# Creatine enhances protein deposition and improves mitochondrial quality in myotubes at fasting state by serving as an energy substrate

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**Research**

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# Abstract

**Background:** There are diverse physio-pathological stimuli, including denervation, fasting, aging, and systematic diseases, which can trigger skeletal muscle atrophy. Creatine (Cr) serves as fast energy buffer in skeletal muscle, thus plays an important role in muscular energy homeostasis and involves in protein metabolism. The aim of the present study was to evaluate the supplemental effect of Cr on protein accumulation at malnutrition state mimicked by *in vitro* serum-free culture. We also hypothesized that Cr functions as a source of energy to contribute the protein deposition at fasting state.

**Methods:** We used serum-free medium to mimic the fasting state. The primary myoblast were obtained from the breast muscle of specific-pathogen-free (SPF) chicks. The protein synthesis rate, ATP level, and mitochondrial function of myotubes were measured. The mammalian target of rapamycin (mTOR)/P70S6 kinase (P70S6K) and ubiquitin proteasome (UP) pathways were evaluated.

**Results:** Cr treatment alleviated myotube atrophy and enhanced protein deposition by inhibiting these genes expression involved in UP pathway. Meanwhile, the present result indicated that Cr supplementation increased ATP level, mitochondrial membrane potential (MMP), upregualted the transcriptional coactivators peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) expression, and decreased reactive oxygen species (ROS) accumulation, suggesting that Cr improves mitochondrial quality. Glucose treatment increased protein content and myotube diameter. Meanwhile, glucose supplementation improved mitochondrial quality indicated by the observation of increased myotube ATP level, MMP and PGC1α expression and decreased ROS accumulation. In the present of glucose, however, the favorable effect of Cr on protein content and myotube diameter disappeared.

**Conclusions:** The present results suggest that at fasting state, Cr take a favorable effect on protein deposition via suppression of UP pathway and amelioration of mitochondrial quality of myotubes by serving as an energy substrate. The result highlights the potential clinical application for the modulation of muscle atrophy at fasting state.


# Background

Skeletal muscle contains creatine (Cr) in concentrations up to 20-40 mM [1, 2]. Cr plays an important role in muscle energy homeostasis via ATP-phosphocreatine phosphoryl exchange mediated by creatine kinase (CK) and the phosphorylated creatine (PCr) serves as an energy buffer for ATP replenishment [3, 4]. Cr supplementation elevate muscle Cr and PCr contents and improve muscle energy status in humans [5], rats [6)], and chickens [7].

Cr can increase muscle mass and prevent disease-induced muscle atrophy and reduce apoptosis [8-11]. The metabolic disorder in Cr is associated with muscle weakness and atrophy [12]. Systemic Cr depletion results in mitochondrial dysfunction and intracellular energy deficiency, as well as structural and physiological abnormalities, which could be reversed by oral Cr administration [13]. In the model of Cr transporter knockout mouse, PCr deficiency in skeletal muscle associates with enhanced protein

degradation and muscle atrophy [14, 15]. Mammalian target of rapamycin (mTOR)/P70S6 kinase (P70S6K) pathway, a vital component of the anabolic protein synthesis machinery [16-18], is a crucial regulator of skeletal muscle hypertrophy and atrophy [19, 20]. Cr supplementation enhances myotube differentiation by activating mTOR signal pathway [21, 22], which, however, is not observed in older adults [23]. Hence, the mechanism of Cr on muscle protein metabolism especially at malnutrition state remains to be elucidated.

Mitochondrial dysfunction potentially contributing to muscle protein degradation, muscle fatigue, decreased endurance and muscle weakness are commonly seen in the mitochondrial myopathies [24]. The transcriptional coactivators peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) contributes to the control of skeletal muscle mass and energy metabolism through their coordinated transcriptional regulation of genes involved in mitochondrial biogenesis and fusion [25-27], protection against protein degradation, inflammation and chronic disease [28-30]. The mRNA expression of PGC1α in muscle decreases during disuse atrophy and various other types of muscle wasting [31]. Cr increases PGC1α expression, which, in turn, promotes mitochondrial functions, inhibits protein degradation, and delays the progression of sarcopenia [32]. Cr also plays a role in the promotion of mitochondrial biogenesis and slightly augments mitochondrial mass under oxidative stress conditions [33].

In mammals, the fast-twitch glycolytic fibres are more susceptible to age-related atrophy than slow-twitch oxidative fibres [34, 35] In chicken, the breast muscle primarily comprises of the fast-twitch glycolytic fibres, which made chicken myotube an interesting model. The aim of the present study was to evaluate the supplemental effect of Cr on protein accumulation at malnutrition state mimicked by *in vitro* serum-free culture. The primary myoblast were obtained from the breast muscle of SPF chicks. The protein synthesis rate, ATP content, and mitochondrial function of myotubes were measured. The mTOR/P70S6K and ubiquitin proteasome (UP) pathways were evaluated.

# Materials And Methods

### Cell culture

The specific-pathogen-free (SPF) chicken eggs were obtained from a commercial supplier (Jinan SAIS Poultry CO, Ltd., Jinan, China). The eggs were incubated in an incubator at 37 °C. Embryonic myoblasts of chicken were prepared according to Yablonka and Nameroff [36]. Briefly, at embryonic D 15, the breast muscle tissues (*M. pectoralis major*) were obtained, cut into pieces, and digested with 1% streptomyces protease. The myoblasts were purified by percoll density gradient centrifugation for 40 mins at 3000 *rpm*. Then, the myoblasts were seeded in 6-well plates, cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Solarbio, Beijing, China) with 16% fetal bovine serum (Gibco, Grand Island, NY, US) and 1% penicillin/ streptomycin (Solarbio, Beijing, China), and maintained at 37 °C in a humidified atmosphere with 5% $CO_2$. When the cells reached approximately 95% confluence and formed myotubes, cells were subjected to the treatments.

## Myotubes treatments

In the treatment, the myotubes were cultured in serum-free medium to mimic the fasting state [37, 38]. Cr was supplemented in the culture medium at a dose of 5, 10, or 20 mM (Aladdin, Shanghai, China) for 24 h according to the Cr level in chicken breast muscle tissues [39].

To further evaluate the physiological role of Cr, chicken myotubes were subjected to the following treatments: Cr (10 mM) with or without glucose (0, 5 or 25 mM; Aladdin, Shanghai, China) for 24 h in DMEM (Solarbio, Beijing, China; 1.0 g/L glucose).

## Protein content

Protein concentration was assayed using a Bradford protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol, and absorbance was measured at 595 nm with a microplate reader (Elx808, Bio-Tek, Winooski, VT). The protein content was calculated using the protein concentration and the volume of lysis buffer (0.1 mL), and expressed as the μg protein per well.

## Myotube diameter

The myotube diameter was measured by using myosin heavy chain (MHC) staining [37]. Briefly, myotubes were grown and differentiated on glass coverslips. After supplemented with different treatments as mentioned above, the chicken myotubes were then fixed, permeabilized, and incubated overnight with anti-MHC (1:1000) (Abcam, US). After antibody removal and washing, the slips were incubated for 4 h with 1:1000 affinity-purified Alexa Fluor 488 dye-conjugated goat anti-mouse antibody (Beyotime, Shanghai, China). Antibody was again removed with washing, then the slips were incubated for 3 min with 4',6-diamidino-2-phenylindole (DAPI) dye (Beyotime, Shanghai, China). The slips were observed under a confocal fluorescence microscope (Dragonfly, Andor, Belfast, UK) and photographed. Average myotube diameter was calculated using Image J from 10 myotubes per visual field and 10 visual fields were recorded from each of the different sample. Myotubes were defined as all multinucleated cells positive for the MHC stain and containing at least three nuclei [37].

## Cell viability assay (CCK8)

The myoblasts were cultured in 96-well plates. After the cells differentiated and formed myotubes, the myotubes were incubated in DMEM supplemented with different treatments as above. Then, the medium was replaced with 100 μL of fresh medium, and 10 μL of CCK-8 dye solution was added to each well, which was incubated at 37 °C for 2 hours. Absorbance was measured at 450 nm with a microplate reader (Elx808, Bio-Tek, Winooski, VT). The mean optical density (OD) of six wells for each treatment group was used to calculated the cell viability percentage.

## Protein synthesis rate analysis

The protein synthesis rate was determined using a nonradioactive method [40, 41]. After treatment with Cr, puromycin (10 μm; Beyotime, Shanghai, China) was added to the culture media for an additional 30 min, and protein were extracted from myotubes and used for subsequent analysis. The newly synthesized proteins were labeled with puromycin and were subsequently detected with an anti-puromycin antibody. The accumulation of puromycin-conjugated peptides into nascent peptide chains reflected the rate of protein synthesis.

### Measurement of ATP

ATP content in myotubes was determined with commerical kit (S0026, Beyotime, Shanghai, China). The chemi-luminescence signal was read with a fluorescence microplate system (BioTek Instruments, Winooski, VT, USA) and the data were normalized for protein concentration.

### Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP) of myotubes was determined with commercial kit (C2006, Beyotime, Shanghai, China). The chemi-luminescence signal was detected using a fluorescence microplate system (BioTek Instruments, Winooski, VT, USA). The CCCP, a mitochondrial electron transport chain inhibitor, was used to verify the feasibility of this method. The results were presented as fluorescence value and normalized for control group.

### Reactive oxygen species (ROS) concentrations

Intracellular ROS concentrations were detected by a fluorescent molecular probe (2',7'-dichlorodihydrofluorescein diacetate, DCFH-DA, S0033S, Beyotime, Shanghai, China). DCFH-DA is a non-polar dye and is converted into the polar derivative DCFH by cellular esterases. DCFH is non-fluorescent and switches to highly fluorescent DCF, a fluorescent compound (kexicitation=498nm; kemission=522nm), when oxidized by intracellular ROS or other peroxides [42]. Briefly, myotubes were grown and differentiated on glass coverslips, and cultured in DMEM containing 10 μm DCFH-DA for 30 min after Cr or glucose treatment and then the cells were washed with PBS to remove unincorporated probes, maintained in normal DMEM. Images of cells under transmitted and fluorescence illumination were obtained under a confocal fluorescence microscopy (Dragonfly, Andor, Belfast, UK) and photographed. The cells were cultured in 96-well black microtitre plates, after the myotubes were incubated in DMEM supplemented with different treatments as above. Then, fluorescence signals were detected using a fluorescence microplate system (BioTek Instruments, Winooski, VT, USA) with an excitation wavelength of 498 nm and an absorption wavelength of 522 nm. The results were presented as fluorescence value and normalized for control group.

### RNA extraction and quantitative real-time PCR analyses

Total RNA was extracted from the cell using TRIzol (Invitrogen, USA). The RNA concentration was measured by spectrophotometry (Eppendorf, Germany), and RNA purity verified by calculating the ratio between the absorbance values at 260 and 280 nm (A260/280 ≈ 1.75-2.01). Then 1 μg RNA was reverse-

transcribed to cDNA using DNase I (Invitrogen, USA) according to the manufacturer's protocol. Real-time PCR was performed using ABI Quant Studio 5 PCR machine (Applied Biosystems; Thermo, United States). Following the manufacturer's protocol, the cDNA was amplified in a 20 μL PCR reaction system containing 0.2 μmol/L of each specific primer (Sangon, Shanghai, China) and the SYBR Green master mix (Roche, Germany). The primer were designed with Primer 6.0 software, and were based on published target sequences (Table 1). Primer against GAPDH was used as internal controls, and all of the mRNA values were normalized with the differences between individual samples. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.

| Gene | Sequence (5′–3′) | Accession. no | Product(bp) |
|---|---|---|---|
| MuRF1-F | GCCAAGCAGCTCATTAAAACG | XM_015297755.2 | 81 |
| MuRF1-R | CATGTTCTCATAGCCTTGCTCAAT | | |
| Atrogin1-F | AGGCCGCAGTGTGTTGTTCT | XM_015283108.2 | 81 |
| Atrogin1-R | GTGTGAATGGCTGGTTGCAT | | |
| MSTN-F | GCTTTTGATGAGACTGGACGAG | NM_001001461.1 | 173 |
| MSTN-R | AGCGGGTAGCGACAACATC | | |
| FoXOl-F | TCTGGTCAGGAGGGAAATGG | NM_204328.1 | 60 |
| FoXOl-R | GCTTGCAGGCCACTTTGAG | | |
| WWP1-F | GGAAGAGCCACTGTAGACTTGAG | XM_015282817.2 | 189 |
| WWP1-R | GGTTGCTGGTGAACTGAGATTTGT | | |
| mTOR-F | GAAGTCCTGCGCGAGCATAAG | XM_417614.6 | 92 |
| mTOR-R | TTTGTGTCCATCAGCCTCCAGT | | |
| P70S6K-F | ATTCGATCACCTCGCAGATTCATAG | XM_015295787.2 | 111 |
| P70S6K-R | AGTATTTGATGCGCTGGCAGAAG | | |
| 4EBP1-F | TTCCCACCAGCCAGGCTTAC | XM_424384.6 | 142 |
| 4EBP1-R | GTGTATTCACACCCACACGGAGA | | |
| IGF-1-F | TGTACTGTGCTCCAATAAAGC | NM_001004384.2 | 127 |
| IGF-1-R | CTGTTTCCTGTGTTCCCTCTACTTG | | |
| IGF-1R-F | TTCAGGAACCAAAGGGCGA | NM_205032.1 | 158 |
| IGF-1R-R | TGTAATCTGGAGGGCGATACC | | |
| PGC-1α-F | GACTCAGGTGTCAATGGAAGTG | NM_001006457.1 | 272 |
| PGC-1α-R | ATCAGAACAAGCCCTGTGGT | | |
| GAPDH-F | ACATGGCATCCAAGGAGTGAG | NM_204305.1 | 144 |
| GAPDH-R | GGGGAGACAGAAGGGAACAGA | | |

**Table. 1** qRT-PCR primer sequences.

### Protein preparation and western blotting

The cells were washed three times briefly with PBS and collected in 0.1 mL of RIPA (radio immunoprecipitation assay) lysis buffer (Beyotime, Shanghai, China). The operation was kept on ice to prevent protein degradation. The homogenate was centrifuged at 12000 g for 10 min at 4°C, and the supernatant was collected. Afterwards, the protein concentration of supernatants was assayed using a BCA protein assay kit (Beyotime, Shanghai, China) according to manufacturer's instructions, and absorbance was measured at 570 nm with a microplate reader (Elx808, Bio-Tek, Winooski, VT). An equal amount of proteins (20 μg) was separated by 8% or 12% SDS polyacrylamide gels (Bio-Rad, Richmond, 246 CA) and the proteins were transferred onto polyvinylidene fluoride membrane (Millipore, United States) at 200 mA for 2 h in a Tris-glycine buffer with 20% anhydrous ethanol at 4 °C. Then membranes were blocked with western blocking buffer (Beyotime, Shanghai, China) for 1 h at room temperature. The membranes were incubated with specific primary antibodies at 4 °C with gentle shaking overnight. The following primary antibodies were used: anti-mTOR, anti-phospho-P70S6K (Thr 389), anti-P70S6K, anti-phospho-4EBP1 (Thr 37/46), anti-4EBP1, anti-phospho-FoXO1/3a, anti-FoXO1 (Cell Signaling Technologies, Danvers, MA, US), anti-MHC, anti-IGF1, anti-phospho-mTOR (Thr 2446), anti-MuRF1, anti-Atrogin1, anti-MSTN, anti-PGC1α (Abcam, Cambridge, MA, US), anti-WWP1 (Affinity Biosciences, US), anti-mouse puromycin (Kerafast, Boston, MA, US), anti-tubulin (Beyotime, Shanghai, China). The membrane was washed with Tris-buffered saline/Tween buffer for three times at 10 min, then the membranes were incubated with secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG, 1:1000; Beyotime, Shanghai, China) for 4 h at 4 °C. After washing, membranes were then visualized by exposure to Hyperfilm ECL (Beyotime, Shanghai, China). When two different proteins had the same or similar molecular weight (including the phosphorylated and total levels of each protein), we used different membranes to separately detect them. In contrast, when two proteins were of different molecular weights (>15 kD), the membranes was blocked again and incubated with another antibody after one protein was detected. Western blots were developed and quantified using BioSpectrum 810 with VisionWorks LS 7.1 software (UVP LLC). The band intensity was normalized to the tubulin band in the same sample.

### Statistical analysis

The data were presented as the means ± SD (n = 6). All the data were analyzed with one-way ANOVA by using STATISTICAL ANALYSIS Software (version 8e; SAS Institute, Cary, NC, United States). Differences between the means were evaluated using Tukey's honestly significant difference test. Differences were considered as significant at $P<0.05$.

## Results

### Effect of fasting on myotubes

The protein content was decreased by 17.15% and 27.75% ($P$<0.001, Fig. 1A), the cell viability was reduced by 58.12% and 65.70% ($P$<0.001, Fig. 1B), and the myotube diameter was reduced by 17.79% and 31.22% ($P$<0.001, Fig. 1C, D) for 12 h and 24 h, relative to its level at 0 h, respectively. After 24 h fasting, the mRNA expression of genes related to protein catabolism *MuRF1*, *Atrogin1*, *MSTN*, *FoXO1*, and *WWP1* was elevated ($P$<0.01, Fig. 1E) as well as the expression of genes involved in protein anabolism *mTOR*, *4EBP1*, *IGF1*, and *IGF1R* (Fig. 1F).

### Effect of Cr supplementation on protein metabolism

Cr treatment significantly increased protein content, cell viability and myotube diameter, compared with control ($P$<0.001, Fig. 2A, B, C, D). The MHC protein expression level was increased by Cr treatment in a dose dependent manner ($P$<0.001, Fig. 2E, F).

In contrast, the protein synthesis rate was not changed by Cr treatment ($P$>0.05, Fig. 3A, B). The mRNA levels of *IGF1* and *IGF1R* were not influenced ($P$>0.05) by Cr as well as the protein levels of IGF1, total and phosphorylated mTOR, P70S6K, and 4EBP1 (Fig. 3C-H).

Cr treatment significantly decreased the mRNA expression levels of *FoXO1*, *MuRF1*, *Atrogin1*, *MSTN*, and *WWP1* compared with control ($P$<0.05, Fig. 4A-E). In contrast, Cr increased p-FoXO1/3a protein expression level ($P$<0.001, Fig. 4F, G) and meanwhile inhibited the protein expression of Atrogin1 ($P$<0.05, Fig. 4F, I), MSTN ($P$<0.05, Fig. 4F, J), and WWP1 ($P$<0.05, Fig. 4F, K) at 20 mM Cr group. No detectable influence was observed on MuRF1 protein level ($P$>0.05, Fig. 4F, H).

### Effect of Cr supplementation on ATP content and mitochondrial function

Cr treatment increased ATP content ($P$<0.001) and MMP ($P$<0.05), while decreased ROS formation ($P$<0.001) compared with control cells (Fig. 5A-D). Meanwhile, Cr supplementation upregulated *PGC1α* mRNA ($P$<0.01, Fig. 5E) and protein expression ($P$<0.01, Fig. 5F, G) compared with control cells.

### Effect of combined treatment of Cr with glucose

The role of Cr was further evaluated in the present of different glucose concentrations (0, 5 mM, 25 mM). Compared to control, Cr or glucose treatment increased protein content, cell viability, myotube diameter, and MHC protein level ($P$<0.05, Fig. 6A-F). In the presence of 5 and 25 mM glucose, however, Cr treatment had no obvious influences on protein content, cell viability, myotube diameter, and MHC protein level ($P$>0.05).

Glucose treatment at 25 mM decreased expression of genes *FoXO1* ($P$<0.001), *MuRF1* ($P$<0.05), *Atrogin1* ($P$<0.05), *MSTN* ($P$<0.01), and *WWP1* ($P$<0.05) compared to control (Fig. 7A-E). In the presence of glucose, the significantly decreased ($P$<0.05) expression of *FoXO1* and *Atrogin1* by Cr treatment was not observed ($P$>0.05, Fig. 7A, C). Adversely, *MSTN* expression was declined by Cr in the present of 5 mM glucose ($P$=0.094) and 25 mM glucose ($P$<0.05, Fig. 7D), while *MuRF1* mRNA level was increased by Cr in 25 mM glucose group ($P$<0.05, Fig. 7B).

Glucose treatment at 25 mM significantly increased p-FoXO1/3a/T-FoXO1 ($P$<0.01), declined MuRF1 ($P$<0.05), Atrogin1 ($P$<0.05), MSTN ($P$<0.01) and WWP1 ($P$<0.01) protein expression, compared to control (Fig. 7G-K). The suppression effect of Cr on Atrogin1 ($P$<0.05) and WWP1 ($P$<0.01) was not detected in the present of glucose ($P$>0.05, Fig. 7I, K). In contrast, in the presence of 25 mM glucose, Cr treatment increased MuRF1 protein expression ($P$<0.01, Fig. 7H).

Glucose treatment increased ATP and decreased ROS levels compared to control ($P$<0.001, Fig. 8A, C, D). In the presence of glucose, the stimulating effect of Cr on ATP was not changed ($P$<0.01), whereas the suppression effect on ROS formation ($P$<0.05) was not observed ($P$>0.05). 25 mM glucose treatment decreased the mRNA expression levels of *PGC1α* ($P$<0.05), compared with control. The increased *PGC1α* ($P$<0.001) expressions by Cr were not detected in the presence of glucose ($P$>0.05, Fig. 8E). In contrast, 5 mM and 25 mM glucose supplementation improved PGC1α protein level compared with control ($P$<0.01, Fig. 8F, G). The significantly increased effect of Cr on PGC1α protein expression ($P$<0.05) was not detected in the presence of glucose ($P$>0.05).

# Discussion

In the present study, we investigated the role of Cr on muscle protein metabolism and mitochondrial function *in vitro* cultured chicken myotubes at fasting condition. The results indicated that Cr supplementation alleviated myotube atrophy mainly by inhibiting UP pathway. Cr enhances ATP content, suppresses ROS accumulation, and improves mitochondrial quality. In the present of glucose, however, the beneficial effects of Cr were diminished, suggesting that Cr mainly play a role of energy source at fasting state.

### Cr alleviate myotube atrophy by inhibiting ubiquitin proteasome pathway under fasting condition

Skeletal muscle atrophy can be induced by a diverse array of external stimuli factors such as inactivity, malnutrition, and inflammation [18]. In the present study, we used serum-free DMEM medium to mimic the fasting state. The decreased protein content, cell viability, and myotube diameter after 12-h serum-free culture indicated that muscle atrophy model was successfully built up, in line with the previous works [37, 38].

Cr treatment increased in protein content and myotube diameter, in line with the work by Deldicque et al. [21], who reported that Cr supplementation increased the myotube diameter and MHC expression in C2C12 cells. Similarly, O'Connor et al. [8] reported that Cr enhanced myonuclear addition and myotube growth. Hence, the present result demonstrates that Cr enhances protein accumulation in myotubes at fasting state.

The balance between protein synthesis and degradation determine the protein content in myoblasts [43]. The skeletal muscle atrophy model shares a basic feature of an imbalance of muscle protein synthesis and degradation [43]. Therefore, we firstly evaluated protein synthesis rate and the status of mTOR pathway. In C2C12 cells, Cr enhanced muscle mass by increasing muscle protein synthesis and increased

phosphorylation state of P70S6K, whereas 4EBP1 remained unchanged [21]. Cr supplementation enhanced protein synthesis in primary myotube cultures derived from Duroc and Landrace pigs [22]. In the contrast to the previous studies, the present study was conducted at serum-free condition to mimic fasting state. The present results showed that Cr supplementation had no effects on protein synthesis rate, indicating that Cr has no beneficial influence on protein synthesis at starving state. Meanwhile, the unchanged phosphorylation of mTOR and P70S6K by Cr treatment indicated the state of mTOR pathway is not activated. In older adults, although the total amount of mTOR is upregulated by supplement of L-Carnitine, Cr, and leucine, the phosphorylation of mTOR and other components of translation-signaling pathway were not changed [23]. Collectively, the result implies that favorable effect of Cr on muscular protein synthesis and the activation of mTOR pathway depend on the nutritional conditions and physiological state.

The protein degradation occurs mainly through the UP pathway in cells and approximately 40% ~ 50% of proteins are degraded via the pathway [44, 45]. The genes Mafbx/Atrogin1 and MuRF1, both encoding E3 ubiquitin ligases would increase the expression through upregulate FoXO transcription factors [18, 45]. UP pathway is the major protein degradation pathway in cells and plays a major role in signal transduction associated with cellular physiology [46, 47]. FoXO family transcription factors play a critical role in this loss of cell protein, and when activated, causing expression of the atrophy-related ubiquitin ligases Atrogin1 and MuRF1 and profound loss of muscle mass [31, 45]. MSTN is essential for the negative regulation of skeletal muscle growth [48]. In the present study, the p-FoXO1/3a was increased by Cr treatment, suggesting that the suppression of FoXO1 pathway. Meanwhile, Cr treatment downregulated *MuRF1*, *Atrogin1*, *WWP1*, and *MSTN* mRNA expression and decreased the protein expression level of Atrogin1, MSTN, and WWP1, indicating that Cr treatment suppresses proteolysis. This result was in line with previous report showing that Cr supplementation decreased Atrogin1 expression and rescued the atrophic effects of MSTN in C2C12 myotubes [49]. Low-dose Cr supplementation increases lean tissue mass and reduces muscle protein degradation during resistance training in older men [50]. Hence, the result suggests that Cr alleviates myotube atrophy, at starving status, mainly by suppressing UP pathway.

### Cr enhances intracellular ATP content and improves mitochondrial quality

PCr system serves as rapid energy buffer to maintain constant ATP levels and mediates subcellular high energy phosphate transfer [3, 4]. The present results indicated that Cr supplementation increased ATP level, indicating that Cr meliorates myotube energy status. Under normal energy condition (cultured with 10% fetal bovine serum), in L6 rat skeletal myoblasts and C2C12 cells, Cr increases Cr and PCr contents, but the ATP content of the cells was not affected [6, 21, 51]. The result indicates the Cr functions as an energy buffer to improve cellular energy status.

Mitochondrial dysfunction potentially contributing to muscle protein degradation [24]. PGC1α, the prime regulator of mitochondrial quality and oxidative metabolism, is reduced when skeletal muscle atrophy and perturbed metabolic function occur [29, 52, 53]. Cr supplementation has been shown to protect

mitochondria function in C2C12 cells [9, 33]. In line with the present study, the present results indicated that Cr supplementation increased MMP level, upregulated PGC1α mRNA and protein expression, and decreased ROS accumulation, suggesting that Cr improves mitochondrial function. In mammals, the activation of PGC1α regulates multiple physiological pathways, such as protein degradation, ROS levels, inflammation and apoptosis [30]. PGC1α protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription [31]. In myotubes, PGC1α over-expression inhibits protein degradation without affecting protein synthesis [29]. Hence, the increased PGC1α expression by Cr may contribute to the suppressed proteolysis.

### Cr functions mainly as an energy source at fasting state

Cr plays an important role in muscular energy homeostasis and involves in protein metabolism. We further investigated that if Cr functions as a source of energy to contribute the protein accumulation at fasting condition. The present result showed that glucose treatment increased protein content and myotube diameter, indicating that glucose could stimulate myotube development. This result was in line with the previous works by Nedachi et al. [54], who reported that glucose treatment (22.5 mM) increase myotube formation in C2C12 cells. High glucose (25 mM) increased protein accumulation compared with a physiological glucose concentration (5.5 mM) in cultured cardiomyocytes [55]. Mitochondrial function in the skeletal muscle has been modulated in a wide variety of energy conditions [56]. The present results indicated that glucose supplementation increased myotube ATP level, while the effect of Cr on ATP content was weaken as glucose concentration increased (0 mM: +355%; 5 mM: +285%; 25 mM: +121%). Glucose treatment improved mitochondrial quality indicated by the observation of increased MMP and PGC1α expression and decreased ROS accumulation. In the present of glucose, however, the favorable effect of Cr on protein content and myotube diameter disappeared. Simultaneously, the decreased protein levels of Atrogin1 and WWP1 by Cr was masked by the presence of glucose. Collectively, the result suggests that Cr, at starving state, plays a beneficial effect on myotube development via functioning mainly as an energy source.

## Conclusions

The present result indicates that, at fasting state, Cr take a favorable effect on protein accumulation via suppression of UP pathway and amelioration of mitochondrial function of myotubes by serving as an energy supplier. The result highlights the potential clinical application for the modulation of muscle atrophy at fasting state.

## List Of Abbreviations

Cr: creatine; UP: ubiquitin proteasome; PCr: phosphorylated creatine; MMP: mitochondrial membrane potential; PGC-1α: transcriptional coactivators peroxisome proliferator-activated receptor-γ coactivator-1α; ROS: reactive oxygen species; mTOR: mammalian target of rapamycin; P70S6K: P70S6 kinase; MHC:

myosin heavy chain; SPF: specific-pathogen-free; CK: creatine kinase; OD: mean optical density; DMEM: Dulbecco's modified Eagle's medium

# Declarations


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### Authors' contributions

MS and HL designed the research. MS performed experiments, analyzed, and interpreted the data. HJ, JZ and XW participated in manuscript preparation. MS and HL interpreted the results, edited, reviewed, and approved the final version of the manuscript. HL had primary responsibility for final content. All authors read and approved the final manuscript.


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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Competing interests

The authors have declared that they have no conflicts of interest.

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# References

1. Markus W, Rima KD. Cr and creatinine metabolism. Physiological Reviews. 2000; 80: 1107-1213.

2. Tossenberger J, Rademacher M, Németh K, Halas V, Lemme A. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. Poult Sci. 2016; 95: 2058-2067.

3. Wallimann T, Tokarska-Schlattner M, Schlattner U. The Cr kinase system and pleiotropic effects of Cr. Amino Acids. 2011; 40: 1271-1296.

4. Sestili P, Barbieri E, Stocchi V. Effects of Cr in skeletal muscle cells and in myoblasts differentiating under normal or oxidatively stressing conditions. Mini Rev Med Chem. 2016; 16: 4-11.

5. Harris RC, Söderlund K, Hultman E. Elevation of creatine in resting and exercise muscle of normal subjects by creatine supplementation. Clin Sci (Lond). 1992; 83: 367-374.

6. Ceddia RB, Sweeney G. Creatine supplementation increases glucose oxidation and AMPK phosphorylation and reduces lactate production in L6 rat skeletal muscle cells. Journal of Physiology. 2004; 555: 409-421.

7. Wang XF, Zhu XD, Li YJ, Liu Y, Li JL, Gao F, Zhou GH, Zhang L. Effect of dietary creatine monohydrate supplementation on muscle lipid peroxidation and antioxidant capacity of transported broilers in summer. Poult Sci. 2015; 94: 2797-2804.

8. O'Connor RS, Steeds CM, Wiseman RW, Pavlath GK. Phospho Cr as an energy source for actin cytoskeletal rearrangements during myoblast fusion. J Physiol. 2008; 15: 2841-2853.

Sestili P, Barbieri E, Martinelli C, Battistelli M, Guescini M, Vallorani L, Casadei L, D'Emilio A, Falcieri E, Piccoli G, Agostini D, Annibalini G, Paolillo M, Gioacchini AM, Stocchi V. Cr supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts. Mol Nutr Food Res. 2010; 53: 1187-1204.

10. Tarnopolsky MA. Cr as a therapeutic strategy for myopathies. Amino Acids. 2011; 40: 1397-1407.

11. Chilibeck PD, Kaviani M, Candow DG, Zello GA. Effect of Cr supplementation during resistance training on lean tissue mass and muscular strength in older adults: a meta-analysis. Open Access J Sports Med. 2017; 8: 213-226.

12. Stockebrand M, Sasani A, Das D, Hornig S, Hermans-Borgmeyer I, Lake HA, Isbrandt D, Lygate CA, Heerschap A, Neu A, Choe CU. A mouse model of Cr transporter deficiency reveals impaired motor

function and muscle energy metabolism. Front Physiol. 2018; 9: 773.

13. Nabuurs CI, Choe CU, Veltien A, Kan HE, van Loon LJ, Rodenburg RJ, Matschke J, Wieringa B, Kemp GJ, Isbrandt D, Heerschap A. Disturbed energy metabolism and muscular dystrophy caused by pure creatine deficiency are reversible by creatine intake. J Physiol. 2013; 591(2): 571-92.

14. Russell AP, Foletta VC, Snow RJ, Wadley GD. Skeletal muscle mitochondria: a major player in exercise, health and disease. Biochim Biophys Acta. 2014; 1840: 1276-1284.

15. Baroncelli, L, Molinaro, A, Cacciante, F, Alessandri, MG, Napoli, D, Putignano E, Tola J, Leuzzi V, Cioni G, Pizzorusso T. A mouse model for creatine transporter deficiency reveals early onset cognitive impairment and neuropathology associated with brain aging. Hum. Mol. Genet. 2016; 25: 4186-4200.

16. Hornberger TA, Chien S. Mechanical stimuli and nutrients regulate rapamycin-sensitive signaling through distinct mechanisms in skeletal muscle. J Cell Biochem. 2006; 97: 1207-1216.

17. Shimizu N, Yoshikawa N, Ito N, Maruyama T, Suzuki Y, Takeda S, Nakae J, Tagata Y, Nishitani S, Takehana K, Sano M, Fukuda K, Suematsu M, Morimoto C, Tanaka H. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab. 2011; 13: 170-182.

18. Wang R, Jiao H, Zhao J, Wang X, Lin H. L-Arginine enhances protein synthesis by phosphorylating mTOR (Thr 2446) in a nitric oxide-dependent manner in C2C12 cells. Oxid Med Cell Longev. 2018; 26: 1-13.

19. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol. 2001; 3: 1014-1019.

20. You JS, Anderson GB, Dooley MS, Hornberger TA. The role of mTOR signaling in the regulation of protein synthesis and muscle mass during immobilization in mice. Dis Model Mech. 2015; 8: 1059-1069.

21. Deldicque L, Theisen D, Bertrand L, Hespel P, Hue L, Francaux M. Cr enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways. Am J Physiol Cell Physiol. 2007; 293: C1263-1271.

22. Young JF, Bertram HC, Theil PK, Petersen AG, Poulsen KA, Rasmussen M, Malmendal A, Nielsen NC, Vestergaard M, Oksbjerg N. In vitro and in vivo studies of Cr monohydrate supplementation to Duroc and Landrace pigs. Meat Sci. 2007; 76: 342-351.

23. Evans M, Guthrie N, Pezzullo J, Sanli T, Fielding RA, Bellamine A. Efficacy of a novel formulation of L-Carnitine, Cr, and leucine on lean body mass and functional muscle strength in healthy older adults: a randomized, double-blind placebo-controlled study. Nutr Metab (Lond). 2017; 14: 7.

24.Russell, AP, Ghobrial, L, Wright, CR, Lamon, S, Brown, EL, Kon, M, Skelton MR, Snow RJ. Creatine transporter (SLC6A8) knockout mice display an increased capacity for in vitro creatine biosynthesis in skeletal muscle. Front Physiol. 2014; 5: 314.

25. Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. Cell metabolism. 2005; 1: 361-370.

26. Liesa M, Borda-d'Agua B, Medina-Gómez G, Lelliott CJ, Paz JC, Rojo M, Palacín M, Vidal-Puig A, Zorzano A. Mitochondrial fusion is increased by the nuclear coactivator PGC-1β. Plos One. 2008; 3: e3613.

27. Shao D, Liu Y, Liu X, Zhu L, Cui Y, Cui A, Qiao A, Kong X, Liu Y, Chen Q, Gupta N, Fang F, Chang Y. PGC-1β-regulated mitochondrial biogenesis and function in myotubes is mediated by NRF-1 and ERR α. Mitochondrion. 2010; 10: 516-527.

28. Handschin C, Spiegelman BM. The role of exercise and PGC1α in inflammation and chronic disease. Nature. 2008; 454: 463-469.

29. Brault JJ, Jespersen JG, Goldberg AL. Peroxisome proliferator-activated receptor γ coactivator 1α or 1β overexpression inhibits muscle protein degradation, induction of ubiquitin ligases, and disuse atrophy. Journal of Biological Chemistry. 2010; 285: 19460-19471.

30. Schnyder S, Handschin C. Skeletal muscle as an endocrine organ: PGC-1α, myokines and exercise. Bone. 2015; 80: 115-125.

31. Sandri, M, Lin, J, Handschin, C, Yang, W, Arany, ZP, Lecker, SH, Goldberg, AL, and Spiegelman, BM. PGC-1alpha protects skeletal muscle from atrophy by suppressing FoxO3 action and atrophy-specific gene transcription. Proc Natl Acad Sci USA. 2006; 103: 16260-16265.

32. Candow DG, Chilibeck PD, Forbes SC. Cr supplementation and aging musculoskeletal health. Endocrine. 2014; 45: 354-361.

33. Barbieri E, Guescini M, Calcabrini C, Vallorani L, Diaz AR, Fimognari C, Canonico B, Luchetti F, Papa S, Battistelli M, Falcieri E, Romanello V, Sandri M, Stocchi V, Ciacci C, Sestili P. Cr prevents the structural and functional damage to mitochondria in myogenic, oxidatively stressed C2C12 cells and restores their differentiation capacity. Oxid Med Cell Longev. 2016; 2016: 1-12.

34. Larsson L, Biral D, Campione M, Schiaffino S. An age-related type IIB to IIX myosin heavy chain switching in rat skeletal muscle. Acta Physiol Scand. 1993; 147(2): 227-34.

35. Ferraro E, Pin F, Gorini S, Pontecorvo L, Ferri A, Mollace V, Costelli P, Rosano G. Improvement of skeletal muscle performance in ageing by the metabolic modulator Trimetazidine. J Cachexia Sarcopenia Muscle. 2016; 7:449-57.

36. Yablonka-Reuveni Z, Nameroff M. Separation and partial characterization of fibroblast-like cells from embryonic tissue using density centrifugation. Histochemistry. 1987; 87: 27-38.

37. Qiu, J, Fang, Q, Xu, T, Wu, C, Xu, L, Wang, L, Yang, X, Yu, S, Zhang, Q, Ding, F, and Sun, H. Mechanistic role of reactive oxygen species and therapeutic potential of antioxidants in denervation- or fasting-Induced skeletal muscle atrophy. Frontiers in physiology.2018; 9: 215.

38. Xu, Y, Wang, X, Geng, N, Zhu, Y, Zhang, S, Liu, Y, & Liu, J. Mitophagy is involved in chromium (VI)-induced mitochondria damage in DF-1 cells. Ecotoxicology and environmental safety.2020; 194: 110414.

39. Michiels J, Maertens L, Buyse J, Lemme A, Rademacher M, Dierick NA, De Smet S. Supplementation of guanidine acetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. Poult Sci. 2012; 91: 402-412.

40. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009; 6: 275-277.

41. Wang R, Li K, Wang H, Jiao H, Wang X, Zhao J, Lin H. Endogenous cse/hydrogen sulfide system regulates the effects of glucocorticoids and insulin on muscle protein synthesis. Oxidative Medicine and Cellular Longevity. 2019, 9752698.

42. Gomes A, Fernandes E, Lima JL. Fluorescence probes used for detection of reactive oxygen species. J Biochem Biophys Methods. 2005; 65: 45-80.

43. Breen L, Phillips SM. Skeletal muscle protein metabolism in the elderly: Interventions to counteract the 'anabolic resistance' of ageing. Nutr Metab (Lond). 2011; 8: 68.

44. Bodine SC, Glass DJ. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science. 2001; 294: 1704-1708.

45. Baptista IL, Silva WJ, Artioli GG, Guilherme JP, Leal ML, Aoki MS, Miyabara EH, Moriscot AS. Leucine and HMB differentially modulate proteasome system in skeletal muscle under different sarcopenic conditions. PLoS One. 2013; 8: e76752.

46. Lecker SH, Solomon V, Mitch WE, Goldberg AL. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J Nutr. 1999; 129: 227S-237S.

47. Burkhardt D. Role of proteasomes in disease. BMC Biochemistry. 2007; 8.

48. Wang R, Jiao H, Zhao J, Wang X, Lin H. Glucocorticoids enhance muscle proteolysis through a myostatin-dependent pathway at the early stage. PLoS One. 2016; 11: e0156225.

49. Mobley CB, Fox CD, Ferguson BS, Amin RH, Dalbo VJ, Baier S, Rathmacher JA, Wilson JM, Roberts MD. L-leucine, beta-hydroxy-beta-methylbutyric acid (HMB) and Cr monohydrate prevent myostatin-

induced Akirin-1/Mighty mRNA down-regulation and myotube atrophy. J Int Soc Sports Nutr. 2014; 11: 38.

50. Candow DG, Little JP, Chilibeck PD, Abeysekara S, Zello GA, Kazachkov M, Cornish SM, Yu PH. Low-dose creatine combined with protein during resistance training in older men. Med Sci Sports Exerc. 2008; 40: 1645-1652.

51. Alfieri RR, Bonelli MA, Cavazzoni A, Brigotti M, Fumarola C, Sestili P, Mozzoni P, De Palma G, Mutti A, Carnicelli D, Vacondio F, Silva C, Borghetti AF, Wheeler KP, Petronini PG. Creatine as a compatible osmolyte in muscle cells exposed to hypertonic stress. J Physiol. 2006; 576: 391-401.

52. Soyal S, Krempler F, Oberkofler H, Patsch W. PGC-1α: a potent transcriptional cofactor involved in the pathogenesis of type 2 diabetes. Diabetologia. 2006; 49: 1477-1488.

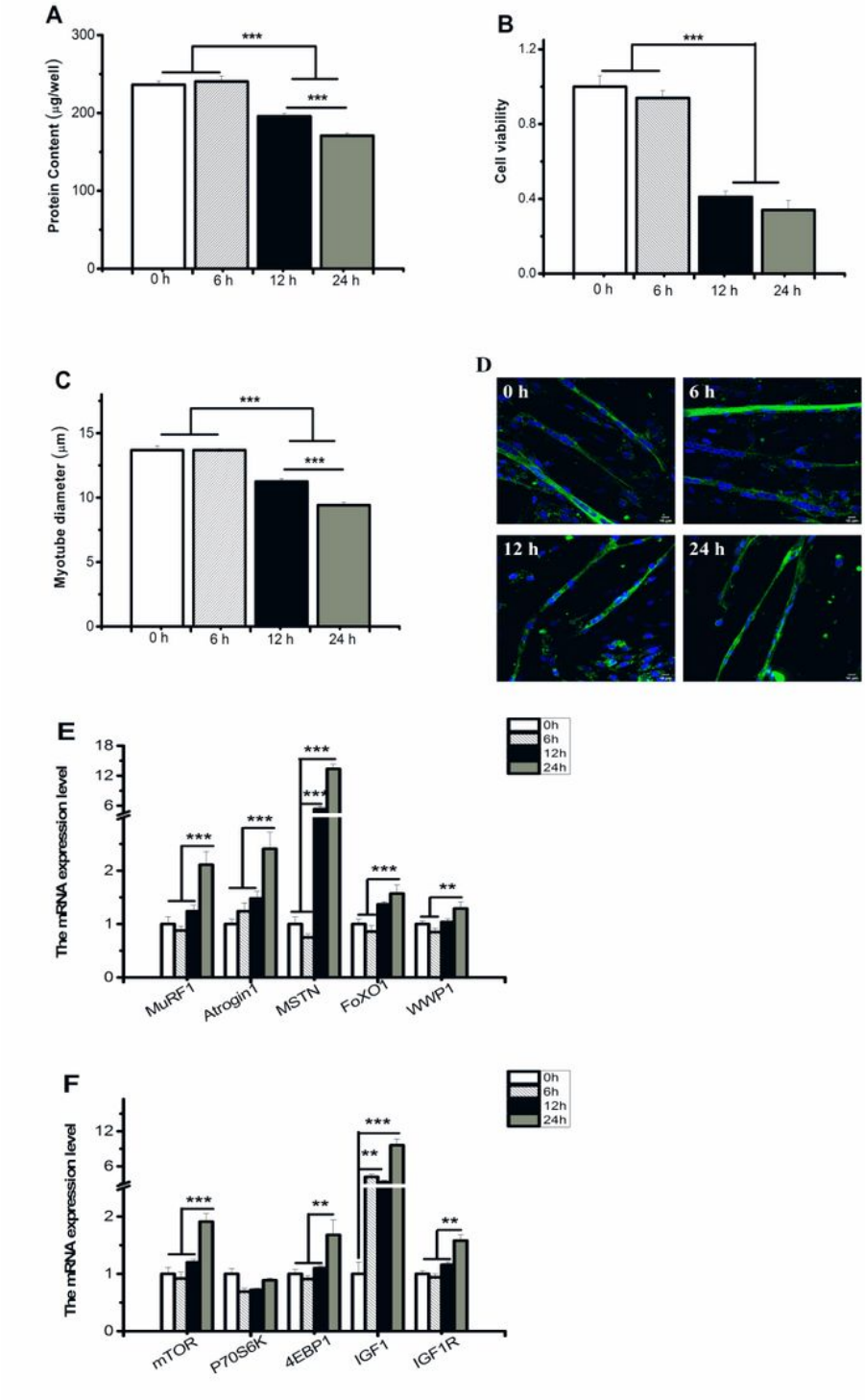
53. Joseph AM, Adhihetty PJ, Buford TW, Wohlgemuth SE, Lees HA, Nguyen LM, Aranda JM, Sandesara BD, Pahor M, Manini TM, Marzetti E, Leeuwenburgh C. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. Aging Cell. 2012; 11: 801-809.

54. Nedachi T, Kadotani A, Ariga M, Katagiri H, Kanzaki M. Ambient glucose levels qualify the potency of insulin myogenic actions by regulating SIRT1 and FoxO3a in C2C12 myocytes. Am J Physiol Endocrinol Metab. 2008; 294: 668-678.

55. Yeshao W, Gu J, Peng X, Nairn AC, Nadler JL. Elevated glucose activates protein synthesis in cultured cardiac myocytes. Metabolism. 2005; 54: 1453-60.

56. Civitarese AE, Smith SR, Ravussin E. Diet, energy metabolism and mitochondrial biogenesis. Current Opinion in Clinical Nutrition & Metabolic Care. 2007; 10: 679-687.

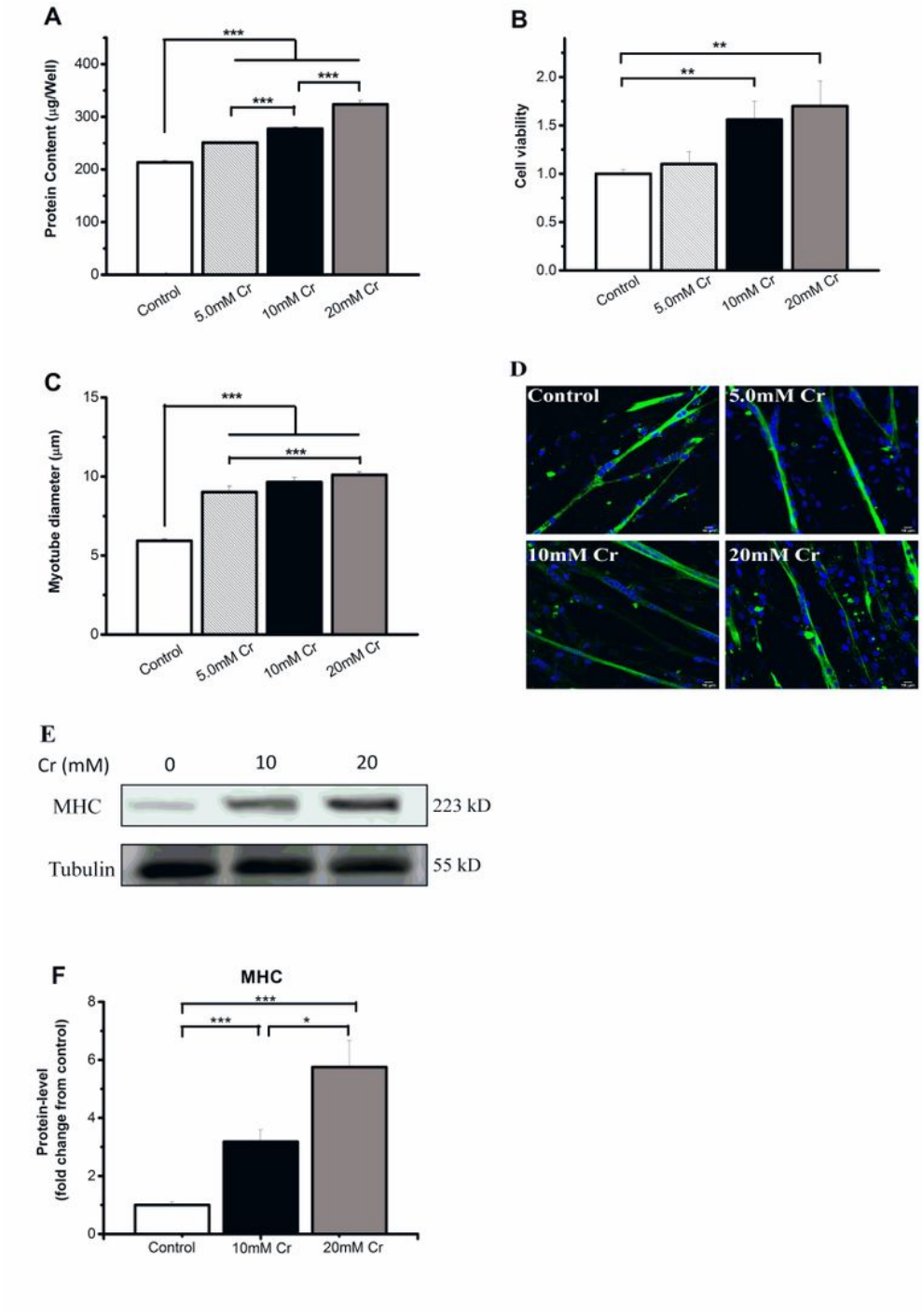
# Figures




## Figure 1

Effect of fasting condition on the development of myotubes. Protein content (A), cell viability (B), myotube diameter (C) and representative MHC staining images (D) were determined under fasting condition (no serum) for different time points (0, 6, 12, or 24 h). The mRNA expression level of protein ubiquitination degradation (E) and protein anabolism (F) related genes were performed by real-time PCR,

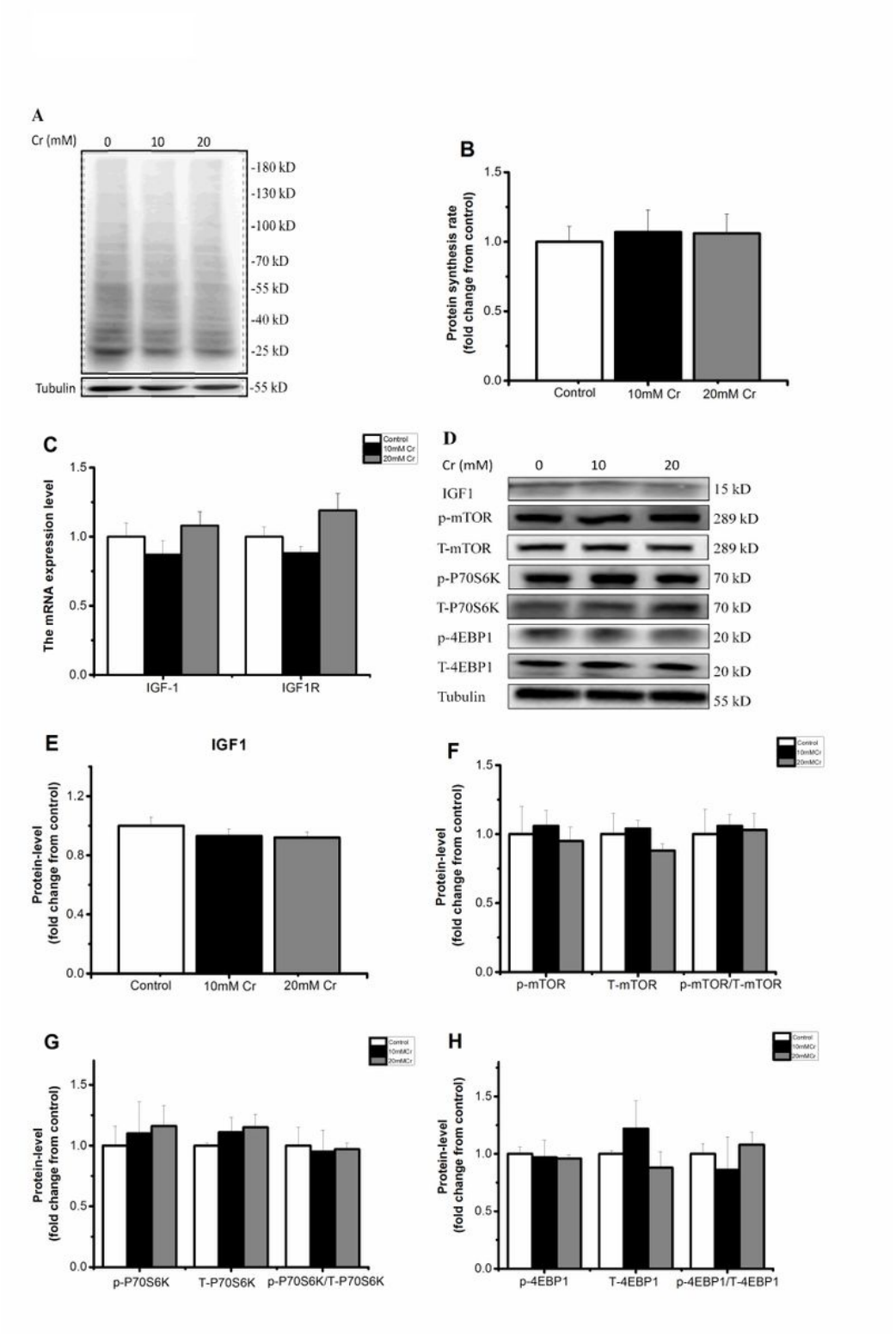
and each target transcript was related to that of the reference gene (GAPDH). Data are expressed as the means ± SD (n = 6). **P < 0.01 and ***P < 0.001 compared with other time points cells.




## Figure 2

Effect of Cr supplementation on the development of myotubes under fasting con-dition. The protein content (A), cell viability (B), myotube diameter (C), representa-tive MHC staining images (D), and the

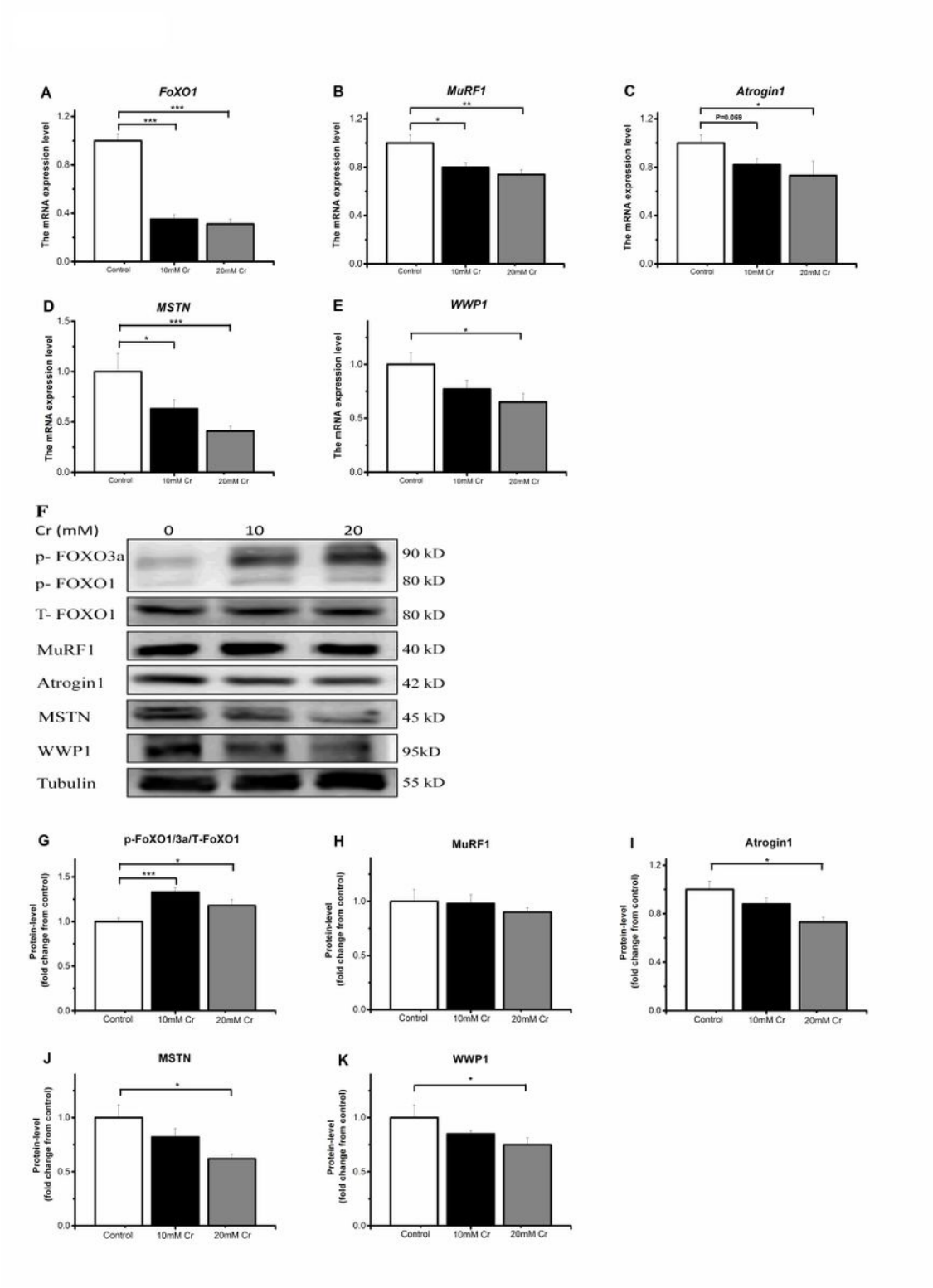
protein level of MHC (E, F). Data are present-ed as the means ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.




## Figure 3

Effect of Cr supplementation on protein synthesis rate and mTOR pathway of myotubes. The protein synthesis rate were evaluated after treatment with Cr and sup-plementation with puromycin (10 μM) for 30 min in the cell-free supernatant (A, B); The mRNA expression levels of IGF1 and IGF1R (C); the protein

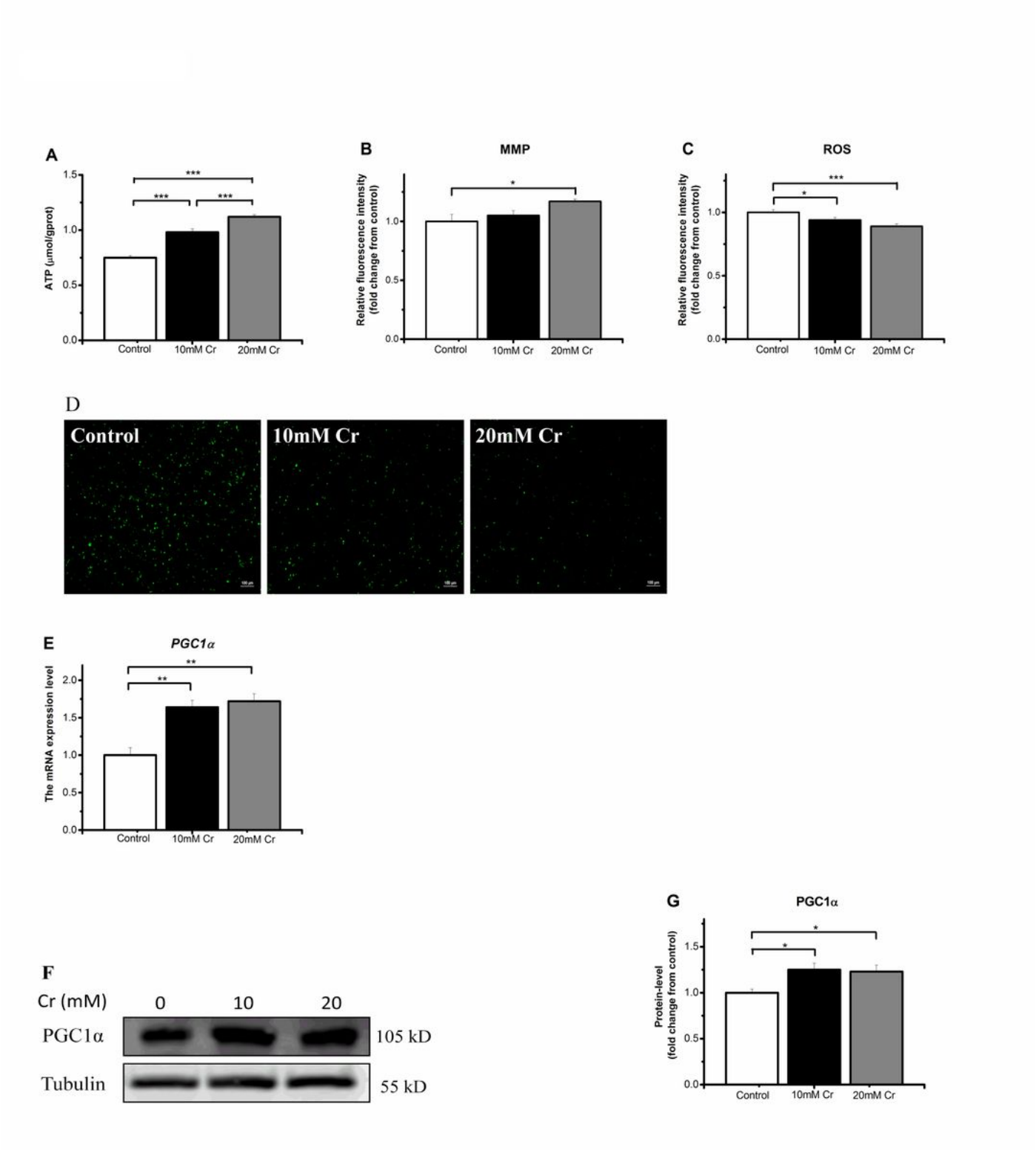
levels of IGF1 (D, E); mTOR (D, F); P70S6K (D, G); and 4EBP1 (D, H). Data are presented as the means ± SD (n = 6).




## Figure 4

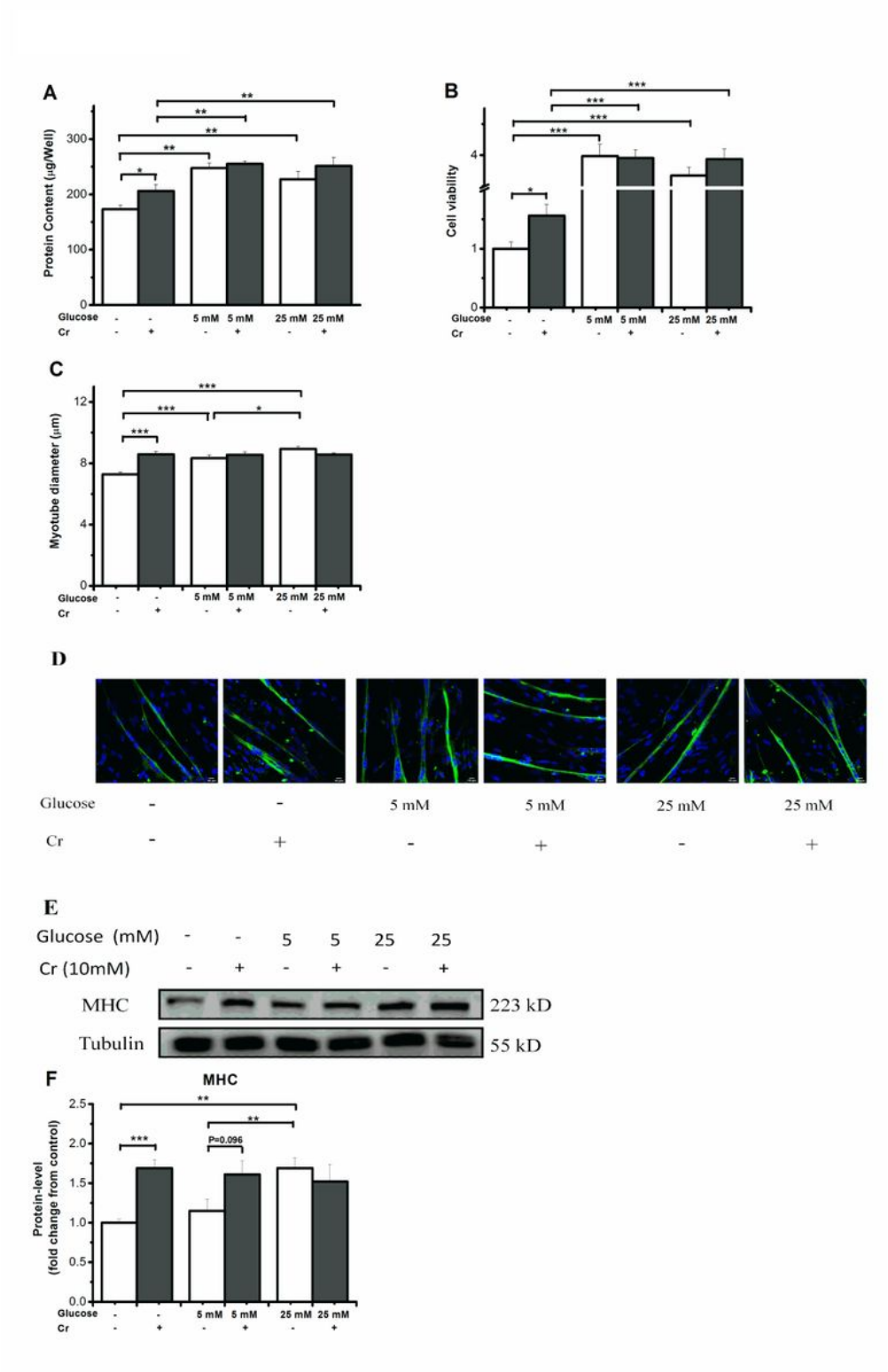
Effect of Cr supplementation on ubiquitin proteasome pathway of myotubes. The mRNA expression levels of FoXO1 (A), MuRF1 (B), Atrogin1 (C), MSTN (D), and WWP1 (E); the protein levels of p-

FoXO1/3a/T-FoXO1 (F, G), MuRF1 (F, H), Atrogin1 (F, I), MSTN (F, J), and WWP1 (F, K). Data are presented as the means ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.



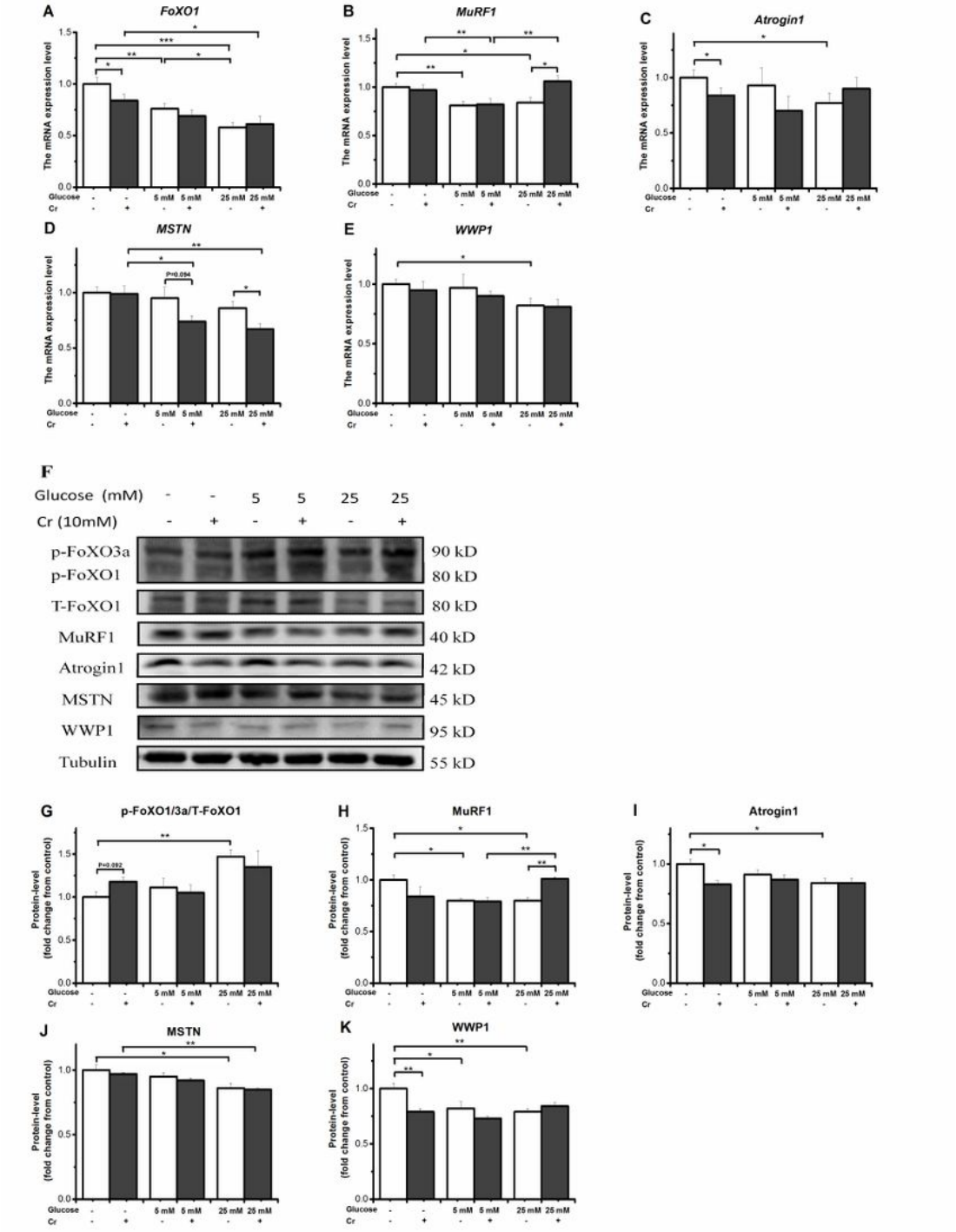

## Figure 5

Effect of Cr supplementation on mitochondrial quality of myotubes. ATP content (A), MMP (B), ROS level (C, D), the mRNA (E) and protein (F, G) expression level of PGC1α. Data are presented as the means ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.



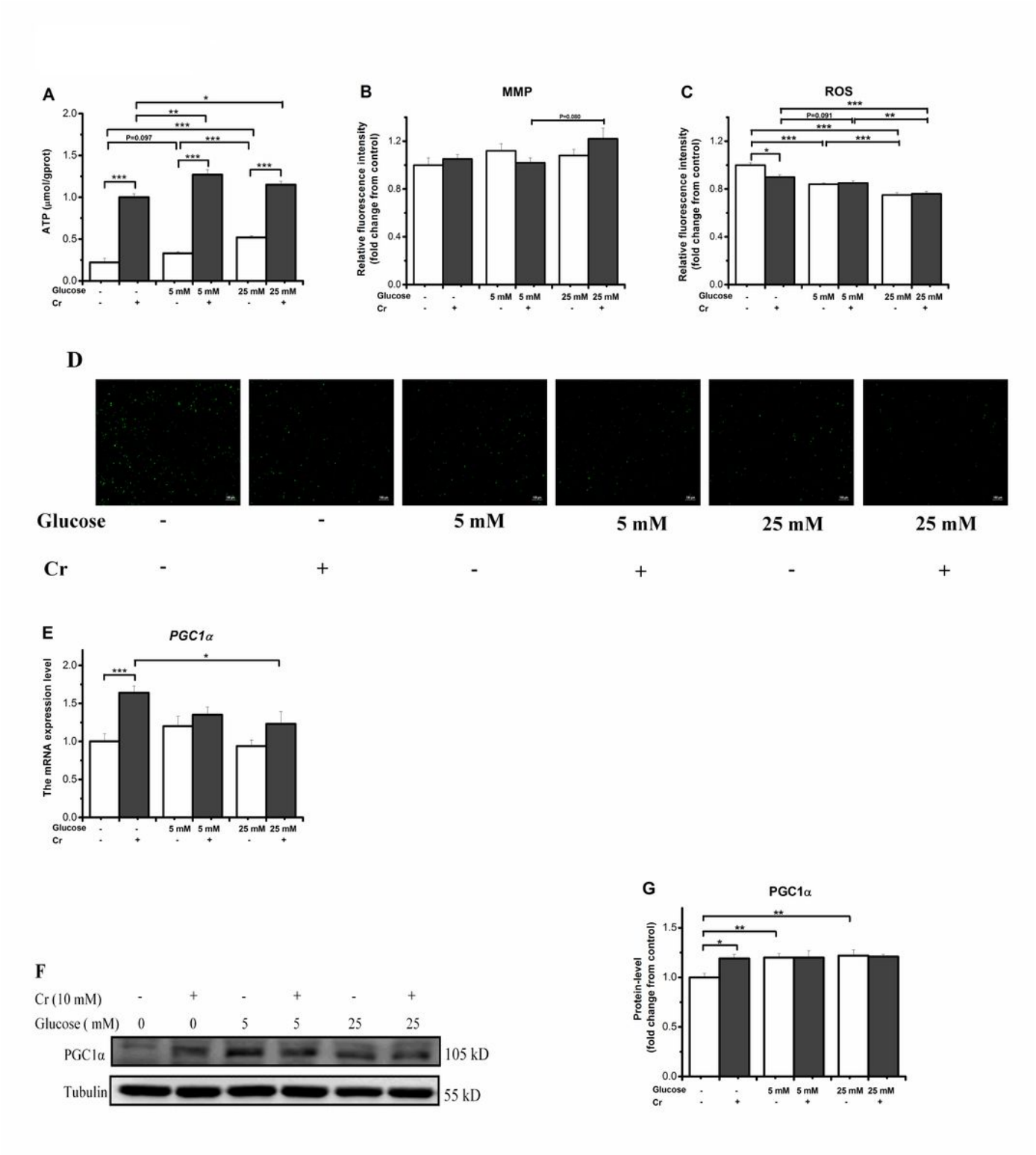

## Figure 6

Effect of Cr and glucose supplementation on the development of myotubes under fasting condition. The protein content (A), cell viability (B), myotube diameter (C), MHC staining images (D), and the protein level of MHC (E, F). Data are presented as the means ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001.




## Figure 7

Effect of Cr and glucose supplementation on ubiquitin proteasome pathway of myotubes. The mRNA levels of FoXO1 (A), MuRF1 (B), Atrogin1 (C), MSTN (D), and WWP1 (E); the protein levels of p-FoXO1/3a/T-FoXO1 (F, G), MuRF1 (F, H), Atrogin1 (F, I), MSTN (F, J) and WWP1 (F, K). Data are presented as the means ± SD (n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001.




## Figure 8

Effect of Cr and glucose supplementation on mitochondrial quality of myotubes. ATP content (A), MMP (B), ROS level (C, D), the mRNA (E) and protein (F, G) ex-pression level of PGC1α. Data are presented as the means ± SD (n = 6). *P < 0.05, **P < 0.01 and ***P < 0.001.

# Supplementary Files

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