

Effects of Nitric Oxide on C2C12 Cell Inflammatory Responses and Notexin-Induced Muscle Injury

Efectos del Óxido Nítrico en las Respuestas Inflamatorias de las Células C2C12 y la Lesión Muscular Inducida por Notexina

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NIU, S. L.; JIN, X. F.; YANG, T.; CHEN, Z. H.; WANG, G. D.; LIANG, Y. & YAN, J. Effects of nitric oxide on C2C12 cell inflammatory responses and notexin-induced muscle injury. *Int. J. Morphol.*, 40(1):251-260, 2022.

SUMMARY: Skeletal muscle injury is an acute inflammatory condition caused by an inflammatory response. To reduce inflammatory cell infiltration and relieve skeletal muscle injury, efficient treatment is urgently needed. Nitric oxide is a free radical molecule reported to have anti-inflammatory effects. In this study, we showed that NO could inhibit the inflammatory response of C2C12 cells in vitro and protect rat skeletal muscle injury from notexin in vivo. NO synthase inhibitor (L-NG-Nitroarginine Methyl Ester/L-NAME) and NO donor (sodium nitroprusside dehydrate ?SNP) were used to explore the vital role of lipopolysaccharides (LPS) in LPS-stimulated C2C12 myoblasts. The expression of IL-18 and IL-1b was upregulated by L-NAME and downregulated by SNP, as indicated by the ELISA results. NO can reduce ASC, Caspase-1, and NLRP3 mRNA and protein levels. Furthermore, NO was detected in the rat model. The results of immunohistochemical staining showed that the production of DMD decreased. We conducted qRT-PCR and western blotting to detect the expression of Jo-1, Mi-2, TLR2, and TLR4 on day 6 post injury following treatment with L-NAME and SNP. The expression of Jo-1, Mi-2, TLR2, and TLR4 was upregulated by L-NAME and significantly reversed by SNP. NO can alleviate C2C12 cell inflammatory responses and protect rat skeletal muscle injury from notexin.

KEY WORDS: Nitric oxide; Muscle injury; C2C12; Inflammasome; Inflammation.

INTRODUCTION

Skeletal muscle is the largest muscle tissue of the human body and consists of contractile muscle cells and striated muscle tissue. In addition, skeletal muscles are the primary store of energy in the body and account for approximately 40 % of total body weight (Smith *et al.*, 2008; Blanc *et al.*, 2020). To maintain a normal body structure, more than 600 pieces of elastic muscle tissues are attached to the bones and distributed in the trunk and limbs. Skeletal muscles can help the body perform daily activities and exercise sessions. However, prolonged and intense exercise may lead to injuries (Kääriäinen *et al.*, 2001; Warren *et al.*, 2007). Moreover, skeletal muscle injury can cause muscle contraction and extension dysfunction, which can be accompanied by varying degrees of pain,

leading to decreased quality of life. Recently, inflammation has been recognized as the core mechanism of skeletal muscle injury (Yeow *et al.*, 2002). Studies have shown that myoblast cells in skeletal muscle can promote the activation and release of intracellular inflammasomes and accelerate skeletal muscle injury under the stimulation of inflammation.

Myoblasts are mononuclear cells that are found between the sarcolemma and the basement membrane (Wang *et al.*, 2018). New research has suggested that myoblasts are the prerequisite cells of skeletal muscle that originate from the mesoderm. Myoblasts are also involved in muscle growth and regeneration. When skeletal muscle

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is injured, myoblasts exert biological functions under specific stimuli and promote the proliferation and differentiation of myoblasts through regulatory factors (Yamashita *et al.*, 2016; Liu *et al.*, 2019; Pan *et al.*, 2019). Myoblast cells can successfully integrate damaged myocardial tissue in experiments and generate striated muscle tissue to restore the contractile function of the mouse myocardium (Yu *et al.*, 2020). Myoblast cells can also fuse with damaged muscle fibers to form new fibers to repair skeletal muscle injury (Srikuea *et al.*, 2012). However, the repair of skeletal muscle injury may be inhibited by negative regulatory factors that can inhibit the proliferation and differentiation of myoblast cells.

Nitric oxide (NO) is a free radical molecule that has a variety of biological functions (Filippin *et al.*, 2009; Bruand & Meilhoc, 2019) and participates in the metabolic regulation of various tissues. A large number of inducible NO synthase (NOS) and superoxide anion free radicals can be produced during complex inflammatory responses in the body. Studies have suggested that NO can act on smooth muscle cells, dilate blood vessels, and improve blood perfusion in local tissues to reduce tissue damage (De Palma & Clementi, 2012). Meanwhile, a large amount of NO is detected in the articular fluid of patients with arthritis and it has been shown to induce the release of a variety of inflammatory mediators to alleviate articular cartilage damage. Although myoblasts can synthesize and secrete intracellular inflammasomes to promote skeletal muscle injury, NO has been shown to inhibit inflammatory formation and activation in other experiments (Kaminski & Andrade, 2001; Kim, 2015). However, it has not been reported that NO can repair skeletal muscle injury by inhibiting the formation of specific inflammatory bodies in myoblast cells (Filippin *et al.*, 2011; Guo *et al.*, 2019). This study was designed to investigate the specific inhibitory effect of NO on the formation and activation of inflammatory bodies using C2C12 cells and a skeletal muscle injury rat model.

MATERIAL AND METHOD

Reagents. C2C12 cells were purchased from Fenghui Biotechnology Company Ltd. (Hunan, China). Dulbecco's modified Eagle's medium (DMEM, high glucose) and 0.25 % trypsin-EDTA were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Primary antibodies against ASC, cysteinyl aspartate specific proteinase-1 (Caspase-1), NOD-like receptor family, pyrin domain containing 3 (NLRP3), Toll-like receptor-2 (TLR2), and Toll-like receptor-4 (TLR4) were purchased from Bo'ao sen

Biological Ltd. (Beijing, China). Primary antibodies against ACTB were purchased from Sangon Biological Company, Ltd. (Shanghai, China). Cytokine enzyme-linked immunosorbent assay (ELISA) was purchased from Lianke Biological Company Ltd. (Zhuhai, China). TRIzol reagent, reagents for RNA reverse transcription, and quantitative polymerase chain reaction (PCR) were obtained from Abcam Inc. (Cambridge, MA, USA).

Cell culture. Frozen cells were removed from the liquid nitrogen and immediately placed in a 37 °C water bath. Cells were rapidly rocked to ensure they quickly thawed (2 min), after which they were added to 9 mL of complete medium. Following centrifugation, the supernatant was discarded, and the cells were inoculated into a culture flask with Hyclone high glucose medium containing 1 % penicillin-streptomycin and 10 % fetal bovine serum and placed in a 37 °C incubator in a 5 % CO₂ atmosphere.

Cellular inflammatory model and cell viability assay. Cell viability assays were performed using the Cell Counting kit-8 assay (CCK8, Quanshi Gold). C2C12 cells of good growth state and a confluence rate of 90 % were prepared into 5 × 10⁴ cells/mL cell suspension with complete medium. Cells were cultured in 96-well plates and then incubated in the presence or absence of lipopolysaccharide (LPS, 100 ng/mL) for 24 h. Finally, 100 mL of CCK-8 solution was added to the 96-well plate after discarding the medium and incubated for 1 h. The optical density (OD) value was measured using a microplate reader with absorbance at 450 nm (Bio-Rad, China).

Real-time PCR. Total RNA was extracted from C2C12 cells and quadriceps muscle tissue of rats using TRIzol reagent or tissue-specific RNA extraction kit according to the manufacturer's instructions and then reverse transcribed to cDNA according to the mRNA concentration. Quantitative real-time PCR was amplified using Bio-Rad under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a dissolution curve at 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s.

The following primers were used: b-actin (human): forward: CCCATCTATGAGGGTTACGC, reverse: TTTAATGTCACGCACGATTTTC; Jo-1 (human): forward: GCTGCTGTCCCTCCGTTATG, reverse: CCGGCGATACACCTTTGCTA; Mi-2 (human): forward, TGCCCTCTCTCCCTCCAGATT; reverse, GGGAGCTTCTGATCGTTCGTC; TLR2 (human): forward: TGGCCACAGGACTCAAGAGCA, reverse: AGGCCTATCACAGCCATCAAGA; TLR4 (human): forward: CTACCTCGAGTGGGAGGACA, reverse: TCCTCAGGTCAAAGTTGTTGCT.

Protein extraction and western blotting. Total proteins were extracted from C2C12 cells and quadriceps muscle tissues by RIPA lysis buffer and PMSF (100:1) mixture after place in a humid chamber for 1 h and then high-speed cryogenic centrifugation (12000 g/min, 15 min, 4 °C). Protein concentrations were measured using a BCA assay kit (Quanshi Gold, Beijing, China). Equal amounts of protein were added with an appropriate amount of 5 % SDS-PAGE loading buffer and heated in boiling water at 100 °C for 5 min to fully denature. Protein fractions were separated by 8–12 % SDS-PAGE and transferred to PVDF membranes. After blocking with 5 % non-fat milk in TBS containing 0.1 % Tween-20 (TBST) for 1 h at room temperature, they were then incubated with primary antibodies overnight at 4 °C. Membranes were washed three times with 5 % TBST for 5 min and incubated in goat anti-rabbit IgG or goat anti-mouse IgG for 1 h at 25 °C. The membranes were washed again with TBST and then detected using ECL.

Cytokine ELISA. The cells were treated with or without LPS and then cultured at 37 °C in a 5 % CO₂ atmosphere. The supernatant was collected and added to detection buffer and diluted antibody within 15 min for the examination of inflammatory cytokines. The concentrations of IL-1b, IL-18, and NO were measured using different rat ELISA kits, according to the manufacturer's instructions.

Animal model and experimental design. Male Sprague-Dawley rats were purchased from the Animal Laboratory Center at Xinjiang Medical University. Following one week of acclimatization, rats weighing 280–320 g were subjected to a skeletal muscle injury by intramuscular injection of 10 mL notexin (25 mg/mL), and then the model was confirmed by HE staining. Rats were randomly divided into control, notexin, notexin + L-NAME, and notexin + SNP groups. Each group was randomly divided into one, three, and six subgroups with eight rats in each subgroup. Rats were then sacrificed; serum samples were taken and the quadriceps femoris tissue harvested.

Histological analysis. Rat quadriceps femoris muscles were fixed with 4 % paraformaldehyde for 48 h, dehydrated with conventional gradient ethanol, and embedded in paraffin. The coated wax block was sliced into tissue sections and placed in a 65 °C incubator for baking for 1.5–2 h. Tissue sections soaked in xylene were dewaxed and hydrated with anhydrous alcohol and distilled water. Finally, the tissue sections were stained with HE.

Immunohistochemical staining. Dewaxed and hydrated tissue sections were boiled in a pressure cooker with citrate buffer solution (pH 6.0) and soaked for 10 min. Endogenous peroxidase was removed with 3 % H₂O₂ water and washed

with phosphate-buffered saline three times. The tissue sections were incubated in primary antibody DMD at 4 °C overnight and then incubated with the secondary antibody at 25 °C for 20 min. Finally, the DMD protein was examined under a microscope.

Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). SPSS19.0 software was used for statistical analysis of data in each group. One-way ANOVA was used for statistical analysis, and a t-test was used to compare the same group at different times. Statistical significance was set at $p < 0.05$.

RESULTS

Effect of LPS on C2C12 viability and the expression of IL-18 and IL-1b in C2C12 cells. Cells were cultured with or without LPS (100 ng/mL) for 6 h, and then the OD value at 450 nm was detected using an enzyme plate analyzer. Results showed that cell proliferation was significantly reduced after LPS treatment ($p < 0.01$) (Fig. 1). The number of cells was also significantly reduced after LPS intervention, as observed under a microscope (Fig. 1). However, the concentrations of IL-18 and IL-1b were increased in the cell supernatants after LPS stimulation ($p < 0.05$). These results suggest that LPS can simulate the inflammatory model of cellular skeletal injury (Fig. 1).

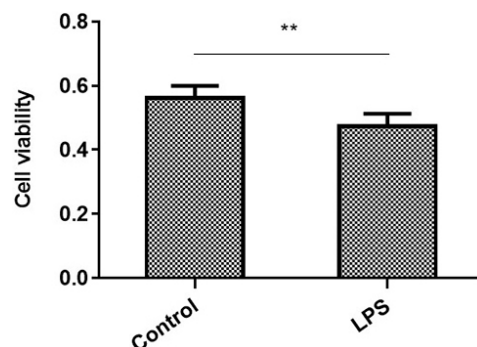


Fig. 1 Cells were cultured with or without LPS 100ng/ml for 6 h and then OD value at 450nm was detected by enzyme plate analyzer. The results showed that the cell proliferation was significantly reduced after LPS intervention ($p < 0.01$).

Effect of L-NAME and SNP on IL-18 and IL-1b expression in C2C12 cells. C2C12 myoblasts were cultured in the presence or absence of LPS (100 ng/mL) and then treated with L-NAME (2.7 mg/mL) and SNP (8.94 mg/mL) for 6 h. Interestingly, we found that the expression of IL-18 and IL-1b was upregulated by L-NAME and, on the contrary, downregulated by SNP ($p < 0.05$) (Fig. 2).

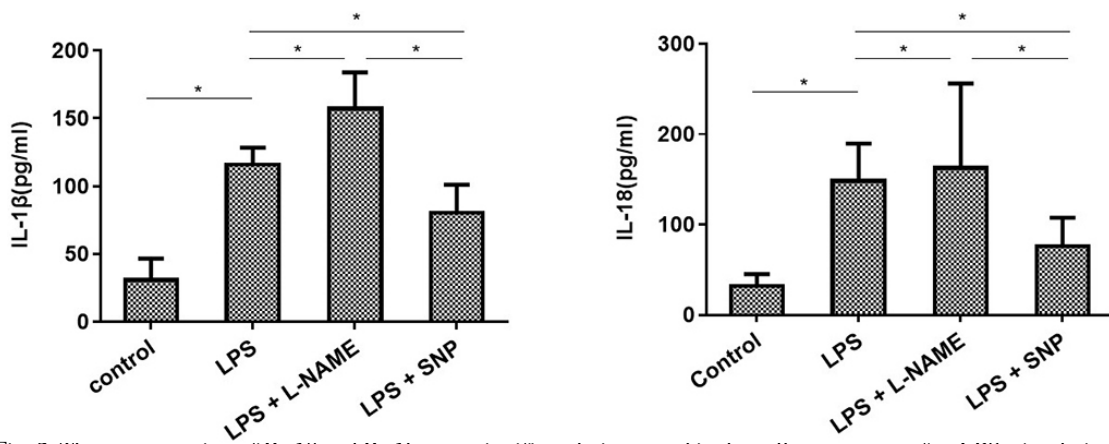


Fig. 2. The concentration of IL-18 and IL-1b were significantly increased in the cell supernatant after LPS stimulation. The expressions of IL-18 and IL-1b were up-regulated by L-NAME and, on the contrary, down-regulated by SNP ($p < 0.05$).

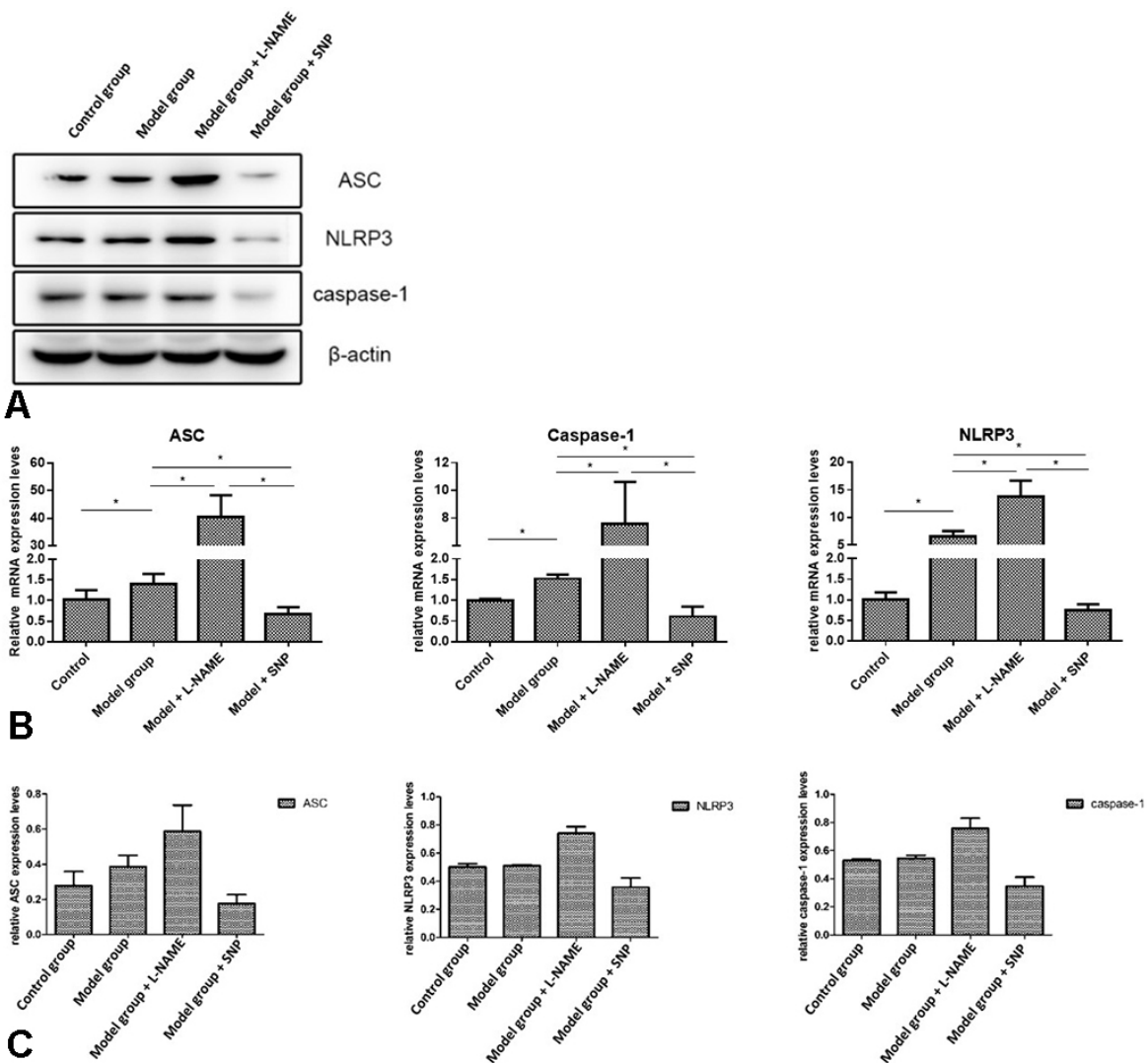


Fig. 3. Comparison of ASC, Caspase-1 and NLRP3 expression level by qRT-PCR (B) and Western blot (C). The results showed that the expressions of ASC, Caspase-1 and NLRP3 were up-regulated by L-NAME and significantly reversed by SNP ($p < 0.05$).

SNP downregulates ASC, Caspase-1 and NLRP3 levels in C2C12 cells. To further investigate the effect of NO on the skeletal muscle injury inflammatory response in vitro, cells were cultured with or without LPS and then treated with L-NAME or SNP for 6 h. We performed qRT-PCR to analyze the levels of ASC, Caspase-1, and NLRP3. In addition, we measured the levels of ASC, Caspase-1, and NLRP3 by western blotting (Fig. 3). The results showed that the expression of ASC, Caspase-1, and NLRP3 was upregulated by L-NAME and significantly reversed by SNP. Subsequently, the influence of IL-1b and IL-18 content in the supernatant was detected by ELISA. We found that the expression of IL-18 and IL-1b was upregulated by L-NAME. However, the levels of IL-18 and IL-1b were consistently

observed in the supernatants with SNP.

Notexin-induced skeletal muscle injury in rat model. In the control group, the gross structure of the voluntary muscle did not show obvious abnormalities at 1, 3, or 6 days. However, following notexin treatment, we found that muscle fiber atrophy, dissolution, rupture, necrosis were increased. We also found that some muscle fiber nuclei were increased, such as the nucleus, and vesicular nuclei. Furthermore, we found hyperplasia of fibrous tissue among some muscle fibers with infiltration of adipose tissue, focal infiltration of lymphocytes, and a few hemorrhagic foci. More importantly, we found that the lesions at days 1 and 6 were milder than those at day 3 (Fig. 4).

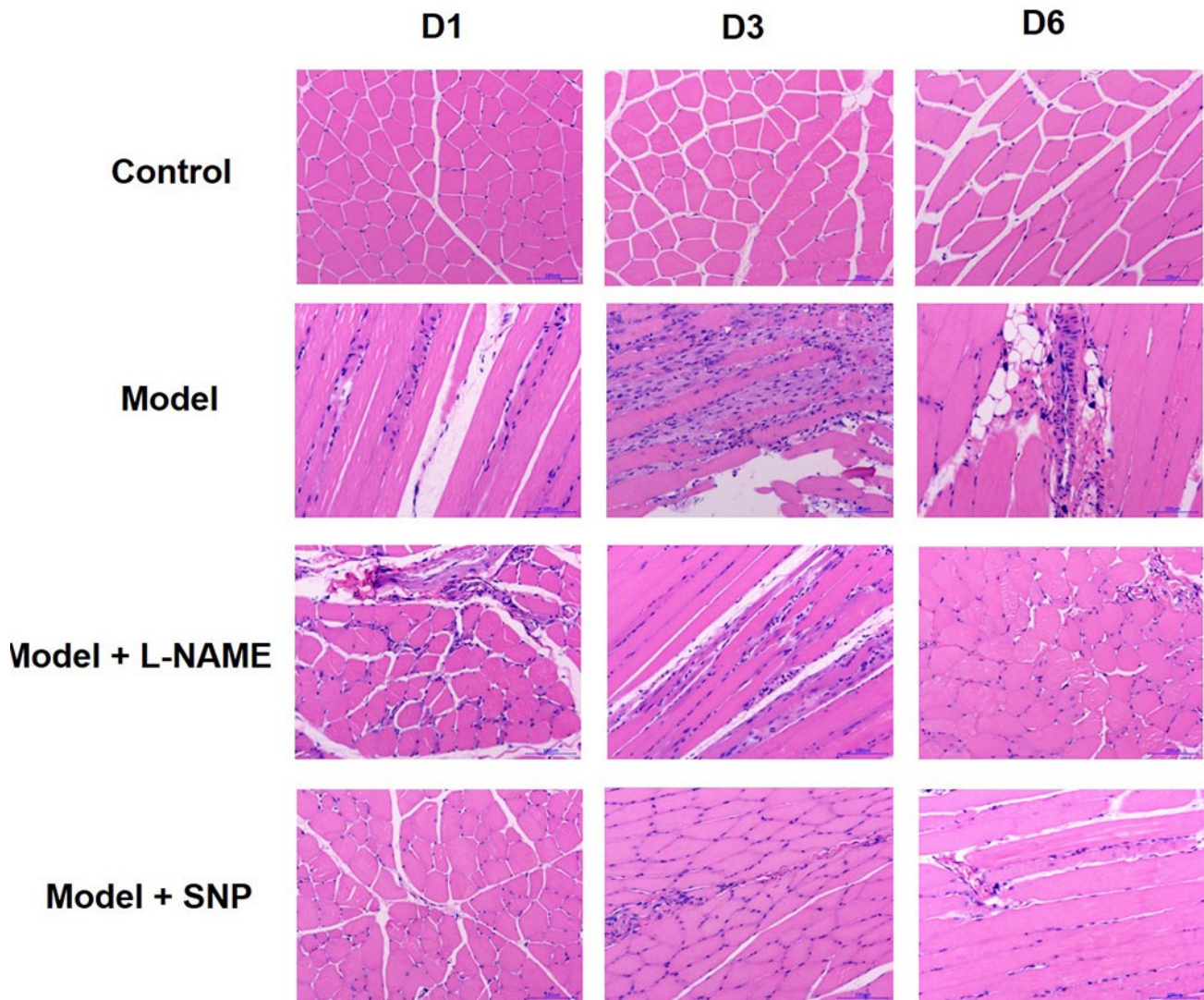


Fig. 4. Through HE and immunohistochemical staining, some muscle fibers of the rhabdoid muscle were atrophied and the muscle space was significantly widened. What's more, part of the muscle tissue dissolved, ruptured, necrotic and large area of inflammatory cells exuded, as well as fibrous tissue hyperplasia among some muscle fibers on day 3. The necrotic muscle fibers were replaced by a large number of muscle fibers with central nucleus on day 6.

SNP relieve the skeletal muscle injury in the notexin-induced rat model. NO is an important protective factor against the skeletal muscle injury inflammatory response in vitro. We next employed HE, immunohistochemical staining, and ELISA to verify the protective effect of skeletal muscle injury in a rat model. Rats were injected intraperitoneally with L-NAME (100 µg/kg) and SNP (0.5 mg/kg) after intramuscular administration of notexin. As shown in Figure 6, the results indicated that SNP treatment resulted in a significant downregulation of DMD protein levels and

inhibited expression of IL-18 and IL-1b at day 6. In contrast, L-NAME promoted the production of DMD protein and the levels of IL-18 and IL-1b at day 6. Next, we conducted qRT-PCR and western blotting to detect the expression of Jo-1, Mi-2, TLR2, and TLR4 on day 6 post injury following treatment with L-NAME and SNP and investigated whether SNP can protect rat skeletal muscle injury from notexin. As expected, the expression of Jo-1, Mi-2, TLR2, and TLR4 was upregulated by L-NAME and significantly reversed by SNP (Fig. 7).

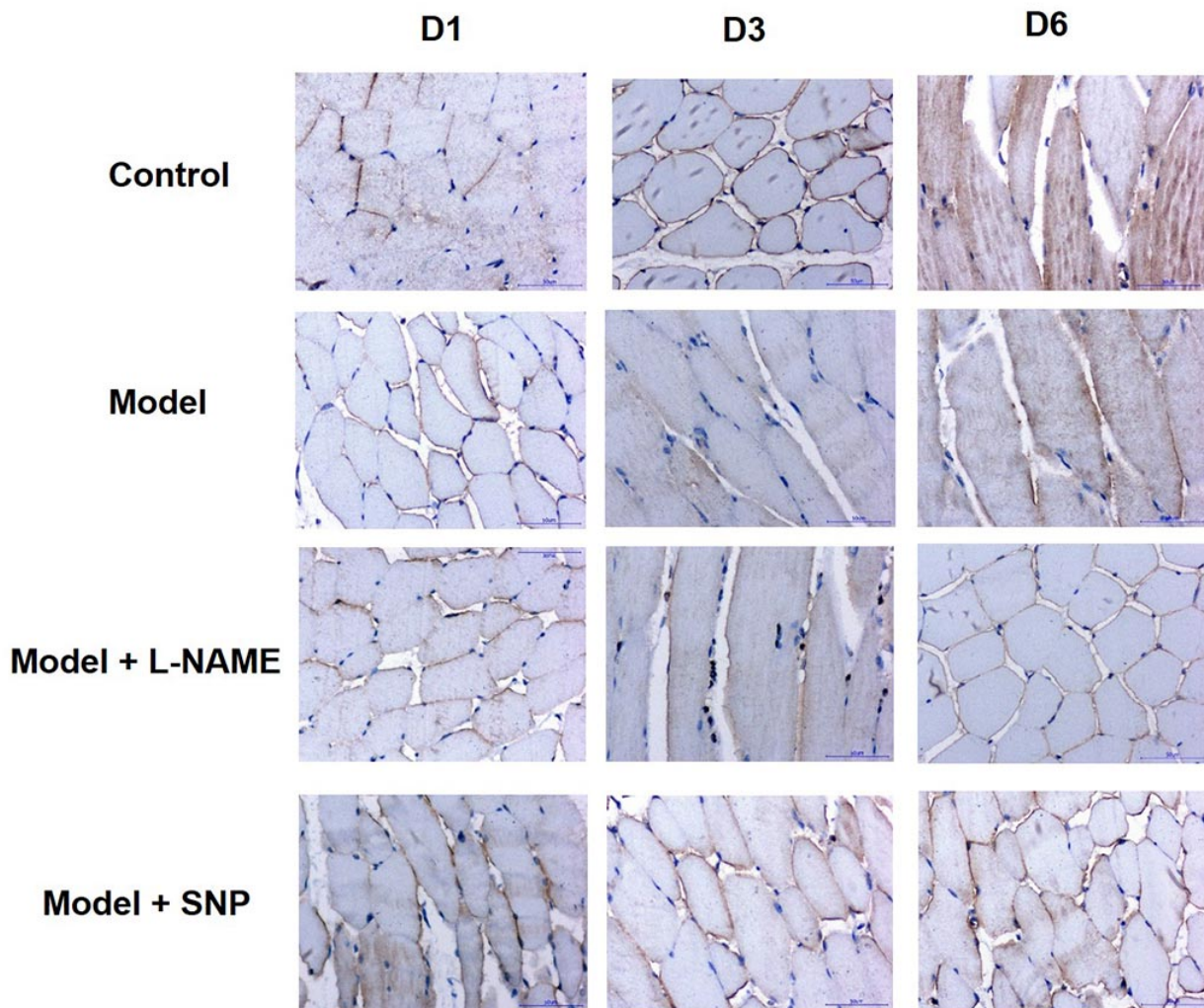


Fig. 5. Effect of L-NAME and SNP treatment with Notexin induced skeletal muscle injury rats model. The results indicated that SNP treatment resulted in a significant down-regulation of proteins level of DMD and the pathological changes in the Notexin+L-NAME group were more serious than those in the Notexin+SNP group at 1, 3, 6 day.

DISCUSSION

Inflammation is an essential process associated with the injury and repair of skeletal muscles. Studies have shown

that severe inflammatory responses are not conducive to the repair and regeneration of muscle injuries (Tidball & Villalta,

2010; Tsutsui *et al.*, 2011; Yamamoto *et al.*, 2020). Toxic substances such as peroxide and superoxide, which are released during inflammation, can dissolve cell membranes and damage skeletal muscle fibers. Continued inflammatory exudation can lead to secondary damage to the skeletal muscles. Therefore, the coordination of the inflammatory response and repair relationship after skeletal muscle injury is particularly important. NO can activate skeletal muscle myoblasts and participate in skeletal muscle regeneration in

the process of acute and chronic injury of skeletal muscle (Qi *et al.*, 2004; Zimiani *et al.*, 2005; Wang *et al.*, 2009; Kawashima *et al.*, 2020). Exploring the molecular inflammation mechanism is conducive to understanding the mechanism by which NO alleviates skeletal muscle injury. In this study, the therapeutic effects of NO on the inflammatory response after muscle injury were evaluated in both an LPS-treated C2C12 cell model and a notexin-induced skeletal muscle injury rat model.

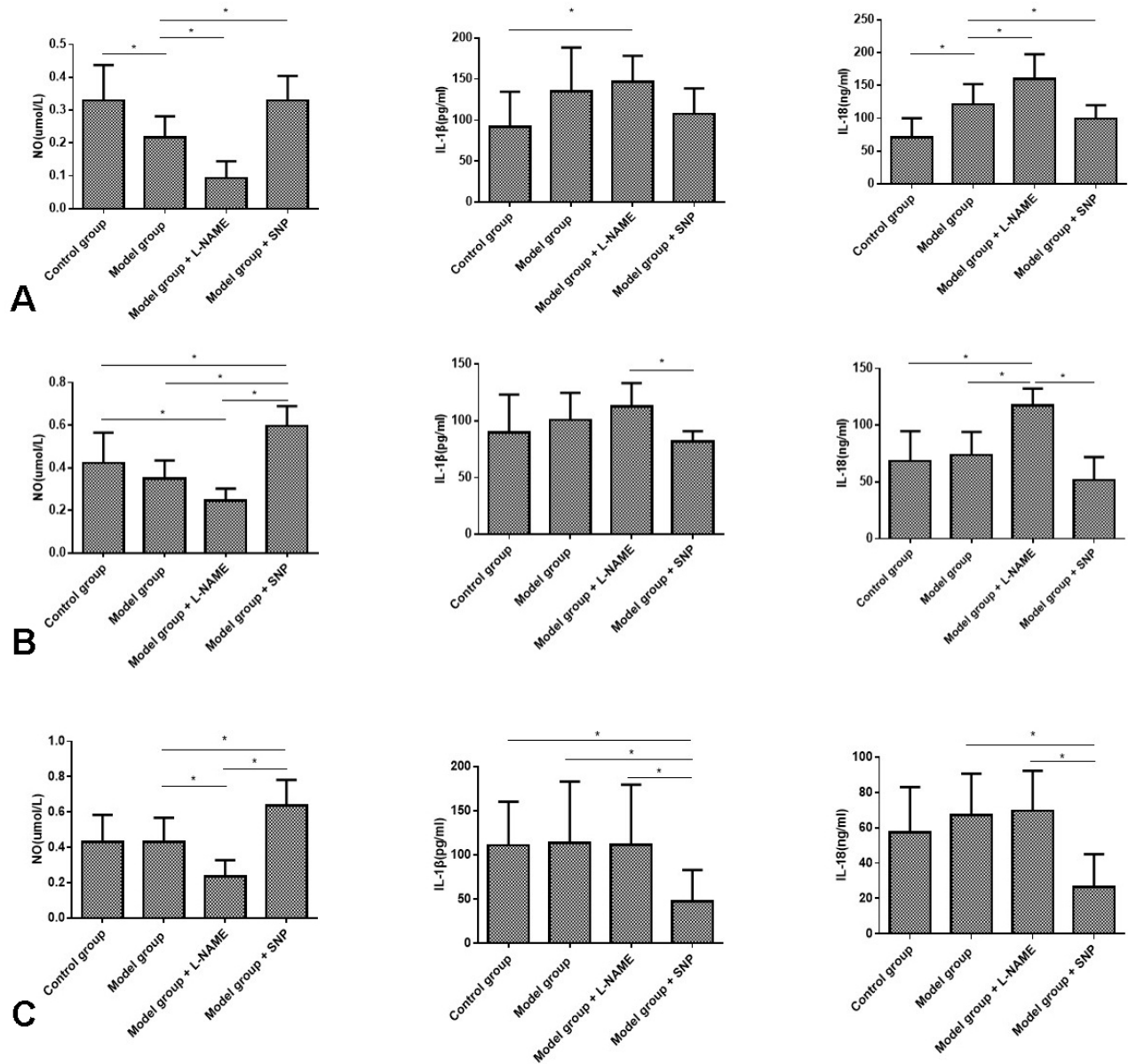


Fig. 6. We used ELISA to verify whether the protective effect skeletal muscle injury in rats model. Rats were injected intraperitoneally with L-NAME(100ug/kg) and SNP(0.5mg/kg) after intramuscular injection with Notexin. We found that the expressions of IL-18 and IL-1b were down-regulated bySNP at day 1 (A).The results indicated that L-NAME promoted the levels of IL-18, IL-1b. In contrast, SNP inhibited the expression of IL-18, IL-1b at day 3. (B).More importantly, we found that the expressions of IL-18, IL-1b was decreased more obvious at day 6 than day 3 (C).

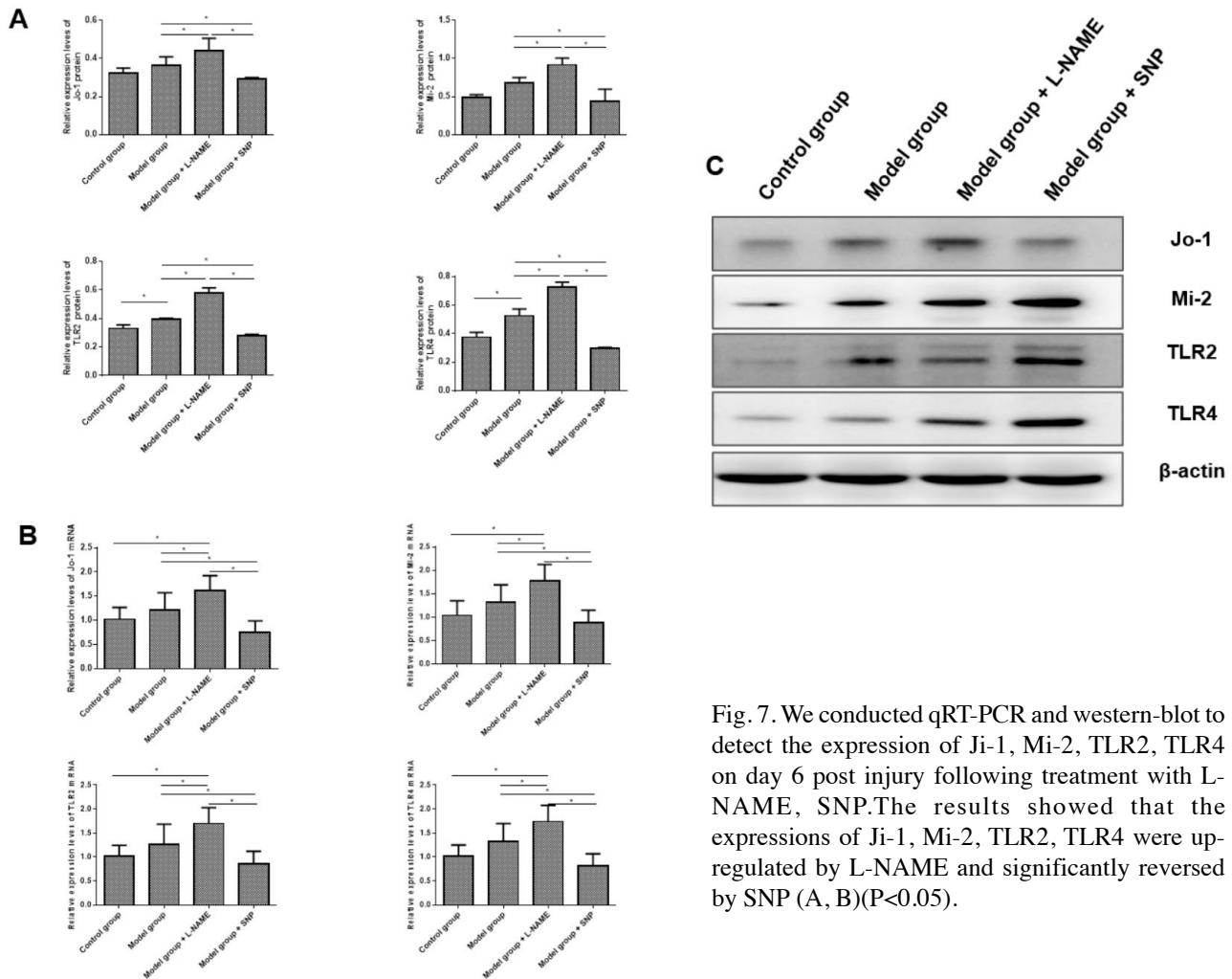


Fig. 7. We conducted qRT-PCR and western-blot to detect the expression of Ji-1, Mi-2, TLR2, TLR4 on day 6 post injury following treatment with L-NAME, SNP. The results showed that the expressions of Ji-1, Mi-2, TLR2, TLR4 were up-regulated by L-NAME and significantly reversed by SNP (A, B) ($P < 0.05$).

First, C2C12 cells were stimulated with LPS to construct a skeletal muscle injury model in vitro, and IL-1b and IL-18 in cell supernatants were detected by ELISA to verify the successful construction of the inflammatory cell model. The results showed that the proliferation of C2C12 cells was significantly inhibited, and the cellular IL-1b and IL-18 were highly expressed. Studies have indicated that the NO antagonist L-NAME promotes the expression of C2C12 cell antigen, while SNP has the opposite effect, suggesting that NO may be involved in the downregulation of skeletal muscle inflammation (Frost *et al.*, 2004; Boyd *et al.*, 2006; Kumar *et al.*, 2017; He *et al.*, 2018; Phua *et al.*, 2020). The apoptosis and inflammation related proteins ASC, caspase 1, and NLRP3 were detected by qRT-PCR and western blotting after L-NAME and SNP, respectively, in the C2C12 supernatant. The mRNA expression was increased in the LPS and LPS + L-NAME groups but reduced in the LPS + SNP group. These results were similar to those of

western blotting. It is speculated that NO downregulates the inflammatory response through activation of the NLRP3 inflammasome (Ikebe *et al.*, 2002; Duma *et al.*, 2011; Jing *et al.*, 2019).

Through HE and immunohistochemical staining, some muscle fibers of the rhabdoid muscle were atrophied, and the muscle space was significantly widened. Furthermore, some of the muscle tissues were dissolved, ruptured, and a large area of inflammatory cells exuded on day 3. However, the necrotic muscle fibers were replaced by a large number of muscle fibers with a central nucleus on day 6. Interestingly, the notexin + SNP group suggested that muscle fiber atrophy and muscle hyperplasia, as well as fibrous tissue hyperplasia between muscle fibers, lymphocyte infiltration was reduced compared with the notexin group on days 1, 3, and 6. The pathological changes in the notexin + L-NAME group were more pronounced than those in the

notexin + SNP group at each time point. These results indicate that NO can inhibit the inflammatory response in notexin-induced skeletal muscle necrosis and inflammatory exudation (Sayan *et al.*, 2001; Gunji *et al.*, 2003; Nguyen & Tidball, 2003).

Since skeletal muscle necrosis and inflammatory exudation induced by notexin were completely replaced by regenerated muscle at day 6, we chose this day as the sampling time point for the in vitro experiment. The levels of Jo-1, MI-2, TLR2, and TLR4 were significantly upregulated in the notexin + L-NAME group and downregulated in the notexin + SNP group.

In this study, we found that NO could inhibit the inflammatory response of C2C12 cells in vitro and protect rat skeletal muscle injury from notexin in vivo. We conducted qRT-PCR and western blotting to detect the expression of IL-18, IL-1b, ASC, Caspase-1, and NLRP3. These inflammasomes were upregulated by L-NAME and downregulated by SNP. In addition, the production of DMD protein was decreased, as shown by immunohistochemical staining, and the expression of Jo-1, Mi-2, TLR2, and TLR4 was upregulated by L-NAME and significantly reversed by SNP. Furthermore, SNP relieved skeletal muscle injury in a notexin-induced rat model. Overall, these results suggest that NO can alleviate inflammatory responses due to skeletal muscle injury.

NIU, S. L.; JIN, X. F.; YANG, T.; CHEN, Z. H.; WANG, G. D.; LIANG, Y. & YAN, J. Efectos del óxido nítrico en las respuestas inflamatorias de las células C2C12 y la lesión muscular inducida por notexina. *Int. J. Morphol.*, 40(1):251-260, 2022.

RESUMEN: La lesión del músculo esquelético es una afección inflamatoria aguda causada por una respuesta inflamatoria. Para reducir la infiltración de células inflamatorias y aliviar la lesión del músculo esquelético es necesario un tratamiento eficaz. El óxido nítrico es una molécula de radicales libres que tiene efectos antiinflamatorios. En este estudio, demostramos que el ON podría inhibir la respuesta inflamatoria de las células C2C12 *in vitro* y proteger la lesión del músculo esquelético de rata de la notexina *in vivo*. El inhibidor de ON sintasa (L-NG-nitroarginina metil este, L-NAME) y el donante de ON (nitroprusiato de sodio deshidratado, SNP) se utilizaron para explorar el papel vital de los lipopolisacáridos (LPS) en los mioblastos C2C12 estimulados por LPS. La expresión de IL-18 e IL-1b fue regulada positivamente por L-NAME y regulada negativamente por SNP, como indican los resultados de ELISA. El ON puede reducir los niveles de proteína y ARNm de ASC, Caspasa-1 y NLRP3. Además, se detectó ON en el modelo de rata. Los resultados de la tinción inmunohistoquímica mostraron que disminuyó la producción de DMD. Realizamos qRT-PCR y transferencia Western para detectar la expresión de Jo-1, Mi-2, TLR2 y TLR4 el día 6 después de la

lesión después del tratamiento con L-NAME y SNP. La expresión de Jo-1, Mi-2, TLR2 y TLR4 fue regulada positivamente por L-NAME y significativamente revertida por SNP. El ON puede aliviar las respuestas inflamatorias de las células C2C12 en ratas, y proteger la lesión del músculo esquelético de la notexina.

PALABRAS CLAVE: Óxido nítrico; Lesión muscular; C2C12; inflammasoma; Inflamación

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