Research Note: Creatine monohydrate alleviates protein breakdown induced by corticosterone via inhibiting ubiquitin proteasome pathway in chicken myotubes

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ABSTRACT Stress is a common problem diminishing the muscle development of broilers. Creatine (**Cr**), an energy buffer in skeletal muscle, plays a fundamental role in muscle physiology. This study aimed to evaluate the effect of Cr monohydrate **(CMH)** on protein breakdown in chicken myotubes challenged by corticosterone **(CORT)** in vitro. The morphology of myotube was measured and the activation of ubiquitin proteasome (**UP**) pathway was determined. The result showed that CORT treatment decreased myotube diameter ($P < 0.05$), increased 3-methyl-histidine **(3M-His)** content in medium, enhanced the mRNA expression levels of *muscle ring finger1* **(*MuRF1*)** and *Atrogin1* ($P < 0.001$), and Atrogin1 protein level ($P < 0.05$) compared with control. By contrast, CMH increased myotube diameter ($P < 0.05$) and myosin heavy chain (**MHC**) expression ($P < 0.001$), whereas decreased 3M-His and the mRNA and protein levels of Atrogin1 ($P < 0.05$), compared to control. In the present of CMH, the decreased myotube diameter and increased 3M-His, mRNA levels of *MuRF1* and *Atrogin1*, and Atrogin1 protein level by CORT were partially relieved ($P < 0.05$). Hence, the result suggests that CMH alleviates CORT-induced protein breakdown by suppressing Atrogin1 expression in chicken myotubes. The result highlights the potential application of CMH in regulating muscle protein catabolism in chickens under stress.

Key words: creatine monohydrate, corticosterone, ubiquitin proteasome pathway, chicken myotube, protein breakdown

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INTRODUCTION

Creatine (**Cr**), serving as fast energy buffer, plays an important role in the organs with high-energy demand such as skeletal muscle. The concentration of Cr in skeletal muscle depends on types of muscle fiber (Kushmerick et al., 1992). Cr supplementation have a number of beneficial effects such as increase muscle mass and performance, prevent disease-induced muscle atrophy, and reduce apoptosis (Wyss and Kaddurah-Daouk, 2000). In the model of Cr transporter (**CrT**) knockout mouse, phosphocreatine (**PCr**) deficiency in skeletal muscle associated with impaired motor function and muscle mass (Stockebrand et al., 2018).

Stress is a common problem to enhance the protein breakdown and suppress the development of skeletal muscle of broilers (Gao et al., 2008). Glucocorticoids (**GCs**), as the final effectors of the hypothalamic-pituitary-adrenal **(HPA)** axis, participate in the control of whole-body homeostasis and birds response to stress. Corticosterone (**CORT**) and dexamethasone, a synthetic glucocorticoid exhibiting a high affinity for glucocorticoid receptors, has been used to mimic in vivo and in vitro stress conditions in chickens (Menconi et al., 2008; Wang et al., 2016). The ubiquitin-proteasome (**UP**) pathway is the major protein degradation pathway, and 40 to 50% of protein are degraded via the pathway (Menconi et al., 2008). GCs stimulate E3 ubiquitin ligases muscle ring finger1 **(MuRF1)** and Atrogin1 expression, and leads to activation of protein degradation (Menconi et al., 2008; Wang et al., 2016). Dietary

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addition of Cr monohydrate (**CMH**) ameliorated transport-induced rapid muscle glycolysis and reduction of meat quality (Zhang et al., 2017). Recently, our result indicates that CMH mainly takes a favorable effect on protein accumulation via suppression of the UP pathway in a starvation state (Sun et al., 2022). Under stress condition, however, the regulating effect of Cr on muscle protein catabolism in myofibers of chicken remains unclear. Hence, we hypothesized that CMH supplementation is beneficial to protein accretion in CORT-challenged chicken myotubes.

The present study aimed to investigate if CMH supplementation could alleviate protein breakdown induced by CORT in chicken myotubes. The CORT-induced stress effect was evaluated with the protein content, myotube diameter, and myosin heavy chain (**MHC**) expression in myotubes. The myofibrillar protein breakdown induced by CORT was assessed by 3-methyl-histidine (**3M-His**) released in medium and the expression of genes related to UP pathway.

MATERIALS AND METHODS

All procedures in the study were approved by the Animal Care Committee of Shandong Agricultural University and were performed in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China).

Cell Culture

The specific-pathogen-free 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 previous studies (Sun et al., 2022). Briefly, at embryonic D 15, the *M. pectoralis major* muscle tissues 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 (1×10^6/mL), cultured in Dulbecco's modified Eagle's medium (**DMEM**, Solarbio, Beijing, China; 1.0 g/L glucose) with 16% fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Solarbio, Beijing, China), and maintained at 37°C in a humidified atmosphere with 5% CO_2. When cells proliferated (72 h) and differentiated (24 h) to form myotubes, cells were subjected to treatments.

Myotube Treatments

In the present study, the myotubes were subjected to the following treatments: 10 mM CMH (C425001, Aladdin, Shanghai, China), 0.2 μm CORT (C104537, Aladdin), the combined treatment of CMH and CORT. The myotubes were cultured in serum-free DMEM medium for 24 h. The concentration of CMH and treatment time were determined based on our previous work (Sun et al., 2022). After treatment, the myotubes were examined by morphometric or biochemical assays.

Myotube Diameter

The myotubes were grown and differentiated on glass coverslips. After supplementation with different treatments as mentioned above, the slips were observed and photographed under a microscope (Dragonfly, Andor, Belfast, UK) at 200× magnification, and analyzed with Image J software, which allows the location of measurements and the myotubes to be measured. The myotube diameter was determined at 3 points along the length of the myotube in a blinded fashion, and the average diameter per myotube was expressed as the mean of 3 measurements. At least 100 myotubes were measured per replicate, with 6 replicates from each of the treatment group.

Cr Content

The Cr content in medium was determined by the HPLC method. Cr (C0780, Sigma-Aldrich Inc, MI) was used as standard. Briefly, the DMEM medium (serum free) was collected after treatment with CMH, then the 1.0 mL medium was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, MA). Aliquots of 20 μL were injected using an auto sampler. Chromatographic separation was performed on a Waters Alliance HPLC system 2695 (Waters Corporation, Milford, MA) with a Waters SunFire C18 column (250 mm × 4.6 mm i.d., 5 μm). UV detection was accomplished at a wavelength of 210 nm. Methanol and H_2O (1:9; v/v) were used as the mobile phase at a flow rate of 1.0 mL/min.

Protein Content

Protein concentration was determined by 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 by using protein concentration and volume of lysis buffer (0.1 mL), and expressed as the μg protein per well.

3M-His Content

The DMEM medium (serum free) was collected, then the 3M-His content in medium was measured by the HPLC method after derivatization of fluorescein with a treatment of perchloric acid and heating. 3M-His (N137204, Aladdin) was used as standard. Aliquots of 20 μL were injected using an autosampler. Chromatographic separation was performed on a Waters Alliance HPLC system 2695 (Waters Corporation) with a Waters SunFire C18 column (250 mm × 4.6 mm i.d., 5 μm). The fluorescence signals detection was

accomplished with an excitation wavelength of 365 nm and an absorption wavelength of 460 nm. 10mM/L sodium phosphate buffer and acetonitrile (7:3; v/v) were used as the mobile phase at a flow rate of 1.0 mL/min.

RNA Extraction and RT-PCR Analyses

Total RNA was extracted from the cell using TRIzol (Invitrogen, Carlsbad, California, USA). The RNA concentration was measured by spectrophotometry (Eppendorf, Hamburg, Germany), and RNA purity verified by calculating the ratio between the absorbance values at 260 and 280 nm (A260/280 $\approx$ 1.75−2.01). Then 1μg RNA was reverse-transcribed to cDNA using Prime ScriptTM RT Master Mix (DRR019A, Takara, Dalian, China) according to the manufacturer's protocol. Real-time PCR was performed using ABI Quant Studio 5 PCR machine (Applied Biosystems, Thermo, Waltham, MA, USA). 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, Basel, Germany). The primers were designed with Primer 6.0 software. The primers were as follows: CrT: CTGGAAGGGCGTCAAGTCG and reverse, CTTGGACCAGTCAGGCTTCAG; FoXO1: forward, TCTGGTCAGGAGGGAAATGG and reverse, GCTTGCAGGCCACTTTGAG; MuRF1: forward, GCCAAGCAGCTCATTAAAACG and reverse, CATGTTCTCATAGCCTTGCTCA-AT; Atrogin1: forward, AGGCCGCAGTGTGTTGTTCT and reverse, GTGTGAATGGCTGGTTGCAT; MSTN: forward, GCTTTTGATGAGACTGGACGAG and reverse, AGCGGGTAGCGACAACATC; GAPDH: forward, ACATGGCATCCAAGGAGTGAG and reverse, GGGGAGACAGAAGGGAACAG-A. 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.

Protein Preparation and Western Blotting

The cells were washed three times briefly with PBS and collected in 0.1 mL of radio immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) containing 1% protease and phosphatase inhibitors (NCM Biotech, Suzhou, China). The operation was kept on ice to prevent protein degradation. The homogenate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. Afterward, the protein concentration of supernatants was assayed using a BCA protein assay kit (Beyotime) according to manufacturer's instructions. An equal amount of proteins (20 μg) was separated by 8% SDS polyacrylamide gels (Bio-Rad, Richmond, CA) and the proteins were transferred onto polyvinylidene fluoride microporous membrane (Millipore) 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) for 1 h at room temperature. The membranes were incubated with specific primary antibodies at 4°C with gentle shaking overnight. The primary antibodies in the present study have been used in poultry studies (Sun et al., 2022). The following primary antibodies were used: anti-MHC (ab51263), anti-MuRF1 (ab183094), anti-Atrogin1 (ab168372), anti-MSTN (ab98337), anti-Tubulin (ab6046) (Abcam, Cambridge, MA). 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/mouse, Beyotime, Shanghai, China) for 4 h at 4°C. After washing, membranes were then visualized by exposure to Hyperfilm ECL (Beyotime). Protein molecular weight markers (NCM Biotech, Suzhou, China) were used to calculate the molecular weights of the proteins in each sample. Western blots were developed and quantified using BioSpectrum 810 with Vision Works 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). Prior to analysis, all data were examined for the homogeneity and normal distribution plots of variances among the treatments by using UNIVARIATE procedure. Statistical analysis was performed using SAS statistical software (SAS version 8.1, Cary, NC). A two-way ANOVA model was conducted to estimate the main effects of CMH and CORT treatments, as well as their interaction effects. Differences between the means were evaluated using Tukey's honestly significant difference test. Differences were considered as significant at $P < 0.05$.

RESULTS AND DISCUSSION

CMH Alleviates Protein Hydrolysis Induced by CORT

Compared to control, CMH treatment increased myotube diameter ($P < 0.001$, Figures 1A and 1B), CrT mRNA expression ($P < 0.01$, Figure 1C), protein content ($P < 0.01$, Figure 1D), MHC protein expression ($P < 0.001$, Figures 1F and 1G), and declined 3M-His content in medium ($P < 0.05$, Figure 1E). Cr is usually used for increasing muscle mass and performance (Menezes et al., 2007). The present study was in line with our previous work (Sun et al., 2022). CMH stimulates protein synthesis with the involvement of mTOR/P70S6K pathway at normal state, whereas takes a favorable effect on protein accumulation via suppression of UP pathway mainly by serving as an energy supplier under starvation condition (Sun et al., 2022).

In previous study, CMH has been proved to be beneficial to the transport-induced rapid muscle glycolysis and reduction of meat quality (Zhang et al., 2017). Moreover, stress is a common problem to enhance the protein

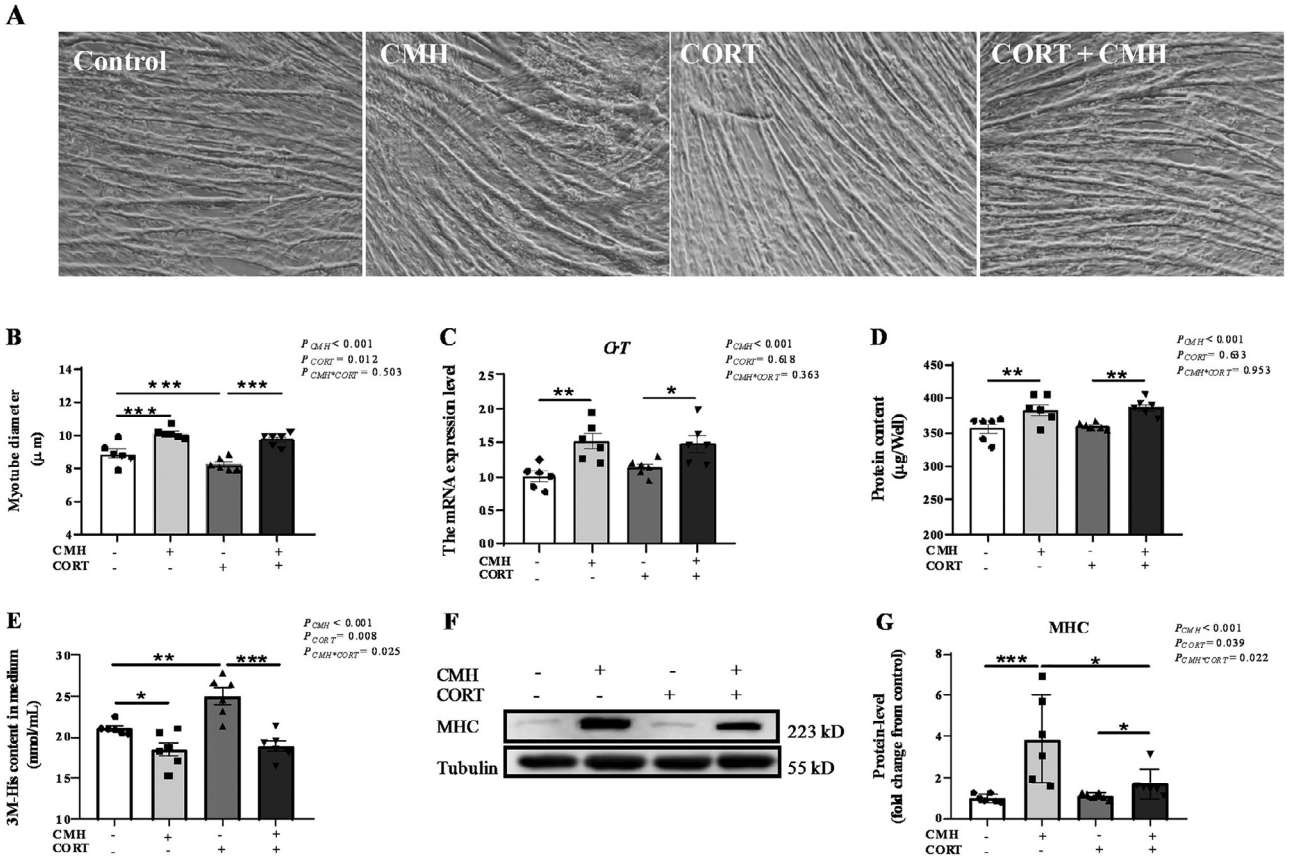

Figure 1. Effect of CMH supplementation on protein hydrolysis of myotubes under CORT condition. The representative chicken myotube images (A, 200×, Scale bar = 10 μm), myotube diameter (B), the mRNA expression of *CrT* (C), protein content (D), 3M-His content in medium (E) and the protein level of MHC (F, G) were evaluated. Data are presented as the means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Abbreviations: CMH, creatine monohydrate; CORT, corticosterone; MHC, myosin heavy chain; 3M-His, 3-methyl-histidine.

breakdown and suppress the development of skeletal muscle of broilers (Gao et al., 2008). Therefore, we used CORT to treat myotubes to establish an in vitro model of stress condition. The results showed that CORT supplementation significantly declined myotube diameter ($P < 0.05$), MHC protein expression ($P < 0.05$), and increased the myofibrillar protein degradation marker 3M-His content in medium ($P < 0.01$), indicating that CORT induced protein breakdown. In the previous research, Cr has been shown to prevent disease-induced protein degradation, improve rehabilitation and afford mild antioxidant activity (Wyss and Kaddurah-Daouk, 2000). Compared to CORT treatment, CMH increased myotube diameter ($P < 0.001$), *CrT* mRNA expression ($P < 0.05$), protein content ($P < 0.01$), and MHC protein expression ($P < 0.05$), and decreased 3M-His content in medium ($P < 0.001$). In this research, after treatment with CMH for 24 h, the retention rate of Cr by myotubes was 70.14 ± 0.12% with or without the CORT supplementation. 3M-His is mainly located in the myosin and actin in myofibrillar protein and is not reuse for protein synthesis. Therefore, 3M-His release from myotubes into medium shows myofibrillar protein degradation. In the present study, there was an interaction of CMH and CORT treatments on 3M-His content in medium ($P < 0.05$) and MHC protein expression ($P < 0.05$). Hence, the result indicates that CMH alleviates protein breakdown caused by CORT in myotubes. Consistent with our results, Menezes et al. (2007) had reported that CMH supplementation attenuates CORT-induced muscle wasting and impairment of exercise performance in rats.

CMH Alleviates Protein Hydrolysis Induced by CORT by Inhibiting UP Pathway

The UP pathway is the major protein degradation pathway in cells and plays a major role in signal transduction associated with stress. Glucocorticoids were important mediators of muscle proteolysis and were found to upregulate UP pathway dependent protein degradation in skeletal muscle (Menconi et al., 2008). Therefore, we next examined whether UP pathway is involved in the regulating effect of CMH on CORT induced muscle protein hydrolysis. CORT treatment upregulated the mRNA expression levels of *MuRF1* ($P < 0.001$, Figure 2B) and *Atrogin1* ($P < 0.001$, Figure 2C), and increased the Atrogin1 protein level ($P < 0.01$, Figures 2E and 2G) compared with control. In contrast, CORT had no detectable influence on MuRF1 and MSTN protein levels ($P > 0.05$, Figures 2E, 2F, 2H). This result

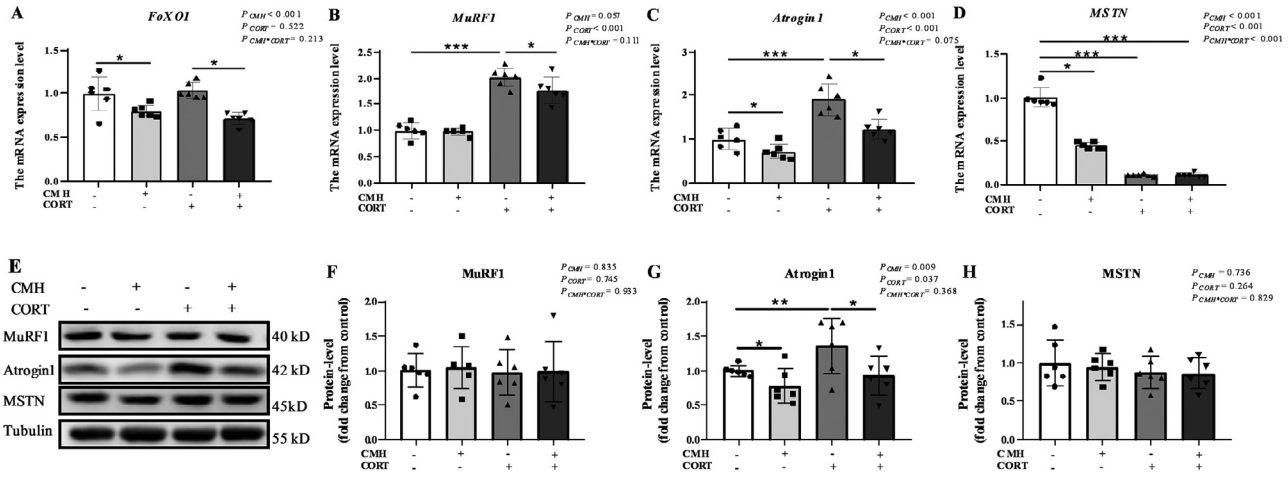

Figure 2. Effect of CMH supplementation on UP pathway related genes expression of myotubes under CORT condition. The mRNA expression of *FoXO1* (A), *MuRF1* (B), *Atrogin1* (C), and *MSTN* (D) were determined and the protein expression of MuRF1 (E, F), Atrogin1 (E, G), and MSTN (E, H) were also evaluated. Data are presented as the means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Abbreviations: CMH, creatine monohydrate; CORT, corticosterone.

was in accordance the study in C2C12 myotubes by Menconi et al. (2008), who reported that DEX (0.05 or 1 μM) or CORT (0.1 or 1 μM) increase *Atrogin1* mRNA levels but has no influence on *MuRF1* expression.

In the present of CMH, the increased mRNA expression levels of *MuRF1* ($P < 0.05$) and *Atrogin1* ($P < 0.05$), and increased Atrogin1 protein expression ($P < 0.05$) by CORT were significantly partially restored, compared with CORT treated cells. There was interaction of CMH and CORT treatments on *MSTN* expression ($P < 0.001$, Figure 2D) and the suppression of CMH on *MSTN* expression was dismissed in the presence of CORT. CMH significantly decreased the mRNA expression levels of *FoXO1* ($P < 0.05$, Figure 2A), *Atrogin1* ($P < 0.05$), and *MSTN* ($P < 0.05$), and declined Atrogin1 protein expression ($P < 0.05$), compared to control cells, indicating that CMH treatment suppresses proteolysis by inhibiting UP pathway related genes expression. CMH supplementation reverse MSTN induced decrease in myotube diameter by inhibiting Atrogin1 expression, however, there was no effect on MuRF1 expression in C2C12 cells (Mobley et al., 2014).

In conclusion, the present result suggests that CMH alleviates protein breakdown and enhances protein accretion under CORT challenged condition in chicken myotubes, mainly due to downregulation of these genes expression involved in the UP pathway.

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DISCLOSURES

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

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