Progression of Secondary Injury After Musculoskeletal Trauma—A Window of Opportunity?

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Context: Acute musculoskeletal-injury management largely focuses on inhibiting secondary injury, although the data describing secondary injury and the timeline for its progression are sparse. Objective: To describe the timeline and early progression of secondary injury in skeletal muscle over the first 5 h after blunt trauma. Design: A controlled laboratory study with 2 independent variables (injury status and postinjury time point) in a 2 x 21 factorial. Setting: University research laboratory. Subjects: 168 male Sprague Dawley rats (250 to 275 g). Interventions: Uniform blunt-contusion injury was caused to the right triceps surae using a drop-weight method; the contralateral limb served as an uninjured control. Both triceps surae were excised and flash frozen at 21 intervals across 5 h postinjury (8 animals, each 15 min). Main Outcome Measures: Cytochrome-c oxidase activity via reduction of triphenyltetrazolium chloride (TTC) to triphenylformazan. Results: There was an interaction effect ($P = .041$) between and main effects for both injury status ($P < .0005$) and postinjury time point ($P = .038$). In the first 30 min after injury, uninjured tissues did not differ from injured tissues, and both displayed TTC reduction rates in the vicinity of 7.1 ± 0.94 μg · mg⁻¹ · h⁻¹. Statistical differences between uninjured and injured tissues became evident starting at 30 min. TTC reduction for uninjured tissues did not change, but injured tissues declined in a roughly linear fashion across the entire 5-h period to 4.8 ± 1.04 μg · mg⁻¹ · h⁻¹. Conclusions: Cytochrome-c oxidase activity, an indicator of oxidative phosphorylation and mitochondrial viability, is diminished by events that follow muscle trauma. Loss of this enzymatic activity becomes statistically evident at 30 min postinjury and continues linearly for at least 5 h. This suggests that secondary injury is a slowly developing problem of more than 5 h duration. A window of opportunity for intervention may lie somewhere within the first 30 min after injury.

Keywords: acute injury, mitochondria, oxidative phosphorylation

The goals for early acute management for musculoskeletal trauma in sports injuries are to provide a degree of relief from the initial pain and other unpleasant
sequelae and to minimize the risk of further damage to the injured tissues. Efforts to minimize further damage typically include both protection or immobilization of the injury and application of cold and compression. The logical paradigm evident in these efforts is that the greater the quantity of damaged or necrotic tissue, the longer the time required for its removal and repair and, ultimately, the longer the time before restoration of normal function.

An important tenet in this paradigm for injury management is that the damage to the tissue results from both primary and secondary sources. Primary sources are the initial injurious events themselves and are beyond our ability to modify once the injury has occurred. The secondary sources, however, are an important target of our acute treatments and appear to be modifiable. Secondary causes have been suggested to possibly include autoimmune injury resulting from the acute inflammatory response (also referred to as secondary enzymatic injury), as well as ischemic or metabolic injury (originally called secondary hypoxic injury). In the latter form, there is some evidence to indicate that the secondary injury is related to the loss of cellular aerobic metabolism through damage to the mitochondria.

To modify the secondary causes, it logically follows that we must provide interventions in a timely fashion before the secondary damage has been done. Although there are several articles that have begun to describe musculoskeletal secondary injury and its modification through acutely timed interventions, there are none that have described the critical time course for these interventions or even the time course for the progression of secondary injury itself. The purpose of this study was to provide some of this foundational knowledge by describing the timeline and early progression of secondary injury in skeletal muscle in the first 5 hours after blunt trauma. We did this with hopes of defining a potential window of opportunity for initiating care that might be the target for future research. The notion that there might be such a window comes from our earlier work, in which we demonstrated a substantial retardation of secondary injury with the use of continuous cryotherapy. In that work, however, it was not clear when in time secondary injury took place. In this study, we focused on the mitochondrial sources of secondary injury by examining flux through the oxidative phosphorylation pathway as indicated by the activity of one of this pathway’s key enzymes, cytochrome-c oxidase. We have previously demonstrated that posttrauma changes in this pathway are indicative of secondary injury.

**Methods**

**Design**

This was a controlled laboratory study in a rodent model with 2 independent variables. The first independent variable was injury status with 2 levels (injured and control). The second was postinjury time point with 21 levels starting at the time of injury and covering the first 5 hours postinjury in 15-minute increments. Eight animals were studied at each time point, with one hind limb serving as the control condition and the other as the injured condition. The dependent variable studied was the activity of cytochrome-c oxidase, a key enzyme in the oxidative phosphorylation pathway. Cytochrome-c oxidase activity was measured through its reduction of triphenyltetrazolium chloride (TTC) to triphenylformazan (formazan red) using
a colorimetric assay. All animal care and use procedures were approved by the university’s Institutional Laboratory Animal Care and Use Committee.

Participants

168 male Sprague Dawley rats (Harlan Laboratories, Indianapolis IN) with body weights ranging from 250 to 275 g were studied. All animals were housed 2 per cage, fed and watered ad libitum on commercial rat chow, and kept on a 12-hour light–dark cycle. All animals were accommodated to the laboratory and human handling for approximately 1 week before beginning the experiments. Eight animals were used at each time point in the study.

Procedures

All animals received the experimental injury to one hind limb, with the opposite hind limb serving as a matched control. Each animal was anesthetized with sodium pentobarbital (40 mg/kg of body weight, IP) and supplemented as necessary (20 mg/kg of body weight, IP) for the duration of the experiment (up to 5 h). Under anesthesia, the right calf of each animal received a uniform blunt contusion injury produced using a drop-mass model. Muscle contusion injury was produced by a mass falling through a clear Lucite guide tube (Figure 1). The mass (171 g) was dropped from a 110-cm height onto the top of an impactor (6.4-mm radius) that was resting on the midbelly of the gastrocnemius muscle. We have used this device to

Figure 1 — Novel drop-mass-injury device. The mass was dropped 110 cm through the clear tube onto the impactor. The semispherical head of the impactor rod rested on the belly of the gastrocnemius.
produce uniform injury in several previous studies. No fractures occurred during the injury production. The left calf was used as a control and was not injured. After injury, animals were returned to their containers, where they rested in a side-lying position. Body temperature was monitored using a rectal temperature probe and maintained with a warming pad.

Eight animals were sacrificed at each time point beginning immediately after injury and at 15-minute intervals for the 5 hours after injury. For each animal, the entire triceps surae were excised bilaterally before the animal was euthanized, and the tissue was immediately flash frozen with liquid nitrogen. The frozen tissue samples were stored at –80°C until analysis. At the time of analysis, the tissue samples were weighed, minced, and homogenized in 3 mL of 0.25-M sucrose to isolate mitochondria. Additional sucrose was added to make a 20% homogenate by weight. The homogenate was filtered, and any fascia fragments were weighed and subtracted from the sample weight. Protein content of the sample was determined using a modified version of the method of Lowry.

Cytochrome-c oxidase activity was examined through an assay for the reduction of TTC to triphenylformazan (formazan red) that we have previously used and that was initially described by Belkin et al. Briefly, muscle homogenate mixed with an equal volume of 0.15% TTC in 0.033-M phosphate buffer was incubated for 60 minutes at 39°C. Then, after the reaction was stopped by adding acetone, mixing, and centrifuging, absorbance of the formazan-containing supernatant was measured at 485 nm. The triplicate measurements for each sample were averaged and converted to concentrations of triphenylformazan using a regression equation calculated from a standard curve. The concentrations were normalized to the mass of the sample and to the duration of incubation to produce enzymatic activity expressed in micrograms of TTC reduced per milligram of muscle per hour.

Statistical Analysis

Data were analyzed using SPSS 14.0 software with an ANOVA in a mixed-model 2 × 21 factorial (injury status × time point). Injury status was treated as a fixed, within-subject factor because one limb served as the control condition and the other was the injured condition. Time point was treated as a random, between-subjects factor because the time points were representative of the time continuum after injury rather than discrete points of interest. Experimentwise error rate was set a priori at α = .05. Sidak-corrected pairwise comparisons were used as post hoc tests as needed.

Results

The time course we observed for cytochrome-c oxidase activity after blunt-contusion injury is presented in Figure 2. We observed an interaction effect (P = .041) between injury status and postinjury time point, as well as a main effect for both injury status (P < .0005) and postinjury time point (P = .038). It appears that cytochrome oxidase activity begins declining within the first few minutes after injury, but it took a little time before it became statistically different than the control. In fact, for the first 30 minutes after injury, uninjured tissues did not statistically differ from injured tissues, and both displayed cytochrome-c oxidase activity in the vicinity of 7.1 ±
Differences between uninjured and injured tissues became statistically evident starting at 30 minutes, although activity in the injured tissues appears to have been already declining at the 15-minute time point. Cytochrome-c oxidase activity for uninjured tissues did not change across the time course of the study, but that in injured tissues declined in a roughly linear fashion across the entire 5-hour period to $4.8 \pm 1.04 \mu g \cdot mg^{-1} \cdot h^{-1}$ at our final sample. This equates to a 32% reduction in activity across the 5 hours studied.

**Discussion**

The purpose of this study was to describe the timeline and early progression of secondary injury in skeletal muscle in the first 5 hours after blunt trauma. We hoped that by doing so, we might better understand the “when does it occur” question about secondary injury that we and others have previously identified as being important. The ultimate goal of this line of questioning is a clinical one—to determine the time frame for effective intervention to suppress secondary injury. This study is a step in this direction, but additional steps are still required. If we know at what point secondary injury has already occurred, then, by extension, we know at what point it becomes too late to intervene to try to suppress it. The window of opportunity for attempting to suppress secondary injury would logically be somewhere between the initiation of secondary injury and the point at which it becomes meaningful. We believe that we have very roughly identified a potential window through this study, but it will require future
work to confirm whether interventions applied during this window are clinically effective.

The sports-injury model proposed by Knight\(^6\) 34 years ago and later expanded on in his writing\(^1,20\) has become the predominant paradigm for understanding injury and its resolution to restore function and return athletes to competition. In this model, Knight describes the total quantity of damage in musculoskeletal injury in terms of being the sum of primary and secondary injury. Primary injury he described as the initial, tissue-level ultrastructural damage\(^1,6,20\) such as strains, sprains, contusions, and fractures. It is not modifiable because it occurs before the initiation of any injury management. He described secondary injury as additional damage to otherwise uninjured tissues that results from the physiologic response to the primary injury. Secondary injury was thought to be modified by acute interventions.\(^1,6,20\) Knight’s primary–secondary distinction still holds true even though methodological and theoretical advances have shown some of the details of his model to be incomplete or out of date.

A major hole in our understanding of secondary injury relates to its time course.\(^4\) We know that interventions applied immediately after primary injury are capable of suppressing secondary injury.\(^3,5\) Although clinicians, if present, are able to apply interventions rapidly after injury, there are certainly situations when no care is available initially and there is a delay in care. We do not know whether delayed interventions have potential for benefit, nor do we even know the point in time when secondary injury becomes measurable or ends. This lack of this foundational knowledge limits our understanding of when our interventions have the best chance of being effective.

In this study, we observed that secondary injury, as indicated by the loss of cytochrome-c oxidase activity as mitochondria fail, may begin in the first few minutes after injury and becomes statistically evident by 30 minutes (Figure 2). From this we conclude that acute interventions aimed at limiting secondary injury are likely to be most effective if initiated immediately and certainly at some time within the first 30 minutes. Furthermore, in examining Figure 2, because there may be some degree of mitochondrial function loss at the 15-minute mark (although not statistically different from uninjured controls because of within-group variability and small numbers of animals), it may even be advisable to begin interventions before the 15-minute point. Other investigations\(^3,5\) have demonstrated meaningful suppression of secondary injury when immediate cryotherapy is used.

In addition to noting the onset of secondary injury, we observed that the loss of cytochrome-c oxidase activity continues linearly for at least 5 hours (to the endpoint of our study) without having reached a limit. At the 5-hour point, it had declined 32\% compared with uninjured control tissues, which remained essentially unchanged. From these observations, it is apparent that the processes that produce secondary injury continue for at least 5 hours after what we would characterize as a blunt contusion of moderate severity. The implication of this observation is that it may be useful to continue interventions over a multihour period. To date, there are no studies that have definitively outlined the best practices for how long such interventions should continue, but this study is a first step. It is logical that because secondary injury appears to worsen over a time period of more than 5 hours, interventions aimed at minimizing secondary injury may be useful across at least the same period of time.
Limitations

We designed this study with 2 specific aims. The first and most important was to perform a descriptive study in which we identify the early timeline for the progression of secondary injury in skeletal muscle for the first 5 hours after blunt-contusion injury. The second goal was to identify the limits of a theoretical window of opportunity for initiating acute treatments that might limit secondary injury. It was not our purpose and is not possible to conclude from this study the effectiveness of treatments initiated in such a window; this is an important limitation of this work.

Another important limitation is that we only examined a single approach to secondary injury by looking only at the activity of a single enzyme in a key pathway for mitochondrial function. Although this approach has been used previously, it is not the only available one. There are hundreds of interacting agents in the inflammatory and injury processes, and this study’s limited scope does not take these other players into consideration. Additional work using a triangulated approach to quantifying secondary injury will be imperative as this area of research grows. A similar limitation involves the model we used. We elected to use a drop-mass model to produce our injury. Although others have used this model, a great deal of muscle-injury research is being conducted using a strain-injury model. It is unclear whether strain injury produces secondary injury similar to what we observed with contusion injury. To date, the very limited body of secondary-injury research has only used contusion/crush injury or freezing injury. Furthermore, all this research has been performed in outbred rat models. We are making a leap of faith that similar effects will occur in our human patients.

Conclusions

The purpose of this study was to attempt to fill a hole in our knowledge of the timing and progression of secondary injury in skeletal muscle over the first 5 hours after blunt-contusion trauma. A secondary purpose was to attempt to define a window of opportunity for initiating acute care of these injuries that could be studied in future research. We observed that cytochrome-c oxidase activity, an indicator of mitochondrial function and marker of secondary injury, declined linearly by 32% across the 5 hours after trauma. This may prove to be a useful guideline for the duration of intervention programs after acute trauma. We observed that the decline in activity continues some time beyond our 5-hour study duration. Therefore, we cannot draw any conclusion about the endpoint for the treatment window of opportunity. However, we can draw some limited inference about the opening of this window. The decline in enzymatic activity appears to begin almost immediately, and by 30 minutes its magnitude is such that enzymatic activity is statistically different than in uninjured tissues. From this we conclude that a window of opportunity for intervention may open somewhere within the first 30 minutes after injury, providing additional evidence to support long-held clinical notions about the importance of early interventions. These conclusions will require confirmation in future interventional studies and randomized clinical trials before they can be truly useful.
Acknowledgments

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References
