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NOTE

Anatomy

Glycerol induces early fibrosis in regenerating rat skeletal muscle

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ABSTRACT. Glycerol has been recently used to induce muscle adiposity in mice. However, its effects on the rat muscles have not been investigated previously. Therefore, we investigated the regeneration outcomes of rat muscles following glycerol-induced injury at different time points. Glycerol injection induced myofiber degeneration with extensive inflammatory infiltration on day 4 followed by appearance of regenerating myotubes on day 7 after injury without adipocyte infiltration. Meanwhile, a significant collagen deposition at early stage of regeneration that increased together with persistent inflammatory infiltration up to day 14 after injury indicates impaired regeneration. In conclusion, glycerol injury in rats is more suitable as a fibrosis-inducing model than in mice due to earlier and higher accumulation of fibrous tissue with lacking adipogenesis.

KEY WORDS: fibrosis, glycerol injury, muscle regeneration, rat

Mice have been extensively used as a model to study muscle regeneration [4, 6, 12, 14, 22, 23], while only a limited number of studies have investigated this process in rats [19, 24, 25]. It is known, however, that muscle regeneration following the same type of injury shows differences between species [3]. For example, myotoxic damage by bupivacaine causes complete fiber necrosis in rat muscles [31], while necrosis is only observed in 50% of muscle fibers in mice [23]. Rats and mice also showed different responses to contraction-induced [24] and denervation injuries [3]. Therefore, it is essential to choose a suitable animal model, taking into account species-specific features [11].

Intramuscular injection of glycerol is one of the most commonly used experimental models to study acute kidney injury in rats [9, 30]. Recently, glycerol-induced muscle injury has been used as a unique model to induce muscle regeneration and adiposity [12, 13, 22]. Notably, Kawai *et al.* [7] reported that degenerative changes following glycerol-induced injury are similar to those observed in Duchenne muscular dystrophy (DMD), a genetic disorder characterized by repetitive cycles of muscle necrosis and regeneration [29] with muscle fibrosis [8] and adipocyte infiltration [27], suggesting a similarity in their mechanism.

Our previous studies revealed that glycerol injection damages the cell membrane of myofibers and disrupts its osmotic property. This results in myofiber degeneration followed by regeneration accompanied by adipocyte infiltration and fibrous tissue accumulation in late stage of regeneration in mice [14, 15]. However, to our knowledge, there is no information about the response of the rat muscle to glycerol-induced injury. Therefore, we investigated whether glycerol has the same effect on rat and mouse muscles. Glycerol-injured rat muscles responded in a way that was different from that previously recorded in mouse muscles following glycerol-induced injury.

The experimental protocols used in the present study were in accordance with the guidelines of the Animal Research Committee, Tottori University, Japan (approval number 15-T-24). Adult Wistar male rats (n=30, 8 weeks old, 200–220 g body weight; CLEA Japan, Tokyo, Japan), were anesthetized with sodium pentobarbital intraperitoneally (0.02 mg/g body weight). Equal volumes of glycerol (Wako, Osaka, Japan) and sterile phosphate buffered saline (PBS), pH 7.4, were mixed, and 500 μl of the resulting 50% glycerol solution were injected into the left tibialis anterior (TA) muscle of the rats as previously described in mice [15]. Briefly, the anterolateral aspect of the leg region of left hind limb was shaved and disinfected. Then glycerol was injected along the length of the TA muscle while removing the needle. The contralateral right TA muscle was left intact and served as the non-injured control. Animals were sacrificed by overdose of the inhalation anesthetic isoflurane (Intervet, Osaka, Japan), followed by decapitation. The injured (left) and non-injured control (right) TA muscles were collected at 4, 7 and 14 days after injury

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(five animals per time point) which represent degeneration, early regeneration, and late regeneration phases, respectively [14]. Paraffin sections were stained with hematoxylin and eosin to evaluate morphology and perform morphometric measurements. The stained sections were examined on an Olympus inverted microscope (IX71, Olympus, Tokyo, Japan) and images acquired on a digital camera (DP71, Olympus). Morphometric analysis was carried out with the Image J software (National Institutes of Health, Bethesda, MD, U.S.A.). For evaluation of muscle regeneration, we measured the minor axis diameters (smallest diameters) of the regenerating myotubes (with central nuclei) in injured muscles, and mature myofibers (with peripheral nuclei) in non-injured muscles (about 150 myofibers/ myotubes in each TA) [32]. Muscle sections were incubated with picrosirius red solution, a mixture of 0.1% Sirius red (Polysciences, Warrington, PA, U.S.A.) and a saturated aqueous solution of picric acid (Katayama Chemicals, Osaka, Japan), for 1 hr. At least three non-overlapping fields, measuring 583.839 μm^2, were analyzed per section and three sections per animal were observed. The Sirius red-positive area was measured, and fibrosis index was calculated as the ratio of the Sirius red-positive area to the total muscle area in each field [2]. The mean value for each group of animals was reported. Statistical analyses were performed using SPSS software, version 21 (IBM SPSS Statistics, Chicago, IL, U.S.A.). One-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was used to compare the data with those of the control. All data were expressed as mean ± standard deviation (SD), and differences were considered statistically significant at $P<0.05$.

HE-stained sections were analyzed by light microscopy to evaluate muscle morphology after a glycerol-induced injury of the TA muscle. Myofiber swelling and degeneration, loss of muscle architecture, and extensive mononuclear cellular infiltration were detected on day 4 after injury. Regeneration of injured muscle started on day 7 after injury, as indicated by the appearance of newly regenerating myotubes that were small in size and had central nuclei (Fig. 1A). The average myotube diameter was 13.3 ± 4.0 μm on day 7 (47.4% of the average diameter of the myofibers in the non-injured muscle, $P<0.05$) compared to 28.0 ± 7.1 μm in non-injured muscle, with 70.4% of myotubes being less than 15 μm in diameter (Fig. 1B). On day 14 after injury, the average myotube diameter increased to be 20.1 ± 5.8 μm (72.0% of the average diameter of the myofibers in the non-injured muscle, $P<0.05$). However, it still remained significantly smaller than the average myofiber diameter in the non-injured muscle, with about 10% of the myotubes being less than 15 μm in diameter. A considerable infiltration of inflammatory cells and many damaged myofibers were still observed on day 14 after injury, in addition to the widening of the interstitial spaces. No adipocytes were detected at any time point. Progressive collagen deposition was observed in injured TA muscles with advanced regeneration (Fig. 1A). Collagen fibers were distributed between newly regenerated myotubes. The fibrosis index of the injured muscle was 29.8 ± 3.9% and 36.2 ± 3.6% on day 7 and 14, respectively, [approximately 4.7-fold and 5.7-fold higher than that of the non-injured muscle on day 7 and 14, respectively ($P<0.05$)] compared to 6.3 ± 1.9% in non-injured muscle (Fig. 1B).

Intramuscular glycerol injection has mainly been used as a model to study acute kidney injury in rats [9, 30]. Recently, several studies used glycerol-induced muscle injury to induce regeneration and adiposity in mouse skeletal muscles [12, 22] and rabbit skeletal muscles [7]. However, the effect of glycerol-induced injury in rat skeletal muscles is still unclear. Therefore, in this study we aimed to study the tissue response and regeneration outcomes of rat muscles in response to glycerol-induced injury.

Rat TA muscle showed signs of degeneration with extensive infiltration of mononuclear cells on day 4 after injury, while regenerating myotubes were detected by day 7. Previous mouse studies showed a similar time course after glycerol-induced muscle injury [12, 14]. However, necrotic myofibers and numerous inflammatory cells could be detected in glycerol-injured TA muscle on day 14 after injury, which suggests impaired regeneration [20]. This is further supported by our morphometric results showing the presence of a large number of myotubes with small diameters (less than 15 μm). In addition, the average myotube diameters were 47 and 72% of the average myofiber diameters in non-injured rat muscles at day 7 and 14, respectively, compared to 56 and 80% of the average myofiber diameters in non-injured mouse muscles at the same time points [14].

Our results showed a significant progressive increase in the fibrosis index after injury compared with that in the non-injured muscle. This is in agreement with the results of Pereira *et al.* [21], who reported a significant accumulation of fibrotic tissue after cryolesion-induced injury in the rat TA muscle. Our previous study showed that glycerol induces mild fibrous tissue accumulation at early stage of regeneration (day 7 after injury) that increases progressively at late stage of regeneration (at day 14 after injury) [14]. In contrast to our previous findings in mice, the fibrosis index of the injured rat muscle was approximately 4.7-fold and 5.7-fold higher than that of the non-injured muscle on day 7 and 14 after injury, respectively, compared to 3.4-fold and 5.5-fold higher than that of the non-injured muscle at the same time point in mice [14]. It was reported that rats have a higher ability to produce scar tissue following trauma compared with mice [3]. Persistent inflammatory response alters the extracellular environment and increases the secretion of various inflammatory cytokines, which contribute to muscle fibrosis [5]. Excessive deposition of collagen fibers hinders normal muscle repair following injury [17]. It also reduces the amount of target muscle available for repair, which impairs the effectiveness of cell and gene therapies [8]. Taken together, glycerol induced collagen deposition at early stage of regeneration that persisted up to 2 weeks after glycerol-induced injury in rats.

No adipocytes could be detected in glycerol-injured rat muscles at any time point. In contrast to these findings, adipocyte infiltration appears by day 7 in mouse [16, 26] and rabbit [7] muscles, and persists up to 4 weeks after glycerol-induced injury [22]. This difference could be explained in light of the hypothesis that different animal species [3] and different animal strains [10, 18] show different reactions to the same injury during the regeneration process. Moreover, degeneration and regeneration responses differs between fast and slow rat muscles subjected to crush-induced injury [1]. It has been shown that both fibrosis and adipocytes are differentiated from a common mesenchymal progenitor cells residing within skeletal muscle [28]. However, adipocyte differentiation depends on muscle environment following injury [26]. Taken together, it is suggested that muscle environment developed following glycerol-induced injury rats might inhibited the differentiation of adipocytes. Further research is required to clarify this point.

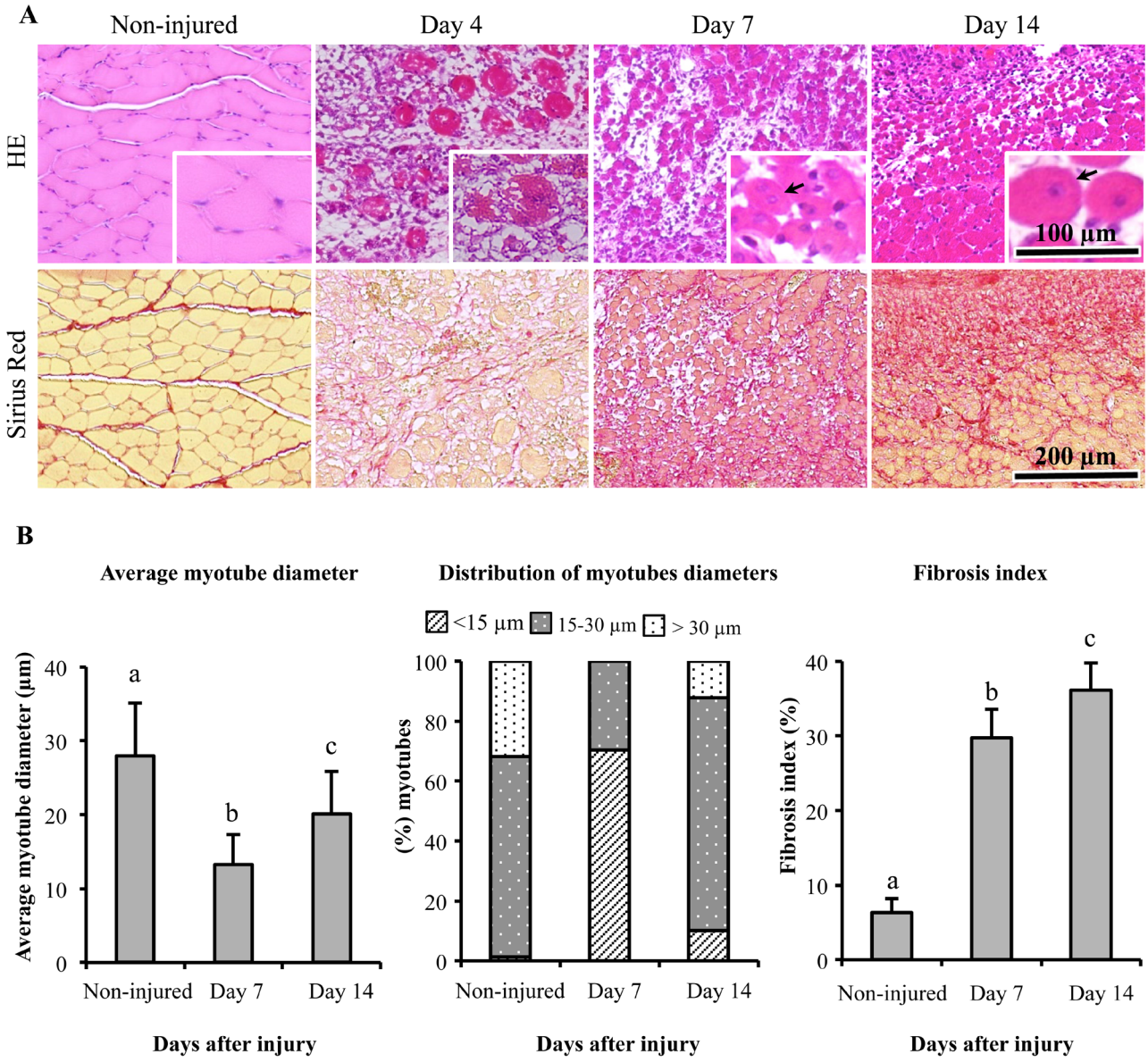

Fig. 1. Analysis of muscle response after glycerol-induced injury at different time points in the rat TA muscle. (A) Histological analysis of muscle sections from non-injured and glycerol-injured TA muscles on days 4, 7 and 14 stained with hematoxylin and eosin (HE, upper panel), Sirius red (lower panel). The inset shows a higher magnification, note the swollen myofiber at day 4 and regenerating myotubes (arrows) at day 7 and 14. (B) Glycerol injury affects the average myotube diameter in the regenerating TA muscle compared with that in the non-injured muscle, the distribution of myotube diameters at different time points (as % of total myotubes) and fibrosis index computed as the ratio of the fibrosis area to the total area. Data are expressed as means ± SD and significant difference is indicated in different letters (P<0.05).

In conclusion, our data showed that rat muscles responded in a way that was different from that previously recorded in mouse muscles following glycerol-induced injury. Glycerol injection induces myofiber degeneration with extensive mononuclear cellular infiltration followed by regenerative changes. Although glycerol is used to induce muscle adipogenesis in mice, glycerol-injured rat muscles regenerated without adipocyte infiltration. Meanwhile, a significant collagen deposition at early stage of regeneration that increases together with persistent inflammatory infiltration up to day 14 after injury indicates impaired regeneration. Therefore, glycerol injury in rats is more suitable as a fibrosis-inducing model than in mice due to earlier and higher accumulation of fibrous tissue with lacking adipogenesis. Further studies are recommended to investigate the possible factors responsible for the differences of muscle response to glycerol-induced injury between rat and mouse.

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