



Research Article

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A novel methods for protective role against reproductive toxicity of carbofuren in male rats using palm pollen grains and vanadyl(II) folate as a new compound

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ABSTRACT

The acute toxicity (LD_{50}) of insecticides carbofuran and its effects on male reproduction in rats were carried out. Carbofuran was given orally to dose (2.4 mg kg⁻¹ b.wt, corresponding to 1/25 LD_{50}) alone and in combination with date palm pollen grains extracts (60 mg/ kgb.wt) and vanadylfolate (50 mg /kg b.wt). Fertility index, weight of sexual organs, semen picture, serum testosterone level and activity of lactate dehydrogenase (LDH), acid phosphatase enzymes and fructose content in serum. Results showed that there was a correlation between carbofuran administration and the significant decrease of the fertility index, weight of the testes and accessory male sexual glands, serum testosterone level and sperm cell abnormality. Carbofuran increased significantly lactate dehydrogenase and acid phosphate activity and decreased the fructose content in the serum. In contrast Co-administration of date palm pollen grains or vanadylfolate to carbofuran treated rats restored almost of these biochemical parameters to normal levels and alleviates the toxic effects of carbofuran on reproductive functions in male rats.

Keywords: Carbofuran; date palm pollen grains; vanadylfolate; fertility; testes; testsosterone.

INTRODUCTION

Pesticides widely used to control agricultural pests and insects causing public health hazards. An insecticide causes toxicity for animals and human which usually occurs either from direct exposure to insecticides or indirectly from contaminated feeds or water by such chemicals. Prolonged exposure to insecticides causes chronic malignant tumours, neurological syndrome, immunosuppressive action, teratogenic effects, abortion and decreased male fertility in experimental animals [1]. Carbamates derivatives of carbamic acid represent a large variety of compounds which have some field applications as insecticides, fungicides and herbicides. Many of these chemicals are potential neurotoxins, particularly following occupational, accidental or intentional exposure. These compounds cause reversible carbamylation of the acetylcholinesterase enzyme allowing accumulation of acetylcholine [2]. Carbofuran (2,3-dihydro-2,2-dimethyl -7- benzofuran-5-ylmethyl carbamate) was a broad spectrum carbamate pesticide that kills insects, mites and nematodes on contact or after ingestion in many of agricultural crops, including maize, rice, potatoes, alfalfa and grapes. Carbofuran decreased libido and sperm number in rabbits. This pesticide also disrupts testicular morphology and alters activities of enzymes associated with specific cell types of testes [3]. Chemical derived from plant are used to relieve sexual dysfunction and they have sex enhancing potentials. These phytochemicals increase sexual potency, libido and sexual pleasure [4]. Date palm pollen (DPP) grains is widely used as folk remedy for curing male infertility in traditional medicine. Approximately, 1000 tons of

(DPP) are produced every year by millions of palm trees grown in the Arabian regions. DPP was reported to promote fertility in women in ancient Egypt [5]. Also DPP extracts contain estrogenic compounds, oestrogen-gonadstimulating compound that can improve male infertility. Reports have also showed that DPP contain sterols and other agents that might influence male fertility (6). Also DPP contain concentrations of phytochemicals and nutrients and are rich in flavonoids, carotenoids and phytosterols. Moreover, they are good source of protein, amino acids, vitamins, dietary fiber, enzymes, hormones minerals and fatty acids. [7]. Folate is important for normal fertility in men and women as it is necessary during cell division and growth period such as infancy and pregnancy and in males folate is necessary for spermatogenesis [2]. Vanadyl(II) sulfate improves insulin sensitivity in diabetes type 2 and there are increasing in oxidative damage in stomach and spleen tissues in diabetic patients and vanadyl(II) sulfate has a positive effect on the oxidative stress via its antioxidant property at the same time low doses from vanadyl(II) sulfate activating the functions of reproductive system in male human and various animal species [8].

The present work aimed to determine the acute oral LD₅₀ of carbofuran insecticides and to examine its toxic effects alone and in combination with date palm pollen grains extracts (DPP) or with vanadyl(II) folate as a new compound on male fertility in rats. The biochemical alterations in the testes of male rats exposed to carbofuran may have implications in managing humans with accidental exposures to such compounds.

EXPERIMENTAL SECTION

2.1. Test insecticide

Carbofuran technical grade (75% wp) was obtained from El-Wattanya Company for pesticides and chemicals, Egypt.

2.2. Preparation of palm pollen extracts

Find powder of palm pollen grains were purchased from local markets in Taif, Saudi Arabia. The water suspension was prepared by mixing 0.5 gram of pollen grains within 10 mL of sterile saline with vigorous shaking and vortexing. Then the solution kept at water bath at 60 °C for 90 minutes. This is followed by sonication within ultrasound probe (6 kHz) for 30 seconds. Then samples were stored at 3°C overnight, followed by centrifugation at 2500 G for 10 minutes. The clear supernatant as then separated into clear tubes and stored at refrigerator until use.

2.3. Synthesis of oxovanadium (IV) folate complex

Folic acid (2.21 g, 5 mmol) was added to 50 mL methanol and ammonium carbonate (2 mmol) to adjust pH at 7-8, then 15 mL methanolic solution of (1.63 g, 10 mmol) of VOSO₄ was added with continuously stirring, after that the mixture was warmed at about ~ 75 °C and then neutralized. The mixture was left overnight until precipitated, the greenish brown precipitate was settling down then turns to dark green by time, filtered off, washed several times by minimum amounts of hot methanol and dried under vacuum over anhydrous CaCl₂. The oxovanadium(IV) folate solid complex resulted has a yield about 68 %.

2.4. Animals

Male and female rats of Albino rates weighting 150 ± 10 g and 16-18 weeks of age were supplied from the farm of general organization of serum and vaccine (Helwan farm). Animals were maintained at the animal care facility in toxicology department, central laboratories of agricultural pesticides, Dokki, Egypt in plastic cages under controlled temperature (23 ± 2° C), 12-h light/dark cycle and 50 ±5% relative humidity water and food were available ad libitum. Rats were acclimatized to the laboratory environment for two weeks prior to the start experiments.

2.5. Acute toxicity experiment

For estimating the LD₅₀ of carbofuran in 50 male albino rats were distributed into five groups each containing 10 animals. Rats were given orally by stomach tube, the tested insecticides in graded doses. Toxic symptoms and the number of rats that died in each group after 48 h observation were recorded. The LD₅₀ of carbofuran was then calculated according to the method described in Gad and weil [9].

2.6. Experimental design

After the period of acclimation animals were divided into six groups with 25 animals in each. The first group was used as control. The animals of control group were orally given dimethylsulphoxide (DMSO) (4ml/kg b.w). The second male group was orally treated with carbofuran (2.4 mg/kg b.w) about 1/25 LD₅₀. The third male group was orally treated with date palm pollen grains extracts (60 mg/ kg b.w), fourth group was treated with combination of carbofuran (2.4 mg/ kg b.w) and date palm pollen grains extract (DDP) (60 mg/kg b.w). fifth group was treated with vanadylfolate (50 mg/kg b.w) dissolved in dimethylsulphoxide (DMSO) as a solvent and the six group was treated with combination carbofuran (2.4 mg/ kg b.w) and vanadylfolate (50 mg/kg b.w) .The duration of the oral

administration during the experiments lasted for 70 days for completion of the spermatogenic cycle and maturation of sperms in epididymis [10].

2.7. Mating and fertility indexes

After the end of the treatment course, males of control and experimental groups of treated rats (n= 25/ group), were mated 1:1 with untreated proven fertile, with regular estrous cycle females for 5 days (complete one estrous cycle). Mating was confirmed by the presence of vaginal plugs or deposition of spermatozoan at the vaginal orifice upon vaginal examination. The day that a vaginal plug was found was considered day 0 of gestation. Then mating and fertility indexes were estimated and recorded.

2.8. Sperm quantity and quality

2.8. Testicular sperm count

Immediately after dissection, one testis of each rat was placed in 1 ml phosphate buffer (pH 7.4). Tunica albuginea was cut by surgical blades, removed and the remaining seminiferous tubules were mechanically minced using surgical blades in 1 ml phosphate buffer. The testicular cell suspension was pipetted several times to make a homogenous cell suspension. One drop of the suspension was placed on the "Haemocytometer chamber" (Neubauer improved, Feinoptik Bad Blankenburg, Germany) and testicular sperm suspension was evaluated as million sperm cells per ml of suspension under 200X magnification using phase contrast microscope and the sperm were counted manually. Testicular sperm count was measured by (11)

2-8-1. Sperm motility analysis:

The sperm were collected as quickly as possible after a rat was sacrificed. The cauda epididymis was placed in 1 ml of 37°C phosphate buffer saline solution and cut by surgical blades into approximately 1 mm³ pieces. The solution was pipetted several times to homogenize the sperm suspension. One drop of the suspension was placed on a slide, covered by a cover slip, and evaluated under a phase contrast microscope at 200 magnifications. The sperm were categorized on the basis of their motility as "motile" or "immotile". The results were recorded as percentage of sperm motility. (Uzunhisarcikli *et al.*, 2007).

2-8-2. Sperm morphology:

To determine the percentage of morphologically abnormal spermatozoa, one drop of the suspension was spread on a clean slide. The slides were stained with a mixture of 1.67% eosin and 10% nigrosin in 0.1M sodium citrate for viewing under a light microscope at 400-magnification. A total of 300 spermatozoa were examined on each slide [1800 cells in each group (n=5)], and the head, tail and total abnormality rates of spermatozoa were expressed as percentage (12) and seminal smears were also prepared for the microscopic examination of sperm cell abnormality and Seminal smears were also prepared for the microscopic examination of sperm cell abnormality

2-9. Biochemical assays

At the end of the 70th day of the treatment course, blood samples were collected from anaesthetized males of all groups by puncturing the retro-orbital venous plexus with a fine sterilized glass capillary tube into heparin-coated and dry tube. The gathered blood left for 20 min at room temperature, then centrifuged at 3000 rpm (600 g) for 10 min for the separation of sera. The sera were kept in a deep freezer (-20 °C) until analyses of certain biochemical parameters. The biochemical measurements were performed according to the details given in the kit's instructions.

2.9.1. Testosterone determination

Serum samples of the treated male rats were used for estimating testosterone concentration using radioimmunoassay (RIA) method. After incubation, the liquid contents in the tubes were withdrawn and the bound radioactivity was determined using gamma counter according to method described by [2].

2.9.2. Measurement of some enzyme activities related fertility

Activities of serum lactate dehydrogenase (LDH), acid phosphatase and fructose content were measured spectrophotometry using commercial kits (Boehringer Mannheim, GmbH, Mannheim Germany).

2.10. Statistical analysis

Analysis of data was performed by using SPSS (Version 15). Results are expressed as Mean ± standard error (M ± S.E.) Statistical differences were determined by Duncan test for multiple comparisons after ANOVA. p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The results of acute toxicity study revealed that oral LD₅₀ of carbofuran insecticide in male rats was 10.2 mg Kg /b.wt. The toxic symptoms were muscular tremors, abdominal cramps, sweating, muscle in coordination and irregular respiration and heart rate. These symptoms were seen during the first 24h post administration. This results was nearly similar to the mentioned by [13] who showed that carbofuran is highly toxic insecticide via the oral route ,with reported oral LD₅₀ values ranged from 6 to 18 mg/Kg in rats and in various other species including rabbit ,cat and dog for 3-19 mg/ Kg. This difference between both results could be attributed to difference in the strain of rats used and /or due to sex difference. As recorded in Table 1 oral administration of carbofuran at dose 2.4 mg Kg / b.wt for 70 successive days caused significant decreases in the weight of testes, seminal vesicles and prostate glands as compared with normal control group. Co-administration of DPP extract (60mg /Kg b.wt) with carbofuran (2.4 mg/Kg b.wt) or vanadylfolate at (50mg/ Kg b.wt) with carbofuran .Significantly increased the weight of testes, seminal vesicles and prostate gland as compared to control group.

The administration of carbofuran brought about marked alteration in the weight of testes. The reduction in the testicular weight reflects regressive changes in seminiferous tubules. Reduction in the number of spermatogenic elements and spermatozoa leads to reduction in the weight of testes. Similar reduction in the weight of testes was also observed by [14]. Also the increases in weight gains observed in epididymis, testes and seminal vesicles when treatment with DPP may be due to fluid resorption effects of estradiol which come from phytoestrogen component in (DPP) .Also (DPP) contain flavonoid components which have scavenging properties that have positive effects on the sperm quality and male reproductive activity [5]. At the same time vanadylfolate has an ameliorating effect on the oxidative stress via its antioxidant property due to its components from vanadyl sulphate and folic acid [15].

Data present in Table 2 and Fig 1 showed that administration of carbofuran at 2.4 mg/Kg b.wt for 70 successive days to male rats caused significant decreases in sperm cell concentration and percentage of total sperm abnormalities. Morphological sperm abnormalities frequently seen in seminal sears of rats given carbofuran were mainly represented by detached head, double head and without head as shown in Fig 1. Co-administration of DPP and vanadylfolate with the tested dose of carbofuran improved these changes and caused significantly increased in sperm concentration and motility but decrease abnormality of sperms compared to carbofuran group and this means that DPP and vanadylfolate minimized the reproductive toxicity of carbofuran.

Table (1): Effect of oral administration of carbofuran, date palm pollen grains (DDP), vanadylfolate and their combination for 70 days on the weight of sexual organs in male rats

Groups	Testes	Epididimides	Prostate	Seminal vesicles
Control	2.186±0.0830	0.756±0.0144	0.606±0.0346	1.798±0.0244
Carbofuran (1/25 LD ₅₀)	1.626±0.0687 ^(a,b)	0.586±0.0108 ^(a,b)	0.390±0.00707 ^(a,b)	0.960±0.010 ^(a,b)
DPP (60 mg/kg-1 b.wt)	2.340±0.0598	0.794±0.0238	0.568±0.0124	1.798±0.0213
Vanadylfolate (50 mg/kg-1 b.wt)	2.328±0.0476	0.770±0.0170	0.538±0.00663	1.816±0.0108
Carbofuran (1/25LD ₅₀)+ DPP(60mg/Kg /b.w)	1.958±0.0500	0.712±0.0271	0.448±0.0116	1.382±0.0263
Carbofuran (1/25 LD ₅₀) +Vanadylfolate (50mg/Kg-b.wt)	1.954±0.0677	0.682±0.00860	0.436±0.00678	1.350±0.0192

The data are presented as mean ± SE, n=25

(a) Significant difference as compared with control group ($P \leq 0.05$).

(b) Significant difference as compared with Carbofuran group ($P \leq 0.05$).

Sperm motility is an important functional measurement to predict sperm fertilizing capacity. Any negative impact on motility would seriously affect fertilizing ability. In the present study carbofuran caused inhibition of sperm motility this may be due to low level of ATP content. Also sperm motility may be affected by altered enzymatic activities of oxidative phosphorylatic process. Oxidative phosphorylatic process is required for ATP production, a source for energy for the forward movement of spermatozoa. Full ATP pool is crucial for normal spermatozoa movement and a slight deprivation of ATP leads to reduction in motility. Sperm count is considered to be one of the important factors that affect fertility [17]. Toxicants have direct effect on sertoli cell function, which appears to be involved in the control of spermiation and when disturbed caused epithelial disorganization and subsequent tubular atrophy [18]. Also [5] reported that the reduction in the weight of the sex organs is primary indicator of possible changes in androgen status. Some recent reports suggested that exposure to toxicants decreases testicular sperm count and increases sperm abnormalities which could be related to low levels of serum testosterone. The present study considers the first study that used vanadylfolate and DPP against the toxicity of carbofuran. The male rats were given combination of DPP and carbofuran have fertility index 68% and combination of vanadylfolate and carbofuran have fertility index 77% while the fertility index in the rats treated with carbofuran only was 53% as compared to control group as shown in Table 3 these may be due to proactive effects of DPP and antioxidant property of vanadylfolate. Data present in Table 4 showed significant decrease in serum testosterone concentration in rats treated with carbofuran compared to control. While DPP and vanadylfolate significantly increased testosterone level and mitigated the negative effects of carbofuran. This finding was in agreement with that reported

by [18] who recorded a significant decrease in the level of serum testosterone of methomyl in toxicated rats. At the same time treatments with combinations of DDP and carbofuran or vanadylfolate and carbofuran significantly increased the testosterone level in serum of the treated rats because the main compounds of DDP are flavonoids, steroids, saponins and estradiol. These components can increase sexual behaviour and stimulate endogenous testosterone levels by raising the level of luteinizing hormones (LH) [4].

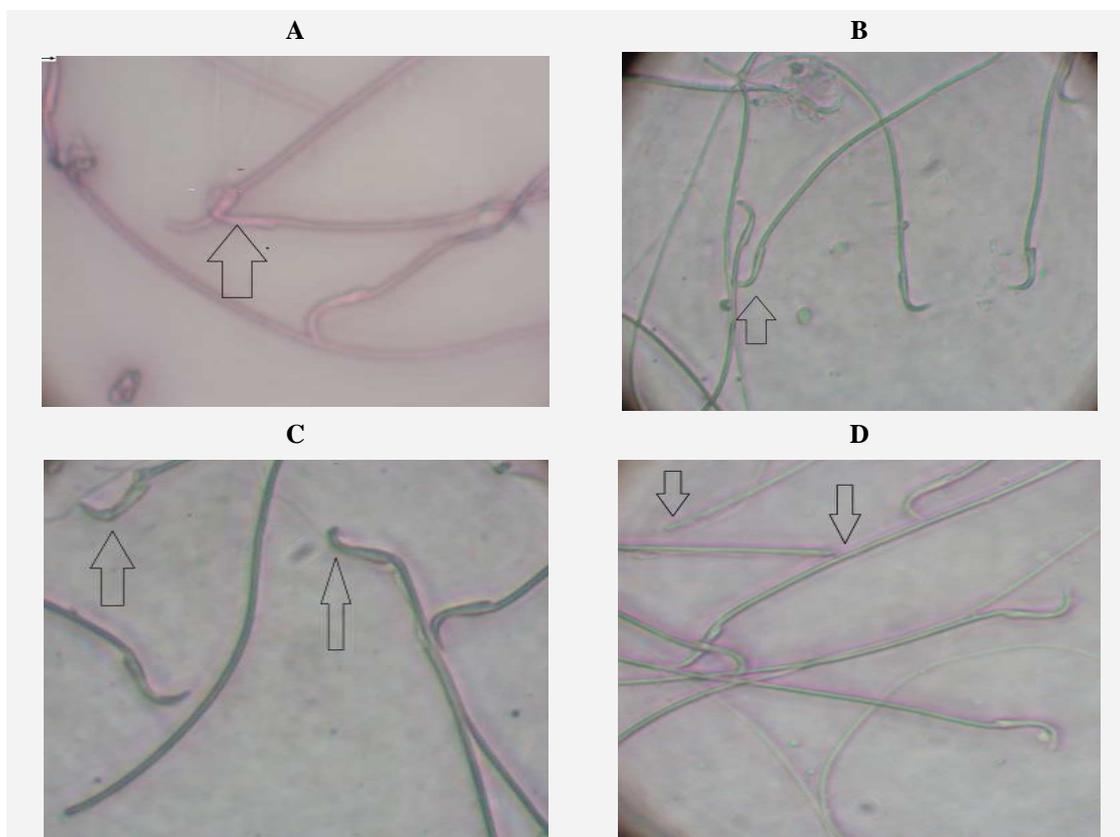


Fig. (1): Seminal smear from crude epididymis of a Rat given 1/25 of carbofuran showing (A) Deformed head (B) Hooked head (C) Banana head (D) Without head

Table (2): Sperm characteristics in male rats after oral administration of carbofuran, date palm pollen grains (DPP), vanadylfolate and their combination for 70 days

Groups	Count/ Epididymis $\times 10^{-6}$	Motility (%)	Progressive motility	Abnormal form
Control	93.406 \pm 1.097	61.476 \pm 0.628	35.010 \pm 0.771	13.278 \pm 1.016
Carbofuran (1/25 LD ₅₀)	59.292 \pm 1.152 ^(a,b)	25.372 \pm 1.389 ^(a,b)	16.752 \pm 1.53 ^(a,b)	40.188 \pm 0.671 ^(a,b)
DPP (60mg/kg b.wt)	92.390 \pm 0.701	58.616 \pm 1.011	34.474 \pm 0.953	15.778 \pm 0.491
Vanadylfolate (50mg/kg b.wt)	78.260 \pm 0.681	49.536 \pm 0.592	29.326 \pm 0.684	22.368 \pm 0.732
Carbofuran (1/25LD ₅₀)+DPP(60mg/Kg/b.wt)	90.946 \pm 0.677	57.428 \pm 0.819	31.542 \pm 0.293	17.300 \pm 0.886
Carbofuran (1/25LD ₅₀)+ Vanadylfolate (50mg/Kg/b.wt)	76.312 \pm 0.932	43.474 \pm 1.301	28.388 \pm 0.730	24.560 \pm 1.699

The data are presented as mean \pm SE, n=25

(a) Significant difference as compared with control group ($P \leq 0.05$).

(b) Significant difference as compared with Carbofuran group ($P \leq 0.05$).

Also coadministration of vanadylfolate and carbofuran decreased its reproductive toxicity and increased testosterone level because folic acid is necessary for normal fertility in men and women and in males foliate is important for spermatogenesis process [2]. Also vanadyl sulphate has sexual and antioxidant activity at low dose and folic acid decrease the toxicity of vanadyl sulphate and vanadyl sulphate at highly dose depletion of protein content corresponds to the inhibition of spermatogenesis and suppressed Leydig cell function [15]. Results in Table 5 showed that the activities of lactate dehydrogenase and acid phosphatase enzymes were increase after treatment with carbofuran and decreased after treatments with DPP or vanadylfolate also fructose content was decreased after treatment with carbofuran and increased with different treatments. These results are in accordance with those reported by [19] who observed a significant inhibition in lactate dehydrogenase activity after treatment with toxicant

in rats. Also lactate dehydrogenase (LDH) is an intracellular enzyme and a sole marker of liver injury it increased after toxicant administration.

Table (3): Functional fertility parameters of male rats after oral administration of carbofuran date palm pollen grains (DPP), vanadylfolate and their combination for 70 days

Groups	Number of males that used for mating	Mating index (%)	Fertility index%
Control	25	25/25(100)	25/25(100)
Carbofuran (1/25 LD ₅₀)	25	13/25(52%)	7/13(53%)
DPP (60 mg/kg-1 b.wt)	25	25/25(100)	25/25(100)
Vanadylfolate (50mg/kg b.wt)	25	19/25(76%)	13/19(68%)
Carbofuran (1/25LD ₅₀) +DPP(60mg/K1b.w)	25	25/25(100)	25/25(100)
Carbofuran (1/25LD ₅₀) + vanadylfolate (50mg/Kg-b.wt)	25	18/25 (72%)	14/18(77%)

Mating index (%) = Number of males inseminated females /total number of males cohobited with females x100

Fertility index (%) =Number of cohobited females becoming pregnant /number of non pregnant with evidence of vaginal plug x 100

(a) Significant difference as compared with control group (P≤0.05).

(b) Significant difference as compared with Carbofuran group (P≤0.05).

Testicular acid phosphatase is localized in the sertoli cells and the leydig cells and is an accurate marker of specific stages of spermatogenesis and activity correlates with the activity of testosterone. Also phosphatases in semen play an important role in phosphorylation processes in sperm metabolism [20].

Fructose is secreted by the seminal vesicles which are about 84% of the total fructose of the body and it was decreased during toxicity, which may reduce the viability of spermatozoa due to improper nutrition and fructose is the principal seminal sugar, anaerobic conditions [21].

Table (4): Serum testosterone of male rats after oral administered with carbofuran, date palm pollen grains (DPP), vanadylfolate and their combination for 70 days

Groups	Testosterone level (ng/dl)
Control	6.268 ±0.0885
Carbofuran (1/25 LD ₅₀)	3.166 ±0.0218 ^(a,b)
DPP (60 mg/kg-1 b.wt)	7.192 ±0.0227
Vanadylfolate (50 mg/kg b.wt)	4.106 ±0.0445
Carbofuran (1/25 LD ₅₀) + DPP(60 mg/K1b.w)	6.922 ±0.0565
Carbofuran (1/25 LD ₅₀) + Vanadylfolate (50mg/Kg-1b.wt)	4.012±0.0654

The data are presented as mean ± SE, n=5

(a) Significant difference as compared with control group (P≤0.05).

(b) Significant difference as compared with Carbofuran group (P≤0.05).

Table (5): The effect of carbofuran alone and its combination with date palm pollen grains (DPP) or vanadylfolate on serum lactate dehydrogenase (LDH), acid phosphatase activity and fructose content

Groups	LDH (U/L)	Acid phosphatase (U/L)	Fructose
Control	1247.396 ± 21.496	42.874 ± 0.803	165.70 ± 2.574
Carbofuran (1/25 LD ₅₀)	2292.998 ± 17.104 ^(a,b)	68.540 ± 0.847 ^(a,b)	88.180 ± 0.616 ^(a,b)
DPP (60 mg/kg-1 b.wt)	1250.788 ± 27.242	43.782 ± 1.328	163.196 ± 2.573
Vanadylfolate (50 mg/kg b.wt)	1800.820 ± 13.616	51.626 ± 1.061	154.730 ± 3.195
Carbofuran (1/25 LD ₅₀) + DPP (60mg/K1b.w)	1867.740 ± 13.154	43.410 ± 1.039	164.83 ± 1.627
Carbofuran (1/25 LD ₅₀) + Vanadylfolate (50 mg/Kg-1b.wt)	1240.704 ± 16.591	56.522 ± 0.659	136.83 ± 2.608

The data are presented as mean ± SE, n=5

(a) Significant difference as compared with control group (P≤0.05).

(b) Significant difference as compared with Carbofuran group (P≤0.05).

CONCLUSION

The present results showed that the insecticide carbofuran is a highly toxic compound which caused deterioration in semen quality, decrease of fertility indexes as well as decreased the enzyme activities of serum and serum testosterone level. Co-administration of date palm pollen grains and vanadylfolate with carbofuran antagonizes its reproductive toxicity of male rats and improved all the biochemical parameters. Therefore, the present study elucidated the therapeutic effects of DPP and vanadylfolate administered in combination with carbofuran to minimize its reproductive toxicity.

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