caspase-3 activity assay**

Caspase-3 activity in the cells was detected using a caspase-3 Activity assay kit (C1115, Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Briefly, after incubating as stated above, the cells were collected and lysed with lysis buffer on ice. The cell lysate was subsequently centrifuged (16 000 × g, 10 min, 4°C), and then the supernatant was collected. Protein concentrations were measured using a bichinchoninic acid assay kit (P0012, Beyotime Institute of Biotechnology). The acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) was added to the supernatant. The absorbance was measured at 405 nm with a microplate reader, and the data were normalised by protein concentration.

**Measurement of mitochondrial membrane potential and ADP/ATP ratio**

The mitochondrial membrane potential (MMP) was detected by a JC-1 kit (C2006, Beyotime Institute of Biotechnology) according to the manufacturer's instructions. In brief, after the treatment, muscle cells were collected and incubated with JC-1 working solution at 37°C for 20 min. After that, the cells were washed by JC-1 dye buffer twice. The fluorescence intensity was immediately measured using fluorescence microplate reader. The ratio of red fluorescence intensity to green fluorescence intensity was used to represent the level of MMP. The ratio of ADP:ATP was accurately measured using fluorescence microplate reader. The ratio of red fluorescence intensity to green fluorescence intensity was used to represent the level of MMP. The ratio of ADP:ATP was calculated by the ΔΔCt method using β-actin gene expression as reference. All the primers used in the present study were listed in Table 1.

**Glucose uptake assay**

Cells were seeded in 96-well plates at a density of 5 × 10³ cells/well and incubated at 25°C for 24 h. Then, the cells were incubated as stated above. After the 24-h incubation, the cells were washed with PBS and treated with 100 nM 2-((N-7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxy-D-glucose (2-NBDG) (Invitrogen, Carlsbad, CA, USA) (a fluorescent indicator for direct glucose uptake measurement) for 30 min. Then cells were washed twice with ice-cold PBS, and intracellular fluorescence intensity was measured with a fluorescence microplate reader at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The values were normalised on cell proliferation by flow cytometry within 1 h.

**Western blot analysis**

After treatment, the medium was removed, and cells in 6-well plates were washed three times with 2 ml PBS per well and lysed in 1 ml Trizol (Invitrogen). RNA from cells was extracted by Trizol and quantified on a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific). Reverse transcription was performed using PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time, Takara). The quantity of cDNA for each transcript was analysed on the ABI7500 system (Applied Biosystems) using TB Green Fast qPCR Mix (Takara, Shiga, Japan). The present study analysed target genes including: optic atrophy protein-1 (opa1), mitofusin 1 (mfn-1), mitofusin 2 (mfn-2), glycogen synthase (gsyn: muscle type), glycogen phosphorylase (bggym: muscle type), phosphofructokinase (pffk), pyruvate kinase (pk), myostatin-1 (mstn-1), muscle RING-finger protein 1 (mrf1), muscle atrophy F-box protein (mabx), myoblast determination protein (myod), myogenin (myog), muscle-specific regulatory factor 4 (mrf4) and myogenic factor 5 (myf5). The primers for gene expression analysis were obtained from the previous studies(15–17). Relative quantities of target genes were calculated by the ΔΔCt method using β-actin gene expression as reference. All the primers used in the present study were listed in Table 1.

**Cell glycogen analysis and periodic acid-schiff assay**

To analyse cell glycogen concentration, cells were seeded in 6-well plates at a density of 1 × 10⁵ cells/well and incubated at 23°C for 24 h. The cells were incubated as stated above for another 24 h. After incubation, cells were washed with PBS, and then the glycogen content was determined by using a glycogen content assay kit (BC0345, Solarbio Science and Technology Co., Ltd.) according to the manufacturer's instructions. Glycogen staining was performed with a periodic acid-Schiff staining kit (G1360, Solarbio Science and Technology Co., Ltd.) according to the manufacturer's protocol.

**Gene expression**

After treatment, the medium was removed, and cells in 6-well plates were washed three times with 2 ml PBS per well and lysed in 200 μl radioimmunoprecipitation lysis buffer (Solarbio Science and Technology Co., Ltd.) supplemented with protease and phosphatase inhibitor cocktail (Roche) at 0°C for 30 min. Homogenates were centrifuged at 12 000 g for 10 min at 4°C, and the protein concentration in the supernatant was determined using a bichinchoninic acid protein assay kit. Nuclear protein and mitochondria protein were extracted using a NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Fisher Scientific) and a Cell Mitochondria Isolation Kit (C6001, Beyotime Institute of Biotechnology) according to the manufacturers' instructions, respectively. Equal amounts of protein were separated by SDS-PAGE and transferred to 0.45 μm PVDF membrane (Millipore). Incubation with the primary antibody was performed overnight at 4°C. The primary antibodies used were phospho-AMP-activated protein kinase (AMPK) (Thr172) (dilution 1:1000, Beyotime Institute of Biotechnology, cat. No. AA393). AMPK (dilution 1:1000, Cell Signaling Technology Inc., Cat. No. 5831), phospho-mTOR (Ser2448) (dilution 1:1000, Cell Signaling Technology Inc., Cat. No. 2971), phospho-mTOR (Ser2448) (dilution 1:1000, Cell Signaling Technology Inc., Cat. No. 2972), and phospho-4EBP-1 (Thr37/46) (dilution 1:500, Cell Signaling Technology Inc., Cat. No. AP5159), nuclear factor erythroid 2-related factor 2 (Nrf2) (dilution 1:500, Cell Signaling Technology Co., Ltd.) according to the manufacturer's protocol.
The high glucose treatment significantly reduced the MMP (P < 0.05), and mannitol did not affect the MMP of olive muscle cells (P > 0.05) (Fig. 1(b)). The ADP/ATP ratio (Fig. 1(c)) was significantly increased in HG group compared with the Control group (P < 0.05), while the addition of mannitol did not change it significantly (P > 0.05).

### 2-NBDG uptake in cells

It was found that, after the 24-h incubation, high glucose condition significantly induced glucose uptake in olive flounder muscle cells (P < 0.05). No significant difference was found between the Mannitol group and the Control group (P > 0.05) (Fig. 1(d)).

### Relative quantification of reactive oxygen species level in cells

The fluorescence intensity of fluorescent 2,7-dichlorofluorescein (oxidation product of DCFH-DA by hydrogen peroxide) in the HG group was significantly higher than that in the Control group (P < 0.05). No difference was found between the Mannitol group and the Control group (P > 0.05) (Fig. 2(f)). The fluorescence intensity represents the level of ROS production, this result showed that high glucose could increase the production of ROS in cultured olive flounder muscle cells. Meanwhile, more intense fluorescence signal was found in the HG group (Fig. 2(f)). The fluorescence micrographs also showed that the intracellular ROS level was induced by high glucose condition.

### Apoptosis detection of cells

After annexin V and propidium iodide double staining, induction of cell apoptosis by high glucose was detected and quantified by flow cytometry. Apoptosis of cells in different groups was shown in four-quadrant diagram (Fig. 2(h)–(j)). The average percentages of apoptotic cells were 0.75%, 0.86% and 9.06% in the Control group, the Mannitol group and the HG group, respectively (Fig. 2(k)). The apoptotic percentage in the HG group was significantly higher than that in the Control group, the Mannitol group and the Control group (P < 0.05). As the fluorescence intensity of annexin V and PI was significantly increased in HG group compared with the Control group (P < 0.05), the annexin V and PI positive apoptosis cells were also significantly increased in HG group compared with the Control group (P < 0.05). No significant difference was found between the Mannitol group and the Control group (P > 0.05).

### Statistical analysis

All statistical evaluations were analysed by software SPSS 22.0 (IBM Corp.). The cell viability in different medium glucose concentration was analysed by ANOVA followed by Tukey’s multiple range tests. Other statistical analyses were analysed by t test compared with the Control group. All data were expressed as means ± SEM. Differences were considered significant when P < 0.05.

### Results

#### Changes in mitochondrial membrane potential and relative ADP/ATP ratio in cells

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
<th>Product length</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>optic atrophy protein1 (opa1)</td>
<td>CAGTGCCGAGGAGTTGAGCC</td>
<td>TCACGGTACTGATGACGCT</td>
<td>191 bp</td>
<td>MK757585</td>
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<tr>
<td>mitofusin 1 (mnf-1)</td>
<td>CGGTATGGCCGACGACACTA</td>
<td>AGACGCTCTGTTGGAGGT</td>
<td>82 bp</td>
<td>MK757584</td>
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<tr>
<td>mitofusin 2 (mnf-2)</td>
<td>TTGTGACAGGTCGTCATG</td>
<td>CAACCCACTGCTCTGCAG</td>
<td>85 bp</td>
<td>MK757586</td>
</tr>
<tr>
<td>glycojen synthase (gysm)</td>
<td>GAGGAGCAGCAGGACGACCC</td>
<td>TTACAGCAGTACGACGCC</td>
<td>80 bp</td>
<td>MN201568</td>
</tr>
<tr>
<td>glycojen phosphorylase (pygsm)</td>
<td>AACAAAGCCGAGGAGGTCG</td>
<td>TTCATGACAGGAGGACGCG</td>
<td>74 bp</td>
<td>MN201569</td>
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<tr>
<td>Phosphofructokinase (pfk)</td>
<td>TTGTGATCGAGGAGGTTCC</td>
<td>ATTTGGTCTGATGGTGCC</td>
<td>116 bp</td>
<td>MN173857</td>
</tr>
<tr>
<td>pyruvate kinase (pk)</td>
<td>GCTTGAGTAAAGCACCGAGGAGG</td>
<td>CTCCTGATCGCTGAGGACGCG</td>
<td>155 bp</td>
<td>MK453387</td>
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<tr>
<td>myostatin (mstn-1)</td>
<td>TTGTGAGGACTGGTGGCTGCGG</td>
<td>GCAGACATGCATGGCGGAGGGGTA</td>
<td>172 bp</td>
<td>DQ184914</td>
</tr>
<tr>
<td>muscle RING-finger protein 1 (mrf-1)</td>
<td>TTGTGAGGACTGGTGGCTGCGG</td>
<td>GCAGACATGCATGGCGGAGGGGTA</td>
<td>172 bp</td>
<td>DQ184914</td>
</tr>
<tr>
<td>muscle atrophy F-box protein (mafb)</td>
<td>GCAACGCCCATCAGCTGACTCG</td>
<td>CTTCTGAGGGAGGAGAGAAATAG</td>
<td>152 bp</td>
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<tr>
<td>Myogenin (myo)</td>
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<td>GACGCTCTCCTTCCTCCATCG</td>
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<tr>
<td>muscle-specific regulatory factor 4</td>
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<td>GACCTTGAGGAGGGCCACATGA</td>
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<td>myogenic factor 5 (myf5)</td>
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<td>TGCACTTACTGTCGAGCCACACT</td>
<td>167 bp</td>
<td>DQ872515</td>
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<td>beta-actin (β-actin)</td>
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<td>CTCATGGCACACGCGGACCT</td>
<td>161 bp</td>
<td>H836788</td>
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</tbody>
</table>

marker of apoptosis. No difference was found between the Mannitol group and the Control group.

**Glycogen concentration in cells**

The cells in the HG group had a significantly higher glycogen concentration than those in the Control group ($P < 0.05$). No significant difference was detected between the cells in Mannitol group and cells in the Control group ($P > 0.05$) (Fig. 2(o)). To show intracellular glycogen concentration results more intuitively, periodic acid-Schiff staining was used to analyse the intracellular glycogen concentration. The high glucose-treated cells stained more positively for glycogen (Fig. 2(l)–(n)). High glucose treatment increased the glycogen concentration in olive flounder muscle cells.

**Relative expressions of mitochondria-related genes, glycogen metabolism genes, protein degradation genes and myogenic regulatory factors**

After treated with high glucose, the mRNA levels of optic atrophy protein 1 (opa1) and mitofusin 2 (mfn-2) were significantly decreased ($P < 0.05$), while mitofusin 1 (mfn-1) expression remained unchanged ($P > 0.05$) (Fig. 3(a)). The expressions of glycogen metabolism genes were also affected by high glucose treatment. In the HG group, the mRNA level of glycogen

![Image](https://doi.org/10.1017/S0007114521002634) Published online by Cambridge University Press
Fig. 3. Effect of high glucose on gene expressions. (a) Relative expressions of mitochondria-related genes after the high glucose treatment in olive flounder muscle cells. (b) Relative expressions of glycogen metabolism genes after the high glucose treatment in olive flounder muscle cells. (c) Relative expressions of protein degradation gene after the high glucose treatment in olive flounder muscle cells. (d) Relative gene expressions of myogenic regulatory factors (MRF) after the high glucose treatment in olive flounder muscle cells. Results are represented as mean ± SD (n = 3). * means significantly different compared with the Control group.

**Discussion**

Mitochondria plays a vital part in energy production in fish skeletal muscle. Besides being the main sites for energy production, mitochondria are also the major regulators of apoptosis. The mitochondrial dysfunction and oxidative stress-mediated mitochondrial injuries play important roles in cell apoptosis. MMP is a central bioenergy parameter controlling ATP synthesis, respiratory rate and ROS production. It is controlled by electron transfer and proton leakage. Loss of MMP is one of the causes of mitochondrial dysfunction, and the loss of MMP caused by high glucose treatment has been reported. Meanwhile, decreased MMP indicates mitochondrial damage and the early phase of apoptosis. In the present study, high glucose treatment significantly decreased the MMP in olive flounder muscle cells. It is indicated that the mitochondrial function was affected by high glucose. Mitochondria are the major source of ROS generation. When damaged by oxidative stress, mitochondria produce more superoxide. As the key responders of the stress, mitochondria are the most immediate targets of the oxidative damage inflicted by ROS. ROS can elicit mitochondrial dysfunction and loss of ATP in cells, in turn, mitochondrial dysfunction leads to excessive accumulation of ROS and ATP consumption. Oxidative stress was proved to shift mitochondria towards consuming rather than synthesising ATP, which may be one of the reasons for the decrease of ATP/ADP ratio under high glucose condition. In the present study, cells treated with high glucose accumulated significantly higher amount of ROS. Meanwhile, the
**Fig. 4.** The results of Western blot analysis. High glucose increased the release of mitochondrial CytC as well as increases the nuclear translocation of Nrf2 (a–e). High glucose increased the expressions of PGC-1α, GLUT4, UCP2 and cleaved caspase-3 (f–j). High glucose increased the phosphorylation level of AMPK as well as inhibits the mTOR signalling (k–o). Results are represented as mean ± SD (n 3). * means significantly different compared with the Control group.
Effects of glucose on flounder muscle cells.

ADP/ATP ratio was significantly increased in the HG group, which is a manifestation of mitochondrial dysfunction. Optic atrophy protein 1 (OPA1), mitofusin 1 (MFN1) and mitofusin 2 (MFN2) are three proteins required for mitochondrial fusion and modulating mitochondrial architecture. OPA1 is associated with better mitochondrial function and energy efficiency. It can also reduce the ROS generated by mitochondria. Mitofusins were found to increase the mitochondrial energetic efficiency and MMP(35). Previous study found that Mfn2 deficiency induces mitochondrial dysfunction and ROS production(36). In the present study, the decreased expressions of opta1 and mfn2 were accompanied by increased ROS content in the HG group. This is consistent with the results of previous studies(37,38). It is suggested that high glucose might negatively affect mitochondrial function partly by downregulating the mRNA expressions of mitochondrial fusion proteins.

The damage of the mitochondrial membrane by ROS leads to leakage of Cytc followed by cell apoptosis. Cytc is essential for the mitochondrial electron transport and cell apoptosis. The release of Cytc from mitochondria to cytoplasm is a key step in the apoptotic cascade. ROS mediates MMP loss and mitochondrial permeability transition, then the Cytc is released into the cytoplasm and results in the activation of caspase cascades, thus lead to cell apoptosis(39). Caspase proteases family has a vital catalytic role in apoptosis, and caspase-3 is the most critical one. The activity of caspase-3 would give an index to the process of apoptosis. The present study found that high glucose treatment induced the expression of cleaved caspase-3 (active form) and increased the activity of caspase-3, which are indexes to the process of apoptosis. Actually, the results of apoptosis determined by flow cytometer also confirmed the increased cell apoptosis in the HG group. Moreover, high glucose condition was proved to cause ROS overproduction by increasing metabolic input into mitochondria and disrupting the electron transport chain(33). Overproduction of ROS could lead to oxidative damage of lipids, DNA and proteins, as well as lead to cell and tissue injury by apoptosis and mitochondrial dysfunction(33). As ROS(10) and cell apoptosis(39) were both reported to affect muscle quality partly by downregulating the mRNA expressions of mitochondrial fusion proteins.

The glycogen content in cells was induced by the high glucose treatment in the present study. PGC-1α has the ability to enhance the muscle glycogen storage by increasing the expression of GLUT4 and decreasing the expression of glycogen phosphorylase(44). It is reported that glucose synthase and glycogen phosphorylase are the two major enzymes affecting the muscle glycogen metabolism. The glycogen synthesis and glycogen decomposition in muscle are promoted by glycogen synthase and glycogen phosphorylase, respectively(45). It was found in the present study that the expressions of PGC-1α and GLUT4 in the HG group were significantly increased. The mRNA expression of gysm was decreased, while the mRNA expression of gysm was upregulated in the HG group. The glucose accumulation in the cells of the HG group might be partly due to the increased glucose uptake, upregulation of glycogen synthesis and the reduced glycogen decomposition capacity. PGC-1α is a downstream factor of AMPK, and the activation of AMPK can increase the expression of PGC-1α(46). In this study, the activation level of AMPK was also increased in the HG group. Considering all these results, it was suggested that high glucose might lead to muscle glycogen accumulation by affecting glucose uptake and glycogen metabolism in fish via the activation of AMPK/PGC-1α. At the same time, the increased glycogen content will inevitably affect the glycolysis potential and the pH of fish muscle(45), thus having a negative impact on muscle texture. The growth and development of skeletal muscle requires activation of myogenic regulatory factors, including MyoD, Myf5, MyoG and Mrf4. MyoD and Myf5 regulate proliferation of myoblasts, while MyoG and Mrf4 control the differentiation and the fusion of myoblasts(47). Myostatin (MSTN) is a negative regulator of muscle growth. It has been shown that MSTN can decrease the mRNA levels of myogenic regulatory factors in fish(48). High glucose has been reported to inhibit myogenesis of C2C12 mouse myoblast cells by decreasing intracellular levels of MyoD and MyoG and increasing the cellular content of...
MSTN\(^{(47)}\). In the present study, high glucose significantly increased the mRNA level of \textit{mstn-1}. At the same time, the gene expressions of \textit{myod}, \textit{myog} and \textit{myf5} were significantly decreased in the HG group. The C2C12 cells and the cells used in the present study are both muscle derived. These results indicate that the myogenesis of olive flounder muscle cells was suppressed by high glucose treatment. High glucose level might be an abnormal nutritional condition that can inhibit the differentiation of myoblasts and finally reduce the number of muscle fibers\(^{(47)}\) in olive flounder. Concurrently, the mRNA expressions of \textit{murf-1} and \textit{mafbx} were significantly increased in the HG group. MuRF-1 and MAfbx are two ubiquitin ligases that participate in fish muscle atrophy. Research in C2C12 myotubes showed that oxidative stress increases the expressions of MAfbx and MuRF1\(^{(14)}\). Besides, MSTN was also reported to increase the gene expressions of \textit{mafbx} and \textit{murf-1}\(^{(48)}\). In the present study, increased oxidative stress was proved to exist in the HG group by the enhanced ROS level. The mRNA expressions of \textit{murf-1} and \textit{mafbx} might be stimulated by the increased ROS level and the upregulated \textit{mstn-1} expression. The increased expressions of these two E3 ubiquitin ligases (\textit{murf-1} and \textit{mafbx}) may contribute to the protein degradation of olive flounder muscle cells under high glucose condition.

mTOR is a serine-threonine kinase, it controls translation, protein stability and transcription. The mTOR/p70S6K/4EBP-1 signalling pathway is essential for cell growth and protein synthesis; p70S6K and 4EBP-1 are two downstream effectors of mTOR that promote protein synthesis\(^{(49)}\). In the present study, the activation state of mTOR in olive flounder muscle cells was analysed. Results showed that the phosphorylation level of mTOR was reduced by high glucose treatment. At the same time, the phosphorylation levels of 4EBP-1 and S6 were also downregulated in the HG group. S6 is a primary substrate of p70S6K, and its phosphorylation level reflects the phosphorylation level of p70S6K\(^{(50)}\). Thus, it can be concluded that high glucose inhibits the activation of mTOR/p70S6K/4EBP-1 signalling pathway, which could inhibit the protein synthesis and growth of olive flounder muscle cells. Similar results were found in the studies of other cells, in which the high glucose treatment significantly inhibited the activation of mTOR\(^{(60)}\). High glucose was shown to be a nutritional state unfavourable to the growth of olive flounder muscle cells. Apart from the inhibited mTOR signalling pathway, the AMPK activity and \textit{mstn-1} expression were increased in the HG group. AMPK and MSTN were proved to be upstream regulatory elements of mTOR. Studies have identified that both the activation of AMPK and MSTN result in the inhibition of mTOR signalling pathway, thus suppressing protein synthesis\(^{(51,52)}\). Therefore, high glucose could inhibit mTOR activity through AMPK and MSTN-1. It is reasonable to infer that high glucose has negative effects on the growth, protein synthesis and myogenesis of olive flounder muscle cells. These results are consistent with the growth decline of some cultured fish under high dietary carbohydrate level\(^{(2,53)}\) and explain this phenomenon to a certain extent at the cellular level. Previous studies found that high dietary carbohydrate level led to growth inhibition and inflammation in olive flounder. Accompanied with these changes, one of the main physiological indicators in the olive flounder fed with high carbohydrate diet is the significantly higher blood glucose level compared with the fish fed with lower carbohydrate diet\(^{(17,54,55)}\). In the present study, 33 mM of medium glucose concentration led to a significant decrease in cell viability, which is a critical \textit{in vitro} parameter, so this concentration of medium glucose may mimic the adverse physiological conditions of blood glucose change caused by high carbohydrate diet in olive flounder, and the present study may provide a cell-based insight into the effect of high glucose on olive flounder.

In conclusion, high glucose increased apoptosis, glycogen accumulation and inhibited protein synthesis in primary cultured muscle cells of olive flounder. Based on the results from the present study, the underlying mechanisms could be concluded as follows: High glucose caused damage to the mitochondrial health and function thus induced the mitochondria mediated...
apoptotic signalling pathway. It might lead to muscle glycogen accumulation by affecting glucose uptake and glycogen metabolism. In addition, high glucose has negative effects on the activity of mTOR signalling pathway and the expressions of myogenic regulatory factors. It also induced the expressions mstr1 and E3 ubiquitin ligases (murf-1 and mafs), thus inhibiting the growth, protein synthesis and myogenesis of olive flounder muscle cells (Fig. 5).

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