Longitudinal evaluation of local muscle condition in a rat model of gastrocnemius muscle injury using an in vivo imaging system

（ラット筋挫傷モデル(LP-iDOPE腹腔内投与)におけるIn vivoイメージャーを用いた局所の経時的評価）

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Longitudinal evaluation of local muscle conditions in a rat model of gastrocnemius muscle injury using an in vivo imaging system

Running title: Longitudinal evaluation of muscle injury

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Abstract

This study aimed to evaluate the time course of local changes during the acute phase of gastrocnemius muscle strain, in a rat model, using an in vivo imaging system. Thirty-eight, 8-week-old Sprague–Dawley male rats were used in our study. Experimental injury of the right gastrocnemius muscle was achieved using the drop-mass method. After inducing muscle injury, a liposomally formulated indocyanine green derivative (LP-iDOPE, 7 mg/kg) was injected intraperitoneally. We evaluated the muscle injuries using in vivo imaging, histological examinations, and enzyme-linked immunosorbent assays. The fluorescence peaked approximately 18 h after the injury, and decreased thereafter. Histological examinations revealed that repair of the injured tissue occurred between 18 and 24 h after injury. Quantitative analyses for various cytokines demonstrated significant elevations of interleukin-6 and tumor necrosis factor-α at 3 and 18 h post-injury, respectively. The time course of fluorescence intensity, measured using in vivo imaging, demonstrated that the changes in cytokine levels and histopathologic characteristics were consistent. Specifically, these changes reached peaked 18 h post-injury, followed by trends toward recovery.

Keywords: local muscle condition; rat model of muscle injury; in vivo imaging; muscle injury; gastrocnemius muscle strain

Introduction

Muscle strain is a common injury, accounting for 10–50% of all sport-related injuries.[1] In this type of injury, muscle fibers are damaged by external pressure; such injuries often occur during contact sports such as football, rugby, and American football. Typically, these injuries develop on the frontal surface of the thigh. Generally, they are managed through follow-up observation, without treatment. In a limited number of top athletes who require or achieve early recovery, a few early diagnostic techniques, such as ultrasound,[2] magnetic resonance imaging,[3,4] etc., are available; however, these intensive diagnostic approaches are not common.

Owing to the limited number of examples studied, several factors involved in the acute local changes occurring during muscle strain remain unknown. However, recent muscle pain studies have revealed that cytokines play important roles during the transition from the acute to chronic pain phase. Typically, inappropriate treatment during the early phase of injury results in chronic pain in approximately 30% of patients.[5] This remains true for the treatment of muscle injuries. Therefore, appropriate treatment during the early phase is considered critical for preventing the transition to chronic pain, although the effect of the treatment may depend on the severity of the injury.

The few studies that have evaluated the time course of local changes at the injury site during the early phase have not elucidated the underlying mechanisms of muscle strain injuries.

Thus, since numerous factors regarding the underlying mechanisms remain unknown, a core treatment procedure has not been established for muscle strain. As a result, clear correlations between the severity of such injuries and local changes are not available, and muscle strain injuries tend to be left unmanaged.[6] In general, recovery of muscle function during early rehabilitation can be important. However, according to a prior study in rabbits, post-injury muscles clearly show an inferior contraction and stretch rupture strength. Furthermore, secondary muscle damage was reported to occur following excessive exercise, which also generates edema and pain. Therefore, excessive rehabilitation during the early recovery period carries a high risk of secondary damage, and there are many unknowns regarding the timing and level of appropriate rehabilitation.[7,8] Therefore, an evaluation of the time course of local early phase changes in a strained muscle is important to clarify its underlying mechanisms.

In an in vivo imaging system, a charge-coupled device camera detects near-infrared fluorescence via a near-infrared wavelength laser, enabling professionals to evaluate the accumulation of fluorescence by photographing the body after the injection of a fluorescence-inducing material.[9] LP-iDOPE is a liposomally formulated indocyanine green derivative (iDOPE: a lipid conjugated with indocyanine green) that is used as a near-infrared probe.[10] LP-iDOPE is also a nanoparticle that selectively accumulates in cancerous and inflammatory tissues through an enhanced permeation and retention effect (Fig. 1).[11] By

optimizing this unique feature of LP-iDOPE, the measurement of fluorescence intensity allows the visualization of cancerous and inflammatory tissues using an in vivo imaging system.[12] This system has been applied clinically, and some of its applications, such as imaging for sentinel lymph nodes in breast cancer, are covered by insurance in Japan.[13] However, no previous studies have evaluated tissue inflammation in strained muscles.

The present study aimed to evaluate the time course of local changes, using an in vivo imaging system, during the acute phase of gastrocnemius muscle strain in a rat model.

Methods

The ethics committees of our University approved the animal protocols used in this study, and we followed the National Institutes of Health (Bethesda, MD, USA) Guidelines for the Care and Use of Laboratory Animals (1996 revision). Thirty-eight, 8-week-old Sprague–Dawley male rats were used in our study. The rats' mean body weight was approximately 250 g at the time of muscle injury.

Before inducing the injury, all rats were anesthetized with ethyl ether. If a withdrawal reflex was still present after the initial ether exposure, additional anesthesia was administered until no reflex response was noted. Experimental injury of the right gastrocnemius muscle was achieved using the drop-mass method; no skin incisions were made.[14] A 115-g mass was dropped from a 1-m height on to each rat's medial gastrocnemius muscle of the right leg (Fig. 2). The

gastrocnemius muscle of the left leg was used as a non-injured control site. After the muscle was injured, LP-iDOPE (7 mg/kg) was intraperitoneally injected. We used the following 3 approaches to evaluate the muscle injury site.

Experiment 1: In Vivo Imaging

We visually evaluated the concentration of the fluorescent probe and measured the fluorescence intensities at the sites of the muscle of injury (right side) at 6, 12, 18, and 24 h, post-injury, in 14 rats, using an in vivo imaging system (In-Vivo FX PRO® Imaging System; Bruker, Billerica, MA, USA). The imaging system was equipped with a light-emitting diode that emits light at a near-infrared wavelength of 760 nm; a charge-coupled device image detector; and an optical high-pass filter, placed in front of the charged-couple device, to efficiently detect fluorescent signals. This system also comprised a camera unit; a controller that operated the camera unit; and a remote control that controlled the light-emitting diode intensity, video gain, and offset. Each fluorescence image was sent to a digital video processor for real-time display on a television monitor.

Experiment 2: Histologic Examination

The muscle injury site (right side) and the non-injury site (left side) were dissected from both hind limbs under anesthesia with sodium pentobarbital (40 mg/kg, intraperitoneally) at 12, 18, and 24 h (9 rats; 3 rats per time period) post-injury and were perfused transcardially with

0.9% saline, followed by 500 mL of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Each formalin fixed tissue specimen was embedded in paraffin, after a 14-h dehydration procedure in a series of increasing ethanol concentrations in a tissue processor (Tissue-Tek V.I.P M1500, Sakura Finetek Japan, Tokyo, Japan). Subsequently, 4-μm-thick sections were cut from the paraffin blocks using a sliding microtome (LS113, Yamato Kohki Industrial, Saitama, Japan) and placed on glass slides (#5116, Muto Pure Chemicals, Tokyo, Japan). The sections were stained with Mayer's hematoxylin (Muto Pure Chemicals) for 5 min after deparaffinization with xylene and ethanol. After washing with distilled water, these sections were dipped in 0.1% ammonium solution several times, washed again, and stained with an alcoholic 1% eosin and phloxine solution for 20 s. The sections were finally overlaid with mounting medium (Entellan New, Merck, Darmstadt, Germany) after dehydration through a graded series of ethanol and xylene mixtures. The slides were observed using a microscope (BH20, Olympus, Tokyo, Japan) by a professional animal pathologist, and the findings were semiquantitatively evaluated. We evaluated the presence/absence of histologic degeneration, bleeding, and neutrophil recruitment at each slice. Furthermore, for the identification of neutrophils, immunohistochemical staining, using anti-myeloperoxidase rabbit polyclonal antibodies (A0398, DAKO, Glostrup, Denmark) was performed. We also compared the histologic changes, over time, at the injury site (right side) against the non-injury site (left side).

Experiment 3: Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of inflammatory mediators at the muscle injury site were analyzed at 3, 6, 12, 18, and 24 h (15 rats; 3 rats per time period) after the muscle injury. The resected gastrocnemius muscle samples from the injured and non-injured sites were frozen in liquid nitrogen, pulverized or homogenized, and digested in tissue lysis reagent. Levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α at the muscle injury site were quantified using ELISA, according to the manufacturer's protocols. Tissue protein levels were determined (Bio-Rad, Hercules, CA, USA), and inflammatory mediator levels were normalized to tissue protein levels.

Statistical Analysis

Fluorescence values at the injured muscle sites were compared, between groups, at 6, 12, 18, and 24 h, post-injury, using Student's *t*-test. Inflammatory mediator levels at the injury and control sites were compared at 3, 6, 12, 18, and 24 h, post-injury, using Student's *t*-test. P-values < 0.05 were considered statistically significant.

Results

Result 1: In Vivo Imaging

The average fluorescence values at the muscle injury sites were: 3671 pixel intensity, 6 h after injury; 14,847 pixel intensity, at 12 h; 24,375 pixel intensity, at 18 h; and 12,834 pixel intensity, 24 h after injury. There was no significant difference between the 6- and 12-h

fluorescence values ($p > 0.05$); however, the fluorescence value 18 h after injury was significantly higher than at 12 h ($p < 0.05$), and the fluorescence value at 24 h post-injury was significantly lower than at 18 h ($p < 0.05$) (Fig. 3). These results indicate that the fluorescence peaked approximately 18 h after the injury, and decreased thereafter.

Result 2: Histology

Hematoxylin and eosin staining of the local tissues did not show histologic degeneration 12 h after injury. However, a high degree of histologic degeneration was observed 18 and 24 h after injury. Bleeding was observed around the injury site at 12 h post-injury, extending over the entire layer of tissue, but the volume of bleeding was low. The amount of bleeding 18 h after injury was greater than at 12 h, or 24 h after injury when it was limited to scattered sites between the small muscle bundles. These results indicate that the peak histologic degeneration and bleeding also occurred approximately 18 h after injury. Neutrophil recruitment was not observed 12 h after injury, but the neutrophils were concentrated around the injured muscle tissue (Fig. 4). These results indicate that repair of the injured tissue occurred between 18 and 24 h after injury.

Result 3: ELISA

The IL-6 levels peaked approximately 3 h after muscle injury, and were significantly higher than those at 6, 12, 18, and 24 h after muscle injury ($p < 0.05$); the IL-6 levels decreased

over the experimental period (Fig. 5). There were no significant differences among the TNF-α levels at 3, 6, and 12 h after muscle injury ($p > 0.05$). However, the TNF-α levels at 18 h were significantly higher than those at the other sampling times ($p < 0.05$). Thus, peak TNF-α levels were observed approximately 18 h after muscle injury and decreased thereafter (Fig. 6).

Discussion

In this study, the fluorescence intensity in the muscle strain region, as measured using the in vivo imaging system, peaked approximately 18 h post-injury and decreased thereafter. Histopathologic evaluation of the hematoxylin and eosin-stained sections from the local area revealed edema and hematoma in the muscle tissue immediately after injury, with the severity of degenerative changes in the lesion peaking 18 h post-injury. Additionally, numerous neutrophil aggregates were observed around the crushed muscle fibers 18 and 24 h post-injury, followed by macrophage accumulation, suggesting the onset of tissue regeneration. Crisco et al. previously reported that edema and hematoma occurred in muscle tissues immediately after muscle strain, followed by macrophage accumulation and degeneration, with tissue regeneration occurring approximately 2 days post-injury,[14] consistent with our results.

The quantitative analysis of various cytokines in this study demonstrated a significant elevation of IL-6 and TNF-α levels at 3 and 18 h post-injury, respectively. Thereafter, no significant elevations were observed, relative to levels in the normal, uninjured muscle on the

contralateral leg. Warren et al. also reported that both IL-6 and TNF-α levels were increased at 6 h post-injury, peaked at 24 h, and decreased thereafter.[15]

The time-dependent changes in the morphological lesions and ELISA-measured local cytokine levels described in this report are consistent with the results from previously published literature. The results indicate that hemorrhage and edema occur immediately after the injury, and are accompanied by elevated levels of inflammatory cytokines (e.g., IL-6 and TNF-α), an induction of neutrophil aggregation due to the elevated inflammatory cytokines, and lastly, macrophage accumulation and degeneration indicative of tissue regeneration.[16] According to a study involving inflammatory cell-knockout mice, inflammatory cell infiltrates promote a regulatory mechanism for both positive (repair) and negative (damage) changes through elevated levels of free radicals, growth factors, and chemokines.[17] Studies have also demonstrated that the peaks for both histopathological and cytokine changes are observed during the early phase, i.e., 18 h post-injury.

The time course of fluorescence intensity, measured using an in vivo imaging system, revealed that fluorescence intensity changes were consistent with changes in cytokine levels and histopathologic features at each time point. Specifically, these changes peaked 18 h post-injury and trended towards recovery, thereafter. A fluorescence peak reflects local hemorrhage and edema. According to the past reports, local hemorrhage and edema may lead an accumulation of

inflammatory cells (e.g., neutrophils) and an increase in inflammatory cytokines (e.g., TNF α).[17] Thus, the fluorescence peaks may correspond to accumulations of inflammatory cells and peaking cytokine levels.

Limitations of this study include the small number of time courses investigated and the absence of detailed examinations of the increased fluorescence intensity associated with muscle strain development. In this study, increased fluorescence intensity associated with the development of muscle strain was correlated with the observed local hemorrhage. For demonstrative purposes, the study required the conduct of tissue evaluations using identical specimens over time and then comparing the fluorescence intensity over short periods. However, conducting such tissue time course evaluations, using identical specimens, is difficult in the absence of a methodology similar to that implemented (due to the influence of frequent anesthesia and several muscle resections), and is, therefore, considered a limitation. Furthermore, the absence of muscle function examinations is also considered a limitation. Since behavioral assessments were not conducted in this study, muscle function was difficult to describe.

In this study, we reported localized temporal changes associated with early muscle strain. The in vivo imaging system indicated temporal changes that were similar to the local histological and inflammatory cytokine level findings, suggesting the system's usefulness for evaluating local changes over time. This method is useful because temporal or longitudinal evaluations can be

performed with identical specimens, without sacrificing the animals. Utilizing this advantage, we plan to use the system to conduct evaluations of muscle strain treatment methods in rats. Specifically, we plan to examine the temporal changes associated with compression/cooling as a general treatment for muscle strain. According to past reports, many aspects of this treatment remain to be clarified, such as the appropriate timing of treatment initiation, treatment duration, and number of treatments. Because several time courses are required to elucidate these treatment aspects in animal models, such examinations have not previously been realistic owing to the very large number of animals required. On the other hand, such experiments become feasible if temporal evaluations are possible, with identical specimens, by using the in vivo imaging method reported in this study. From such experiments, efficient muscle strain treatment methods can be expected to be established through optimization of the previously indicated aspects of compression/cooling therapy.

Conclusion

The in vivo imaging system employed in the present study is safe (free of x-ray radiation), cost-effective, compact in size, and has a real-time display. The system has been shown to be applicable for a variety of purposes and represents a promising technique for intraoperative diagnoses. Our results suggest that the in vivo imaging system may be a useful tool for evaluating time-dependent local changes during the early phase of muscle strain in humans.

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Figure Legends

Figure 1: Concentration of fluorescence probes in a rat model of gastrocnemius muscle injury.

Figure 2: Drop mass method. (a) Schematic of the impact apparatus. (b) A 115-g weight was dropped from a 1 m height, through an acrylic guide tube. (c) The weight dropped onto the impactor, dividing the impact over the muscle belly without injuring the overlying skin.

Figure 3: The average fluorescence values at the muscle injury site measured using the in vivo imaging system.

Figure 4: Hematoxylin and eosin staining of local tissues (a–c). Transverse sections showing gastrocnemius muscle fibers. (a) Normal site with no injury, 100×. (b) Injury site, 18 h post-injury; 100×. (c) Injury site, 24 h post-injury; 100×. Immunohistochemical staining used anti-myeloperoxidase rabbit polyclonal antibody (d, e). (d) Injury site, 18 h post-injury; 400×. (e) Injury site, 24 h post-injury; 400×.

Figure 5: Interleukin-6 levels at the site of muscle injury 3, 6, 12, 18, and 24 h post-injury.

Figure 6: Tumor necrosis factor-α levels at the muscle injury site 3, 6, 12, 18, and 24 h after injury.

Figure 1:

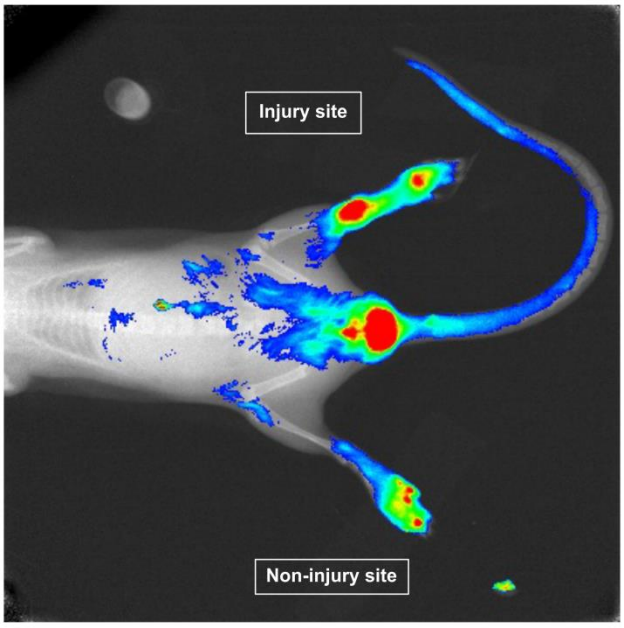
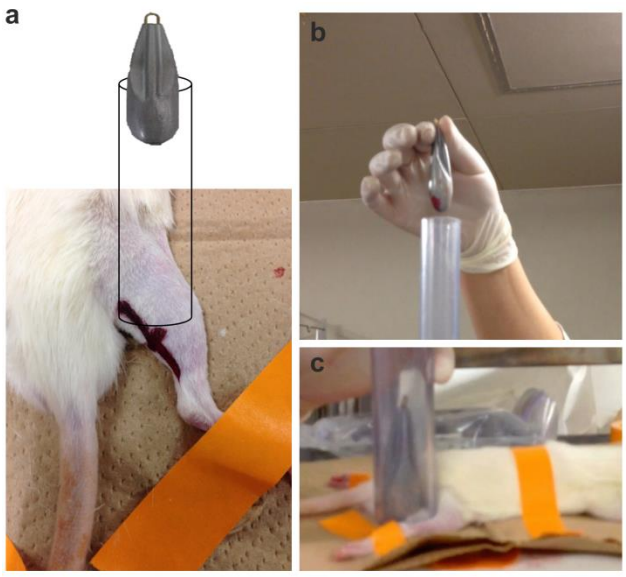


Figure 2:



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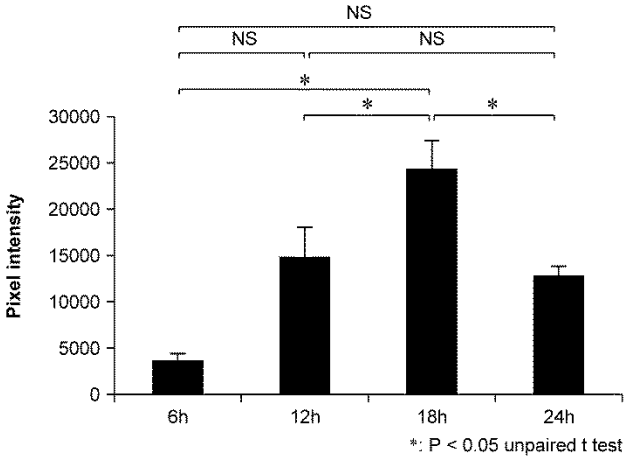
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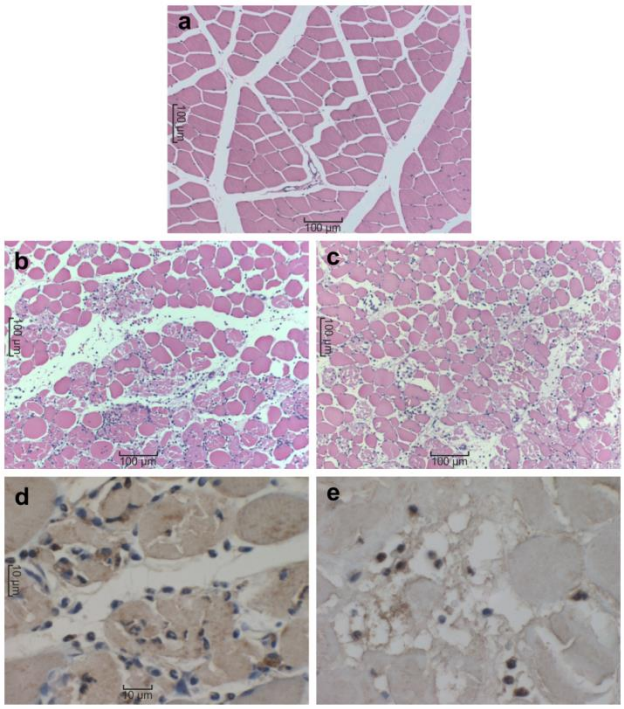

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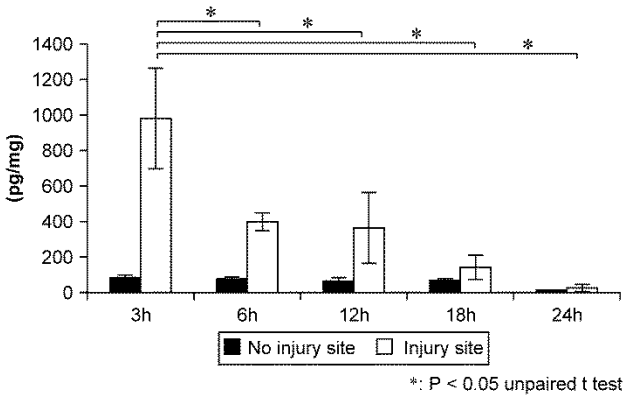
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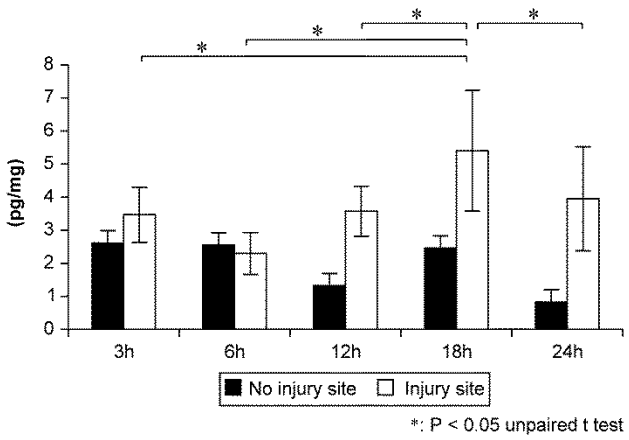
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