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Original Article

The effect of aerobic training and eugenol supplementation on the PI3K/AKT/mTOR pathway in skeletal muscle of male rats poisoned with chlorpyrifos

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Abstract

Background and aims: Chlorpyrifos (CPF) is an insecticide that is widely used in the world. The purpose of this research was to investigate the effect of 4-week aerobic exercise and eugenol supplementation on the phosphatidylinositol-3-kinases/protein kinase/mammalian target of rapamycin (Pi3K/AKT/mTOR) pathway on the skeletal muscle of male rats poisoned with CPF.

Methods: Overall, 12-week-old female rats were used in this experimental research. The rats were randomly divided into 8 groups (8 rats in each group), including healthy control, toxic control, poison solvent, corn oil solvent, poisoned + eugenol, poisoned + aerobic exercise, and poisoned + aerobic exercise + eugenol. Moderate training was in the range of 50-60% VO_2max, including 5 training sessions per week (treadmill). Poisoning was performed with CPF poison with a dose of 3 mg/kg, and the dose of eugenol was determined to be 250 mg/kg.

Results: There was no significant difference between the groups in terms of mTOR and AKT expression ($P=0.369$, $P=0.59$). However, the expression of PI3k in the poisoned control group was lower than that in the healthy control group ($P=0.049$). In addition, the expression of PI3k was higher in the poisoned + eugenol + exercise group compared to the poisoned control group ($P=0.009$). The corn solvent group also had a higher PI3k expression in comparison to the poisoned control group ($P=0.025$). Finally, there was no significant difference among the other groups.

Conclusion: In general, 4 weeks of CPF poisoning caused a significant decrease in PI3K, but it did not have a significant effect on AKT and mTOR. Based on the finding, 4 aerobic exercises and eugenol consumption could significantly increase in PI3K, while it had no significant effect on AKT and mTOR.

Keywords: Pesticide, Aerobic exercise, Chlorpyrifos, PI3K/AKT/mTOR

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Introduction

The use of pesticides and the possibility of their entrance into the human diet have caused many concerns. Organophosphorus is a toxic compound, and different people are always exposed to it; in addition, cancer is one of its side effects (1). Chlorpyrifos (CPF) is an insecticide that is widely used in the world (2). CPF has harmful effects on various organs, including biochemical and blood parameters, the immune system, the liver, and the reproductive system (3,4). Further, studies showed that this poison increases oxidative stress (OS) in different body tissues such as the kidney and liver (4). Furthermore, exposure to this toxin causes a decrease in heart antioxidants, an increase in lipid peroxidation, an increase in heart tissue apoptosis, and heart failure (5). An increase in hepatic and oxidative enzymes due to exposure to this poison has been reported as well.

Hallal et al found that long-term exposure to CPF has a negative effect on the movement and contraction of slow and fast skeletal muscles in rats (6). In another study, it was reported that the chronic exposure of adult male rats to CPF is associated with an increase in the contractile function of the diaphragm and fatigue, which can be related to a disturbance in the catabolic and anabolic hormonal balance and/or a possible change in the excitation-contraction coupling mechanism (7). In this regard, the mammalian target of rapamycin mTORC is known as a key regulator in the control of skeletal muscle mass following contraction and hypertrophy caused by mechanical load (8). MTOR controls anabolic and catabolic signaling in the skeletal muscle, leading to the modulation of muscle hypertrophy (8). The phosphatidylinositol-3-kinases/protein kinase/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway is an intracellular signaling pathway important in cell cycle regulation. Therefore, it has a direct relationship with cell quiescence, proliferation, cancer, and longevity. The activation of PI3K phosphorylates and activates AKT and localizes it to the plasma membrane (9). Jalouli et al demonstrated that exposure to the pyrethroid-based insecticide Allethrin adversely affected follicle structure and function in mouse offspring during adulthood. In particular, Allethrin can induce excessive OS and

incomplete apoptosis related to autophagy, possibly by inactivating the PI3K/AKT/mTOR signaling pathway, and its effects may contribute to ovarian dysfunction and help fertility in female children. Based on their properties, pesticides increase OS, and OS inhibits the PI3K/AKT/mTOR signaling pathway. The inhibition of the PI3K/AKT/mTOR signaling pathway causes autophagy (10).

El-Sherbeeny et al represented that pesticides cause OS in the central nervous system, along with motor dysfunction and neurodegeneration in rodents. Reducing the activity of the PI3K/AKT/mTOR signaling pathway was effective in this case (11). Previous studies have shown the effect of regular exercise and supplementation of medicinal plants on changing the expression of genes in different tissues. Eugenol or 4-allyl-2-methoxyphenol is a molecule that exists in the essential oils of different plants, including cinnamon and cloves. Eugenol contains natural antioxidants with a phenolic structure that play an important role in protecting body tissues against free radicals (12). Research has confirmed the anti-inflammatory, anti-cancer, anti-apoptotic, antioxidant, and anti-OS effects of eugenol (12). According to studies, eugenol has an important protective effect against lipid peroxidation caused by free radicals, and its anti-inflammatory effect has been proven as well (13).

The results of Wang et al indicated that methyl eugenol reduces liver ischemia-reperfusion injury by activating PI3K/AKT signaling. However, in samples poisoned with pesticides, no clear results have been presented in this field (14). On the other hand, the effect of exercise is also highly important. Liu et al reported that regular aerobic exercise for 10 weeks (increase for the first 6 weeks, followed by constant loading for 4 weeks) increased CaMKIIβ carbonylation, Hnrnpa2b1, and modulated apoptosis through CaMK activation and phosphoinositide 3-kinase/protein kinase B/m (15). Kang and Cho found that treadmill exercise improves PI3K/AKT phosphorylation in the cerebral cortex of NSE/htau23 transgenic mice, ameliorating abnormal autophagy by reducing the expression level of mTOR, which controls autophagy activity (16). However, no clear results have been reported about the effect of exercise on samples affected by pesticides. It seems that exercise combined with eugenol has better effects on the PI3K/AKT/mTOR signaling pathway and improves muscle function. Therefore, this research sought to investigate the effect of aerobic training and eugenol supplementation on the AKT/mTOR/PI3K pathway on the skeletal muscle of male rats poisoned with CPF.

Materials and Methods

As mentioned earlier, the current study evaluated the effect of aerobic exercise and eugenol supplementation on the Pi3k/AKT/mTOR pathway in the quadriceps muscle tissues of rats poisoned with CPF. Considering that this study is in the category of interventional studies and the independent variables are manipulated to examine its effect on the dependent variables, it is included in the category of experimental studies. Moreover, the study is experimental and of the first category because the studied population included laboratory mice and all environmental confounding factors were under control.

Animal caring

All the rats were transferred to the animal house of Islamic Azad University, Central Tehran Branch, after being prepared by the Pasteur Institute of Iran. To adapt the animals to the zoo environment and control the disturbing factors, the rats were settled in the environment for 2 weeks before the start of the project.

The zoo environment was regulated and controlled with the following standards.

1. Setting the relative humidity of 50% ± 10%
2. Setting the lighting cycle with a special timer in the form of 12 hours of darkness and 12 hours of light.
3. Setting the temperature at 3 ± 23
4. Using ventilation to remove the unpleasant smell by silent ventilator
5. Keeping 3 rats in each cage for rodents with transparent polycarbonate (dimensions 42*15/26*5)
6. Providing free access to municipal water and food for laboratory mice (plates)

Female rats (rats), 12 weeks old and weighing between 180 and 220 grams were used in this study. All rats were healthy. They were obtained from the Pasteur Institute of Iran and randomly divided into 8 groups of 8 each, including poisoned control, health control, poisoned + exercise, poisoned + eugenol, poisoned + Eugenol + exercise, corn oil solvent, and poison solvent.

Poisoning protocol

Poisoning was performed with CPF poison prepared by a reputable company with a dose of 3 mg/kg. Dimethyl sulfoxide solvent and 9% normal saline were used for dilution and injection. After dissolving, the solution was completely homogenized using a sonicator and intraperitoneal injection. The duration of exposure in the intervention groups was 4 weeks. There were 5 injections during the week and 2 days of rest.

Aerobic exercise protocol

The training was performed for 4 weeks, and for the adaptation of the rats, for further 2 weeks before the start of familiarization exercises with a treadmill for rodents for 20 minutes at a speed of 9 meters per minute. The intensity and duration of training from the first day to the last day were as follows:

The exercise protocol was performed as moderate intensity exercise in the range of 50-60% VO_2max, which included 5 exercise sessions per week (treadmill). The warm-up lasted 5 minutes, followed by 20 minutes of the main training activity and 5 minutes of cool-down. Initially, the speed training started at 16 m/s, and according to the protocol, it increased every week until it

reached 26 m/s on the last day after 4 weeks (17).

Complementary protocol

The supplement used in this plan was eugenol (the active substance of cloves). The corn oil solvent was employed to dilute the supplement based on previous studies.

Based on the study of Singh et al, the selected dose was determined as 250 mg/kg and was fed by gavage to the rats in the supplement group for 4 weeks and 5 days a week.

Tissue removal

In this method, 48 hours after the last intervention, all rats fasted for 8-10 hours, and weight was taken before tissue removal. Anesthesia in this method is by injection and in the long term due to the time-consuming perfusion process and the need to keep the heart beating until the last stage.

The anesthetic drug was a combination of ketamine 10% and xylazine 2%, and the selected dose for ketamine was 100 mg/kg and xylazine 10 mg/kg.

Polymerase chain reaction method

The quantitative polymerase chain reaction (qPCR) method was applied to investigate the expression of genes in the heart tissue. In this study, the reference gene Gapdh was utilized as a control gene, and the expression of other genes was compared with it. To perform this technique, first, the primer design was performed, and then total RNA was extracted from the tissues and converted into complementary (cDNA). Then, the cDNA was amplified by the PCR and analyzed for the expression of the mentioned genes.

RNA extraction was performed by a manual method using Trizol material prepared by Kiazist Company and according to the existing standard protocol for the Trizol method.

The synthesis of cDNAs was performed using a Parstous cDNA synthesis kit (Parstous, Mashhad, Iran, Catalog No. A101161). Furthermore, the primers were designed with Gene Runner, version 6.5.

In addition, the PCR method was performed using Korea's BioFACT kit: 2X Real-Time PCR Master Mix (including SYBR Green, High ROX; cat no. DQ385-40h, Table 1). The results were expressed based on ΔΔCT and considering the *GAPDH* control gene.

Statistical analysis

In this research, the Shapiro-Wilk test was used to check the normality of the data distribution. After determining the normality of the data distribution, a one-way analysis of variance (ANOVA) test and Tukey's post hoc test were employed to check the difference between groups and to determine the place of difference between groups, respectively. All analyzes were performed using SPSS software (version 22) and at the level of $P<0.05$.

Results

ANOVA test results showed that there is no significant difference between the groups in terms of mTOR expression ($P=0.369$, Figure 1 and Table 1). Moreover, as regards AKT expression, no significant difference was found between the groups ($P=0.59$, Figure 2 and Table 2).

Based on ANOVA test results, there was a significant difference between the groups in terms of PI3K expression ($P=0.002$). The expression of PI3k in the poisoned control group was lower than that in the healthy control group ($P=0.049$). Additionally, the expression of PI3k was higher in the poisoned + eugenol + exercise group compared to the poisoned control group ($P=0.009$). The corn solvent group also had a higher PI3k expression in comparison to the poisoned control group ($P=0.025$). There was no significant difference among the other groups (Figure 3 and Tables 2 and 3). Similarly, there was no significant difference in PI3k among the other groups.

Table 1. Primers

	Sequence
mTOR-F	ACTATAGAACCACATGCCACAC
mTOR-R	TGTCCATCAGCCTCCAATTC
r-Akt-f	TGTGGGAAGATGTGTATGAGAA
r-Akt-r	TTGATGAGGCGGTGTGATGGTGA
rPik3r1 F	TTAAACGCGAAGGCAACGA
rPik3r 1R	CAGTCTCCTCCTGCTGTCGAT
GAPDH_F	AACCCATCACCATCTTCCAG
GAPDH_R	CCAGTAGACTCCACGACATAC

Table 2. ANOVA Results for All Makers

Markers	mTOR	AKT	PI3k
Poisoned control	0.012735033 ± 0.007325639	2.96124E-06 ± 3.14689E-06	0.005154423 ± 0.006827594
Health control	0.01469499 ± 0.006596115	1.41509E-05 ± 1.2733E-05	0.010520599 ± 0.006408335
Poisoned + exercise	0.018743195 ± 0.005321472	1.40102E-05 ± 1.66401E-05	0.022032523 ± 0.019346766
Poisoned + eugenol	0.014551187 ± 0.00639053	8.34679E-06 ± 8.04565E-06	0.017627421 ± 0.017627421
Poisoned + Eugenol + exercise	0.022593117 ± 0.018812252	2.216E-05 ± 3.49743E-05	0.039691566 ± 0.019009668
Corn oil solvent	0.023516901 ± 0.008256935	1.67797E-05 ± 1.38927E-05	0.036458477 ± 0.025211589
Poison solvent	0.020427147 ± 0.009288628	1.35485E-05 ± 1.00156E-05	0.013904128 ± 0.009367601
P	0.369	0.599	0.002

Note. ANOVA: Analysis of variance; mTOR: Mammalian target of rapamycin; AKT: Protein kinase; Pi3K: Phosphatidylinositol-3-kinases.

Table 3. Post Hoc Test Result for PI3K

Group	Group	Mean	SD	*P*
Poisoned control	Health control	-0.00537	0.00888	0.99
	Poisoned + exercise	-0.01688	0.00888	0.99
	Poisoned + eugenol	-0.01247	0.00888	0.99
	Poisoned + eugenol + exercise	-0.03454*	0.00888	0.009
Poisoned + exercise	Poisoned + eugenol	0.00441	0.00888	0.99
	Poisoned + eugenol + exercise	-0.01766	0.00888	0.99
Poisoned + eugenol	Poisoned control	0.01247	0.00888	0.99
	Health control	0.00711	0.00888	0.99
	Poisoned + exercise	-0.00441	0.00888	0.99
	Poisoned + eugenol + exercise	-0.02206	0.00888	0.376
Poisoned + Eugenol + exercise	Poisoned control	0.03454*	0.00888	0.009
	Health control	0.02917*	0.00888	0.049
	Poisoned + exercise	0.01766	0.00888	0.99
	Poisoned + eugenol	0.02206	0.00888	0.376
Corn oil solvent	Poisoned control	0.03130*	0.00888	0.025
	Health control	0.02594	0.00888	0.127
	Poisoned + exercise	0.01443	0.00888	0.99
	Poisoned + eugenol	0.01883	0.00888	0.863
	Poisoned + eugenol + exercise	-0.00323	0.00888	0.99
	Poison solvent	0.02255	0.00888	0.329
Poison solvent	Poisoned control	0.00875	0.00888	0.99
	Health control	0.00338	0.00888	0.99
	Poisoned + exercise	-0.00813	0.00888	0.99
	Poisoned + eugenol	-0.00372	0.00888	0.99
	Poisoned + eugenol + exercise	-0.02579	0.00888	0.133
	Corn oil solvent	-0.02255	0.00888	0.329

Note. Pi3K: Phosphatidylinositol-3-kinases; SD: Standard deviation.

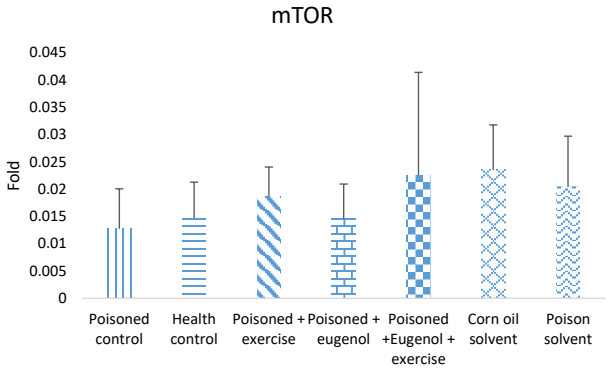

Figure 1. mTOR Expression in All Groups. *Note*. mTOR: Mammalian target of rapamycin.

Discussion

The present research findings demonstrated that the use of CPF pesticide decreased PI3K in the muscle tissue of rats, which is in line with the result of Jalouli et al. PI3K inhibition was reduced in their research (10). These findings suggest that the inhibition of PI3K may be involved in CPF-induced autophagy, and this process may be regulated by increased OS (10). Zhu et al also found that reactive oxygen species accumulation contributes to pesticide-induced apoptosis and autophagy through the inactivation of the PI3K pathway in TM3 Leydig cells (18). On the other hand, our results revealed that aerobic exercise combined with eugenol supplementation increased PI3k in poisoned rats. Wu et al concluded that the non-exercise group displayed significantly lower phosphorylation of AKT, but exercise activates PI3K by decreasing the expression of 5α reductase type 1 in PCOS mice (17). Wang et al investigated the effects of different intensity exercises and pesticide exposure on insulin receptor substance/PI3K/AKT signaling pathways. They reported that pesticides decreased PI3K signaling pathway activity, but high-intensity exercise increased it (19).

Exercise and eugenol supplementation appear to affect PI3K in several ways. Pesticides cause OS (20). OS can inhibit or decrease PI3K activity (21). Therefore, it can be considered that aerobic exercise and eugenol supplementation have increased the expression of PI3K in the muscle tissue by increasing antioxidants and reducing OS (21-24). Further, exercise through insulin-like growth factor-1 can also affect PI3K (25).

On the other hand, our results showed that although poisoning the rats decreased the expression of AKT and mTOR in the muscle tissue of the rats, these changes were not significant. It seems that the short duration of

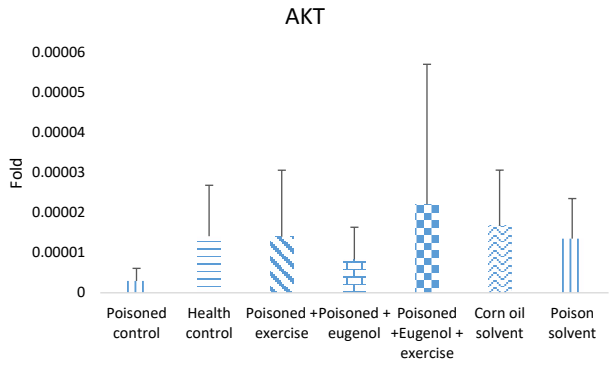

Figure 2. AKT Expression in All Groups. *Note*. AKT: Protein kinase.

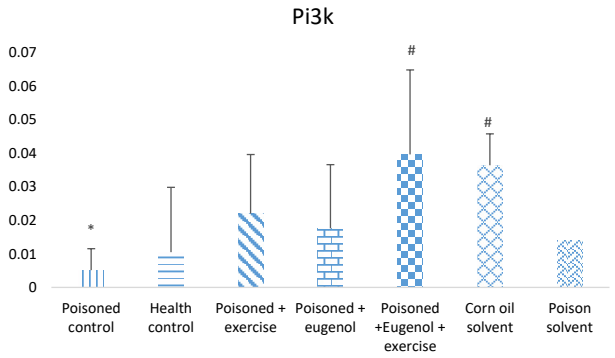

Figure 3. PI3K Expression in All Groups. *Note*. Pi3K: Phosphatidylinositol-3-kinases; * Significant compared to health control; # Significant in comparison to poisoned control.

the research period is one of the factors affecting this process. Furthermore, the amount and dose of CPF can be considered effective in the absence of significant changes in AKT and mTOR in the poisoned control group. Moreover, our results demonstrated that exercise and eugenol supplementation increased AKT and mTOR in interaction with each other, but these changes were not significant either. Considering that no similar research has focused on the effect of exercise and eugenol on the expression of AKT and mTOR in samples poisoned with CPF, it is impossible to refer to consistent or inconsistent results. Aghaei Bahman Beglou et al reported a significant change regarding the effect of intermittent exercise on the expression of AKT and mTOR in the heart tissue of diabetic samples (26). However, Kang and Cho found increased expression of AKT and mTOR (16). Sherafati Moghadam et al concluded that 4 weeks of intermittent training significantly increased mTOR in the soleus muscle of diabetic rats, but it did not cause a significant change in AKT. They found other signaling pathways effective in increasing mTOR (27).

Conclusion

According to our findings, CPF poisoning only caused a significant decrease in PI3K while no significant change in the expression of AKT and mTOR in the skeletal muscle of male rats. It seems that there was not enough time to induce the effects of CPF. Moreover, exercise and eugenol only increased PI3K, but had no significant effect on AKT and mTOR expression. In this case, short training time or adequate training intensity and eugenol dosage can be considered effective.

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Authors' Contribution

Conceptualization: Mohadeseh Behnam Moghadam.
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Writing–review & editing: Mohammad Ali Azarbayjani, Hasan Matinhomaee.

Competing Interests

The authors have no conflict of interest.

Ethical Approval

This article has been approved by the Ethics Committee of the Islamic Azad University Marvdasht Branch with the code IR.IAU.M.REC.1401.018.

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