

BMB Reports – Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-21-132

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Article Type: Article

Keywords: cisplatin; mitochondrial function; autophagy; skeletal muscle; cancer

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Running Title: Cisplatin affects mitochondria and autophagy.

Keywords: cisplatin, mitochondrial function, autophagy, skeletal muscle, cancer

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ABSTRACT

Cisplatin is widely known as an anti-cancer drug. However, the effects of cisplatin on mitochondrial function and autophagy-related proteins in the skeletal muscle are unclear. The purpose of this study was to investigate the effect of different doses of cisplatin on mitochondrial function and autophagy-related protein levels in the skeletal muscle of rats. Eight-week-old male Wistar rats (n = 24) were assigned to one of three groups; the first group was administered a saline placebo (CON, n = 10), and the second and third groups were given 0.1 mg/kg body weight (BW) (n = 6), and 0.5 mg/kg BW (n = 8) of cisplatin, respectively. The group that had been administered 0.5 mg cisplatin exhibited a reduced BW, skeletal muscle tissue weight, and mitochondrial function and upregulated levels of autophagy-related proteins, including LC3II, Beclin 1, and BNIP3. Moreover, this group had a high LC3 II/I ratio in the skeletal muscle; i.e., the administration of a high dose of cisplatin decreased the muscle mass and mitochondrial function and increased the levels of autophagy-related proteins. These results, thus, suggest that reducing mitochondrial dysfunction and autophagy pathways may be important for preventing skeletal muscle atrophy following cisplatin administration.

INTRODUCTION

Cisplatin—cis-diamine-dichloroplatinum II—is an effective anti-cancer drug (1, 2). The basic mode of treatment is cisplatin-induced DNA modification, activating damage recognition and repair of cancer DNA, cell-cycle arrest, and cell apoptosis (3). Although cisplatin treatment is highly effective, many patients suffer from severe side effects, especially muscle atrophy (4)(5).

An animal model of cancer cachexia revealed that intraperitoneal injection of 1–3 mg/kg body weight (BW) (hereafter, mg/kg BW) of cisplatin for several days caused weight loss and muscle atrophy (4, 6-10). Clinical trials have also indicated that skeletal muscle mass significantly decreased during chemotherapy in patients with advanced gastric cancer (11). Many previous studies have clarified that cisplatin-induced skeletal muscle atrophy or dysfunction is mainly caused by alterations in autophagy (12) and mitochondrial dysfunction (4, 12-15). It is widely accepted that the specific mechanism that helps maintain the muscle mass is supported by the IGF-1/PI3K/AKT/mTOR pathway (16). However, there is still a lack of studies that elucidate the effect of different concentrations of cisplatin administered on mitochondrial function, autophagy, and muscle mass in the skeletal muscles of animals.

Considering the significant mitochondrial dysfunction caused by cisplatin administration, it is clear that retaining mitochondrial function and quality control are critical for maintaining the muscle mass (17). A previous study showed that cisplatin toxicity affected oxidative phosphorylation, as measured by the respiratory control ratio (RCR), and decreased the respiratory capacity of cisplatin-administered cells (15). Mitochondrial dysfunction in skeletal muscle fibers of cisplatin-treated animals affected mitochondrial fusion and fission proteins, such as dynamin-related protein 1 (Drp 1), and caused muscle atrophy (18). The results of this study

indicated that cisplatin administration decreased mitochondrial biogenesis and skeletal muscle mass. Many other *in-vitro* studies have shown the effects of cisplatin treatment on levels of proteins related to mitochondrial biogenesis and indicated that mitochondrial dysfunction in cisplatin administration is associated with cisplatin toxicity (13) and muscle atrophy (19). Therefore, it is necessary to directly analyze mitochondrial function in skeletal muscles following cisplatin administration.

In addition to mitochondrial function, autophagy is a major metabolic regulator that protects against the side effects of chemotherapy involving cisplatin-administration (20). Autophagy in skeletal muscles is associated with muscle fiber regeneration (21) and modulates various molecular signaling pathways, as a chemosensitizer, in cisplatin-administered cancer cells (22). In a cisplatin-induced cachexia model, cisplatin-administration in fast-twitch skeletal muscle (e.g., gastrocnemius) increased cell-autophagy markers (LC3 II, p62, and Beclin 1) by down-regulating AKT and FOXO3a (6); LC3 II specially affects the autophagosome residual protein and mitochondrial function (23-25). However, most studies that analyzed autophagy in cancer cells were performed *in vitro*. Although cisplatin is associated with a severe side effect, i.e., muscle atrophy, very few studies have investigated the effects of varying doses of cisplatin on skeletal muscles.

The purpose of this study was to investigate the effects of different doses of cisplatin, including 0.1 mg/kg BW of cisplatin, 0.5 mg/kg BW of cisplatin, and a control (CON; saline), on mitochondrial function and autophagy-related protein levels in skeletal muscles. Based on the study results, we conclude that high doses of cisplatin (0.5 mg/ kg BW) decreased mitochondrial function and downregulated the levels of key modular autophagy-related LC3 II proteins (compared to CON and 0.1 mg/kg BW cisplatin).

RESULTS

Effect of cisplatin reduces BW and gastrocnemius muscle tissue weight

The **BW** varied significantly between the groups ($F(1.64, 14.73) = 63.58, p < 0.001$) and based on time ($F(1.86, 16.74) = 38.09, p < 0.001$). The variation **in** BW with time was significantly different among the groups ($F(1.46, 9.46) = 183.7, p < 0.001$). From the baseline up to 14 days, the BW changes differed for the three groups; after 14 days of cisplatin administration, the group administered 0.5 mg/kg BW showed a significant reduction in BW (Figure 1a). The **tissue weight (g) of the gastrocnemius (GAS) skeletal muscle** was also significantly different among the three groups ($F = 43.98, p < 0.001$). The BW of the 0.5 mg group ($n = 9, 2.20 \pm 0.13$) was significantly lower than that of the CON ($n = 8, 3.42 \pm 0.08, F = 11.91, p < 0.001$) and 0.1 mg groups ($n = 5, 3.40 \pm 0.07, F = 10.21, p < 0.001$) (Fig. 1C). **Moreover, the** normalization of GAS muscle tissue **based on** BW did not **reveal** a significant difference ($F = 0.17, p = 0.84$) between the CON ($n = 6, 1.04 \pm 0.02$), 0.1 mg ($n = 4, 1.04 \pm 0.02$), and 0.5 mg ($n = 6, 1.03 \pm 0.01$) groups (Fig. 1D).

Effect of cisplatin on mitochondrial function in skeletal muscle

As shown in Figure 2A-D, the levels of all mitochondrial functions namely glutamate (GM) (Fig. 2A, $F = 3.80, p < 0.05$), adenosine di-phosphate (ADP) (Fig. 2B, $F = 18.99, p < 0.001$), **and** succinate (SUCC) (Fig. 2C, $F = 9.93, p < 0.01$), and **the** respiratory control ratio RCR (Fig. 2D, $F = 7.08, p < 0.01$) **significantly differed** among the CON, 0.1 mg, and 0.5 mg groups. **The mitochondria** in the GAS skeletal muscle showed significantly reduced ADP levels (Fig. 2B) in the 0.5 mg group ($n = 8, 4.42 \pm 1.05$) compared to **those in** the CON ($n = 8, 28.01 \pm 3.11, p < 0.001$) and 0.1 mg groups ($n = 5, 15.84 \pm 4.76, p < 0.05$). As shown in Figure 2A, the GM levels **were significantly decreased only** in the 0.5 mg group ($n = 7, 2.27 \pm 0.59, p < 0.05$) **than**

in the CON ($n = 8$, 5.95 ± 1.01) group. The SUCC levels (Fig. 2C) were also lower in the 0.5 mg group ($n = 8$, 11.06 ± 2.45 , $p < 0.001$) than in the CON group ($n = 8$, 44.82 ± 7.27). The RCR level (Fig. 2D) in the 0.5 mg group ($n = 7$, 2.23 ± 0.24) was the lowest among the three groups (CON ($n = 8$, 5.10 ± 0.39 , $p < 0.01$) and 0.1 mg ($n = 5$, 4.94 ± 1.27 , $p < 0.05$) groups).

Effect of cisplatin on autophagy-related proteins in skeletal muscle

Injection of cisplatin (< 0.1 mg/kg BW) in the GAS muscle did not cause changes in the levels of autophagy-related proteins, while 0.5 mg/kg BW cisplatin injection significantly activated the conversion of LC3II to LC3I (Fig. 3E, $F(2, 21) = 37.63$, $p < 0.001$), increasing the LC3II/LC3I ratio (Fig. 3B, $F(2, 21) = 14.01$, $p < 0.001$). The expression of Beclin 1 (Fig. 3C, $F(2, 18) = 25.98$, $p < 0.001$) and BNIP3 (Fig. 3D, $F(2, 20) = 42.13$, $p < 0.001$) increased significantly in the 0.5 mg group, but there was no change in Beclin 1 expression in the 0.1 mg group. The p62 content did not change with cisplatin administration (Fig. 3F, $F(2, 21) = 0.73$, $p = 0.49$).

Correlation between RCR and autophagy-related proteins in skeletal muscle

As seen in Figure 4, significant correlations were observed between RCR (ADP/GM) and LC3 II/GAPDH (Fig. 4A, $r = -0.61$, 95 % CI = $-0.83, -0.22$, $p < 0.01$), the LC3 II/I ratio (Fig. 4B $r = -0.57$, 95 % CI = $-0.81 - 0.15$, $p < 0.05$), Beclin 1/GAPDH (Fig. 4D, $r = -0.83$, 95 % CI = $-0.94 - 0.59$, $p < 0.001$), and BNIP3/GAPDH (Fig. 4E, $r = -0.55$, 95 % CI = $-0.80 - 0.12$, $p < 0.05$). However, the correlation between RCR and p62 was not significant (Fig. 4C, $r = -.21$, 95 % CI = $-60 - 0.26$, $p > 0.05$). The strong correlation of Beclin1 and LC3II with RCR indicated that high levels of RCR resulted in lower levels of autophagy-related proteins.

DISCUSSION

In this study, differences in skeletal muscle tissue weight, BW, mitochondrial function, and autophagy-related protein levels in GAS skeletal muscles among the CON, 0.1 mg, and 0.5 mg groups were analyzed. The results showed that the skeletal muscle tissue weight, BW, and mitochondrial function (GM, ADP, SUCC, and RCR) were reduced in the 0.5 mg cisplatin group. In addition, the levels of autophagy-related proteins (Beclin 1, BNIP3, and LC3II) in skeletal muscles were increased in the 0.5 mg group. These results indicate that the administration of 0.5 mg/kg BW of cisplatin decreased the muscle weight, BW, and mitochondrial function and increased the levels of autophagy-related proteins.

Cisplatin administration is associated with severe skeletal muscle dysfunction (4). Among the various forms of cisplatin-induced muscle dysfunction, the most relevant is muscle mass loss (5). This study found that the reduction in BW and muscle tissue weight was higher in the 0.5 mg group than in the CON and 0.1 mg groups (Fig. 1B and C). In addition, the normalization of skeletal muscle tissue based on BW did not reveal any significant differences among the three groups, indicating that the administration of higher cisplatin doses (up to 0.5 mg) results in muscle atrophy (Figure 1D). These findings may indicate a specific molecular mechanism, i.e., cisplatin-induced muscle atrophy may be specifically related to the transcript-level expression of muscle atrophy F-box (MAFbx), muscle RING fiber-1 (MuRF1), and Atrogin-1 (3, 6). Muscle mass is also regulated through the IGF-1/PI3K/AKT/mTOR pathway, preventing muscle atrophy (26). However, in this study, we did not analyze the specific muscle atrophy levels associated with cisplatin administration, and further studies are needed to elucidate the influence of these factors at different cisplatin concentrations.

Mitochondria are dynamic organelles whose function and morphology is altered through fission and fusion (27). Mitochondrial dysfunction following cisplatin administration resulted in damaged mtDNA, which decreased mitochondrial biogenesis and activity (28). Our results revealed a decrease in GM, ADP, SUCC, and RCR levels in the 0.5 mg group (Fig. 2). In addition, the RCR, an indicator of oxidative phosphorylation, dramatically decreased in the 0.5 mg group. It may be inferred that treatment of wild type cells with cisplatin resulted in decreased RCR (15). These results indicated that at high concentrations, cisplatin induced a lower capacity for oxidation and ATP turnover and increased the proton peak in mitochondria (4, 15, 29). Moreover, our findings showed that cisplatin-induced toxicity can result in metabolic dysfunction (30) and production of mitochondrial reactive oxygen species (ROS). The lowest SUCC levels in the 0.5 mg group in this study were related to skeletal muscle apoptosis (31). Considering our results as a whole, it can be inferred that high doses of cisplatin can cause mitochondrial dysfunction, including decreased RCR levels, leading to ROS production and skeletal muscle cell apoptosis.

The highest levels of autophagy-related proteins, such as LC3 II, Beclin 1, and BNIP3, and LC3 II/I ratio were observed in the 0.5 mg group (Fig. 3). Autophagy is a major metabolic regulator of resistance to chemotherapy (20). Inhibition of autophagy increases cisplatin-induced apoptotic cell death (32). Our findings are similar to those of previous studies that observed mitochondrial dysfunction (Fig. 2) and upregulation of Beclin 1 and LC3 II (Fig. 3C and E) in cisplatin-induced cachexia of the skeletal muscle (18). In addition, a previous study indicated that C2C12 muscle cells treated with 50 μ M cisplatin for 24 h exhibited upregulated expression of Beclin 1, BNIP3, and muscle atrophy-related atrogin-1 (33). These results are consistent with our findings, which showed that cisplatin (0.5 mg) increased muscle atrophy

and the expression of autophagy-related proteins (Fig. 1) in the skeletal muscle. effect of cisplatin administration and muscle atrophy and mitochondrial dysfunction is mediated through AKT phosphorylation that results in dephosphorylation of FOXO3a, which then migrates to the nucleus to activate the transcription of autophagy-specific genes (e.g., LC3 II and Beclin1) and causes muscle atrophy (Murf 1 and BNIP3) (4, 34, 35). Overall, our findings support the results of previous studies that show that administration of high doses of cisplatin can cause an increase in the levels of autophagy-related proteins (LC3 II, Beclin 1, and BNIP3) as well as muscle atrophy. However, our study did not consider a cancer cachexia animal model, and further studies are required to analyze mitochondrial function, skeletal muscle morphology, and autophagy response in such a model.

Mitochondrial function, especially RCR(ADP/GM), is associated with autophagy-related proteins (Fig. 4). Mitochondrial function is affected by autophagy in various metabolic diseases (36). An acute kidney injury model administered with cisplatin showed that an increased level of LC3 II was associated with mitochondrial dysfunction in the context of increased ROS levels and decreased levels of Drp1, Opa1, ATP5a, Sirt3, and Ndufs4 (37). Our findings supported these results, which showed an increase in the RCR, indicating that mitochondrial function influenced the reduced levels of autophagy-related proteins, including LC3 II, Beclin 1, and BNIP3, and the LC3 II/I ratio in cisplatin-administered skeletal muscle. The results suggest that activation of extracellular signal-regulated kinase by cisplatin decreased the LC3 II levels (38). However, we did not quantify the mitochondrial protein levels; therefore, further studies are required to analyze mitochondrial protein levels and function by comparing autophagy-related functions.

This study revealed that administration of cisplatin in rat models affected the skeletal muscle weight, BW, mitochondrial function, and autophagy-related protein levels in skeletal muscles. The highest dose of cisplatin (i.e., 0.5 mg/kg BW) induced remarkably deleterious effects, with a decreased muscle mass and mitochondrial dysfunction and increased levels of autophagy-related proteins. These results suggest that cisplatin administration for cancer treatment may be safer and more beneficial when used in combination with additional agents that reduce mitochondrial dysfunction and autophagy pathway activity to prevent skeletal muscle dysfunction, thereby providing further insights to develop novel therapeutic approaches.

MATERIALS AND METHODS

Materials and methods are available in the supplemental materials.

ACKNOWLEDGMENTS

This research was funded by the Ministry of Education of the Republic of Korea and the National Research Foundation of Korea (NRF-2018S1A5A8027802), Ministry of Science and ICT of the Republic of Korea (2018R1A2A3074998), the Basic Research Lab Program (2020R1A4A1018943), Ministry of Science, ICT, and Future Planning of the Republic of Korea (2020M3A9D803866011). We thank Dr. Jeong Rim Ko for performing cisplatin-administered animal models.

CONFLICTS OF INTEREST

There are no conflicts of interest or financial disclosures to declare.

FIGURE LEGENDS

Figure 1. Experimental procedure; body weight (BW, g), GAS skeletal muscle tissue weight (GAS, g), and GAS normalized by BW among CON (n = 10), 0.1 mg (n = 6), and 0.5 mg (n = 10) groups. (A) Experimental procedure. (B) Changes in BW over 15-days (time) for each group. (C) Differences in GAS of CON (n = 8) and 0.1 mg (n = 5) groups as compared to the 0.5 mg (n = 9) group. (D) Levels of normalization of GAS by BW in CON (n = 6), 0.1 mg (n = 4) and 0.5 mg (n = 7) groups. Values were determined by densitometric quantification and are shown as the mean \pm standard error of the mean (SEM). Outliers in each group were excluded. CON, saline control; 0.1 mg, 0.1 mg/kg BW of cisplatin administered in skeletal muscle; 0.5 mg, 0.5 mg/kg BW of cisplatin administered in skeletal muscle. Statistical methods used were one and two-way repeated ANOVA with the factor time for BW and one-way ANOVA with Tukey's post-hoc test for GAS and normalization of GAS and BW analyses. Units are arbitrary. *** $p < 0.001$ vs Time and Group in (B) *** $p < 0.001$ vs CON and ### $p < 0.001$ vs 0.1 mg in (C).

Figure 2. Effect of cisplatin on mitochondrial function in skeletal muscle. (A) GM levels. (B) ADP levels. (C) SUCC levels. (D) RCR levels. Values were determined by densitometric quantification and are shown as the mean \pm standard error of the mean (SEM). Outliers in each group were excluded. CON, saline control; 0.1 mg, 0.1 mg/kg BW of cisplatin; and 0.5 mg, 0.5 mg/kg BW of cisplatin, administered in skeletal muscle. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. BW, body weight. Units are arbitrary. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs CON and # $p < 0.05$ vs 0.1 mg.

Figure 3. Effect of cisplatin on autophagy-related proteins in skeletal muscle. (A) Representative images of western blots. (B) LC3II/I ratio. (C) Beclin1/GADPH. (D) BNIP3/GADPH. (E) LC3II/GADPH (F) p62/GADPDH. Values were determined by densitometric quantification and are shown as the mean \pm standard error of the mean (SEM). BW, body weight; GAS, gastrocnemius; CON, saline control (n = 10), 0.1 mg, 0.1 mg/kg BW of cisplatin (n = 6), and 0.5 mg, 0.5 mg/kg BW of cisplatin (n = 8), administered in skeletal muscle. Statistical analysis was performed using one-way ANOVA with Tukey's post-hoc test. Units are arbitrary. *** $p < 0.001$ vs CON and ## $p < 0.01$, ### $p < 0.001$ vs 0.1 mg.

Figure 4. Correlation between mitochondrial function RCR and the expression of autophagy-related proteins in the GAS skeletal muscle. Correlation of RCR with (A) LC3II/GAPDH, (B) LC3II/I ratio, (C) p62/GAPDH, (D) Beclin 1/GADPH, and (E) BNIP3/GADPH. Values were determined by densitometric quantification and are shown as the mean \pm standard error of the mean (SEM). Extreme outliers were excluded. Statistical analysis was performed using Pearson's correlation coefficient (R).

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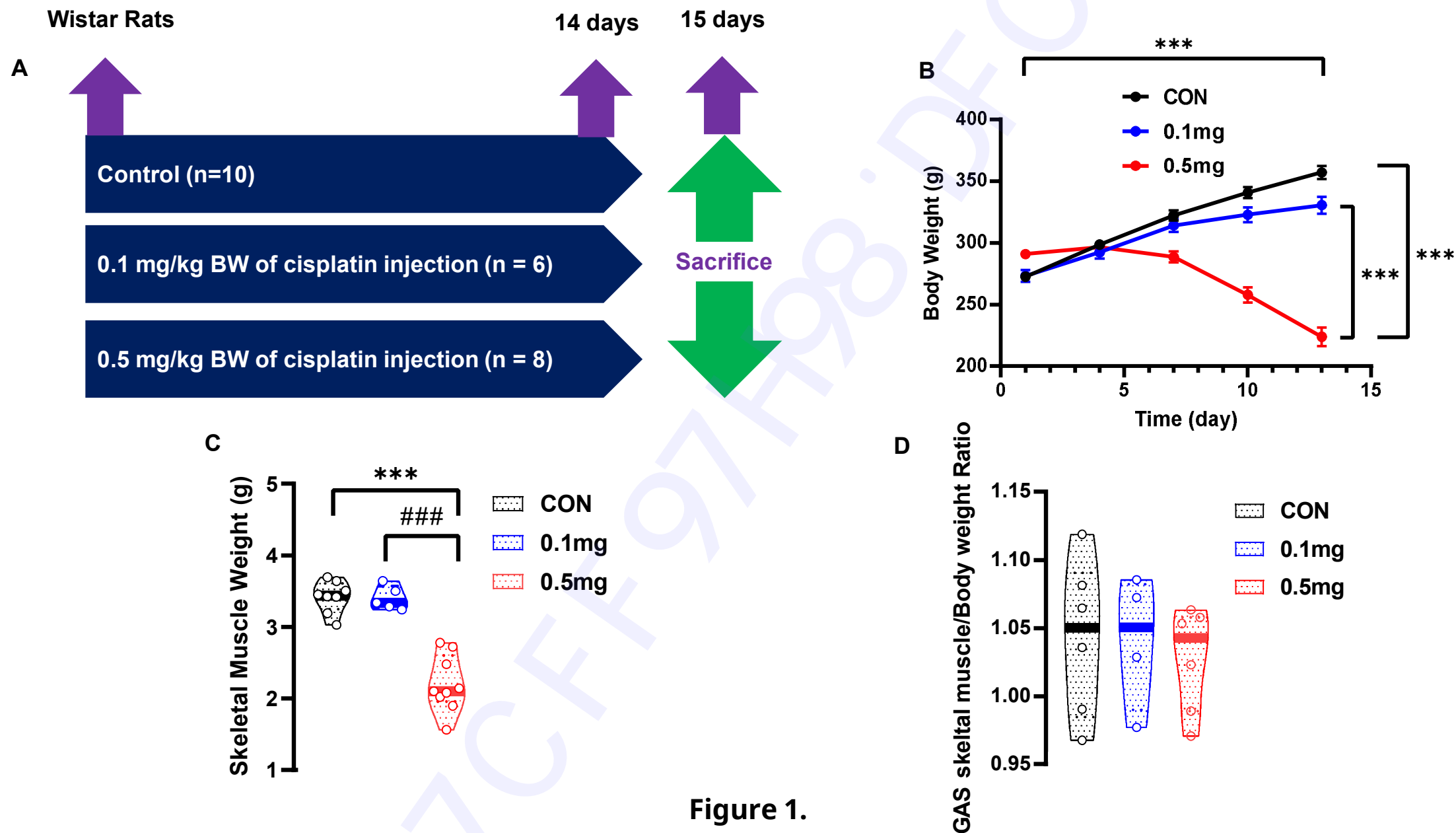
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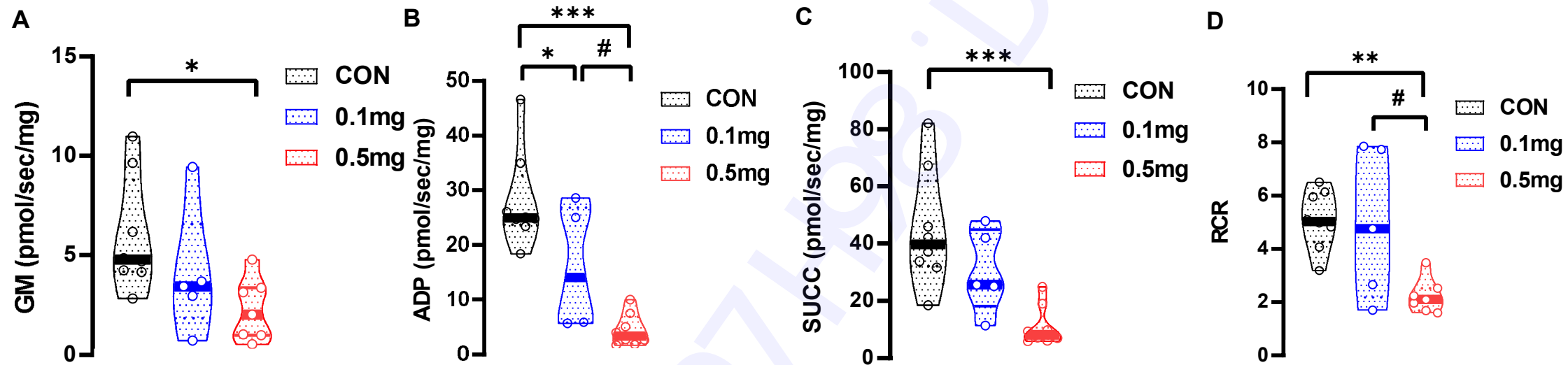


Figure 2.

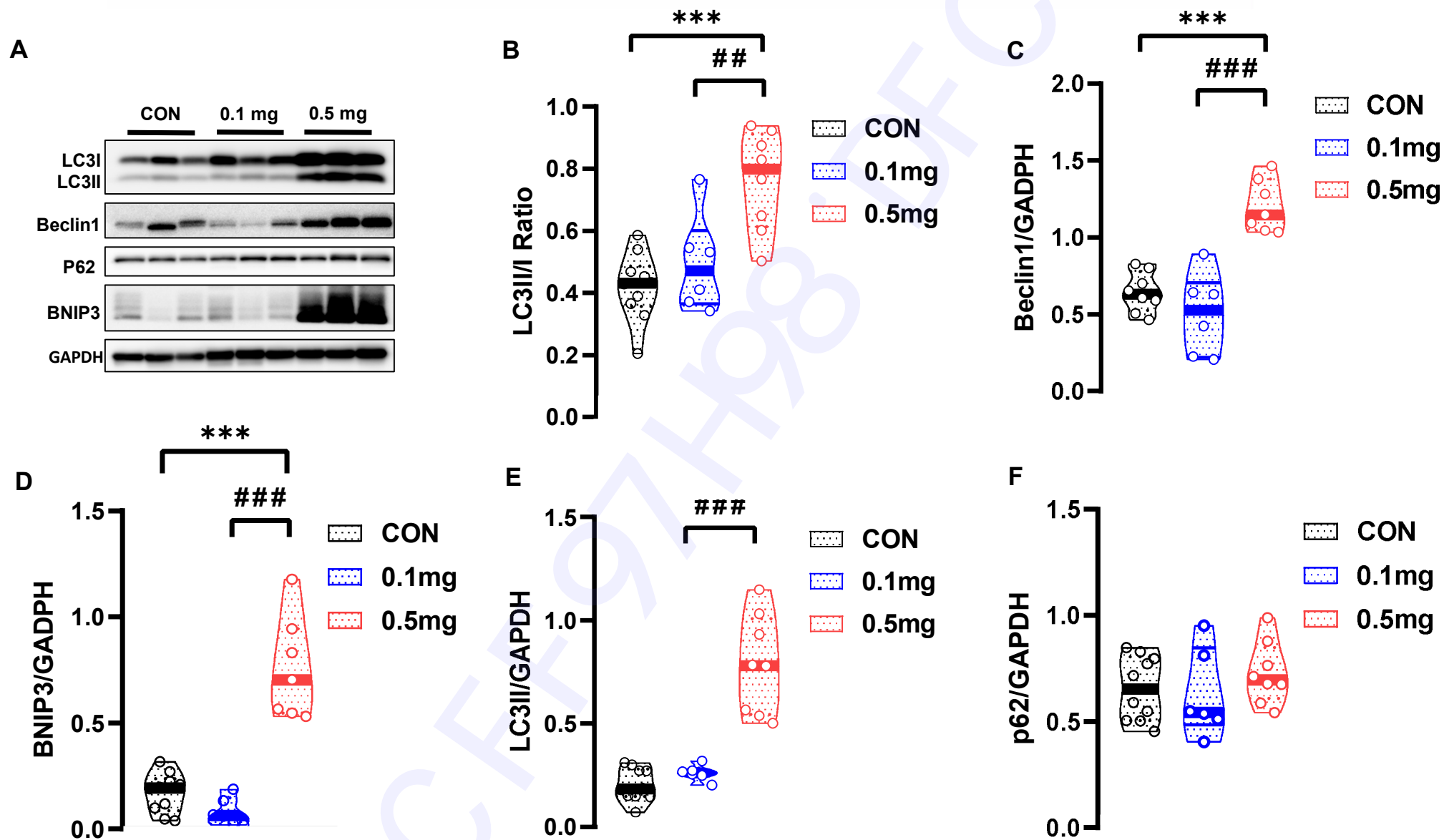
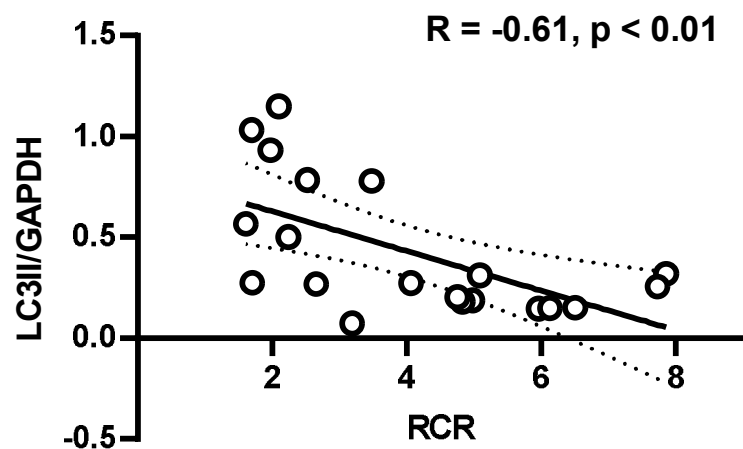
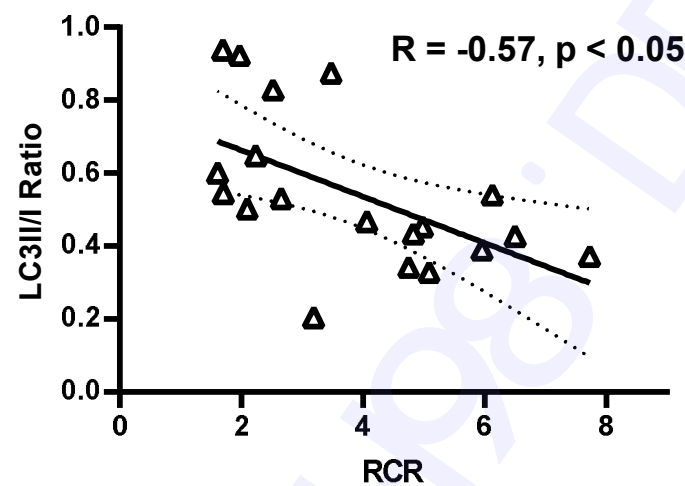
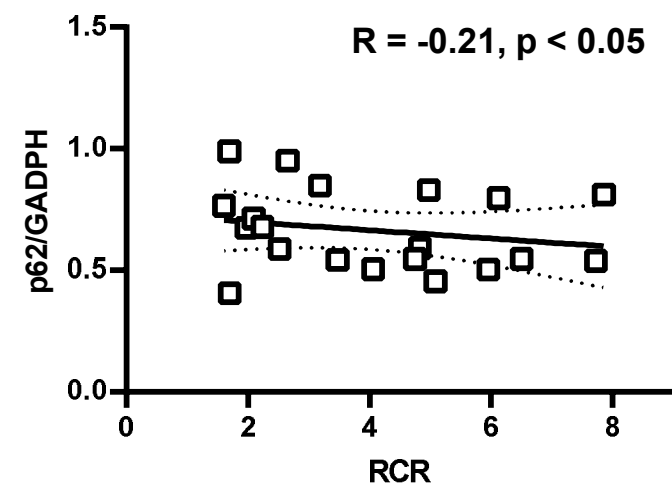
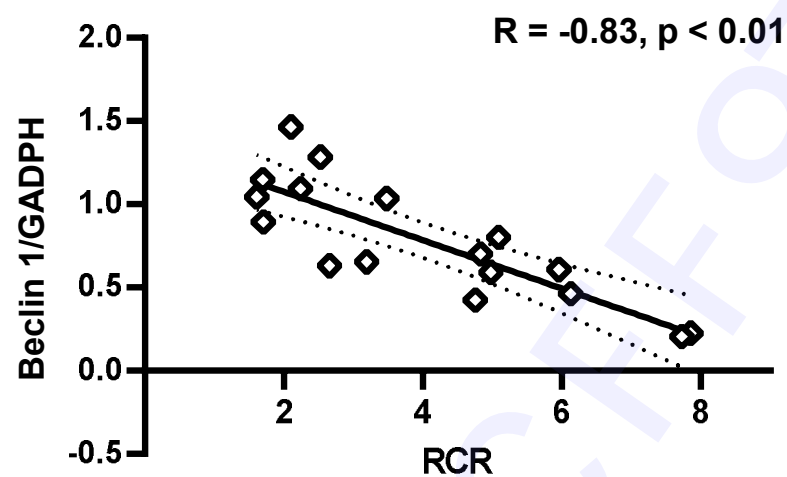
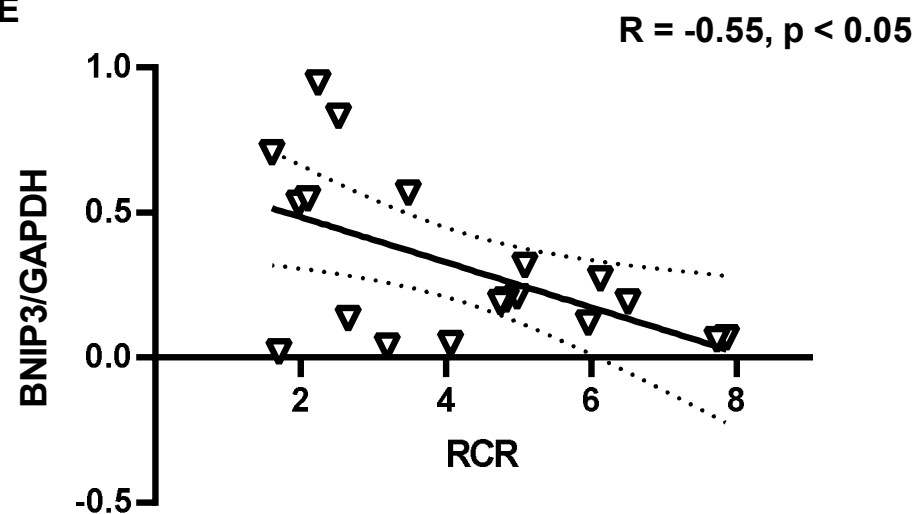


Figure 3.

A**B****C****D****E****Figure 4.**

MATERIAL AND METHODS

Experimental design

Seven-week-old male Wistar rats were sourced from the Orient Bio Company (Seongnam-si, Gyeonggi-do, Korea). The animals were preserved at a temperature of 20 ± 2.5 °C under a 12-hour light-dark cycle with free access to food and water. The animals were adapted for one week prior to the start of the study. After one week, the rats were assigned to three groups (hereafter referred to as CON group, 0.1 mg group, and 0.5 mg group), which were administered a saline placebo (CON, $n = 10$), 0.1 mg/kg BW of cisplatin ($n = 6$), and 0.5 mg/kg BW of cisplatin ($n = 8$), respectively, via intraperitoneal injection once daily for 14 days. After 14 days, the rats were euthanized, weighed, and the gastrocnemius muscle was collected. All experimental procedures were approved by the Institutional Animal USE and Care Committee of Inje University (NO: 2018-013). Figure 1A illustrates the experimental procedure.

Tissue preparation

White gastrocnemius (GAS, type IIb fiber) muscles were collected within 24 hours after death. All muscle tissues were isolated and washed with phosphate-buffered saline. After washing, all muscle tissues were dried, weighed, and stored in -80 °C until analysis.

Measurement of mitochondrial function

Mitochondrial function analysis included measurement of adenosine di-phosphate (ADP), glutamate (GM), succinate (SUCC), and the respiratory control ratio (RCR: ADP/GM). The GAS muscles were separated in ice cold buffer composed of 7.23 mM K_2EGTA , 2.77 mM Ca K_2EGTA , 20 mM imidazole, 0.5 mM DTT, 20 mM taurine, 5.7 mM ATP, 14.3 mM

phosphocreatine, 6.56 mM MgCl₂·6H₂O, and 50 mM 2-(N-morpholino) ethane sulfonic acid. The separated myofibers were subjected to mitochondrial permeabilization using saponin. Following permeabilization for 30 min at 4 °C, the myofibers were washed for at least 15 min before mitochondrial function analysis.

Western blot

Total protein was extracted from the GAS muscle tissue using RIPA buffer (ThermoFisher Scientific #89900, Waltham, MA, USA), supplemented with a phosphatase inhibitor cocktail (Sigma-Aldrich #490685001, St. Louis, MO, USA) and a protease inhibitor cocktail (Roche #4693159001, Basel, Switzerland). Proteins were separated using 10–16% trisglycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose (NC) membranes via iBlot 2 transfer stacks (Invitrogen #IB23002, Carlsbad, CA, USA). We used the following primary antibodies: LC3 II and I (Sigma-Aldrich #L7543, St. Louis, MO, USA), p62 (Sigma-Aldrich #126M4796V, St. Louis, MO, USA), BNIP3 (Cell Signaling Technology Inc. #3769, Danvers, MA, USA), and Beclin 1 (Cell Signaling Technology #3738, Danvers, MA, USA).

The membranes were probed overnight with primary antibodies diluted to 1:1000 with Tris-buffered saline containing Tween 20 (TBST) (Biosesang #HT2007, Seongnam-si, Gyeonggi-do, Korea) and 5% skim milk at 4 °C. Then, the membranes were incubated with a peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology Inc. #7074, Danvers, MA, USA), and protein bands were detected using Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma #WBKLS0500, Burlington, MA, USA). The band densities were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology Inc. #2118, Danvers, MA, USA) as an internal control.

Statistical Analysis

All data are presented as the mean \pm standard error of the mean (SEM). Statistical analyses were performed using Prim 9.1.2 (GraphPad, San Diego, CA, USA). A normality test was performed among the three experimental groups using the Kolmogorov-Smirnov test ($p > 0.05$). One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used for the analysis. In addition, the BW (g) was analyzed using two-way repeated measures ANOVA with time factors and Tukey's Honest Significant Difference (HSD) test for post-hoc analysis. Pearson correlation coefficients (r) between RCR and autophagy-related proteins (LC3 II, LC3II/I ratio, p62, Beclin 1, and BNIP3) were calculated. $p < 0.05$ was considered significant.