TOXICOPATHOLOGY OF INDUCED CARBOFURAN TOXICITY IN WISTAR RATS (*Rattus norvegicus*)

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ABSTRACT

In present study, 24 female wistar rats were equally divided in to four groups viz A, B, C and D. Group A served as control, while Group B, C and D rats were orally administered Carbofuran @, 0.35, 0.70 and 1.40 mg/kg b. wt., respectively up to 28 days. The clinical symptoms were characterized by weakness, dullness, depression, tremor, salivation, immobilization, diarrhea and dose dependent decreased in body weight at 28th day post treatment in treatment group. A statistically increased in values of HCT, MCV, absolute neutrophils, glucose, total protein, ALT, AST, ALP and creatinine; and a decreased in value of TLC count, absolute lymphocytes, acetyle cholinesterase and MCHC were observed in all groups. Congestion was observed in heart, brain, liver and lung in high dose group and confirmed by histopathological examination. It is concluded that carbofuran at very high dose cause adverse effect in rat.

KEY WORDS: Biochemical, carbofuran, hematology, patho-morphology, rat.

INTRODUCTION

Carbofuran (2, 3-dihydro-2, 2-dimethyl-7-benzofuranyl Methyl-carbamate) is a broad spectrum carbamate pesticide, insecticides and nematicide that kills insects, mites, and nematodes on contact or after ingestion. In 1945, the first soil-acting carbamate herbicides were discovered by British workers. Carbofuran was first registered in the United States in 1969 and is classified as a restricted use pesticide. It is marketed under the trade names Furadan, by FMC Corporation and Curater, among several others. Carbofuran is a white crystalline solid with a slightly phenolic odour. It is used against soil and foliar pests of field, fruits, vegetables, and forest crops. Carbofuran is soluble in water and moderately persistent in the soil (Hayes, 1993). It is rapidly absorbed, metabolized and eliminated. It is highly toxic by inhalation and ingestion, while moderately toxic by dermal absorption. Because of its broad spectrum activity, it is widely used for the control of insects, mites, pests and nematode (Sharma, 2006).

Carbofuran is very highly toxic to freshwater, estuarine/marine fish, birds and mammals on an acute basis, and highly toxic on a sub-acute basis. A chronic effect level could not be established due to the fact that all

concentrations tested caused mortality in the test subjects (USEPA, 1991). Chronic toxicity testing on laboratory rats showed reduced offspring survival and body weight reductions (Baron, 1991). Chronic tests showed reproductive effects. Liver is primary site involved in the metabolism of carbofuran. Secondly kidney and reproductive functions have been reported to be adversely affected with carbofuran exposure to rats (Kareem *et al.,* 2007)

Extensive epidemiological studies have been carried out in human population. However, such studies in laboratory animal are lacking to assess and address the hazards due to carbofuran exposure. Very few researchers tried to correlate hemato-biochemical alterations, oxidative stress and patho-morphological changes in laboratory animals especially in rats, as they considered as a suitable animal model. Hence, looking to the paucity of information, the present investigation was undertaken to study the clinical symptoms, toxic manifestation, hematological changes, biochemical variation, oxidative stress, patho-morphological alteration that occurs due to various doses of carbofuran.

MATERIALS AND METHODS

***Study area*:** The present study was carried out in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar.

***Ethical approval*:** The Institutional Animals Ethical Committee (IAEC) approved the experimental protocol, which met the national guidelines as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Animals: Wistar rats females, procured from Cadila Pharmaceuticals Ltd, Dholaka, Gujarat, India were housed in polycarbonate cages as group of three animals during the quarantine, acclimatization and study periods. Females were non pregnant and nulliparous. Standard diet (amrut rodent diet) was provided ad libitum daily. Potable water was supplied, ad libitum, via an automatic watering bottle. Animal care, housing, and environmental conditions were according to recommendation stated in the International Guideline for Care and Use of Laboratory Animals before and during study period.

Experimental design: Carbofuran (3 % G, encapsulated) obtained from open market and used for inducing toxicity in rats. Study was design according OECD test guideline 407 (OECD, 2008). All the rats were randomly divided into 4 different groups. Each group comprised 6 female wistar rats. The groups were numbered as group A, B, C and D. The LD_{50} (14.0 mg/kg b.wt) of carbofuran for rat was used to calculate dosage for various groups. Group A (vehicle control); 0.5 ml groundnut, Group B (low dose); 0.35 mg/kg b. wt (LD_{50}/40), Group C (mid dose); 0.70 mg/kg b. wt (LD_{50}/20) and group D (high dose); 1.40 mg/kg b. wt (LD_{50}/10) orally by gavaging for 28 days. Aliquots of each target concentration were prepared by an appropriate amount of carbofuran (according to dose concentration) dissolved in the final volume groundnut oil (4ml/kg or 0.4ml/100 g body weight) by stirring until a solution was formed.

Parameters evaluated:

***Clinical observations*:** Observations for morbidity and mortality were made twice daily throughout the study. Observations during acclimation were recorded once on day. From Day 1 to

Day 28, clinical observations were recorded at least twice on each day of dosing (pre-dose and post-dose).

***Body weights*:** Body weights were recorded for all animals on days 1, 7, 14, 21 and 28. Recently taken body weights were used for dose volume determinations.

***Clinical pathology*:** Clinical pathology evaluation was carried out at termination (Day 29) in all groups. Blood was collected from all surviving animals/group for hematology and clinical chemistry. Blood was collected by retro-orbital plexus puncture using glass capillaries in to pre-labeled collection vials. Vial for collection and separation of samples were labeled with animal identification and type of analysis. For hematology and clinical chemistry, approximately 0.5 ml and 1.5 ml of blood, respectively was collected in 10 per cent K_2 EDTA (1.6 mg/ml of blood). Samples were analyzed on same day of dosing. All animal were fasted overnight (approximate 16 hours) before blood collection.

***Hematology*:** Whole blood collected for hematology in 10 per cent K_2 EDTA was used to analyze all parameters by auto blood analyzer (Medonic CA 620/530 VET, Boule Medical AB, Sweden). Hematology analysis included total leucocytes count (TLC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, differential leukocyte counts (neutrophils, monocytes, eosinophils, basophils, lymphocytes).

Biochemistry: Whole blood collected for biochemistry in 10 per cent K_2 EDTA was used to analyze all parameters except lipid peroxidase by using Merck Kits (Merck Specialties Pvt. Ltd., Ambernath) by Clinical Analyzer (Systronics, Ahmedabad). Clinical chemistry analysis included alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total protein, and albumin, glucose, creatine and acetyl cholinesterase.

***Necropsy*:** At the end of dosing period on Day 29, all surviving animals of all groups, examined externally, sacrificed by CO_2 asphyxiation and decapitation and were subjected to necropsy and detailed gross pathology evaluation. All the animals were fasted overnight (approximate 16 hours) prior to necropsy. Carcasses were observed externally for any abnormality, and cranial, thoracic and visceral cavities were opened and examined macroscopically. Brain, heart, intestine, kidneys, liver, lungs, lymph nodes (mandibular, and mesenteric), ovaries, salivary gland (mandibular), spleen, stomach (fore stomach, glandular), thymus and trachea were fixed in 10 per cent neutral-buffered formalin for minimum 24 hours.

***Organ weights*:** Brain, heart, kidneys, liver, lungs, ovaries and thymus were weighted after trimming off the adherent tissue/s and fat from all groups.

***Histopathology*:** Tissues/organs fixed during necropsy were trimmed and processed, embedded in paraffin blocks, sectioned at four to six microns, mounted on glass microscope slides and stained with hematoxylin and eosin.

***Data analysis*:** The statistical analysis of data generated on various parameters were subjected to statistical analysis using completely randomized design (Snedecor and Cochran, 1980) and using CD values compared the treatment means. Since, the CD permits comparison of two consecutive treatment mean after arranging treatment mean in ascending or

descending order, it was thought worthwhile to compare treatment mean with all other treatment mean (Overall comparison). Hence, Duncan's New Multiple Range Test (Steel eand Torrie, 1984) was used for the same.

RESULTS AND DISCUSSION

Clinical observations

No any mortality was found in any groups during study period. Females dosed at 1.40 mg/kg b. wt exhibited weakness, dullness, depression, tremor, salivation, immobilization and diarrhea. Females dosed at 0.70 mg/kg b. wt were exhibited weakness, dullness and depression. No clinical sign was observed at dose of 0.35 mg/kg b. wt. Clinical signs observed in present study might be due to inhibition of acetyl cholinesterase lead to change in behavior as reported by Goad *et al.* (2004) and Satar *et al.* (2005).

Body weights

Significantly decreased in mean body weights in all carbofuran treated animals were observed as compared to vehicle control animals. There was no significant reduction in body weight of rats up to 8th day except high dose group (Table 1). Significant ($P < 0.05$) decrease in body weight was observed in rats of low, mid and high dosed groups from 15th day onwards. The most significant ($P < 0.05$) dose dependant reduction was observed in high dosed (180.333±2.679) group rats at 29th day post treatment followed by mid dose (201.667±2.108) and low dose (208.000±3.183) as compared to vehicle control (261.000±4.683). Significant decrease of body weight in present study might be due to dose dependant generalized toxicity of carbofuran on rat as reported by Pant *et al.* (1995), Luty *et al.* (2001) and Gera *et al.* (2009) or due to diarrhea observed in present study as reported by Baligar and Kaliwal, (2002).

Hematology

On day 29, statistically significant decreased in TLC, absolute lymphocytes count and MCHC value were observed in 0.35 mg/kg b. wt or higher dose groups as compared to vehicle control. Increased mean values of HCT, MCV, absolute neutrophils and monocytes counts were noted in the treated groups (Table 2). Increased in HB, PCV, MCV might be due to dehydration observed due to diarrhea and sweating as reported by USEPA (1986). Present finding of decrease of MCHC value might be due to inhibition of erythropoiesis was coincided with finding of Rehman *et al.* (2006) @ 75, 112.5 and 150 mg /kg b. wt in carbofuran intoxicated birds. Present finding of decrease of WBC value might be due to immunosuppressive effects of carbofuran was reported by Brkic *et al.* (2008) @ 25, 100 and 400 ppm in carbofuran intoxicated rats, Patil *et al.* (2008) @ in 1, 2 and 4 mg/kg b. wt and Garg *et al.* (2009) @ 4 mg/kg b. in methomyl intoxicated rats. Present finding of increase in neutrophil and monocytes; and decrease lymphocytes might be due to generalized toxicity of carbofuran and metabolic disturbance occurred as reported by Garg *et al.* (2009) @ 4 mg/kg b. wt in methomyl intoxicated rats.

Clinical chemistry

On day 29, statistically significant increased in values of glucose, total protein, ALT, AST, ALP and creatinine were observed in mid and higher dose groups. Statistically significant decreased in acetyl cholinesterase was noticed in animals of all treated groups compared to vehicle control animals (Table 3). It indicated oxidative stress in animals. Serum transaminase activity is known as toxicity marker in study of hepatotoxicity induced by chemicals.

Change in clinical chemistry parameters is reported by Al-Shinnawy (2008), Patil *et al.* (2008) and Munglang *et al.* (2009) and might be due to chemical injury to tissue. Inhibition of acetylcholinesterase activity might be due to inhibition of cholinesterase enzyme at electric switching centers (synapse) leading to nervous manifestation and prevention of breakdown of acetylcholine as advocated by Satar *et al.* (2005), Brkic *et al.* (2008) and Kamboj *et al.* (2008) in carbofuran toxicity.

Organ weigh

Dose dependent decreased in relative organ weights of brain, kidney, liver, ovary, thymus and spleen in mid and higher dosed groups were noticed as compared to vehicle control. There was dose dependent decreased in relative organ weight of ovary in all treated groups (Table 4). Decrease in weight of liver, brain and kidney might be due to generalized in decrease in body weight as advocated by EPA (1986). Present finding of decrease in weight of spleen weight might be due to decrease viability of spleenocytes because of oxidative stress and was coincided with finding of Lohitnavy and Sinhaseni (1998) in methomyl (at 6 and 8 mg/kg) intoxicated rats. Present finding of decrease of weight of ovary might be due to decrease developing follicles and graffian follicles, increase atretic follicles and was reported by Baligar and Kaliwal (2002) in carbofuran (1.3 mg/kg body weight) intoxicated mice, Baligar and Kaliwal (2004) in carbofuran (1.3 mg/kg body weight) intoxicated mice and Ksheeragar and Kaliwal (2008) in carbosulfan (48 mg/kg body weight) intoxicated rats. Decrease in weight of thymus may be due to decrease lymphocytes in cortex and it is reported by Jeong *et al.* (1999).

Pathomorphology

At time of necropsy, congestion was observed in heart, brain, liver and lung (Fig. 1, 2, 3 and 4) in high dose group. Microscopic changes were observed in the liver, spleen, thymus, ovary, kidney, brain, lung, stomach and intestine of high dose group. Microscopic examination of liver showed moderate congestion in central vein, basophilic cytoplasm, proliferation of kupffer cells with dilatation of sinusoid was observed (Fig. 5 and 6). Lymphocytic depletion was observed in white pulp of spleen (Fig. 7). Microscopic examination of kidney revealed dilated tubules with mineralization and protenious materials (Fig. 8). In lung, multifocal hemorrhage was seen with emphysema and sloughing of bronchiolar epithelial cell lining with disruption of alveolar septa (Fig. 9). Few developing follicles, small corpora lutea and many atretic follicles and hemorrhage were observed in ovary (Fig. 10 and 11). Depletion of lymphocytes and phagocytosis with starry sky appearance was observed in thymus (Fig. 12). Disruption of villi of gastric mucosa of stomach (Fig. 13) and intestine; and foci of lymphocytic infiltration in intestine was observed (Fig. 14). Subpial hemorrhage was shown in brain (Fig. 15). Depletion of lymphocytes in spleen in present study might be due to, immunosupression nature of compound, oxidative stress and free redical damage in cellular component as advocated by Lohitnavy and Sinhanseni (1998) and Radad *et al.* (2009). Present finding in liver was coincided with finding of Radad *et al.* (2009) @ 2 mg/kg b.wt in methomyl intoxicated rats and Munglang *et al.*, (2009) @ 200 mg per kg body weight in carbaryl intoxicated rats and Ksheerasagar and Kaliwal (2006) @ 48 mg/kg b. wt in carbosulfan

intoxicated mice. Lesions in lung in present study might be due to extensive storage of carbofuran in lung and excretion in expired air as advocated by Radad *et al.* (2009). Lesions in ovary in present study might be due to hormonal imbalance occurred in toxicity as a result of direct and indirect effects on ovary or hypothalamo-hypopysial ovarian axis as advocated by Ksheerasagar and Kaliwal (2008).

CONCLUSION

Carbofuran administration by gavage up to 28 consecutive days produced toxicity in rats, which is exhibited as loss of body weight, alteration in normal hematology and biochemical values and histopathlogical alteration in various visceral organs in high, mid or low carbofuran dosed group. No-Observed Adverse Effect Level (NOAEL) is not established in present study.

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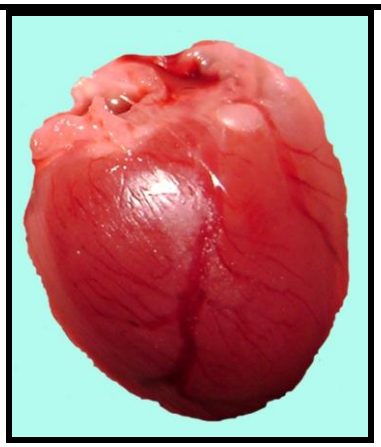

Figure 1*: Heart of group D showing severe congestion with round border*

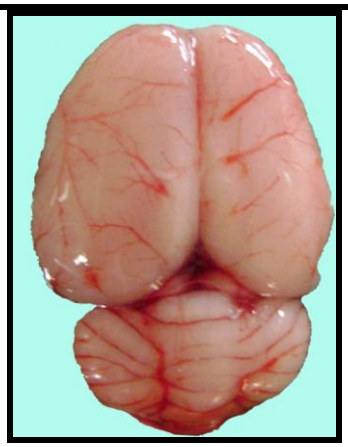

Figure 2:*Brain of group D showing sever congestion.*

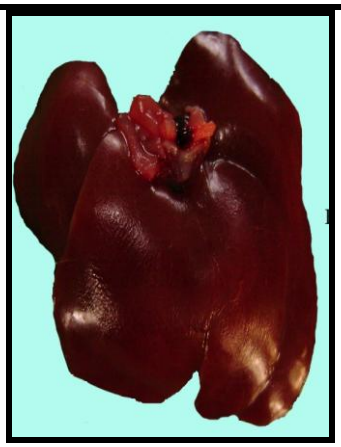

Figure 3: *Liver of group D showing sever congestion*

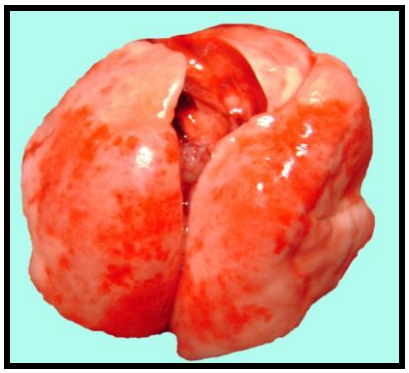

Figure 4: *lung of group D showing sever congestion*

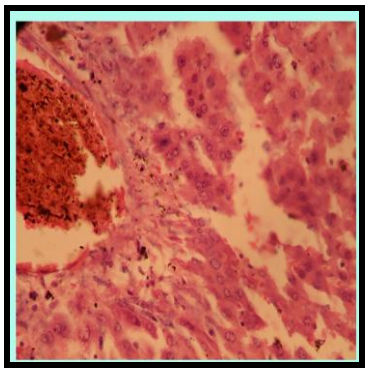

Figure 5*: liver of group D showing moderate congestion in central vein and basophilic cytoplasm (H.Ex400)*

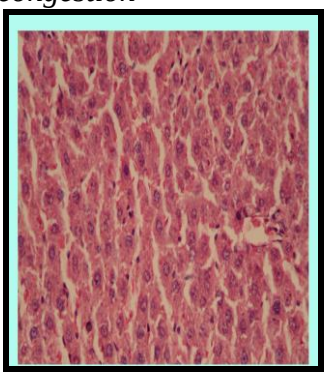

Figure 6*: liver of group D showing proliferation of kupffer cells with dilatation of sinusoid wasobserved (H.E.x100)*

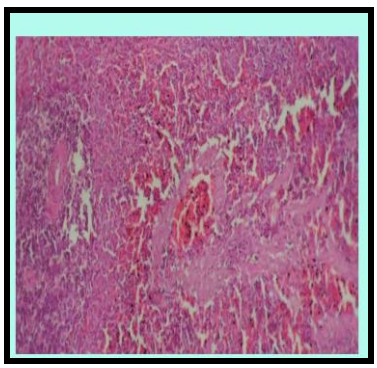

Figure 7*: spleen of group D showing lymphocytic depletion in white pulp (H.E.x100)*

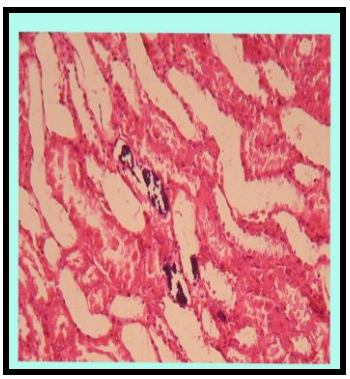

Figure 8*: Kidney of group D showing dilated tubules with mineralization and protenious materials (H.Ex400)*

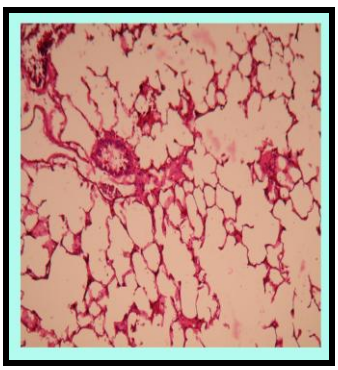

Figure 9*: Lung of group D showing multifocal hemorrhage with emphysema and sloughing of bronchiolar epithelial cell lining with disruption of alveolar septa* (H.E.x100)

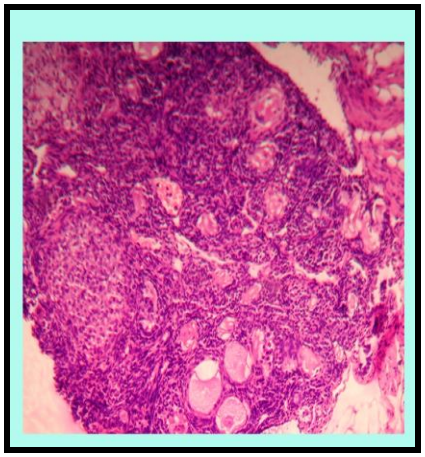

Figure 10:*Ovary of group D showing few developing follicles, small corpora lutea, many atretic follicles and hemorrhage (H.Ex400)*

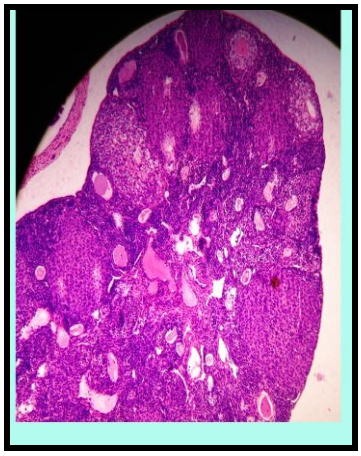

Figure 11*:Ovary of group D showing few developing follicles, small corpora lutea and many atretic follicles (H.E.x100)*

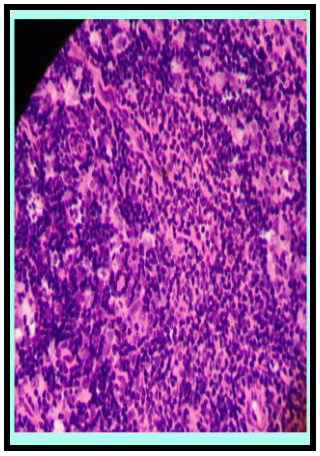

Figure 12*: Thymus of group D showing depletion of lymphocytes and phagocytosis with starry sky appearance (H.E.x100)*

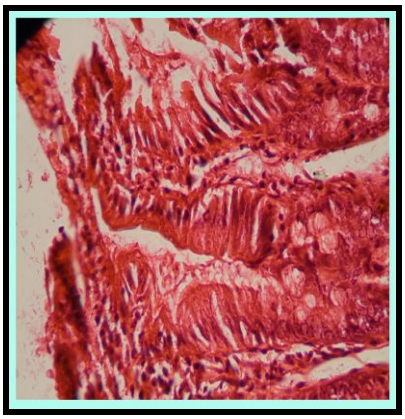

Figure 13*: Stomach of group D showing disruption of villi (H.Ex400)*

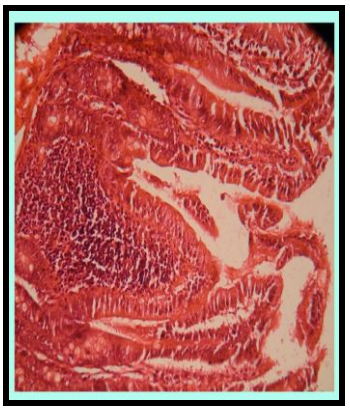

Figure 14*: Intestine of group D showing disruption of villi with lymphocytic infiltration (H.Ex400)*

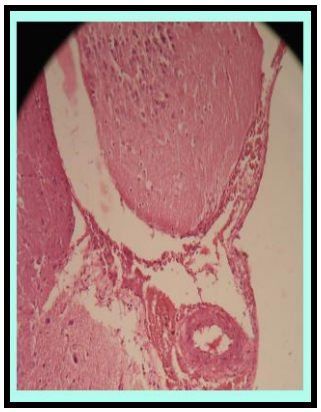

Figure 15*: Brain of group D showing Subpial hemorrhage (H.E.x100)*

Table 1: Effect of carbofuran on Hb, WBC, PCV, MCV, MCH, MCHC,Platelets (Mean ± S.E.) of different experimental group of rats (n=6).

Groups	**Days**				
	1	**8th Day**	**15th Day**	**22nd Day**	**28th Day**
A (control)	230.833 [a] ± 4.070	237.000 [a] ± 3.715	245.500 a ± 4.638	251.167 [a] ± 5.659	261.000 [a] ±4.683
B	226.667 [a] ± 2.044	225.000 [a] ± 1.789	222.167 [b] ± 2.638	218.500 [b] ± 5.071	208.000 [b] ± 3.183
C	235.000 [a] ± 3.011	229.000 [a] ± 2.206	223.500 [b] ± 1.408	208.000 [b] ± 1.506	201.667 [b] ±2.108
D	238.333 [a] ± 3.333	224.167 [b] ± 3.745	215.000 [b] ± 4.082	193.000 [c] ± 3.376	180.333 [c] ±.2.679

❖***Superscripts are to be read column wise for mean comparison.***

❖***Mean with similar superscripts in column do not differ significantly (P < 0.05).***

Table 2: Effect of carbofuran on Hb, WBC, PCV, MCV, MCH, MCHC,Platelets (Mean ± S.E,) of different experimental group of rats (n=6)

Parameters	Groups	Days	
		0 Days	29 Days
Hb (g/dl)	A (control)	15.63 ± 0.338[a]	15.06 ± 0.194[a]
	B	15.8 ± 0.314[a]	13.43 ± 1.115[a]
	C	15.68 ± 0.266[a]	15.65 ± 0.312[a]
	D	15.68 ± 0.215[a]	16.18 ± 0.282[b]
TLC (thousand/ cumm)	A (control)	7.62 ± 0.571[a]	9.72 ± 0.322[a]
	B	8.77 ± 1.04[a]	7.27 ± 0.397[b]
	C	7.85 ± 0.541[a]	5.75 ± 0.491[c]
	D	7.92 ± 0.882[a]	4.18 ± 0.605[c]
PCV (%)	A (control)	44.53 ± 1.315[a]	42.06 ± 0.545[a]
	B	45.15 ± 0.927[a]	60.76 ± 5.187[b]
	C	44.20 ±0.946[a]	70.78 ± 1.227[c]
	D	44.05 ± 0.575[a]	72.61 ± 1.332[c]
MCV (fl)	A (control)	50.79 ± 1.143[a]	50.23 ± 2.087[a]
	B	50.26 ± 1.313[a]	83.65 ± 0.985[b]
	C	52.48 ± 1.393[a]	81.55 ± 1.261[b]
	D	50.43 ± 1.459[a]	82.38 ± 0.589[b]
MCH (pg)	A (control)	17.86 ± 0.537[a]	17.96 ± 0.767[a]
	B	17.35 ± 0.703[a]	18.60 ± 0.751[a]
	C	18.81 ± 0.503[a]	18.0 ± 0.304[a]
	D	17.95 ± 0.514[a]	18.41 ± 0.104[a]
MCHC (%)	A (control)	35.15 ± 0.433[a]	35.78 ± 0.180[a]
	B	35.00 ± 0.419[a]	22.23 ± 0.823[b]
	C	35.47 ± 0.312[a]	22.08 ± 0.087[b]
	D	35.59 ± 0.189[a]	22.26 ± 0.080[b]
Plateletes (x 10^3/ ul)	A (control)	934.16 ± 37.464[a]	888.83 ± 22.928[a]
	B	848.66 ± 39.160[a]	982.00 ± 215.645[a]
	C	819.50 ± 42.474[a]	820.00 ± 99.940[a]
	D	918.83 ± 61.308[a]	987.50 ± 37.939[a]

❖***Superscripts are to be read column wise for mean comparison.***
❖***Mean with similar superscripts in column do not differ significantly (P < 0.05).***

Table 3: Effect of carbofuran on plasma biochemical parameters (Mean ± S.E,) of different experimental group of rats (n=6)

Parameters	Groups	**Days**	
		0 Days	**29 Days**
ALT (IU/L)	A (control)	46.613 ± 1.412 [a]	51.018 ± 1.539 [a]
	B	53.901 ± 2.339 [a]	53.802 ± 2.995 [a]
	C	51.399 ± 2.642 [a]	78.533 ± 2.594 [b]
	D	50.020 ± 2.695 [a]	92.470 ± 2.245 [c]
AST (IU/L)	A (control)	116.241 ± 6.130 [a]	120.763 ± 4.667 [a]
	B	132.744 ± 10.104 [a]	117.053 ± 2.646 [a]
	C	122.279 ± 9.793 [a]	136.657 ± 4.416 [b]
	D	117.106 ± 2.802 [a]	157.698 ± 4.190 [c]
ALP (IU/L)	A (control)	111.907 ± 3.673 [a]	128.188 ± 6.536 [a]
	B	112.543 ± 4.982 [a]	137.260 ± 4.629 [a]
	C	136.935 ± 8.697 [a]	175.066 ± 2.559 [b]
	D	127.467 ± 4.503 [a]	265.734 ± 12.821 [c]
Creatinine (mg/dl)	A (control)	0.673 ± 0.016 [a]	0.684 ± 0.027 [a]
	B	0.661 ± 0.018 [a]	0.725 ± 0.043 [a]
	C	0.645 ± 0.013 [a]	1.039 ± 0.073 [a]
	D	0.689 ± 0.016 [a]	2.463 ± 0.402 [b]
Glucose (mg/dl)	A (control)	95.208 ± 2.394 [a]	103.364 ± 4.388 [a]
	B	94.561 ± 2.004 [a]	121.285 ± 3.190 [b]
	C	95.218 ± 2.476 [a]	130.289 ± 2.246 [b]
	D	92.342 ± 1.204 [a]	184.422 ± 5.900 [c]
Total Protein (g/dl)	A (control)	6.638 ± 0.126 [a]	7.061 ± 0.068 [a]
	B	7.069 ± 0.066 [a]	7.284 ± 0.322 [a]
	C	7.050 ± 0.094 [a]	8.986 ± 0.154 [b]
	D	7.061 ± 0.173 [a]	9.874 ± 0.207 [c]
Acetyl Cholinesterase (IU/L)	A (control)	5775.762 ± 198.847 [a]	5912.851 ± 240.475 [a]
	B	5484.675 ± 180.681 [a]	5327.697 ± 102.516 [a]
	C	5595.454± 150.500 [a]	4293.570 ± 378.151 [b]
	D	5978.980 ± 179.973 [a]	3145.592 ± 210.334 [c]

- ***Superscripts are to be read column wise for mean comparison.***
- ***Mean with similar superscripts in column do not differ significantly (P < 0.05).***

Table 4: Relative organ weights (Mean ± S.E., g.) of various organs in female rats of different experimental groups of rats (n = 6 rats)

Groups	Brain	Liver	Kidney	Heart	Ovary with fallopian tube	Thymus	Lung	Spleen
A (control)	2.261^{a} ± 0.059	10.681^{a} ± 0.369	2.275^{a} ± 0.081	1.250^{a} ± 0.037	0.151^{a} ± 0.008	0.666^{a} ± 0.024	1.626^{a} ± 0.033	0.453^{a} ± 0.017
B	2.275^{a} ± 0.050	10.065^{a} ± 0.246	2.150^{a} ± 0.051	1.248^{a} ± 0.022	0.130^{b} ± 0.004	0.631^{a} ± 0.028	1.535^{a} ± 0.024	0.418^{a} ± 0.022
C	1.955^{b} ± 0.045	8.985^{b} ± 0.256	1.92^{b} ± 0.032	1.221^{a} ± 0.026	0.105^{c} ± 0.005	0.473^{b} ± 0.021	1.548^{b} ± 0.021	0.311^{b} ± 0.021
D	1.681^{c} ± 0.049	7.680^{c} ± 0.227	1.533^{c} ± 0.059	1.220^{a} ± 0.025	0.075^{d} ± 0.008	0.365^{c} ± 0.015	1.563^{c} ± 0.024	0.253^{b} ± 0.027

❖***Superscripts are to be read column wise for mean comparison.***

❖***Mean with similar superscripts in column do not differ significantly (P < 0.05)***

[MS received: August 5, 2014] *[MS accepted: September 3, 2014]*