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RISK ASSESSMENT OF CHLORPYRIFOS-METHYL USING 21-DAY SUBACUTE DIETARY STUDY IN THE JAPANESE QUAIL (*Coturnix coturnix japonica)*

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ABSTRACT: *The risk assessment of the organophosphate pesticide, chlorpyrifos-methyl (CPM), was conducted to determine the probability of adverse effects occurring from exposure to CPM in the Japanese quail (Coturnix japonica). The quail were exposed to sublethal levels, 1/10, 1/100 and 1/1000 LC_{50} of CPM, for 21-day dietary toxicity test, based on OECD workshop report. At 1/10 LC_{50}, quail body weight was significantly decreased by 22.7% than that of the control. However, absolute weight of liver from quail exposed to 1/10 and 1/100 LC_{50} showed significant increases than that of the control. Kidney weight was also increased at the lower dose, 1/1000 LC_{50}, while showed a significant decrease at the higher dose, 1/10 LC_{50}. Data on hepatic function showed increased levels of ALT and ALP at concentrations of 1/10 and 1/100 LC_{50}. Total protein levels were also increased at these doses. In kidney function parameters, data showed an obvious increase in concentrations of uric acid, at 1/10 and 1/100 LC_{50} without significant change in creatinine level due to pesticide exposure. The data obtained verified the toxic hazard of chlorpyrifos methyl, at concentration used, on Japanese quail that could be an excellent bird model for monitoring the toxicological risks of pesticides in Egypt.*

Key words: *Dietary toxicity test; Japanese quail; Chlorpyrifos-methyl*

INTRODUCTION

The organophosphorus pesticide (OP) chlorpyrifos-methyl [O,O-dimethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is widely used in agriculture, horticulture and in the home worldwide. This compound is registered in 1985 for use on stored grain for protection of stored food, feed oil, and seed grains against injury from stored grain weevils, moths, borers, beetles and mealworms including granary weevil, rice weevil, red flour beetle, confused flour beetle, lessor grain borers, seed treatment, grain bin and warehouse. Even though OP pesticides, including chlorpyrifos-methyl, degrade rapidly in the environment, they may exert lethal and sublethal effects on birds immediately after field application (White *et al.,* 1982).

According to US EPA categories, chlorpyrifos-methyl belongs to the category III, moderately toxic group (acute toxicity in rats, oral LD50 is 2814 mg/kg b.w.) (US EPA, 2002). Toxicity of the pesticide to birds is moderate. Considering both the acute (technical: LD50=923 mg/kg b.w.; formulation: LD50= 227 mg/kg b.w.) and dietary toxicity (LC50=2010 ppm) to the bobwhite quail, *Colinus virginianus*, it appears that the quail is one of the more sensitive avian species that has been extensively evaluated (Smith, 1987; Hudson *et al.,* 1984; Johnson *et al.,* 2001). The Japanese quail, *Coturnix japonica*, were chosen as a test species in order to detect and evaluate the toxicities of the OPs, isazofos and pyraclofos, in Korea, because they are widespread in both the forests and countryside, are small enough to handle, and are easily obtained from local suppliers (Seok *et al.,* 2008).

Field effects of chlorpyrifos on birds are not at all decisive. For this reason, additional wildlife studies on chlorpyrifos have been strongly recommended (Smith, 1987). Few studies aiming at a better understanding of the impact of chlorpyrifos-methyl in birds have been conducted. Booth *et al.* (2005)

concluded in their studies that application of chlorpyrifos to turf at 3.4 and 6.7 kg ai/ha is not expected to have chronic deleterious effects on populations of the bobwhite quail grazing on treated grass or seeds, provided there is an abundant supply of seeds for the quail to eat.

Accordingly, the objectives of the present study were to verify the toxic hazard of chlorpyrifos-methyl on Japanese quail and to provide an additional data for quail using the 21-day dietary toxicity test that is based on the OECD workshop report and EPA guidelines (US EPA, 1996; OECD, 1996). In access of guideline requirements, liver and kidney function parameters, beside serum steroid hormones were investigated.

MATERIALS AND METHODS

Animals and animal husbandry

Male and female, 6-week-old Japanese quail (*C. coturnix japonica*) weighting 130-150 g were obtained from a local supplier (Poultry farm, Sakha, Kafr-Elsheikh, Egypt). The birds were allowed to adapt to the laboratory conditions for a period of 2 weeks before the experiment commenced. The quail were kept at room temperature (25±2 °C), 60±10%,relative humidity, and received ~ 10-h light/14-h dark cycles. The birds received commercial layer feed obtained from commercial local supplier and tap water ad libitum. The birds were divided into equal four groups with 4 males and 4 females for each group. The experimental groups I-III were received sublethal doses of chlorpyrifos-methyl, where group IV was fed on untreated diet and served as the control. Diets were prepared by dissolving chlorpyrifos-methyl in corn oil, then thoroughly mixing this solution into the layer feed (7ml dissolved solution with pesticide in corn oil/kg feed). Diet for the control group was prepared likewise, but with only corn oil.

Test substance and dosage

Commercially formulation of chlorpyrifos-methyl [*O,O*-dimethyl *O*-3,5,6-trichloro-2-pyridylphosphorothioate], RELDAN®, 25% EC (Dow AgroSciences LLC), was used in this study for subacute avian toxicity tests.

Repeated dose 21-day subacute dietary assay

Sub-Acute dietary Toxicity tests were carried out based on procedure described by The Office of Prevention, Pesticides and Toxic Substances (OPPTS) guideline (OPPTS, 1996). In this experiment, the quail were divided into four groups with four pairs (males and females, weighing 130-150 g) for each group. The birds in groups I, II and III were exposed to diet treated with selected sublethal doses of chlorpyrifos-methyl, these doses were equal to 1/10, 1/100, and 1/1000 LC50, respectively, where the birds in group IV were exposed to diet treated only with the vehicle (corn oil) and served as the control. After treatment, each group was observed for an additional week to detect any potential recovery from the pesticide treatment. The birds were monitoring daily for clinical signs and toxic symptoms. Body weight and growth gain was also recorded every 3 days. On the 21th day of treatment, the birds were sacrificed and the organs (liver and kidney) from each animal were removed and weighed.

Blood serum biochemical parameters

Blood samples were collected by heart puncture using a syringe. Serum was obtained by centrifugation for 15 min at ~1000-2000 RCF. Concentrations of total protein, alkaline phosphatase (ALP), transaminases (aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured in blood serum for hepatic function. To evaluate the kidney function, concentrations of uric acid and creatinine were measured. The colorimetric method was used for analysis. All chemical-kits were purchased from Alkan Pharma Co. S.A.E., Egypt, and the procedure for each assay was followed as described by the manufacturer's manual.

Statistical analysis

Data were expressed as means±S.D. The significant difference between each group was analyzed through the Duncan's multiple range test (CoStat, CoHort Software) at level $p < 0.05$..

Results

The mean body weights of quail in the groups fed on diet treated with 1/10, 1/100 and 1/1000 LC50 of chlorpyrifos-methyl significantly decreased by 22.7, 14.1 and 13.5 % than that of the untreated control group, respectively (Table 1). However, the mean weights of liver from quail in the groups fed on diet treated with 1/10 and 1/100 LC50 significantly increased (5.4 and 4.2 g, respectively) compared to the control group (3.4 g) (Table 1). Data also indicated that the relative values of liver weights to the animal body weights were significantly increased in these two groups, mentioning3.3 and 2.5 %, respectively, compared to 1.6 % in the control. It was obvious that feeding on the lower dose, 1/1000 LC50 of chlorpyrifos-methyl resulted in a significant increase in the mean weights of kidney, with 4.67 g, compared to the control, 3.4 g (Table 1). The relative value of kidney weight to the animal body weight was also increased with 2.56, compared to that of the control, 1.52 %. However, feeding on the higher dose, 1/10 LC50, decreased the mean weight of kidney, 2.25 g with a 1.38 % relative to the body weight, compared to that of the control.

Data of kidney function revealed a significant increase in uric acid levels, 8.83 and 6.98 mg/dl, in serum from the quail fed on diet treated with the higher doses, 1/10 and 1/100 LC50 of chlorpyrifos-methyl compared to 3.79 mg/dl in the control, indicating 133 and 84.2 % increase than the control, respectively (Table 2). Level of creatinine was also significantly increased in case of quail exposed to the higher dose, 1/10 LC50 (0.13 mg/dl) compared to that of the untreated control (0.10 mg/dl) indicating 30 % increase than the control. However, the levels of creatinine at dose of 1/1000 LC50 was not significantly different than the control (Table 2). The protein levels were significantly increased, 3.22, 2.59 and 2.48 IU/L, at all doses 1/10, 1/100 and 1\1000 of LC50, respectively, compared to that of the control (2.05 IU/L).

Data of liver function indicated that there was a significant increase of ALT (alanine aminotransferase) concentrations at the sublethal doses, 1/10, 1/100, and 1/1000 LC50 with 379, 365, 402 IU/L, respectively, compared to that of the control, 263 IU/L (Table 3). The levels of AST (aspartate aminotransferase) were also found to significantly decrease at the higher dose, 1/10 LC50 with 4.0 IU/L, compared to the values obtained from the control, 7.0 IU/L. The ALP (alkaline phosphatase) concentrations were found to significantly increase at the higher doses, 1/10 and 1/100 LC50, with 872 and 642 IU/L, respectively, compared to the level obtained from the control group, 541 IU/L (Table 3).

Table 1: Mean values of body weights of Japanese quail males at termination and absolute and relative liver and kidney weights after repeated feeding for 21 days on diets treated with 1/10, 1/100 and 1/1000 of LC_{50} of chlorpyrifos-methyl compared to the untreated control[a]

concentration 1/x of LC_{50} [mg/kg diet]				
	0 (n=4)	1/10 (n=4)	1/100 (n=4)	1/1000 (n=4)
Body weight at termination (g)	210.5 ± 7.6a	162.7 ± 5.7c (22.7)[b]	180.8 ± 3.5bc (14.10)[b]	182 ± 4.9bc (13.53)[b]
Liver weight (g)	3.40 ± 2.60d	5.4 ± 2.19a	4.20 ± 3.1bc	3.9± 2.17cd
Liver/body (%)	1.62 %	3.31 %	2.54 %	2.14 %
Kidney weight (g)	3.20±1.21bc	2.25±0.92d	3.15±1.86bc	4.67±2.20a
Kidney/body (%)	1.52 %	1.38%	2.74 %	2.56 %

[a]Values are means of 4 adult males for each treatment. Means followed by the same letter(s) within each row are not significantly different (Least Significant Difference (LSD) test; $P < 0.05$.

[b]Values in parentheses refer to reduction % of body weight relative to the control.

Table 2: Uric acid, creatinine and total protein levels in serum from Japanese quail males fed for 21 days on diet treated with 1/10, 1/100 and 1/1000 of LC_{50} of chlorpyrifos-methyl in comparison with the untreated control[a]

Treatment 1/x of LC_{50}	Uric acid (mg/dl)	Creatinine (mg/dl)	Total protein (IU/L)
Control	3.79 ± 2.60b	0.10 ± 0.21c	2.05 ± 0.20c
1/10	8.83 ± 2.19a	0.13 ± 0.04a	3.22 ±1.33a
1/100	6.98 ± 2.1a	0.07 ± 0.03d	2.59 ± 1.19b
1/1000	4.25 ± 2.17b	0.11± 0.05bc	2.48 ±1.08b

[a]Values are means of 4 adult males for each concentration- and control-treatments. Means± SD followed by the same letter(s) within each column are not significantly different (Least Significant Difference (LSD) test; $P < 0.05$).

Table 3: Concentrations of ALT, AST and ALP in serum from Japanese quail males fed for 21 days on diet treated with 1/10, 1/100 and 1/1000 of LC_{50} of chlorpyrifos-methyl in comparison with the untreated control[a]

Treatment 1/x of LC_{50}	**ALT (IU/L)**	**AST (IU/L)**	**ALP (IU/L)**
Control	263 ± 2.60c	7 ± 3.21a	541± 3.15c
1/10	379 ± 3.19b	4 ± 1.92b	872 ± 4.25a
1/100	365 ± 2.1cbc	6 ± 1.86a	642 ± 3.88b
1/1000	402 ± 2.71a	6 ± 2.20a	524 ± 4.25c

[a]Values are means of 4 adult males for each concentration- and control-treatments. Means± SD followed by the same letter(s) within each column are not significantly different (Least Significant Difference (LSD) test; $P < 0.05$).

Discussion

The clinical signs observed in the quqil treated by OP pesticides were apparently due to inhibition of acetylcholine esterase (AChE) resulting in accumulation of acetylcholine in the cholinergic receptors of the peripheral and central nervous systems (Eaton *et al*., 2008). The present study also showed that 21-day repeated dietary subacute exposure caused a comparatively lower body weight gain compared to the control groups, despite the fact that we did not observe an appreciable differences in the level of food consumption in between the groups. It seemed that the rate of decrease in body weight was dose- and exposure time-dependant. The 21-day LC50 of the toxicity tests in similar studies carried by Seok *et al*. (2008) on the quail indicated that OP insecticides, isazofos and pyraclofos, with dosages of 50, 100, and 200 mg/diet kg/day, resulted in significant decrease, during the 21-day test, in daily food intake with a subsequent reduction in body weight compared to the respective control group. Food consumption data were used for the estimation of threshold concentrations (100 and 200 mg/diet kg groups of isazofos- or pyraclofos treated quail) at which death occurred. The food consumption and clinical signs are important criteria and ecological attributes necessary for generating reliable data that can be used for predicting how pesticides affect quail under field conditions (Seok *et al*., 2008).

The present study also indicated that administration of CPF-M at 21 day- repeated subacute dietary treatment significantly increased the liver weights at the higher doses used, 1/10 and 1/100 LC50. However, the kidney weights were significantly reduced at the higher concentration used, 1/10, of tested pesticide and increased significantly at the lower concentration, 1/100 LD50. These findings were in agreement with results obtained from Grote

et al. (2006) who revealed an increase in liver weights in both quail sexes at the high concentration of fungicide, fentin hydroxide . The effect on liver weight in quail might be an indication of either the induction of OP pesticide metabolism as a result of increased substrate availability or minor hepatotoxic effects of pesticide on birds. Also, this result agreed with findings from previous studies (Ambali *et al*., 2007; Ambali, 2009; Mansour and Mossa, 2010). Apart from oxidative stress, CPF has been shown to inhibit the activity of cholesteryl ester hydrolase (Civen *et al*., 1977), an enzyme that is essential in response to stress. Similarly, the cholinergic stress recorded in the CPF group may have contributed to the level of stress, hence the lower body weight gain. Therefore, the comparatively low body weight changes in the CPF group may have been due to toxic stress, oxidative stress and cholinergic stress OP insecticides may cause an increase in AST enzyme activity (Gomes *et al*., 1999; Kalender *et al*., 2005). In our study, CPF-M at 1/10 and 1/100 LC50 caused a significant increase in the activities of ALT compared to that of the control. This result was in agreement with results obtained from Ambali *et al*. (2011) who revealed a significant increase in the activities of AST and ALT in the Wistar rats exposed to subchronic-CPF only signalling hepatic damage. AST is a sensitive marker of liver damage, even if the damage is of a subclinical nature (Kauppinen, 1984; Meyer and Harvey, 1998). AST is an important indicator of liver damage in clinical studies. AST was found to be secreted into the blood during hepatocellular injury (Kalender *et al*., 2005). On the other hand, ALT is a more specific marker of hepatic injury (Ballantyne, 1988) and the increase in its activity in the CPF group indicates hepatic damage. ALT and AST are enzymes produced by the hepatocytes, where they are involved in the metabolism of amino acids and synthesis of proteins. In dying or damaged cells, these enzymes leak into the blood stream (Mansour and Mossa, 2010). Changes in AST levels might differ depending on the exposure time and dose. This increase occurs in parallel to time (Seok *et al*., 2008). CPF-M administration, at 1/10 and 1/100 LC_{50} caused also a significant ALP activity compared to the enzyme activity obtained from the control group. The significant increase in ALP activity in the CPF group is also an indication of hepatotoxicity (Ambali *et al*., 2011). However, the enzyme is not liver specific as it is also associated with muscle, bones and intestines. The elevation of AST, ALT and ALP activities elicited in the CPF group indicating hepatotoxicty has been reported in previous studies (Goel *et al*., 2005; Ambali, 2009; Mansour and Mossa, 2010). The elevation in the liver enzymes activities may be due to liver dysfunction with consequent reduction in their biosynthesis and altered membrane permeability permiting enzyme leakages into the serum (Mansour and Mossa, 2010). The liver is susceptible to damage because of direct exposure to toxic products due to its role in the detoxicification of metabolic by-products and xenobiotics.

In addition, the present study revealed obviously the significant increase in serum uric acid concentrations at 1/10 and 1/100 LC50, compared to the control group. The increase in serum uric acid concentration is a demonstration of impairment of kidney function since the organ primarily excrete urea in the urine. This result agrees with findings of Ambali *et al*. (2007, 2010, and 2011). Repeated OP pesticide, chlorpyrifos exposure has been shown to cause glomeruli and tubular degeneration partly due to oxidative stress (Ambali, 2009), thereby impairing renal functions. Ambali *et al*. (2011) indicated that pretreatment with zinc resulted in a slight decrease in urea concentration indicating its apparent protective effect. This protective effect may be due to its antioxidant properties, which protect the renal tissue from lipoperoxidative changes, thereby improving renal functions.

The clinical signs, biochemical assays examination reported above are important criteria and ecological attributes necessary for generating reliable data that can be used for predicting how pesticides affect quail under field conditions. This study also confirms the utility of the OECD workshop report (OECD, 1996) in Japanses quail. In

addition, Japanese quail could be an excellent bird model for monitoring the toxicological risks of pesticides in Egypt.

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تقدير مخاطر مبيد كلوربيريفوس ميثيل في حالة دراسات التغذية شبة الحادة لمدة ٢١ يوم لطائر السمان

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الملخص العربي

أجرى تقيم مخاطر المبيد الفسفورى كلوروبيريفوس ميثيل لتقدير الآثار السيئة الناتجة عن تعريض طائر السمان حيث تم تعريض الطائر للمبيد فى صورة جرعات شبة مميتة ١٠/١ ،١٠٠/١، ١٠٠٠/١ من التركيز النصفي القاتل LC50 في اختبار سمية غذائية لمدة ٢١ يوم متتالية استنادا إلى تقرير ورشة العمل أوضحت النتائج أنة عند تركيز ١٠/١ من ال LC50 فان وزن الجسم للسمان ينخفض معنويا معنويا عن المقارنة بنسبة ٢٢.٧%عن المقارنة ،هذا بينما الوزن الكلى للكبد يزداد معنويا عن المقارنة عند التعرض (التغذية) على تركيزات ١٠٠/١٠,١/١ من ال LC50 كذلك يزداد معنويا وزن الكلية عند التركيزات المنخفضة ١٠٠٠/١ من ال LC50 بينما ينخفض معنويا مع التركيز الأعلى ١٠/١ من الLC50 هذا وقد أوضحت بيانات وظائف الكبد زيادة في مستويات ALP,ALT عند تركيزات ١٠/١ ،١٠٠/١ من ال LC50. كذلك يزداد مستوى البروتين الكلى عند هذه التركيزات بينما أظهرت نتائج وظائف الكلية زيادة واضحة في تركيز حمض اليوريك عند تركيزات ١٠/١ .١٠٠/١ من ال LC50 بدون اى تغير معنوي في مستوى الكرياتينين نتيجة التعرض للمبيد الحشري ، وتؤكد النتائج المتحصل عليها مخاطر مبيد الكلوروبيريفوس ميثيل بالتركيزات المستخدمة على طائر السمان والذي يعتبر نموذج مثالي ممتاز لتوضيح المخاطر السامة لمبيدات الآفات في مصر.