Original Research Article

Muscle-protective effect of carnosine against dexamethasone-induced muscle atrophy in C2C12 myotube

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Running Head: Carnosine mitigates dexamethasone-induced muscle atrophy.

Summary

This study investigated the protective effect of carnosine and its components (L-histidine and β-alanine [HA]) against dexamethasone (Dex)-induced muscle atrophy in C2C12 myotubes. Myotubes were treated with Dex (10 μM) to induce muscle atrophy manifested by decreased myotube diameter, low myosin heavy chain content, and increased expression of muscle atrophy–associated ubiquitin ligases (atrogin-1, MuRF-1, and Cbl-b). Carnosine (20 mM) treatment significantly improved the myotube diameter and MyHC protein expression level in Dex-treated C2C12 myotubes. It also downregulated the expression of atrogin-1, MuRF-1, and Cbl-b and suppressed the expression of Forkhead box O (FoxO3a) mediated by Dex. Furthermore, reactive oxygen species production was increased by Dex but was ameliorated by carnosine treatment. However, HA (20 mM), the component of carnosine, treatment was found ineffective in preventing Dex-induced protein damage. Therefore, based on above results it can be suggested that carnosine could be a potential therapeutic agent to prevent Dex-induced muscle atrophy compared to its components HA.

Keywords: carnosine, dexamethasone, muscle atrophy, ubiquitin ligase, C_2C_{12} myotube

Abbreviations:

Cbl-b, casitas B-lineage lymphoma proto-oncogene-b; Dex, dexamethasone; DMEM, Dulbecco's Modified Eagle's Medium; FoxO, forkhead box O; IGF-1, insulin-like growth factor-1; MAFbx1, muscle atrophy F-box protein 1; MuRF-1, muscle ring finger protein 1; MyHC, myosin heavy chain; H2-DCFDA, 2′,7′-dicholorofluorescein diacetate; ROS, reactive oxygen species; SOD1, superoxide dismutase-1 HA, L-histidine and β-alanine; Akt, Protien kinase B; P-Akt, phosphoryalted protien kinase B.

Introduction

Skeletal muscle is an important organ in the body and is associated with maintaining posture, movement, metabolism, respiration, temperature, and more (1). Generally, the equilibrium between muscle protein synthesis and muscle proteolysis regulates skeletal muscle mass. However, numerous physiopathological conditions, such as disuse, ageing, systematic diseases, malnutrition, and prolonged glucocorticoid treatment, disturb this equilibrium, leading to decreased skeletal muscle mass and functions, that is, muscle atrophy (2). Various agents, such as androgens, synthetic drugs, and natural compounds, have been trialled as possible treatments; however, considering their numerous side effects and lower specificity, more effective agents are needed against muscle atrophy (3). Therefore, finding compounds with potent anti-atrophic potential is crucial.

Glucocorticoids are synthetic drugs used to treat inflammation-mediated diseases; however, extended use or a higher dose of this drug causes several side effects, including osteoporosis, hyperglycemia, and muscle atrophy (4). Dexamethasone (Dex), a potent long-acting synthetic glucocorticoid, favors muscle protein degradation by activating the ubiquitin–proteasome system and suppressing the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (P13K/Akt/mTOR) pathway, causing impaired protein synthesis (5). In brief, after binding to glucocorticoid receptor, Dex increases muscle-specific ubiquitin E3 ligases such as muscle atrophy F-box (MAFbx/atrogin-1) and muscle atrophy ring finger-1 (MuRF1) via forehead box O3 (FoxO3a) (6). Foxo3a is also regulated via protein kinase B/Akt . The phosphorylation of Akt inactivates FoxO3a activity and vice versa (7, 8). Furthermore, Dex impairs mitochondrial activity and induces oxidative stress in the skeletal muscle. Oxidative stress manifested by increased ROS production and reduced antioxidant defense triggers MAFbx/atrogin-1 and MuRF-1 expression by enhancing the expression of Casitas B-lineage lymphoma-b (Cbl-b) (9). Increased Cbl-b levels cause the degradation of insulin receptor

substrate-1 (IRS-1), thereby negatively regulating the insulin-like growth factor-1 (IGF-1) pathway (10). Subsequently, disturbed IGF-1 signaling enhances the expression of atrogin-1 and MuRF-1 via FoxO3a, resulting in muscle mass damage (10).

Carnosine is a dipeptide comprised of the amino acids L-histidine and β-alanine (HA), mainly found in meats like beef, chicken pork and other sources like prawn, tuna, salmon, trout etc. [11-13]. Carnosine is naturally produced from L-histidine and β-alanine in the human body, whereas only β-alanine is synthesized in the human liver [14]. Carnosine is distributed in several tissues, such as the heart, brain, and muscles. The skeletal muscle reportedly contains the highest concentration of carnosine, which serves as a physiological buffer, an enzyme regulator, a sarcoplasmic reticulum calcium regulator, and an antioxidant [15, 16]. Carnosine taken from dietary sources or supplements to the body shows cardioprotective, anti-inflammatory, anti-obesity, antidiabetic, and anti-aging effects [17, 18]. It also regulates the protein metabolism of skeletal muscles and ameliorate the loss of skeletal muscle proteins under hypobaric hypoxia [19]. However, the effect of carnosine and its metabolites (HA) on Dex-induced muscle atrophy remains uninvestigated. Therefore, this study aimed to investigate the effect of carnosine and HA on Dex-mediated muscle atrophy in C2C12 cells.

Materials and Methods

Reagents and chemicals

We obtained carnosine from Hamari Chemicals Ltd. (Osaka, Japan), HA from Fujifilm (Osaka, Japan), Dex from Sigma-Aldrich (St. Louis, MO), and Isogen™ from Nippon Gene (Tokyo, Japan). All other chemicals and reagents used were of analytical grade.

Cell culture

C2C12 cells originating from the American Type Culture Collection were obtained and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and 1%

98 penicillin/streptomycin solution (Nacalai Tesuque, Kyoto, Japan) at 37℃ in 5% CO_2
99 humidified air. Cells were incubated until reaching 90%–100% confluency. Then, DMEM
100 containing 2% horse serum (Thermo Fisher Scientific) was applied to differentiate the cells
101 into myotubes. At every 48 h intervals, media were changed. Fully differentiated myotubes
102 were used in this experiment. Myotubes were divided into four groups: a) control, treated with
103 a vehicle (Milli-Q water); b) Dex, treated with dexamethasone (10 μM); c) Dex + carnosine,
104 treated with Dex and carnosine (20 mM); and d) Dex + HA, treated with Dex and HA (20 mM).
105 Carnosine alone was also treated and perfomed the immustaining and western blotting to
106 anlayse the myoutube diameter and other biochemical changes. Carnosine and HA at 20 mM
107 dose were pretreated for 1 h separately, followed by treatment with 10 μM of Dex for 24 h to
108 induce muscle atrophy. Milli-Q water was used as a vehicle for both carnosine and Dex. The
109 dose of carnosine (20 mM) was selected based on our random study of two independent doses
110 of carnosine in which 20 mM was found to be more effective in suppressing the ubiquitin
111 ligases expression. Moreover, Ishibashi et al. recently showed that carnosine in the dose of 1
112 mM to 50 mM neither decrease the cell viability nor induce any toxicity in the C2C12 cells [20],
113 therefore using 20 mM of carnosine in C2C12 is assumed safe. Finally, cells were harvested,
114 processed, and subjected to quantitative real-time polymerase chain reaction (qRT-PCR) and
115 Western blot analysis.

116 **Myotube diameter measurement**

117 The diameter of C2C12 myotubes was analyzed by two ways. a) Myotubes were anlyze
118 capturing normal myotubes micrograph. Myotubes were grown on 6-well plates and capturing
119 the images of normal myotubes by using a phase-contrast microscope (BIOREVO, BZ-9000;
120 Keyence, Osaka, Japan) at 20 × magnification, as described by [21]. b) Myotubes were anlyze
121 capturing MyHC stained myotubes micrograph. For immunostainging of MyHC, myotubes
122 were grown on 48-well plates and immunostained with MF20 (Thermo Fisher Scientific) as a

primary antibody (1:1 dilution) and incubated for 18 hr at 4°C followed by anti mouse Alexa 568 (Thermo Fisher Scientific) (1:1000) treatment as a secoundary antibody for 1h at room temperature. The images per experimental group captured by using a phase-contrast microscope and randomly measured 100 myotubes from 10 micrographs. We chose the thickest part of each myotube for diameter measurement using the BZ-II analyzer software (Keyence).

qRT-PCR

We conducted qRT-PCR on the Step One Plus™ Real-Time PCR system (Applied Biosystem, CA, USA) using the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix™ (Agilent, Texas, USA). Briefly, we used Isogen™ (Nippon Gene, Tokyo, Japan) to isolate total RNA from the cells. Isolated RNA was then measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), and 1 μg of RNA was reverse-transcribed into cDNA. Atrogin-1, MuRF-1, Cbl-b, SOD1 and Catalase were measured by qRT-PCR. Table 1 shows the primers with their sequences used in this study. The internal standard used was 18S ribosomal RNA.

Western blot analysis

We used a lysis buffer containing NaCl (150 mM), ethylenediaminetetraacetic acid (5 mM), Tris-HCl (50 mM, pH 7.5), NaF (10 mM), Na_3VO_4 (2 mM), 1% Triton-X-100, a protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland), and MG-132 (10 μM). Protein concentrations in the cell homogenates were measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), making bovine serum albumin as the standard, in accordance with the manufacturer's protocol. Electrophoresis was conducted using 10 μg of protein per lane on an 8% sodium dodecyl sulfate polyacrylamide gel under 300 V for approximately 1 h, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked by Milli-Q water containing 4% block ACE™ powder (DS Pharma Biomedical Co. Ltd., Osaka, Japan) for 1 h. and secondary antibodies were incubated overnight at 4°C and 1 h at room temperature, respectively. A C-DiGit scanner (LI-COR Biosciences, Lincoln, NE) was used for

densitometry analysis of the blots. Antibodies such as total, fast and slow-type myosin heavy chain (MyHC), alpha-tubulin (Sigma), FoxO3a, Akt, Phosphorylated Akt (P-Akt) and rabbit IgG (Cell Signaling Technology, Danvers, MA), as well as phosphorylated FoxO3a (Invitrogen, Thermo Fisher Scientific) were used. Total, Fast and Slow type MyHC (1:10,000), alpha-tubulin (1:1000), FoxO3a (1:1000), P- FoxO3a (1:1000), total Akt (1:1000), P- Akt (1:1000), IgG mouse and IgG rabbit secondary antiobody (1:5000) has been used.

ROS measurement

To measure the ROS in myotubes, we grew myotubes on 96-well plates. Next, we treated them with Dex and carnosine or HA for scheduled time. Thereafter, cells were washed and incubated them with 2′,7′-dicholorofluorescein diacetate (H_2-DCFDA, 5 μM) reagent in Hank's balanced salt solution (HBSS) for 1 h at 37°C according to the manufacturer's protocol. The myotubes were then washed twice with HBSS. Fluorescence was determined using a microplate reader (Infinite M Nano™; Tecan, Mannedorf, Switzerland) at excitation and emission wavelengths of 495 and 527 nm, respectively. The results were recorded at 0, 3, 6, 12, and 24 h.

Statistical analysis

All the results are presented as mean ± SD. Results were analyzed by one–way analysis of variance, followed by the Tukey's post hoc test using the GraphPad Prism Software version 9.3.1 (GraphPad Software Inc., San Diego, CA, USA). A *P*-value less than 0.05 was considered statistically significant.

Results

Muscle-protective effect of carnosine on Dex-induced myotube atrophy

The diameter of Dex-treated myotubes was significantly reduced compared with that of the control myotubes both in normal and in MyHC-immunostained myotubes (Fig. 1A & 1B). Interestingly, Dex-induced myotube diameter reduction was effectively attenuated by carnosine (20 mM) treatment but not by HA treatment in both the experiments. Carnosin alone

treatemnt did not affect the myotube diamter and showed similar results as of control myotubes (Fig. 1B).

Effect of carnosine on MyHC protein expression

The expression of fast- type, slow-type MyHC and total MyHC proteins was analyzed by Western blotting to elucidate the effect of carnosine and HA on Dex-induced muscle protein degradation. Fast-type MyHC is mostly affected by glucocorticoids compared with slow-type MyHC [22]. Consistently in our study, Fast-type MyHC was significantly decreased in Dex-treated C2C12 myotubes compared to control group. Nonetheless, this reduction was effectively reversed by carnosine treatment (Fig. 2A). However, HA treatment was found ineffective in preventing Dex-induced protein damage (Fig. 2A). Carnosin treatment alone showed similar experssion of protien as of control group (Fig. 2A).

The expression of slow-type MyHC was only slightly changed by Dex treatment compared with that of control group. Carnosine administration tended to increase the slow type MyHC protein's level but not significant (Fig. 2B). The expression of slow-type MyHC in HA treated myotube was similar to that in Dex treated myotube (Fig. 2B). Carnsoine treatemnent alone did not show any noticeable change compared to control group (Fig. 2B).

The western blotting of the total MyHC protein showed that Dex significanty decreased the total MyHC compared to control, however treatment of Carnosine effectvely prevented Dex-induced reduction of total MyHC protein (Fig. 2C). Carnosine treatment alone had no significant changes compared to control group (Fig. 2C).

Muscle-protective effect of carnosine on the mRNA expression of ubiquitin ligases

Given that ubiquitin ligases mediate protein degradation by binding ubiquitin to the target protein [23], we investigated carnosine's effect on the expression of muscle atrophy–associated ubiquitin ligases (Atrogin-1, MuRF-1, and Cbl-b) in response to Dex by qRT-PCR. Dex administration significantly increased the mRNA expression of these ubiquitin ligases

198 compared with the control group. However, their increased expression was significantly
199 attenuated by carnosine treatment (Fig. 3A–C). Conversely, HA treatment failed to suppress it
200 (Fig. 3A–C).

201 **Muscle-protective effect of carnosine on the protein expression of transcription factors**

202 Considering that FoxO3a is the upstream regulator of ubiquitin ligases atrogin-1 and MuRF-1
203 [24], the expression of both the total and phosphorylated FoxO3a (P-FoxO3a) was investigated
204 (Fig. 4A). Dex treatment significantly increased the total FoxO3a expression but decreased the
205 phosphorylated FoxO3a expression, consistent with other studies in C2C12 myotubes [6].
206 However, the expression of total FoxO3a was significantly suppressed in the carnosine-treated
207 group compared with that in the Dex-treated group. The Dex-mediated phosphorylation of
208 FoxO3a in C2C12 myotubes was also reversed by carnosine treatment (Fig. 4A). Unlike
209 carnosine, HA showed a similar expression of P-FoxO3a as Dex treated group (Fig. 4A).
210 Carnosine alone treatment showed similar expression of total FoxO3a and P-FoxO3a compared
211 to control myotubes (Fig. 4A).
212 To elaborate the mechanism of FoxO3a activation, western blotting of Akt and P-Akt was
213 performed. The hypophosphorylation of Akt activates FoxO3a, while its hyperphosphorylation
214 inactivates FoxO3a leading to redcued ubiquitin ligaese expression (8). We found that Dex
215 treatment significantly suppressed P-Akt compared to control, while carnosine treatment along
216 with Dex increased the P-Akt level compared to Dex group (Fig. 4B). Carnosine treatment
217 alone had similar effect as like control group (Fig. 4B).

218 **Muscle-protective effect of carnosine on oxidative stress**

219 Given that ROS plays an important role in predisposing muscular atrophy, we investigated the
220 effect of carnosine against Dex-mediated ROS accumulation. The ROS level in C2C12
221 myotubes was measured at 0, 3, 6, 12, and 24 h of Dex treatment with 1 h pretreatment of
222 carnosine or HA. At 3 and 6 h, Dex-treated myotubes showed significantly higher ROS levels

compared to the control group, but the carnosine-treated group concomitantly scavenged the accumulated Dex-induced ROS (Fig. 5A). HA treatment could not inhibit such accumulation (Fig. 5A). The expression of antioxidants such as superoxide dismutase-1 (SOD1) and catalase were measured as oxidative stress markers. Both antioxidants decreased significantly compared with those in the control group (Fig. 5A & 5B). The carnosine-treated group showed significantly increased levels of these antioxidants compared with the Dex-treated group (Fig. 5B–C).

Discussion

Multifarious pharmacological effects of carnosine have been reported in various disease models *in vitro* and *in vivo*, but its effect on Dex-induced muscle atrophy is scarce. This study has found that carnosine efficiently attenuates muscle atrophy induced by Dex by suppressing muscle protein degrading molecules, especially ubiquitin ligases Atrogin-1, MuRF-1 and Cbl-b. It is also effective in suppressing ROS generated by Dex. Additionally, the dipeptide (Carnosine) is more effective than its components L-histidine and β-alanine in attenuating muscle atrophy. Various peptides and dipeptides exert beneficial effects, including the alleviation of muscle atrophy and improvement of muscle health [25-27]. Carnosine is an endogenous dipeptide chiefly found in the skeletal muscle and is associated with the regulation of muscle contractile functions and fatigue resistance [28, 29]. Conversely, decreased carnosine level affects weight maintenance and protein metabolism in the skeletal muscle, indicating that carnosine level is associated with skeletal muscle protein metabolism [30]. Various pathophysiological conditions, including disuse, obesity, diabetes, cancer, chronic kidney disease, and aging, can decrease carnosine expression [31].

Carnosine has been reported to be a very safe dipeptide in dietary and supplemental forms. There is no significant report of side effects documented except paresthesia, which is a benign sensation of numbness and tingling when circulating β-alanin concentration exceeds 100 μM

[32]. Recently Ishida et al have shown that carnosine up to the dose of 50 mM neither induces any toxicity nor decreases the viability of C2C12 cells[20]. Moreover, an *in-vivo* experiment in rats reported that intravenous injection of carnosine up to 2000 mg/ Kg body weight exhibited no toxicity[33]. Furthermore, a human clinical study to assess the health benefit of carnosine in the dose of 500 mg daily for 6 months was found to improve physical performance and enhance quality of life with no adverse effects [34]. Overall, carnosine is a safe bioactive food component that has various health benefits outweighing the side effects.

Carnosine is a bioavailable dietary peptide that reaches the blood in an intact form *via* absorption through the gastrointestinal tract; however, its oral bioavailability is limited due to its poor basolateral transport [34]. It is absorbed in the human small intestine on the apical side by a specific peptide transporter PEPT1 or PEPT2 [35]. In enterocytes, carnosine is hydrolyzed by Carnosinase-2 (CN2) [32]. Carnosine crosses the basolateral membrane of enterocytes to the blood by a proton-coupled transporter. In the blood, carnosine is hydrolysed by CN1 and the half-life of carnosine in the human serum is under 5 min [36]. A human cohort study examined the bioavailability of carnosine after the consumption of 200 g of beef that contains 268 mg of carnosine. The blood was collected at a regular time gap from the subjects. Carnosine was detected in the blood as early as 15 minutes after ingestion. Carnosine concentrations in serum peaked at 3.5 hours post-ingestion and went below detection levels in the blood after 5.5 hours[37].

Dex administration causes muscle atrophy through different pathways (5). Notable among them is the ubiquitin–proteasome system in which proteins are ubiquitinated and degraded with the upregulation of various ubiquitin ligases, including atrogin-1, MuRF-1, and Cbl-b. FoxO3a, a transcription factor, regulates the transcriptional activity of muscle-specific ubiquitin ligases [5, 38]. Dex causes FoxO3a hypophosphorylation, allowing it to enter the nucleus and promote the upregulation of atrogin-1 and MuRF-1; meanwhile, FoxO3a hyperphosphorylation prevents its

entry into the nucleus and the upregulation of such ubiquitin ligases [39, 40]. Moreover, Akt plays vital role in regulating FoxO3a expression. Activation of Akt causes phosphorylation of Foxo3a and vice-versa (7). In our study, Dex increased total FoxO3a levels and caused its hypophosphorylation, but carnosine effectively inhibited FoxO3a activity, as manifested by a decrease in total FoxO3a level and increased phosphorylation state. Additionally, Carnosine increased phosphorylaiton of Akt leading to hyperphosphorylation of FoxO3a, that prevents its entry into nucelus and subsequently suppressed ubiquitin ligaese expression. Therefore, we suggest that the protective effect of carnosine in decreasing atrogin-1 and MuRF-1 expression was mediated via downregulation of FoxO3a, which was due to Akt activation by carnosine.

Previously, we reported that Dex-induced atrophy is associated with ROS accumulation, which was mediated by the glucocorticoid receptor [21]. ROS induces catabolism by promoting the ubiquitination of muscle proteins through the increased expression of ubiquitin ligases [41]. Additionally, ROS stimulates the expression of the oxidative stress–sensitive ubiquitin ligase Cbl-b (9), which degrades the IRS-1 by its ubiquitination and interrupts IGF-1 signaling (10). By interrupting IGF-1 signaling, FoxOs and FoxO-mediated ubiquitin ligases are activated. Carnosine may be a potent antioxidant agent [42]. In our study, carnosine significantly reduced ROS accumulation induced by Dex in C2C12 myotubes. Consequently, Dex-mediated Cbl-b expression was significantly suppressed. SOD1 and catalase are two important endogenous antioxidant enzymes that contribute greatly against the harmful effects of ROS. SOD dismutases superoxide radicals to hydrogen peroxide, which is then broken into water and molecular oxygen by catalase [43]. Carnosine treatment has been shown to boost the levels of endogenous antioxidants in C2C12 cells.

Fast and slow types of MyHC are important proteins in the skeletal muscle. Fast-type MyHC is more affected by Dex than the slow-type [22]. In the present study, Dex administration significantly decreased total and fast-type MyHC levels without any significant changes in

slow-type MyHC expression. Interestingly, carnosine effectively suppressed total and fast-type MyHC reduction, possibly because of the suppression of muscle proteolytic ubiquitin ligases. Therefore, carnosine treatment could diminish the effects of Dex-induced muscle atrophy.

Conclusions

Carnosine can attenuate muscle atrophy in C2C12 cells by suppressing FoxO3a and its downstream molecules activating protein kinase-B/Akt. Moreover, as a potent antioxidant, it could suppress ROS accumulation induced by Dex. ROS inhibition downregulated Cbl-b expression and Cbl-b-mediated muscle wasting. Thus, these results pave the way for further extensive research of carnosine for its ascribed therapeutic properties in vivo.

Author contribution

T.N: Conceptualization, Writing review & editing, Supervision, Project administration & Funding acquisition; MMR: Conceptualization, Methodology, Investigation, Data curation, Writing original draft; AU: Methodology, Data curation, Writing original draft; T.U: Methodology, Validation and Formal analysis; H.M: Validation and Formal analysis, Writing review & editing; Y.Y: Validation and Formal analysis. All authors have read and agreed to the published version of the manuscript.

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Dr. Hiroki Moriwaki and Mr. Yusuke Yasukawa work at Hamari Chemcals, Ltd and Hamari Nutritional Sciences, Ltd, respectively. All others authors have no conflict of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Figure Captions

Fig. 1. Muscle-protective effect of carnosine on myotube diameter in C2C12 cells. (A) The diameter of myotubes was analyzed by using a phase-contrast microscope. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 20 mM carnosine or HA as a pretreatment for 1 h. (B) The diameter of MyHC-stained myotubes was analyzed by immunostainging technique and then capture the immage using a phase-contrast microscope. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 20 mM carnosine or HA as a pretreatment for 1 h followed by immunstating as discussed in materials and mehtod section. Carnosine alone was also treated to the mytoubes undergone immunostaining. The results are expressed as means ± SD (n = 100 per group). Scale, 100 μm. ****$P < 0.0001$. Dex, dexamethasone; CR, carnosine; and HA, L-histidine and β-alanine.

Fig. 2. Muscle-protective effect of carnosine on the protein expression of (A) Fast-type MyHC, (B) Slow-type MyHC and (C) Total MyHC. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 20 mM carnosine or HA as a pretreatment for 1 h. Carnsoine alone was also treated and western blot was perfomed compared to control myotubes and shown in right side of corresponding figures (2A, 2B & 2C). The results are expressed as means ± SD (n = 3 per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. MMSTD, molecular mass standard; Dex, dexamethasone; CR, carnosine; and HA, L-histidine and β-alanine.

Fig. 3. Muscle-protective effect of carnosine on the mRNA expression of ubiquitin ligases (A) MAFbx1/atrogin-1 (B) MuRF-1 and (C) Cbl-b. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 20 mM carnosine or HA as a pretreatment for 1 h. The results are presented as means ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Dex, dexamethasone; CR, carnosine; and HA, L-histidine and β-alanine.

Fig. 4. Muscle-protective effect of carnosine on the protein expression of (A) Total Foxo3a and P-Foxo3a (B) Total Akt and P-Akt. Myotubes were treated with 10 μM dexamethasone for 24 h with or without 20 mM carnosine or HA as a pretreatment for 1 h. Carnsoine alone was also treated and western blot was perfomed compared to control myotubes and shown in right side of corresponding figures (4A & 4B). The results are expressed as means ± SD ($n = 3$ per group).). *$P < 0.05$ **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. MMSTD, molecular mass standard; Dex, dexamethasone; CR, carnosine; and HA, L-histidine and β-alanine.

Fig. 5. Muscle-protective effect of carnosine on oxidative stress in C2C12 cells. ROS production was measured over 24 h. (A) Dex-induced ROS production. Myotubes were pretreated with or without 20 mM of carnosine or HA for 1 h, followed by Dex treatment at different time period (0, 3, 6, 12, and 24 h). The results are presented as means ± SD ($n = 3$). *$P < 0.05$ Dex compared to control group, #$P < 0.05$ Dex compared to Dex + CR group. The mRNA expression of (B) SOD1 and (C) catalase. Myotubes were pretreated with or without

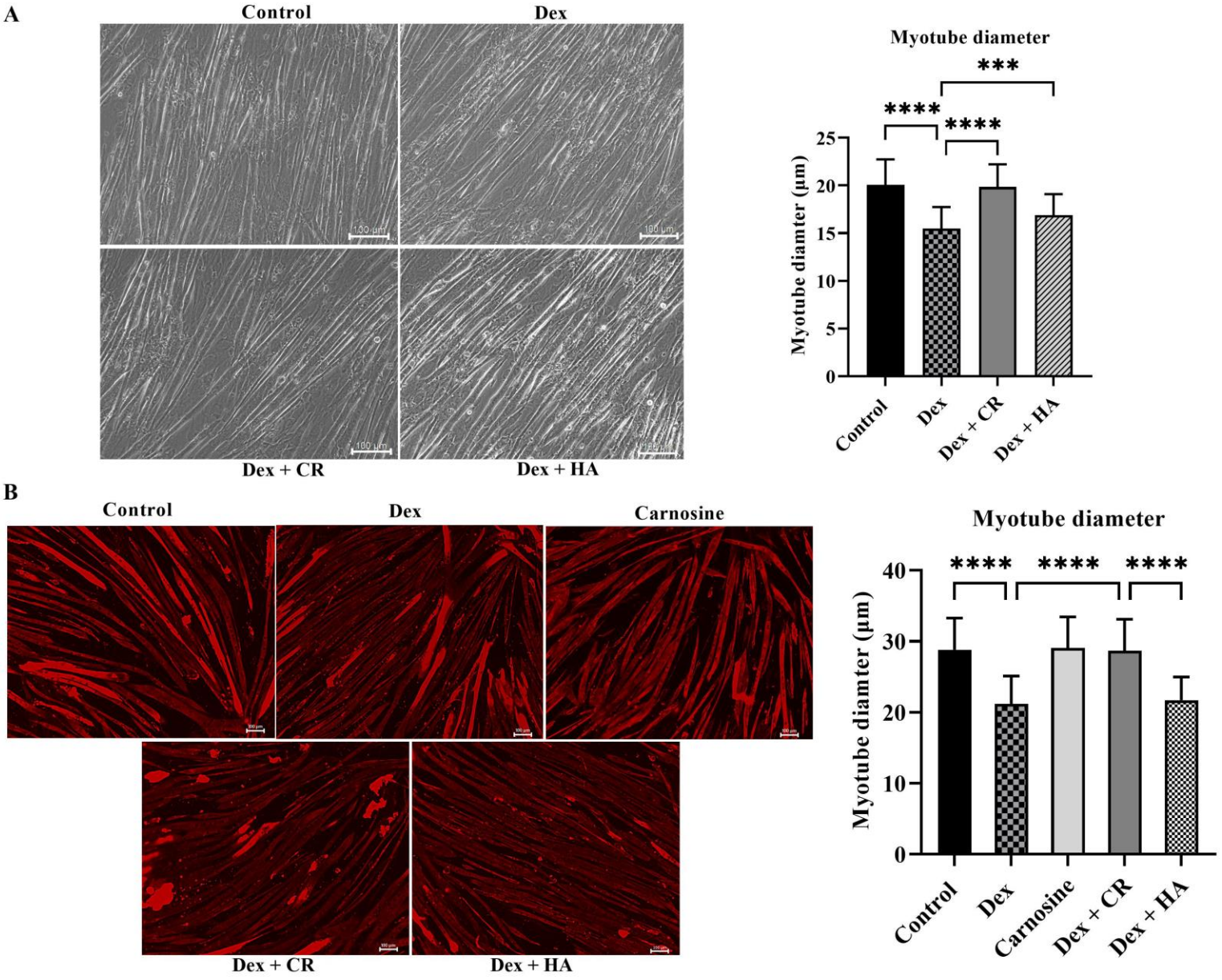
20 mM of Carnosine or HA for 1 h followed by Dex tratment for 24 h. The results are presented as means ± SD (n = 3). *P < 0.05, **P < 0.01

Table 1. Primers used for polymerase chain reaction.

Target gene		Sequence	Length (bp)
MAFbx1/atrogin-1	S	GGCGGACGGCTGGAA	101
	AS	CAGATTCTCCTTACTGTATACCTCCTTGT	
MuRF-1	S	TGTCTGGAGGTCGTTTCCG	183
	AS	CTCGTCTTCGTGTTCCTTGC	
Cbl-b	S	GAGCCTCGCAGGACTATGAC	222
	AS	CTGGCCACTTCCACGTTATT	
SOD1	S	ACCAGTGCAGGACCTCATTTTAA	78
	AS	TCTCCAACATGCCTCTCTTCATC	
Catalase	S	ATGGCTTTTGACCCAAGCAA	69
	AS	CGGCCCTGAAGCTTTTTGT	
18Sr	S	CATTCGAACGTCTGCCCTA	119
	AS	CCTGCTGCCTTCCTTGGA	

18Sr, 18S ribosomal RNA; Cbl-b, Casitas B-lineage lymphoma proto-oncogene-b; MAFbx1, muscle atrophy F-box protein 1; MuRF-1, muscle ring finger protein-1; SOD1, superoxide dismutase 1.

Fig. 1


A
Control
Dex
Dex + CR
Dex + HA
Myotube diameter
***
****
****
Myotube diamter (µm)
25
20
15
10
5
0
Control
Dex
Dex + CR
Dex + HA
B
Control
Dex
Carnosine
Dex + CR
Dex + HA
Myotube diameter
****
****
****
Myotube diamter (µm)
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30
20
10
0
Control
Dex
Carnosine
Dex + CR
Dex + HA

Fig. 2

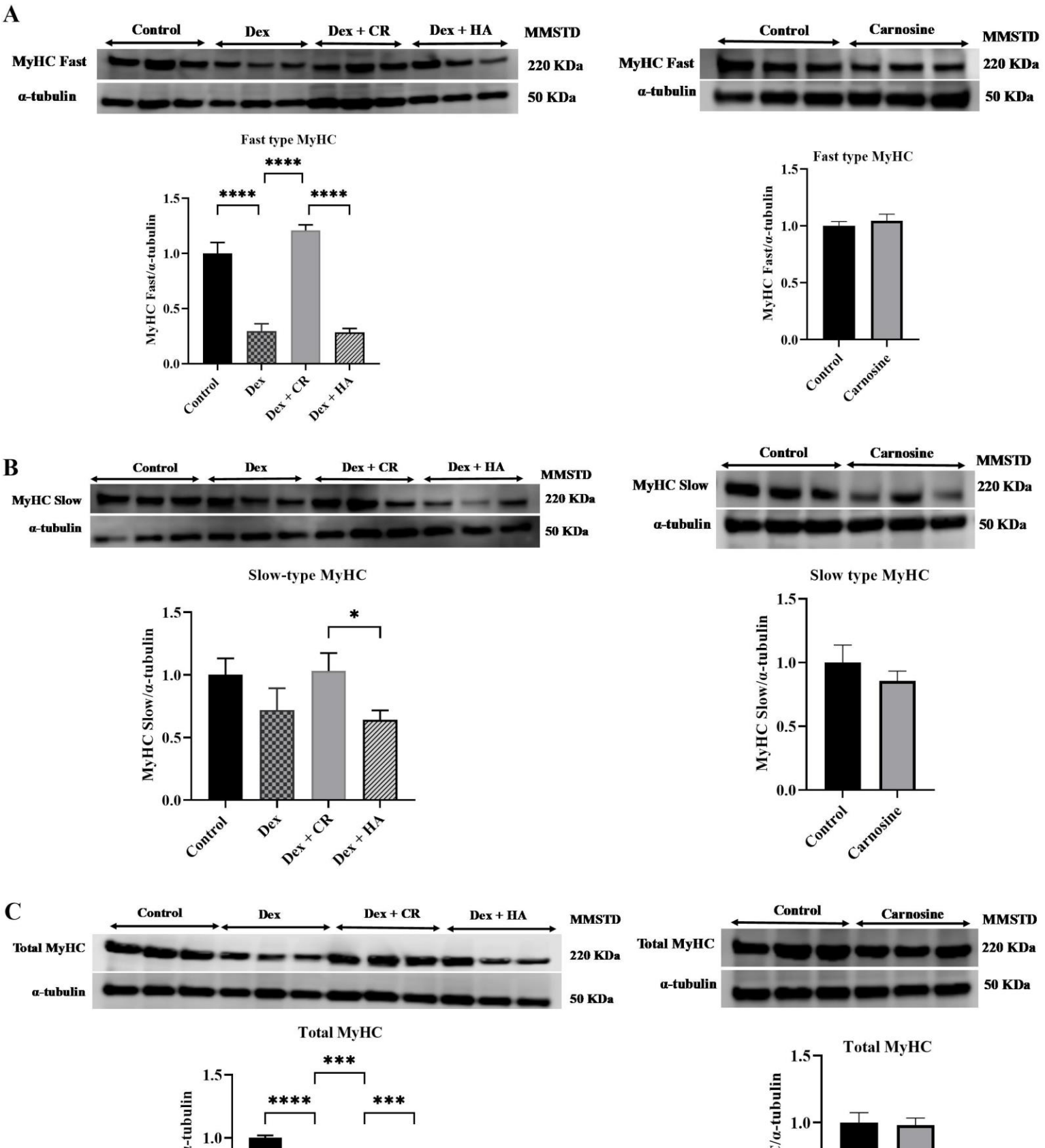

A
Control
Dex
Dex + CR
Dex + HA
MMSTD
MyHC Fast
220 KDa
α-tubulin
50 KDa
Fast type MyHC
MyHC Fast/α-tubulin
****
****
****
1.5
1.0
0.5
0.0
Control
Dex
Dex + CR
Dex + HA
Control
Carnosine
MMSTD
MyHC Fast
220 KDa
α-tubulin
50 KDa
Fast type MyHC
MyHC Fast/α-tubulin
1.5
1.0
0.5
0.0
Control
Carnosine
B
Control
Dex
Dex + CR
Dex + HA
MMSTD
MyHC Slow
220 KDa
α-tubulin
50 KDa
Slow-type MyHC
MyHC Slow/α-tubulin
*
1.5
1.0
0.5
0.0
Control
Dex
Dex + CR
Dex + HA
Control
Carnosine
MMSTD
MyHC Slow
220 KDa
α-tubulin
50 KDa
Slow type MyHC
MyHC Slow/α-tubulin
1.5
1.0
0.5
0.0
Control
Carnosine
C
Control
Dex
Dex + CR
Dex + HA
MMSTD
Total MyHC
220 KDa
α-tubulin
50 KDa
Total MyHC
***
****
***
1.5
1.0
Control
Carnosine
MMSTD
Total MyHC
220 KDa
α-tubulin
50 KDa
Total MyHC
1.5
1.0

Fig. 3

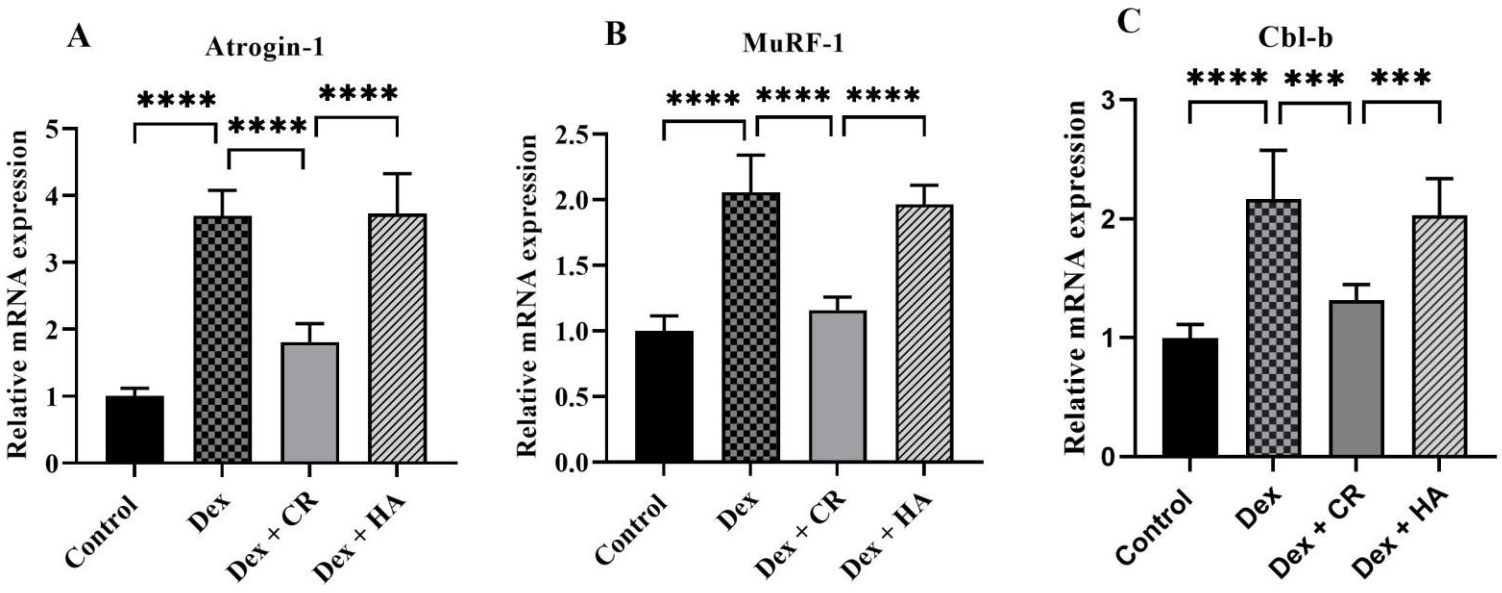

A
Atrogin-1
Relative mRNA expression
0
1
2
3
4
5
****
****
****
Control
Dex
Dex + CR
Dex + HA
B
MuRF-1
Relative mRNA expression
0.0
0.5
1.0
1.5
2.0
2.5
**** **** ****
Control
Dex
Dex + CR
Dex + HA
C
Cbl-b
Relative mRNA expression
0
1
2
3
**** *** ***
Control
Dex
Dex + CR
Dex + HA

Fig. 4

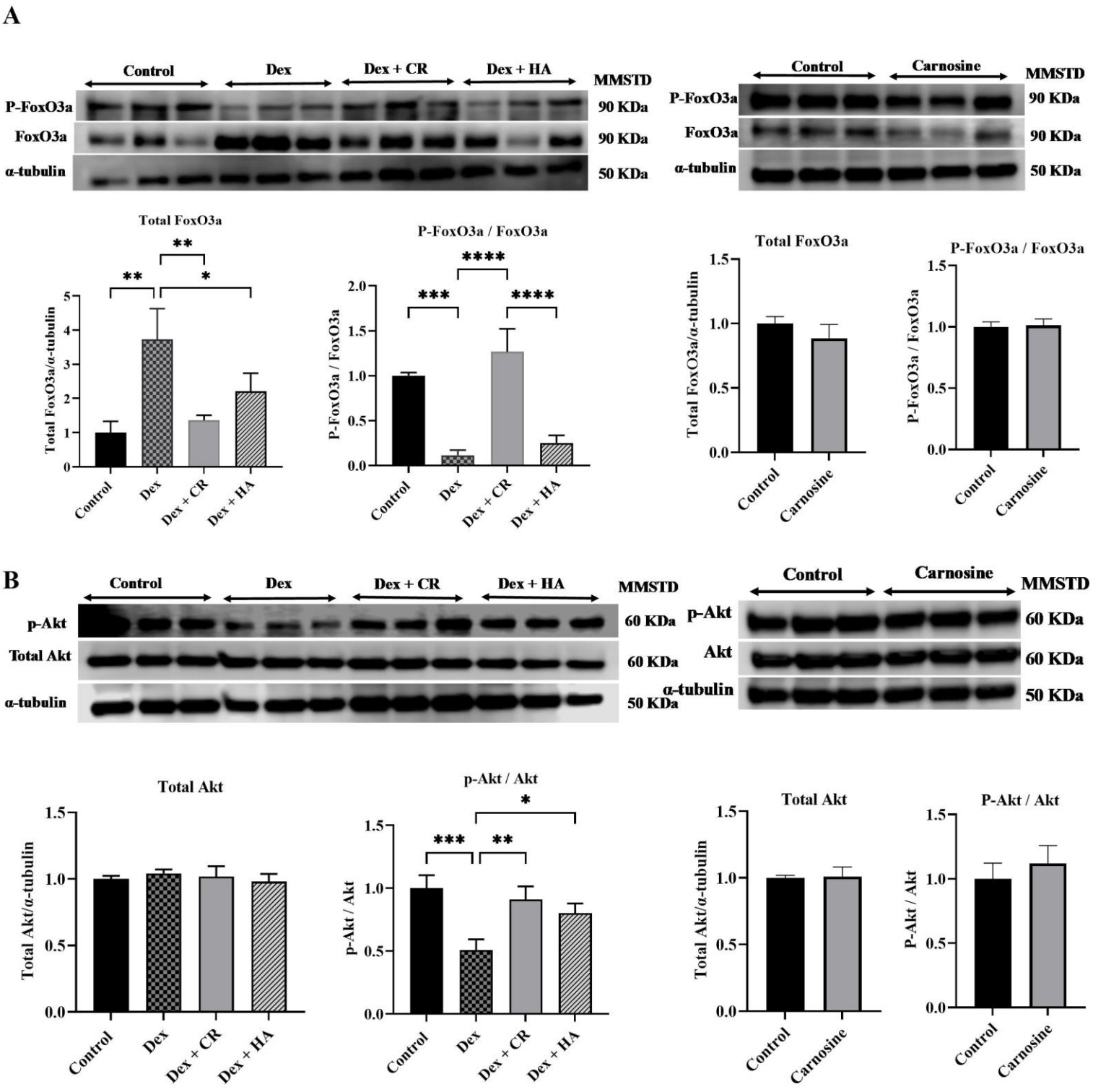
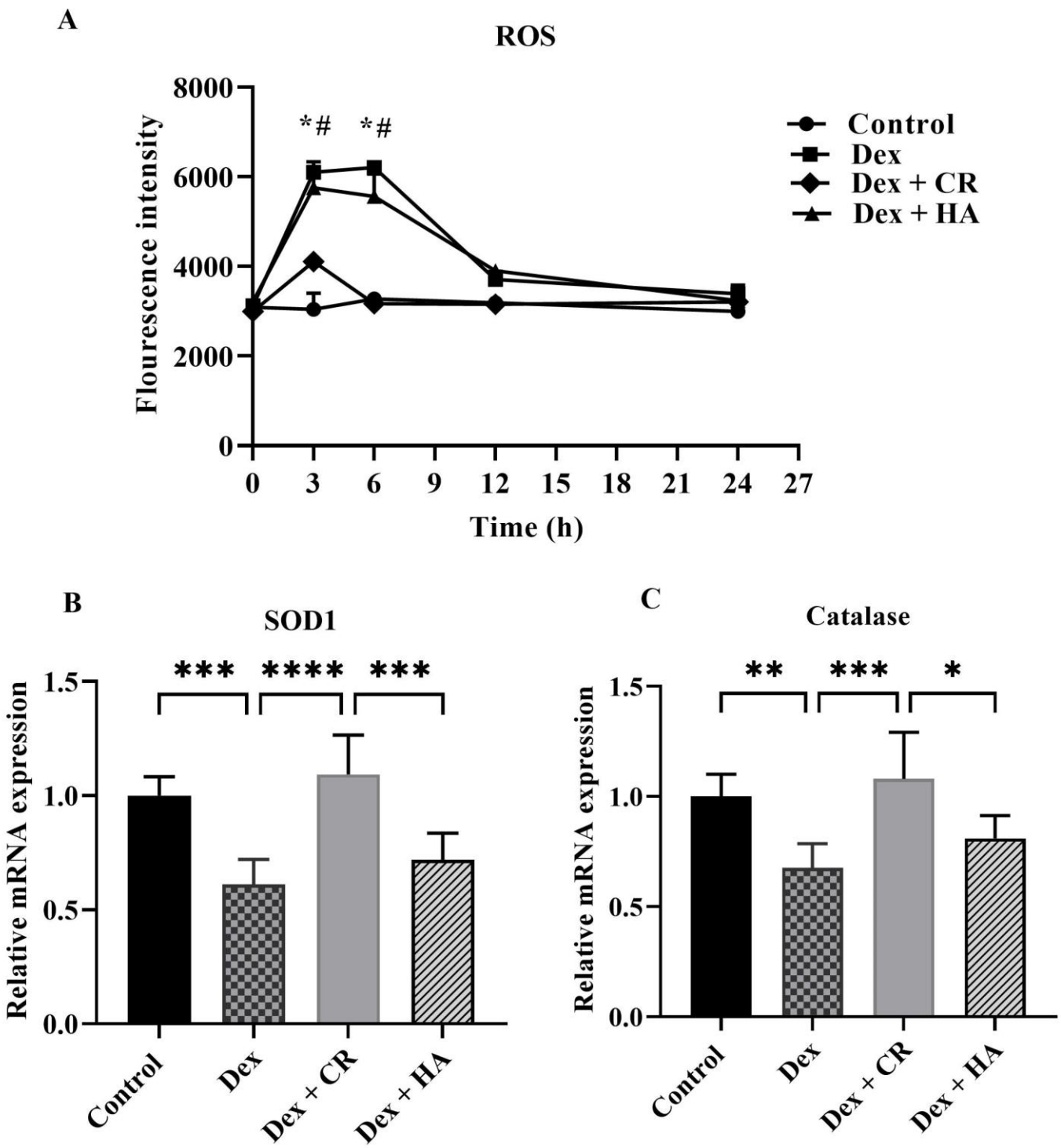

A
Control
Dex
Dex + CR
Dex + HA
MMSTD
P-FoxO3a
FoxO3a
α-tubulin
90 KDa
90 KDa
50 KDa
Control
Carnosine
MMSTD
P-FoxO3a
FoxO3a
α-tubulin
90 KDa
90 KDa
50 KDa
Total FoxO3a
Total FoxO3a/α-tubulin
P-FoxO3a / FoxO3a
P-FoxO3a / FoxO3a
Total FoxO3a
Total FoxO3a/α-tubulin
P-FoxO3a / FoxO3a
P-FoxO3a / FoxO3a
Control
Dex
Dex + CR
Dex + HA
Control
Carnosine
B
Control
Dex
Dex + CR
Dex + HA
MMSTD
p-Akt
Total Akt
α-tubulin
60 KDa
60 KDa
50 KDa
Control
Carnosine
MMSTD
p-Akt
Akt
α-tubulin
60 KDa
60 KDa
50 KDa
Total Akt
Total Akt/α-tubulin
p-Akt / Akt
p-Akt / Akt
Total Akt
Total Akt/α-tubulin
P-Akt / Akt
P-Akt / Akt
Control
Dex
Dex + CR
Dex + HA
Control
Carnosine

Fig. 5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ROS
Flourescence intensity
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6000
4000
2000
0
*#
*#
0
3
6
9
12
15
18
21
24
27
Time (h)
Control
Dex
Dex + CR
Dex + HA
B
SOD1
Relative mRNA expression
1.5
1.0
0.5
0.0
***
****
***
Control
Dex
Dex + CR
Dex + HA
C
Catalase
Relative mRNA expression
1.5
1.0
0.5
0.0
**
***
*
Control
Dex
Dex + CR
Dex + HA