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The effects of overtraining protocol on markers of oxidative stress and inflammation

Amriansyah Syetiawinanda[1,2*], Muchsin Doewes[1,3], Bambang Purwanto[1,4], Soetrisno[1,5], Agus Kristiyanto[3], Eti Poncorini Pamungkasari[1,6]

ABSTRACT

Background: Overtraining can elevate reactive oxygen species (ROS), suppress endogenous antioxidant activity, and trigger systemic inflammation. Monitoring oxidative stress and inflammation is essential to prevent muscle damage. This study aimed to examine the effects of an overtraining protocol on oxidative stress and inflammatory markers MDA, IL-6, NO, and SOD in male Wistar rats.

Methods: The experimental was conducted over two weeks using male Wistar rats at the Bioscience Institute Laboratory, Universitas Brawijaya, Malang, Indonesia as a preliminary study. Three rats were examined to assess the effects of overtraining on MDA, IL-6, NO, and SOD levels. Observations were made one day after overtraining protocol for the intervention group value (KP) and six weeks after overtraining for the control group value (K1).

Results: Three male rats underwent an overtraining protocol, and blood samples were collected one day post-overtraining (KP) to assess MDA, IL-6, NO, and SOD levels. The mean values at KP were MDA: 370.3 ± 10.3, IL-6: 5.9 ± 0.7, NO: 61.0 ± 8.4, and SOD: 0.05 ± 0.03. At six weeks post-overtraining (K1), MDA (192.8 ± 10.3), IL-6 (3.3 ± 1.9), and NO (41.7 ± 21.6) levels were lower, while SOD (0.3 ± 0.2) was higher compared to KP.

Conclusion: Overtraining can elevate ROS and inflammatory responses, as indicated by increased MDA, IL-6 levels, and NO, along with reduced SOD expression.

Keywords: antioxidants, inflammation, overtraining, reactive oxygen species.
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[1]Doctoral Program in Medical Science, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia;
[2]Physiotherapy Study Program, Faculty of Physiotherapy, Universitas Esa Unggul, Jakarta, Indonesia;
[3]Faculty of Sports, Universitas Sebelas Maret, Surakarta, Indonesia;
[4]Department of Internal Medicine, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia;
[5]Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia;
[6]Department of Public Health and Preventive Medicine, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia.

*Corresponding author:
Amriansyah Syetiawinanda;
Doctoral Program in Medical Science, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia and Physiotherapy Study Program, Faculty of Physiotherapy, Universitas Esa Unggul, Jakarta, Indonesia;
amriansyah@esaunggul.ac.id

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INTRODUCTION

Exercise plays a vital role in human health due to its numerous benefits.[1,2] It involves regular activity performed at specific durations and intensities to maintain physical fitness. Research supports its use in preventive, therapeutic, and rehabilitative programs aimed at preserving and enhancing health.[3,4]

Athletes are individuals who compete in sports requiring physical strength, speed, or endurance.[5] Sports training is designed to enhance performance, adaptability, and physical fitness to achieve competitive success. Overtraining is a common strategy used to push the body beyond normal limits for optimal results.[6] However, without adequate recovery, it can lead to overtraining syndrome, ultimately impairing performance.[7]

Overtraining significantly increases the production of reactive oxygen species (ROS), reduces endogenous antioxidant activity, alters nitric oxide (NO) levels, and activates systemic inflammation. Numerous studies have confirmed that high-intensity, high-frequency exercise without adequate recovery elevates ROS levels. Excessive ROS can damage lipids, proteins, and DNA, ultimately leading to cellular and tissue dysfunction.

Under normal conditions, the body's antioxidant defense system such as superoxide dismutase (SOD) neutralizes reactive oxygen species (ROS). However, during overtraining, this system shows reduced activity, compromising the body's ability to counteract oxidative stress.[10,11] This decline increases susceptibility to cellular damage, reflected by elevated levels of oxidative biomarkers like malondialdehyde (MDA). Overtraining also triggers inflammatory pathways, marked by higher levels of proinflammatory cytokines such as interleukin-6 (IL-6). Therefore, this study aimed to investigate the effects of an overtraining protocol on markers of oxidative stress and inflammation, including MDA, IL-6, nitric oxide (NO), and SOD, in male Wistar rats.

METHODS

This preliminary study employed an experimental design and was conducted in October 2024 at the Bioscience Institute Laboratory, Universitas Brawijaya, Malang, Indonesia, over a two-week period using three male Wistar rats. All animals underwent both overtraining and control conditions in a repeated-measures design. Initially, one day after post-overtraining blood samples were taken to serve as the intervention condition (KP). After a six-week washout period without any additional treatment or exercise, the same rats underwent blood test (K1) to evaluate the effects of overtraining.

Overtraining was induced using a treadmill-based exhaustive exercise protocol. Rats ran at a 5° downhill incline, with speeds starting at 12–27 m/min and increasing by 3 m/min every 10 minutes until exhaustion (Figure 1). Exhaustion was defined as the animal's inability to leave the fatigue zone (the proximal end of the treadmill) for 10 consecutive seconds. This protocol was carried out daily for two consecutive weeks.

At each time point (post-overtraining and control), blood samples (totaling 16 mL) were collected under general anesthesia. The samples were centrifuged at 3,000 rpm for 15 minutes at 4°C to separate the serum, which was then aliquoted (≥500 µL per sample) and stored at −80°C until analysis. Prior to testing, samples were thawed at room temperature for 30 minutes.

Markers of oxidative stress and inflammation MDA, IL-6, NO, and SOD were measured using ELISA kits (eBioscience Co., San Diego, CA, USA) following the manufacturer's protocol. Briefly, 100 µL of each sample, standard, control, and blank was added to antibody-coated 48-well plates and incubated at room temperature for 2 hours. Plates were washed three times, followed by incubation with 100 µL of biotin-conjugated detection antibody for 1 hour. After an additional wash, 100 µL of streptavidin–horseradish peroxidase (SA-HRP) was added and incubated for 30 minutes in the dark. The reaction was developed with 100 µL of TMB substrate for 10–15 minutes and stopped using 100 µL of stop solution. Absorbance was measured at 450 nm using a microplate reader. All assays were performed in triplicate to ensure reliability. This study was approved by the Ethics Committee of Universitas Brawijaya, Malang, Indonesia (No. 122-KEP-UB-2024). All animal procedures complied with established ethical guidelines for the care and use of laboratory animals.

RESULTS

A preliminary study was conducted to evaluate the effects of an overtraining protocol on markers of oxidative stress and inflammation. Three male rats underwent the protocol, and blood samples were collected one day post-overtraining (KP) to measure MDA, IL-6, NO, and SOD levels. The results showed elevated levels of MDA (370.3±10.3), IL-6 (5.9±0.7), and NO (61.0±8.4), and reduced SOD (0.05±0.03) compared to values at six weeks post-overtraining (K1): MDA (192.8±10.3), IL-6 (3.3±1.9), NO (41.7±21.6), and SOD (0.3±0.2) (Figure 2). These findings confirm that the overtraining protocol effectively induced oxidative stress and inflammation, supporting its validity for use in the main study.

DISCUSSION

This preliminary study examined the effects of overtraining on oxidative stress, antioxidant response, and inflammation in male Wistar rats. The findings indicated that MDA, a common marker of oxidative

Figure 1. Rats ran on treadmill with exhaustive exercise protocol.

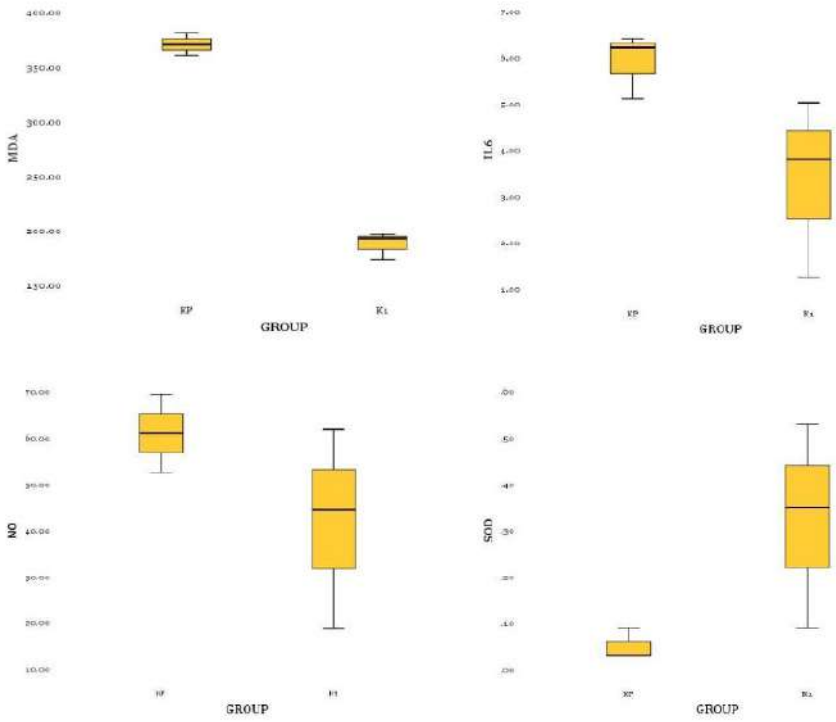

Figure 2. Mean differences of MDA, IL-6, NO, SOD variables. KP: preliminary group; K1: negative control group.

stress, increased during prolonged intense exercise, suggesting that elevated ROS levels exceeded the body's antioxidant capacity and led to oxidative damage in muscle cells.[13]

Under overtraining conditions, NO levels become dysregulated as exercise intensity and volume exceed the body's recovery capacity. This imbalance between NO production and degradation is linked to oxidative stress and systemic inflammation. Excessive NO, particularly from inducible nitric oxide synthase (iNOS), can generate harmful free radicals like peroxynitrite, which are toxic to tissues.[14]

SOD is a key antioxidant enzyme that catalyzes the conversion of superoxide radicals into hydrogen peroxide and oxygen.[15] In conditions of overtraining, SOD activity tends to decline, indicating a fatigued antioxidant system, which exacerbates oxidative stress and accelerates the onset of overtraining symptoms.[16]

Overtraining can elevate levels of IL-6, a pro-inflammatory cytokine. Under prolonged overtraining conditions, IL-6 tends to increase continuously, indicating systemic stress and low-grade inflammation.[17,18] This condition showed that IL-6 not only functioned as a marker of acute inflammation but also reflected physiological dysregulation due to excessive exercise stress.[19,20]

This study has several limitations. First, it included only male rats, limiting generalizability to females due to potential sex-related physiological differences. Second, the rats, being nocturnal, were trained during their inactive (light) phase, which may have influenced their physiological and stress responses.[21] Prior research shows that stress markers like corticosterone vary diurnally, with heightened levels during the active (dark) phase, suggesting that training timing may have confounded the results. Third, the two-week intervention may be insufficient to reveal cumulative or dose-dependent effects of prolonged or varied exercise intensities. Lastly, while the rat model offers insights into biological mechanisms, direct translation to humans is limited by interspecies differences in muscle physiology, exercise adaptation, and systemic responses.

Future studies should address these limitations to enhance the relevance and depth of findings. Including female rats would allow investigation of sex-based physiological responses to overtraining. Conducting training during the animals' active (dark) phase may yield more accurate insights aligned with their circadian rhythms. Extending the exercise duration and varying intensity could clarify cumulative or dose-dependent effects. Investigating post-intervention recovery periods may reveal the reversibility of oxidative stress and inflammation. Finally, studies in humans or human-relevant models are essential to improve translational relevance given interspecies differences.

CONCLUSION

In conclusion, this study demonstrated that an overtraining protocol in male Wistar rats led to elevated oxidative stress and inflammation, as evidenced by increased MDA and IL-6 levels and decreased NO and SOD expression. These findings suggest that excessive training without adequate recovery may disrupt redox balance and promote inflammatory responses, underscoring the need for proper training load management to prevent physiological harm.

ETHICS APPROVAL

This study was approved by the Ethics Committee of Universitas Brawijaya, Malang, Indonesia (No. 122-KEP-UB-2024). All animal procedures complied with established ethical guidelines for the care and use of laboratory animals.

CONFLICT INTERESTS

All the authors declare that there are no conflicts of interest.

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AUTHORS CONTRIBUTIONS

AS designed the study, collected and processed the data, and drafted the initial manuscript. MD, BP, SS, AK, and EP contributed to study design and manuscript revision.

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