



Sex-specific relationships between muscle damage and regenerative myofiber characteristics post CTX injury

Naitik Jhanwar¹, Andrea Alfaro-Chacon²

1. All Saints Cathedral School, 12th grade, naitikjhanwar@gmail.com
2. University at Buffalo, abalfaro@buffalo.edu

Abstract

Skeletal muscle regeneration following acute injury relies on highly coordinated interactions between inflammatory signaling and muscle tissue responses. Following myofiber necrosis, immune cells infiltrate the damaged ECM and secrete growth factors like Amphiregulin (Areg), which can promote satellite cell proliferation and muscle repair. However, how biological sex and Areg genotype interact to influence these regenerative processes remains poorly defined. We examined whether sex and Areg genotype modulate the relationship between tissue damage and histological measures of regeneration. It was hypothesized that in a mouse model of CTX-induced injury, female and male mice across WT and Areg KO genotypes would exhibit sex- and genotype-dependent differences in the relationship between damaged area and regenerative myofiber characteristics (size and central nuclei). Twenty-seven mice were divided across four cohorts: WT males, WT females, Areg KO males, and KO females. Following CTX-induced injury, Tibialis Anterior sections were harvested at day 5, stained with WGA and DAPI, imaged, and analyzed using FIJI to quantify morphometric parameters. Pearson correlation analyses were performed separately for each group. A strong inverse correlation between percent damaged area and median CSA was observed only in WT males ($r = -0.89$, $p = 0.019$). Conversely, percent damaged area positively correlated with percent central nuclei in WT females ($r = 0.87$, $p = 0.011$) and KO females ($r = 0.83$, $p = 0.041$). These findings suggest that Areg signaling and biological sex distinctively shape regenerative relationships, underscoring the need for multivariate criteria when evaluating muscle repair.

1. Introduction

The skeletal muscle regeneration process is a highly internally coordinated process that can be generalized into five main phases: muscle degeneration, inflammation, regeneration, maturation, and functional recovery. After myofiber degeneration and necrosis, damaged tissue must be cleared and replaced with new muscle fibers while preserving overall muscle architecture. This process takes place during inflammation, regeneration, and maturation. During the inflammatory phase, immune cells infiltrate the injury site and remove cellular debris. Neutrophils arrive post-injury and are followed by macrophages, which transition from a pro-inflammatory (M1) phenotype to a pro-regenerative (M2) phenotype, signaling the start of the regeneration phase. This shift is facilitated by the secretion of growth factors and signaling molecules that support tissue repair and muscle regeneration (1,2).

One signaling molecule in this shift is Amphiregulin (Areg), a ligand of the epidermal growth factor receptor (EGFR) that contributes to muscle healing (3). As the inflammation phase ends, Areg is secreted by immune cells involved in tissue repair, including regulatory T (Treg) cells and M1 macrophages (4). Areg can influence nearby cells within the regenerating muscle microenvironment, such as promoting proliferation of muscle satellite cells. Areg-mediated EGFR signaling has been linked to satellite cell activation and proliferation, helping enable the transition from inflammation to regeneration.

Muscle regeneration depends largely on satellite cells (SCs), resident muscle stem cells that remain quiescent under normal conditions that become activated following injury. Activated SCs proliferate and differentiate into myoblasts that repair damaged fibers or form new myofibers. Regenerating myofibers commonly exhibit centrally located nuclei, a histological marker of active muscle repair. Other cell types, including fibro-adipogenic progenitors (FAPs), also contribute to regeneration by supporting satellite cell activity. However, this must be carefully maintained as well because excessive or prolonged FAP activation can contribute to fibrosis and impaired muscle function. Successful regeneration depends on precisely coordinated interactions among immune cells, satellite cells, and the surrounding tissue environment.

Understanding these repair mechanisms is clinically important because the disruption of normal regeneration contributes to chronic muscle disorders. Diseases like muscular dystrophy, polymyositis, and dermatomyositis expose muscle tissue to prolonged inflammatory signaling without transition to regeneration, impairing progressive muscle function (5). Persistent inflammation may hinder communication between immune cells and satellite cells, while reducing the effectiveness of tissue repair. Identifying factors that influence regenerative responses may improve our understanding of both normal healing and pathological muscle degeneration. One biological variable that is important to investigate is sex-related difference, which may influence these responses to injury.

To investigate skeletal muscle regeneration under controlled conditions, researchers often use cardiotoxin (CTX)-induced injury models. CTX is a myotoxin from the venom of *Naja pallida* that causes rapid and easily reproducible degeneration of skeletal muscle fibers. Unlike many forms of physical injury by trauma, CTX produces sterile and controlled damage while preserving structures necessary for regeneration like the vasculature, innervation, basal lamina, and

satellite cells of the tissue. This makes CTX useful for studying the biological mechanisms that govern muscle repair.

About 1-2 days post intramuscular administration, CTX induces widespread myofiber necrosis and triggers a regenerative response. Because the underlying regenerative mechanisms remain intact, the satellite cell activation, immune-cell infiltration, and new myofiber formation occur normally (6). This reproducibility allows researchers to compare regenerative outcomes across experimental groups by minimizing potential variation caused by inconsistent injury severity. In addition, the regenerative timeline following CTX injury is well characterized: by approximately days 5 to 7 post-injury, satellite cell proliferation and early myofiber formation are prominent. This period is useful for evaluating indices of muscle regeneration such as myofiber size and central nucleation (7).

Among the skeletal muscles used in CTX injury models, the tibialis anterior (TA) muscle is frequently selected because it is relatively superficial (Figure 1), easily accessible for injection, and well suited for histological analysis (8,9). The TA also exhibits a consistent injury profile following CTX administration, which explains its widespread use in studies of muscle regeneration (10). Histological assessment of TA muscle allows investigators to evaluate both tissue damage and regenerative activity at the cellular level.

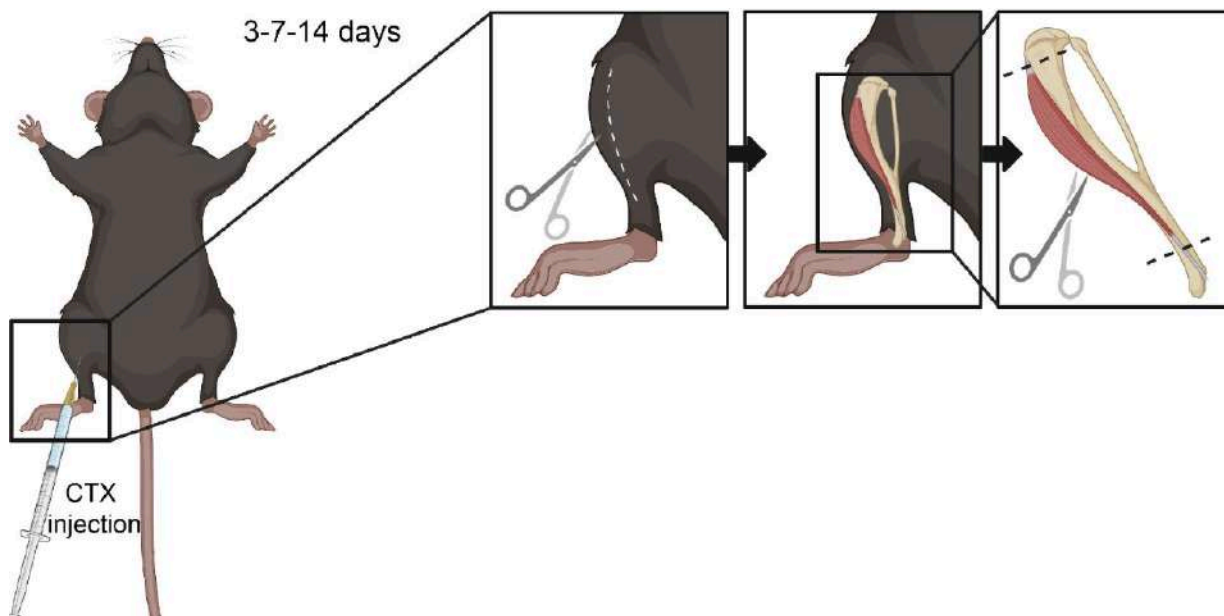


Figure 1. The tibialis anterior muscle in mice. Diagram showing the location and accessibility of the TA muscle in mice for intramuscular injection.

Several staining approaches are commonly used to quantify regenerative outcomes. Wheat germ agglutinin (WGA) is a wheat grain protein visualized in green that binds specifically to N-acetylglucosamine and sialic acid residues, two components abundant in the sarcolemma and extracellular matrix of the cell. It allows researchers to visualize myofiber boundaries and measure cross-sectional area (CSA) (11). DAPI is a fluorescent stain visualized in blue that binds to adenine-thymine rich regions in DNA and provides a means of visualizing nuclei within

muscle tissue (12). Together, these stains enable quantification of damaged tissue area, myofiber size, and central nucleation (Figure 2), three commonly used indicators of muscle regeneration (11).

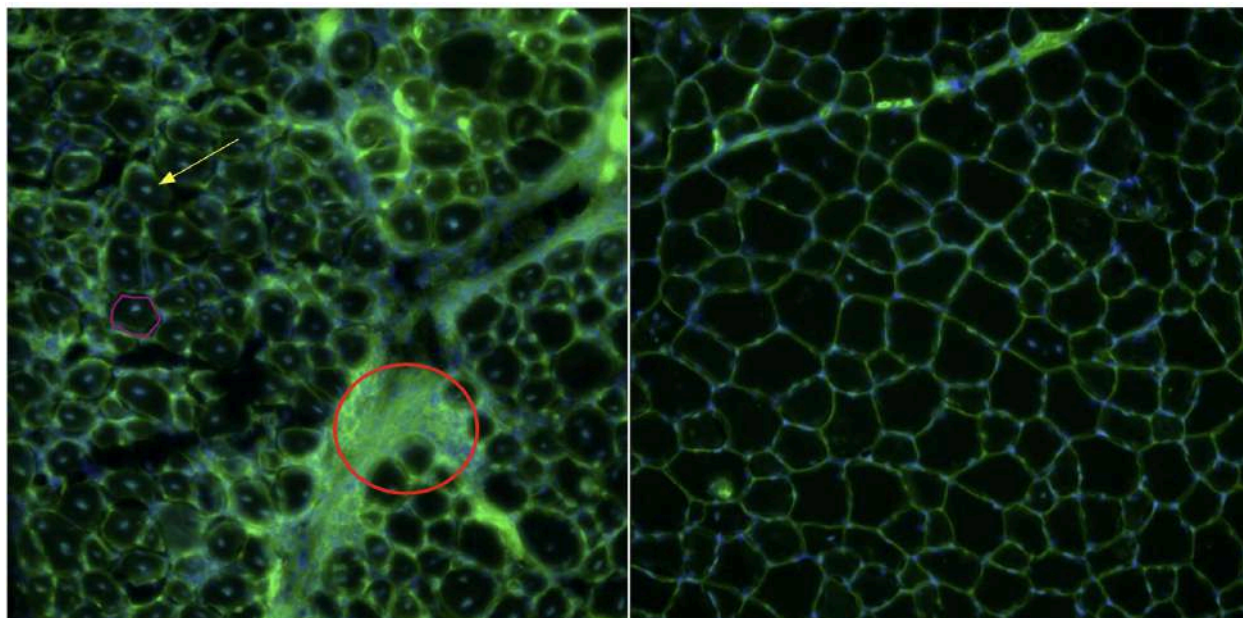


Figure 2a (left) and Figure 2b (right). FIJI images of cross-sectioned muscle. Depiction of cross-sectioned muscle from an injured and uninjured mouse. Common measurements of muscle repair are the damaged area (red circle), central nuclei (yellow arrow), and size of myofiber (magenta polygon) in Figure 2a. In the non-damaged muscle in Figure 2b, there is no damaged area, the myofibers' area is homogenous, and the myofibers with central nuclei are minimal.

Despite advances in understanding the biology of skeletal muscle repair, a gap remains in our knowledge of how biological sex influences the relationship between injury severity and regenerative outcomes. Historically, many similar studies have relied predominantly on male animal models, leaving potential sex-specific differences in regenerative responses insufficiently characterized. In addition, the influence of Areg signaling on these relationships remains incompletely understood. In this study, muscle regeneration was evaluated using histological measures of damaged area, myofiber cross-sectional area, and central nucleation in both wild-type (WT) and Areg knockout (KO) mice. We sought to answer the following research question: In a mouse model of Cardiotoxin (CTX)-induced inflammatory skeletal muscle injury, how does sex influence the correlation between the extent of tissue injury and indices of regeneration? We hypothesized that in a mouse model of CTX-induced inflammatory skeletal muscle injury, female and male mice would differ in the relationship between damaged area and regenerative myofiber characteristics (size and central nuclei), reflecting sex-specific inflammatory and reparative responses.

2. Results

Animal Descriptive Baseline and Morphometrics

To characterize the overall response to CTX- induced injury, we first examined the experimental cohort and the main morphological features of muscle regeneration post-injury.

Experimental Cohort and Morphological Assessment

A total of 27 mice (8-10 week old) were included across 4 experimental groups: 6 WT males, 7 WT females, 8 Areg KO males, and 6 Areg KO females. To evaluate the correlation of different parameters of muscle regeneration in a CTX model, we ensured that all mice were adults with fully mature, responsive satellite cell populations at the time of injury induction. Direct intramuscular delivery was chosen to confine the myotoxin strictly to the TA muscle tissue, preventing systemic diffusion and avoiding systemic or cardiac toxicity. This localized approach allowed the mice to remain healthy, mobile, and physiologically stable throughout the study.

An optimal concentration of the toxin was administered to induce widespread, uniform myofiber necrosis throughout the single-compartment TA muscle belly without causing excessive fluid pressure that could mechanically disrupt the intramuscular structures. Controlling the extent and severity of the initial lesion in this manner ensured that any variations quantified during image analysis, such as differences in myofiber cross-sectional area or central nuclei, can be confidently attributed to true biological differences in sex-specific regenerative response rather than artifacts of inconsistent injury delivery.

Muscle Damage and Regeneration Parameters

To establish baseline parameters of injury severity and regenerative response, we quantified the percentage of damaged tissue area, the proportion of muscle fibers containing central nuclei, and the median myofiber cross-sectional area (CSA) across wild-type (WT) and Areg knockout (KO) male and female cohorts (Figure 3).

Percentage of Damaged Tissue Area (Figure 3a): WT females exhibited the highest average percentage of tissue damage (~25%), followed by WT males (~18%) and KO females (~20%). KO males displayed the lowest overall damaged area (~11%). Percentage of Muscle Fibers with Central Nuclei (Figure 3b): Central nucleation rates remained relatively consistent across all four experimental groups, with cohort averages hovering between ~28% and ~33%. Median Myofiber Cross-Sectional Area (Figure 3c): KO males demonstrated the largest median myofiber CSA (~1700 μm^2). The remaining three cohorts, WT males, WT females, and KO females, exhibited lower and comparable median fiber sizes, averaging around ~1400 μm^2 .

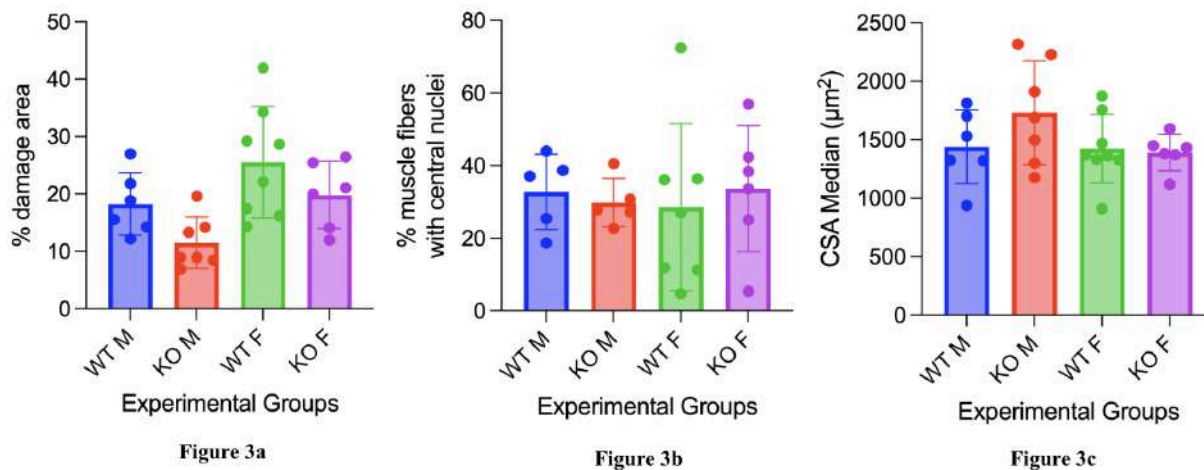


Figure 3. Baseline morphometric parameters in cardiotoxin-injured TA muscle. Quantification of (a) percent damaged tissue area, (b) percent muscle fibers containing central nuclei, and (c) median myofiber cross-sectional area (in square micrometers) across WT male (blue), KO male (red), WT female (green), and KO female (purple) cohorts. Each dot represents an individual biological replicate.

Primary Correlation Analyses: Injury Severity vs. Regeneration

Correlation analyses were used to assess the extent of CTX-induced damage with regenerative changes in the TA muscle.

Correlation 1 - Percent Damaged Area vs. Myofiber CSA

To determine whether muscle injury was associated with impaired muscle fiber growth, we evaluated the relationship between the percentage of damaged muscle area and median myofiber CSA (Figure 4). A strong, statistically significant inverse correlation was observed in WT males ($r = -0.89$, $p = 0.019$), indicating that higher muscle damage was associated with smaller muscle myofibers. A moderate negative association was observed in KO females ($r = -0.71$, $p = 0.117$), while WT females demonstrated a weaker inverse relationship ($r = -0.47$, $p = 0.285$). In contrast, KO males displayed a positive regression trend ($r = 0.39$, $p = 0.345$). Although the direction and correlation strength varied across the groups, only the WT male cohort reached statistical significance ($p < 0.05$), indicating that when the percentage damaged is higher, the area of each myofiber is decreased. The data indicates a strong inverse relationship between tissue damage and myofiber size in WT males, whereas a similar relationship is not statistically supported across other groups with the current sample size.

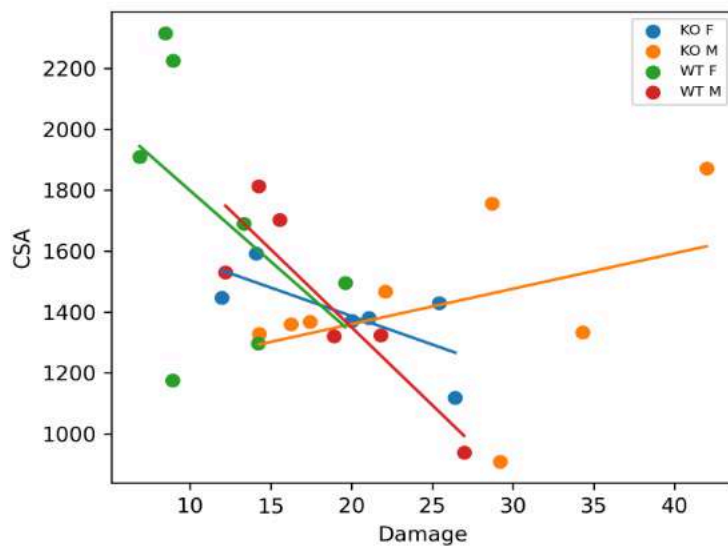


Figure 4. Association between the percentage of damaged area versus myofiber CSA. Each point in the scatter plot represents one biological replicate. Solid lines indicate linear regression. Pearson correlation coefficients (r) and coefficients of determination (r^2) were calculated independently for each group.

Correlation 2 - Central Nuclei per Myofiber vs. Myofiber CSA

To determine whether muscle regeneration was associated with myofiber size, we examined the relationship between the number of centrally nucleated fibers per myofiber and median CSA. Negative correlations were observed across all experimental groups (Figure 5), with the strongest trend seen in WT females ($r = -0.65$, $p = 0.112$) and WT males ($r = -0.61$, $p = 0.272$). Similar negative associations were observed in KO females ($r = -0.57$, $p = 0.238$) and KO males ($r = -0.53$, $p = 0.354$). Although the groups showed a similar overall trend of association, none of the correlations had a significant p-value ($p > 0.05$). Therefore, the data suggests a higher central nucleation tendency occurring associated with lower myofiber CSA, but this relationship is not statistically supported with the present sample size.

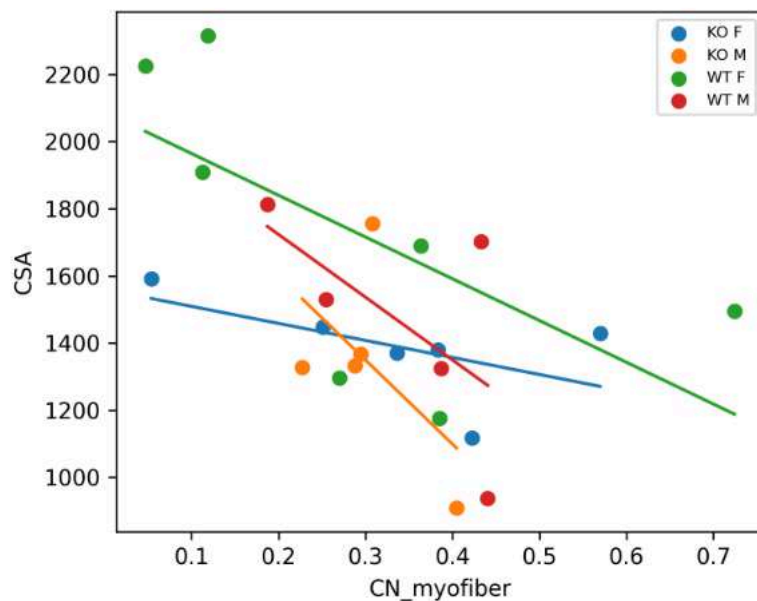


Figure 5. Association between central nuclei per myofiber versus myofiber CSA. Each point in the association represents an individual mouse. Linear regression lines are shown for each experimental group.

Correlation 3 - Percent Central Nuclei vs. Myofiber CSA

We next evaluated whether the percentage of muscle fibers containing central nuclei was associated with fiber size (Figure 6). Moderate to strong inverse correlations were observed across all groups, with WT males displaying the strongest relationship ($r = -0.77$, $p = 0.126$), followed closely by WT females ($r = -0.64$, $p = 0.126$), KO females ($r = -0.57$, $p = 0.238$), and KO males ($r = -0.52$, $p = 0.368$). Although every group exhibited a consistent negative trend, suggesting that muscles undergoing more extensive active regeneration tend to contain smaller myofibers. None of the correlations reached statistical significance ($p > 0.05$). Consequently, while the directional trend is uniform across genotypes and sexes, it was not statistically supported within the present sample size.

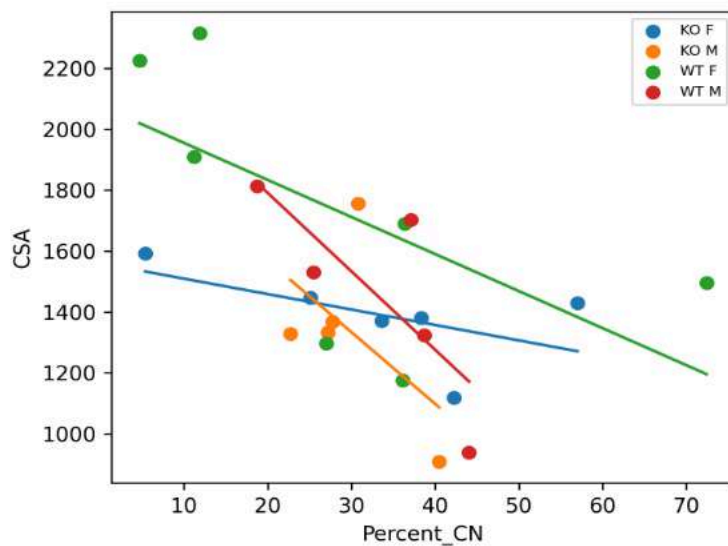


Figure 6. Association between percent central nuclei versus myofiber CSA. Each point in the association represents an individual mouse. Linear regression analyses were performed independently for each sex and genotype.

Correlation 4 - Percent Damaged Area vs. Percent Central Nuclei

To determine whether localized muscle injury promotes active regeneration, we examined the relationship between the percentage of damaged muscle area and the percentage of centrally nucleated fibers (Figure 7). Strong positive correlations were observed in WT females ($r = 0.87$, $p = 0.011$), KO females ($r = 0.83$, $P = 0.041$), and WT males ($R = 0.82$, $P = 0.088$). KO males also exhibited a moderate positive correlation ($R = 0.51$, $P = 0.379$). Because the regressions for both female cohorts (WT and KO) reached statistical significance ($P < 0.05$), the data indicates that greater tissue damage was significantly associated with heightened regenerative activity. In contrast, while positive directional trends were present in male cohorts, these relationships did not achieve statistical significance, suggesting that the tight alignment between injury severity and central nucleation may be more pronounced or reliably maintained in females. The observed associations differed between Areg WT and Areg KO mice. Additional mechanistic studies are needed to determine whether these differences are directly mediated by Areg signaling. Table 1 is a comparative summary of sex-specific and genotype-specific trends across all of the correlation analyses.

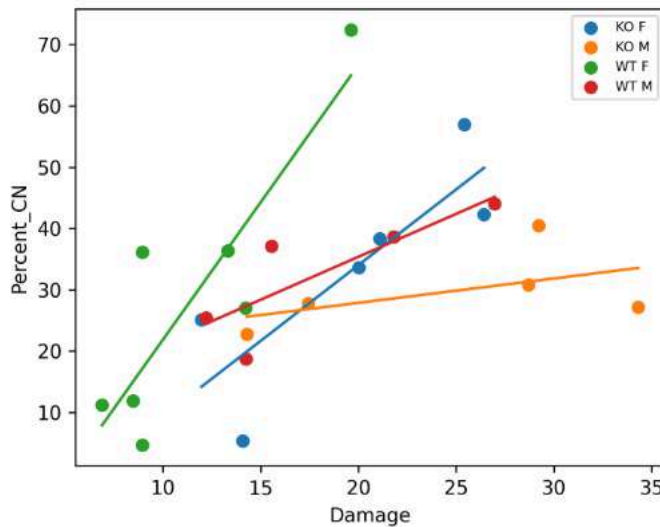


Figure 7. Association between percent central nuclei versus percentage of damaged area. Each point in the scatter plot represents an individual mouse. Linear regression analyses were performed for each sex and genotype to view the trend of data.

3. Discussion

The significant inverse correlation between tissue damage and median myofiber CSA observed in WT males suggests that greater CTX-induced injury suppressed fiber growth in unmanipulated male muscle. This relationship was weaker in WT females, moderate in Areg KO females, and absent in KO males. The reversal of this pattern in KO males (with a positive trend) suggests that the lack of Areg alters the typical relationship between initial tissue damage and muscle remodeling.

In contrast, the relationships comparing central nucleation against myofiber CSA were consistent across all four groups. Whether examining the percentage of centrally nucleated fibers or the number of central nuclei per fiber, negative correlations were consistent, supporting the established observation that regenerating fibers are generally smaller and contain centrally positioned nuclei during early repair. This relationship is biologically expected, since muscle injury activates SCs to form myofibers with centrally located nuclei. However, none of these correlations reached statistical significance, indicating that central nucleation alone may not fully statistically define differences in fiber growth at this sample size.

A different pattern was proven when percent damaged area was compared against the percentage of centrally nucleated fibers. WT and KO females both showed significant positive correlations, indicating that greater tissue damage was statistically associated with a greater proportion of regenerating fibers at day 5 following CTX injury. Although male cohorts had the same positive trend, these relationships were not statistically significant. Together, these findings show that although central nucleation markers reflect standard biology, the relationship between injury severity, regeneration, and myofiber growth may differ according to sex and Areg

genotypes. Because several associations didn't achieve statistical significance, these patterns should be interpreted with caution and confirmed with significance in larger cohorts.

Regenerative capacities and myofiber size dynamics have been supported to be impacted by biological sex. Research has reported sex-related differences in satellite cell function and regenerative potential following injury (13). It has also been shown that muscle regeneration can be influenced by sex-specific biological environments (14). These findings support the importance of considering male and female muscle when studying regeneration. In relevance with the present study, the relationships among damage, central nucleation, and CSA were not identical across sexes.

The inverse relationship observed between tissue damage and myofiber size is also consistent with established models of CTX-induced regeneration. Studies showed that CTX injury initially triggers myofiber necrosis followed by myotube formation, resulting in reduced average fiber size during early repair (6,10). The significant negative correlation observed in WT males demonstrates this model. KO females showed a similar but non-significant trend, whereas KO males showed a positive trend. Although these findings do not demonstrate a direct sex-specific effect of Areg due to non-significance, they suggest that Areg influences how tissue damage relates to post-injury fiber growth.

The differences between the Areg genotype groups can also be considered in relation to previous work examining immunological muscle repair. It has been previously demonstrated that regulatory T cell-derived Areg increases SC proliferation and muscle repair after injury (3). Our findings are consistent with a role for Areg in regeneration, but they don't state Areg loss always contributes to a decline in regenerative output. Central nucleation remained relatively similar across WT and KO groups, while the relationship between damage and CSA varied. Notably, KO females maintained a negative damage-CSA trend whereas KO males did not. This suggests that the effects of Areg loss may depend on the broader biological environment rather than producing identical effects across all groups. Because no downstream signaling pathways were measured, the cellular mechanisms underlying these patterns remain to be determined.

Several broader implications arise from these findings. First, the results support consideration of biological sex as a meaningful variable in studies of skeletal muscle regeneration. The differing correlation patterns observed between males and females indicate that similar levels of injury may not be associated with identical regenerative responses. Second, the findings provide additional reason for investigating the Areg-EGFR pathway during muscle repair. Understanding how this pathway contributes to post-injury remodeling may improve knowledge of muscle healing due to Areg's link to satellite cell function and regenerative signaling. However, the present study did not directly measure EGFR activity or other downstream pathways, so additional experimental work is required before drawing such conclusions.

These observations may also be useful for investigating chronic muscle disorders from impaired regeneration. Conditions like muscular dystrophy and age-related muscle loss involve repeated cycles of degeneration and repair that reduce regenerative capacity. The relationship between tissue damage and fiber growth observed in Areg-deficient mice highlights the importance of immune-muscle communication during regeneration. Further investigation of Areg signaling

could provide insight into mechanisms that influence muscle repair in both acute and chronic diseases, driving therapeutic treatments.

An additional finding of this study is that different histological markers did not always change together. Central nucleation, fiber size, and tissue damage provided different pieces of information about regeneration. Central nucleation, for instance, was relatively similar across groups despite differences in damage-area and CSA relationships. This suggests that reliance on a single regenerative marker could overlook important aspects of tissue repair. Combining measures of damage, myofiber morphology, and nuclei positioning provides a more detailed assessment of regeneration than any individual marker alone.

Several limitations should also be acknowledged. The sample size was small, which might have reduced statistical power and contributed to the non-significance in several correlations. In addition, measurements were collected at only one timepoint following CTX injury. Muscle regeneration is a dynamic process, and analysis at additional stages of recovery would provide a better, more complete understanding of how sex and Areg influence repair over time.

Future studies should include larger cohorts and multiple post-injury timepoints to determine whether the trends observed here persist throughout regeneration. Sampling at points such as days 3, 7, 14, and 28 post CTX injury could show whether the altered damage-growth relationship observed in KO males reflects delayed maturation, accelerated remodeling, or a regenerative deficit. Additional studies incorporating single-cell RNA sequencing or direct analysis of EGFR-related signaling pathways may also help identify the cellular mechanisms underlying the sex- and genotype-dependent patterns observed in this study. Because EGFR binds multiple ligands besides Areg, future work should additionally examine whether compensatory signaling from other ligands contributes to regeneration in Areg-deficient muscle.

Overall, these findings highlight the necessity of incorporating biological sex as a fundamental variable when evaluating muscle regeneration. Our findings demonstrate that male and female muscles exhibit distinct damage vs. growth correlation profiles and distinct sensitivities to Areg loss, which emphasizes that muscle repair mechanisms operate through sex-specific regulatory networks. Recognizing and accounting for these underlying sex-specific differences will be essential for developing targeted therapeutic strategies and improving preclinical models of musculoskeletal recovery.

Relationship	Group	n	R	R ²	P value
Damage vs. CSA	WT M	6	-0.886	0.786	0.0186
	KO M	8	0.386	0.149	0.345
	WT F	7	-0.472	0.222	0.285
	KO F	6	-0.706	0.498	0.117
Central nuclei/myofiber vs. CSA	WT M	5	-0.613	0.375	0.272
	KO M	5	-0.534	0.286	0.354
	WT F	7	-0.652	0.426	0.112
	KO F	6	-0.569	0.324	0.238
% Central nuclei vs. CSA	WT M	5	-0.773	0.597	0.126
	KO M	5	-0.521	0.271	0.368
	WT F	7	-0.635	0.403	0.126
	KO F	6	-0.569	0.324	0.238
Damage vs. % Central nuclei	WT M	5	0.822	0.675	0.088
	KO M	5	0.51	0.261	0.379
	WT F	7	0.869	0.755	0.0111
	KO F	6	0.831	0.69	0.0405

Table 1. Summary of the statistical metrics of all four correlation analyses. (From left to right) n values are representative of the number of mice in each genotype. r and r² values representing the accuracy of each regression are in subsequent columns. p-values to see for statistical significance are in the last column.

4. Materials and Methods

Animals and Experimental Groups

All experimental procedures were conducted using young adult C57BL/6 mice aged between 8 and 10 weeks. To evaluate the roles of biological sex and Amphiregulin (Areg) expression in acute muscle repair, the study cohort comprised both wild-type (WT) and Areg knockout (KO) animals across both sexes. The experimental groups consisted of 6 WT male mice, 7 WT female mice, 8 KO male mice, and 6 KO female mice, giving a total of 27 animals. Standardizing the age range between 8 and 10 weeks ensured that all mice were within a consistent

post-developmental adult stage with fully mature, responsive satellite cell populations at the time of injury induction.

Eight- to ten-week-old male and female C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

CTX-Induced Tibialis Anterior Muscle Injury

To establish a standardized model of acute skeletal muscle injury, a 30 µg/mL dose of cardiotoxin (CTX) was administered via direct intramuscular injection into the belly of the TA muscle. By standardizing this exact injection volume across all 27 experimental mice, an equal baseline of initial tissue damage was established regardless of sex or Areg genotype.

Tissue Collection and Cryosectioning

Five days after CTX injection, the TA muscles were harvested and immediately snap-frozen. The frozen tissue was then cryosectioned at a thickness of 20 µm for subsequent staining and fluorescent imaging.

Day 5 post-injury was selected to assess early muscle regeneration, including the appearance of centrally nucleated myofibers (15).

WGA and DAPI Staining

Following tissue cryosectioning, the TA muscle sections underwent dual-fluorescent staining using wheat germ agglutinin (WGA) and DAPI to visualize and delineate myofiber boundaries and cellular nuclei, respectively.

Sections were incubated with WGA at a dilution of 1:500 for 30 minutes at 37°C. This provides the necessary heat and time for optimal binding of the lectin to N-acetylglucosamine and sialic acid residues along the sarcolemma and extracellular matrix. Sections were subsequently stained with DAPI at a dilution of 1:1000 for 2 minutes at room temperature, to visualize myonuclei without over-saturating the fluorescent signal.

Fluorescence Imaging

To capture high-resolution cross-sectional images of the stained tissue, slides were imaged using a Leica DMI8 fluorescence microscope equipped with a 20x magnification. For each TA muscle section, non-overlapping fields of view were captured using filter sets appropriate for the fluorescent WGA and DAPI signals. WGA fluorescence was used to delineate individual myofiber boundaries while DAPI fluorescence was used to identify nuclei.

Imaging at 20x magnification provided an optimal balance between field of view and spatial resolution, capturing enough individual myofibers per image to assess tissue structure while maintaining the high optical clarity required to accurately identify centrally located nuclei and measure individual myofiber cross-sectional areas.

FIJI Image Analysis

Digital image analysis was performed using FIJI (an expanded distribution of ImageJ software) to quantify muscle damage and regenerative outcomes from the fluorescence images. Using the dual-channel fluorescent images, specific metrics were extracted to evaluate tissue architecture and repair progress for each image.

First, the total count of myofibers exhibiting centrally located nuclei was determined to calculate the proportion of actively regenerating fibers within each field of view. The number and percentage of centrally nucleated myofibers were quantified as an indicator of muscle regeneration.

Next, the percentage of damaged muscle area was quantified relative to the total cross-sectional muscle area by measuring regions where sarcolemma WGA staining revealed membrane disruption, necrotic debris, or expanding extracellular matrix compared to intact tissue.

Finally, individual myofiber boundaries defined by WGA fluorescence were traced to measure the CSA of each fiber in square micrometers (μm^2). The number of central nuclei per myofiber was also recorded for the correlation analysis.

Statistical and Correlation Analysis

Correlation analyses were performed to examine the relationships between tissue injury severity, myofiber morphology, and active regeneration. Four specific correlation analyses were performed: Percent damaged area versus myofiber CSA, central nuclei per myofiber versus myofiber CSA, percent central nuclei versus myofiber CSA, and percent damaged area versus percent central nuclei.

These correlation analyses were used to identify associations between muscle injury, myofiber morphology, and regeneration. Because correlation does not establish causation, the results were interpreted as relationships between variables rather than evidence that one variable directly caused changes in another. Correlations were evaluated separately across the experimental groups to determine whether the relationships differed between male and female mice and between WT and Areg KO animals.

References

1. Choi, et al. "Hepatocyte Growth Factor Regulates Macrophage Transition to the M2 Phenotype and Promotes Murine Skeletal Muscle Regeneration." *Frontiers in Physiology*, vol. 10, 25 July 2019, doi:10.3389/fphys.2019.00914.
2. Rigamonti, et al. "Macrophage Plasticity in Skeletal Muscle Repair." *BioMed Research International*, vol. 2014, 2014, pp. 1–9, doi:10.1155/2014/560629.
3. Burzyn, et al. "A Special Population of Regulatory T Cells Potentiates Muscle Repair." *Cell*, vol. 155, no. 6, Dec. 2013, pp. 1282–1295, doi:10.1016/j.cell.2013.10.054.
4. Gama, et al. "Role of Regulatory T Cells in Skeletal Muscle Regeneration: A Systematic Review." *Biomolecules*, vol. 12, no. 6, 11 June 2022, p. 817, doi:10.3390/biom12060817.
5. Deprez, et al. "Impaired Muscle Stem Cell Function and Abnormal Myogenesis in Acquired Myopathies." *Bioscience Reports*, vol. 43, no. 1, Jan. 2023, doi:10.1042/bsr20220284.
6. Wang, et al. "Skeletal Muscle Regeneration in Cardiotoxin-Induced Muscle Injury Models." *International Journal of Molecular Sciences*, vol. 23, no. 21, 2 Nov. 2022, p. 13380, doi:10.3390/ijms232113380.
7. Fu, et al. "Induction of Skeletal Muscle Injury by Intramuscular Injection of Cardiotoxin in Mouse." *BIO-PROTOCOL*, vol. 13, no. 9, 2023, doi:10.21769/bioprotoc.4668.
8. Feno, et al. "Myonecrosis Induction by Intramuscular Injection of CTX." *BIO-PROTOCOL*, vol. 13, no. 0, 2023, doi:10.21769/bioprotoc.4587.
9. Guardiola, et al. "Induction of Acute Skeletal Muscle Regeneration by Cardiotoxin Injection." *Journal of Visualized Experiments*, no. 119, 1 Jan. 2017, doi:10.3791/54515.
10. Hardy, et al. "Comparative Study of Injury Models for Studying Muscle Regeneration in Mice." *PLOS ONE*, vol. 11, no. 1, 25 Jan. 2016, doi:10.1371/journal.pone.0147198.
11. Kostrominova, Tatiana Y. "Application of WGA Lectin Staining for Visualization of the Connective Tissue in Skeletal Muscle, Bone, and Ligament/Tendon Studies." *Microscopy Research and Technique*, vol. 74, no. 1, 22 Dec. 2010, pp. 18–22, doi:10.1002/jemt.20865.
12. Kapuscinski, Jan. "Dapi: A DNA-Specific Fluorescent Probe." *Biotechnic & Histochemistry*, vol. 70, no. 5, Jan. 1995, pp. 220–233, doi:10.3109/10520299509108199.
13. Deasy, et al. "A Role for Cell Sex in Stem Cell-Mediated Skeletal Muscle Regeneration: Female Cells Have Higher Muscle Regeneration Efficiency." *The Journal of Cell Biology*, vol. 177, no. 1, 9 Apr. 2007, pp. 73–86, doi:10.1083/jcb.200612094.
14. Bahri, et al. "The Panniculus Carnosus Muscle: A Novel Model of Striated Muscle Regeneration That Exhibits Sex Differences in the MDX Mouse." *Scientific Reports*, vol. 9, no. 1, 4 Nov. 2019, doi:10.1038/s41598-019-52071-2.
15. Pizza, et al. "Regenerating Myofibers after an Acute Muscle Injury: What Do We Really Know about Them?" *International Journal of Molecular Sciences*, vol. 24, no. 16, 8 Aug. 2023, p. 12545, doi:10.3390/ijms241612545.