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Effects of Aerobic Exercise Training, Pumpkin Seed Oil and Chickpea Supplementation on Gene Expression of Antioxidant Enzymes in the Liver and Muscle Tissues of Male Rats

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A B S T R A C T

Introduction: There is ample evidence regarding the protective effects of antioxidant vitamins and minerals against oxidative stress. The discovery of zinc's protective role in countering free-radical formation and oxidative stress has instigated extensive research into the antioxidant properties of zinc and its involvement in the antioxidant defense system. Furthermore, the utilization of plants with antioxidant properties has gained increasing attention among researchers. This study aims to investigate the impact of six weeks of aerobic exercise training, along with supplementation of pumpkin seed oil and chickpeas, on the gene expression of antioxidant enzymes in the liver and muscle tissues of male rats.

Materials & Methods: To achieve this objective, 36 Wistar rats were divided into six groups: control (C), training (T), chickpea (Ch), chickpea + training (Ch+T), pumpkin seed oil (P), and pumpkin seed oil + training (P+T). At the conclusion of the training and supplementation period, the gene expression of the antioxidant enzymes glutathione peroxidase 1 (GPX1) and catalase (CAT) were assessed. Data were subjected to one-way analysis of variance at a significance level of $P < 0.05$.

Results: The results revealed that in all the groups involving pumpkin seed oil and chickpea supplementation, in both liver and muscle tissues, a non-significant decrease in the expression of the enzymes CAT and GPX1 was observed when compared to the control and exercise training groups.

Conclusion: To a certain extent, the use of pumpkin seed oil and chickpeas may enhance the expression of antioxidant enzymes.

Keywords: Zinc, Pumpkin seed oil, Chickpea, Catalase (CAT), Glutathione peroxidase (GPX)

➢ **How to cite this paper**

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Introduction

Zinc is an essential trace element with antioxidant properties, crucial for combating and mitigating oxidative stress (1). It plays a pivotal role in cell survival and contributes to the structure and function of numerous proteins (2). In its natural state, zinc exists as a non-redox-active divalent cation (Zn2+) under physiological conditions (3). The response to oxidative stress and reactive oxygen species (ROS) occurs when zinc binds to sulfur (thiolate) in cysteine, forming zinc thiolate, converting zinc into its active reduced state. Oxidants interact with thiolate, releasing zinc in its free form, thereby generating a zinc signal (4). Zinc also enhances the antioxidant defense system and influences the activity of various enzymes, including antioxidant enzymes. As a constituent of the active site in the antioxidant enzyme superoxide dismutase (SOD) and a factor in reducing oxidases' activity, zinc plays a crucial role in balancing pro- and antioxidants within cells. Additionally, it may regulate the activation of antioxidant transcription factors and the expression of antioxidant genes (5,6).

Oxidative stress, characterized by an overproduction of reactive oxygen/nitrogen species (ROS/RNS), known as prooxidants, and a deficiency in enzymatic and non-enzymatic antioxidants, is a well-documented condition (1). The body's antioxidant defense system detoxifies highly reactive molecules, such as free radicals, to prevent membrane damage and skin harm (7). Antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), play a pivotal role in this process (1).

One common physiological condition linked to increased oxidative stress is moderate to high-intensity, short-term physical activity, which generates excess reactive oxygen species, causing damage to various tissues in the body (8). The heightened mitochondrial oxygen consumption and electron transfer during intense physical activity lead to oxidative stress and ROS formation. Intense exercise increases oxygen demand by 10 to 15 times (7). Recently, phytochemicals found in plants have garnered significant attention for their role in preventing diseases related to oxidative stress. These phytochemicals, such as phenols and phenoids, act as antioxidants, neutralizing active oxygen species and scavenging free radicals (9).

Pumpkin seed oil (*Cucurbita pepo L.*) is a dark green and flavorful oil with a hazelnut-like taste (10). Pumpkin seeds are rich in nutrients and bioactive compounds, making them valuable due to their potential biological and medicinal properties, which offer various health benefits. They are a source of nutrients, including beta-carotene, α-tocopherol, B vitamins, lutein, phytosterols, and other minerals like iron, manganese, magnesium, zinc, and more (11). Chickpeas (*Cicer arietinum L.*) are also recognized as nutrient-rich legumes, containing protein, dietary fiber, and essential micronutrients, including iron, zinc, magnesium, and calcium (13). These legumes are known for their phytochemical content, which includes flavonoids, carotenoids, phenolic acids, acetylbones, and lignans, providing protection against damage caused by reactive oxygen species (14).

Zinc deficiency has been shown to increase the formation of free radicals

during exercise and negatively affect antioxidant activity. Therefore, exploring the potential of zinc supplementation to inhibit the formation of reactive oxygen radicals during exercise is not only significant in understanding the connection between zinc and the antioxidant system but also holds implications for the health and performance of athletes (7). Numerous clinical trials have examined the effectiveness of zinc supplementation in improving antioxidant biomarkers and reducing oxidative stress, including glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD) (15).

It's worth noting that the body cannot store zinc, necessitating a consistent dietary intake of this essential element. Given the potential for zinc depletion in athletes, there is a growing need for zinc supplementation due to its sensitive role. This study aims to investigate the impact of herbal antioxidants containing zinc on the antioxidant system. Considering zinc's presence in the structure of SOD, we hypothesize that these supplements can enhance the efficiency of this enzyme. Whether they can also positively influence the function of other important antioxidant enzymes, such as catalase and glutathione peroxidase, in two crucial metabolic tissues during exercise, is a question that motivates our current research, particularly given reports of the presence of other elements in these supplements.

Materials and Methods

Animals

This study involved 36 male Wistar rats (150–180 g, eight weeks of age) obtained from the Laboratory Animal Centre of Babol University in Babol, Mazandaran, Iran. The "IR.UMZ.REC.1400.029" for animal experiments, and ethical guidelines for the care and use of laboratory animals provided by the Institutional Animal Care and Use Committee of Mazandaran University, were followed. The rats were housed at a temperature of 20-22 °C with a 12:12 dark-light cycle. Throughout the study, the rats had ad libitum access to food and water. After a one-week adaptation period, the animals were randomly assigned to one of six groups, each consisting of six rats: control group (C), exercise training group (T) with a running speed of 25 m/min, chickpea control group (Ch), chickpea + exercise training group (Ch+T), pumpkin seed oil control group (P), and pumpkin seed oil + exercise training group (P+T).

Exercise Training Protocol

In this study, a modified protocol from Howarth et al. (16) and Ghanbari Niaki (17) was employed. The training program consisted of 60 minutes of daily running on a rat treadmill at a speed of 25 m/min, five days a week for six weeks. This exercise protocol targeted a maximum oxygen consumption of 65% of a medium intensity level. The subjects initiated the training program at a speed of 10 meters per minute and progressed to a speed of 25 meters per minute. The control groups also engaged in a 10-minute treadmill session at a speed of 10 m/min once a week to ensure equal conditions and avoid inactivity. To motivate running, a mild electric shock was positioned at the end of the treadmill belt. To eliminate any potential effects of the electric shock on the research outcomes, animals were conditioned with sound during their treadmill familiarization stage to

prevent them from approaching and resting at the device's end.

Pumpkin Seed Oil and Chickpea Supplement

Supplements were administered to the rats via specific gavage. Rats received 4 ml/kg/day of cold-pressed pumpkin seed oil and 2 g/kg/day of chickpea flour dissolved in saline for a duration of 6 weeks. The control groups received saline once a week in the same calculated ratio to mitigate gavage-related stress. Weekly re-weighing was performed to ensure precise dosing per kilogram of body weight.

Tissue Collection

At the end of the training period, liver and muscle tissues from the rats were collected. For this purpose, the rats were anesthetized by intraperitoneal injection of a mixture of ketamine (30-50 mg/kg) and xylazine (3-5 mg/kg) following a 10-12 hour fasting period. The abdominal cavity was opened, and liver tissue and Gastrocnemius muscle were removed for gene expression analysis.

RNA Extraction

The extraction process followed the kit instructions from DENA Zist's animal tissue RNA extraction kit (Mashhad, Iran). Briefly, 100 mg of homogenized tissue sample and 1 ml of AR1 buffer plus 2 µl of 2-mercaptoethanol were mixed in RNase- and DNase-free microtubes. After a 5-minute incubation, the resulting solution was transferred to a 1.5 ml tube. To this tube, 350 µl of pre-warmed AR2 (70°C) was added, vortexed, and then centrifuged at 4000 rpm for 5 minutes at 4°C. The supernatant (~500 µl) was transferred to a new microfuge tube. Next, 235 µl of 95% ethanol was added to the tube and centrifuged at 1000 RPM for 2 minutes at 23-25°C. The flowthrough was discarded, and 550 µl of AR3 was added to the spin column, followed by another centrifugation step (1000 RPM for 2 minutes at 23-25°C). This process was repeated, and the eluted RNA sample, containing total RNA, was stored at -80°C for quality control tests.

Quantitative Real-Time PCR for Assessment of CAT and GPX1 Expression Level

To assess the expression levels of *CAT* and *GPX1*, RNA extracted from liver and skeletal muscle tissues was converted into cDNA using the Oligo-dT primer following the kit's instructions (Yekta Tajhiz Azma, Iran). Quantitative real-time PCR was conducted using the SYBR® Green kit (Ampliqon, Denmark) on a Rotor Gene Corbett 6000 system. Beta-actin was used as the internal control gene, and the primer sequences were designed using Primer Premier software and synthesized by Bioneer company, South Korea. The primer sequences are detailed in Table 1.

Table 1. The sequences of primers used in Real Time PCR process

Gene	Primer sequence(5'-3')	Accession number	Product size (bp)
GPX1	F-5´-AGTGCGAGGTGAATGGTGAGA-3´ R-5´-CCAGGAAATGTCGTTGCG-3´	NM_030826.4	146

CAT	F-5'-ATCAGGTTACTTTCTTGTTCAGCG-3' R-5'-TGATGCCCTGGTCAGTCTTG-3'	NM_012520.2	147
ACTB	F-5'GTGTGACGTTGACATCCGTAAAGAC-3' R-5'-TGCTAGGAGCCAGGGCAGTAAT-3'	NM_031144.3	119

After examining the threshold cycle (CT) values obtained from the biological and technical replications of each treatment, each group was analyzed and normalized to beta actin using the following equation:

$$\Delta CT\ (_{\text{in treated and control samples}}) = CT_{\text{target gene}} - CT_{\text{beta-actin}}$$

The fold change in expression was calculated as $2^{-\Delta\Delta CT}$, where

$$\Delta\Delta CT = \Delta CT_{\text{target gene in treated sample}} - \Delta CT_{\text{target gene in control sample}}$$

Statistical analyses

All data are presented as the mean ± SEM. Statistical analyses were conducted using Prism GraphPad version 8.0.2. One-way analysis of variance (ANOVA) was employed to assess differences between the groups, and the LSD test (Least Significant Difference) was used to examine pairwise differences in means between groups. A significance level of $P < 0.05$ was considered statistically significant.

Results

The results of one-way analysis of variance on *CAT* and *GPX1* gene expression in liver tissue of rats indicated that aerobic exercise, owing to its oxidant properties, led to a reduction in the expression of the catalase and glutathione peroxidase genes in the liver. However, this reduction was not statistically significant.

The analysis of *CAT* gene expression levels in liver tissue revealed a non-significant difference ($P > 0.05$) with down-regulation in the pumpkin oil control and pumpkin oil exercise training groups compared to the control group. In the case of chickpea groups, both the chickpea control group ($P = 0.05$) and chickpea exercise training group ($P = 0.12$) exhibited decreases compared to the control group, although these decreases were not statistically significant. Furthermore, the chickpea exercise training group showed a decrease when compared to the exercise training group but displayed a non-significant increase compared to the control group of chickpeas. Overall, the pumpkin oil groups appeared to be more effective in enhancing catalase gene expression compared to the chickpea groups.

The analysis of *GPX* gene expression showed a significant decrease ($P < 0.05$) in the pumpkin oil control group ($P = 0.02$) when compared to the control group. There was a non-significant decrease observed in the pumpkin oil exercise training group compared to the control group. Similarly, a slight, non-significant decrease was noticed when comparing the exercise training group with the training group. In assessing the effectiveness of exercise and supplementation in groups consuming chickpea supplements, the chickpea

control group exhibited a non-significant decrease, while the chickpea exercise training group (P = 0.00) showed a significant decrease compared to the control group. Additionally, there was no significant difference (P > 0.05) observed between the chickpea training group and exercise group.

Figure 1 illustrates the fold changes in glutathione peroxidase and catalase gene expression in liver tissue within the study groups.

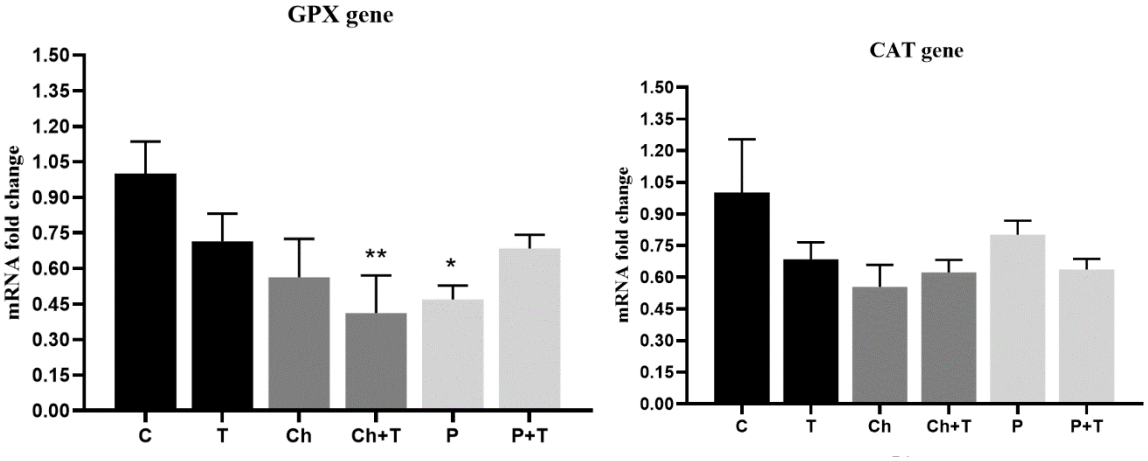

Figure 1. Fold Change in *CAT* and *GPX1* Expression Levels in the Liver After 6 Weeks of Moderate Exercise Training in Rats. Each group consists of 6 rats, and the data are expressed as mean ± SEM. The groups include control (C), training (T), chickpea (Ch), chickpea + training (Ch+T), pumpkin seed oil (P), and pumpkin seed oil + training (P+T). *P < 0.05 vs. the C group.

In the analysis of *CAT* gene expression in muscle tissue, the study results indicated no significant difference (P > 0.05) across groups. Exercise and supplementation with pumpkin seed oil did not significantly differ from the control and exercise training groups. However, both groups receiving chickpea supplementation (chickpea control and chickpea exercise training) showed down-regulation compared to the control and exercise training groups. Notably, only the reduction in the chickpea control group (P = 0.00) was statistically significant when compared to the exercise training group. In summary, pumpkin seed oil had a more pronounced effect in up-regulating the *CAT* gene compared to chickpea supplementation, although both supplements had lower effects than the saline control groups.

As depicted in Figure 2, both the pumpkin oil control and pumpkin oil exercise training groups exhibited down-regulation in glutathione peroxidase gene expression when compared to the control and exercise training groups. However, these differences were not statistically significant. The exercise training group also displayed a non-significant decrease in glutathione peroxidase gene expression compared to the control group. Notably, the pumpkin oil exercise training group showed an increase compared to the pumpkin oil control group, though this increase was not significant.

Furthermore, data from the chickpea supplement groups revealed a decrease in the expression of the glutathione peroxidase gene in both groups (chickpea control and chickpea exercise training) when compared to the control and exercise training groups. Interestingly, the chickpea exercise training group exhibited an

up-regulation in glutathione peroxidase gene expression in muscle tissue compared to the chickpea control group, though this change was not statistically significant.

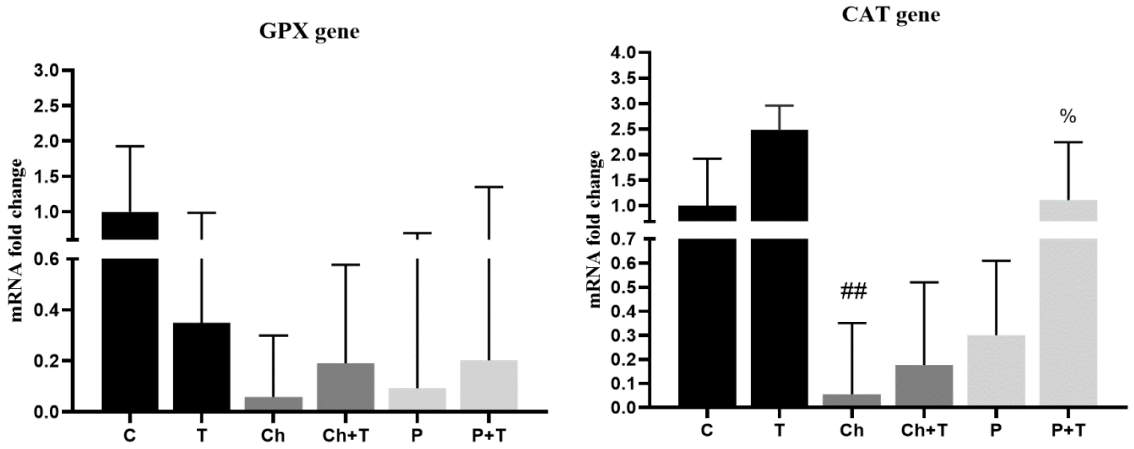

Figure 2. Fold Change of *CAT* and *GPX1* Expression Levels in Muscle after 6 Weeks of Moderate Exercise Training in Rats. Each group consists of 6 rats, and data are expressed as mean ± SEM. Control (**C**), Training (**T**), Chickpea (**Ch**), Chickpea + Training (**Ch+T**), Pumpkin Seed Oil (**P**), and Pumpkin Seed Oil + Training (**P+T**). #P < 0.05 vs. Exercise Training (**T**) group, %P < 0.05 vs. the Ch group.

Discussion

In this study, we observed that a six-week program of moderate-intensity aerobic exercise led to reduced expression of antioxidant enzymes, specifically catalase and glutathione peroxidase, in liver tissue when compared to the control group (8). In contrast, muscle tissue displayed a decrease in glutathione peroxidase but an increase in catalase gene expression within the exercise training group.

Exercise is known to generate reactive oxygen species (ROS), with high-intensity aerobic exercise generating more ROS (8). The activity of antioxidant enzymes follows a cascade mechanism where reduced enzyme activity leads to increased oxidants, ROS, and active nitrogen species, subsequently triggering the activity of other antioxidant enzymes (18). Superoxide dismutase (SOD) is the first line of defense in the endogenous antioxidant system, converting superoxide radicals (O2•-) to hydrogen peroxide (H2O2). H2O2, which is also harmful to cells, can be metabolized through various pathways, including conversion to water by glutathione peroxidase (GPX) or being removed by catalase (CAT) if the H2O2 production exceeds GPX's capacity (19).

The relatively higher expression of catalase in muscle tissue may indicate its reduced role in neutralizing free radicals, especially within exercised muscles (18).

Typically, chronic exercise enhances both enzymatic and non-enzymatic antioxidant defenses, leading to adaptation in the exercise response and improved protection against ROS. However, the lack of adaptation observed in our study may be attributed to the relatively short duration of the training program, which lasted only six weeks. Studies on adaptation to exercise have shown that chronic exercise, such as six weeks of cycling training, can increase the concentration and activity of glutathione (GSH), SOD, and CAT, while maintaining their levels (19). It's worth noting that studies have

reported varied findings, suggesting that the effects of exercise on oxidative stress markers are influenced by exercise type, intensity, and the specific oxidative damage markers used for measurement (20).

In various studies, researchers have demonstrated that the collaboration between antioxidant supplements and endogenous antioxidants enhances the skeletal muscle's ability to neutralize reactive oxygen species. The use of antioxidant supplements in conjunction with physical activity can reduce the harmful effects of exercise-induced oxidative stress, bolster the antioxidant defense system, and maximize the positive effects of physical activity (21). Zinc, an essential trace mineral with antioxidant properties, plays a crucial role as a micronutrient in reducing free radicals. It is involved in the structure and catalytic function of antioxidant enzymes (4).

Our study yielded intriguing results. It revealed an increase in the expression of catalase and glutathione peroxidase genes in muscle tissue following the consumption of two supplements: pumpkin seed oil and chickpeas, combined with moderate-intensity exercise. This suggests the antioxidant potential of these supplements in combating exercise-induced oxidants (12-14). This effect may be partly attributed to zinc's antioxidant properties.

Pumpkin seed oil, rich in vitamins E and B, carotene, and selenium, plays a crucial antioxidant role (26). In contrast, chickpea supplementation led to an increase in catalase expression compared to glutathione peroxidase in liver tissue. This could be related to chickpeas' higher iron content, an essential component of catalase (27).

Clinical trials investigating the effects of external antioxidants have produced contradictory results, underscoring the importance of maintaining the balance between ROS production and neutralization in any complementary therapy (30).

For future research, measuring tissue zinc levels may provide a deeper understanding of antioxidant mechanisms in the context of antioxidant supplementation and exercise.

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Conflict of Interests

There are no conflicts of interest in the present study.

Authors' Contributions

AGh.N: developed the original idea and the protocol, abstracted and analyzed data, provided study supervision and served as the guarantor. AGh: contributed to the development of the protocol, abstracted data, and prepared the manuscript. KhN: was involved in the acquisition of data, critically revised the manuscript for important intellectual content, and conducted statistical analysis.

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