Original Article

Increased Adipocyte Area in Injured Muscle With Aging and Impaired Remodeling in Female Mice

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Abstract

We demonstrated that young male and female mice similarly regenerated injured skeletal muscle; however, female mice transiently increased adipocyte area within regenerated muscle in a sex hormone-dependent manner. We extended these observations to investigate the effect of aging and sex on sarcopenia and muscle regeneration. Cardiotoxin injury to the tibialis anterior muscle of young, middle, and old-aged C57Bl/6J male and female mice was used to measure regenerated myofiber cross-sectional area (CSA), adipocyte area, residual necrosis, and inflammatory cell recruitment. Baseline (uninjured) myofiber CSA was decreased in old mice of both sexes compared to young and middle-aged mice. Regenerated CSA was similar in male mice in all age groups until baseline CSA was attained but decreased in middle and old age female mice compared to young females. Furthermore, adipocyte area within regenerated muscle was transiently increased in young females compared to young males and these sex-dependent increases persisted in middle and old age female mice and were associated with increased *Pparg*. Young female mice had more pro-inflammatory monocytes/macrophages in regenerating muscle than young male mice and increased Sca-1⁺CD45⁺ cells. In conclusion, sex and age influence pro-inflammatory cell recruitment, muscle regeneration, and adipocyte area following skeletal muscle injury.

Keywords: Sarcopenia—Muscle regeneration—Monocyte/macrophage

Skeletal muscle regeneration is an essential part of healing following injury. This complex process includes clearance of necrotic tissue, regeneration of muscle fibers with a time-dependent increase in myocyte cross-sectional area (CSA), and the accumulation of adipocytes in the area of injury (1). Previous studies investigated male and female differences in muscle regenerative capacity following injury as well as differences between young and old mice. Many suggested that females have a regenerative advantage based on estrogen increasing activation and proliferation of satellite cells (2,3); the primary progenitor cells responsible for muscle regeneration (4). Furthermore, transplanting female versus male stem cells into males increased the efficiency of male muscle regeneration (5). Alternatively, our lab

demonstrated no female advantage for regenerated myofiber CSA and increased intermuscular adipocyte area in young female compared to male mice (1). In aging muscle, reduced satellite cell proliferation and differentiation contributed to decreased regenerative capacity (6). Insulin-like growth factor-1 improved satellite cell proliferation in aging muscle and protected against progressive weakness (7,8). However, controversy exists regarding the role of sex and age in regeneration and whether these factors may have an additive effect. Understanding sex- and age-related differences in regeneration could be used to develop sex- and age-specific treatment strategies for patients with muscle injuries, providing insight into the effect of sarcopenia on regenerative capacity.

In addition to affecting satellite cell function, cytokines have profound effects on inflammatory cell recruitment. Following injury, neutrophils constitute the earliest population of phagocytic inflammatory cells, secreting pro-inflammatory cytokines that attract monocytes/macrophages. Circulating blood monocytes mobilized from the bone marrow or spleen are recruited to damaged tissues and can be classified into two populations of pro-inflammatory Ly6C^{hi} and anti-inflammatory Ly6C^{lo} monocytes (9). Tissue monocytes differentiate into macrophage populations that change over time (10,11). Macrophages exhibit remarkable plasticity and can be classified as either classically (M1) or alternatively activated (M2) macrophages, exhibiting either pro-inflammatory or anti-inflammatory activity, respectively (12). M1 macrophages arise from exposure to interferon- γ and lipopolysaccharide, resulting in increased pro-inflammatory cytokines such as tumor necrosis factor- α , iNOS activity, production of reactive oxygen species (12), and expression of Ly6C (11,13). In contrast, M2 macrophages express high levels of arginase-1 (13), the mannose receptor (CD206), and CD301 (14). Targeted depletion of macrophages during different phases of muscle regeneration resulted in altered phenotypes, with the greatest impairments in muscle regeneration being associated with decreased anti-inflammatory macrophages (11).

Impairments in muscle regeneration have also been associated with sarcopenia (15), or age-related loss of lean muscle mass (16) and sex differences in sarcopenia have been identified. Transcriptional regulation of mitochondrial structure and function, muscle protein remodeling, and muscle development played a larger role in the development of sarcopenia in women versus men (17). In rats, soleus muscle weight to body weight ratio declined steadily with age in males while females retained a constant ratio until 26 months in association with differences in regulation of p70 ribosomal protein S6 kinase, protein kinase B (Akt), and mammalian target of rapamycin (18).

While sarcopenia is associated with decreased strength, adjusting for the decreased muscle size suggests that other factors contribute to declining strength with aging (19). Intramuscular adipocytes are increased with aging (20) and may contribute to the loss of muscle strength in older adults (16,19). In addition, intermuscular adipocytes in injured muscle were associated with impaired muscle regeneration in multiple mouse models (11,21,22). While older literature suggested that satellite cells could differentiate into adipocytes (23), more recent studies demonstrated that intermuscular fat emanated from a population of fibrocyte/adipocyte progenitors (FAPs) that reside in muscle (24–26). After injury, FAPs proliferate, interact with myoblasts, and eventually return to quiescent state. With impaired regeneration, FAPs differentiate and generate the components of the fibro-fatty tissue. However, the role of sex and age in the accumulation of adipocytes after injury has not been comprehensively studied.

Based on the reported female (2,3,27) and youth (6,28,29) advantages related to regenerative capacity, we hypothesized that female sex and young age would confer advantages in regeneration of myofiber CSA, adipocyte area in injured muscle, and elimination of residual necrosis after injury. The purpose of this study was to determine the effect of age and sex on differences in muscle regeneration and sarcopenia.

Materials and Methods

Experimental Animals

C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine); male and female mice in three groups: young 4–6,

middle 12–19, and old 25–30 months were studied. A very old 32–33 months group of male mice was also included. All procedures complied with the National Institutes of Health Animal Care and Use Guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the South Texas Veterans Health Care System.

Mouse CTX Model

Myonecrosis was induced by the intramuscular injection of cardiotoxin (CTX) (Calbiochem, San Diego, CA), as previously described (21,22,30). Two and four, 50- μ l CTX [2.5 μ M in normal saline] injections were delivered uniformly into the muscles of the right hind limb anterior and posterior compartments, respectively. Baseline mice did not receive intramuscular injections and were used as controls. Additional study information is available in the [Supplementary Materials and Methods](#).

Results

Sex- and Age-dependent Differences in Body Weight, Anterior Compartment Weight, and Myofiber CSA

At baseline (control, uninjured mice), body and anterior compartment weights were increased ($p < .001$) in male mice compared to females of all corresponding ages (Figure 1). In middle-age female mice, body and anterior compartment weights were increased ($p \leq .02$) compared to young and old females (Figure 1A and B). In contrast, male mice of all ages had similar body and anterior compartment weights. Of note, the anterior compartment weight of very old male mice was reduced ($p \leq .005$) compared to younger male mice (Figure 1B) despite similar body weight among all ages of male mice (Figure 1A).

Similar to muscle weight, the myofiber CSA in tibialis anterior muscle of male mice was larger ($p \leq .01$) than female mice at corresponding ages (Figure 1C). In both male and female mice, myofiber CSA was smaller ($p \leq .008$) in old age group compared to young and middle-aged animals (Figure 1C).

Absence of Residual Myocyte Necrosis in Young Female Mice 7 Days After CTX Injury

The average injury induced by CTX ranged from 84% to 98% of the CSA of the tibialis anterior muscle and was similar between male and female mice as well as between all three age groups. Although robust myocyte regeneration occurred within 7 days after injury, focal areas of residual myonecrosis were associated with mononuclear cell infiltrates among all sex and age groups of mice (Figure 2A–D). The area of residual necrotic tissue at 7 days postinjury was similar between male and female mice at each age (Figure 2E). While there was no age-dependent difference in residual necrosis among male mice, young female mice had less ($p < .001$) residual necrosis than middle and old females, indicating that young female mice were able to remove necrotic tissue faster than middle and old age animals.

Sex-dependent Differences in Muscle Regeneration and Adipocyte Area After CTX-induced Injury

The time course of skeletal muscle regeneration varied among male and female mice. In females, CSA of regenerated myofibers was decreased ($p \leq .04$) compared to baseline (uninjured) myofiber size at Days 7–21 postinjury in young, at Days 7–28 in middle, and at Days 7 and 21 in old mice (Figure 3A–C). The CSA of regenerated

Figure 1. Effect of sex and age on body weight, anterior compartment (AC) weight, and myofiber cross-sectional area (CSA) in the tibialis anterior muscle. Baseline (noninjured) male and female mice of different ages included young (4–6 months), middle-aged (12–15 months), old (25–30 months), and for males only, very old (32–33 months) animals. (A) body weight, (B) AC weight, and (C) CSA of tibialis anterior muscle fibers. Data presented as mean $\pm$ SE; $n = 7$ –12 mice/sex/age group; # $p \leq .01$ male vs. female at the corresponding age group, * $p \leq .02$ compared to middle-aged females; ^ $p \leq .008$ compared to old age group for each sex; § $p \leq .005$ compared to very old male mice.

Figure 2. Effect of sex and age on residual myonecrosis at Day 7 following cardiotoxin injury. (A–D) Histological detail of residual necrosis within regenerated tibialis anterior muscle obtained at 7 days postinjury in young and old, male and female mice. Note regenerated myocytes with centrally located nuclei and infiltrates of mononuclear cells surrounding residual necrotic myofibers (asterisks). Arrows indicate adipocytes. (E) The extent of injury and residual necrosis in the tibialis anterior muscle was determined by histomorphometry in specimen collected from male and female mice at 3 different ages: young (4–6 months), middle (12–15 months), and old (25–30 months). Data presented as mean $\pm$ SE; $n = 7$ –12 mice/sex/age group; * $p < .001$ compared to young female mice.

myofibers at 28 days postinjury in young female mice exceeded ($p = .02$) baseline fiber size (Figure 3A). In males, CSA of regenerated myofibers was decreased ($p \leq .04$) compared to baseline at Days 7–21 in mice of all ages (Figure 3A–C). Regardless of the age in male mice, regenerated myofiber size was similar to baseline by Day 28. In contrast, middle-aged female mice did not attain baseline fiber size by Day 28.

Figure 3. Effect of sex and age on the progressive growth of regenerated myofibers and the intermuscular adipocyte area. The CSA of myocytes and area (%) of adipocytes in the tibialis anterior muscle of male and female mice of different ages included young (4–6 months), middle (12–15 months), and old (25–30 months) obtained at baseline (no injury, Day 0) and at various time (days) after injury. Myofiber CSA in (A) young, (B) middle, and (C) old age male and female mice. Adipocyte area (%) within injured/regenerated muscle in (D) young, (E) middle, and (F) old age male and female mice. Data presented as mean $\pm$ SE; $n = 6$ –13 mice/sex/age group/time point; * $p \leq .04$ compared to baseline for each sex; # $p \leq .05$ male vs female at the corresponding time point.

Regenerated myofiber CSA in male mice was increased ($p \leq .03$) compared to young female mice at Days 21–28 after injury (Figure 3A). In middle-aged mice, myofiber CSA was increased in male ($p \leq .05$) compared to female mice at Days 14–28 postinjury (Figure 3B). In contrast, old male mice had increased ($p \leq .01$) regenerated myofiber size compared to female mice only at Day 7 after injury (Figure 3C).

The area of adipocytes within regenerated muscle was increased ($p < .001$) compared to baseline in both sexes in all three age groups at all postinjury time points (Figure 3D–F). Young female mice had an increased ($p = .01$) adipocyte area compared to young male mice

only at Day 14 postinjury (Figure 3D). Further, compared to male mice, adipocyte area was also increased in female mice in middle and old age animals at Days 14–28 ($p \leq .01$) and Days 21–28 ($p < .001$), respectively (Figure 3). Thus, muscle regeneration in females resulted in increased adipocyte area compared to males.

Inflammatory Cell Accumulation in Injured Skeletal Muscle of Young Mice

Given the increased adipocyte area in female mice and previous reports indicating sex-dependent differences in the accumulation

of macrophage subsets in male versus female mice (31), inflammatory cells and the phenotype of monocyte and macrophage subsets in CTX-injured muscle were quantified by flow cytometry. The total cells recovered from injured muscle were comparable in both sexes (Figure 4A) except for an increase ($p = .008$) in female mice at Day 3 compared to males. Minimal inflammatory cells were present in baseline muscle in both sexes (data not shown). In both female and male mice, neutrophil recruitment after injury (Figure 4B) followed similar trends; maximal at Day 2 after injury and decreased thereafter.

In contrast to the similarities in neutrophil recruitment, total monocytes (Figure 4C) were increased ($p < .001$) at Day 2 in female compared to male mice; this elevation was due almost exclusively to an increase ($p < .001$) in the Ly6C⁺ monocyte subset (Figure 4E and G) as the Ly6C⁻ cells were comparable between male and female mice.

Total macrophages, defined as CD11b⁺F4/80⁺ cells, were similar in male and female mice, peaking at Day 3 (Figure 4D, see Supplementary Figure S1 for gating strategy); however, there were differences in macrophage subsets. Ly6C, CD206, and CD301 were used for further phenotypic analysis of the CD11b⁺F4/80⁺ cell populations (Figure 4F and H). CD206 and CD301 were co-expressed on macrophages (data not shown) and exhibited similar kinetics when combined with Ly6C (Figure 4F and H). The main macrophage subsets in male and female mice were Ly6C⁺ or Ly6C⁻ with minimal expression of CD206 or CD301. In both male and female mice, the predominant macrophage population at Days 1–2 postinjury was Ly6C⁺CD206⁻ or Ly6C⁺CD301⁻ cells; subsequently, the double negative cell population (Ly6C⁻CD206⁻ or Ly6C⁻CD301⁻ cells) predominated. Interestingly, only one cell population, Ly6C⁺CD206⁻ (Figure 4F) or Ly6C⁺CD301⁻ (Figure 4H), macrophage subsets were increased ($p \leq .008$) at Day 3 in female compared to male mice. Thus, female mice had increased Ly6C⁺ monocytes and macrophages compared to male mice.

Age-dependent Differences in Muscle Regeneration After CTX Injury

Data from Figure 3 were also used to compare age-dependent differences in muscle regeneration (Supplementary Figure S2). Regenerated myofiber CSA in old female mice was decreased ($p \leq .05$) compared to young female mice at all times after injury and compared ($p \leq .04$) to middle-aged females at Days 21 and 28 (Supplementary Figure S2A). The myofiber CSA of middle-aged females was decreased ($p \leq .04$) compared to young female mice at Days 7, 21, and 28 (Supplementary Figure S2A). Similarly, the regenerated myofiber CSA of old male mice was decreased ($p < .001$) compared to young males and also decreased ($p \leq .002$) compared to middle-aged male mice at Days 21 and 28 after injury (Supplementary Figure S2B). CSA of middle-aged males was smaller ($p = .02$) than young male mice at Day 7 after injury. Because decreased CSA was present in both sexes in old age, it was not surprising that middle and old age female mice had smaller regenerated myofibers at almost all times following injury compared to young females. In contrast, the size of regenerating myofibers in old, middle, and young male mice was similar through Day 14. Thereafter, the myofiber CSA of old male mice was reduced compared to middle and young male mice, which progressively increased in size to attain their baseline size at Day 28 (Figure 3C and Supplementary Figure S2B). To account for the differences in baseline myofiber size between the age groups and sexes, regenerated myofiber CSA was also shown as a percentage of baseline myofiber CSA for females (Supplementary Figure S2C)

and males (Supplementary Figure S2D). Middle-aged female mice regenerated CSA as a percentage of baseline was decreased at all postinjury time points compared to both young and old-aged mice. In contrast, male mice of all three age groups exhibited similar regenerated myofiber CSA as a percentage of baseline, reaching baseline fiber size by Day 28.

Similar to myofiber CSA, female mice also had age-dependent changes in adipocyte area within regenerated muscle. In female mice, adipocyte area was increased ($p \leq .02$) in middle-aged and old mice compared to young mice at Days 7, 21, and 28 (Supplementary Figure S2E). Adipocyte area was decreased ($p = .03$) in old females compared to middle-aged females at Day 14 (Supplementary Figure S2C). In males, adipocyte area was increased ($p = .006$) in middle-aged mice compared to young mice, and decreased ($p = .01$) in old mice compared to middle-aged mice at Day 28 (Supplementary Figure S2F). Thus, while there was increased adipocyte area within regenerated muscle in young female mice at Day 14, adipocyte area was progressively reduced thereafter. In contrast, middle and old age female mice retained an increased adipocyte area through 28 days (Supplementary Figure S2E). For male mice in all three age groups, adipocyte area was maximal at 7 days and decreased with time after skeletal muscle injury (Figure 5A–H and Supplementary Figure S2F).

Increased Sca-1⁺CD45⁻ Cells in Young Female Mice

Adipogenic progenitor cells reside in skeletal muscle (24,25). With the increased adipocyte area present in young female compared to male mice, we hypothesized that female mice would have increased Sca-1⁺CD45⁻ cells. With injury, Sca-1⁺CD45⁻ cells (Figure 5I) increased over time in both sexes with increased ($p = .05$) Sca-1⁺CD45⁻ cells in female compared to male mice at Day 5 after injury (Figure 5J). Thus, compared to young males, young female mice had increased pro-inflammatory Ly6C⁺ monocytes and macrophages (Figure 2) that preceded an increase in Sca-1⁺CD45⁻ cells (Figure 5) and subsequent increase in adipocyte area (Figure 3).

Decreased Sca-1⁺CD45⁻ Cells and Macrophages With Shift Toward a Pro-inflammatory Phenotype in Middle-aged Female Mice

Given the differences in the Day 5 time point in Sca-1⁺CD45⁻ progenitor cells in young mice, we performed flow cytometry on middle-aged mice to compare inflammatory and progenitor cell profiles to young mice (Figures 4 and 5). The total cells recovered from injured muscle at Day 5 in middle-aged male and female total cells were comparable but decreased ($p \leq .04$) in both sexes in middle-aged compared to young mice (Figure 6A). While neutrophil (Figure 6B) and monocyte (Figure 6C) recruitment after injury were similar between the age groups and sexes, there was a shift in the monocyte subsets in female mice. Young and middle-aged male mice had similar numbers of Ly6C⁺ and Ly6C⁻ cells; however, Ly6C⁻ monocytes predominated in young females while Ly6C⁺ monocytes represented the majority in middle-aged females (Figure 6E). Ratios of Ly6C⁻/Ly6C⁺ cells in young males, middle-aged males, young females, and middle-aged females were 1.1, 0.9, 2.0, and 0.7, respectively. Thus, while total monocytes were similar, there was a shift toward a pro-inflammatory monocyte phenotype in middle-aged female mice.

Total macrophages defined as CD11b⁺F4/80⁺ cells (Figure 6D) were similar in young and middle-aged males but were decreased in middle-aged females compared to young females ($p < .001$) and middle-aged males ($p \leq .02$). Macrophage subsets demonstrated a shift toward a more

Figure 4. Inflammatory cell recruitment in young male and female mice after injury. Cells isolated from cardiotoxin-injured muscles of the anterior and posterior compartments of the hind limbs representing all the muscle below the knee but not including the foot were analyzed by flow cytometry. (A) total cells, (B) neutrophils (CD11b⁺Ly6G⁺), (C) monocytes (CD11b⁺(CD90/B220/CD49/NK1.1/Ly6G)⁻(F4/80/I-A^b/CD11c)⁻Ly6C⁻), (D) macrophages (CD11b⁺F4/80⁺), (E and G) monocyte subsets, and (F and H) macrophage subsets. Data presented as mean $\pm$ SE, $n = 3$ mice/sex/time point; * $p \leq .008$ male vs female at the corresponding time point, † $p < .001$ Ly6C⁺ monocyte subset male vs female at the corresponding time point, * $p \leq .008$ for Ly6C⁺CD206⁻ or Ly6C⁺CD301⁻ macrophage subset male vs female at the corresponding time point.

pro-inflammatory phenotype in both sexes of middle-aged mice. Similar to young mice, the main macrophage subsets in middle-aged mice were Ly6C⁺ or Ly6C⁻ with minimal expression of CD206 or CD301. Middle-aged mice had increased Ly6C⁺ (CD206⁻ or CD301⁻) macrophages and decreased Ly6C⁻ (CD206⁻ or CD301⁻) macrophages compared to

young mice in both sexes (Figure 6F and G). While the decreased Ly6C⁻ (CD206⁻ or CD301⁻) macrophages occurred in both sexes of middle-aged mice, the decreases in middle-aged females were more pronounced. Middle-aged to young ratios for Ly6C⁺ (CD206⁻ or CD301⁻) were 2.3 and 1.6 and for Ly6C⁻ (CD206⁻ or CD301⁻) were 0.6 and 0.2 for male

Figure 5. Adipocyte area and Sca-1⁺CD45⁻ cells in injured and regenerated tibialis anterior muscle. (A–H) Histological appearance of regenerating muscle. (I and J) Cells isolated from cardiotoxin-injured muscle were analyzed by flow cytometry; isotype controls were used to determine optimal antibody concentrations. (I) Gating strategy for Sca-1⁺CD45⁻ cells; gates (left panel) were set using fluorescence minus one controls. After gating out debris and dead cells based on forward vs side scatter and propidium iodide, respectively, Sca-1⁺CD45⁻ cells from a female at Day 5 (right panel) were identified, (J) Sca-1⁺CD45⁻ cells in young male and female mice. Data represented as mean $\pm$ SE, $n = 3$ mice/sex/time point; * $p = .05$ male vs female at the corresponding time point.

and female mice, respectively. Taken altogether, middle-aged female mice had decreased macrophages at Day 5 compared to both middle-aged males and young females and macrophage subsets in middle-aged mice exhibited a more pro-inflammatory phenotype than young mice.

Similar to macrophages, while young and middle-aged male mice had similar numbers of Sca-1⁺CD45⁻ cells (Figure 6H), middle-aged females had decreased ($p < .001$) Sca-1⁺CD45⁻ cells compared to young females. The decreased total cells at Day 5 in middle-aged females were predominately secondary to decreases in macrophages and Sca-1⁺CD45⁻ cells.

Age- and Sex-related Increased Tissue *Pparg* and *Cebpa* Expression

To determine molecular events that may drive the age- and sex-related differences in adipocyte area during muscle regeneration, expression of adipogenic transcription factors *Pparg* and *Cebpa* (32)

were measured across sex, age, and time points at baseline and following CTX injury. Expression of *Pparg* and *Cebpa* (Figure 7) both increased ($p \leq .005$) above baseline at Day 7 for both sexes and all age groups following injury, thereby, mirroring the increased adipocyte area present at Day 7 (Supplementary Figure S2E and F). Consistent with the age-related increase in adipocyte area of injured female mice (Supplementary Figure S2E), *Pparg* (Figure 7A) and to a lesser extent *Cebpa* (Figure 7B) exhibited age-related increased expression at Day 7 ($p \leq .005$) and Day 21 (*Pparg* only, $p \leq .005$) following injury. Interestingly, this trend was not readily observed in the male mice; consistent with the lack of age-related adipocyte area increases in the middle and old compared to young males as was present in female mice (Supplementary Figure S2E and F). While baseline *Pparg* was increased ($p \leq .04$) in middle and old females compared to males, the sex differences in the expression of *Pparg* (Figure 7A) and *Cebpa* (Figure 7B) were increased in old mice at Day 7 ($p \leq .001$) and Day 21

Figure 6. Inflammatory cell recruitment and Sca-1⁺CD45⁻ cells in young and middle-aged mice 5 days after injury. Cells isolated from cardiotoxin-injured muscles of the anterior and posterior compartments of the hind limbs representing all the muscle below the knee but not including the foot were analyzed by flow cytometry in male and female mice of different ages included young (4–6 months, $n = 3/\text{sex}$) and middle (12 months for males and 19 months for females, $n = 4/\text{sex}$). (A) Total cells, (B) neutrophils (CD11b⁺Ly6G⁺), (C) monocytes (CD11b⁺(CD90/B220/CD49/NK1.1/Ly6G)⁻(F4/80/L-A^b/CD11c)⁻Ly6C⁺), (D) macrophages (CD11b⁺F4/80⁺), (E) monocyte subsets, (F and G) macrophage subsets, and (H) Sca-1⁺CD45⁻ cells. Data presented as mean $\pm$ SE, $n = 3\text{--}4$ mice/sex/age group; $\dagger p \leq .04$ compared to young for the corresponding sex, $\# p \leq .04$ male vs female at the corresponding age group, $* p = .04$ for Ly6C⁺CD206⁻ macrophage subset compared to young for the corresponding sex, $\S p \leq .02$ for Ly6C⁻CD206⁻ or Ly6C⁻CD301⁻ macrophage subset compared to young for the corresponding sex, $^{\wedge} p = .03$ for Ly6C⁻CD301⁻ macrophage subset compared to middle-aged males.

(*Pparg* only, $p \leq .03$). However, the expression of these transcription factors was comparable across sex in young mice and was consistent with the transient increase in adipocyte area at Day 14 in young female compared to male mice (Figure 3D).

Discussion

This study investigated the effect of both sex and age on skeletal muscle regeneration after extensive injury. Despite previous

literature reporting regenerative advantages for female rodents (2,3,5,27) and young rodents (6,33–35), we found that the regenerative capacity of muscle, as measured by CSA, was remarkably similar across all ages in males, but was impaired in middle-aged and old female mice. Furthermore, while all males exhibited transient increases in adipocyte area after injury, middle-aged and old-aged females exhibited stable increases in intermuscular adipocyte area compared to males and young females. This increased adipocyte area coincided with increased expression of transcription factors that

Figure 7. Effect of sex and age on expression of transcription factors *Pparg* and *Cebpa* at baseline, 7, and 21 days after injury. Whole tissue RNA isolated from muscles of the hind limb anterior compartment at baseline (uninjured) or at Day 7 or 21 after cardiotoxin injury were analyzed by quantitative RT-PCR in male and female mice of different ages including young (4–6 months), middle (12–15 months), old (25–30 months). The ranges of raw Ct values for each transcript were: *Ubc* 20.5 – 26.8, *Pparg* 22.9 – 29.7 and *Cebpa* 23.8 – 32.9. (A) *Pparg* and (B) *Cebpa*. Data presented as mean $2^{-\Delta Ct} \pm SE$, $n = 4$ –7 mice/sex/age group; * $p < .04$ compared to young for the corresponding time point and sex, # $p < .04$ male vs female at the corresponding time point and age group, † $p < .04$ compared to baseline for the corresponding age group and sex, ‡ $p < .04$ compared to middle-aged for the corresponding time point and sex.

promote adipogenesis, *Pparg* and *Cebpa* (32) in old age female mice. In addition, young females had increased Ly6C⁺ pro-inflammatory monocytes/macrophages preceding the increased Sca-1⁺CD45⁺ cells compared to young male mice. Taken altogether, these findings suggest a role for sex-specific differences and age-related changes in myofiber regeneration, intermuscular adipocyte area, and inflammatory cells after injury.

Different time points for the histomorphometry and flow cytometry studies were chosen based upon our extensive experience with the cardiotoxin injury model (1,11,21). In young mice of both sexes, baseline fiber size was restored by Day 28 (see Figure 3A). To determine if the myofiber CSA size increases were impaired, it was necessary to use later time points such as Days 14 through 28. Inflammation, however, occurs early in the course of injury/regeneration, was mostly resolved by Day 7 (21) and was similar to baseline values by Day 14. Furthermore, ablating macrophages in the early stages of regeneration (Days 2 and 3) resulted in impaired muscle regeneration at later (Day 21) time points (11). Therefore, we chose early time points for the flow cytometry studies and later time points to determine the myofiber CSA and adipocyte area.

Previous studies were consistent with our results in that male mice have larger myofiber CSA, muscle mass, and body mass compared to females at baseline (1,36). Furthermore, baseline muscle mass, body weight and muscle fiber size decreased with age in mice, consistent with sarcopenia (15,16,37). However, body weight did not significantly decline with age and AC weight was only significantly decreased in the very old (32–33 months) male mice. Alternatively, baseline myofiber size decreased more rapidly with age and was significantly decreased in both old and very old males suggesting that myofiber CSA is a more sensitive measurement of sarcopenia

in aging mice. This finding was consistent with a prior study performed on gastrocnemius muscles of aged male rats; a reduction in CSA was more closely associated with weakness compared to reduction in muscle weight (38). Female mice demonstrated a different pattern with increased body and AC weight in middle-aged mice with similar body and AC weight between young and old mice. In contrast to body and AC weight, baseline CSA was decreased in old compared to young and middle-aged females, providing further support that CSA is a more sensitive measurement of sarcopenia than muscle weight.

This study demonstrated that male mice in all age groups had similar abilities to clear necrotic tissue; however, young females demonstrated an improved ability to clear residual necrosis compared to middle and old females. While female mice do not undergo formal menopause similar to humans (39), perhaps the difference between young and old females could be explained by hormonal changes in aging female mice that do not occur in males (40). For example, previous studies suggested estrogen provides a protective effect against injury and oxidative damage; age-related reduction of estrogen could contribute to the increased residual necrotic tissue observed in middle and old female mice (41). Reduced estrogen with ageing contributed to the loss of lean body mass in human females (42) and could likewise affect muscle regeneration. Elevated levels of interleukin (IL)-6 in aged mice contributed to slower recovery of muscle following injury and estrogen administration lowered levels of IL-6, improving recovery (43).

In contrast to our results in old-aged males demonstrating no impairment in muscle regeneration using myofiber CSA, many studies have shown decreased regeneration with aging using satellite cell function as a measure of regenerative capacity. Older male rats exhibited impaired satellite cell proliferation and increased atrophy following muscle disuse (6). Additional studies also suggested a decline in proliferative potential of satellite cells with aging (44–46), while insulin-like growth factor-1 (7,8) and testosterone (47) have been implicated in improving satellite cell proliferation and therefore muscle regeneration in aging rodents. Interestingly, defects in satellite cell proliferation may greatly depend upon the environment. Aged mice parabiotically paired with young mice restored proliferative capacity of satellite cells (46). Our study, on the other hand, used myofiber CSA as a measure of regeneration, yielding a different result in aged male versus female mice. CSA is a commonly used measure of sarcopenia (15,48). By using CSA as a measure of regeneration as well as sarcopenia, we could directly compare CSA as a baseline and regenerative measure within and between age groups and sexes. Despite many studies describing impaired muscle regeneration based on satellite cell function, we found that regeneration was unimpaired in old-aged males despite decreased baseline CSA. Old-aged females, however, demonstrated impaired regeneration based on CSA, suggesting that CSA may be a more sensitive indicator of muscle regeneration impairment than satellite cell function.

Our previous study compared muscle regeneration in young mice demonstrated similar regenerated CSA following injury between males and females after extensive injury (1), which was inconsistent with other literature that demonstrated a female advantage in a milder form of exercise-induced injury (41). The female advantage has been attributed to estrogen stimulation of satellite cells and increased proliferation of female muscle-derived stem cells (2,3,5). However, our study indicated a male advantage in regeneration with aging. Middle-aged males regenerated in a pattern very similar to that of young males. Old male regenerated myofiber CSA was similar to that of middle and young males

until the smaller, sarcopenic baseline myofiber CSA was reached. However, all three age groups achieved baseline fiber size at the same time point, Day 28. These results contradict previous studies, which demonstrated impaired regenerative capacity in old male rodents attributed to a decline in satellite cell numbers (35,49) and proliferation (33,34). In contrast to males, middle-aged females mirrored the regeneration pattern of old females with smaller regenerated myofiber size compared to young females throughout the regeneration process. Furthermore, middle-aged females did not achieve baseline fiber size by Day 28, while middle-aged male mice did. This contrast between males and females suggests a decoupling of sarcopenia and muscle regeneration. While both old males and females exhibited decreased baseline CSA, older males were able to regenerate in a pattern similar to that of young males, while the regenerated myofiber size in middle-aged and old females lagged behind that of young females. However, both sexes were able to reach the decreased baseline CSA present in old males and females, suggesting that sarcopenia may not be linked to regenerative capacity (37,50–52). While poorly understood, many factors control muscle fiber size (53). As myofibers regenerated to the same size as their respective baselines, it is intriguing to speculate that there is an inherent set point for fiber size that decreases with age and despite maintenance of regenerative capacity, regulates maximal attainable myofiber size in old mice. Muscle injury with impaired regeneration has been proposed as a potential mechanism for the development of sarcopenia (54), although not supported by the results of the current or previous studies (50,51).

Estrogen has also been associated with differences in neutrophil recruitment after muscle injury (55). Myeloperoxidase activity, an indicator of neutrophil infiltration, was increased in the muscles of male, but not in female rats, compared to controls 1 day after exercised-induced muscle injury. Interestingly, administering estrogen to male rats prior to injury resulted in similar myeloperoxidase activity as controls (55). Our previous study comparing young male and female mice after CTX, a more severe injury model than exercise, demonstrated similar neutrophil recruitment in males and females at Days 3 and 7 after injury (1), which was consistent with the current study.

Monocytes/macrophages are also important inflammatory cells in skeletal muscle regeneration (11). Additionally, the importance of age to the capability of macrophages to promote regeneration was demonstrated as CD11b⁺ macrophages obtained from young mice, as opposed to those obtained from old mice, recovered youthful proliferation of satellite cells derived from old mice (56). A previous study demonstrated similar numbers of CD11b⁺ cells were recruited to injured muscle in young male and female mice; however, differences in macrophage subsets were present (31). Our study was consistent with these observations in that CD11b⁺F4/80⁺ macrophages were similar between young male and female mice (Figure 4D); however, females exhibited increased Ly6C⁺ macrophage subset compared to males (Figure 4F and H). Furthermore, monocyte recruitment, and specifically the Ly6C⁺ monocyte subset, was increased in female compared to male mice after muscle injury (Figure 4C, E, and G). Our previous study in young mice did not delineate monocytes versus macrophages nor study subset populations between male and female mice (1). Ly6C⁺ macrophages can produce increased pro-inflammatory cytokines such as tumor necrosis factor- α and IL-6 (11,57), suggesting that young female muscle regeneration occurred in the presence of increased pro-inflammatory mediators. To our knowledge, this is the first study that used flow cytometry as a direct measurement to investigate the effect of age and sex on the number

of macrophages and their phenotypes in injured skeletal muscle. Our study demonstrated that total macrophages were decreased in middle-aged females compared to young females with similar numbers of macrophages in young and middle-aged males. However, macrophage subsets in both sexes of middle-aged mice switched toward a more pro-inflammatory phenotype; consistent with previous studies that demonstrated pro-inflammatory markers were increased in elderly humans/animals (58,59). Thus, middle-aged mice exhibited differences in inflammatory cell recruitment and the more pronounced differences in females may have contributed to the impairments in muscle regeneration.

Sex- and age-specific differences were also present in adipocyte area after injury. Male adipocyte area transiently peaked at Day 7 and rapidly decreased in all age groups. In contrast, young females had increased adipocyte area from 7 to 14 days that decreased while middle-aged and old age females maintained increased adipocyte area throughout the time course of the experiment. The expression of two transcription factors that promote adipogenesis, *Pparg* and *Cebpa*, were elevated at 7 days after injury in both male and female mice across all ages, consistent with the increase in adipocyte area. Expression of *Pparg* and *Cebpa* exhibited an age-related increase in female mice that was most distinct in the old at Day 7 (for both *Pparg* and *Cebpa*) and Day 21 (*Pparg* only). The abundance and maintenance of *Pparg* expression at both Days 7 and 21 compared to *Cebpa* suggests a more prominent role in age- and sex-related differences in adipocyte area consistent with previous publications demonstrating that *Cebpa* acts through induction of *Pparg* and was not required to promote adipogenesis (32). Thus, sustained elevations of *Pparg* in old female mice may be a mechanism for the elevated adipocyte area present in old females after CTX injury.

Adipocyte area in skeletal muscle has been observed in several conditions and was inversely related with the regenerative capacity of muscle (60,61). However, the origin of the adipocytes remained elusive until recent studies showed that adipocytes can originate from FAP (24). In the current study, Sca-1⁺CD45⁺ cells were increased in young female compared to male mice at Day 5 after injury and were preceded by increased Ly6C⁺ pro-inflammatory monocytes/macrophages at Days 2 and 3, respectively. In contrast, middle-aged female mice had decreased Sca-1⁺CD45⁺ cells compared to young females and while macrophages were also decreased, the monocytes and macrophage subsets in middle-aged females exhibited a more pronounced pro-inflammatory phenotype. It is intriguing to speculate whether the increased Ly6C⁺ monocytes and macrophages in young female mice may induce a pro-inflammatory state in injured muscle that may be responsible for the increased adipocyte area in young female compared to male mice. IL-4 and IL-13 signaling, hallmarks of alternative, anti-inflammatory activation, enhanced FAP proliferation to support muscle regeneration while inhibiting FAP differentiation into adipocytes (62). Interestingly, while both glycerol- and CTX-induced muscle injury resulted in adipocytes in injured muscle, glycerol-induced injury had increased adipocyte numbers and adipocyte area while exhibiting a stronger pro-inflammatory signature compared to CTX injury (61). Interestingly, regenerating muscle injured by either glycerol or CTX exhibited similar trends in adipocyte numbers and area (61). A limitation of our study was that we only measured adipocyte area, and therefore could not distinguish between differences in adipocyte numbers versus size. Nevertheless, crosstalk between FAPs, Sca-1⁺CD45⁺ cells, and inflammatory cells may regulate the extent of intermuscular adipocyte area in injured muscle.

While the current study used a variety of measures to assess sarcopenia and muscle regeneration, muscle function was not directly assessed. Muscle function is difficult to measure in rodent models; limited studies have analyzed functional quality in regenerated muscle. While young mice recovered fiber number and tetanic force after injury by 28 days, older mice did not recover tetanic force even after 60 days (63). Other studies demonstrated that old fibers were much more susceptible to injury, perhaps due to the degeneration of fibers and replacement with weaker fibers with age (64) or due to decreased protein turnover or protection against free radicals in aged muscle (65). Heat shock protein 70 was decreased in older mice and contributed to the inability of older mice to recover the maximum tetanic force following injury (66). Finally, recovery of function following injury in aged male and female mice demonstrated that both sexes of old mice did not recover muscle mass or maximum force following injury, unlike the full recovery in younger mice (67). In contrast, extensor digitorum longus muscle function was similar in young and old-aged female mice after notexin injury with robust regeneration in old mice (51).

While functional studies remain limited, many previous studies have documented a decline in baseline muscle function with age. Loss of slow and fast muscle units with age as well as atrophy of muscle fibers contributed to declining strength and flexibility (15) and decreased tetanic force (68). Neurologic changes contributed to weakness associated with aging (16). The importance of environment was demonstrated by improved regeneration and greater isometric contractile force measured from muscle transplants into young versus old male rats (69). Given the paucity of functional studies in aged rodent models of regeneration, we can only speculate on the functional significance of impaired muscle regeneration in aged female mice demonstrated in the current study of severe muscle injury. That both old male and female mice exhibited decreased baseline CSA, but only females had impaired muscle regeneration present in middle-aged female mice, prior to the onset of sarcopenia, further suggests a decoupling of regeneration and sarcopenia (50–52). On the other hand, baseline fiber size in old versus young mice was 70% in females versus 80% in males, thus the decrease in baseline CSA was more severe in female mice. It is interesting to note that C57BL/6 male mice live longer than female (70,71), yet in other inbred strains, female mice live longer than males (70). Perhaps the impaired muscle regeneration with injury in females contributed to decreased longevity and may contribute to the accelerated sarcopenia in old-aged female compared to male mice in the current study.

In conclusion, age and sex effect muscle regeneration and adipocyte area after severe injury in skeletal muscle. Males demonstrated an advantage over females both with increased CSA regenerative capacity with age and increased ability to clear intermuscular adipocytes following injury. These differences might be related to differences in macrophage recruitment between males and females. Interestingly, increased adipocyte area in young female mice was associated with increased proinflammatory monocytes/macrophages and Sca-1⁺CD45⁺ cells compared to young males. These differences suggest that strategies for muscle regeneration can be tailored for sex and age to improve outcomes.

Supplementary Material

Please visit the article online at <http://gerontologist.oxfordjournals.org/> to view supplementary material.

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