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Effect of carbofuran on quantitative and qualitative alterations in haemolymph of larva of *Oryctes rhinoceros* L. (Coleoptera: Scarabaeidae)

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ABSTRACT: Investigation to evaluate the toxicity of carbofuran pesticides on haematological parameters of third instar larvae of *Oryctes rhinoceros* L. Indicated alterations in total haemocyte count and differential haemocyte count for toxicity assessment. Various doses of carbofuran (0.05g, 0.010g and 0.015 g) applied on insect through oral route and its impact after 24 hours of its application revealed that various doses of carbofuran exert specific alterations in both total and differential haemocytes of insect haemolymph.

KEYWORDS: carbofuran,changes, *Oryctes rhinoceros*, toxicity, haemocytes

Haemocytes and immune responses are considered to be potential indicators of toxicity in insects. Hence the study of changes in either the total or part of insect haemolymph is a proper system for detecting effects of toxic substances. Toxic substances induce an irreversible cytopoiesis of the host's haemocytes (Vladimir *et al.*, 1991).There are very little study on the haemograms of insects exposed to toxic substances and the role of haemocytes and direct detoxification of pesticides. Cytopoiesis has been proven in insects exposed to lethal doses of arsenates, nicotine dichloro-diethyl ether, carbon tetrachloride and DDT (Vladimir *et al.*, 1991).

Carbofuran is a very toxic pesticide widely used by farmers and registered for more than 25 crops in India. As a result of widespread use, air, water and food are polluted with carbofuran and its metabolites (Bushway *et al.,* 1992; Kross *et al.,* 1992; Waite *et al.,* 1992). Carbofuran has high toxicity to human through the oral and inhalation routes of exposure affecting the nervous system It is highly toxic to birds, bees, fish and non- target species due to its high acute toxicity. The residues of these chemical have been reported in plant, soil and water there for its use has been restricted or banned in many countries (Goulart *et al.,* 2015). Present investigation is focused to evaluate the toxicity of carbofuran pesticides on haematological parameters of third instar larvae of *Oryctes rhinoceros* L. to understand the impacts on circulatory system of insects.

Third instar larvae of coconut beetle, *O. rhinoceros* one of the important economic pests of coconut palm having long life span, voracious feeding habit, sensitivity to insecticides or any control agent and ease in rearing and handling the larvae of *Oryctes rhinoceros* being excellent experimental animal was used as test animal. Various stages of larvae

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were collected from the local manure pits and reared in the laboratory on cow dung which formed the stock. In the present study the third instar larvae, those have long lifespan, peculiar voracious feeding habit and ease of rearing were considered for the isolation from the stock and each larva kept separately in plastic containers with cow dung as feed.

Carbofuran is broad spectrum commercial grade carbamate insecticide used for control of insects, mites and nematodes and being also used against soil and foliar pests of field, fruits, vegetables and forest crops. Carbofuran is highly toxic by inhalation or ingestion and moderately toxic by dermal absorption.

The chemical name of carbofuran is 2, 3- dihydro 2, 2- dimethyl-7- benzofuranyl methyl carbamate. Formulations of carbofuran include flowable or granular formGranular form like Furadan 3G used in the present study is usually prepared by mixing technical grade materials with silica based particles in required proportions. Pure form is a white crystalline solid with slight phenolic odour. It has a melting point of 153-154^{0}C. It is slightly soluble in water. It is highly soluble in N- methyl-2-pyrrolindone, dimethyl formaldehyde, dimethyl sulfoxide, acetone, aectonitrite, methylene chloride, cyclohexanone, benzene and xylene. It is stable under alkaline conditions. Degradation of carbofuran in soil takes place by microbial action. In water, direct photolysis and photo irradiation via hydroxyl radical, 2-hydroxy furadan and furadan phenol are the major pathways of degradation. In the air, degradation occurs by photolysis. Half-life in water is 5.1 weeks at pH 7.0 and 1.2 hours at pH 10 and in the soil several days to over three months (HSDB, 1998)

Three doses of the of the toxicity *viz*., 0.05, 0.010 and 0.015g doses of carbofuran mixed with 30g cow dung each was kept in containers for a day. Then actively feeding larvae after 30-35 days of moulting with an average weight of 9.6 $\pm$ 0.01 g were introduced into each experimental container with four replications along with a control without furadan. Pesticide dose was chosen based on result of preliminary continuous bioassays and probit analysis, (Finney, 1971). Behavioural changes and toxic sighs were recorded daily.

Haemolymph was collected from both treated and control larvae. A puncture was made on the body wall so as to draw the exuded haemolymph using a capillary tube and kept in eppendorf tubes containing a few crystals of phenylthiourea (Wyatt and Pan, 1978) to prevent melanisation. The collected samples were used immediately, for analysis.

Haemolymph smear was prepared according to the method of Arnold and Hinks (1979). The smear of haemolymph was prepared by placing a drop of freshly extracted haemolymph on a clean glass slide and a thin uniform smear was drawn by using a rectangular cover slip at 45 degrees. After air drying for few minutes, the smear fixed in methanol and stained with Giemsa's stain for 5 min., washed in double distilled water and mounted in DPX.

Total haemocyte count (THC) was done by the method of Gosh and Roy (1984). A Newbaur Haemocytometer was used for this purpose (Witting, 1966). The haemolymph was initially collected on micro slides. This haemolymph was taken into a clear WBC pipette filling up to the mark 0.5. Care was taken to avoid air bubbles. The blood sticking to the tip of the pipette was wiped out. Dilution medium (Turk's dilution fluid) was pipette up to the mark 1.1. The haemolymph was allowed to mix thoroughly with the dilution medium. The fluid from the lower end of the pipette was discarded. The counting chamber of the haemocytometer was charged and the preparation was kept aside for the haemocytes to settle. The haemocytes were counted from the four corners squares with the aid of a microscope. Total number of haemocytes was calculated by multiplying the average number of cells in one chamber with the volume of one square of haemocytometer and dilution factor, *i.e.,* average number of cells in one chamber X10 X 20. Following formula of Jones (1962) was adapted for calculations:

$$\frac{\text{Haemocytes in 1mm Squares X dilution X depths at the Chamber}}{\text{number of 1mm square counted}}$$

Haemolymph smears prepared from each experimental larva were examined under a light

microscope, and all cells were counted. The total cells were counted and the percentage of each kind of haemocytes Prohaemocytes (PRC), Plasmatocytes (PLC), Granulocytes (GRC), Adipocytes (ADC), Spherulocytes (SPC) and Oenocytes (OEC) were calculated to arrive the differential haemocytes. All the data were analysed statistically at $p < 0.005$. The significance was calculated by using ANOVA.

Differential Haemocyte Count (DHC) of control and exposed to carbofuran for 24 hours

Differential Haemocyte Count in experiment and control larvae is given in Table 1. Proportion of the PRC in the control was 46.00 ± 3.79per cent while 18.33 ± 1.45 PLC, 20.0 ± 0.58 GRC , 6.67 ± 0.88 ADC, 5.67 ± 1.45 SPC and 3.33 ± 0.88 OEC indicating the population size of PRC was the highest followed by GRC and PLC. The least population was observed in OEC followed by SPC and ADC. When the larvae exposed to 0.005g carbofuran for 24 h the PRC, PLC and GRC were 17.67 ±1.45, 9.00 ± 1.15 and 60.0 ± 2.31 per cent respectively expressing a steep elevation of GRC count over control as against PLC showing a significant reduction in their population. Proportion of the other cell types were 7.00 ± 0.58, 4.66 ± 0.33 and 1.67 ± 0.33 per cent respectively for ADC, SPC and OEC. A slight decrement was noted in the count of SPC and OEC from control value. Exposing to the higher dose of to 0.01g carbofuran, the mean population of PRC was 13.33 ± 0.88 per cent while PLC- 8.00 ± 1.53, GRC- 68.33 ± 0.88 , ADC - 4.67 ± 0.88, SPC - 3.33 ± 0.67 and OEC- 2.33 ± 0.88 per cent wherein, GRC showed steep significant elevation as against the decrement of ADC, SPC and OEC though statistically insignificant.

However 0.015 g carbofuran treated larvae haemolymph exhibited that the mean counts of haemocytes were PRC- 11.00±1.15 per cent of PRC, 6.33±0.88 PLC, 72.00±1.73 GRC, - 5.33± 0.33 ADC, 4.00 ± 0.58 SPC and 1.33 ± 0.33 per cent OEC respectively indicating significant increase in case of PRC, PLC and GRC, however, insignificant in the case of ADC, SPC and OEC count. Overall assessment expressed a significant sharp increase of granulocytes due to exposure larvae corresponding with increase in dose of carbofuran as against other haemocytes got decreased over control.

Cytological study of haemolymph of *O. rhinoceros* larvae-control and exposed to carbofuran

Haemocytes of control larvae comprised of PRCs, PLCs, GRCs, ADCs, SPRs and OECs (Fig. 1). PRCs found in numerous groups were small round cells with dense homogenous cytoplasm and large nucleus. PLCs were spindle shaped cells with a centrally placed round nucleus and surrounding of abundant cytoplasm. GRCs were observed as large spherical or oval cells having more granular cytoplasm and centrally located round or elongated nucleus. ADCs were small round or slightly elongated and centrally or eccentrically located nucleus. The cytoplasm contained characteristic small to large refringent fat droplets and other non-lipid granules in addition to vacuoles. SPCs were round with small central or eccentric nucleus. A number of spherules are found in the cytoplasm. These cells are larger than granulocytes. OECs were small to large oval or spherical cells with a granular, thick, homogenous cytoplasm and centrally located small round nucleus.

High total haemocytes counts with moderate increase in granulocytes were found in the larvae exposed to 0.005g carbofuran for 24h (Fig. 2). Degeneration and nuclear pyknosis was observed in granulocytes. Reactive changes were observed in PLCs whose cytoplasm was darkly stained. Smears of the larvae exposed to 0.010g carbofuran for 24 h showed mild decrease in total haemocytes with increase in number of GRCs which were degenerated with distorted shape having irregular nucleus. Some of them showed enlargement. (Fig. 3).

Blood smear of larvae exposed to 0.015g exhibited mild decrease in total haemocytes with increase in GRCs which were more basidophilic with thickened granules. Clustering of cells with abnormal staining

was observed with ruptured cell membrane and distorted cell shape (Fig. 4). Carbofuran exposed larvae showed a significant decrease of total haemocytes and various cytopathological changes, particularly an increase in GRC sand increased cellular damage compared to control.

The haemocytes of insects constitute a complex system of cells circulate in the haemolymph which play an essential role in immunity against invading substances through coagulation, phagocytosis, encapsulation and detoxification process. Haemocytes are several types and their primary functions also include storage and distribution of nutritive materials. Six types of haemocytes were identified in the haemolymph of *O. rhinoceros* larvae such as PRC, GRC, PLC, SPC, OEC and ADC (Annie, 1995). In the present study their response to different doses of carbofuran at various time periods were investigated. A drastic change in total haemocytes (THC) with various histopathological changes in haemocyte morphology was observed due to carbofuran intoxication. This is because haemocytes are known to respond to various intrinsic or extrinsic factors. Under adverse conditions and at the time of experimental stress, numbers of haemocytes were reported to get increased (Shapiro, 1979, Gupta, 1985). However, the present investigation indicated a decrease in the total haemocytes due to carbofuran treatment which could be the result of degeneration of pathological cells caused by toxicity of carbofuran.

Number of haemocytes is a key factor in compaction of the encapsulation of invading foreign bodies (Sendi and Salehi, 2010). Moreover, PLC and GRC have been found to be most sensitive and the main phagocytic haemocytes in most of the insect studied (Crossley 1964, Arnold, 1970; Neuvarth, 1974; Akain and Sato, 1978). In this sense, differential heamocyte count (DHC) is more meaningful, because, present investigation found sharp increase of GRC in the haemolymph of treated larvae and other haemocytes showed numerical decline over control. Such an increase in the population size of GRC might be connected with the growing demand for cellular immunity (Gupta, 1985, 1986, 1991). This is because granular

Table 1.Total and differential haemocytes in the haemolymph of control *Oryctes rhinoceros* larvae exposed to carbofuran for 24 h.

Doses of carbofuran (g/larvae)	Total haemocyte count (THC) (cu/mm))	Differential haemocyte count (DHC) (%)					
		Prohae-mocyte (PRC)	Plasma-tocyte (PLC)	Granulocyte (GRC)	Adipocyte (ADC)	Sperulocyte (SPC)	Oenocyte (OEC)
Control	7973.33± 1065.85a	46.00± 3.79a	18.33± 1.45a	20.0± 0.58a	6.67± 0.88a	5.67± 1.45a	3.33± 0.88a
0.005	13766.6± 523.87b**	17.67± 1.45 b**	9.00± 1.15 b**	60.0± 2.31b**	7.00± 0.58a	4.66± 0.33 a	1.67± 0.33a
0.010	6563.33± 545.60a	13.33± 0.88 c **	8.00± 1.53c**	68.33± 0.88c**	4.67± 0.88 a	3.33± 0.67 a	2.33± 0.88 a
0.015	3933.33± 348.01d*	11.00± 1.15 d **	6.33± 0.88d**	72.00± 1.73d**	5.33± 0.33 a	4.00± 0.58 a	1.33± 0.33 a
ANOVA	F=37.78 P=.000	F=56.83 P=.000	F=17.73 P=.001	F=242.37 P=.000	F=2.43 P=0.141	F=1.32 P=0.333	F=1.75 P = 0.234

Values are the mean of five observations ±SE
Common alphabets denote insignificant difference between control and doses
Different alphabets denote significant difference between control and doses
(*) significant at 5% level (**) significant at 1% level

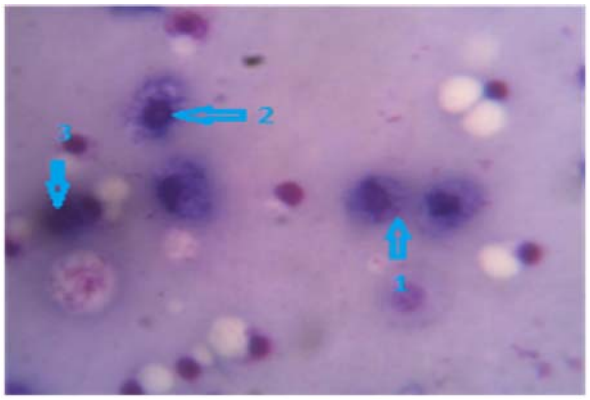

Fig. 1. Blood smear shows heamocytes of control larvae X40x

1. Proheamocyte
2. Plasmatocyte
3. Adipocyte
4. Sperulocyte

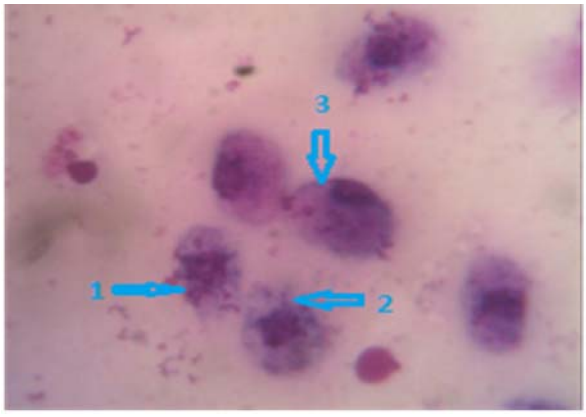

Fig. 2. Blood smear shows heamocytes of 0.005g Carbofuron/ 24 hours

1. GRC shows degeneration
2. GRC shows nuclear pykinosis
3. PLC shows darkly stained cytoplasam

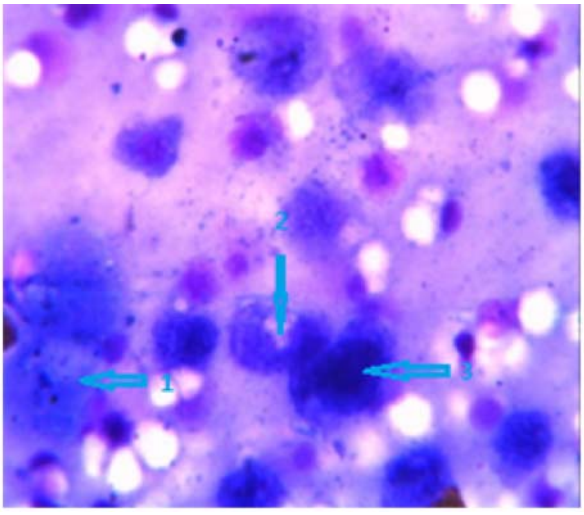

Fig. 3. Blood smear shows heamocytes of 0.010g Carbofuran /24 hours

1. Distortion in cell shape
2. Degeneratative changes with irregular nucleus
3. GRC shows cellular enlargement

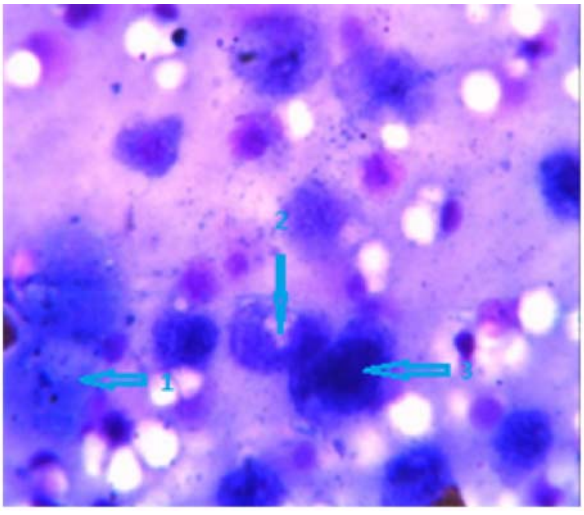

Fig. 4. Blood smear shows heamocytes of 0.015g Carbofuron/ 24 hours

1. GRC shows clustering with ruptured cell membrane
2. Ruptured cell membrane and distorted cell shape
3. Basidophillic with thickened granules

haemocytes are primarily involved in body defence activities (Ambrose and George, 1996). A study of *Atemisia annua* extract in *Eurygaster integriceps* revealed an alteration in the number of hemocytes and their phagocytic activity (Zibaee and Bandani, 2010).

Pathological study of haemolymph indicated dose dependent cellular degeneration due to carbofuran treatment. Numerous changes in cytoplasm and nucleus observed in haemolymph of larvae treated with the highest dose. Vacuolization of cytoplasm and cellular clumbing were the feature of high dose of carbofuran (0.015g) treated larvae as result of aggregation of several cells due to loss of their cells boundaries. Similar pathological systems were reported by using some of the insecticides (Yeager and Manson, 1942; Gupta and Sutherland, 1968; Zaidi and Khan, 1977; Azam and Ilyas, 1986; Younes, *et al.*, 1999; Haq, *et al.,* 2005; Sendi and

Salehi, 2010). Phytochemicals like plumbagin and neem induced similar changes (Sharma *et al.,* 2003). *O. rhinoceros* larvae exhibited a high cytological response to carbofuran indicating that carbofuran exerted peculiar changes in the circulatory system of insect.

Several reports were published on the impact of insecticides in altering the number and morphology of insect heamolymph. Electron microscopic studies of *Spodopteralitura* Fabricious larvae treated with neem gold and *Artemisia calamus* oil found cytoplasmic projections andrapid regeneration in granulocytes. Vacuolization in the cytoplasm and degeneration in organelles in PLCs and GRCs leading to degenerative transformation and degranulation were observed within a period of 48 hours of exposure resulting in the disintegration of immunity-building mechanism (Sharma *et al.,* 2003, 2008). In the present study similar observation could be observed in *O. rhinoceros* treated with different doses of carbofuran.

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