Ischemia Reperfusion Induces Fibrosis Formation in the Skeletal Muscle of Mice

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INTRODUCTION
Extremity trauma constitutes the majority of war wounds, and is a significant problem in civilian medicine (1). A large portion of these involve muscle trauma in the form of ischemia reperfusion injury (I/R) caused by vascular trauma, tourniquet use and/or compartment syndrome. I/R results in inflammation, edema, and oxidative stress, resulting in a slower healing process (2).

When muscle injuries are left untreated they can develop fibrosis, an excessive accumulation of connective tissue that causes scars to form in tissue after several weeks (3). This scar tissue has a negative impact on the ability of muscle to regenerate and regain its function. The goal of this study was to investigate tissue damage and fibrosis development in a tourniquet induced IR mouse model. Our previous research has explored antifibrosis agents to improve muscle healing by preventing fibrotic tissue development. Understanding the timeline of fibrotic development from IR injury will determine the timing at which potential therapies may be administered to reverse the scar tissue formation.

METHODS
Tourniquet Application: Mice were anesthetized with 2% isoflurane gas prior to and for the duration of tourniquet application. A single, randomly selected hind limb was elevated, and a pneumatic tourniquet (D.E. Hokanson, Inc.) was wrapped snugly against the proximal portion of the limb and inflated to 250 mm Hg by the portable Tourniquet System (Delfi Medical Innovations, Inc.) to ensure complete occlusion of blood flow to the limb for a duration of 2 hours. Body temperature was maintained at 37°C with the use of a water heated surgical bed during this procedure. After 2 hours, the pneumatic tourniquet was removed. Animals were recovered from anesthesia and allowed to regain consciousness before returning to their home cages. In 3, 5, 7, 10, 14, 21, and 28 days following the initial tourniquet application, five mice from each of the time points were sacrificed and their muscles of the uninjured contralateral limb served as controls.

Muscle Tissue Isolation and Analysis: Following isolation and excision, muscles were frozen by submerging it in liquid nitrogen. Serial transverse frozen sections (10 μm) were collected from the belly of the muscles. Slides were then stained with Masson’s modified Trichrome Stain (MmTS) Kit (K7298 IMEB, Inc.). Five randomly selected muscle sections were imaged and analyzed to assess the area percentage of red (non-injured muscle) and blue (tissue damage) using CellProfiler.

RESULTS
Assessment of the tourniquet injury model from 0 to 7 days after initial application: Tissue damage from 0 to 7 days was assessed using MmTS as shown in Figure 1. Scarring does not occur until at least 10 days after injury, therefore the blue region is considered the afflicted area containing both collagen deposition, the infiltration of inflammatory cells and cellular/ECM debris. Above illustrates the progression of tissue damage into the muscle tissue of the TA (figures 1A-1D) and the GM (figures 1E-1H) after the application of the tourniquet. Non-injured samples show only native collagen present, (figures 1A & 1E), whereas 3 (figures 1B & 1F), 5 (figures 1C & 1G), and 7 (figures 1D & 1H) days after the tourniquet has been applied to the leg, the amount of tissue damage has dramatically increased. All images were taken at 10x magnification.

Assessment of the tourniquet injury model from 10 to 28 days after initial application: Collagen deposition was assessed using MmTS as shown in Figure 2. Ten days after the initial injury, fibrotic tissue (blue) development begins and accumulates around dead blood vessels as well as throughout the myofibers as shown in figures 2A-H. Fibrotic development is observed for the TA muscle as shown in figures 2A-D and the GM muscle as shown in figures 2E-H. Fibrotic development is shown at 10 (figures 2A & 2E), 14 (figures 2B & 2F), 21 (figures 2C & 2G) and 28 (figures 2D & 2H) days. All images were taken at 10x magnification.

DISCUSSION
The aims of this study were to establish a mouse IR injury model, measuring the magnitude of tissue damage and fibrosis formation over a time period of 28 days in both the TA and GM muscle. These results showed that the maximal tissue damage occurred at 5 days after tourniquet application as observed in figure 1. By 10 days after the tourniquet application, fibrotic scar tissue had begun forming as illustrated in figure 2. The collagen deposition during fibrosis maintained the same percentage from the 10 to 28 day time period for both muscle types; however, at the end of 28 days, collagen deposition in the TA muscle was lower than the GM muscle. It is not clear from the data why the pattern is different between these muscles. Regardless, these results demonstrate that two hours of tourniquet induced I/R causes a severe degree of fibrosis in a mouse model. This characterization will prove the basis of future work aimed at exploring agents to reverse the fibrotic process and improve soft tissue healing from IR injury.

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REFERENCES