Research Article

Effects of Long Exposure to Low Dose of Chlorpyrifos on Total Cholesterol Levels in Wistar Rats

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Abstract

Prolonged exposure to chlorpyrifos, a commonly used pesticide, can lead to pesticide residues on fruits and alter metabolic processes in the body. This study aims to examine the effect of prolonged low dose chlorpyrifos exposure on total cholesterol levels in wistar rats. A posttest-only randomized control group design was employed. 30 wistar rats were assigned to one of five groups: a normal control group (KN) and four groups exposed to low dose chlorpyrifos for 7 days (K1), 14 days (K2), 28 days (K3), and 56 days (K4). This research was conducted from February to May 2023 at the Faculty of Medicine, University of Jember. The results from the LSD post hoc test show significant differences between the normal control group and the K1 and K2 exposure groups. Moreover, significant differences were observed between the K3 exposure group and the K1 and K2 exposure groups, as well as between the K3 exposure group and the K2 exposure groups. In conclusion, exposure to low chlorpyrifos doses significantly increases total cholesterol levels at 7 and 14 days, followed by a decrease at 28 days and a subsequent increase at 56 days of exposure.
Keywords: *Insecticide, pesticide, organophosphate, cardiovascular disease.*

Efek Pajanan Lama Klorpirifos Dosis Rendah terhadap Kadar Kolesterol Total Tikus Wistar

Abstrak

Pajanan klorpirifos, pestitida yang umum digunakan dalam jangka waktu lama dapat meninggalkan residu pestisida di buah dan menyebabkan perubahan metabolisme tubuh. Penelitian ini bertujuan untuk menguji pengaruh pajanan klorpirifos dosis rendah dalam waktu lama terhadap kadar kolesterol total pada tikus wistar menggunakan desain penelitian kelompok kontrol acak posttest-only. Sebanyak 30 ekor tikus wistar digunakan dan dibagi menjadi lima kelompok yaitu kelompok kontrol normal (KN) dan empat kelompok yang diberi klorpirifos dosis rendah selama 7 hari (K1), 14 hari (K2), 28 hari (K3), dan 56 hari (K4). Penelitian ini dilaksanakan pada bulan Februari sampai Mei 2023. Hasil uji LSD post hoc menunjukkan perbedaan bermakna antara kelompok kontrol normal dengan kelompok pajanan K1 dan K2. Selain itu, didapatkan perbedaan bermakna antara kelompok pajanan K3 dengan kelompok pajanan K1 dan K2, serta kelompok pajanan K3 dengan kelompok pajanan K2. Disimpulkan pajanan klorpirifos dosis rendah meningkatkan kadar kolesterol total secara bermakna pada pajanan 7 hari dan 14 hari, diikuti dengan penurunan pada 28 hari dan peningkatan kembali pada 56 hari pajanan.
Kata kunci: *insektisida, pestisida, organofosfat, penyakit kardiovaskular.*

Introduction

Chlorpyrifos is ranked first as the most widely used organophosphate in the world at 40%, according to the Environmental Protection Agency (EPA).[1] Chlorpyrifos in food has been banned in the European Union and the USA in 2020. Yet, it remains the leading choice of organophosphates by farmers in developing countries, including Indonesia. Based on data from the Ministry of Agriculture of the Republic of Indonesia, chlorpyrifos ranks first as the active ingredient for the most organophosphate trademarks that are 44 brands out of 168 registered brands (26.19%).[2,3] The repeated use of chlorpyrifos leaves a lot of pesticide residue in the fruit and vegetable products that people usually consume. A research study of 315 samples of agricultural products concluded that pesticide residues are found in 47% of fresh product samples and 7% of processed food samples.[4] In addition, the European Food Safety Authority (EFSA) stated that chlorpyrifos residue in agricultural products often exceeds the maximum residue limit (MRL), which has been set as 1 mg/kg (1/150 LD50).[5]

Clinical and epidemiological studies show that exposure to low dose chlorpyrifos residues in humans causes chronic degenerative diseases, such as coronary heart disease and stroke, which are the highest causes of death in Indonesia. Chlorpyrifos residue causes health problems due to its effect on forming reactive oxidative species (ROS). ROS accumulation triggers oxidative stress conditions, resulting in several changes in lipid metabolism due to an increase in the hormones cortisol and epinephrine. Changes in lipid metabolism led to increased lipolysis of fat deposits in adipose tissue, which increases total cholesterol levels.[6-8]

Research by Kondakala et al,[9] showed that acute exposure to low chlorpyrifos doses (2 mg/kg) for 12 hours, combined with a normal diet, increases triglyceride levels in wistar rats. However, a study by Uchendu et al[10] found that subacute exposure to high doses of chlorpyrifos (8.5 mg/kg) for 21 days does not significantly change total cholesterol levels. In contrast, a study by Elsharkawy et al[11] reported that subchronic exposure to high doses of chlorpyrifos (30 mg/kg), probed weekly for 90 days, shows total cholesterol and triglyceride levels increase. Research on the effects of low dose chlorpyrifos exposure on total cholesterol levels remains limited and yields inconsistent results, highlighting the need for further time series studies. This study aims to address this gap by conducting a time series analysis to standardize dosing and monitor total cholesterol levels at regular intervals after low dose chlorpyrifos exposur.

Methods

Research Design

This research used a posttest-only randomized control group design. This study was conducted from February to May 2023 at the Animal House, Pharmacology Laboratory, and Biochemistry Laboratory, Faculty of Medicine, University of Jember. A total of 30 male wistar rats (*Rattus norvegicus*), aged 2–3 months with body weights ranging from 120-170 g were used. The rats were divided into five groups: one control group (KN) and four treatment groups based on exposure duration. The control group was given 5 mL/kg BW of normal saline solution (+5% tween20) orally via a gastric tube for 56 days. Meanwhile, the treatment group was administered chlorpyrifos 5 mg/kg (1/30 LD50) orally by gastric tube for 7 days (K1), 14 days (K2), 28 days (K3), and 56 days (K4). Low doses of chlorpyrifos were given to simulate the amount of residues typically found in vegetables and fruits consumed by humans, as chlorpyrifos is commonly used in agriculture to control pests in these crops.[12] According to a study by Noushi[13], a low dose is 1/30 of the LD50. The oral LD50 of chlorpyrifos in rats was reported to be 150 mg/kg BW. Based on this, a low dose of chlorpyrifos is 5 mg/kg BW.[12-14]

Material and Preparation of Chlorpyrifos Solution

This study used chlorpyrifos Pestanal analytical standard (Sigma Aldrich), standard saline, tween20, aquadest, CHOD-PAP reagent, standard solution, and 30 male wistar rats. The standard analytical chlorpyrifos (Sigma Aldrich) was weighed 150 grams, then dissolved with 5 ml Tween20, and 1000 ml normal saline was added. The mixture was then thoroughly homogenized using a hot plate magnetic stirrer.

Termination and Measurement of Total Cholesterol Levels

After the treatment, the rats were euthanized through an intraperitoneal injection of pentobarbital at a dose of 200 mg/kg BW. Blood was collected intracardially from the atrium and subsequently centrifuged to obtain serum. The serum was then used to measure total cholesterol levels using the CHOD-PAP (cholesterol oxidase-para aminophenazone) method. A total of 1000 µl of CHOD-PAP reagent, 10 µl of Wistar rat serum,

and 10 µl of standard solution were added to each labeled microtube and then homogenized using a vortex. The mixture was incubated for 10 minutes at 20–25°C. Cholesterol is oxidized to produce peroxide which is then colored with four amino antipyrine and phenol to form quinoneimine which is purple red. Furthermore, readings are carried out using a digital spectrophotometer with a wavelength of 500 nm and units of mg/dL.

Data Analysis and Ethical Approval

The total cholesterol level data were analyzed using one-way ANOVA with a 95% confidence level (α=0.05), followed by the LSD post hoc test to identify significant differences between groups. This study obtained ethical approval from the Ethics Commission of the Faculty of Medicine, University of Jember (ethical approval number 1751/H25.1.11/KE/2023).

Results

Total cholesterol levels were measured using the CHOD-PAP method, with readings taken on a digital spectrophotometer at a wavelength of 500 nm, expressed in mg/dL. The average total cholesterol levels showed a gradual increase from the normal control group (47.978±4.985 mg/dL) to the 7-day (64.665±7.515 mg/dL) and 14-day exposure groups (67.147±12.57 mg/dL), followed by a decrease in the 28-day exposure group (39.483±15.562 mg/dL), and then an increase again in the 56-day exposure group (54.573±7.482 mg/dL).

The average total cholesterol levels showed a gradual increase from the normal control group to the 7-day and 14-day exposure groups, followed by a decrease in the 28-day exposure group, and then an increase again in the 56-day exposure group. The detailed average cholesterol levels and corresponding serial graphs are presented in Figure 1.

The results of the normality and homogeneity tests indicated that the data were normally distributed ($p>0.05$). Subsequently, the data were analyzed using one-way ANOVA, yielding a $p<0.001$, indicating a significant difference in total cholesterol levels between at least two groups. The analysis was further refined with the LSD post hoc test to identify the specific groups with significant differences ($p<0.05$).

The LSD post hoc test revealed a significant difference between the normal control group and the groups exposed to chlorpyrifos for 7 days ($p=0.010$) and 14 days ($p=0.004$). Moreover, significant differences were observed between the 28-day exposure group compared to the 7-day and 14-day exposure groups ($p<0.001$), as well as between the 56-day exposure group compared to the 14-day ($p=0.046$) and 28-day exposure groups ($p=0.018$). Detailed results of the LSD post hoc test are presented in Table 1.

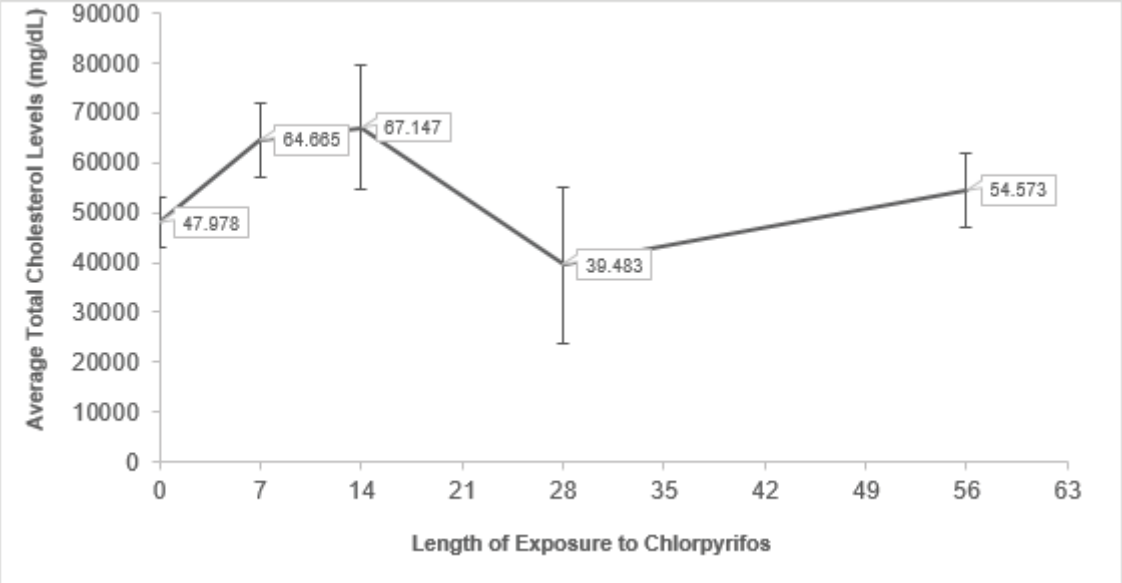

Figure 1. Serial Graph of Total Cholesterol Levels of the Control and Chlorpyrifos Exposure Groups

Table 1. Post Hoct Test Result for Total Cholesterol Levels

Group	Control (KN)	Seven Days (K1)	14 Days (K2)	28 Days (K3)	56 Days (K4)
Control (KN)	NC	0.010*	0.004*	0.168	0.281
Seven days (K1)	0.010*	NC	0.682	0.000*	0.104
14 days (K2)	0.004*	0.682	NC	0.000*	0.046*
28 days (K3)	0.168	0.000*	0.000*	NC	0.018*
56 days (K4)	0.281	0.104	0.046*	0.018*	NC

*significantly different (p<0.05); NC:not compared; KN; control group

Discussion

The total cholesterol levels of all treatment groups exposed to a low dose of 5 mg/kg BW chlorpyrifos showed an increase on 7-day (acute) and 14-day (subacute), followed by a decrease on the 28-day (subchronic), and a subsequent increase on the 56-day (subchronic). Total cholesterol levels increased significantly in the 7-day and 14-day exposure groups compared to the normal control group, with levels of 64.665 ±7.515 mg/dL and 67.147±12.570 mg/dL, respectively.

When chlorpyrifos enters the human body, it is metabolized in the liver by cytochrome P450 enzymes into its bioactive form, chlorpyrifos-oxon (CPO). The imbalance between ROS production and levels of enzymatic antioxidants in mitochondria leads to oxidative stress in microglia, which are stress-responsive cells in the central nervous system. This oxidative stress activates the HPA axis and the sympathetic nervous system. Activation of the HPA axis increases cortisol binding to its receptors in adipose tissue, while sympathetic nervous system stimulation enhances hormone-sensitive lipase activity through elevated epinephrine secretion. Together, these mechanisms promote lipolysis of triglyceride reserve fat deposits in adipose tissue and trigger an increase in total cholesterol levels.[16,17] The findings are consistent with research by Kondakala et al[9] who demonstrated that giving wistar rats acute exposure to low doses of 2 mg/kg BW of chlorpyrifos for 12 hours significantly increases total cholesterol levels. In addition, a study by Tanvir et al[18] shows subacute exposure to low doses of 5 mg/kg BW for 21 days significantly increases total cholesterol levels in wistar rats.

Total cholesterol levels in the 28-day subchronic exposure group did not significantly increase compared to the control group. In fact, the average cholesterol level in this group decreased significantly compared to the 7-day and 14-day exposure groups. A potential explanation for this reduction is the negative feedback mechanism of the anterior pituitary gland, which may reduce adrenocorticotropic hormone (ACTH) levels, leading to decreased cortisol secretion. This negative feedback likely inhibits ACTH secretion by the anterior pituitary, causing shrinkage of the fasciculata layer in the adrenal cortex. The shrinkage occurs due to the loss of the function of ACTH as a growth stimulator and secretion of the zone so that the secretion of the hormone cortisol decreases. Negative feedback by the anterior pituitary also inhibits CRH expression in the PVN, leading to the decrease of CRH-initiated ACTH secretion.[19] Moreover, the decrease in the synthesis and secretion of the hormone cortisol in the adrenal cortex causes a decrease in the lipolysis of fat deposits in adipose tissue so that total cholesterol levels also decrease. Research conducted by Zhang et al[20] proved that giving zebrafish organophosphate monocrotophos at 100 µg/L for 14 days can increase plasma cortisol levels in fish, but these levels decrease on day 21. Another study by Ibrahim et al[21] found that exposure to low dose chlorpyrifos of 0.5 mg/L in freshwater fish increases total cholesterol levels on days 7, 14, and 21 but decreases on days 28. Although it shows an increase in total cholesterol levels on day 21, the percentage of increased total cholesterol levels decreases when compared to days 7 (221.9%), 14 (83.6%), and 21 (19.8%).

In this study, following a decrease in total cholesterol levels at 28 days of subchronic exposure, a significant increase was observed at 56 days of exposure. The average total cholesterol level of the 56-day exposure group was 54.573±7.482 mg/dL, which exceeds the upper limit of the normal total cholesterol level of the wistar rats (10−54 mg/dL). These findings indicate that total cholesterol levels increase gradually, despite the prior decrease. The reincrease in total cholesterol levels is caused by a rise in ACTH secretion, as sufficient amounts of ACTH are stored in the secretory granules. The

temporary inhibition of ACTH secretion allows the anterior pituitary time to synthesize and store ACTH, a process that can vary from hours to days depending on the activity of each individual's HPA axis, ACTH levels, and cortisol, which are influenced by genetic factors.[22,23]

Research by Rich et al[24] on European starlings subjected to stressors every 30-45 minutes per day for three weeks showed a decrease in cortisol levels on the day 20, followed by a gradual increase thereafter. However, a study by Fang et al.[25] showed that exposure to a low dose (3 mg/kg BW) of chlorpyrifos in wistar rats for nine weeks did not significantly increase total cholesterol levels, though the levels were still higher than the control group. These results are caused by increased adipocyte cell size and a surge in hormone progesterone levels during pregnancy, which causes cholesterol synthesis to increase.[26]

Increased total cholesterol levels are associated with an increased risk of cardiovascular disease, such as atherosclerosis. Although the 28-day (K3) and 56-day (K4) subchronic chlorpyrifos exposure group did not significantly increase total cholesterol levels, it does not mean that the wistar rats are free from atherosclerotic plaque formation in the arteries. The imbalance between ROS production and the antioxidant defence system leads to decreased nitric oxide (NO) bioavailability, impaired vascular tone, and other collective endothelial changes that trigger endothelial dysfunction in arteries. Endothelial dysfunction allows LDL to attach and initiate the atherosclerotic process to form atheromatous plaques. Moreover, research by Progression of Early Subclinical Atherosclerosis (PESA) revealed that 49.7% of 740 participants, all of whom were free from risk factors for cardiovascular disease, were found to have subclinical atherosclerotic plaques. However, lipid profile assessment alone cannot be relied upon to determine the presence or extent of atherosclerosis, as they serve only as indicators of cardiovascular disease risk.[27,28]

A limitation of this study is the absence of a treatment group exposed to chlorpyrifos at 5 mg/kg BW for a chronic duration (≥90 days). A research by Yano et al,[29] which involved administering 5 mg/kg BW of chlorpyrifos to wistar rats for 13 weeks, revealed histopathological changes, including vacuolation of parenchymal cells and lipid accumulation in the zona fasciculata of the adrenal cortex. The lipid accumulation in the adrenal cortex shows that the adrenal glands are overworked, leading to increased cortisol production and secretion. Therefore, further studies are needed to examine the long-term effects of chronic exposure to chlorpyrifos to better understand the long-term impact of low dose chlorpyrifos exposure on total cholesterol levels.

Conclusion

Low dose chlorpyrifos exposure exerts a prolonged effect on total cholesterol levels in wistar rats during both acute and subacute phases. Acute exposure for 7 days and subacute exposure for 14 days significantly elevated total cholesterol levels. However, levels decreased during 28 days of subchronic exposure, followed by an increase at 56 days of subchronic exposure.

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