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A 30% Volumetric Muscle Loss Does Not Result in Sustained Functional Deficits after a 90-Day Recovery in Rats

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Abstract

Volumetric muscle loss (VML) is defined as the loss of skeletal muscle tissue which exceeds the body's repair capabilities leading to sustained functional deficits over time. Some etiologies leading to VML include traumatic injuries, congenital diseases, and degenerative myopathies. Currently, the lack of standardized animal models prevents an appropriate estimation of the severity of injury capable of exceeding self-regeneration. Recent work in our laboratory has shown that a 30% VML does not create a sustained functional loss in rats after 3 months. Therefore, the purpose of this study was to evaluate the percentage threshold of muscle loss that results in permanent functional deficits. We surgically created models of 30, 40, and 50% VML injuries in the tibialis anterior (TA) of rats, and subsequently evaluated TA function and structure after a 90-day recovery period. TA muscle force production was measured *in situ* by stimulating the sciatic nerve to obtain a maximum tetanic force. Results revealed that the maximum force produced by rats with a 30% VML was not significantly different from the uninjured muscle, while the maximum force of the 40% and 50% VML groups was significantly lower in comparison to the uninjured muscle. Overall, this study further supports our observations, suggesting that a 30% VML rat model is not suitable for VML studies. Thus, increasing VML percentages might provide an improved standardized and clinically relevant model for VML that produces a long-term deficit in muscle self-regeneration, while providing a strong base for future tissue engineering techniques in medicine.

Lay Summary

The lack of a standardized animal model limits studies addressing volumetric muscle loss. Previous work in our laboratory has shown that a loss of 30% muscle mass does not create a prolonged deficit in the muscle's functional properties. Thus, the purpose of this study was to determine the percentage of muscle loss necessary to produce a long-term deficit in the functional properties of the injured muscle. Our results suggest that a minimum of 40% muscle loss is needed to produce a significant sustained deficit in muscle force production.

Future work in rats will involve the use of a 40% or more VML injury. We will focus on addressing these larger VML injuries by implanting engineered muscle tissue into the injury site to restore muscle function to pre-injury levels.

Introduction

Multiple situations, including traumatic injuries, congenital diseases, and degenerative myopathies, can lead to a severe loss of skeletal muscle. Volumetric muscle loss (VML) is defined as the loss of muscle tissue that exceeds the body's threshold to repair and reestablish its functional properties [1]. Even though skeletal muscle has a great capacity for regeneration in response to injury [2-4], VML injuries – like ones associated with trauma injuries – could result in the inability of the body to fully regenerate muscle and regain function.

Currently, VML treatments focus on replacing the lost skeletal muscle tissue via either a reflected muscle graft or an autologous muscle transfer. These treatments, however, are limited by donor site morbidity and tissue availability [5]. In response to these limitations, researchers are developing new methods to treat VML and combat the limitations of traditional surgical approaches. Specifically, our laboratory has developed scaffold-free, multi-phasic, tissue-engineered skeletal muscle units (SMUs) composed of engineered skeletal muscle constructs fused to engineered bone-tendon anchors. In a previous study, the efficacy of our SMUs was tested in a rat model with an acute 30% VML deficit in the tibialis anterior (TA) muscle followed by a 28-day recovery time [5, 6]. Our VML model in the rat consists of surgically removing a longitudinal section from the lateral aspect of the tibialis anterior (TA) muscle. Consistent with other VML models in the rodent hindlimb, we showed that our model of a 30% VML resulted in a sustained functional deficit after a recovery period of 28 days.

While many studies show variable deficits using a variety of VML injuries, there is a lack of a standardized animal model for the study of VML and the range of injuries includes longitudinal and horizontal incisions, and punch biopsies. While these models of VML vary in the severity of functional deficit, they all exceed self-regeneration capacity of muscle following a 28-day recovery [7, 8, 9]. However, recent work from our laboratory has shown that a 30% VML is not sufficient to create a sustained functional loss in rats. Using our model for VML followed by a 3-month recovery, all VMLs, even the no-repair group, regained 100% function. This data suggests the need for a new animal model that mimics the long-term effects of VML observed in humans. Therefore, the purpose of this study was to create and evaluate different percentages of VML injury – 30%, 40%, and 50% in rat tibialis anterior (TA) – to establish which percentage of muscle loss results in a permanent functional deficit. After a 90-day recovery period, we evaluated the function and structure of the muscle.

Methodology

Animal model and animal care

This study was conducted using 130-150g female Fischer-344 rats, obtained from Charles River Laboratories, Inc. (Wilmington, MA). All animals were acclimated to our colony conditions for 3 months to mature and to reach appropriate experimental weight of 160-180g. The animals were fed Purina Rodent Chow 5001 laboratory chow and water *ad libitum*. All surgical procedures were performed in an aseptic environment, with animals in a deep plane of anesthesia induced by isoflurane or intraperitoneal injections of sodium pentobarbital (65mg/kg). Supplemental doses of sodium pentobarbital were administered as required to maintain an adequate depth of anesthesia. All animal care and animal surgery procedures were in accordance with The Guide for Care and Use of Laboratory Animals [10] and the protocol was approved by the Institutional Animal Care and Use Committee.

Surgical Procedures

Animals were randomly assigned to one of four experimental groups: 1) Uninjured control, 2) 30% VML, 3) 40% VML, and 4) 50% VML. The amount of muscle removed was calculated by multiplying the desired percentage of VML by the rat's estimated TA mass. To estimate the mass of the TA muscles, we used historical data from our past studies on TA muscle mass per body weight. Thus, we took the current body mass of the rats at the time of surgery and multiplied it by 0.1%. Following induction of anesthesia with isoflurane, an incision was made along the left lower hindlimb exposing the TA muscle. A longitudinal incision was made to remove ~30%, 40% or 50% of the TA volume. The resected muscle was blotted and weighed to quantify the mass of muscle removed. The surgical site was then closed with surgical staples, and buprenorphine was administered immediately post-surgery at a dose of 0.03mg/kg.

TA contractile properties measurements

Following 90 days of recovery, isometric contractile properties of the TA were measured *in situ* as previously described by Larkin *et al.* [11]. Briefly, rats were anesthetized with an injection of sodium pentobarbital (65mg/kg) with supplemental injections given to maintain an adequate level of anesthesia throughout the procedure. The TA muscle was isolated from surrounding muscle and connective tissue using great care not to damage the nerve and/or blood vessels during the dissection. A 2-0 silk suture was tied around the distal tendon, and the distal tendon was severed. The animal was then placed on a temperature-controlled platform warmed to maintain body temperature at 37°C. The knee was securely fixed to a post with a wire passed through the femoral head. The distal tendon of the TA muscle was then tied to the lever arm of a servomotor (model 305B; Aurora Scientific) using the 2-0 suture silk. A continual drip of saline warmed to 37°C was administered to the TA muscle to maintain muscle temperature and to prevent desiccation. Muscle contractions were elicited by stimulation of the sciatic nerve using a bipolar platinum wire electrode. The voltage of single 0.2ms stimulation pulses was adjusted to give a maximum isometric twitch. Subsequently, muscle length was adjusted to the optimal length (L_0), the length at which twitch force was maximal. With the muscle held at L_0 , 300ms trains of stimulus pulses were applied at increasing stimulation frequencies until the maxim isometric tetanic force (P_0)

was achieved. Measures of L_0 were taken and used to calculate functional cross-sectional area (CSA) as described previously [11].

After force measurements were completed, both the VML and contralateral TA muscles were removed, and deeply anesthetized rats were euthanized by overdose of sodium pentobarbital followed by bilateral pneumothorax. TA muscles were trimmed of their tendons, blotted, and weighed. Muscle fibers length was calculated by multiplying L_0 by a ratio of fiber length to TA muscle length described previously [12]. Functional CSA was calculated by dividing the muscle mass (mg) by the product of muscle fiber length (mm) and the density of mammalian skeletal muscle, 1.06 g/cm^3 . Specific force (N/cm^2) was calculated by dividing P_0 by the functional CSA for each muscle. Immediately after muscle mass was measured, muscles were coated in tissue-freezing medium, frozen in dry ice-chilled isopentane, and stored at -80°C until being analyzed for histology.

Histological & immunohistochemical Analysis

The frozen TA muscle samples were sectioned cross-sectionally at $10\mu\text{m}$, mounted on Superfrost Plus microscopy slides (Fisher, cat. No. 12-550-15) and used for histological analysis. Sections were stained for general morphology with hematoxylin and eosin (H&E). Masson's trichrome (Polysciences, Inc., cat. 25088-1) was used to assess the degree of fibrosis within the injury site. The sections were examined and photographed with an Olympus BX-51 microscope. Midbelly sections of the TA were imaged and cross-sectional area of the whole muscle was measured using ImageJ. The same H&E-stained midbelly cross-sections were used to measure fiber cross-sectional area. For these measurements, approximately 50 individual H&E-stained fibers were measured using ImageJ in each of three animals per experimental group.

Statistical analysis

Values are presented as mean $\pm$ standard deviation. Measurements of significant differences between groups were performed using GraphPad Prism 7 software. Means were compared using one-way ANOVA and a Tukey post-hoc multiple comparisons test. Differences were considered significant at $p < 0.05$.

Results

Body and muscle mass

Following a 90-day recovery period, the body masses, and VML and contralateral TA muscle masses were compared to determine the effects that the variable percentage of VML had on overall TA muscle mass. The average body masses of the rats from the control, 30% VML, 40% VML, and 50% VML groups were $179 \pm 5.8 \text{ g}$, $175 \pm 5.0 \text{ g}$, $167 \pm 5.8 \text{ g}$, and $172 \pm 9.7 \text{ g}$, respectively, and were not statistically different from each other (Figure 1A), indicating that the increment percentages in VML had no impact on the growth and health of the animals.

All the VML injuries were created on the left TA muscle. As such, the uninjured contralateral right leg was used as a control. Compared to the contralateral (right) TAs, both

the 30% and 40% VML groups showed no statistically significant difference in TA mass ($p=0.1207$, $n=3$ and $p=0.0976$, $n=5$, respectively) (Figure 1B). The 50% VML groups had statistically significant loss of TA mass ($p<0.0001$, $n=6$) compared to the uninjured contralateral TAs. Thus, only the 50% VML group possessed a mass deficit after a 90-day recovery period.

Contractile properties

Regarding functional outcomes of the VML injuries, isometric tetanic force (P_o) measurements of TA muscles following a 30% VML was $P_o=5362.46 \pm 372.50\mu\text{N}$ on average and was not significantly different ($p=0.3674$) from the P_o of $6193.03 \pm 410.87\mu\text{N}$ produced by the uninjured muscle of the control group (Figure 2A). The isometric tetanic force from the 40% and 50% VML groups was $4669.93 \pm 743.05\mu\text{N}$ ($p=0.0136$) and $4741.78 \pm 861.67\mu\text{N}$ ($p=0.0141$), respectively, and was significantly lower in comparison to the uninjured control group (Figure 2A). There was no significant difference in the isometric tetanic forces between the VML injured groups (30% VML vs. 40% VML had $p=0.5178$, 30% VML vs. 50% VML had $p=0.5788$, and 40% VML vs. 50% VML had $p=0.9980$).

Average specific force calculated for the TAs of the control, 30% VML, 40% VML, and 50% VML groups were $27.56 \pm 0.75 \text{ N/cm}^2$, $29.92 \pm 2.35 \text{ N/cm}^2$, $25.13 \pm 4.05 \text{ N/cm}^2$, $27.90 \pm 2.29 \text{ N/cm}^2$, respectively, and were not statistically different when compared to the control group (30% VML $p=0.7057$; 40% VML $p=0.5814$; and 50% VML $p=0.9975$, Figure 2B) suggesting that the intrinsic properties of the VML recovered muscle resembled those of healthy muscle.

Histological analysis

H&E staining of the control TA muscles and VML groups was performed to visualize the gross morphology of the muscles after the 90-day recovery period (Figure 3A-D). Masson's Trichrome staining was performed to evaluate the degree of fibrosis within the injury site (Figure 3E-H). Upon analysis of the Masson's Trichrome staining, none of the VML groups showed fibrotic tissue in the injury site. In both stains, it is evident that the 30%, 40%, and 50% VML groups all exhibit gross morphological differences in the lateral aspect of the TA relative to the control.

Measures of whole TA cross-sectional area revealed that uninjured control muscle had an average cross-sectional area of $27.47 \pm 1.61 \text{ mm}^2$. In comparison, the 30% VML group had an average cross-sectional area of $22.23 \pm 1.20 \text{ mm}^2$, representing a 19.09% decrease ($p=0.0067$) (Figure 4A). Moreover, the 40% VML and the 50% VML groups experienced an even greater decrease in the muscle's cross-sectional area than the 30% VML group. The average muscle cross-sectional area for the 40% VML and 50% VML groups was $21.29 \pm 2.17 \text{ mm}^2$ and $19.48 \pm 2.31 \text{ mm}^2$, respectively, (Figure 4A). There was no difference in the average muscle cross-sectional area between the 40% VML and 50% VML groups. When compared to uninjured TA muscle, muscle cross-sectional area of the 40% and 50% VML groups was reduced by 22.49% ($p=0.0005$) and 29.10% ($p<0.0001$), respectively (Figure 4A).

Regarding the size of the individual muscle fibers, the average CSA for the muscle fibers of the uninjured control, 30% VML, 40% VML, and 50% VML groups were not significantly different from each other ($p=0.2068$, $n=3$ per group) (Figure 4B). The average muscle fiber cross-sectional areas for the uninjured control, 30% VML, 40% VML and 50% VML groups were $3440.89 \pm 1298.76 \text{ mm}^2$, $2510.75 \pm 822.42 \text{ mm}^2$, $3200.20 \pm 768.24 \text{ mm}^2$ and $2501.21 \pm 1019.70 \text{ mm}^2$, respectively. This lack of a significant difference between the VML groups and the uninjured control suggests the presence of healthy, mature muscle fibers in all of the VML groups.

Discussion

The purpose of this study was to evaluate the effect of VML size on recovery of muscle structure and function to establish a percentage of muscle loss that results in a permanent functional deficit in rats, similarly to VML injuries observed in humans. Currently, VML studies that use rats try to create muscle loss ranging between 20% and 30% [3, 5, 9]. Previous work in our laboratory has shown that when recovery periods surpass 28 days, rats with 30% VML injuries fully regained force production capabilities in their injured TA muscle. We hypothesized that after three months we would see no force deficit in the rats that experienced a 30% VML in their left TA, while the 40% VML and 50% VML groups would sustain a significant force deficit. Additionally, we hypothesized the amount of fibrotic tissue would increase as the percentage of VML increased and be a contributing factor to the force deficit.

After a 90-day recovery period, there were no significant differences in body mass between the control, 30% VML, 40% VML and 50% VML groups. This indicates that the different VML injuries did not have any adverse effects on the growth and health of the animals. When comparing the muscle mass of the 30% and 40% VML groups to their respective contralateral legs, the 30% and 40% groups did not show a statistically significant muscle mass deficit. In contrast, the 50% VML group did not recover muscle mass after the 90-day recovery period and exhibited a significant muscle mass reduction when compared to its contralateral leg.

As seen by previous work in our laboratory, the maximum tetanic force produced by a 30% VML group was not statistically different from the uninjured control group, suggesting complete force recovery of the 30% injured TA muscle. In contrast, both the 40% and 50% VML groups showed significant deficit in maximum tetanic force production in comparison to the uninjured group. This decreased force production in both groups suggests the incapability of the muscles to recover from the injuries, and thus creating a sustained deficit. Again, our data suggests that using our model of VML, a 30% VML does not create a permanent functional deficit in the TA muscles of rats. On the contrary, the TA muscles of both 40% VML and 50% VML groups had a significant reduction in force production when compared to the uninjured control muscle, indicating that TA muscles suffering a 40% VML or greater will have a persistent functional deficit after a 90-day recovery.

Histological analysis utilizing Masson's Trichrome showed that there was no fibrotic tissue deposition in the injury areas for the 30%, 40%, and 50% VML groups, suggesting the

presence of healthy muscle tissue. Our model of VML represents the type of injury that would be induced by surgical means, such as during a tumor excision, rather than a traumatic injury. This could explain why no fibrotic tissue was noted in any of the experimental groups. In addition, there was no significant difference in muscle fiber cross-sectional area between groups, suggesting the presence of healthy muscle fibers. The surgical vs. traumatic nature of our model is also corroborated by the specific force data that showed no differences from normal muscle, indicating the remaining muscle is healthy. Thus, while gross morphological changes on the lateral aspect of the TA muscle were present in the 40% and 50% VML groups, the absence of fibrotic tissue indicates that the remaining muscle was healthy and that simply the loss of muscle mass alone explains the deficit in maximum force capabilities in this VML model.

Conclusion

Overall, this study supported our hypothesis that in rats, using our VML model, a 30% loss of muscle is not severe enough to create a functional deficit that is sustained after a 90-day recovery. While the 30% VML group did lose 13.4% of force capability on average, this was not significantly different from the uninjured control muscle. In contrast, the 40% VML and 50% VML lost more than 20% of force production capability on average, suggesting that a minimum of a 40% VML in a rat model must be created to elicit the clinically relevant, long-term functional deficits observed in human VML injuries.

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Figure 1. Body mass and TA mass after 90-day recovery period.

(A) Body mass was measured to evaluate the effect of the injury on the rat's body mass. There were no significant differences in body weight in the 30%, 40%, and 50% VML groups relative to the control group ($p=0.8621$, $p=0.1007$, $p=0.3410$, respectively), indicating that the VML injuries did not impact the overall health of the animals. (B) Comparing left (injured) TA mass with uninjured contralateral TA mass, only the 50% VML group experienced a significant difference in mass ($p<0.0001$), indicating that at least a 50% VML is needed to result in a sustained mass deficit after 90 days. Significant values are identified by ** and no significance with NS.

Figure 2. Comparison of maximum tetanic force (P_0) and specific force.

(A) After a 90-day recovery period, maximum tetanic force production was measured for each of the VML groups. The 30% VML group produced a maximum force that was not significantly different from the control group ($p=0.3674$). Both the 40% VML and the 50% VML groups produced significantly lower forces compared to the control ($p=0.0136$ and $p=0.0141$, respectively). (B) There was no significant difference in specific forces between groups ($p=0.2010$). The average specific force produced by each group was equivalent to literature values for healthy skeletal muscle, indicating the presence of healthy muscle fibers. Significant values are identified by ** and not significant by NS.

Figure 3. H&E and Masson's Trichrome staining.

(A-D) H&E staining shows variations in morphology in all VML groups relative to the control. The 30% (B), 40% (C), and 50% VML (D) groups all exhibit morphological differences (black arrows) in the lateral aspect of the TA relative to the control (A). (E-H) Trichrome stains do not show evidence of differences in tissue fibrosis when comparing the uninjured control muscle group (E) with the 30% (F), 40% (G), and 50% (H) VML groups. Orientation of sections: S=superficial, D=deep, M=medial, and L=lateral. Black arrows indicate site where the different VML injuries were created. Scale bars = 1mm.

Figure 4. Cross-sectional area of TA muscle and muscle fibers.

(A) The 30%, 40%, and 50% VML groups all had a significantly lower average TA midbelly cross-sectional area compared to the uninjured control ($p=0.0067$, $p=0.0005$, and $p<0.0001$, respectively). (B) In contrast, there was no significant difference in the average fiber cross-sectional area between groups ($p=0.2068$, $n=3$ per group).