Influence of Heat Treatment on Muscle Recovery after Skeletal Muscle Injury in Rats: Histological and Immunohistochemical Studies

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Abstract

Background: Muscle injuries represent a great part of athletic injuries. The repairing of skeletal muscle after injury is highly influenced by its regenerative response that may be affected by thermotherapy. Aim: This research examined the consequence of heat therapy on muscle recovery after skeletal muscle injury in rats. Materials and Methods: Forty-five male adult albino rats were classified into three groups: control, cardiotoxin-injected without heat (nonheating group), and cardiotoxin-injected with heat (heating group). Muscle injury was caused by the injection of cardiotoxin intramuscularly into the tibialis anterior muscles. Heating treatment (40°C for 20 min) was started immediately after the injury. Subsequent observations were performed at day 1, 3, and 7 after injury, including histological imaging and vimentin immunostaining expression. Results: In the heating group, the regenerating myotubes, having two or more central nuclei, first looked at 3 days after muscle injury, while in the nonheating group, the regenerating fibers were first observed at 7 days after muscle injury. Immunohistochemically, the vimentin reactions were absent in control muscle fibers but were identified in regenerating muscle fiber of the heating group earlier than in the nonheating group. Conclusion: Starting of heat treatment immediately after muscle injury promoted the regeneration of muscle fibers.

Keywords: Cardiotoxin, heat, muscle injury, regeneration, vimentin

Introduction

Healthy skeletal muscle is very important for human life. Their proper function permits stability of the joint and body parts during movement.[1] The skeletal muscle injuries are relatively common among athletes. The ideal physical condition in sports persons has always been subject of research for scientists.[2] Within the field of sports medicine and physiotherapy, there has been incredible effort in encouraging skeletal muscle rehabilitation.

Many options are used in the treatment of muscle injuries including both pharmacological and nonpharmacological approaches. Nonpharmacological treatment strategies include heat or cold therapy. Heat and cold therapy are often recommended to alleviate edema, pain, and disability associated with muscle injury.[1] Various types of muscle injuries can be successfully treated with cold therapy, thermotherapy, or a combination of both.[3] Ice contact has been well approved as a first-aid management for athletic injuries. Ice is usually employed in sports rehabilitation to reduce pain and inflammation related to injuries.[4] Recent research suggested that ice treatment has a deleterious influence on skeletal muscle recovery following injury.[5] On the other hand, several studies have shown that heat treatment is one of the effective stimuli on the skeletal muscle. Previous researchers[7] suggest that warm water soaking could be more effective than cold water to decrease muscle rigidity.
Thermostherapy that is frequently used comprises hot packs and hot water, doings through skin contact.\cite{10} Heat application leads to blood vessels dilatation and increase in blood flow to injured area which should cause recovery to happen more rapidly.\cite{11} Local heat application is stated to be a harmless and authentic method for the management of muscle injuries in persons.\cite{12} Single or repeated application of heat treatment following muscle injury accelerated muscle regeneration and prevented fibrosis.\cite{13,14} Moreover, heat treatment accelerates the growing of wasted muscle \cite{15,16} and enhances proliferative potential of the muscle.\cite{17,18}

Skeletal muscle controls body movements through highly systematized cylindrical muscle fibers. Myofibers contain contractile myofibrils that are composed of sarcomeres arranged between two Z-lines. Sarcomeres consist mainly of myosin and actin filaments.\cite{19,20} The Z-lines are the attachment sites of titin, α-actinin, vimentin, and desmin.\cite{21} Vimentin employs a chief role in maintaining muscle construction and is considered an effective indicator for muscle regeneration.\cite{22} During myotubes differentiation, vimentin progressively fades in the sarcoplasm of mature muscle fibers.\cite{21}

The effect of heat treatment on the recovery of myofibers, including the expressions of vimentin, remains lacking up till now. Hence, this work studied the effect of heating on the muscle differentiation and expression of vimentin in regenerating fibers.

**Materials and Methods**

**Animals**

Forty-five male albino rats, weighing 250–300 g each, were used in the present study. They were handled according to the guidelines and ethics of the animal protocol of faculty of medicine, Tanta University, Egypt. They were housed in well-ventilated stainless steel cages, at normal room temperature and 12-h light/dark cycle with strict care and hygienic measures. All rats were freely provided with water *ad libitum* and standard rat chow.

**Experiment design**

After 2 weeks of acclimatization, the rats were classified into three groups, 15 rats per each group.

- **Control group:** It was subdivided into two subgroups; six animals were left untreated, whereas the others were given a single intramuscular injection of saline at the same volume and time of cardiotoxin injection (0.3 mL saline) in the right tibialis anterior (TA) muscle
- **Nonheating group:** Cardiotoxin-injected animals without heat application
- **Heating group:** Cardiotoxin-injected animals with heat application.

At 1, 3, and 7 days (5 rats per each period) after muscle injury, animals were anesthetized and the muscle specimens were collected.

**Muscle injury**

Induction of muscle injury was made by the injection of cardiotoxin in saline (Sigma, St. Louis, MO, USA) intramuscularly as a single dose of 0.3 mL of 10 μM into the proximal, middle, and distal area of the right TA muscle of the rats (about 0.1 mL for each region). All processes were achieved under anesthesia by the injection of pentobarbital sodium (60 mg/kg BW) intraperitoneally.\cite{23} Similarly, the same amount of saline was injected into the right TA of control rats.

**Heat treatment**

Five minutes after cardiotoxin injection and while the rat was still anesthetized, hot water bottle (40°C) was put on the skin above the injured TA muscle in the heating group. The hot water bottle was applied for 20 min for one time on the day of injury.\cite{13}

**Histological and immunohistochemical study**

**Collection of tissue samples for histological examination**

The animals were anesthetized by ether inhalation; the TA muscle of the right leg of each rat was dissected and cut into smaller pieces. The specimens were fixed in 10% formalin, then dehydrated in the ascending grades of ethyl alcohol, and cleared in xylene. The specimens were impregnated and embedded in pure molten paraffin wax, sectioned on a rotary microtome at 5 μm thicknesses, and mounted on an albuminized glass slide. Finally, the sections were stained with hematoxylin and eosin (H and E) for studying general structure.

**Immunohistochemistry protocol**

Some paraffin sections were mounted on charged glass slides for immunohistochemical localization of vimentin intermediate filaments. The paraffin wax was removed from the sections by xylene, rehydrated in the descending grades of alcohol, and then dipped in 3% H2O2 at 37°C for 10 min. After that, the sections were washed in phosphate-buffered saline. Sections were blocked in normal goat serum at 37°C for 10 min, and the solution was removed. Then, mouse anti-human vimentin monoclonal antibody (Vim 3B4, 1:200; Dakopatts, CA, USA) was used to identify the immune reactivity. The sections were counterstained using hematoxylin, dehydrated in the ascending grades of alcohol, cleared in xylene, and mounted in Canada balsam.\cite{24}

**Semi-thin sections**

Longitudinal muscle strips from the TA muscle from all groups were collected, trimmed into small pieces approximately 0.5 mm3, and immediately fixed by immersion in 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at 0°C–4°C for 8 h. Postfixation was carried out in 1% osmium tetroxide, dehydrated in the ascending grades of ethyl alcohol, cleared in propylene oxide, and embedded in epoxy resin then left for 24 h in an oven at 40°C for resin polymerization. Semi-thin sections from muscles were cut with ultramicrotome and stained with toluidine blue.\cite{25}

**Results**

**Hematoxylin and eosin-stained sections**

**Control group**

Longitudinal section of the right TA muscle of both control subgroups revealed the normal structure of the skeletal muscle.
The muscle fibers appeared parallel and cylindrical in shape. They have multiple flat elongated nuclei and acidophilic sarcoplasm [Figure 1].

**Nonheating and heating groups**

At 1 day after injury, examination of the nonheating group revealed severe loss of muscle architecture with loss of transverse striations [Figure 2]. The muscle fibers of the heating group showed segmental degeneration; parts of them appeared swollen with wavy contour and pale staining sarcoplasm [Figure 3].

In the rats sacrificed at 3 days after injury, most muscle fibers in the nonheating group were affected and degeneration of the muscle fibers extended to massive destruction. The contour of muscle fibers became unclear [Figure 4]. The heating group showed regenerating myotubes with centrally located nuclei that appeared side by side with the degenerated muscle fibers, as well as the appearance of mononuclear cell infiltration [Figure 5].

The muscles of both nonheating and heating groups at 7 days after injury showed regenerating muscle fibers that were more obvious in the heating group than in the nonheating one [Figures 6 and 7]. In the nonheating group, dilated capillaries were observed near the regenerating fibers and splitting of some myofibers was also appeared [Figure 6].

**Toluidine blue-stained sections**

**Control group**

Examination of semi-thin section of longitudinal section of the right TA muscle showed parallel and cylindrical myofiber with sarcoplasm containing many myofibrils. Regular arrangement of alternating dark (A) and light (I) bands was noticed. At the middle of the light band, Z-line was also seen. Flat peripheral nuclei were present beneath the sarcolemma [Figure 8].

**Nonheating and heating groups**

The degenerating muscle fibers were observed in both nonheating and heating groups at 1 day after injury, but the...
Degree of degeneration was more severe in the nonheating group than in the heating group. The fibers of the nonheating group appeared with irregular contour. The arrangement of light and dark bands completely disrupted with complete loss of transverse striations in some areas. The neighboring myofibers were widely apart [Figure 9]. However, in the heating group, some fibers showed regular sarcolemma with unclear transverse striation [Figure 10].

Three days after injury, the nonheating group showed progressive disarrangement of the internal structure of muscle fibers [Figure 11], while in the heating group, the regenerating fibers appeared in the form of myotubes surrounded by many capillaries. The myotubes retained 2–3 central nuclei with prominent nucleoli [Figure 12]. However, these myotubes were not yet found anywhere in the nonheating group at the same time.

At 7 days after injury, multiple small regenerating muscle fibers were observed in the nonheating group surrounded by many capillaries. Each fiber has one central nucleus [Figure 13]. The regenerating myotubes in the heating group were obviously more mature than those in the nonheating group [Figure 14].

Vimentin-immunostained sections

Control group

No vimentin immunoreactivity was detectable in the sarcoplasm of normal muscle fibers of both control subgroups [Figure 15].

Nonheating and heating groups

At 1 day after injury, vimentin immunostaining was absent in the sarcoplasm of muscle fibers of both nonheating [Figure 16] and heating groups, but it is detected as brown deposits in the interstitial tissue between the muscle fibers of heating groups [Figure 17].

Three days after injury, the muscle fibers immunoreactive to vimentin was absent in the nonheating group [Figure 18]. On
Figure 9: A longitudinal section of the muscle of nonheating group at 1 day showing loss of muscle architecture, focal loss of transverse striations in many parts of muscle fibers (S) (Toluidine blue, ×1000)

Figure 10: A longitudinal section of the muscle of heating group at 1 day showing indefinite striations in some affected fibers (S) (Toluidine blue, ×1000)

Figure 11: A longitudinal section of the muscle of nonheating group at 3 days showing progressive disarrangement of the internal structure of muscle fibers (f). Outlines of the degenerating muscle fibers became unclear (star) (Toluidine blue, ×1000)

Figure 12: A longitudinal section of the muscle of heating group at 3 days showing the regenerating fibers in the form of myotubes (R) with centrally located nuclei (N) containing prominent nucleoli. Congested capillaries (c) (Toluidine blue, ×1000)

Figure 13: A longitudinal section of the muscle of nonheating group at 7 days showing the regenerating fibers with preserved banding pattern and central nuclei (R). Capillaries (c) (Toluidine blue, ×1000)

Figure 14: A longitudinal section of the muscle of heating group at 7 days showing regenerating fibers closely related to each other (R) (Toluidine blue, ×1000)
the other hand, the muscle fibers immunoreactive to vimentin start to increase in number in the heating group [Figure 19]. Vimentin was predominantly localized in the perinuclear area [Figure 20].

Seven days after injury, sarcoplasm of some muscle fibers of the nonheating group started to show immunoreactivity to vimentin [Figure 21]. By comparing the heating group of this period with the pervious group, the number of muscle fibers immunoreactive to vimentin was progressively decrease when passing from 3 to 7 days groups [Figure 22].

**Discussion**

Skeletal muscle injuries are relatively common among athletes. The most common noninvasive management after muscle injuries is the application of controlled temperature on the harmed skeletal muscle. The present study employed a skeletal muscle injury model and studied the effect of heat treatment on the muscle recovery and regeneration.

In the current study, cardiotoxin was used for induction of muscle injury as it is considered a simple method for the induction of muscle damage. Cardiotoxin isolated from snake venom toxins and causes degeneration of muscle fibers, which finally activates the myofibers regeneration.[27] Cardiotoxins make acute muscle injury by damaging plasma membrane of skeletal myofiber without causing injury to its blood supply.[28] Thus, cardiotoxin has become helpful method for investigating different details of the process of muscle regeneration.[29,30]

Only the male rats were used in this research to exclude the influence of estrogen hormone on the recovery of muscle, because multiple studies proved that estrogen has been shown to play an important role in muscle regeneration and motivation of satellite cells.[31-35]
The results detected in this work appeared in the form of muscle degeneration and regeneration. The degenerated myofibers were interchanged by centrally located nuclei in regenerating muscle fibers, which appeared more earlier in the heating group than in the nonheating group at day 3 following injury. This indicated that satellite cells were committed into the myoblast pathway to differentiate into myotubes and eventually formed new muscle cells. Regeneration of muscle fibers depends on reestablishment of the blood supply necessary for the interchange of nutrition and the shaping of mature myofibers.\(^{[42]}\) The development of new blood vessels and myogenesis are combined by interacting endothelial cells and muscle satellite cells during skeletal muscle regeneration.\(^{[43,44]}\) Endothelial cells have paracrine effects and a direct interaction with satellite cells by secreting many factors, including hepatocyte growth factor, vascular endothelial growth factor, and angiopoietin-1, which accelerate the process of regeneration.\(^{[45-48]}\)

In this study, many capillaries were observed around the regenerating fibers. This result is in agreement with a previous work reporting that, during development of regenerating muscle fibers, the number of capillaries around each myofiber was increased.\(^{[49]}\) Other investigators\(^{[41]}\) also found that, in the presence of adequate capillary ingrowth, satellite cells proliferated into myotubes and eventually formed new muscle cells. Regeneration of muscle fibers depends on reestablishment of the blood supply necessary for the interchange of nutrition and the shaping of mature myofibers.\(^{[42]}\) The development of new blood vessels and myogenesis are combined by interacting endothelial cells and muscle satellite cells during skeletal muscle regeneration.\(^{[43,44]}\) Endothelial cells have paracrine effects and a direct interaction with satellite cells by secreting many factors, including hepatocyte growth factor, vascular endothelial growth factor, and angiopoietin-1, which accelerate the process of regeneration.\(^{[45-48]}\)

In the current study, splitting of muscle fibers was detected in the nonheating group at 7 days after injury. Previous study\(^{[49]}\) indicated that the splitting fibers are a transient response.
probably acquired from satellite cells and are not derived from true splitting of pre-existing fibers. In line with this, multiple studies demonstrated that splitting of the skeletal myofibers plays some role in the regeneration of damaged skeletal muscles.[50-52] On the other hand, previous researchers[53] explained splitting of fibers as a consequence of inadequate fusion of regenerative skeletal muscle fibers.

Vimentin is an intermediate filament demonstrated in the skeletal myofibers, and it is also found in mesenchymal tissue.[54] In the present study, vimentin was negative in the sarcoplasm of control muscle fibers but was recognized during the early stages of regeneration, and then, it was decreased until it was no longer noticeable in mature regenerated fibers. The same results were previously reported by researchers[55] who found that vimentin was lacking in control muscle fibers, but it was recognized in triggered satellite cells.[55] In addition, other investigators[56] demonstrated that more vimentin was present in less differentiated myocytes and vimentin diminished gradually from the undifferentiated myoblast to differentiated myocytes. They also added that vimentin might provide a basis for the generation of myofibrils. Previous study[57] also reported that vimentin is expressed at very low levels in mature myocytes, although its expressions are raised in regenerating muscle fibers in reaction to both injury and disease.

The perinuclear position of vimentin observed in this work was previously demonstrated by researchers[58] who found that vimentin filaments anchored on nuclear pore complex and may be involved in nuclear transportation. Recently, other study[59] found that vimentin intermediate filaments attach with the nuclear membrane by membrane proteins creating a link between nucleoskeleton and cytoskeleton complex. Furthermore, vimentin expression levels are correlated with nuclear stability and chromatin reorganization.[60]

**Conclusion**

The results of this work showed that exposure to heat immediately after muscle injury hastens the muscle regeneration. There is a need for further research toward optimizing the most favorable muscle temperature and duration of application on the injured area. Further studies are warranted to decide whether the number of heat application (once or repeated) accelerates the muscle recovery. Such knowledge would help in the establishment of many heat treatment protocols for both sporting and clinical cases.

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**Conflicts of interest**

There are no conflicts of interest.

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El-Sheikh, et al.: Histological effect of heat stress on injured muscle

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