Systemic and Local Phenotypes of Barium Chloride Induced Skeletal Muscle Injury in Mice

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Skeletal muscle regeneration in mice has traditionally been studied using local freeze burn or snake venom injection models. More recently, a barium chloride ($BaCl_2$)-induced muscle injury model has been established and is gaining popularity due to the relatively simple procedure and accessibility to required reagents. Here we sought to characterize the local and systemic effects of $BaCl_2$-induced muscle injury. For this study, a 1.2% $BaCl_2$ solution was locally administered to the tibialis anterior (TA) muscle and local and systemic phenotypes were analyzed at different timepoints. When 50 μL of the solution was injected unilaterally in the TA muscle, no mortality was observed. However, when 100 μL of the solution was injected, 50% of the mice died within 24 h. Serum analysis of the mice injected with 50 μL of $BaCl_2$ solution at days 1 and 7 revealed changes resembling rhabdomyolysis. At day 1 post-injection of 50 μL of the $BaCl_2$ solution, acute suppurative inflammation was observed in gross examination of the TA muscle, while extensive hemorrhagic necrosis was revealed on histological examination. At day 7, regenerated myofibers with centralized nuclei appeared with the resolution of acute inflammatory infiltration and the muscle tissue displayed molecular signatures consistent with myofiber differentiation. The overall muscle injury and regeneration phenotypes in the $BaCl_2$-induced muscle injury model were similar to those of the well-established freeze burn or snake venom injection models. Taken together, the $BaCl_2$-induced muscle injury model is comparable to conventional muscle injury and regeneration models, with considerations for possible systemic effects. **(Ann Geriatr Med Res 2019;23:83-89)**

Key Words: Muscle regeneration, Barium chloride, Phenotype

INTRODUCTION

In living organisms, tissue injury and repair processes have evolved to restore tissue homeostasis as a response to damage and insults arising from both extrinsic and intrinsic factors.[1] Unlike many non-vertebrates and some vertebrates such as salamanders, the tissue repair capabilities in mammals are relatively limited. Tissue regeneration processes in vertebrates require adult stem cells, which are maintained and regulated through complex interactions between the stem cells and cellular and humoral components of the surrounding milieu,[2] and these interactions are often affected by biological events such as aging. In clinical medicine, understanding how appropriate tissue regeneration processes occur without organ function impairment and systemic adverse effects has important implications in the prognosis and treatment of disease.[3]

Skeletal muscle is one of the most widely studied tissues in regenerative biology due to its accessibility to experimental injury models and its ability to regenerate efficiently in a relatively short timeframe of 14 days in laboratory mice.[4,5] Furthermore, the clinical importance of alterations in skeletal muscle in older adults provides strong motivation to study skeletal muscle regeneration processes within the context of aging.[6] In the pathophysiology of sarcopenia, a common geriatric condition defined by reduced muscle strength, performance and/or muscle mass, multifaceted causes including sedentary lifestyle, poor nutrition, hormonal changes with aging, and systemic inflammation have been reported to accelerate muscle wasting in older adults.[7-10] Biologically, one of the contributing factors for sarcopenia is a decreased ability of aged muscle to regenerate following injury,[11,12] prompting research to study the effects of aging on muscle stem cells and its paracrine milieu in muscle regeneration processes after injury.

In studying the skeletal muscle regeneration process, animal models commonly use direct injuries to individual

muscles such as the tibialis anterior (TA) or extensor digitorum longus (EDL).[13,14] Historically, the local injection of snake venoms such as cardiotoxin or notexin, which induce prolonged depolarization and disruption of cellular membranes, has been a popular and common method to induce muscle injury and regeneration.[5] In the typical course of this muscle injury model, exudative acute inflammation with mononuclear cell infiltration occurs 12–24 h after toxin injection, with subsequent myogenic proliferation and new myotube formation with centralized nuclei occurring 5–6 days after the initial injury. Thereafter, myofiber maturation occurs until 10–14 days after the initial injury.[5,15]

A skeletal muscle injury model provided by the local injection of barium chloride solution (BaCl$_2$) has recently been established and is gaining popularity as a model for studying muscle regeneration.[16] BaCl$_2$ has advantages over previous reagents to induce muscle injury due to its ease of purchasing and transporting without the regulatory restrictions that apply to snake venoms. BaCl$_2$ has been traditionally used as a potassium channel blocker in experimental physiology[17] and is thought to induce myonecrosis in muscle tissue by eliciting prolonged depolarization of myofibers, analogous to snake venoms. However, compared to snake venoms, the detailed local effects of BaCl$_2$ across a time course, including both gross and microscopic morphology, have been less studied. In addition, although its systemic toxicity[18] (median lethal dose [LD50] of 19.2 mg Ba^{2+}/kg in ICR mice) associated with arrhythmogenic effects has been reported,[19] BaCl$_2$-induced muscle injury models have been largely considered a locally specific method for the study of muscle regeneration. Therefore, this study sought to assess the acute systemic effects after BaCl$_2$ injection in mouse TA muscle along and to perform a detailed analysis of local phenotypes during injury repair along a temporal scale.

MATERIALS AND METHODS

Animals

The animal experiments in this study were approved by the Institutional Animal Care and Use Committee at the Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea (approval number of KA2015-34). Male C57BL6/J mice were maintained in a specific pathogen-free animal facility at KAIST and were housed at room temperature (24°C) under a 12 h light/dark cycle. All mice were allowed access to water and a standard chow diet *ad libitum*. Mice between 12 and 15 weeks of age were used for the experiments.

Muscle Injury Procedure

All muscle injury procedures were performed under inhaled anesthesia using sevoflurane with a mixture of oxygen with room air. In studying acute survival associated with BaCl$_2$ injection, 100 μL or 50 μL of a 1.2% BaCl$_2$ solution

dissolved in phosphate buffered saline (PBS) was injected to the TA muscles of sedated mice. Since we anecdotally observed that mice inadvertently given a higher dose of BaCl$_2$ solution deceased one day after injection, we selected 100 μL and 50 μL doses for survival analysis. For the study of the local phenotypes and levels of serum markers, 50 μL of a 1.2% BaCl$_2$ solution dissolved in PBS was injected into the TA muscles of sedated mice. The contralateral hindlimbs served as untreated controls.

Blood Chemistry

At day 0, as a control group before BaCl$_2$ injection, and at days 1 and 7 after BaCl$_2$ injection, whole blood was collected in serum-separating tubes by cardiac puncture. From the separated serum after centrifugation, levels of chemical markers including aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, creatinine kinase (CK), creatinine, glucose, C-reactive protein (CRP), and lactate dehydrogenase (LDH) were measured using a Cobas 8000 modular analyzer system (Roche, Basel, Switzerland) according to the manufacturer's protocol.

Tissue Harvest, Histological Analysis, and RNA Extraction

Upon euthanasia and sacrifice of the mice, the TA muscles were quickly harvested at 1, 3, 5, 7 days after BaCl$_2$ injection. For histological analysis, the muscles were fixed in neutral buffered formalin overnight and then processed and embedded in paraffin for hematoxylin and eosin staining. Total RNA from TA muscles 7 days after BaCl$_2$ injection was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After extraction, RNA quality was assessed on a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RNA integrity numbers (RINs) above 7.0 were used for RNA sequencing.

RNA Sequencing

For RNA sequencing, libraries were prepared with an Illumina TruSeq Sample Preparation Kit and sequenced on a NovaSeq6000 system (Illumina, San Diego, CA, USA) with multiplexing to produce more than 40 million reads per sample. The reads were converted to fragments per kilobase of transcript per million (FPKM) after alignment to the reference mouse genome. We considered differentially expressed genes with at least two-fold changes in expression values between groups.

Protein Extraction and Western Blot Analysis

Protein lysates were prepared from TA muscles in control mice and test mice 1, 4, and 7 days after BaCl$_2$ injection using RIPA lysis buffer (Thermo Fischer Scientific, Wilmington, DE, USA) containing a protease inhibitor cocktail. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific); the samples were denatured in sodium dodecyl sulfate (SDS) loading

buffer, separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The transferred membranes were examined through Ponceau S staining. Following blocking with 3% bovine serum albumin for 30 min, the membranes were incubated overnight at 4°C with anti-β-catenin (1:1,000, #8480), anti-phospho-ERK (1:1,000, #9101), anti-ERK (1:1,000, #9102), anti-phospho-S6 (1:1,000, #2211), anti-S6 (1:1,000, #2217), and anti-α-tubulin (1:2,000, #2144) antibodies. After incubation with horseradish peroxidate (HRP)-conjugated host-specific secondary antibody, the resulting blot was visualized on a ChemiDoc XRS+ system (Biorad, Hercules, CA, USA). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical Analysis

For statistical comparison, values between groups were compared using independent t-tests. For acute systemic toxicity, survival analysis was performed with log-rank tests and Kaplan–Meier plots. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA), with two-sided p-values <0.05 considered statistically significant.

RESULTS

Systemic Effects of the BaCl$_2$-Induced Muscle Injury Model

We first assessed for possible acute systemic toxicity with local BaCl$_2$ injection of TA muscle. Upon injection of 50 μL or 100 μL of a 1.2% BaCl$_2$ solution, four of eight mice in the 100 μL group died within 24 h after injection, while none of the eight mice in the 50 μL group died (Fig. 1A). Observation of the mice until 15 days after injection revealed no subacute mortality after the initial 24 h in both groups.

Gross examination of separated serum color showed a bright red color at days 1 and 7 after injection of 50 μL BaCl$_2$ to the TA muscle (Fig. 1B), indicative of myoglobinemia. Accordingly, significant increases in serum CK and LDH levels were observed at days 1 and 7, compared to those in the uninjected controls (Fig. 1C). There were similar patterns of increased LDH and AST at days 1 and 7, tracking with serum CK levels, suggesting that the increase in AST and LDH was due to acute myonecrosis. There was no statistical difference in ALT creatinine levels between the control, day 1, and day 7 groups (Fig. 1C). The serum CRP levels were less than 0.5 mg/L in all three groups (data not shown).
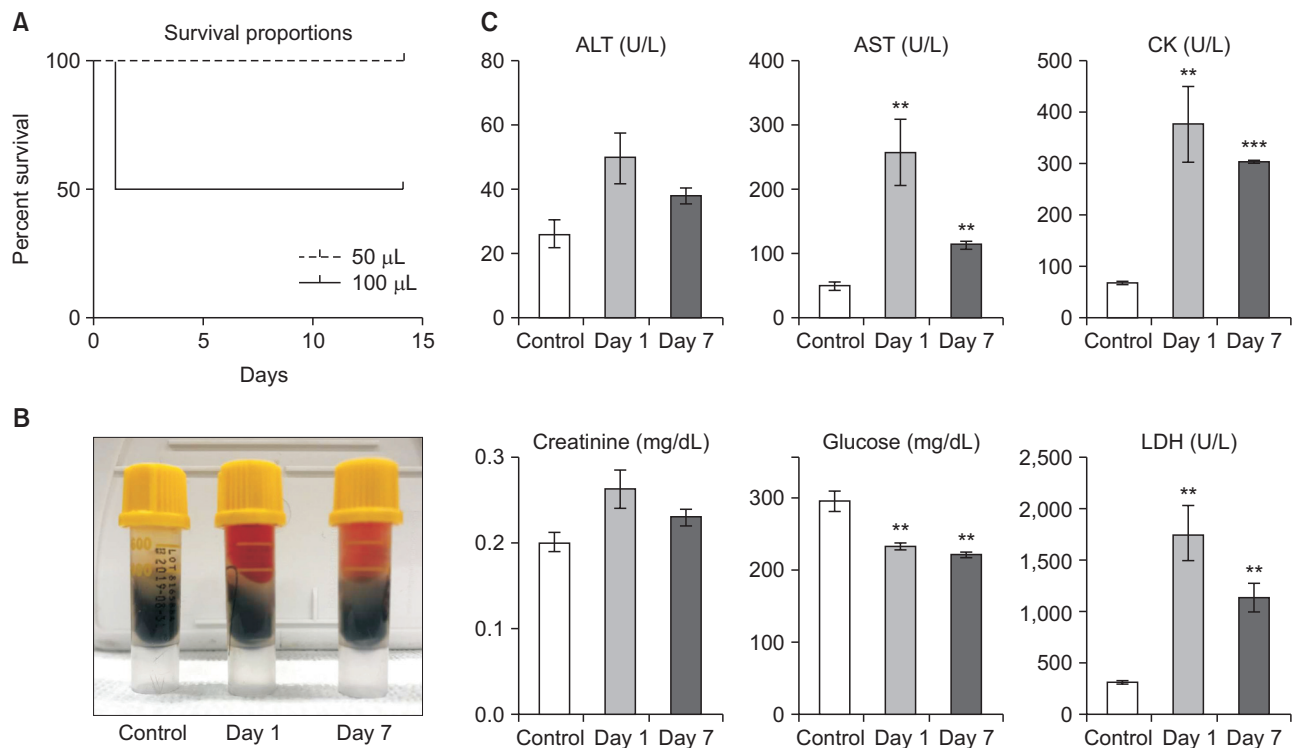
Fig. 1. Systemic phenotypes of the barium chloride (BaCl$_2$)-induced muscle injury model. (A) Kaplan–Meier survival plot of mice that underwent BaCl$_2$ injection of the ipsilateral tibialis anterior muscle (n=8 for each group, log-rank p-value <0.001). (B) Red-colored serum at days 1 and 7 after BaCl$_2$ injection after centrifugation. (C) Analysis of serum biochemical markers from mice at days 1 and 7 after BaCl$_2$ injection, with uninjured mice (control). ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatinine kinase; LDH, lactate dehydrogenase. **p<0.01, ***p<0.001.

Local Gross and Microscopic Morphology in the BaCl$_2$-Induced Muscle Injury Model

To examine the temporal changes in the gross and histological morphologies of TA muscle after BaCl$_2$-induced muscle injury, we harvested TA muscle at 1, 3, 5, and 7 days after local BaCl$_2$ injection (50 μL) and performed hematoxylin and eosin staining (Fig. 2). Grossly, hemorrhagic edema peaked at day 1 and gradually subsided during the regeneration process. Histologically, hemorrhagic inflammation with pronounced exudation of red blood cells was observed at day 1 and mononuclear infiltration peaked at day 3. At day 5, large numbers of small myofibers with centralized nuclei appeared and mononuclear infiltration was mostly cleared at day 7.

Molecular Signatures of Muscle Regeneration in the BacI$_2$-Induced Muscle Injury Model

We assessed the gene expression profile of muscle regeneration from RNA sequencing data comparing regenerating TA muscles at 7 days after BaCl$_2$-induced injury to uninjured muscles (n=4 for each group). The expression profiles of genes reflecting proliferating cells showed mixed results, with *Pax7* and *Myf5* significantly increased at day 7 but *Myod1*, *Foxk1*, *Des* showing no significant difference when compared to uninjured muscles (Table 1). In contrast, genes reflecting differentiating muscle stem cells (satellite cells) including *Fgfr4*, *Myh3*, *Atp2a2*, *Tnnt1*,
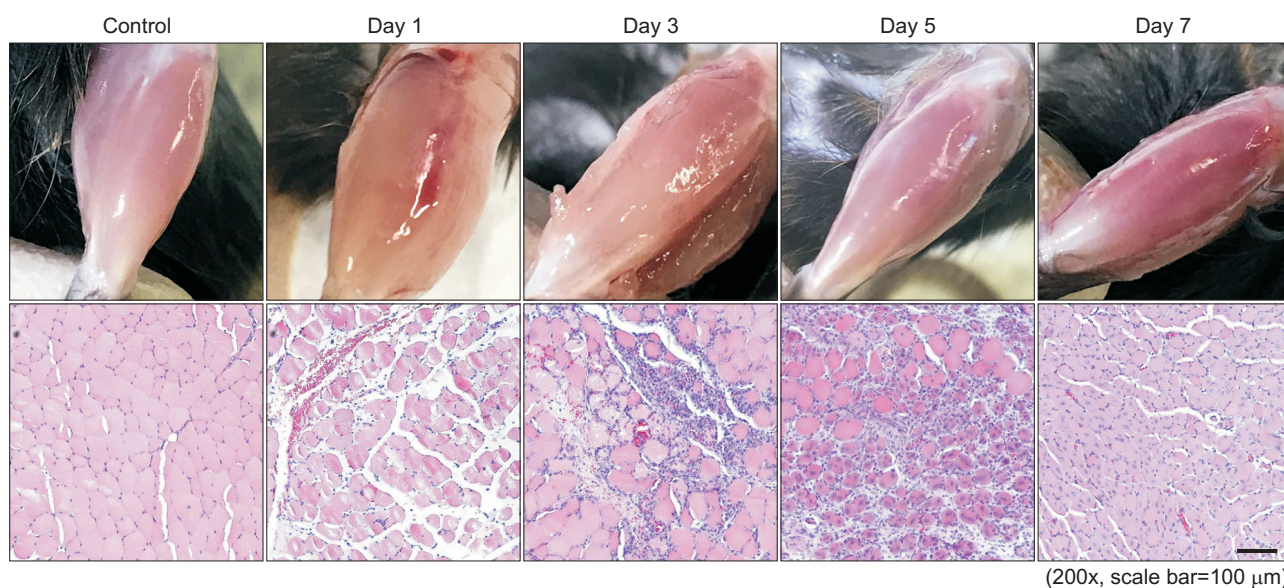

Fig. 2. Representative gross morphology and hematoxylin and eosin-stained sections of tibialis anterior muscle at days 1, 3, 5, and 7 after barium chloride (BaCl$_2$) injection, with uninjured muscle (control).

Table 1. Expression of selected genes from RNA sequencing, comparing tibialis anterior muscles at 7 days after BaCl$_2$ injection to uninjured tibialis anterior muscles (n=4 for each group)

Gene ID	Gene name	Log$_2$FC	Log$_2$FC SE	p-value
Markers of proliferating satellite cells				
ENSMUSG00000028736	*Pax7*	1.91	0.28	2.41×10^{-10}
ENSMUSG00000000435	*Myf5*	2.23	0.28	2.15×10^{-13}
ENSMUSG00000009471	*Myod1*	0.42	0.24	0.163
ENSMUSG00000056493	*Foxk1*	-0.27	0.38	0.630
ENSMUSG00000026208	*Des*	0.34	0.28	0.374
Markers of differentiating satellite cells				
ENSMUSG00000005320	*Fgfr4*	2.14	0.31	1.64×10^{-10}
ENSMUSG00000020908	*Myh3*	6.66	0.86	6.51×10^{-13}
ENSMUSG00000029467	*Atp2a2*	1.70	0.48	0.002
ENSMUSG00000064179	*Tnnt1*	2.27	0.52	9.93×10^{-5}
ENSMUSG00000048583	*Igf2*	5.94	0.30	1.09×10^{-84}
ENSMUSG00000027107	*Chrna1*	2.36	0.25	4.55×10^{-19}
ENSMUSG00000028364	*Tnc*	4.61	0.61	2.40×10^{-12}
ENSMUSG00000026459	*Myog*	4.26	0.35	6.48×10^{-32}

FC, fold change; SE, standard error.
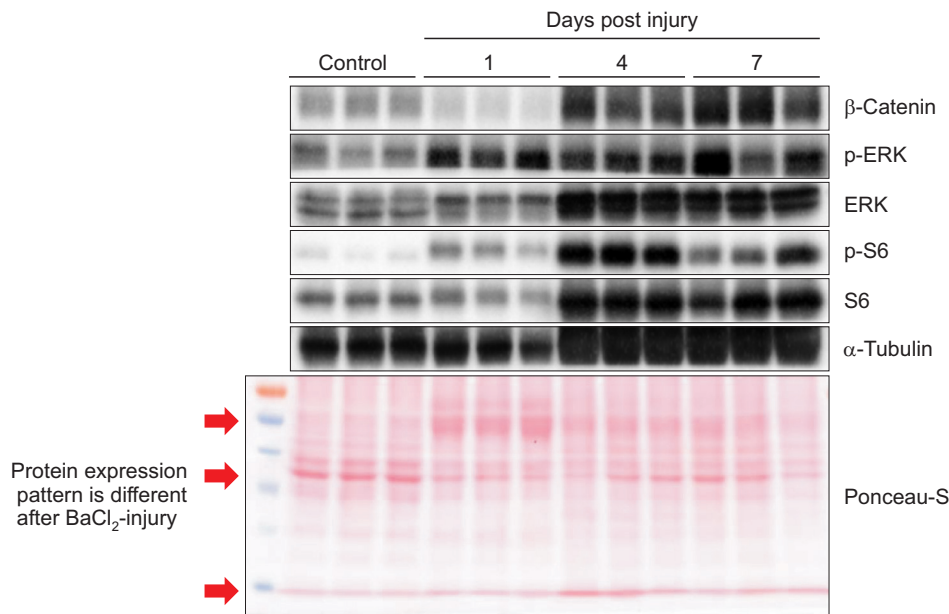
Fig. 3. Protein expression in tibialis anterior muscle at days 1, 4, and 7 after barium chloride ($BaCl_2$) injection compared to uninjured muscle (control), examined by western blotting. α-Tubulin was used as the loading control (n=3 for each group).

Igf2, *Chrna1*, *Tnc*, and *Myog* were consistently activated at day 7.

To assess cellular proliferation and protein synthesis activity at days 1, 4, and 7 after $BaCl_2$ injection, western blot analysis was performed against proteins known to be activated during muscle regeneration. The levels of β-catenin, phospho-β-catenin, phospho-S6, and phospho-ERK were increased in the $BaCl_2$-injected muscle at days 4 and 7 compared to those in control muscle (Fig. 3).

DISCUSSION

This study assessed the systemic and local phenotypes of the $BaCl_2$-induced muscle injury model. Using varying $BaCl_2$ doses to induce muscle injury, we found that the experimental window in terms of injection dosage was limited as acute mortality in 50% of mice was observed when 100 μL of a 1.2% $BaCl_2$ solution was injected into TA muscles. Also, systemic rhabdomyolysis was observed based on serum color and blood chemistry after $BaCl_2$-induced TA injury. Locally, the injury and regeneration processes of $BaCl_2$ were largely similar to the previously reported phenotypes produced by injecting snake venoms.

In most biological studies, muscle injury and regeneration models have been proposed as a strictly tissue-specific, local method to study regenerating mechanisms focusing on muscle stem cells and their local environments.[2,5] Although many studies have analyzed the effect of non-tissue autonomous factors such as aging,[20] endocrine alterations,[21] and systemic inflammation[22] on muscle regeneration models of laboratory animals, studies on the systemic effects of muscle injury have been mostly confined to human clinical studies.[23] As a result, the possible systemic effects of various muscle injury models in mice have been not been carefully assessed. In the present study, we observed two distinct systemic effects of $BaCl_2$ injection model.

First, high-dose $BaCl_2$ injection, with a two-fold increase in dosage, resulted in 50% of the mice dying within 1 day after injection. Since $BaCl_2$ is a known arrhythmogenic agent acting as potassium channel blocker with an LD50 of 19.2 mg Ba^{2+}/kg in ICR mice,[18,24] 100 μL of 1.2% $BaCl_2$ corresponds to 31.8 mg Ba^{2+}/kg, which might have been enough to cause fatal arrhythmia. Moreover, in standard experiments of $BaCl_2$-induced muscle injury using 50 μL of a 1.2% $BaCl_2$ solution (15.9 mg Ba^{2+}/kg), mice may suffer cardiac arrhythmias which may cause alterations in systemic physiology compared to that in uninjected control mice, although these arrhythmias may not lead to mortality. This effect on survival is likely specific to $BaCl_2$ as other muscle injury methods do not report such systemic effects and the mechanisms of muscle injury are different.

Second, rhabdomyolysis-like features were observed in the analysis of serum at days 1 and 7 after $BaCl_2$ injection. After centrifugation, the serum supernatants presented with a bright red color at days 1 and 7, with elevated serum CK and LDH levels at days 1 and 7, suggesting myoglobulinemia with myonecrosis similar to what is observed in clinical snake venom poisoning.[25] Although the possibility of acute kidney injury was low, with serum creatinine levels not elevated significantly by $BaCl_2$ injection, myoglobinemia may cause systemic responses that may eventually affect the muscle regeneration process, which requires the precise orchestration of inflammatory cells, local paracrine environment, and muscle stem cells.[26,27] These systemic effects are unavoidable in in vivo muscle injury models since all muscle injury and regeneration models accompany acute, severe myonecrosis in the mouse hindlimb.

Local phenotypic characterization of injury and regen-

eration after BaCl$_2$ injection revealed temporal changes in histological features similar to those in previous models of muscle regeneration, with suppurative inflammation at day 1, cellular proliferation at day 3, myotube formation at day 5, and myotube maturation at day 7 and beyond.[5,15,28] Assessment of protein and RNA expressions at day 7 showed upregulation of differentiation and maturation markers in accordance with previous reports.[5,15] Based on these findings, BaCl$_2$-induced muscle injury models are largely similar to other injury models for the study of muscle regeneration.

Although we identified the systemic effects of the BaCl$_2$-induced muscle injury model that were less considered in previous studies, this study has several limitations. First, we were not able to directly compare BaCl$_2$ to cardiotoxin, the most popular venom used in muscle injury experiments, due to logistic issues with local regulations. Also, we did not confirm that the acute mortality after high-dose BaCl$_2$ injection was actually due to fatal arrhythmia. This limiting aspect may be resolved by performing electrocardiogram studies during the acute observation periods after BaCl$_2$ injection.

However, with clinical and systemic perspectives on muscle regeneration, we observed that muscle injury and regeneration following BaCl$_2$ injection occurred with possible efferent signals that may influence other organs, which may, in turn, cause indirect effects on muscle regeneration phenotypes. In particular, the effects of crosstalk between injured/repairing muscle and other organ systems should be considered when studying alterations of muscle regeneration within the context of aging.

CONFLICTS OF INTEREST DISCLOSURES

The researchers claim no conflicts of interest.

ACKNOWLEDGMENTS

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