EXERCISE ACCELERATES CUTANEOUS WOUND HEALING AND DECREASES WOUND INFLAMMATION IN AGED MICE

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Running Head: exercise and wound healing

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Abstract. This study purpose was to determine the effect of exercise on wound healing and inflammation in young (three months) and old (18 months) female Balb/cByJ mice. Mice were assigned to either exercise (EX) or sedentary control (CON) groups. EX mice were run on a motorized treadmill at a moderate intensity for 30 min per day for eight days. All mice were given four full thickness dermal wounds and the rate of wound closure was assessed daily for 10 days. Four months later, the aged mice were re-randomized to treatment, wounded again in different locations, and wounds were harvested at 1, 3, or 5 days post-wounding. Wound tissue was analyzed for interleukin-1 beta (IL-1β), interleukin-6 (IL-6), keratinocyte chemoattractant (KC), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor alpha (TNF-α) protein. Myeloperoxidase (MPO) activity and F4/80 mRNA were assessed as an indirect measure of neutrophil and macrophage content, respectively. There was a trend (p = 0.10) for exercise to reduce wound size in young mice, and exercise significantly (p < 0.05) decreased wound size in old mice. TNF-α, KC, and MCP-1 were significantly (p < 0.05) lower in wounds from EX old mice when compared to CON. No group differences were found for wound IL-1β or IL-6, MPO activity, or F4/80 mRNA. Our data suggest that exercise accelerates the wound healing process in old mice. This improved healing response in the old mice may be the result of an exercise-induced anti-inflammatory response in the wound.
Introduction

The clinical impact of delayed wound healing seen in the aged population is priced at over $9 billion per year in the United States (5). Beyond just the economic impact, impaired wound healing causes increased pain and suffering for the elderly, as well as decreased mobility, which can further exacerbate the inactivity and related diseases. In addition to normal aging, prolonged psychological (24) or physical (32) stress can severely delay wound healing. There are many factors that appear to speed wound healing, including topical hormones (4), norepinephrine (19), hyperbaric oxygen therapy (14), and growth factors (13). Many of these same treatment modalities may occur naturally as a result of exercise. The extent to which regular exercise alters wound healing has not been extensively addressed.

In a recent preliminary investigation, exercise improved cutaneous wound healing in older adults (10). In that study, subjects exercised at 70% of their maximum heart rate one hour a day for three months, and a standard wound healed almost 10 days faster in those who exercised when compared to sedentary controls. While the mechanism(s) responsible for this effect was not elucidated, the authors suggested that the acceleration of wound healing could be due to an enhanced neuroendocrine response, and suggested further investigation into this hypothesis and evaluation of proinflammatory cytokines in the local wound environment.

Cutaneous wound healing is characterized by an initial inflammatory response, followed by reformation of the epithelial barrier and extracellular matrix deposition. Inflammation is an important process in wound healing resulting in recruitment of neutrophils (PMNs) and macrophages (møs) by way of inflammatory cytokines and
chemokines such as MCP-1 and TNF-α (6). These inflammatory cells are important for healing and necessary in the event of wound infection. However, in many models of wound healing, lower levels of inflammation are associated with faster healing and less scarring. For example, studies have established that wounds created in fetal mice that exhibit lower levels of inflammation not only heal much faster than adult mice, but also heal without scarring, something not seen in adults (28). In addition, PU.1 null mice, lacking mφs and PMNs, have greatly reduced levels of inflammation in their wounds. Despite lacking functional immune cells early in the wound healing process, these mice not only heal well, but quickly and without scar formation (31). In contrast, normal aging is associated with elevated systemic markers of inflammation. Findings are somewhat inconsistent, but there can be up to a fourfold increase in blood markers of inflammation associated with aging (27). Importantly, aging also appears to exaggerate the inflammatory phase of wound healing (2, 5, 30, 38); a finding that may be causally related to slower wound healing in older subjects.

The hypothesis that exercise may improve wound healing by decreasing proinflammatory cytokines comes from studies that have found that physical activity is associated with decreased levels of inflammation (11). In addition, a randomized trial (1) reported that after 12 weeks of training, subjects with stable chronic heart failure had a significant reduction in MCP-1. In an animal study from our lab, we found that prolonged exercise could reduce intratumoral mφ and blood vessel density and slow the growth of an allogeneic tumor (41), suggesting that exercise may reduce inflammatory cell accumulation at sites of chronic inflammation. These studies, in addition to Emery et al.
(10) led to the hypothesis that moderate exercise would speed cutaneous wound healing and that this effect would be related to lower inflammation within the wounds.

**Materials and Methods**

**Subjects.** All experiments were approved by the University of Illinois at Champaign-Urbana Institutional Animal Care and Use Committee. Female Balb/cByJ mice were individually housed and fed ad libitum, and were either 3, 18, or 22 months of age at the time of experimentation. Food intake and body weights were recorded daily. Mice were kept on a reverse light and dark cycle. Exercise sessions were performed at the beginning of the dark cycle (approximately 0900) to commence at the beginning of the animal’s active period. Exercise was performed on a motorized treadmill adapted with lanes for the mice to run in individualized compartments. Mice were exercised 30 min per day at approximately 70% of their VO₂ max as determined for their age group in previous experiments, which corresponded to 18 m/min at 5% grade for young mice and 12 m/min at 5% grade for old mice (29). Exercise began three days prior to wounding and lasted for five days afterwards. This protocol was used to parallel the pattern used in restraint stress and wound healing studies (32). Control mice were deprived of food and water during the exercise sessions and were placed on the treadmill for similar exposure to the noise and vibration of the treadmill without exercising.

**Wounding Procedure.** The well established wounding procedure (12) involved creating full thickness circular dermal wounds on the upper dorsum of the mouse, resulting in two wounds in young mice and four wounds in old mice. The anesthetic Isoflurane (Isoflo®) was administered in 100% oxygen at a flow rate of two to three liters per min continuously by cone mask under a flow hood. A 3.5 mm sterile, disposable punch
biopsy instrument (Fray Products Corp, Buffalo NY) was used to create the wounds. Wounds were applied one hour after the exercise session was completed. At 18 months of age, mice were wounded, photographed for 10 days, then allowed to heal for four months. At 22 months of age, mice were wounded in different locations on the dorsum and wounds harvested one, three, or five days after wounding. Old mice rested between studies to allow them to completely heal, and all mice were re-randomized for the second study. After sacrifice by rapid CO₂ asphyxiation, wounds and surrounding tissue were harvested using a 6.0 mm punch biopsy instrument. Tissue samples were then snap frozen in liquid nitrogen and stored at -80°C until analysis.

Wound size was evaluated daily using digital photoplanimetry, with a 6.5 mm spot as a reference. Photographs were analyzed by NIH ImageJ software. Using this software, the reference spot was used to calibrate the readings, the wound outline was traced, and the wound area measured. Wound closure was represented by wound area. Wounds were evaluated by two independent investigators; one blinded as to group, and wound size data were compared by correlation and independent t tests. The correlation between raters was 0.91 (p < 0.05) indicating good inter-rater reliability, and the wound size values were not significantly different between the two raters (t = 0.39, p = 0.69).

**Wound Cytokine Analysis.** IL-1β, IL-6, KC, MCP-1 and TNF-α protein levels in wound tissue were determined using Bio-Plex™ cytokine assay (Bio-Rad, Hercules, CA) following the manufacturer’s protocol. Briefly, wounds that had been snap frozen in liquid nitrogen and stored at -80°C were homogenized in a cell lysis buffer (Bio-Rad, Hercules, CA). After being sonicated for 15 seconds, homogenates were centrifuged and filtered to remove debris. 50µl of samples or standards were added to 96-well plates
containing antibody-coupled beads. After a 30 min incubation, plates were washed three
times and detection antibodies added. After another 30 min incubation, plates were
washed three times and detection antibodies added. After a 10 min incubation plates were
washed three times and resuspended in assay buffer and the plate was read on a Bio-Plex
suspension array system (Bio-Rad, Hercules, CA). Protein concentration of each sample
was measured by BCA™ protein assay kit (Pierce, Rockford, IL) and cytokine results
were expressed in terms of picograms of cytokine per microgram of total protein.

**Neutrophil and Macrophage Analysis.** As an indirect means of measuring PMN
infiltration to the wound tissue, wounds from CON (n = 6, 5, and 3 on days 1, 3, and 5)
and EX mice (n= 7, 5, and 3 on days 1, 3, and 5) were analyzed for myeloperoxidase
(MPO) activity as previously described (36). To prepare samples, individual wounds
were homogenized in 2.0 mL of 20 mmol/L phosphate buffer, pH 7.4 (Sigma, St. Louis,
MO). Homogenates were centrifuged at 12,000 × g for 45 minutes, and the supernatant
was decanted. The pellets were resuspended in 1.0 mL of 50 mmol/L phosphate buffer
containing 10 mmol/L ethylenediamine tetraacetic acid (Sigma, St. Louis, MO) and 0.5%
hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO). After a freeze-thaw
cycle, the samples were sonicated briefly and incubated a 60°C for 2 hour to release
maximal MPO activity. The samples were centrifuged at 500 x g for 10 min and the
supernatant was transferred to 1.5 mL tubes for storage at −20°C. F4/80 mRNA was used
as an indicator of mφ infiltration into the wound tissue. Approximately 30mg of wound
tissue was used for quantitative RT-PCR analyses. Total RNA was extracted using an
RNeasy Mini Kit (Qiagen, Valencia, CA) and quantified using the Nanodrop®
(Nanodrop Technologies, Wilmington, DE). RT-PCR was performed using the
MX3000P™ Real-Time PCR System using Brilliant® SYBR® Green Master Mix kits, 1-Step (Stratagene, La Jolla, CA). The thermal profiles consisted of 50°C for 30 min for generation of first strand synthesis of cDNA and 10 min at 95°C for denaturing, followed by 40 cycles of 95°C for 30 sec annealing at 60°C for 1 min, and 72°C for 30 sec. GAPDH was used as the housekeeping gene. All duplicate or triplicate Ct values were within 0.5 Ct units of each other. The primer sequences for GAPDH were kindly provided by K. Goralski. The F4/80 primers were designed using Integrated DNA Technologies PrimerQuest® using gene sequences obtained from NCBI GenBank (Table 1). All primer sequences were verified using the NCBI Nucleotide “Blast” feature. All sequences were purchased from MWG Biotech (High Point, NC).

Data Analysis. All data were analyzed using SPSS v14 software (SPSS Inc. 2005). Values are expressed as mean ± sem. Significance level was set at α = 0.05. Differences in wound sizes between groups were determined with repeated measured analysis of variance (ANOVA) for group and time using a general linear model. Differences in cytokine expression between groups were determined using general linear model univariate ANOVA for group and day. Post hoc t-tests with Bonferonni correction were used in the event of significant main effects.

Results

Body Weight and Food Intake. While there were no overall time, treatment or time by treatment effects for body weight in the 10 day period following wounding in young or aged mice (data not shown), we did notice that all mice lost weight on days 1 and 2 post-wounding, with weight re-gain starting on day 3. Mice lost approximately 0.5 g of body weight by day 1 and there was no difference between the treatments. There was also a
Aging Delays and Exercise Speeds Wound Healing in Old Mice. As has been shown previously (2), we found that cutaneous wound healing was significantly delayed in aged (18 months) when compared to young (three months) mice. There was a significant age main effect (F1,31 = 8.0; p = 0.008; Fig 1) and an age x time interaction (F10,22 = 4.4; p = 0.002; Fig 1). In young mice, while there was a tendency for exercise to reduce early cutaneous wound healing rates (Figure 2), we failed to detect significant group (F1,24 = 2.98; p = 0.10) or group x time interaction (F10,15 = 0.66; p = 0.75) effects. In contrast, in old mice that exhibit delayed wound healing; exercise treatment resulted in altered healing (Figure 3). We found a significant group main effect (F1,38 = 6.3; p = 0.02), but no group x time interaction (F6,33 = 1.9; p = 0.11), indicating better healing in the exercise treated group. Upon examination of the data, the exercise effect appeared to occur in the early phases (up to day 6 post-wounding) of wound healing (Figure 3). Indeed, when wound data were expressed in terms of percent of original wound size, the length of time it took for wounds to close by 20% was 2.5 days faster in the aged exercised mice.

Exercise Reduces TNF-α and Proinflammatory Chemokines in Wounds of Aged Mice.

We examined protein concentrations of the proinflammatory cytokines TNF-α and IL-1β along with IL-6 in the wounds of exercised and sedentary mice. Moreover, we chose to examine the chemokines KC and MCP-1 because of their role in attracting PMNs and mços to inflamed sites, respectively. There was a trend towards an exercise-induced reduction in wound IL-1β (group main effect F1,30 = 3.2; p = 0.08) with no day or group x day interaction effects (Figure 4a). There was a significant group main effect (F1,30 =
12.9; p = 0.001) for TNF-α protein levels in wounds, but no day (F_{2,30} = 0.005; p = 0.99) or group x day interaction (F_{2,30} = 0.008; p = 0.99) indicating that TNF-α levels were lower in the wounds of EX when compared to CON (Figure 4b). While wound IL-6 levels increased as a function of time post-wound (day main effect F_{2,30} = 4.15; p = 0.03), there was no significant group main effect (F_{1,30} = 0.16; p = 0.69) or interaction (F_{2,30} = 0.98; p = 0.39) statistic (Figure 4c). We also found that exercise prior to and during wounding significantly (group main effect F_{1,30} = 9.5; p = 0.004) reduced the PMN chemokine KC (Figure 5a). Moreover, exercise also resulted in a significant reduction (group main effect F_{1,30} = 9.5; p = 0.004) in the monocyte chemokine MCP-1 (Figure 5b).

**Exercise did not alter MPO activity or F4/80 expression in aged mice.** To indirectly assess PMN and mϕ infiltration into wounds, we measured MPO activity and F4/80 mRNA, respectively. We found no group (F_{1,19} = 0.03; p = 0.86), day (F_{1,18} = 0.65; p = 0.43), or group by day effects (F_{1,19} = 0.54, p = 0.47) in MPO activity (Figure 6). In addition, there was no group (F_{1,21} = 0.052; p = 0.82), day (F_{2,21} = 1.96; p = 0.17), or group by day interaction (F_{2,21} = 0.49, p = 0.62) in F4/80 mRNA expression (Figure 7).

**Discussion**

Our data add to the growing body of literature that aging inhibits cutaneous wound healing (2, 5, 8, 15, 17, 18, 20, 21, 30, 33, 38). More importantly, this study is the first to report that exercise can improve cutaneous wound healing in aged mice and that the improved healing is associated with decreased levels of TNF-α and proinflammatory chemokines in the wound tissue. Our data are in agreement with the preliminary study of Emery et al. (10) who found that regular exercise improved wound healing in older people. In that study, subjects exercised at 70% of their maximum heart rate for one hour
a day for three months. They found that a standard cutaneous wound, given one month into the intervention, healed (e.g. decreased to 10% of the original wound size) about 25% faster in the exercisers when compared to sedentary controls. In our study, exercise exerted its effect on wound healing early in the healing process. For example, the time it took exercised mice to heal their wounds to 80% of their original size occurred 51% faster than sedentary controls. In contrast, Godbout et al. (16) found that voluntary wheel running after collagenase-induced Achilles tendon injury in rats did not promote tendon healing and was associated with PMN accumulation in the tendon, reduced stiffness and tensile strength. The reasons for the discrepant results of exercise on wound healing between the studies is most likely related to the tissue (e.g. skin vs. tendon) as tendons differ vastly from skin in that they have different functions, a much lower blood supply and cellular content, and are known to heal much more slowly than skin, taking months or years to fully heal. In addition, the mechanical stress was applied directly to the wounded tendons by exercise in Godbout et al. (16), but indirectly to the cutaneous wounds applied in this study.

There are numerous mechanisms that might explain why exercise alters healing rates, including altered neuroendocrine status, oxygen partial pressure, blood flow, or mechanical load. Indeed, oxygen therapy (14), norepinephrine (19), estrogen treatment (4), and mechanical loading (16) can all impact wound healing rates and each can be altered by exercise. Proinflammatory cytokines and chemokines initiate and coordinate the inflammatory phase of wound healing (9, 39). However, it is now well established that uncontrolled or elevated inflammation might be responsible for the age-related delay in wound healing rates (2). Indeed, wounds from aged subjects exhibit exaggerated
inflammation characterized by an early elevation in PMN and elastase (2, 3) concomitant with reduced mφ production of VEGF and FGF and impaired re-epithelialization and angiogenesis (37). Numerous reports suggest that acute (34, 41) and regularly performed (23, 26) exercise can exert anti-inflammatory effects. For example, a recent report from our laboratory demonstrated that exercise resulted in a reduction in the number of intratumoral PMNs and mφs in subcutaneous allogeneic tumors implanted into mice (41). This effect was associated with a reduction in blood vessel density within the tumors and altered growth kinetics.

In this study, we sought to examine whether the exercise-induced alteration in wound healing in aged mice was related to a reduction in inflammatory cytokines and chemokines within wound tissue. We found that exercise significantly reduced TNF-α, KC, and MCP-1 when measured early (days 1, 3 or 5) after wound healing. There was a trend for lowering of wound IL-1β and no differences in IL-6; a cytokine that has both inflammatory and anti-inflammatory properties (40). The finding of reduced inflammatory cytokines and chemokines is important because wounds from aged subjects exhibit increased inflammation (5, 38), and it is thought that this increased inflammation is responsible for delayed healing. Indeed, Dovi et al. (7) reported accelerated cutaneous wound closure in mice that had been depleted of PMNs by neutralizing antibodies. Moreover, topical estrogen therapy, a well-studied treatment for speeding healing, inhibits PMN chemotaxis and adhesion molecule expression (4).

Interestingly, despite an exercise-induced reduction in chemokines, based upon our analysis of PMN (e.g. MPO activity) and mφ (e.g F4/80 mRNA expression) content, our data do not support the contention that exercise decreased mφ and PMN infiltration
into the wound tissue. There are several possible explanations for this apparent discrepancy. First, although these methods of identifying PMNs and mφs in inflamed tissue have been used (7), they are indirect. Histological examination and, importantly, spatial localization of specific cell populations and proteins in wound tissue in exercised subjects needs to be performed and can be considered a limitation in the present study. Second, chemokines in addition to KC and MCP-1 contribute to the signals that recruit neutrophils and mφs to inflamed tissues. Indeed, mφ recruitment to inflamed adipose tissue of obese mice was not prevented by MCP-1 gene deletion, suggesting that other chemokines can attract mφs to tissues (22). Lastly, despite the fact that we found no change in our markers mφ and PMN infiltration, it is possible that the exercise employed in this study altered the phenotype of the recruited cells, especially that of the mφ. The role of mφs in the wound environment is complex, ranging from early arriving inflammatory mφs which are responsible for clearing debris and infection to late arriving alternatively-activated mφs which are responsible for extracellular matrix and blood vessel formation (25). The influence of exercise on wound mφ phenotype and function will require further investigation, but our finding of a significant reduction in the potent pro-inflammatory cytokine TNF-α, which is produced by inflammatory mφs, lends support to the idea that exercise may have resulted in a phenotypic switch in mφ populations. Future studies will need to address the issue of cell phenotype and determine whether anti-inflammatory treatment of wounds (by traditional treatment modalities or by exercise) alters wound susceptibility to infection.

In summary, we have found that moderate exercise applied shortly before and for five days after cutaneous wounding improved the healing response in aged mice that
exhibit delayed healing. This is an important finding because delayed and impaired
wound healing in the elderly has huge financial and personal costs in the United States
and worldwide. We consider the magnitude of the change in wound healing induced by
exercise in this study to be clinically significant because it is similar to the changes seen
in response to other healing strategies (35) and comparable to that in the only other
exercise study on cutaneous wound healing (10). Importantly, we have found that
exercise reduced the expression of inflammatory cytokines and chemokines in the
wounds of aged mice which could be mechanistically linked to faster healing.
Acknowledgements

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References


Table 1. Primer sequences (5’ to 3’) used for qRT-PCR

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<th>Primer</th>
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<tr>
<td>GAPDH F</td>
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</tr>
<tr>
<td>GAPDH R</td>
<td>TTG ATG TTA GTG GGG TCT CGC TCC</td>
</tr>
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<td>F4/80 F</td>
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<tr>
<td>F4/80 R</td>
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Figure legends

Figure 1. Comparison of cutaneous wound healing in young (three months; n=13) and old (18 months; n=20) female Balb/c mice. Old mice had slower wound healing than young mice, especially in the early phases (up to day 6) post-wounding. There was a significant age main effect ($F_{1,31} = 8.0; p = 0.008$) and an age x time interaction ($F_{10,22} = 4.4; p = 0.002$). * $p < 0.05$ vs. young mice.

Figure 2. Comparison of cutaneous wound healing in young sedentary control (n=13) and exercised (n=13) female Balb/c mice. There was a tendency towards smaller wounds in the exercised group early post-wounding (group main effect $F_{1,24} = 2.98; p = 0.10$) but there was no group x time interaction ($F_{10,15} = 0.66; p = 0.75$).

Figure 3. Comparison of wound size in aged exercised (n=20) and control (n=20) female Balb/c mice. While there was no significant group x time interaction ($F_{6,33} = 1.9; p = 0.11$), wounds in exercised mice were smaller than the controls ($F_{1,38} = 6.3; p = 0.02$), especially up to day 6 post-wounding. * $p < 0.05$ vs. sedentary mice.

Figure 4. Effect of exercise on cytokine protein expression in wounds of aged mice (n=5-7 mice/group/day). There was a trend ($p = 0.08$) for an exercise-induced reduction in wound IL-1β with no day or interaction effects (4a). Exercise resulted in a significant ($p = 0.001$) reduction in wound TNF-α at Days 3 and 5 post-wounding (4b). While wound IL-6 increased significantly post-wounding, there was no group or interaction effect (4c). *$p < 0.05$ vs. sedentary control mice.
**Figure 5.** Effect of exercise on chemokine protein expression in wounds of aged mice (n = 5-7 mice/group/day). Wound KC was significantly (p = 0.004) lower in exercised when compared to control mice with no day or day x group interaction effect (5a). Likewise, exercise also significantly (p = 0.004) reduced wound MCP-1 expression (5b). * p < 0.05 vs. sedentary control mice.

**Figure 6.** Effects of exercise on MPO activity in wounds of aged mice (n = 3-7 mice/group/day). There was no significant group x time interaction (F1,19 = 0.54; p = 0.47), as well as no group (F1,19 = 0.03; p = 0.86) or day (F1,18 = 0.65; p = 0.43) effects. * p < 0.05 vs. sedentary control mice.

**Figure 7.** Effects of exercise on F4/80 mRNA expression in wounds of aged mice (n = 2-7 mice/group/day). There was no group (F1,21 = 0.052; p = 0.82), day (F2,21 = 1.96; p =0.17), or group by day interaction (F2,21 = 0.49; p = 0.62).
Figure 1

The graph shows the change in wound size (mm²) over days post-wounding for young and aged individuals. The y-axis represents wound size (mm²) ranging from 0 to 11, while the x-axis represents days post-wounding from -1 to 11. The data points are marked with error bars indicating variability. The graph shows a trend of decreasing wound size over time, with a notable difference between young and aged groups, indicated by the * symbol.
Figure 2

![Graph showing wound size over days post-wounding for control and exercise groups. The graph illustrates a decrease in wound size with time, with the exercise group showing a slightly faster rate of wound closure compared to the control group. The x-axis represents days post-wounding, ranging from -1 to 11, and the y-axis represents wound size in mm², ranging from 0 to 11. The graph includes error bars to indicate variability.](image-url)
Figure 3

![Graph showing wound size over days post-wounding for Control and Exercise groups. The graph indicates a decrease in wound size over time, with Control and Exercise groups converging by Day 11.](image-url)
Figure 4

a) IL-1β (pg/µg protein)

b) TNF-α (pg/µg protein)

c) IL-6 (pg/µg protein)
Figure 5

(a) pg KC/µg protein

(b) pg MCP-1/µg protein

* indicates statistical significance.
Figure 6.

[Bar graph showing MPO Activity (units/mg protein) over different days post-wounding for sedentary and exercised groups.]
Figure 7.

![Graph showing F4/80 mRNA expression (fold change from GAPDH) over days post-wounding for sedentary and exercised groups.]

- **Day Post-wounding**
  - 1: Sedentary (0.06), Exercised (0.03)
  - 3: Sedentary (0.01), Exercised (0.02)
  - 5: Sedentary (0.00), Exercised (0.00)