

# Improving the minced muscle graft for repair of volumetric muscle loss injuries by priming donor tissue

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**INTRODUCTION:** Volumetric muscle loss (VML) is an extreme injury in which the loss of skeletal muscle tissue overwhelms the innate regenerative mechanisms, leading to chronic functional deficits. With no standard rehabilitative or surgical standard of care, VML causes high levels of disability, secondary morbidity, and increased mortality. Minced muscle grafts are a regenerative approach using autologous tissue to supplement components lost in VML, enabling *de novo* muscle regeneration; they are translational and do not require FDA approval, but to date have shown only modest strength recovery. We believe the limited graft-derived functional recovery may be due to a less than optimal satellite cell response to transplantation that can be improved by first priming the cells for regeneration. A single bout of eccentric contractions results in activation and proliferation of satellite cells in both human and rodent muscle, often doubling or tripling the total number. Thus, exposing donor muscle to an eccentric-contraction protocol prior to transplantation should result in a larger number of satellite cells being supplied to the VML-injured donor site. We hypothesized that priming donor muscles using eccentric contracts before transplantation into VML injuries would increase satellite cell activation/proliferation and increase the number of pro-regenerative immune cells that support myogenesis and resolve chronic inflammation. Further, we sought to understand if differences in recovery and regeneration were unique between males and females.

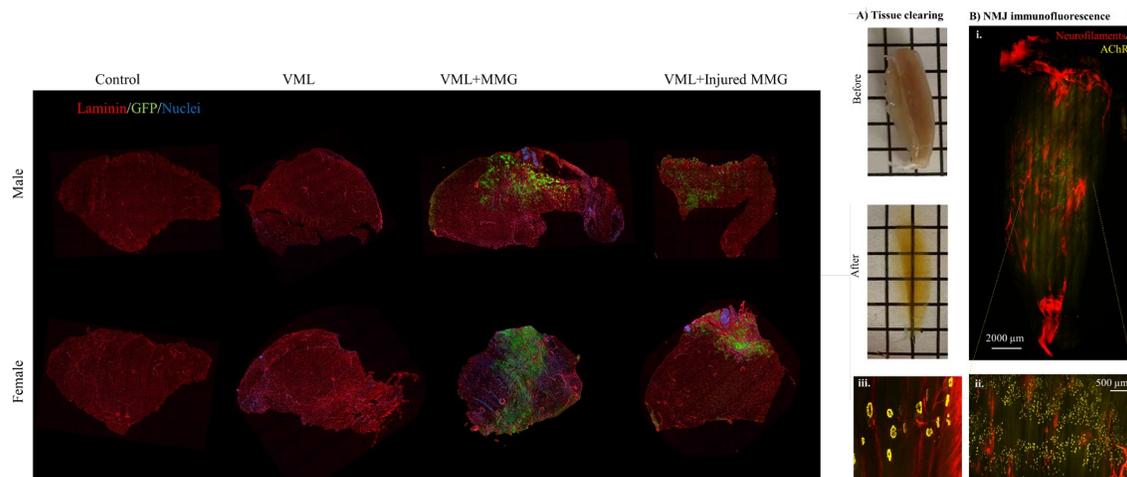
**METHODS:** All experiment protocols and animal care guidelines were approved by the Institutional Animal Care and Use Committee; in compliance with the Animal Welfare Act, the Implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. A total of 47 adult male and female Lewis rats were used across this study. Majority of the rats received VML injuries to the left tibialis anterior (TA) and were randomized to one of three treatments: no repair, minced muscle graft treatment, or primed minced muscle graft treatment (primary experimental groups n=8 per group per sex), contralateral control muscles were also evaluated. A subset of rats were used for donors, with donor muscle subjected to eccentric injury protocol 3 days prior to grafting. GFP-labeled donor tissue enabled tracking of graft-derived myofiber formation. All donors were age and sex matched and inbred Lewis rats ubiquitously expressing GFP. At 8 weeks post-injury, *in vivo* isometric torque was assessed. Tissues were collected for histological, biochemical, and bulk RNA sequencing analyses. A subset of TA muscles underwent tissue clearing and light-sheet microscopy for 3D visualization of graft-derived myofibers and reinnervation. Group differences were evaluated with one- and two-way ANOVAs as necessary.

**RESULTS SECTION:** Eccentric contractions induced immediate donor muscle loss of torque and injury. By 3 days post-priming, there was increased cellular proliferation and immune cell infiltration, indicating early-stage regeneration. Subsequently the donor tissue was successfully harvested, minced and orthotopically transferred to treat the VML injuries. At 8 weeks post-treatment, *in vivo* maximal torque testing revealed enhanced torque in female rats receiving primed grafts, with limited improvement observed in males. The number of *de novo* GFP-expressing fibers was evaluated both cross-sectionally and within the cleared tissue. Surprisingly, the non-primed minced muscle graft treatment resulted in slightly more fibers, and the volume of the grafted area was also slightly greater. Tissue clearing enabled full 3D reconstruction of the TA muscle, uncovering treatment-dependent differences in the neuromuscular junction (NMJ) network. The 3D model offers a more comprehensive view of muscle fiber orientation, often missed by traditional 2D sectioning.

**DISCUSSION:** The sex differences observed in this study highlight the need for further investigation across VML treatment strategies. Tissue clearing and 3D visualization of the TA provided superior characterization compared to traditional 2D sectioning, enabling detailed analysis of NMJ networks and fiber alignment.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Muscle grafts can prove useful in other non-VML situations, such as promoting healing after complex fractures and aiding in reconstruction for conditions like cleft lip/palate. As advancements are made in the use of novel sources of organs for transplantation, such as the ongoing development of human-pig chimera, the use of muscle grafts will likely become a more common clinical practice to treat VML injuries and multiple strategies to enhance outcomes in patients with musculoskeletal trauma need to be evaluated.

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Donor tissue (green) contributed to *de novo* fiber formation in both minced muscle graft treatment groups.

Tissue clearing was successfully performed to enable visualization of innervation in 3D using light sheet microscopy. A) Perfused whole TA muscle shown before and after the SMARTbatch process. B) An immunofluorescent 3D projection of a cleared control TA that is stained for acetyl-choline receptors ( $\alpha$ -bungarotoxin, yellow) presynaptic terminals and neurofilaments (SV2A and 2H3, red). A zoomed in view of the band of innervation in the middle of the TA.