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Effects of Phycocyanin on Oxidative Stress in The Brain of Prolonged Strenuous Exercise Rats

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Abstract

Excessive exercise can lead to oxidative stress which can negatively affect the nervous system. Phycocyanin, a pigment in blue-green algae like spirulina, has potent antioxidant properties that help neutralize free radicals and reduce oxidative stress. This study examined the effects of phycocyanin on oxidative stress in the rat brain induced by prolonged strenuous exercise. The male Sprague-Dawley rats were divided into 5 groups: Control group, exercise group, exercise with low (100 mg/kg BW) or high (200 mg/kg BW) doses of phycocyanin or vitamin C (200 mg/kg BW). After the 8th week of experiment, brain tissue was collected to analyze malondialdehyde levels (MDA) and antioxidant activity in the prefrontal cortex, hippocampus, and amygdala. The results show that excessive exercise tends to increase MDA levels and decrease superoxide dismutase (SOD) levels, although these changes are not statistically significant. However, excessive exercise significantly reduces the antioxidant enzymes catalase (CAT) and glutathione peroxidase (GPx), which may contribute to oxidative stress. Phycocyanin, on the other hand, enhances the antioxidant activity of SOD and CAT, both of which play a critical role in mitigating oxidative damage. Interestingly, phycocyanin does not appear to significantly affect GPx levels. This suggests that phycocyanin may selectively target specific antioxidant pathways while leaving others unaffected.

Keywords: Phycocyanin, Oxidative stress, Antioxidant, Overexercise, Hippocampus, Amygdala, Prefrontal cortex

Introduction

Nowadays, people are placing greater emphasis on health, including diet, lifestyle behaviors, and, particularly, exercise. It is widely recognized that exercise offers numerous health benefits, such as improving physical fitness, reducing blood lipid levels, and lowering risk factors for cardiovascular diseases, stroke, hypertension, and various types of cancer [1]. Exercise has many benefits, but it can also have some negative effects on the body. Intense and prolonged exercise can lead to an overproduction of free radicals-unstable molecules that can damage cells, including neurons. Under normal conditions, the body utilizes antioxidants to neutralize these free radicals, converting them into harmless substances like water and oxygen. However, when the production of free radicals exceeds the body's antioxidant defenses, it can result in oxidative stress, potentially harming brain cells and impairing cognitive functions. Previous studies have reported that excessive exercise can damage the prefrontal cortex, hippocampus, and amygdala, leading

to cell injury and death. This can impair learning and memory, reduce decision-making ability, and increase the risk of neurological disorders such as Alzheimer's disease and amyotrophic lateral sclerosis [2-6].

Phycocyanin is a natural food pigment with a blue color, found in the protein structures of Cyanobacteria, Rhodophyceae, and Cryptophyceae. It possesses pharmacological properties as an antioxidant and anti-inflammatory agent [7]. It can help reduce the risk of various neurological disorders and, importantly, prevent oxidative stress. Phycocyanobilin exerts its effect by forming bonds or donating electrons to free radical molecules, stabilizing their electron structure, thereby protecting cells and reducing oxidative damage [8]. Another mechanism involves phycocyanobilin stimulating the enzyme heme oxygenase-1 (HO-1), which increases the production of bilirubin, a natural antioxidant in the body. This helps eliminate reactive oxygen species (ROS) by inhibiting NADPH oxidase, a key enzyme responsible for generating ROS and free radicals, thereby reducing oxidative stress [9]. While the antioxidant potential of phycocyanin has been explored in various models of oxidative stress, its specific effects on exercise-induced oxidative damage in the brain remain less well understood. Strenuous exercise is known to elevate ROS levels, leading to lipid peroxidation and oxidative damage in neural tissues. However, whether phycocyanin can effectively counteract these effects in the brain following prolonged exercise is still under investigation. This study aims to evaluate the impact of phycocyanin on oxidative stress markers, particularly lipid peroxidation and antioxidant activity, in the brains of rats subjected to exhaustive exercise. By addressing this gap, we can better understand the neuroprotective potential of phycocyanin in exercise-induced oxidative stress.

Materials and methods

Phycocyanin

Phycocyanin was obtained from Xi'an Day Natural Inc. (Xi'an, Shaanxi, China). Its content was analyzed using a spectroscopic method [10], revealing a purity level of 1.9 and a yield of 25 % as described in the previous study [11].

Animals and experimental design

Male Sprague-Dawley rats, aged 4 weeks and weighing 160 - 220 g, were obtained from Nomura Siam International Co., Ltd., Bangkok, Thailand. The animals were housed in the Center for Animal Research at Naresuan University under controlled light-dark cycles (12:12 h) at a temperature of 22 ± 1 °C. This study was approved by the Institutional Animal Care and Use Committee (IACUC) under ethical approval number NU-AE 630605. The rats were categorized into 2 main groups: A non-exercise group (Control or C, n = 8) and 4 exercise groups. Before the experiment, the exercise groups underwent one week of swimming training. These exercise-trained rats were then randomly assigned into 4 groups as follows: E group: Exercise without any additional treatment (n = 8), ELP group: Exercise + low-dose phycocyanin (100 mg/kg BW) (n = 8), EHP Group: Exercise + high-dose phycocyanin (200 mg/kg BW) (n = 8) and VC Group: Exercise + Vitamin C (200 mg/kg BW) as a positive control (n = 8) (**Figure 1**). At the end of the experiment, the animals were euthanized, and the hippocampus, prefrontal cortex, and amygdala were collected to investigate the effects of phycocyanin on oxidative stress.

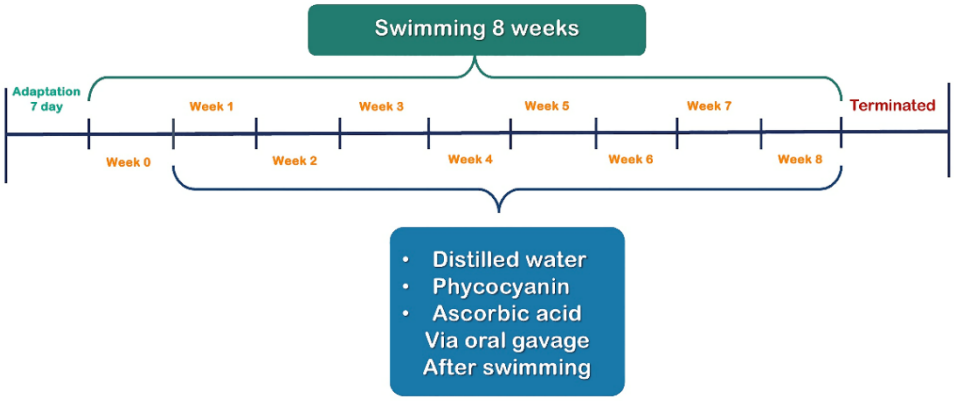

Figure 1 Time period for giving test substances and making the animals swim for excessive exercise.

Prolonged excessive exercise

To induce excessive exercise through swimming, the rats in groups E, ELP, EHP, and VC were placed in underwent a swimming regimen in a 0.50×0.50×0.90 m^3 pool filled with water maintained at 30 ± 5 °C, 5 days a week for 8 weeks. During the 1st week, the rats swam for 30 min per day, which increased to 40 min per day in the 2nd week. From weeks 3 to 5, the duration was extended to 60 min per day, and in weeks 6 to 8, they swam for 75 min daily. The animals were allowed to swim freely without external interference. After each session, they were carefully dried before being returned to their housing cages [12, 13, 14, 15]. This swimming protocol was reported in our previous study [11] and is classified as a model of prolonged excessive exercise.

Biochemical parameters

Tissue preparation

Three parts of the rat brain, prefrontal cortex, hippocampus, and amygdala were collected and stored in a temperature-controlled freezer at –80 °C and later analyzed biochemically. The tissue was homogenized with 0.1X phosphate buffered saline (PBS) (pH 7.4), and centrifuged at 9,000 xg for 20 min at 4 °C [16]. The supernatant was then collected for biochemical analysis.

Measurement of lipid peroxidation levels

The MDA level was determined using the TBARs assay by measuring MDA produced from lipid peroxidation in U/mg protein. The supernatant obtained from brain tissue centrifugation was mixed with prepared solutions: 0.8 % Thiobarbituric acid, 8.1 % sodium dodecyl sulfate, and 20 % acetic acid solution (pH 3.5). 1,1,3-3-Tetramethoxypropane was used as the standard, and distilled water served as the blank. After thorough mixing, the samples were incubated at 95 °C for 1 h in a heat box, then the absorbance was measured at 532 nm using a microplate reader.

Measurement of superoxide dismutase activity

The supernatant was used to measure SOD activity, an antioxidant enzyme that converts superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2). Pyrogallol was used as the substrate due to its ability to undergo auto-oxidation, producing H_2O_2. The prepared pyrogallol solution, 50 nM Tris-EDTA (pH 8.2), and 50 nM Tris-HCl (pH 7.4) were mixed with the supernatant, with pyrogallol serving as the blank. The mixture was then analyzed at 420 nm using a spectrophotometer in kinetic mode for 5 min.

Measurement of catalase activity

The supernatant was used to measure CAT activity, an antioxidant enzyme that breaks down H_2O_2 into water (H_2O) and oxygen (O_2). The prepared solutions 30 % v/v H_2O_2 and catalase assay buffer were mixed with the supernatant, with distilled water used as the blank. The reaction was then measured at 240 nm using a spectrophotometer in kinetic mode for 5 min.

Measurement of glutathione peroxidase activity

The supernatant was used to measure GPx activity, an antioxidant enzyme that converts H_2O_2 into H_2O and

O_2. The prepared solutions of 10 mM Sodium azide (NaN_3), 4 mM Reduced GSH, 2.5 mM H_2O_2, and PBS were mixed with the supernatant and incubated at 37 °C for 5 min. After incubation, 5 % Trichloroacetic acid was added to separate GPx from other substances, followed by the addition of 1.2 mM 5,5'-dithiobis-2-nitrobenzoic acid to produce a yellow-colored compound. The absorbance was then measured at 412 nm using a microplate reader.

Protein quantification using BCA protein assay

The supernatant obtained from brain tissue centrifugation was used to measure protein concentration for normalizing MDA, SOD, and CAT levels per mg of protein. The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) was used for the analysis. The prepared Working Solution was mixed with the supernatant, with bovine serum albumin as the standard and 0.1 M PBS as the blank. The mixture was thoroughly mixed using a plate shaker for 30 s and then incubated at 37 °C for 30 min in a heat box. Finally, the absorbance was measured at 562 nm using a microplate reader. This method first described by Smith *et al.* [17].

Statistical analysis

The data were processed using GraphPad Prism version 8.0.1 and expressed as the mean ± standard error of the mean (SEM). Differences between the control and various treatment groups were evaluated using a one-way analysis of variance (ANOVA), followed by an LSD post hoc test. A *p*-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results and discussion

Effect of phycocyanin on the lipid peroxidation

The levels of MDA in the 3 brain regions (prefrontal cortex, hippocampus, and amygdala) showed no significant differences in the exercise group compared to the control group. However, when comparing the exercise group combined with low-dose and high-dose phycocyanin supplementation, MDA levels in all 3 brain regions were significantly reduced in the ELP and EHP groups compared to the E group ($p < 0.05$, $p < 0.01$) (**Figure 2**).

Previous studies have shown that prolonged exercise (11 weeks) can elevate MDA levels, a key marker of oxidative stress, contributing to lipid peroxidation and potential neuronal degeneration [5, 18]. However, our findings align with research indicating that moderate exercise such as swimming for 2 h [18] or continuous treadmill running at 1.6 km/h for 8 weeks [19], does not significantly alter MDA levels in various brain regions, including the hippocampus and prefrontal cortex. Interestingly, the impact of exercise-induced oxidative stress appears to be region-specific. For instance, studies have reported increased MDA levels in the striatum following a 5-day-per-week exercise regimen for 2 months, likely due to the striatum's high dopaminergic activity and its role in motor control. The striatum contains a high density of dopaminergic neurons, and dopamine metabolism generates ROS, contributing to oxidative stress [20]. Additionally, MDA levels have been found to increase in the medulla and cerebral cortex, whereas no significant changes were observed in the cerebellum, hypothalamus, prefrontal cortex, or hippocampus. The cerebral cortex, which plays a crucial role in synaptic plasticity and neuronal signaling, has high energy demands, leading to increased mitochondrial ROS production during exercise. In contrast, the hippocampus and prefrontal cortex possess robust endogenous antioxidant defense systems, including high levels of SOD, GPx, and CAT, which neutralize ROS and prevent excessive lipid peroxidation [21, 22]. These findings suggest that the brain's response to exercise-induced lipid peroxidation varies depending on the type, intensity, and duration of physical activity, as well as the intrinsic susceptibility of different brain regions to oxidative stress. Additionally, our results indicate that while exercise alone does not significantly alter MDA levels in the prefrontal cortex, hippocampus, or amygdala, the combination of exercise with phycocyanin supplementation led to a notable reduction in MDA levels. This suggests that phycocyanin exerts a protective effect against oxidative stress in the brain.

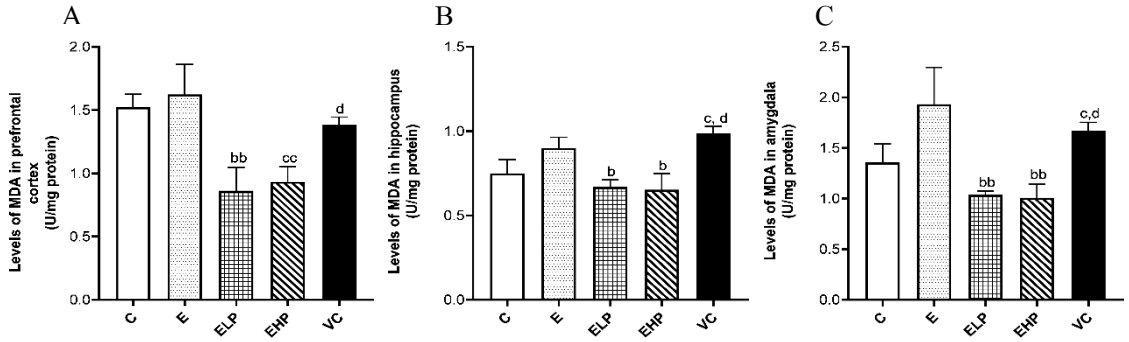

Figure 2 The effect of phycocyanin on levels of MDA in the prefrontal cortex (A), hippocampus (B), and amygdala (C) in rats. Data are represented as the mean ± SEM. $^{bb}p < 0.01$ when compared to the E group, $^{cc}p < 0.01$ when compared to the ELP group, and $^{d}p < 0.05$ when compared to the EHP group.

Effect of phycocyanin on antioxidant enzyme activity

The exercise group showed no significant difference in SOD activity compared to the control group. However, in the group that exercised and received a high dose of phycocyanin, SOD activity was significantly increased in the prefrontal cortex and hippocampus compared to the E group ($p < 0.01$, $p < 0.001$) (**Figure 3**). In contrast, no significant differences in SOD levels were observed in the amygdala among the groups. Additionally, the vitamin C group also exhibited significantly higher SOD activity in the prefrontal cortex and hippocampus compared to the exercise group ($p < 0.05$, $p < 0.01$) (**Figure 3**). These findings suggest that phycocyanin enhances SOD activity in the EHP group, potentially boosting antioxidant enzyme function and reducing oxidative stress-related damage in the prefrontal cortex and hippocampus.

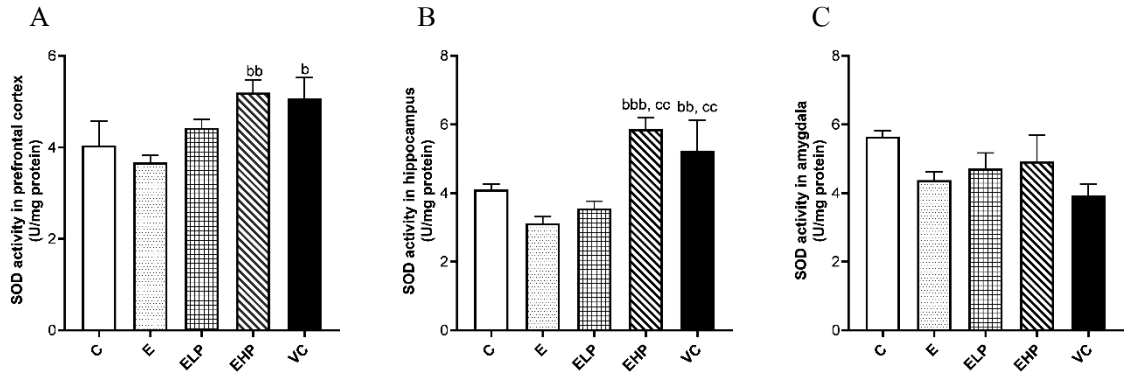

Figure 3 The effect of phycocyanin on SOD activity in the prefrontal cortex, hippocampus, and amygdala in rats. Data are represented as the mean ± SEM. $^{b}p < 0.05$, $^{bb}p < 0.01$, $^{bbb}p < 0.001$ when compared to the E group, and $^{cc}p < 0.01$ when compared to the ELP group.

The CAT activity in exercise group was significantly decreased in the prefrontal cortex, hippocampus, and amygdala compared to the control group ($p < 0.001$) (**Figure 4**). However, when compared to the groups that exercised and received low-dose or high-dose phycocyanin, CAT activity was significantly increased in the hippocampus and amygdala in the EHP group and in the prefrontal cortex in both the ELP and EHP groups compared to the exercise-only group (E) ($p < 0.05$) (**Figure 4**). Additionally, the vitamin C group showed significantly higher CAT activity in all 3 brain regions compared to the exercise group (E) ($p < 0.05$, $p < 0.001$) (**Figure 4**).

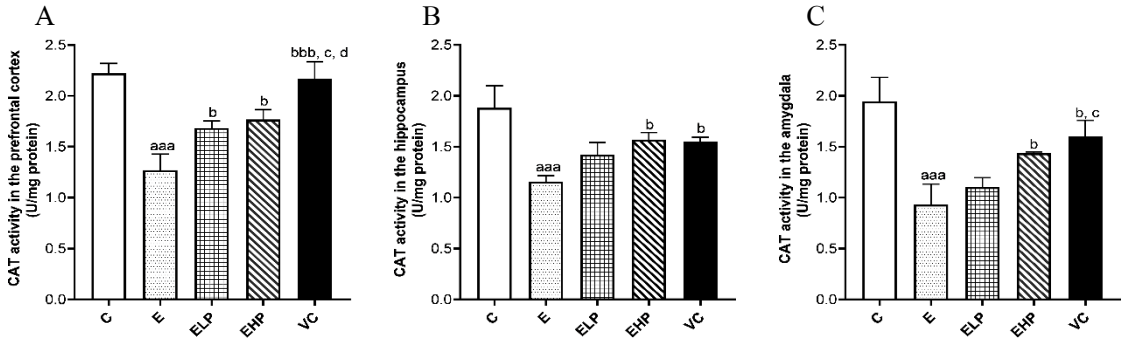

Figure 4 The effect of phycocyanin on CAT activity in the prefrontal cortex, hippocampus, and amygdala in rats. Data are represented as the mean ± SEM. $^{aaa}p < 0.001$ when compared to the control group, $^{b}p < 0.05$ and $^{bbb}p < 0.001$ when compared to the E group, $^{c}p < 0.05$ when compared to the ELP group, and $^{d}p < 0.05$ when compared to the EHP group.

The exercise group showed a significant reduction in GPx activity across all 3 brain regions compared to the control group ($p < 0.05$, $p < 0.01$) (**Figure 5**). However, when compared to the groups that exercised and received low-dose or high-dose phycocyanin, no significant differences in GPx activity were observed in any of the 3 brain regions. In contrast, the vitamin C group showed a significant increase in GPx activity in the prefrontal cortex and hippocampus compared to the exercise group ($p < 0.05$, $p < 0.01$) (**Figure 5**).

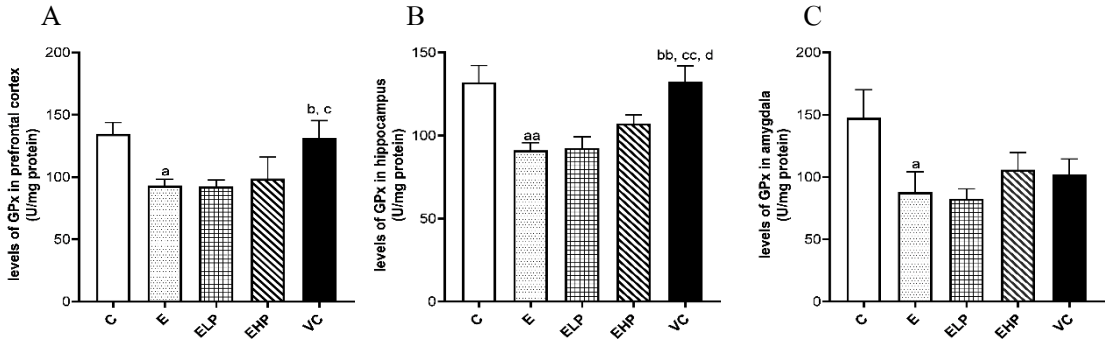

Figure 5 The effect of phycocyanin on GPx activity in the prefrontal cortex, hippocampus, and amygdala in rats. Data are represented as the mean ± SEM. $^{a}p < 0.05$ and $^{aa}p < 0.01$ when compared to the control group, $^{b}p < 0.05$ and $^{bb}p < 0.01$ when compared to the E group, $^{c}p < 0.05$ and $^{cc}p < 0.01$ when compared to the ELP group, and $^{d}p < 0.05$ when compared to the EHP group.

The findings suggest that exercise alone does not significantly alter SOD activity when compared to the control group. However, the combination of exercise with a high dose of phycocyanin significantly increased SOD activity in the prefrontal cortex and hippocampus. This suggests that phycocyanin at a higher dose may enhance the brain's ability to counteract oxidative stress in these regions. The amygdala, however, did not exhibit any significant changes in SOD activity, indicating that the effect of phycocyanin on SOD activity may be region-specific. Similarly, vitamin C supplementation also resulted in a significant increase in SOD activity in the prefrontal cortex and hippocampus, reinforcing the potential role of antioxidant supplementation in mitigating oxidative stress in these critical brain areas. In contrast, strenuous exercise was found to reduce CAT and GPx levels in the prefrontal cortex, hippocampus, and amygdala. These results in accordance with the previous studies that SOD levels in the hippocampus remained unchanged following 7.5 weeks of exercise

[4]. Similarly, different exercise intensities did not significantly affect SOD levels in the prefrontal cortex [21]. Previous reports indicate that SOD functions primarily to eliminate superoxide anions (O_2^-), the 1st ROS generated during cellular respiration. The body maintains stable SOD levels to continuously neutralize O_2^- under conditions of high oxidative stress. Meanwhile, CAT plays a role in detoxifying hydrogen peroxide (H_2O_2), a harmful byproduct of SOD converting O_2^- into H_2O_2. The activity of CAT and GPx tends to fluctuate in response to increasing H_2O_2 levels, with significant changes typically observed under conditions of oxidative stress [23].

Different types of exercise specifically aerobic and anaerobic have distinct effects on oxidative stress and antioxidant enzyme activity in the brain. Aerobic overexercise is known to increase ROS production, initially triggering an upregulation of SOD, CAT, and GPx as a compensatory response. However, with prolonged overexercise, these antioxidant systems become overwhelmed, leading to enzyme depletion and increased oxidative damage [23-25]. This aligns with our study's findings, where exercise alone resulted in a significant decrease in CAT and GPx activity across multiple brain regions, suggesting that prolonged exercise-induced ROS production exceeded the capacity of endogenous antioxidant defenses. Anaerobic overexercise, characterized by short bursts of high-intensity activity, induces localized oxidative stress. This often leads to a transient rise in SOD and CAT activity but may reduce GPx levels due to rapid glutathione depletion [23-25]. In our study, the observed lack of significant changes in SOD activity in the exercise-only group suggests that the compensatory antioxidant response may have been insufficient to counteract the ROS load, especially in brain regions with higher metabolic demands.

The regional variations in oxidative stress and metabolic activity further contribute to differences in antioxidant enzyme responses. The striatum and cerebral cortex, with their high metabolic activity, generate increased ROS, leading to elevated MDA levels a key indicator of lipid peroxidation [26-27]. In contrast, the hippocampus and prefrontal cortex, which exhibit lower metabolic demands, may experience reduced oxidative stress and possess enhanced antioxidant defense mechanisms. These regions have been reported to have elevated levels of SOD and GPx, which effectively mitigate ROS-induced damage and maintain stable MDA levels [23, 26, 27]. This aligns with our findings that phycocyanin supplementation significantly increased SOD and CAT activity in these regions, suggesting that certain brain areas may be more responsive to antioxidant intervention.

Previous studies have demonstrated that phycocyanin exhibits pharmacological properties, including antioxidants, anti-inflammatory, and neuroprotective effects [7]. Phycocyanin can help prevent oxidative stress caused by excessive ROS production and inhibit ROS formation [28]. In this study, both low- and high-dose phycocyanin supplementation significantly reduced MDA levels and enhanced antioxidant activity across all 3 brain regions, suggesting a protective role against oxidative stress and neuronal damage. However, regional variations in neuroprotection may influence antioxidant responses. The prefrontal cortex and hippocampus are particularly vulnerable to oxidative stress due to their high metabolic activity and crucial roles in cognitive function. Increased oxidative stress in these regions may trigger upregulation of antioxidant defense mechanisms, including SOD. In contrast, the amygdala may experience relatively lower oxidative stress levels, leading to a diminished antioxidant response [29-31]. Prior studies have shown that administering phycocyanin at doses ranging from 50 to 200 mg/kg in rodents effectively reduces oxidative stress and enhances antioxidant levels in the brain. Specifically, doses of 100 and 200 mg have been shown to increase the activity of antioxidant enzymes such as SOD and CAT, reinforcing its antioxidant potential and its ability to suppress oxidative stress [32, 33]. While phycocyanin has a high safety profile, it should be used in doses supported by research. In humans, a recommended dosage of 100 mg/kg is considered both safe and effective for preventing oxidative stress [34]. However, phycocyanin is the long-term consequences of high-dose use are not yet fully understood, with some studies suggesting that excessive antioxidant supplementation could disrupt the balance of ROS, potentially leading to adverse effects. Further research is needed to fully understand the implications of long-term supplementation [35].

Conclusions

In summary, phycocyanin supplementation effectively lowers lipid peroxidation and enhances antioxidant enzyme activity, particularly SOD and CAT, in a region-specific manner. These findings suggest that phycocyanin has potential as a neuroprotective supplement against exercise-induced oxidative stress, warranting further research into its therapeutic applications. The findings of this study align with previous research demonstrating that prolonged or intense exercise can lead to increased oxidative stress, as indicated by decreased CAT and GPx activity in the exercise-only group. Studies have suggested that moderate exercise may enhance antioxidant defenses, but excessive or prolonged physical activity can overwhelm these protective mechanisms, leading to oxidative damage. The current study supports this notion by showing a reduction in antioxidant enzyme activity in response to exercise alone. Additionally, the observed increase in SOD and CAT activity with phycocyanin supplementation is consistent with prior research on the antioxidant properties of phycocyanin, which has been reported to enhance enzymatic defenses and reduce oxidative damage in various. These findings offer fresh insights into minimizing damage caused by high-intensity exercise. Future research should further explore optimal exercise regimens and antioxidant supplementation strategies to balance ROS production and antioxidant defense, ultimately promoting brain health and cognitive function.

Acknowledgements

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