RESEARCH ARTICLE

Early Active Exercise Improves MDA, SOD, and GSH Levels without Memory or NO Changes in Wistar Rats

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Abstract

BACKGROUND: Circadian rhythms regulate various physiological processes, including responses to exercise. However, the effects of exercise timing on cognitive function and oxidative stress remain unclear. One key factor in oxidative stress is nitric oxide (NO), an enzyme complex that produces reactive oxygen species (ROS) as part of normal cellular signaling. Excessive NO activity can disrupt redox balance and contribute to neuronal damage. An imbalance favoring oxidative stress can impair memory and learning, while a higher antioxidant capacity supports brain health and cognitive performance. This study was performed to investigate whether early active and late active aerobic exercise differentially impact cognitive function and oxidative stress biomarkers in Wistar rats.

METHODS: Sixteen male Wistar rats were randomly assigned to four groups: early active control, late active control, early active exercise, and late active exercise. The exercise groups underwent treadmill running for seven weeks, five days per week. Cognitive performance was assessed using the novel object recognition (NOR) test, while oxidative stress biomarkers, including malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) were analyzed from brain tissue samples (hippocampus) following already established methods. Meanwhile the NO were assessed using Enzyme-linked Immunosorbent Assay (ELISA).

RESULTS: This study showed that exercise timing did not significantly affect non-spatial memory performance. However, early active exercise led to a significant increase in SOD and GSH levels compared to the control and late active exercise groups, suggesting enhanced antioxidant activity. Conversely, late active exercise did not significantly impact oxidative stress markers. No changes was found in the NO concentration in both exercise timing.

CONCLUSION: These findings suggest that exercise performed during the early active phase may be more beneficial for oxidative stress regulation, potentially contributing to long-term cognitive resilience.

KEYWORDS: circadian rhythm, exercise timing, cognitive function, oxidative stress

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Introduction

The circadian rhythm is a 24-hour internal cycle regulating a wide range of biological processes including sleep-wake cycles, hormone secretion, metabolism and oxidative balance. This rhythm is centrally governed by the suprachiasmatic nucleus (SCN) and synchronized with environmental cues such as light and dark.(1) Disruptions to circadian alignment, through behaviors like shift work or irregular sleep patterns have been linked to increased risk of chronic diseases.(2,3)

Emerging evidence suggests that aerobic exercise not only supports cardiovascular and metabolic health but also modulates circadian clock, but also potentially affecting cognitive by enhancing mempry and learning, as well as affecting and oxidative outcomes depending on the time of day it is performed.(4,5) Exercise timing that classified as early active or late active is defined relative to an organism's internal clock with early active exercise typically performed soon after the start of the activity phase (zeitgeber time 0) and late active exercise occurring toward the end of this phase.(6)

While human studies are often limited by confounding factors such as diet, chronotype and light exposure (7,8), the controlled animal studies offer a clearer opportunity to explore how exercise timing interacts with physiological parameters. In particular, rodent models enable precise control of environmental variables and provide insight into mechanisms such as oxidative stress and memory function. Oxidative stress plays a significant role in age-related cognitive decline. It arises from an imbalance between reactive oxygen species (ROS) and antioxidant defenses with enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase along with molecules like glutathione (GSH) that working to neutralize ROS and maintain redox homeostasis.(9) Nitric oxide (NO), is a primary source of ROS production. Overactivation of NO, often due to stress or aging, leads to elevated oxidative damage and impaired neuronal function.(10,11) Malondialdehyde (MDA) is a key biomarker of lipid peroxidation and oxidative stress, while increased levels of endogenous antioxidants like SOD and GSH are considered protective against cognitive decline.(12,13) Studies have found that higher oxidative balance scores correlate with improved cognitive outcomes.(14,15)

Regular aerobic exercise is known to stimulate adaptive antioxidant responses. Although it transiently elevates ROS production, consistent training enhances antioxidant enzyme activity, reduces MDA levels over time and supports neuroplasticity thereby improving cognition including the non-spatial memory.(14,16,17) Recent studies also indicate that the timing of aerobic exercise may modulate these benefits. Early active exercise has been associated with improved metabolic outcomes and oxidative balance, while late active exercise may align with physical performance peaks but potentially generate a different redox response.(5,18,19) However, the mechanisms through which exercise timing impacts oxidative stress and cognition remain poorly defined. Therefore, this study was performed to understand how exercise timing influences these parameters may provide new insight into optimizing exercise interventions for cognitive and metabolic health through circadian alignment.

Methods

Experimental Animal

Male Wistar rats aged approximately three months, weighing between 160–180 g were obtained from the Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. Because the fluctuating ovarian-hormone milieu of the estrous cycle leads to a larger variance in behavioural and biochemical endpoints, to obtain more homogeneous baseline data and to reduce the number of animals required, only male Wistar rats were used.(20) Using males therefore controls for hormonal cycling without compromising the generalisability of the findings while also aligning our protocol with the majority of prior exercise-neurobiology work in Wistar rats facilitating direct comparison. Throughout the experiment, rats were housed individually in ventilated cages at a controlled temperature of 24–26°C with a 12-hour light/dark cycle and were given *ad libitum* access to food and water. Animal care and experimental procedures were conducted according to ethical guidelines with the UKM Animal Ethics Committee (UKMAEC) approval (FSK/2022/FARAH/26-JAN/1220-JAN.-2022-OCT.-2023). Sixteen rats were randomly divided into four groups which were early active control, late active control, early active exercise and late active exercise. Control groups did not undergo any exercise but were subjected to the same housing and handling conditions as the exercise groups.

Aerobic Exercise Protocol

Before the start of the exercise phase, all rats underwent a 7-day adaptation period to acclimate to their environment and the treadmill exercise equipment. In the last two days

of this period, rats in the exercise groups were familiarized with treadmill running at a speed of 10 m/minute for 5 minutes daily. Following adaptation, the exercise groups began a structured aerobic exercise protocol that lasted for seven consecutive weeks. The exercise protocol included treadmill running at 18 m/minute for 30 minutes per day, 5 days a week.(21) The early active group exercised at 8:00 AM, while the late active group exercised at 8:00 PM. The respective control groups were placed on the treadmill for 30 minutes per day but did not run ensuring consistent environmental exposure. The treadmill (Orchid Scientific & Innovative, Maharashtra, India) was equipped with adjustable speeds to accurately control exercise intensity.

Novel Object Recognition (NOR) Test

This test was designed to assess non-spatial memory performance by observing natural exploratory behaviour of rats, which had an innate tendency to explore novel objects more than familiar ones. The assessment was conducted in soundproof rooms illuminated by a red fluorescent light source exceeding 20 watts to ensure clear video recordings, with a camera positioned above to capture the rats' behaviour. Before the trials, a 3-minute familiarization period was provided to allow the rats acclimatization. During the first trial, the rats were allowed to explore two identical objects, which were a pair of white glass bottles, each 12 cm in height, for 5 minutes. Following a pre-determined intertrial interval, the second trial introduced one familiar object from the first trial and one novel object, which was a black glass bottle also 12 cm in height, with a 6-minute exploration period. Memory performance was measured based on the time spent investigating the novel object during the second trial. A greater exploration time for the novel object compared to the familiar one suggested that the rat remembered the familiar object and was naturally drawn to investigate the new object.

Oxidative Stress Analysis

MDA, SOD, and GSH assessment were performed to determine the impact of varying exercise timings on the oxidative stress biomarkers. MDA was measured to assess lipid peroxidation (22) using the thiobarbituric acid reactive substances (TBARS) method following the established procedure (23). SOD was measured as a key enzymatic antioxidant and was assessed following previous protocol (24), with a slight modification. SOD activity was determined based on the enzyme's capacity to inhibit nitroblue tetrazolium (NBT) reduction, with one unit of activity defined as the amount of enzyme required to achieve 50% inhibition of NBT reduction under the assay conditions. Meanwhile GSH was measured as a non-enzymatic antioxidant (25), and the GSH levels were quantified following the established method (26). At the end of the 8-week exercise intervention, rats were euthanized without anesthesia to prevent hypoxia-related alterations in oxidative stress markers.

Enzyme-linked Immunosorbent Assay (ELISA) Procedure for NO Measurement

Serum levels of endothelial nitric oxide synthase (eNOS/NOS3) were quantified using Rat NOS3/eNOS ELISA kit (Cat. No. PN803814; ELABscience, Wuhan, China), with a detection range of 15.63–1000 pg/mL a sensitivity of 9.38 pg/mL. Briefly, serum samples were brought to room temperature, centrifuged at 3000 rpm for 20 minutes, and diluted accordingly. Measurement of samples, standards and blanks were performed in duplicate and strictly according to the manufacturer's instructions. Upon completion of the reaction by Stop Solution, absorbance was read at 450 nm. eNOS that representing NO concentration was determined using a 4-parameter logistic standard curve. Out-of-range samples were re-assayed with appropriate dilution.

Statistical Analysis

All data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) and are shown as mean±standard error of the mean (SEM). A mixed-model ANOVA was used to assess neurobehavioral outcomes while a two-way ANOVA was used to compare oxidative stress markers between groups. Data were checked to ensure they met all the assumptions prior to the statistical test. Results were considered statistically significant at $p<0.05$.

Results

No Changes in Non-spatial Memory

No significant differences were found between the control and exercise groups ($p>0.05$) regardless of whether the exercise was performed in the early or late active phase across all measured time points (Figure 1). Since the preference percentage ratio reflected non-spatial memory, these results suggested that exercise timing did not significantly affect non-spatial memory performance.

Exercise Decreased the MDA Levels

No significant differences were found in the mean concentration of MDA between the control and exercise

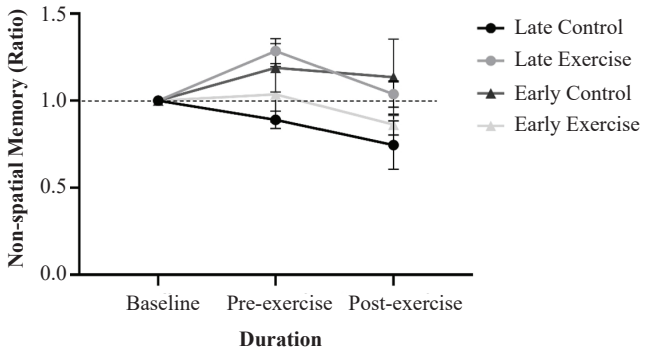

Figure 1. Effects of early and late exercise on non-spatial memory performance over time. Non-spatial memory performance was assessed at three time points: Baseline, Pre-Exercise, and Post-Exercise. Values are expressed relative to baseline (set to 1). Data are presented as mean±SEM (n=4).

groups (p>0.05) regardless of whether exercise was performed in the early or late active phase (Figure 2A). However, the mean MDA concentration in the exercise group appeared higher than in the corresponding control group though this difference was not statistically significant.

No associations were found between non-spatial memory performance (preference percentage) in the exercise group (regardless of exercise timing) and mean MDA concentration (nmol/g protein), although a positive trend was observed (r=0.421, p=0.226) (Figure 2B). These findings suggested that non-spatial memory performance in the exercise group was not directly linked to oxidative stress as indicated by MDA levels under the conditions tested.

Increase of SOD Enzyme Activity Levels in Early Active Exercise

SOD enzyme activity in the early-active exercise group was significantly higher than in both the control group and the late-active exercise group (p<0.05) (Figure 3A), suggesting that early-active exercise may enhance antioxidant activity. In contrast, the late-active exercise group showed no significant difference in SOD activity compared to its control group (p>0.05).

No correlation was observed between non-spatial memory performance (preference percentage) and SOD activity levels (U/mg protein) in exercise group regardless of exercise timing (r=0.178, p=0.623) (Figure 3B).

Increase in GSH Levels in Early Active Exercise

GSH levels in the early-active exercise group were significantly higher than those in both the control group (p<0.05) and the late-active group (Figure 4A). No significant difference in GSH levels was found between the late-active exercise group and the control group. However, the overall pattern in Figure 4A suggested that exercise may increase antioxidant levels, as both exercise groups showed higher GSH levels than the control.

Figure 4B presented the correlation analysis between non-spatial memory performance (preference percentage) and GSH concentration (nmol/g protein) in the exercise group regardless of timing. A positive trend was observed with a borderline significant correlation (r=0.592, p=0.071) suggesting that higher GSH levels may be linked to better non-spatial memory performance.

No Changes in NO Concentration

There are no significant differences in NO concentration between the control and exercise groups regardless of whether exercise was performed in the early or late active phase (Figure 5). These findings suggested that exercise timing did not significantly affect NO levels. The SEM value was 36.480.

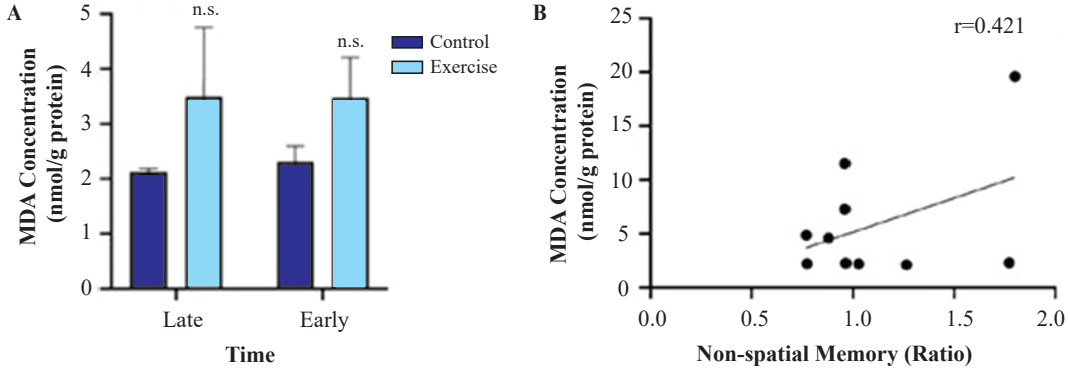

Figure 2. Comparison of mean MDA concentrations in the control and exercise groups and their correlation with preference percentage. A: Mean MDA concentration (nmol/g protein) in control and exercise groups for early active and late active sessions. B: Correlation between mean MDA concentration and preference percentage. Statistical analysis was performed using two-way ANOVA. Data are presented as mean±SEM (n=4). Post hoc: n.s. means non-significant differences compared to controls (late and early active).

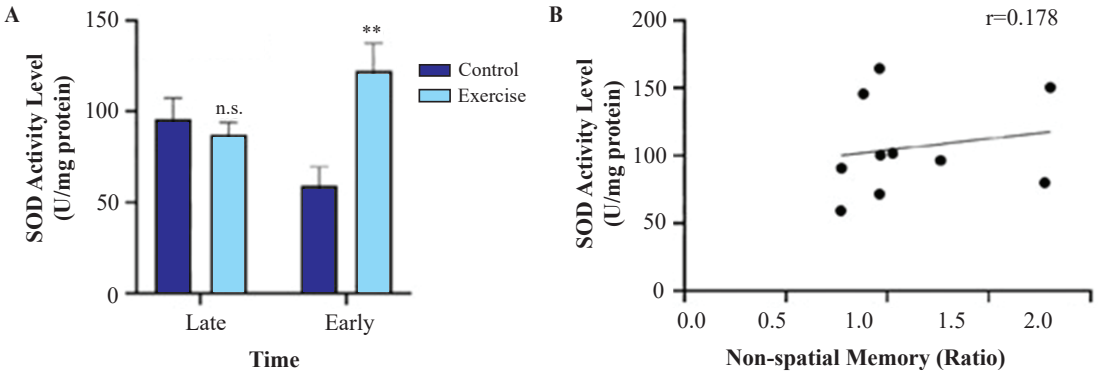

Figure 3. Comparison of mean SOD enzyme activity in the control and exercise groups and their correlation with preference percentage. A: Mean SOD enzyme activity level (U/mg protein) in control and exercise groups for early active and late active sessions. B: Correlation between mean SOD enzyme activity level concentration and preference percentage. Statistical analysis was performed using two-way ANOVA. Data are presented as mean±SEM (n=4). Post hoc: $^{**}p < 0.01$ compared to the control group (late active), n.s. means non-significant differences compared to the control group (late active).

Discussion

This study investigated the effects of early and late active aerobic exercise on non-spatial memory and oxidative stress biomarkers in Wistar rats. While exercise timing did not significantly affect non-spatial memory, early active exercise showed a greater enhancement in antioxidant enzyme activity particularly SOD and GSH compared to late active exercise.(25) These findings suggest that exercise timing may influence the oxidative stress response more than cognitive outcomes.

The absence of significant improvement in non-spatial memory supports earlier findings that the cognitive benefits of exercise are often task-dependent and may require longer interventions.(27,28) Some previous studies have shown that spatial memory and hippocampal plasticity respond more robustly to aerobic exercise often requiring at least 12 weeks of intervention for structural changes to emerge. (29-31)

Oxidative stress is a key factor in aging and neurodegeneration (32), and improving antioxidant defences is critical for brain health (33,34). The increased SOD and GSH levels in the early active group suggest that this timing better aligns with circadian patterns of antioxidant activity. (5) This may reflect the circadian regulation of enzymes such as brain and muscle ARNT-like protein 1 (BMAL1), circadian locomotor output cycles kaput (CLOCK), and Period (PER), which peak in the early active phase and drive antioxidant gene expression.(35-37)

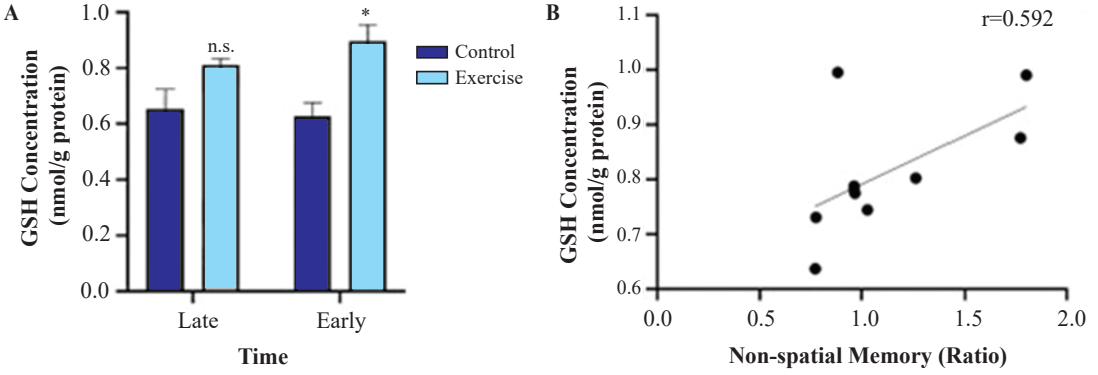

Figure 4. Comparison of mean GSH concentrations in the control and exercise groups and their correlation with preference percentage. A: Mean GSH concentration (nmol/g protein) in control and exercise groups for early-active and late-active sessions. B: Correlation between mean GSH concentration and preference percentage. Statistical analysis was performed using two-way ANOVA. Data are presented as mean±SEM (n=4). Post hoc: $^{**}p < 0.01$ compared to the control group (late active), n.s. means non-significant differences compared to the control group (late active).

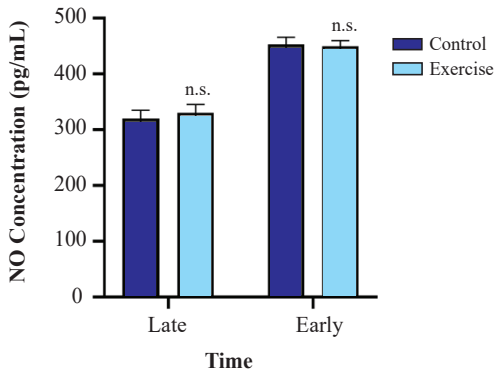

Figure 5. The mean NO concentration in the control and exercise groups. Statistical analysis was performed using two-way ANOVA. Data are presented as mean±SEM (n=4). Post hoc: n.s. means non-significant differences compared to controls (late and early active).

Although MDA levels did not significantly differ between groups, a slight increase in the exercise groups may reflect a transient rise in lipid peroxidation following acute stress consistent with the hormesis theory.(16,17,38) This adaptive mechanism has been observed in both human and rodent studies, where exercise initially increases oxidative markers but ultimately strengthens antioxidant defences.(39,40) The weak correlation between MDA and cognitive outcomes suggests that other mechanisms such as inflammation or neurotrophic signaling that may mediate the relationship between exercise timing and cognition.

A limitation of this study was the small sample size in each group as the number of rats (n=4) was reduced than initially planned. This constraint due to supply limitations from the Universiti Kebangsan Malaysia Animal House restricts the availability of animals. The reduced sample size may have affected the statistical power of the analyses and limited the generalizability of the findings. Additionally, the cognitive assessment was confined to the NOR test that predominantly evaluates non-spatial memory. Other cognitive domains such as spatial memory (using the Morris Water Maze), executive function or working memory were not assessed narrowing the scope of cognitive evaluation of the subject. The study also did not explore molecular signaling pathways such as brain-derived neurotrophic factor (BDNF), cAMP response element-binding protein (CREB) or core circadian genes like BMAL1 and CLOCK nor did it assess structural changes such as hippocampal neurogenesis. The absence of these analyses limits the understanding of the mechanistic underpinnings behind the observed biochemical and behavioural effects. Future studies should incorporate additional neurobiological markers such as BDNF and inflammatory cytokines to elucidate the pathways linking exercise timing, oxidative stress and cognition. Understanding the optimal timing of exercise could have important implications for neuroprotection and cognitive health. Future studies also should explore the underlying molecular mechanisms and assess whether similar effects occur in humans to optimize exercise-based interventions for brain health.

Conclusion

Early active exercise significantly increased antioxidant enzyme levels with higher SOD and GSH concentrations compared to both control and late active exercise groups which indicating an enhanced antioxidant defense. Although MDA levels were slightly elevated in the exercise groups, these changes were not statistically significant. No significant alterations were observed in NO levels across all groups suggesting no effect of exercise timing on this oxidative stress marker. Additionally, exercise timing did not influence the non-spatial memory performance in Wistar rats. These findings suggest that early active exercise is more effective in promoting oxidative stress regulation which potentially contributes to long-term neuroprotection even in the absence of short-term cognitive improvements.

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Authors Contribution

HZ, AF, ND, AH, AM, FW, and NF were involved in the conceptualization of the study. HZ, AF, ND, AH, AM, AHF, FW, NF, and AP were involved in the preparation of methodolgy and formal analysis. HZ, AF, ND, AH, AM, FW, and NF were involved in the data curation. HZ, ND, AH, AM were involved in the study. investigation. Ap interpreted the data. HF and AHF prepared the software used in the study. HZ, ND, AH, AM, and AHF prepared the original draft of the manuscript, while HZ, AF, FW, NF,

and AP were involved in the review end editing manuscript. HZ, ND, AH, and AM prepared the visualization of the data. AF, FW, and FW perforemed supervision and validation of the study. HZ, AF, ND, AH, AM, and FW managed the project administration. AF, FW, and NF were involved in the funding acquisition and resource preparation.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

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