Residues in meat and serological responses of male growing white NewZealand rabbits exposed to either fresh or expired Diazinon®

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Abstract: This study was conducted to investigate the adverse effect of Diazinon on its residues in meat and some serological responses of male growing white NewZealand rabbits. Rabbits were assigned randomly to three symmetric groups (n=20). Treatments were control group G1 (dipping in tap water using the same regime); Diazinon group G2 (1.0 mg Diazinon in 1 liter of water) and a one year expired Diazinon group G3 (1.0 mg expired Diazinon in 1 liter of water). Rabbits were fed commercial pellet diet for fattening. Feeds and water were offered *ad libitum*. Furthermore, vitamins and minerals (1ml/ liter drinking water) were added. The whole body of the animals was dipped in 10 L of Diazinon solution sparing the head for 10 seconds. The dipping of the experimental rabbits was done twice at the first and the second month of the experiment. Data of Diazinon residues in the meat of the experimental rabbits showed lower values in the meat of those exposed to expired Diazinon. Results indicated that dipping the experimental rabbits using the outlined regime had deleterious effects on blood glucose level, both liver and kidney functions and the relevant oxidative biomarkers; furthermore, the exposure to the expired Diazinon was the worst on the rabbits. The present research declared the adverse effects of the exposure of the growing male rabbits to the expired Diazinon more than the fresh Diazinon itself. This research emphasis to avoid the dipping or spraying rabbit 6 months before slaughtering at least and to avoid the use of the expired Diazinon.
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1. Introduction

Diazinon is an oregano- phosphorus insecticide with widespread use in agricultural and veterinary care. Diazinon has been utilized and widely applied as insecticides, helminthicides, ascaricides, nematocides, fungicides and herbicides for 5 decades (Reece and Handson, 1982). Research outlined the harmful effects of the expired Diaznon more than diazinon itself due to the photodegradation of the compound (Ahmadi and Nahri-Niknafs, 2011). The photolysis of diazinon had shown that the main transformation products are its oxygen analogues, diethyl 2-isopropyl-6-methylpyrimidin-4-yl phosphate (diazoxon), its isomer o,o-diethyle s-(2-isopropyle-6-methylpyrimidine-4-yl) thiophosphatr (isodiazinon), and O,O-diethyle O-[2-(1-hydroxy-1-methylethyl)-6-methylpyrimidin-4-yl] thiophosphate (hydroxydiazinon). The primary environmental concerns associated with its use is beings kills, contamination of surface water, plants environment, and impacts on aquatic species (Iyer, 2010, Iyer 2001, Kabir *et al.*, 2008, Indraningsih *et al.*, 2004, Eisler, 1986 and Reece and Handson 1982). Also, the hazardous residues of it and its degradations components that could be found in either milk (El-Kholy *et al.*, 2001 and El-Kholy *et al.* 2000) or meat of farm animals (Kabir *et al.*, 2008 and Eisler, 1986). Toxic effects of Diazinon are due to the inhibition of acetyl cholinesterase. Diazinon® exerts its toxicity by binding its oxygen analog to the neuronal enzyme AChE, resulting in the accumulation of endogenous acetylcholine in nerve tissues and effectors organ (Mayer *et al.*, 1991). Furthermore, it affects mitochondrial membrane transport in rat liver and it disturbs cytochrome P450 system in human liver (Sams *et al.*, 2003 and Kappers *et al.*, 2001). Expired Diazinon is more dangerous than its fresh preparations specially under bad storage circumstances because Diazinon transformation products are more polar than the parent compound, they may be consequently more water soluble, more mobile, and more toxic for animals and human beings consequently the whole environment. Therefore, these compounds would probably be found in higher concentrations than the parent compound (Ahmadi and Nahri-Niknafs 2011). Various toxicological conditions due to diazinon exposure had been reported to cause impairment in liver and kidney functions (Grafft *et al.*, 2002).

Thus, the objectives of this study were to quantify residues of Diazinon® in meat and examine some serological responses in New Zealand male growing rabbits to their exposure to either fresh Diazinon® or a one year expired Diazinon.

2. Materials and methods

Experimental rabbits

This study was conducted in the Rabbit Research Unit, Faculty of Agriculture, and Cairo University, Egypt. Sixty male white New Zealand (NZW) rabbits aging seven months old were used in this study. The rabbits reared in metal batteries with automatic drinkers. The average ambient temperature was ranged between 20.5±0.28 to 28.25±0.13°C and relative humidity ranged between 60.00 ±3.19 to 68.50±2.9 at the rabbitry.

Experimental Ration

Rabbits were fed commercial pellet diet for fattening. The chemical composition of this total mixed ration was estimated according to A.O.A.C. (2000) as shown in table (1). Feeding allowances of the experimental rabbits were formulated according to NRC (1994). Feeds and water were offered *ad libitum*. Furthermore, vitamins and minerals (1ml/ liter drinking water) were added.

Experimental design

After two weeks of adaptation to the feeds, housing and surrounded environment, the experimental rabbits were randomly divided into 3 symmetric groups, each of twenty rabbits, according to the treatments in (Table 2). The stock of Diazinon® (obtained from Ciba Geigy co. Cairo, Egypt) was diluted in tap water to prepare Diazinon solution (1.0 mg Diazinon in 1 liter of water was utilized in G2 group). A one year expired Diazinon was used to prepare a solution (1.0 mg expired Diazinon in 1 liter of water was utilized in G3 group). Each stock is prepared freshly before the use. The LD_{50} of Diazinon in males is 250 mg/kg live body weight (Merck index). For the experimental groups (G2 and G3) the whole body of the rabbits was dipped in 10 L of the solution sparing the head for 10 seconds. The dipping of the experimental rabbits was twice at the first and the second month of the experiment. Usage of Diazinon was approved by the Animal Care Committee according to all guidelines for Purpose of supervision of Experiments on Animals.

Blood sampling

Peripheral blood samples were taken from the marginal vein of the ears. The samples were taken by a 21-gauge butterfly catheter and collected fortnightly into 5 ml Falcon tubes. Blood samples were centrifuged at room temperature using 3500 rpm for 15 minutes. The serum was carefully withdrawn and kept in a deep freezer at -20C° for further analysis (Table 3).

Residues of Diazinon in meat (ppm)

Diazinon was extracted from rabbit meat according to the method described by Toyda *et al.* (1990). Volume of 50 ml acetonitrile was added to 10gm of grinded and homogenized meat and the mixture was vigorously shaken for 10 minutes using a mechanical shaker. The acetonitrile layer was then transferred to a 300 ml Erlenmeyer flask by decantation. The residue was extracted two times with 50ml 70% acetonitrile + water and each extract was filtered through filter paper. One hundred ml water and 2 gm zinc were added to the extract. The mixture was then vigorously shaken for 10 minutes and filtered. The filtrate was added to 200ml 3% sodium chloride and 100ml dichloromethane. The dichloromethane phase was dried under anhydrous sodium sulphate for 30 minutes, then concentrated to 2.5ml and analyzed by G.C. method. The G.C. analysis was done using Hewlett Packard series II 5890 with NB detector at the nitrogen mode and with column 30m length and 320µm diameter; the homogenized samples were injected using an auto-sampler (Hewlett Packard 7613).

Serological parameters in peripheral blood serum

Glucose (mg/dl)

Serum glucose was quantified colorimetrically according to the method described by Trinder (1969). Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid.

A chromogenic oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase (POD), detects the formed hydrogen peroxide (H2O2). The intensity of the color is proportional to the glucose concentration in the sample. Glucose concentration (mg/dl) = A Sample / A Standard × 100 (Standard Conc.).

Superoxide dismutase activity (SOD, U/ml)

Superoxide dismutase (SOD) activity was determined in blood serum according to Nishikimi *et al.* (1972). Superoxide dismutases (SOD) are metalloenzymes that catalyze the dismutase of the Superoxide anion to molecular oxygen and hydrogen peroxide defense mechanism. This assay relies on the ability of enzyme to inhibit the phenazine methosulphate-mediated reduction of nitro blue tetrazolium.

Catalase (U/ml)

Blood serum Catalase activity was determined according to Aebi, (1984). Catalase reactive with a known quantitative of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor. In the presence of peroxidase, remaining H_2O_2 reacts with 3, 5-dichloro-2-hydroxy benzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with color intensity.

Reduced glutathione (GSH, µmol/l)

Reduced glutathione (GSH) was colorimetrically determined according to the method

described by Beutler *et al.* (1963). The method based on the reduction of 5,5` dithiobis (2 - nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Lipid peroxide (μmol/l)

Lipid peroxide (malondialdehyde) determined according to the method of Satoh (1978). Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at temperature of 95°C for 30 min. to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm.

Activity of AST and ALT (U/l)

Colorimetric determination of AST and ALT activity in blood serum were done according to the method of Reitman and Frankel (1957). The keto acid oxaloacetate formed is measured in its derivative form, 2, 4-dinitrophenylhydrazone. The number of units/l of AST activity was calculated using the standard curve. The keto acid pyruvate formed is measured in its derivative form, 2,4-dinitrophenylhydrazone according to Reitman and Frankel (1957). The number of units/l of ALT activity was calculated using the standard curve.

Activity of ALP (IU/l)

Colorimetric determination of alkaline phosphatase activity was done according to the method of Belfield and Goldberg (1971). The phenol liberated is measured in the presence of 4-amino-antipyrine and potassium ferricynide. The presence of sodium arsenate in the reagent stops the enzymatic reaction.

Total bilirubin (mg/dl)

According to Walter and Gerade (1970), the reaction between bilirubin and the diazonium salt of sulphanilic acid produced azobiliruin, which shows a maximum absorption at 535 nm in an acid medium. In the presence of dimethylsulfoxide (DMSO), the total bilirubin participates in the reaction and in the absence of (DMSO), only conjugated bilirubin react.

Urea (mg/dl)

Enzymatic determination of Urea was carried out according to method of Fawcett and Scott (1960). In the alkaline medium, the ammonium ions react with the salicylate and hypochlorite to form green colored indophenols.

Uric acid (mg/dl)

Enzymatic determination of Uric acid was carried out according to the method described by Barham and Trinder (1972). The Uric acid procedure involves the enzymes uricase and peroxidase (POD) in a coupled reaction. The intensity of red color formed is proportional to the uric acid concentration.

Creatinine (mg/dl)

Creatinine was quantified colorimetrically according to the method described by Schirmeister *et al.* (1964). This assay is based on the reaction of creatinine with sodium picrate forming a red complex. The intensity of the color formed is proportional to the creatinine concentration in the sample.

Statistical analysis

Data are expressed as means ± SE. Statistical analysis was performed using the analysis of variance by the general linear model of SAS (SAS, 2002). Repeated measurements (split plot in time) were adjusted according to Netter *et al*. (1985). The significant differences were tested according to Duncan's new multiple test (Duncan, 1955). P-values less than 0.05 were considered statistically significant.

3. Results and Discussion

Data on the residues of Diazinon in the meat of the experimental rabbits presented in table 4. The data revealed that the Diazinon residues in the meat of G2 and G3 of rabbits were 0.160 and 0.0790 ppm, respectively. In addition, rabbits meat of G3 had a lower Diazinon residues ($P<0.05$) compared with other groups. This reduction in Diazinon may be due to the photolysis of the parent compound because of the storage for a period over than the expiry date. In addition, Ahmadi and Nahri-Niknafs (2011) identified that the photoproducts of the Diazinon due to its photodegradation under a simulated solar light. Furthermore, the metabolism of Diazinon inside the animal body affects the presence of its residues in meat. Nakatsugawa *et al.* (1969) mentioned the fate of Diazinon inside the animal body that Diazinon underwent a dual enzymatic oxidation in the liver. The measured residues of Diazinon in the meat of rabbit indicated the long degradation and withdrawal time of such a compound. Our data of Diazinon residues in the meat of rabbits emphasis to avoid the dipping or spraying animals for at least 6 months before slaughtering to avoid the use of the expired Diazinon and the use of freshly prepared compound only, further study is required to quantify the residues of the photoproduct of Diazinon in meat.

Table 5. represents the blood glucose and the oxidative biomarkers of the experimental rabbits as values and percent of changes after Diazinon exposure. In the present study, data revealed that rabbits of G2 and G3 had lower ($P<0.05$) blood serum glucose by 63 and 81 % than the control G1, respectively. Similar observations have been made by the report of Harkness and Wagner (1995) who indicated that the depletion of the glucose in blood needed to eliminate the insecticide from the body due to the use of either fresh or expired Diazinon.

Data on the antioxidant biomarkers [superoxide dismutase (SOD), catalase and glutathione reduced (GSH)] showed an obvious depression in their activities in the rabbits of G2 and G3, but the reduction was more in G2 compared with control group. Rabbits exposed to either fresh or expired Diazinon are deprived from the well-documented role of this antioxidant in the management of oxidative stress and the detoxification, consequently, the accumulation of the free radicals that react with biological molecules and destroy the structure of cells, that eventually causes cancer, liver disease, aging, etc. as documented by Robert et al. (2003).

Although oxidative stress was suggested as an important factor in tissue damage, the importance of oxidative stress has gained recently a wider understanding. It was noticed in this study an elevation in the activity of the lipid peroxide in G2 and G3 compared with control group, which indicates higher lipid peroxidation in these two groups. This Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Such disturbances in these antioxidant biomarkers indicate a considerable hepato-cellular injury compared to control. These results are in accordance with Yehia *et al.* (2007) who reported a hepato-cellular injury due to Diazinon toxicity.

Table 6 presents the activities of transaminases (AST and ALT), ratio between AST and ALT, alkaline phosphatase and billirubin. Data on liver function tests revealed a significant stimulation in AST and ALT activities and increased plasma level of bilirubin in G2 and G3 relative to those of control and reports of Kaneko *et al.* 1997. Also, data showed the effect of Diazinon as a hepatotoxic compound known to cause marked stimulation of serum transaminases (Braide, 1991 and Burk *et al.* (1983). The significant changes in liver function in peripheral blood due to the exposure to Diazinon reveal a damage to the hepatic parenchyma, this may prove the deleterious effect on the physiochemical functions of the liver (Wolf, 1999). The resultant disturbance in the measured oxidative biomarkers could lead to the accumulation of free radicals which can cause oxidative damage to all biomolecules and initiate a chain reaction which results in physiological damage (Ames *et al.*, 1993).

Table 7, represents the concentration of urea, uric acid and creatinine as kidney function tests in the peripheral blood of the experimental rabbits. Data showed an elevation ($P<0.05$) in the concentration of the urea, uric acid and creatinine in G2 and G3 over the control (G1) being the highest in G2, which indicates a disturbances in the renal physiology.

In conclusion, the present study declared the presence of Diazinon residues in rabbit meat being lower in rabbits exposed to expired Diazinon due to the photolysis of the parent compound because of the storage over the expiry date. In addition, the study revealed that the exposure of white NZW male growing rabbits to Diazinon caused adverse effects on the relevant serological parameters that indicate a hepatocytes injury, renal physiology disturbances and the use of an expired Diazinon is more harmful than the fresh compound not efficient in the veterinarian care.

5. Table and Figure Legends:

Table (1): Chemical analysis of the diets fed to the experimental rabbits.

Chemical analysis	%
Moisture	14.21
Dry matter	85.10
Ash	0.65
Crude protein	16.23
Ether extract	3.54
Crude fiber	17.10
NFE	41.11

Vitamins and minerals per 1 ml diluted in 1 liter drinking water contains: Vit. A 50000000 I.U., Vit. D_3 5000000 I.U., Vit. E 40000 mg., Ascorbic acid 100000 mg., Mn 6000 mg., Zn 7200 mg., Fe 1500 mg., Cu 500 mg., I 120 mg., Se 100 mg., Co 100 mg., Mg 1000 mg., Na 14000 mg., K 7500 mg. and P 10000 mg.

Table (2): Experimental conditions

Items	G1	G2 Fresh Diazinon	G3 Expired Diazinon
Numbers of rabbits	20	20	20
Initial body weight (LSM±SE)	1. 465 ± 13.8	1.480 ± 21.7	1.490 ± 12.0
Concentration of Diazinon/ liter	0	1.0 mg	1.0 mg
Schedule of dipping	The first and the second month of the experiment		
Duration of dipping	10 seconds		
Area of dipping	The whole body sparing the head		

Table 3. List of relevant serological analysis

Analysis	Company	Reference
AST	Biodiagnostic	Reitman and Frankel (1957)
ALT	Biodiagnostic	Reitman and Frankel (1957)
Alkaline phosphatase	Biodiagnostic	Belfield and Goldberg (1971)
Total bilirubin	Biodiagnostic	Walter and Gerade (1970)
Serum glucose	Biodiagnostic	Trinder (1969)
Urea	Biodiagnostic	Fawcett and scott (1960)
Uric acid	Biodiagnostic	Barham and Trinder (1972)
Creatinine	Biodiagnostic	Schirmeister *et al.* (1964)
Superoxide dismutase	Biodiagnostic	Nishikimi (1972)
Catalase	Biodiagnostic	Aebi (1984)
Glutathione reduced	Biodiagnostic	Beutler *et al.* (1963)
Lipid peroxide	Biodiagnostic	Satoh (1978)

Table (4): Diazinon residues ($\bar{\chi}$ ±SE) in meat of the experimental rabbits

Item	G1	G2	G3
Diazinon (ppm)	**0.00±0.00**	**0.160^{a}±0.035**	**0.0790^{b}±0.028**

Within the same row, LSM with different superscripts significantly differ at P < 0.05
G1 (control rabbits dipped in tape water), G2 (dipped in fresh diazinon 0.1%), G3 (dipped in expired diazinon 0.1%)

Table 5. Blood glucose and some oxidative biomarkers ($\bar{\chi}$ ±SE) of the experimental rabbit

Treatment	Blood glucose		SOD		Catalase		GSH		Lipid Peroxide	
	mg/dl	%	U/ml	%	U/ml	%	µmol/l	%	µmol/l	%
G1	80.43±4.23^{a}	100	1.920±43.38^{a}	100	9.180±4.74^{a}	100	6.85±0.32^{a}	100	4.71±0.13^{a}	100
G2	50.25±1.64^{b}	62	710±27.70^{b}0.	37	4.682 ±2.19^{b}	51	3.86±0.24^{b}	56	22.60±0.80^{b}	480
G3	64.77±4.98^{c}	81	1.478±26.66^{c}	77	8.086±2.00^{c}	88	5.43±1.71^{c}	79	5.75±0.30^{c}	122

Within the same column, LSM with different superscripts significantly differ at P < 0.05
G1 (control rabbits dipped in tape water), G2 (dipped in fresh diazinon 0.1%), G3 (dipped in expired diazinon 0.1%).

Table 6. Liver function tests ($\bar{\chi}$ ±SE) of the experimental rabbits

Treatment	AST		ALT		AST/ALT		Alkaline Phosphatase		Bilirubin	
	U/L	%	U/L	%	Ratio	%	IU/L	%	mg/dl	%
G1	133.16±7.19^{a}	100	52.41±3.56^{a}	100	2.60^{a}	100	30.63±1.23^{a}	100	0.14±0.02^{a}	100
G2	182.39±8.47^{b}	137	76.71±0.86^{b}	146	2.38^{b}	92	31.87±2.83^{b}	104	0.26±0.04^{b}	186
G3	157.64±19.59^{c}	118	54.72±1.80^{c}	104	2.87^{c}	110	30.69±2.37^{c}	100	0.21±0.02^{c}	150

Within the same column, LSM with different superscripts significantly differ at P < 0.05
G1 (control rabbits dipped in tape water), G2 (dipped in fresh diazinon 0.1%), G3 (dipped in expired diazinon 0.1%)

Table 7. Kidney function tests ($\bar{\chi}$ ±SE) of the experimental rabbits

Treatment	Urea		Uric acid		Creatinine	
	mg/dl	%	mg/dl	%	mg/dl	%
G1	**23.96±2.04^{a}**	**100**	**0.52±0.07^{a}**	**100**	**0.47±0.01^{a}**	**100**
G2	**31.60±2.73^{b}**	**132**	**0.75±0.06^{b}**	**144**	**0.59±0.06^{b}**	**126**
G3	**39.77±0.34^{c}**	**83**	**0.95±0.04^{c}**	**115**	**0.67±0.01^{c}**	**119**

Within the same column, LSM with different superscripts significantly differ at P < 0.05
G1 (control rabbits dipped in tape water), G2 (dipped in fresh diazinon 0.1%), G3 (dipped in expired diazinon 0.1%).

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