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Effects of aerobic exercise and high-intensity interval training on muscle damage in an overtraining rat model

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ABSTRACT

Background: Overtraining is commonly associated with elevated oxidative stress, inflammatory responses, and structural damage to muscle fibers, all of which contribute to a decline in physical performance. This study aimed to assess the impact of aerobic exercise on biomarkers including malondialdehyde (MDA), interleukin-6 (IL-6), caspase-3, nitric oxide (NO), superoxide dismutase (SOD), as well as the integrity of the sarcolemma.

Methods: A six-week experimental study was conducted using 24 rats, which were randomly divided into three groups (n = 8 per group): a negative control group (no treatment), an aerobic exercise group, and a high-intensity interval training (HIIT) group. The levels of biochemical markers, including malondialdehyde (MDA), interleukin-6 (IL-6), nitric oxide (NO), and superoxide dismutase (SOD), were measured using the enzyme-linked immunosorbent assay (ELISA) technique. Additionally, the expression of caspase-3 was determined through immunohistochemical (IHC) analysis. Muscle tissue damage was evaluated by means of histopathological examination. The data were analyzed using a one-way analysis of variance (ANOVA), followed by the Mann-Whitney post hoc test to assess differences between groups.

Results: The findings indicated that aerobic exercise did not result in a statistically significant reduction in malondialdehyde (MDA) ($p = 0.833$), interleukin-6 (IL-6) ($p = 0.800$), nitric oxide (NO) ($p = 0.791$), or superoxide dismutase (SOD) ($p = 0.520$) levels. Nevertheless, the aerobic exercise group exhibited a significantly lower expression of caspase-3 compared to the other groups ($p = 0.023$). Furthermore, aerobic exercise was associated with a significant reduction in muscle tissue damage ($p = 0.001$).

Conclusion: The findings of this study indicate that both aerobic exercise and high-intensity interval training (HIIT) have the potential to be utilized as therapeutic approaches for mitigating exercise-induced muscle damage, primarily through their positive influence on sarcolemma stability. However, additional clinical investigations are necessary to confirm these results and establish their relevance for clinical implementation.

Keywords: aerobic exercise, exercise overtraining, high intensity interval training, muscle damage, sarcolemma integrity.
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INTRODUCTION

Muscle damage is a common consequence of excessive training intensity or volume.[1] This condition is characterized by temporary ultrastructural disruptions of the myofibrils, reductions in muscle strength and power, the occurrence of delayed-onset muscle soreness (DOMS), localized edema, restricted joint mobility in the affected limbs, and the systemic leakage of intracellular muscle components, including creatine kinase (CK) and myoglobin.[2] The mechanical and metabolic stress associated with muscle injury contributes to increased levels of reactive oxygen species (ROS), indicating an imbalance between malondialdehyde (MDA) production and superoxide dismutase (SOD) activity.[3] The inflammatory response accompanying muscle damage is partially regulated by interleukin-6 (IL-6).[4,5] Additionally, chronic overtraining can lead to systemic apoptosis, initiated by cellular stress that promotes the release of cardiolipin and cytochrome c from the mitochondrial intermembrane space, thereby activating caspase-3.[6] Exercise-induced stress is closely linked to elevated ROS production—oxygen-derived molecules that can induce oxidative damage when generated in excess. Interleukin-6 (IL-6), a cytokine involved in both pro- and anti-inflammatory signaling pathways, also serves as a key biomarker of systemic inflammation and muscular stress in response to prolonged or high-intensity exercise.

The intensity and duration of overtraining exert a considerable influence on physiological biomarkers, particularly creatine kinase (CK) and interleukin-6 (IL-6). When excessive physical training is sustained without sufficient rest or recovery, it imposes chronic physiological

strain on both skeletal muscle and the immune system. Creatine kinase, an enzyme predominantly localized within muscle tissue, plays a critical role in catalyzing the conversion of creatine to phosphocreatine, a key substrate for rapid ATP regeneration during muscular contraction. However, microtrauma to muscle fibers commonly induced by high-intensity or high-volume exercise can compromise cellular membrane integrity, leading to the release of CK into the bloodstream and resulting in elevated serum concentrations. These elevated CK levels serve as an indirect biomarker of muscle damage. The greater the training load and its duration, the more pronounced the muscle damage tends to be, thereby increasing circulating CK levels. In cases of pronounced overtraining, CK concentrations may remain persistently high, suggesting impaired muscle recovery or ongoing muscle degradation, which can negatively impact athletic performance and heighten the risk of injury.

Aerobic exercise performed at low to moderate intensity predominantly utilizes oxygen-dependent metabolic pathways for energy production, whereas high-intensity interval training (HIIT) relies more heavily on anaerobic mechanisms. Overtraining induces muscle damage, oxidative stress, and inflammatory responses in skeletal muscle, thereby necessitating supportive therapeutic interventions to promote recovery. Among these, active recovery methods have shown greater effectiveness compared to passive recovery approaches.[7]

Aerobic exercise has been shown to promote the production of nitric oxide (NO), an essential signaling molecule that plays a pivotal role in regulating diverse physiological processes within various tissues, cells, and organ systems.[8] Previous research has also indicated that NO contributes to the upregulation of superoxide dismutase (SOD) activity, a key antioxidant enzyme responsible for the primary defense against oxidative damage induced by reactive oxygen species (ROS).[9,10] Consequently, moderate-intensity continuous training (MICT) may help mitigate oxidative stress by enhancing the expression of endogenous antioxidant enzymes and reducing ROS accumulation in skeletal muscle, adipose tissue, and the vascular endothelium.[11]

High-intensity interval training (HIIT) represents a promising approach for optimizing exercise program development and identifying potential therapeutic targets. Reactive oxygen species (ROS) serve as essential intracellular signaling molecules that modulate skeletal muscle adaptations under both normal and pathological conditions. Specific ROS are acknowledged as key mediators of exercise-induced adaptations, contributing significantly to the regulation of both immediate and long-term skeletal muscle responses to physical training. Importantly, increased concentrations of systemic oxidative stress biomarkers have been positively associated with enhanced physiological adaptations following a six-week exercise intervention in human participants.[12]

Both aerobic exercise and high-intensity interval training (HIIT) promote the secretion of interleukin-6 (IL-6) from skeletal muscle, a process influenced not only by physical activity but also by various internal and external stressors. As a result, IL-6 has been recognized as a key myokine that serves as a biomarker of the muscle stress response.[13] Although conventionally classified as a pro-inflammatory cytokine, IL-6 has been demonstrated to exert beneficial physiological effects, including promoting muscle hypertrophy, regulating glucose homeostasis, and modulating lipid metabolism. On a mechanistic level, IL-6 contributes to the anti-inflammatory effects of exercise by downregulating pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), while simultaneously enhancing the expression of anti-inflammatory cytokines and facilitating the repair of sarcolemmal membrane integrity.[14,15]

METHODS

This experimental study utilized a post-test control group design and was conducted between October 2024 and January 2025 at the Laboratory of the Bioscience Institute, Universitas Brawijaya, Malang, Indonesia. Male Wistar rats (Rattus norvegicus) were used as experimental subjects, with a total of 30 animals randomly assigned into three groups, each housed individually in labeled cages: (1) a negative control group (K1), (2) an aerobic exercise group (K2), and (3) a high-intensity interval training (HIIT) group (K3). Animals in the treatment groups were subjected to their respective exercise regimens for a duration of six weeks to induce an overtraining condition.

A total of twenty-four male Wistar rats (Rattus norvegicus) met the inclusion criteria for this study. Eligible subjects were male rats aged between 12 and 16 weeks, with body weights ranging from 180 to 250 grams, and exhibiting normal, active behavior. Female rats were excluded to eliminate potential hormonal fluctuations and avoid pregnancy-related variables that could confound the results. Prior to the experimental procedures, all animals underwent a one-week acclimatization period in standardized cages (24 × 18 × 16 cm) under controlled environmental conditions, including a 12-hour light/dark cycle, regulated temperature and humidity, and unrestricted access to standard feed and water.

In accordance with established ethical guidelines for the care and use of laboratory animals, all efforts were undertaken to reduce the number of animals utilized while maintaining the scientific rigor of the study. The sample size was calculated based on the research objectives and the available study duration, employing Federer's formula:[16] $(t-1)(n-1) \geq 15$, where t represents the number of treatment groups and n is the number of animals per group.

Sample size calculation yielded 9 rats per group, with 3 additional animals included to account for a 20% dropout rate, totaling 30 rats. During the first two weeks, three rats died (two from the HIIT group, one from the aerobic group), resulting in 8 rats per group (n = 24) for final analysis. This adjusted sample maintained statistical validity while minimizing excess animal use.

Overtraining, characterized by repeated exhaustive exercise beyond recovery capacity, was employed to model exercise-induced muscle damage, oxidative stress (ROS), and inflammation typically linked to high-intensity activity, unfamiliar stretching, and eccentric contractions.[18] To induce an overtraining condition, rats

were subjected to a sustained protocol of exhaustive exercise. The exercise protocol consisted of treadmill running at speeds between 12 and 27 meters per minute on a 5° downhill incline, specifically intended to induce eccentric muscle contractions. The exercise intensity was gradually increased by 3 meters per minute at 10-minute intervals until the animals reached exhaustion. Exhaustion was operationally defined as the inability of the animal to leave the designated fatigue zone for a continuous duration of 10 seconds. This protocol was implemented daily over a two-week period to elicit sufficient physiological strain.[19,20] Eccentric treadmill exercise is widely recognized as a valid and reproducible method for inducing skeletal muscle damage in rodent models.[20-24] When applied with adequate intensity and frequency, this modality reliably elicits significant physiological stress within a relatively short duration. The approach is grounded in the principle of progressive overload, wherein repeated exposure to escalating physical demands stimulates tissue remodeling and structural adaptation.[24]

A total of 16 mL of blood was collected from each animal under general anesthesia. The samples were centrifuged at 3,000 revolutions per minute for 15 minutes at 4°C to separate the serum. A minimum volume of 500 µL of serum per sample was aliquoted and stored at −80°C until subsequent biochemical analyses were performed. Prior to analysis, the serum samples were thawed at room temperature for 30 minutes. The concentrations of malondialdehyde (MDA), interleukin-6 (IL-6), nitric oxide (NO), and superoxide dismutase (SOD) were quantified using commercially available ELISA kits (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's protocols. Briefly, samples, standards, and controls were added to antibody-coated 48-well plates and incubated, followed by sequential addition of biotin-labeled detection antibodies, streptavidin-horseradish peroxidase (SA-HRP), and tetramethylbenzidine (TMB) substrate, with appropriate incubation and washing steps performed between each stage. The reaction was stopped, and absorbance was read at 450 nm. All assays were conducted in triplicate for accuracy.

Paraffin-embedded tissue sections were subjected to deparaffinization using xylene, followed by rehydration through a graded ethanol series (96%, 90%, 70%). Antigen retrieval was conducted using heat-induced epitope retrieval (HIER) with either citrate buffer (pH 6.0) or Tris-EDTA buffer (pH 9.0) for 15 minutes. Subsequently, the slides were rinsed in phosphate-buffered saline (PBS, pH 7.4) for three cycles of 5 minutes each. To prevent non-specific antibody binding, a peroxide blocking reagent was applied, followed by additional PBS washes (pH 7.4, 3 × 5 minutes). Caspase-3 primary antibody was incubated overnight at 4°C, then detected using a CRFTM Antipolyvalent HRP-conjugated secondary antibody. After further PBS washes, DAB substrate was applied for 5 minutes to visualize antigen sites. Slides were rinsed, counterstained with hematoxylin for 5 minutes, and treated with Bluing Reagent for 5 seconds. After dehydration and mounting, antigen expression was assessed under a BX53 light microscope based on staining intensity and distribution.

Statistical analyses were conducted using SPSS software version 24 (IBM, New York, USA), with statistical significance set at $p < 0.05$. The Shapiro–Wilk test was applied to assess the normality of data distribution, while Levene's test was performed to evaluate the homogeneity of variances ($p > 0.05$). For datasets meeting both normality and homogeneity assumptions, a one-way analysis of variance (ANOVA) was carried out, followed by Mann–Whitney post hoc tests where applicable. A p-value below 0.05 was interpreted as statistically significant. These statistical methods were utilized to compare the effects of aerobic exercise and high-intensity interval training on various parameters, including malondialdehyde (MDA), interleukin-6 (IL-6), caspase-3, nitric oxide (NO), superoxide dismutase (SOD), and sarcolemma integrity.

RESULTS

Data are presented as mean ± standard deviation (SD). One-way ANOVA analysis of serum biomarkers, including malondialdehyde (MDA), interleukin-6 (IL-6), nitric oxide (NO), and superoxide dismutase (SOD), revealed no statistically significant differences among the experimental groups. In contrast, histopathological evaluations, such as caspase-3 expression and markers of muscle tissue damage, showed significant differences between the negative control group and both the aerobic exercise and high-intensity interval training (HIIT) groups, as summarized in Table 1. These findings suggest that both aerobic exercise and HIIT similarly influence oxidative stress profiles by lowering MDA and IL-6 concentrations while increasing NO and SOD levels. Furthermore, at the tissue level, both exercise interventions were comparably effective in reducing caspase-3 expression and alleviating muscle fiber damage.

Specifically, malondialdehyde (MDA) levels were reduced in the aerobic exercise group compared to the negative control group. In contrast, the HIIT group demonstrated a slight increase in MDA concentrations relative to the control. A statistically significant difference in MDA levels was observed between the aerobic exercise group (K2) and the HIIT group (K3) ($p < 0.05$). However, neither the aerobic exercise nor the HIIT group exhibited statistically significant differences in MDA levels when compared to the negative control group (K1), as reflected by a p-value of 0.833 (Figure 1).

Interleukin-6 (IL-6) levels in both the aerobic exercise group (K2) and the high-intensity interval training (HIIT) group (K3) demonstrated a downward trend compared to the negative control group (K1). However, IL-6 concentrations in the HIIT group remained slightly higher than those observed in the aerobic exercise group. Despite these patterns, statistical analysis indicated no significant differences in IL-6 levels among the three groups, as evidenced by a p-value of 0.800 (Figure 1).

Similarly, nitric oxide (NO) concentrations exhibited an increasing trend in both the aerobic exercise group (K2) and the high-intensity interval training (HIIT) group (K3) relative to the negative control group (K1), with slightly higher NO levels observed in the aerobic exercise group compared to the HIIT group. Nonetheless, these differences

Table 1. ANOVA test results for the MDA, IL-6, Caspase-3, NO, SOD and Sarcolemma Integrity variable groups

Variables	Group			*P*-value
	Negative Control (K1)	Aerobic Training (K2)	HIIT Training (K3)	
MDA (µg/mL)	182.50 ± 36.04	176.88 ± 30.59	197.59 ± 12.79	0.833
IL-6 (ng/mL)	4.81 ± 2.21	4.18 ± 1.46	4.45 ± 1.48	0.800
Caspase-3	72.50 ± 8.86	48.75 ± 21.00	60.00 ± 11.95	0.023*
NO (ng/mL)	41.59 ± 13.01	45.93 ± 16.37	42.56 ± 9.34	0.791
SOD (ng/mL)	0.36 ± 0.24	0.46 ± 0.20	0.44 ± 0.08	0.520
Muscle Damage	33.17 ± 5.09	16.03 ± 3.44	21.82 ± 8.18	0.001*

HIIT: high-intensity interval training, MDA: malondialdehyde, IL-6: interleukin-6, NO: nitric oxide, SOD: superoxide dismutase
*p<0.05

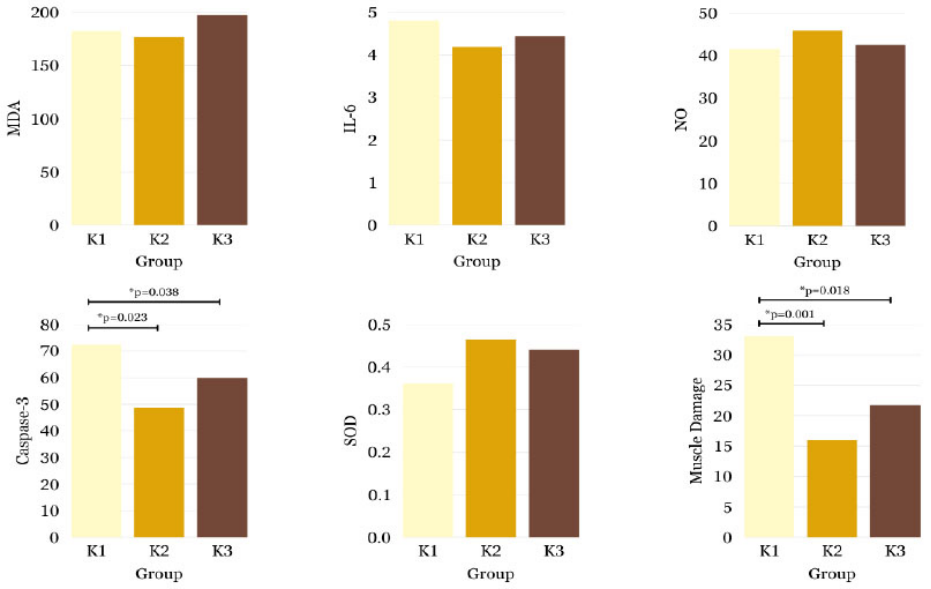

Figure 1. Both treatment groups (K2 and K3) demonstrated a trend toward higher SOD levels compared to the negative control group (K1).

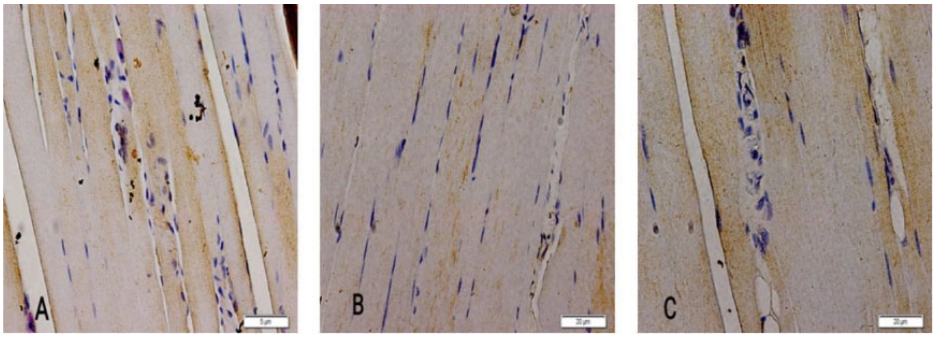

Figure 2. Immunohistochemistry (IHC) between groups. IHC revealed that: (A) The negative control group (K1) exhibited approximately 70% weak to moderate positive staining in the cytoplasm of muscle cells. (B) The aerobic exercise group (K2) showed 30% weak to moderate positive staining. (C) The HIIT group demonstrated 50% weak to moderate positive staining in the cytoplasm of muscle cells.

did not reach statistical significance, as reflected by a *p*-value of 0.791 (Figure 1).

Both the aerobic exercise group (K2) and the high-intensity interval training (HIIT) group (K3) demonstrated significantly reduced Caspase-3 expression levels compared to the negative control group (K1), as shown in Figure 1. The one-way ANOVA analysis yielded a *p*-value of 0.023, indicating a statistically significant difference. Subsequent pairwise comparisons revealed a significant reduction in Caspase-3 expression in both the aerobic exercise group (p = 0.023) and the HIIT group (p = 0.038) when compared to the control group. However, no significant difference was detected between the two exercise groups, suggesting that both interventions were comparably effective in suppressing Caspase-3 expression.

Immunohistochemical analysis of Caspase-3 further confirmed a reduction in apoptotic activity, consistent with decreased structural damage in skeletal muscle tissue. This was visualized in histological sections examined under 40× magnification (Figure 2).

In terms of the antioxidant response, both intervention groups (K2 and K3) demonstrated a tendency toward increased superoxide dismutase (SOD) levels compared to the negative control group (K1), with the aerobic exercise group displaying marginally higher SOD concentrations than the HIIT group. Nevertheless, these differences were not statistically significant, as indicated by a *p*-value of 0.520, as illustrated in Figure 1.

Histopathological evaluation of sarcolemma diameter in the overtraining model revealed significant differences among the groups (p = 0.001). Both the aerobic exercise group (K2) and the high-intensity interval training (HIIT) group (K3) showed a significant reduction in sarcolemma diameter compared to the negative control group (K1). Additionally, the HIIT group (K3) exhibited slightly larger sarcolemma diameters relative to the aerobic exercise group (K2).

Statistical analysis revealed significant differences between both intervention groups and the negative control group, as illustrated in Figure 1. However, no significant differences were detected

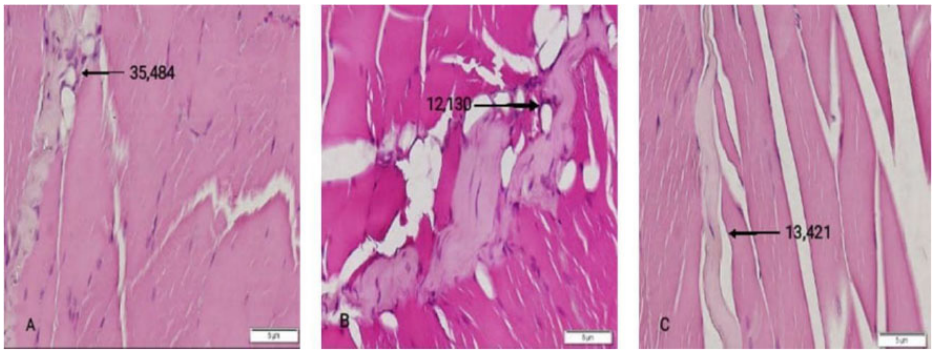

Figure 3. Histopathological picture of skeletal muscle damage, after 6 weeks of treatment, is shown by the diameter of sarcolemmal tissue damage. Both aerobic exercise (K2) and HIIT (K3) effectively reduced muscle tissue damage compared to the negative control (K1), as shown by the smaller average damage diameters.

between the aerobic exercise group (K2) and the high-intensity interval training (HIIT) group (K3) (p = 0.246). These results indicate that both aerobic exercise and HIIT interventions are similarly effective in significantly reducing sarcolemma diameter and improving sarcolemma structural integrity.

Histopathological analysis at 40× magnification revealed mean tissue damage diameters, indicated by the infiltration of inflammatory cells and disruption of the structural matrix, as follows: negative control group (K1) — 35.485 µm, aerobic exercise group (K2) — 12.130 µm, and high-intensity interval training (HIIT) group (K3) — 13.421 µm, as presented in Figure 3.

DISCUSSION

The primary objective of this study was to evaluate the effects of aerobic exercise and high-intensity interval training (HIIT) on muscle damage in a rat model of overtraining. After a six-week intervention period, the results demonstrated no statistically significant differences among the groups regarding reductions in malondialdehyde (MDA) and interleukin-6 (IL-6) levels or increases in nitric oxide (NO) and superoxide dismutase (SOD) concentrations. However, aerobic exercise was associated with a significant decrease in Caspase-3 expression, reflected by a lower prevalence of cytoplasmic apoptosis in muscle cells, along with a marked improvement in sarcolemma integrity, as indicated by a reduction in the diameter of muscle tissue damage.

The human body is equipped with effective antioxidant defense mechanisms, including superoxide dismutase (SOD), which has been shown to increase with regular physical activity due to enhanced oxidative resilience.[25-26] In the current study, malondialdehyde (MDA) levels did not differ significantly between groups, indicating that its concentration remained stable post-exercise.[27-29] This outcome supports previous findings that regular exercise can strengthen antioxidant systems, thereby maintaining stable MDA levels despite initial elevations from oxidative stress.[27,29-31] In trained individuals, MDA concentrations tend to decrease after high-intensity exercise, while remaining unchanged following moderate or low-intensity efforts.[31] Prior studies have noted that MDA levels remain stable in trained individuals after high-intensity exercise but rise significantly in untrained subjects, suggesting that high-intensity interval training (HIIT) can effectively boost antioxidant capacity.[30] Similarly, moderate-intensity continuous training has also been associated with enhanced antioxidant status.[32] Additional research has shown that aerobic training performed five times weekly over eight weeks elevated antioxidant enzyme activity such as SOD, catalase, and glutathione peroxidase while reducing MDA concentrations.[33] Comparable results were observed with aerobic exercise performed three times weekly for 12 weeks, which increased SOD and catalase levels and decreased plasma MDA. Furthermore, aerobic activity has been demonstrated to enhance antioxidant enzymes like Cu/Zn-SOD. A meta-analysis concluded that, regardless of exercise intensity, duration, or population, training generally results in increased antioxidant markers and decreased pro-oxidant levels.[34] These findings confirm the antioxidant-enhancing effects of exercise. Another study comparing HIIT durations of 6 and 12 weeks reported significant improvements in antioxidant capacity with both protocols, with no substantial difference in the extent of improvement between them.[35]

Both aerobic and HIIT interventions led to slight, non-significant reductions in IL-6 levels.[36-37] Exercise-induced inflammation plays a key role in tissue repair and involves systemic cytokine activity, particularly during large muscle group engagement.[38] Studies have shown variable IL-6 responses depending on muscle type, exercise intensity, and modality eccentric and high-intensity exercises tend to produce more IL-6 than low-intensity or concentric ones.[39]

Because elevated IL-6 is linked to muscle damage, a gradual inflammatory response through aerobic exercise may minimize adverse effects. IL-6 may also promote caspase-3 activation via the extrinsic apoptotic pathway.[40] Caspase-3 is essential for muscle remodeling and satellite cell differentiation. Exercise may reduce apoptosis by modulating apoptotic proteins, mitochondrial signaling, and oxidative stress. While both aerobic and HIIT protocols influence apoptosis, aerobic exercise appears more beneficial in regulating caspase-3 and preserving sarcolemma integrity.[41]

This study presents several limitations. Firstly, it was conducted exclusively on male rats, thereby limiting the applicability of the findings to female counterparts. Secondly, the exercise interventions were administered during the daytime, despite the nocturnal nature of rats, which may have influenced physiological responses. The six-week intervention period may also have been insufficient to fully assess the long-term impact or variability in response to differing training intensities and durations. Additionally, the absence of clinical follow-up precludes conclusions about the persistence of observed effects over time. As an animal-based study, the translational relevance to human physiology remains limited, given that

the specific experimental conditions and biological responses in rats may not fully mirror muscle damage and recovery processes in humans following prolonged training.

CONCLUSION

The results of this study suggest that neither aerobic exercise nor high-intensity interval training (HIIT) produced significant changes in reducing malondialdehyde (MDA) and interleukin-6 (IL-6) levels or increasing nitric oxide (NO) and superoxide dismutase (SOD) concentrations. However, both interventions significantly decreased caspase-3 expression and ameliorated muscle damage. Future research should incorporate both male and female subjects, explore a wider range of training protocols, and aim to elucidate sex-specific responses to muscle damage in overtraining models.

ETHICAL CONSIDERATION

This study received ethical approval from the Research Ethics Committee of Universitas Brawijaya, Malang, Indonesia (Approval Number: 122-KEP-UB-2024). All experimental procedures involving animals were conducted in strict accordance with established ethical guidelines and regulations governing the care and use of laboratory animals.

CONFLICT INTEREST

The authors declare no competing interests related to this publication.

AUTHOR CONTRIBUTIONS

AS and AK contributed to the study's conception, design, data collection, and manuscript drafting; MD and EP handled data analysis, interpretation, and critical revision for intellectual content; BP and S supported the literature review, data presentation, and provided administrative assistance throughout the research.

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