Salidroside rescues barium chloride-induced skeletal muscle injury

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Abstract

Skeletal muscle, as an important part of the human body, participates in a variety of body functions. The imbalance of homeostasis in skeletal muscle may be closely related to skeletal muscle growth or atrophy of skeletal muscle and the decrease of muscle function. Salidroside (Sal) is a bioactive component extracted from *Rhodiola rosea*. It has various pharmacological properties, including antioxidant, anti-aging and anti-fatigue properties. However, there are few studies about the effect of Sal on skeletal muscle injury induced by Barium chloride (BaCl2), which damages muscle fibers by depolarizing sarcolemmal and cause Ca2+ overload and membrane rupture. Sal significantly inhibited and reversed the decrease in cross-sectional area and diameter of muscle fibers induced by BaCl2 with HE staining, and increased the expression of myogenic differentiation 1 (MyOD1). Additionally, Sal administration enhances the regeneration potential of satellite cells and accelerates the regenerative process. In other words, Sal shows some degree of protective effects on chemical skeletal muscle injuries.

Introduction

Skeletal muscle is not only one of the largest and most important organs of the human body, but also one of the most dynamic and malleable tissues [1]. For humans, skeletal muscle accounts for about 30–40% of the total body weight [2], contains 50–75% of the whole body protein, and occupies 30–50% of the total protein conversion rate [3]. In general, skeletal muscle maintains multiple body functions by regulating cell and protein metabolism. From a mechanical point of view, the main function of skeletal muscle is to convert chemical energy into mechanical energy, in which strength and power are generated. However, from a metabolic perspective, skeletal muscle plays various roles, including maintaining core temperature and blood sugar levels, and storing amino acids for other tissues such as the skin, brain and heart to synthesize organ-specific proteins. Many different diseases or conditions may lead to muscle atrophy and loss of function, such as cancer, chronic obstructive pulmonary disease, heart failure, aging, denervation, fixation and sepsis [4]. It is a major cause of damage to the body's ability to cope with stress and chronic disease, which leads to organ disorders, failure and even death, and seriously affects the life quality. Unfortunately, the treatment of skeletal muscle atrophy remains a significant challenge. Thus, it is imperative to consider the mechanisms of the tissue homeostasis, maintenance and regeneration for the design of innovative treatment strategies.

Skeletal muscle plays an irreplaceable role in important functions such as exercise, postural support, breathing and thermogenesis. Muscle tissue is mainly composed of long, mitotic multinucleated fibers that irreversibly leave the cell cycle. As indicated by previous studies, lifelong maintenance of muscle tissue is mediated by satellite cells located near muscle fibers[5]. It is thought that the resistance to muscle degeneration may be mediated by the compensation of related structural proteins such as myotrophins or by the higher regeneration potential of satellite cells[6, 7]. Under normal circumstances, adult satellite cells are in resting state, but they can quickly re-enter the cell cycle after getting injured or receiving growth signals. In general, activated satellite cells migrate, proliferate, differentiate and fuse extensively to form regenerated muscle fibers. Importantly, the fate of satellite cells can be greatly

affected by internal and external factors, which is closely related to the dynamic interaction of inflammatory cells, stromal cells, nutritional signals and extracellular matrix (ECM) components. However, it is still a major challenge to understand the complex cellular and molecular interactions of satellite cells and their dynamic microenvironments, especially under pathological conditions. The extracts of *Rhodiola rosea* and salidroside (Sal) are the most abundant active compounds in *Rhodiola rosea* and are suggested to have beneficial effects on mental, behavioral and metabolic disorders [8]. The underlying mechanisms of salidroside's potential protective effect are the regulation of oxidative stress response, inflammation, apoptosis, neural regeneration, neurotransmission, hypothalamic-pituitary-adrenal axis and cholinergic system [9]. Our previous studies have shown that Sal alleviated denervation-induced muscle atrophy by suppressing oxidative stress and inflammation, which are two main molecular mechanisms involved in muscle atrophy[10–12]. Moreover, Sal could mitigate skeletal muscle atrophy in rats with cigarette smoke-induced COPD by up-regulating myogenin and down-regulating myostatin expression[13]. Besides, the absence of side effects makes salidroside potentially attractive as a treatment. However, little is known about molecular target of Sal and clinical trials in this area have not been sufficient. Therefore, further research on the molecular mechanism and clinical application of salidroside is urgent. Currently, the most common models for muscle injury and repair experiments include myotoxicants (neurotoxins, cardiotoxins), chemicals (barium chloride), and physical procedures (frostbite, irradiation, crush, denervation and transplantation). The muscle injury model used in this paper established by injecting barium chloride ($BaCl_2$). Intramuscular injection of $BaCl_2$ leads to muscle fiber damage and localized appearance of PMN ($Ly6G^+$ cells) and macrophages ($CD68^+$ cells) as well as removal of myotubes without reducing the number of monocytes[14]. So far, there are no good drugs to treat $BaCl_2$-induced muscle damage (PGE2 and WNT3a Promote Skeletal Muscle Regeneration after Barium Chloride Damage In-vitro).

Previous studies on skeletal muscle regeneration therapy have focused on the transplantation of healthy myogenic cells into diseased muscles or the possibility of improving the endogenous myogenic potential of satellite cells [15]. Muscle regeneration requires activation of the muscle regulatory gene network. The paired-box 7 (Pax7) is required for satellite cells to generate committed myogenic progenitors[16]. The transcription factors Pax3/7 activate the expression of myogenic factor 5 (Myf5) and myogenic differentiation 1 (MyOD1), that are required for myoblast specification. Myogenin (Myog) governs differentiation into myotubes, then myocyte enhancer factor 2 (Mef2), Myog and MyOD1 regulate terminal muscle differentiation[17]. In this study, we showed Sal treatment could increase the wet weight ratio and muscle fiber cross-sectional area of $BaCl_2$ injured muscle by accelerating the muscle regeneration. That is to say, Sal may be a potential therapeutic candidate for enhancing muscle regeneration post chemical injury, revealing the new use of Sal.

Methods

Animal treatment

In this study, the animal operations were carried out according to the recommendations of the Institutional Animal Care and Utilization Committee of Nantong University and approved by the Institutional Animal Care and Utilization Committee of Nantong University (License No. S20171202-202). Healthy 6-week-old male Institute of Cancer Research (ICR) mice weighing 20g were ordered from the Experimental Animal Center of Nantong University. The mice lived at 24 ℃ and 55 ± 5% humidity and cycled in light / dark for 12 h. Mice were randomly divided into 5 groups: sham operation group (Normal), $BaCl_2$ injury group (Ba-3d, Ba-7d), and Sal treatment post $BaCl_2$ injury group (Ba + Sal-3d, Ba + Sal-7d). Mice were anesthetized with intraperitoneal injection of mixed narcotics (100 mg/kg ketamine plus 10 mg/kg xylazine) before BaCl2 injury. 30 μL 1.2% $BaCl_2$ was injected into the tibialis anterior muscle only once to induce a chemical muscle injury. 20 mg/kg Sal or saline was administrated by intraperitoneal injection following $Bacl_2$ injury and lasted for 3 or 7 consecutive days. The optimal dose of Sal determined by results of our previous work and the pilot study[10].

Sham surgery was performed on the contralateral limb as normal group. The mice were sacrificed under anesthesia through cervical dislocation, and the TA muscle tissue was taken, weighed, and frozen in liquid nitrogen for further study. The wet weight ratio of muscle was calculated by the injury side compared with the contralateral side.

Hematoxylin&eosin (H&E)

The tissue which fixed with 4% paraformaldehyde was embedded in paraffin to prepare paraffin section at a thickness of 6 μm. There are 3 mice per group, 5 slices per muscle. The sections were then dewaxed in xylene and hydrated with graded ethanol series before being stained with haematoxylin and eosin (H&E). After hematoxylin and eosin staining, the morphological characteristics of skeletal muscle cross sections were observed using ImageJ [18].

Immunocytochemical staining

This research prepared the both sides of tibialis anterior muscle samples and fixed them at room temperature with 4% PFA for 30 min, washed with phosphate buffer (PBS) for 15 min, and then blocked with immunostaining buffer (Beyotime, Haimen, China) at room temperature for 1 hour. The anti-eMyHC antibody (AB_528358; Mouse, 1:200, DSHB) and anti-Laminin antibody (ab11575, rabbit, 1:1000, Abcam, Cambridge, UK) were incubated for 3 hours at room temperature. The secondary antibody Donkey anti-goat IgG-Cy3 (Red, Ab97117; 1:500, Sigma, St Louis, MO) and donkey anti-rabbit IgG-FITC (Green, ab6798, 1:1000, Abcam) were further added to allow incubation. Besides, the nucleus was labeled with Hoechst 33342 (Abcam). There are 3 mice per group, 5 slices per muscle. The reaction was observed under the Zeiss imager M2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). ImageJ (Rawak Software Inc., Stuttgart, Germany) was utilized to detect the morphology of tibialis anterior (TA) muscle [19].

Muscle fiber cross-sectional area (CSA) and diameter

CSA of skeletal muscle fibers was detected by laminin staining. The muscle was fixed and frozen quickly. Meanwhile, 10 μm-thick slices were made on a cryo thermostat. The sections were incubated with anti-

laminin antibody (ab11575, Rabbit, 1:1000, Abcam) at 4°C overnight. On the second day, the slices were washed and incubated with Alexa Fluor secondary antibody (Green, ab6798, 1:1000, Abcam) at room temperature for 30 min. Then, the sections were imaged by fluorescence microscope (Zeiss, Germany), and the CSA and diameter of muscle fibers were obtained by ImageJ software (NIH, Bethesda, MD, USA) through blinded analysis of five randomly captured images [20].

Western blot Analysis

The total protein was extracted with RIPA lysis buffer (Beyotime, Nantong). After centrifugation, soluble protein was quantified by BCA kit and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (P0015L, Beyotime, Shanghai, China). Subsequently, the protein in the gel was transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Corp, Billerica, MA). The membrane was blocked with 5% non-fat dry milk (BBI Life Sciences, Shanghai, China) at room temperature for 2 hours, and incubated overnight at 4°C with the anti-MyOD1 antibody (ab203383, Rabbit, 1:1000, Abcam). According to the recommendations of manufacturer, antibody binding was detected with a species-specific secondary antibody bound by horseradish peroxidase (HRP) and goat anti-rabbit IgG H&L (ab205718, 1: 2000, Abcam). Moreover, antibody binding of the membranes was visualized by enhanced chemiluminescence western detection system (Beyotime Biotechnology) and detected by a digital chemiluminescence scanner (Thermo Scientific, Park Ellisville, MO). There are 3 mice per group. The intensities of the immunoblot bands were detected with Image Studio Version software (version 5.2, LI-COR Biosciences) [21].

Statistical Analysis

All the data in this study were analyzed by unpaired Student t test. Additionally, all statistical analyses in this study were carried out using GraphPad Prism software (version 7. 0) (San Diego, California, USA). $P < 0.05$ indicates that the difference is statistically significant [22].

Results

Effects of Sal on skeletal muscle after $BaCl_2$ injury

As is shown in the flow chart of the protocol (Fig. 1A), mouse in the experimental group was injected with $BaCl_2$ in tibialis anterior muscle (TA) for once, then each mouse in treatment group was intraperitoneally injected with 0.4 mg Sal per day. Significant inflammatory infiltration and rhabdomyolysis were observed on the third day after $BaCl_2$ injury [23, 24]. The integrity of TA muscle fibers in Sal treated group was significantly better than that in control group (Fig. 1B). Quantitative histogram suggested that the percentage of regenerating centronuclear fibers (Fig. 1C), the cross-sectional area of tibialis anterior muscle fiber (Fig. 1D), the diameter of tibialis anterior muscle fiber (Fig. 1E) and the wet weight ratio (Fig. 1F) in the Sal treatment group were significantly higher than those in the control group on day 7, revealing that salidroside treatment could promote skeletal muscle regeneration.

Sal enhances the regeneration ability of muscle fibers after $BaCl_2$ injury

eMyHC is a marker of newly forming fibers, which is used to assess muscle regeneration/remodeling. The previous literature suggested that regenerated fibers are formed 7 days after injury, and will grow in size to the moment when the regenerative process is considered finished about 1-month post-injury [25], which was consistent with immunofluorescence staining of eMyHC (Fig. 2A). Laminin is a constituent of the interstitium and basement membrane of cells. Its biological function is the medium for cells to adhere to the matrix and combine with various basement membrane components to regulate cell growth and differentiation. In the Fig. 2A, the cross-sectional morphology of muscle fibers is clearly shown in Laminin staining. Quantitative histogram displayed that the diameter (Fig. 2B) and cross-sectional area of $eMyHC^+$ regenerated fibers (Fig. 2C) in Ba + Sal group were significantly greater than those in group Ba, suggesting that Sal could improve muscle fiber regeneration after $BaCl_2$ injury.

Sal promotes muscle fiber regeneration by activating satellite cells after $BaCl_2$ injury

In order to further study how Sal improve muscle regeneration post $BaCl_2$ injury, we detect the expression changes of MyOD1 in tibialis anterior muscle of mice in different experimental groups. Stationary satellite cells undergo rapid activation after injury[26]. Then myogenic determinant MYOD is up-regulated, which is a key regulator of skeletal muscle differentiation [27]. The upregulation of MyoD1 leads to the activation of myogenin expression and promotes the differetiation of satellite cells into muscle cells. In other words, MyoD1 expression is associated with differentiation and can be used as an effector of the terminal differentiation of established muscle cells [28]. Herein, the expression of MyOD1 increased, promoting the repair of tibialis anterior muscle after injury. After the administration of Sal, the expression of MyOD1 increased significantly compared with the injury group (Fig. 3). MyOD1 and myogenin are regulatory transcription factors expressed early in skeletal muscle differentiation, and could regulate downstream essential genes for muscle fusion and myoblasts [29, 30]. Accordingly, the high expression of myogenic regulatory factor MyOD1 suggested that Sal further enhanced the regeneration ability of tibialis anterior muscle after $BaCl_2$ injury.

Discussion

Skeletal muscle exhibits a high degree of plasticity and adaptability [31]. Chemical damages are usually observed to produce specific and replicable damage phenotypes in vivo so as to ensure the accuracy of follow-up studies. Literatures have shown that $BaCl_2$-induced muscle injury models are comparable to traditional models of muscle injury and regeneration considering possible systemic effects [24, 32]. Local injection of $BaCl_2$ is regarded as an acute injury model for studying skeletal muscle regeneration. It is assumed that $BaCl_2$ induces muscle fiber depolarization, resulting in Ca^{2+} overload, proteolysis and

membrane destruction, because Ba^{2+} inhibits K^+ channels [33, 34]. This suggests that the $BaCl_2$-based chemical damage model could provide us with the convenience of exploring the mechanism of skeletal muscle regeneration. To our surprise, the ratio of wet weight in the muscles 7 days after injury is lower than that of 3 days. There might be two reasons. On one hand, hemorrhagic edema persistent at day 3 and gradually subsided during the regeneration process. One the other hand, hemorrhagic inflammation with mononuclear infiltration peaked at day 3 and mostly cleared at day 7[23, 24]. In this study, the cross-sectional area and mean diameter of new muscle fibers, the number of central nuclear muscle fibers, and the wet-weight ratio of tibialis anterior muscle in the Sal group were significantly higher than those in the untreated group, indicating that Sal treatment could promote skeletal muscle regeneration. What's more, western blot results showed that high expression of myogenic regulator MyOD1 further indicated that Sal enhanced the regenerative potential of tibialis anterior muscle after BaCl2 injury. Of note, the sample size was small. We will expand the sample size and adjust the data analysis method in the subsequent mechanism study to further strictly examine the therapeutic effect of Sal on chemical muscle injury.

Previous study has shown that Sal reduced the levels of MDA, IL-6 and TNF-α in serum and skeletal muscle tissue of rats, and increases the expression of myogenin in skeletal muscle tissue by inhibiting oxidative stress and inflammatory response, increasing antioxidant and regulating the expression of muscular-specific transcription factors [13]. In addition, it has been reported that salidroside has a cell-protective effect. Salidroside promotes the paracrine function and migration potential of the skeletal muscle in hind limb ischemic mice through the simultaneous induction of various factors [35, 36].

However, in many pathological conditions, satellite cells are constantly exposed to an activated environment. The primary culture of myoblasts from malnourished muscles showed much lower proliferative potential than healthy myoblasts in vitro, which was aggravated by aging. Studies have shown that intramuscular injection of Wnt7a contributes to the increase of satellite cell number, muscle fiber size and muscle strength [37, 38]. The pharmacological inhibition of p38 MAPK may be another strategy to reactivate senescent satellite cells. P38 inhibitors have been used in various inflammatory conditions in different clinical trials [5]. Similarly, JAK/STAT inhibitors have been shown to restore the regenerative potential of aging muscle satellite cells [39]. However, the use of systemic inhibitors greatly increases the chance of adverse side-effects, and the interconnection of these pathways complicates the molecular mechanism of satellite cell-mediated skeletal muscle regeneration.

Some evidence suggests that apart from changes in the extracellular environment, internal changes may affect the function of satellite cells [40]. Many proteins have been found as markers of satellite cells, such as Pax3 and Pax7 in the nucleus[26]. In adult muscle, Pax7 is highly and persistently expressed in satellite cells, while Pax3 is usually expressed at a very low level, except in muscle subsets such as the diaphragm. Pax7 deficient mice showed gradual loss of satellite cells, decreased fiber size, and impaired muscle regeneration. Other transcription factors such as myogenic factor 5 (Myf5) and myogenic differentiation 1 (MyOD1) can be specifically expressed by myogenic cells, but not in adult resting satellite cells [28, 41]. These results clearly demonstrate the importance of microenvironment and macroenvironment in regulating the myogenic potential of satellite cells. In this study, western blot was

used to detect the expression of MyOD1 in the tibialis anterior muscle mice in different experimental groups. The muscle satellite cells increased significantly after 3 days of injury, and the expression of MyOD1 also increased, promoting the repair of tibialis anterior muscle after injury. After the administration of Sal, the expression of MyOD1 increased significantly compared with the injury group. It indicates that Sal treatment can further stimulate the activation of satellite cells and improve the regeneration ability of tibialis anterior muscle. More myogenic differentiation markers and multiple more time points post muscle injury are warranted in future's work.

Conclusion

Salidroside administration enhances the regeneration potential of satellite cells and accelerates the regenerative process. To conclude, salidroside provide some degree of protective effects on chemical skeletal muscle injuries.

Declarations

Ethical approval and consent to participate

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal operations were carried out according to the recommendations of the Institutional Animal Care and Utilization Committee of Nantong University and approved by the Institutional Animal Care and Utilization Committee of Nantong University (License No. S20171202-202). The authors have completed the ARRIVE reporting checklist.

Consent for publication

Not applicable.

Competing interest

The authors have no conflicts of interest to declare.

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Author contribution

(I) Conception and Design: S Zhou, H Shen; (II) Administrative support: S Zhou; (III) Provision of study materials or patients: S Zhou, H Shen, Y Shen; (IV) Collection and assembly of data: Y Shen, H Xie, L Wang, H Liu, W Wang, Y Ji, M Yu, Y Shen; (V) Data analysis and interpretation: Y Shen, H Xie, H Shen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Availability of data and materials

The datasets during and/or analysed during the current study is available from the corresponding author on request.

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Figures

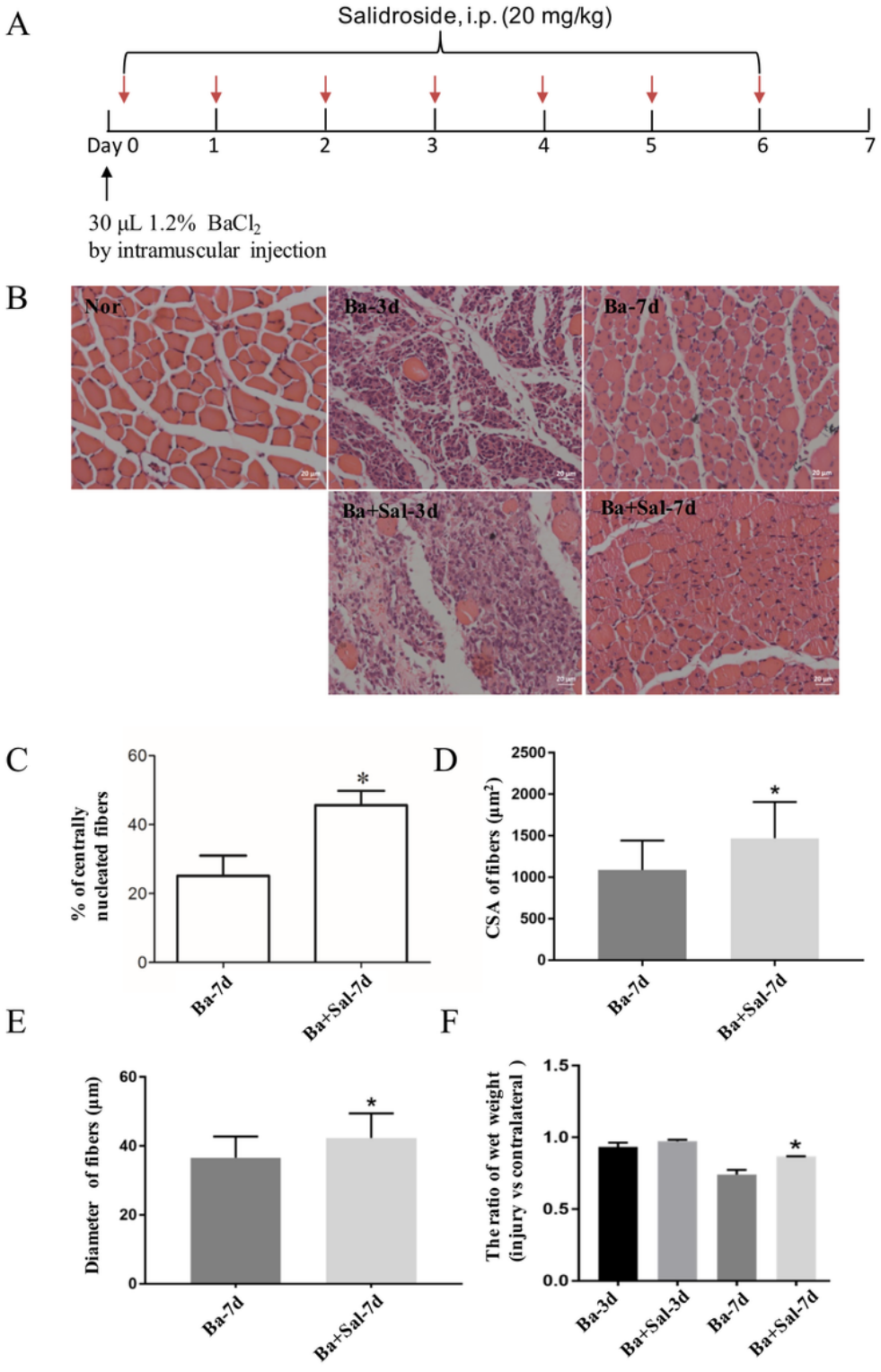

Figure 1

Morphological changes of target muscles after applying Sal to BaCl2 injury. (A) The flow chart of the protocol showed the timing of injury and salidroside administration. (B) HE staining morphological map of tibialis anterior in each group. (Nor group: normal group; Ba: BaCl2 injury group; Ba+Sal: Sal treatment post BaCl2 injury group). Scale bars = 20 μm. (C) Percentage of central nucleated fiber. (D) CSA of muscle fibers. (E) Diameter of muscle fibers. (F) The ratio of muscle wet-weight,the ratio was calculated

by the injury side compared with the contralateral side. *, P < 0.05. Sal, salidroside; Ba, BaCl2; HE, Hematoxylin&eosin. CSA, Cross-sectional area.

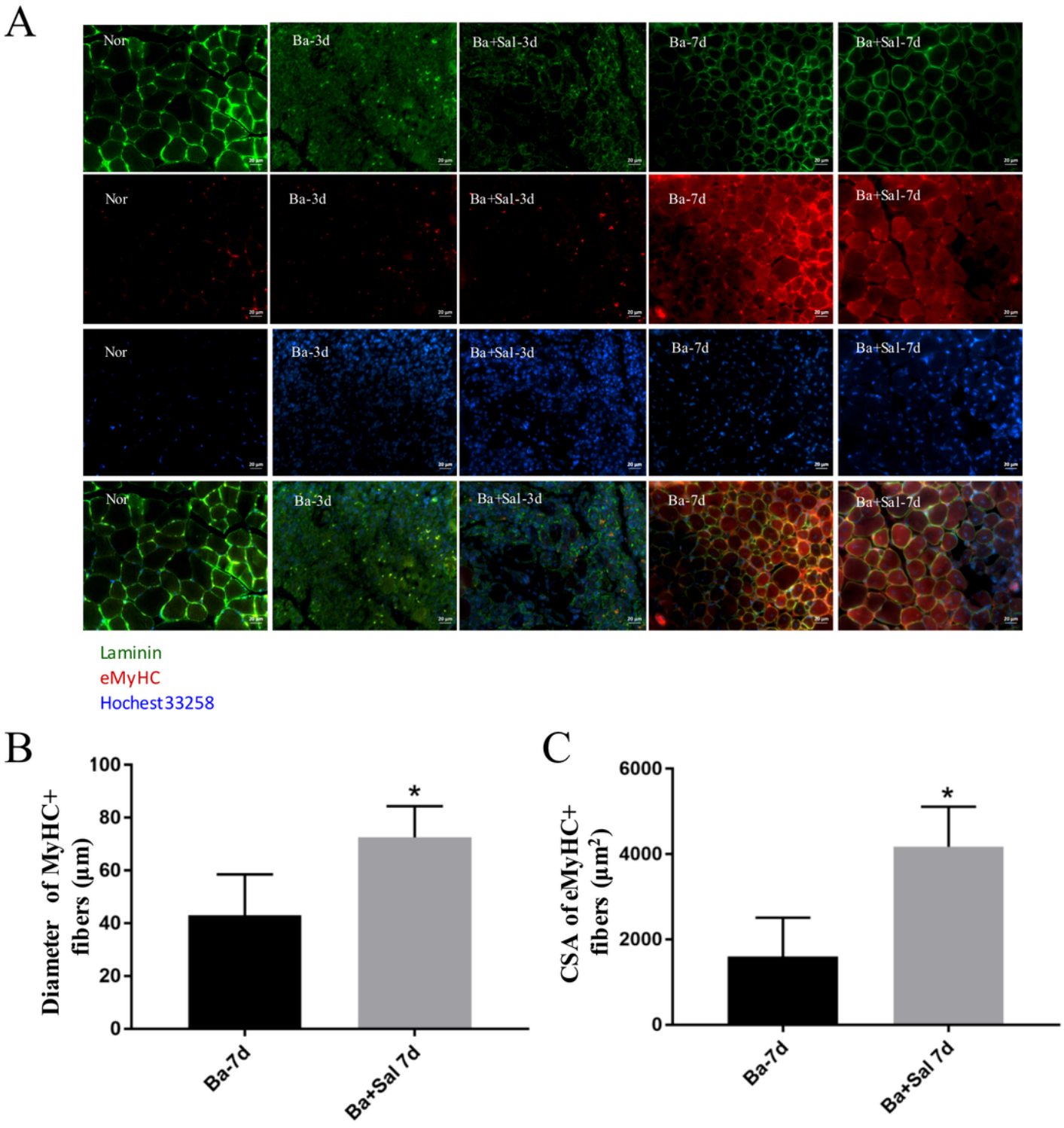

Figure 2

Effect of Sal on muscle fiber regeneration after BaCl2 injury. (A) TA eMyHC and Laminin staining morphological map. Scale bars = 20 μm. (B) Diameter of eMyHC+ fibers, *, P<0.05. (C) CSA of eMyHC+

fibers. *, P<0.05. Sal, salidroside; eMyHC, Embryonic myosin heavy chain; TA, tibialis anterior.

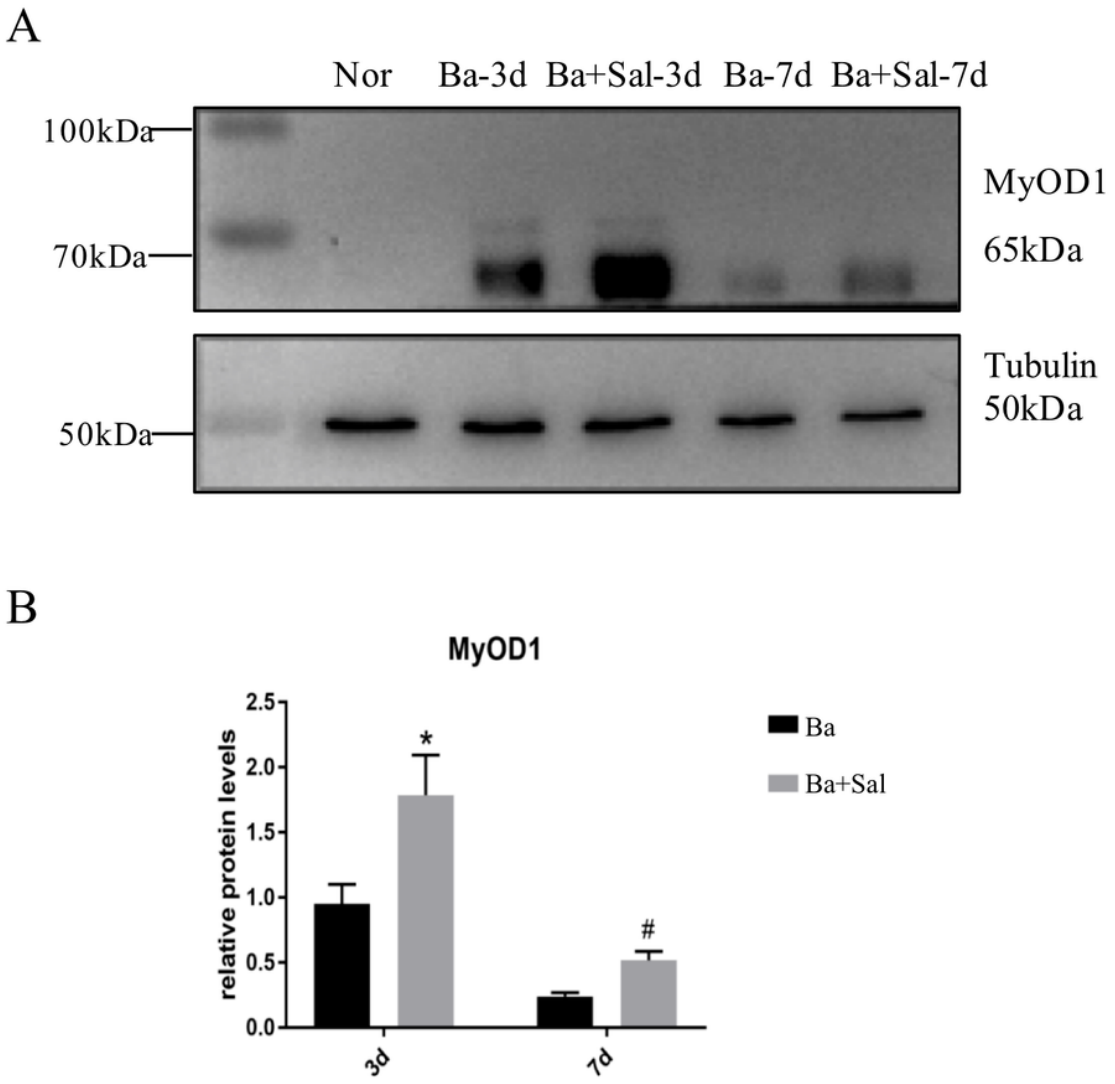

Figure 3

Sal enhances the regeneration ability of muscle fibers after BaCl2 injury. (A) Western blot was used to detect the expression of MyOD1 in tibialis anterior muscle in different experimental groups. Protein quantitative analysis showed that the expression of MyOD1(B) was significantly higher than that of the injury group on the 3rd and 7th day. n=3. * indicates P < 0.05. Sal, salidroside; MyOD1, myogenic differentiation 1.