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In vivo genotoxicity evaluation of carbaryl pesticides using polytene chromosomes of *Anopheles culicifacies* (Diptera: Culicidae)

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Abstract

Carbamate pesticides are in tremendous use in modern agricultural practices due to their relatively low persistence in environment but their impacts on genetic imprints of living organism are well documented by various investigations, therefore genotoxicity evaluation of such pesticides has become crucial before their extensive commercial use. The present research work deals with genotoxicity assessment of a carbamate pesticide *viz:* carbaryl by using genetic material of *Anopheles culicifacies*. To achieve the target, mosquito larvae were exposed to LC_{20} of selected pesticide for duration 24 hours and subsequently larval salivary polytene chromosomes were studied to observe detrimental consequences of carbaryl. The concerned carbamate pesticide was observed to induce different types of structural aberrations such as inversions, translocations, deletions, ectopic pairings, asynapses, chromosomal fusions and breaks. After statistical analysis of obtained data, values were calculated 31.35±9.07 in carbaryl exposed stocks as compared 19.36±4.63 in control groups. Subsequently, statically examination of procured data, from executed parameter, demonstrated significant outcomes, when scrutinized through student't' test ($p<0.05$).

Keywords: Polytene chromosomes, carbaryl and *Anopheles culicifacies.*

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1. Introduction

Numerous synthesized agrochemicals are in tremendous applications, among them, pesticides form prominent portion of usage in developing countries than in developed countries. Pesticides generally act selectively against certain organisms without adversely affecting others. However, the improper implementation of such synthetic chemicals may engender drastic consequences ,therefore, most pesticides are generally remain a toxic risk to living organisms including human beings. The chronic effects of these synthetic chemicals , includes the suppression of the human immune system, asthma, reduced sperm count and vigor, blood and liver diseases, nerve damage, depression, tumour and cancer[1-3]. The genotoxic consequences are considered as most serious side effects of agricultural chemicals because if a chemical reacts to genetic imprints of exposed organism and irreversible damage DNA, it ultimately resulted in occurrence of carcinogenic diseases, reproductive dysfunction, and birth defects. Hence biomonitoring of such synthetic compounds have become a serious awareness topic among scientific communities, as such chemicals find direct application on food crops. Consequently, their genotoxic evaluation has become crucial aspect that should be conducted out on intensive levels so that specific recommended exposure limit of these formulations can be concluded in such a way that predicted concentration for application, should be lethal to target pests but should not have any destructive consequence neither to other species of animals nor to environment.

At present, various *in vivo* and *in vitro* assays based on different protocols, using a wide variety of test organisms

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ranging from bacteria, viruses, fungi, insects, rodents and plants, are in immense applications for genotoxic evaluation of these agrochemical formulations. The objective of the present study was determination of cytotoxicity and clastogenic properties of a commercial carbamate pesticide, carbaryl (n-naphthyl, 1-methyl carbamate), at sublethal dose by using *Anopheles culicifacies* genome. Carbaryl, a polycyclic aromatic carbamate formulation is broad spectrum, systemic compound more pronouncedly employed to controls over 100 species of insects normally infest citrus, fruits, cotton, forests, lawns, nuts, ornamentals and shade trees as well as on poultry, livestock, and pets. This carbamate formulation was introduced in 1956 and was the first carbamate insecticide to be successfully marketed for agricultural and household use. Man gets exposed to it at its manufacturing stage, contact during crop dusting and routine handling. Carbaryl is an insecticide which acts primarily by inhibiting acetylcholinesterase activity which is required for breaking acetylcholine neurotransmitter molecules into choline and acetate group, by carbamoylation of a hydroxyl group of serine moiety in active site of this estrase. Other severe biochemical effect of carbaryl is, it reduces glutathione protein which mainly contributes to the biotransformation of xenobiotics, through reactions involving the glutathione conjugation. Carbaryl has half life period of 28 days and due to its moderately toxic chemical properties U.S. EPA classified it as level II pesticide. Although, numerous *in vitro* and *in vivo* investigation are available in literature review, which provide insight into a variety of chronic genotoxic properties and other deleterious effects of this polycyclic aromatic carbamate chemical, on different test models but only limited studies has been carried out on evaluation of clastogenic and cytotoxic effects on genetic imprints, hence motivate by this reason, present research has been executed.

Currently, a variety of biomarkers are used to assess the potential adverse effects of different environmental mutagens on genetic imprints and reproduction potential, using various innovative techniques, on vast variety of experimental models. Correspondingly, mosquito genome has proved quite efficient analytical material for genotoxicity assessment of various environmental contaminants and has been widely used in various evaluation researches. Various investigators have used larval salivary polytene chromosomes of various *Anopheline* species, as analytical tool for mutagenicity evaluation of various chemical agents like antitumour and anticancerous drugs, aromatic amines, mitostatic drugs and salts of various heavy metals like nickel, mercury and lead[4-8]. In such studies, induced damages to genetic material have been measured in term of increased percentage frequency of chromosomal aberrations. The deleterious effects of these compounds were observed in the form of various types of aberrations such as inversions, translocations, breaks, deletions and asynapses of somatically paired homologues of polytene chromosomes. Furthermore, polytene chromosomes provides an opportunity to detect structural alterations in a magnified manner due to their giant size in which, ectopic pairings and asynapsis of chromosomal regions are some of the unique changes not detectable in ordinary chromosomes of the dividing cells. Additionally, *Anopheles culicifacies* was selected as test organism for present investigation due to its easy availability in nature, adaptability to laboratory conditions, short life span and presence of giant sized polytene chromosomes.

The selected pesticide carbaryl was found potential genotoxic and detrimental reproductive inhibitors in various investigations in different test models[9-10]. During present investigation the induced percentage frequency of various structural aberrations were compared with that of control and statistically level of significance was analyzed by application of student 't' test. The present *in vivo* polytene CA assessment studies has been designed to investigate the correlation between inductions of increased percentage frequency of various aberrant chromosomal configurations in salivary gland chromosomes, with exposure to carbaryl, in comparison to natural population.

2. Materials and Methods

Pesticide tested—Carbaryl pesticide is synthesized from 1-naphthol and methylisocyanate (MIC), the latter being known as the active toxic agent released in the Bhopal disaster happened in 1984 in India. It is a N-methyl carbamate pesticide with IUPAC name 1-naphthyl methyl carbamate, CAS registry

number 63-25-2, molecular formula $C_{12}H_{11}NO_2$ and molecular weight 201.22. For present genotoxicity evaluation study, a 100 gm packet of this pesticide, available under trade name Sevin (M/s S. S. Cropcare Limited, India) was procured from market, and was used as such because the basic motive of present investigation was an evaluation of clastogenic properties of this commercial formulation, which is really going to field. Many investigation with pesticides indicate difference between active as well as inert ingredient with respect to mutagenicity, furthermore main conclusion from these studies pointed out that inert ingredients are more toxicity than mutagenic so such compounds generally increase toxicity of synthetic formulations.

Experimental test organism— Anopheles culicifacies was selected for present investigation as it provides good quality temporary squash preparations of salivary polytene chromosomes. *Anopheles culicifacies* is a principal vector of malaria in rural and peri-urban areas of India[11]. The adults of the species prefer to rest indoors in cattle sheds and human dwellings. *Anopheles culicifacies*, was selected as test organism for present investigation. To achieve the target, the gravid females of this species were collected from a village Nada sahib, about 20 km southeast of Chandigarh, with the help of mouth aspirator and were carried to laboratory in small field collection cage made of mosquito net cloth. Subsequently, those captured gravid female mosquitoes were individually transferring to different test tubes, identified by examined with help of 5X magnification hand lens, by following suitable identification key[12]. After that they were allowed to lay eggs singly on a moist filter paper placed in each test tube placed in mosquito breeding cages at 25 ± 1°C, 70 ± 10% humidity and 12h/12h photoperiods. The eggs obtained from them were allowed to hatch in distilled water filled enamel bowl under controlled conditions of BOD incubator at optimal conditions of temperature and humidity. The larvae hatched from them were reared by feeding them on protein rich diet made from dog biscuit and yeast extract mixed in ratio of 6:4[13 -14]. The feed was gently sprinkled on the surface of rearing medium whose quantity was adjusted according to stage and density of larvae. Rearing medium was changed daily to avoid scum formation for healthy growth of larvae. A colony was reared from them under controlled conditions of laboratory.

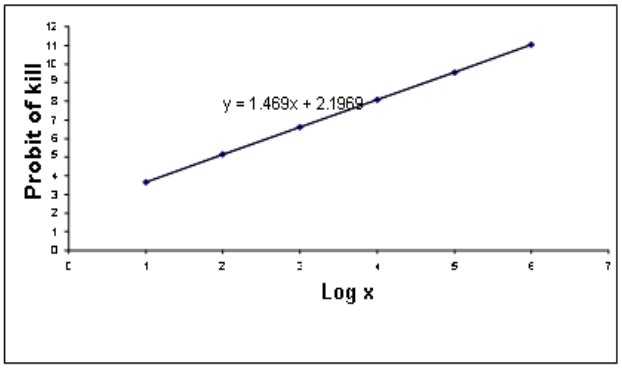


Figure 1. Relationship between the probit of kill and doses of carbaryl for *Anopheles culicifacies.*

Standardization of dose and mode of exposure— LC_{20} was calculated on the basis of mortality of second instar larvae exposed to serial dilution concentrations of stock solution of pesticide (1%) for 24 hours. Desired concentrations of serial dilution were prepared by adding aliquots of the stock solutions in distilled water. To test each of these concentrations, three replicates of twenty larvae were kept simultaneously, with respective controls under controlled conditions of laboratory. The mortality of larvae was monitored after 24 hours and only larvae whose bodies were completely devoid of the exuviae were recorded as alive. On the basis of mortality of larvae in each set the exact value of LC_{20} was calculated by probit analysis[15] which was 14.45 nl/ml µl/ml for *Anopheles culicifacies* during present study as elucidated[16] by Figure 2. The mortality in the control group was taken to be the natural response rate. For polytene chromosomal study , second instar larvae of *Anopheles culicifacies* were given treatment of statistically analyzed LC_{20} for 24 hours, subsequently, they were transferred to distilled water and were reared by feeding on protein rich diet. Present research work was carried out at LC_{20} level as it is sublethal dose which exhibits toxicity but does not drastically reduce the population of test organisms.



Figure 2. Normal compliment of polytene chromosome obtained from control stock of *Anopheles culicifacies.*

Methodologies for polytene chromosome aberrations study — Fourth instar larvae were sacrificed for making temporary squash preparation by following standard staining appropriate procedure [17] with suitable modifications such as dilution of stain and duration of staining. Slides were screened under digital phase contrast microscope and various chromosomal complements with proper spreading and staining were photographed. Those compliments were studied for various types of structural chromosomal aberrations such as inversions, translocations, deletions, ectopic pairings, asynapses, chromosomal fusions and breaks. From obtained data for carbaryl treated stocks and their control, the percentage frequency of various type of aberration were determined. After that the level of significance of induced toxicity was calculated by applying Student 'T' test.

3. Results

During the present course of research work, a total of 58 different types of chromosomal aberrations in the form 30 ectopic pairings, 13 inversions, 13 chromosomal fusions, 1 asynapsis and 1 break were observed in the polytene chromosomes of nontreated larvae with percentage frequencies 29.41, 12.75, 12.75 , 0.98, , 0.98 respectively, whereas, carbaryl treated stocks suffered 94 such aberrations as there were 35 ectopic pairings, 28 inversions, 18 fusions, 6 asynapses, 5 translocations, 2 breaks having percentage frequencies of 34.31, 27.45, 17.65 , 5.88, 4.9 and 1.96 respectively (Fig 3-12). In summary, the frequency of various CA showed significant difference between chemically exposed groups and control groups. Moreover, frequency of normal cells decreased as the frequency of aberrant cells with structural chromosomes aberrations increased during treatment. All this obtained data is specifically illustrated in Figure 4. Statistical analysis of this data corresponds to mean percentage (±SD) of the aberrant chromosomes was significantly higher in treated groups than controls, as indicated by the values 31.35±9.07 in carbaryl exposed stocks as compared 19.36±4.63 in control groups (Table 1). Furthermore, when the obtained values were analyzed statistically, through student't' test (Fig. 13), the apparent discrepancy between in treated and control was found to be highly significant ($p<0.05$).



Figure 3. Chromosomal ectopic pairing in carbaryl treated stock.



Figure 4. Chromosomal ectopic pairing and translocation in carbaryl treated stock.



Figure 5. Chromosomal paracentric heterochromatic inversion in carbaryl treated stock.

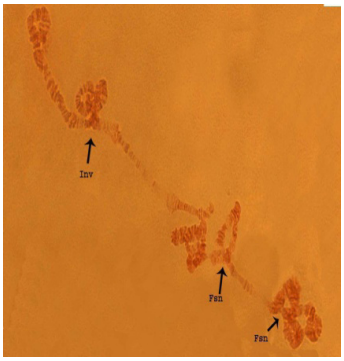


Figure 6. Chromosomal paracentric heterozygous inversion and fusion in carbaryl treated stock.

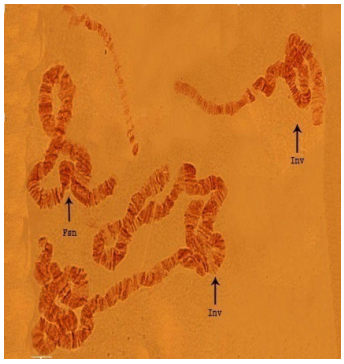


Figure 7. Chromosomal paracentric heterozygous inversion and fusion in carbaryl treated stock.

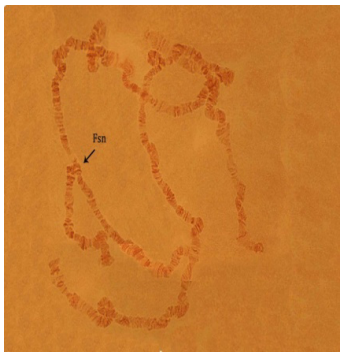


Figure 8. Chromosomal fusion in carbaryl treated stock.

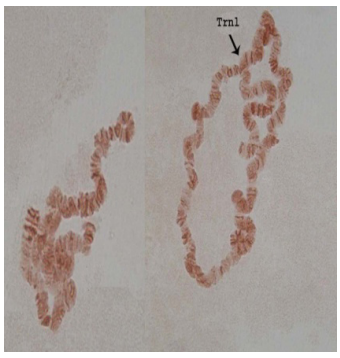


Figure 9. Chromosomal translocation in carbaryl treated stock.

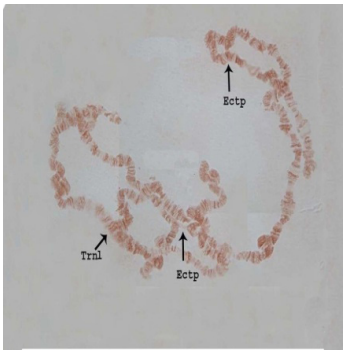


Figure 10. Chromosomal ectopic pairing and translocation in carbaryl treated stock.

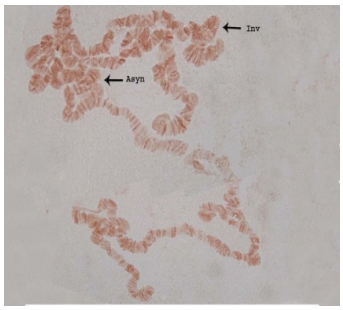


Figure 11. Chromosomal paracentric heterozygous inversion and asynapsis in carbaryl treated stock.

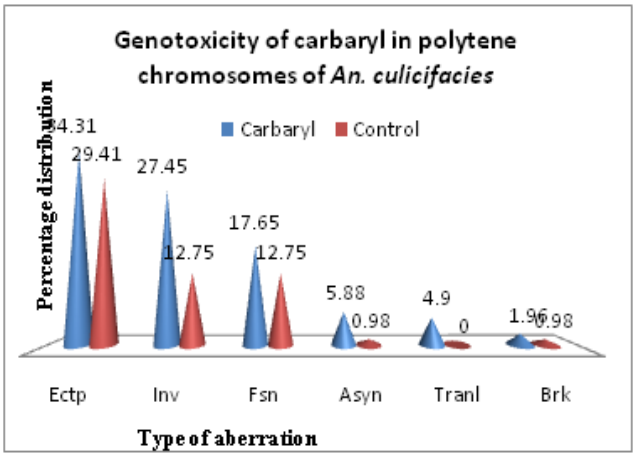


Figure 12. Percentage distribution of chromosomal aberrations in the polytene chromosomes of carbaryl treated stocks. Carbaryl induced maximum incidence of ectopic pairing and minimum number was for chromosomal breaks in treated individuals (Ectp-Ectopic pairing,Inv-Inversion,Fsn-Fusion,Asyn-Asynapsis,Tranl-Translocation,Brk-Break).

Table 1. STATISTICAL ANALYSIS OF ABERRATIONS IN THE CONTROL AND CARBARYL TREATED STOCKS

Nature of stock	Inversion Mean±S.D.	Translocation Mean±S.D	Ectopic pairing Mean±S.D	Asynapsis Mean±S.D	Fusion Mean±S.D	Break Mean±S.D	Total Mean±S.D	't' value
Control	4.34±0.58	-----	10.00±1.73	0.34±0.58	4.34±1.16	0.34±0.58	19.36 ±4.63	3.81
Treated	9.34±2.08	1.67±1.16	11.67±2.52	2.00±1.00	6.00±1.73	0.67±0.58	31.35±9.07	

d.f=4; p<0.05; (S.D.=Standard Deviation, d.f.=degree of freedom)

4. Discussion

Normal compliment of the salivary polytene chromosome preparations from the healthy and active fourth instar larvae present a set of three unequal and well banded elements comprising of a short X-chromosome and two much longer autosomes of unequal size. In majority of the preparations, all the three polytene chromosomes which include right and left arms of chromosomes 2 and 3 (2R, 2L, 3R, 3L) and a long arm of the X-chromosome, are found connected by their centromeric positions to form a heterochromatic mass called chromocenter. Procured data from present research work is presented in Table 1, which indicated the astoundingly elevated incidences of chromosomal aberrations in polytene chromosome cells of treated stocks than control groups. Obtained values apparently demonstrated the association of induced genotoxicity with exposure of pesticide carbaryl in comparison to natural population. Predominate chromosomal aberrations such as ectopic pairing, inversion, fusions, translocation, asynapsis and break were observed in pesticide exposed groups. Additionally, characteristic chromosomal abnormality mainly represented in the form of chromosome translocations were observed only in salivary gland cells of pesticide exposed groups. It was also observed that in genetic material of treated individuals, carbaryl has maximum potential tendency to cause structural CA inform of chromosomal ectopic pairing while lowest frequency of aberrant cells with chromosomal break was discerned from exposed population. After ectopic pairings, chromosomal inversions and fusions were more frequent aberrations as compared to other chromosomal changes. Additionally, few incidences of asynapsis, translocation and chromosomal breaks were also noticed.

Various other researchers have also observed different type of chromosomal aberrations in the polytene chromosomes of different test organisms exposed to environmental mutagens and have concluded that such structural changes to a number of possible reasons. In one study, while studying the effects of lead on the polytene chromosomes of *Glyptotendipes salinus* concluded that the incidence of ectopic pairings was dependent on the organization of the genome in the polytene chromosomes of an organism and interspersed repetitive DNA1[18]. In another study, observed ectopic pairing in the polytene chromosomes treated with quinacrine fluorescent staining[19]. They found the incidence of ectopic pairing between bands of segments 81 F and 83 F of chromosome 3 due to sequence homologies of the heterochromatic DNA bands associated in such associations. Similarly, while studying the effects on the salivary gland polytene chromosomes of *Chironomus riparius* treated with 0.005, 0.01, 0.05 mg/l concentration of copper for 48 h, attributed that chromosomal inversions occurred due to the breakage of chromosomes at certain weak points which resulted in the 180^0 rotation and reinsertion of segment between the two breaks[20]. Recently, one of study had provided general conclusion that most of the natural or induced somatic chromosome rearrangements occur more frequently in those regions of the chromosomes which are composed of repetitive DNA[21]. So it could be concluded that during present genotoxicity evaluation study carbaryl has induced sequence homologies of the heterochromatic DNA bands which resulted in induction of ectopic pairing while reason for chromosomal inversion may be attributed due to the breakage of chromosomes at certain weak points. A translocation takes place when a part of a chromosome breaks and joins with same or another homologous or nonhomologous chromosome. Sometimes, parts of the two chromosomes get exchanged between themselves leading to reciprocal translocation, whereas in an intercalary translocation complete or a part of the chromosome may get inserted into a place along the length of another chromosome. Although translocations are less frequent yet they have been encountered in some species of the subgenus *Cellia* in which the commonest are those in which the

X-chromosome gets inserted into an autosome after suffering a deletion of a small part . This aberration occurs due to association between different DNA rich bands of the same or different chromosomes. When present, it can be easily recognized due to the presence of a thread like connection between bands pairing ectopically. Asynapsis or separation of the intimately synapsed homologues of a polytene chromosome is also an aberrant condition which can occur rarely in nature but more frequently in experimental populations. The environmental factors in nature and treatment with a mutagen in the laboratory can cause these aberrations. A chromosomal fusion can take place between broken ends of the same or different polytene chromosomes . Chromosomal break is the commonest manifestation of a mutagen in which, depending upon the extent of its effect there can be the fragmentation of chromosomes. However, aberrations like inversions, translocations and deletions also need one or more than one simultaneous chromosomal breaks. It could be possible that elevated number of all these abnormalities could be due to interaction of carbaryl with genetic material.

The present investigation is also in accordance to number of other studies available in scientific literature about genotoxicity of carbaryl which provides considerable evidences regarding detrimental consequences on genetic imprints and on reproductive potential of an organism exposed to it. In an earlier *in vitro* study, carbaryl was found to induce unscheduled DNA synthesis in human cell culture and reason behind this effect was the chemical mechanism of metabolism of carbaryl to form an intermediate called diol via epoxide intermediate, characteristically similar to other potential carcinogenic compounds [21]. This synthetic chemical was reported to disturbed spermatogenesis, loss of sperms, detachment of germ cells from the basement membrane and accumulation of cellular mass in the lumen of seminiferous tubules in rats. This happened due to degeneration of leydig cells which ultimately lowered the normal level of testosterone hormone[22] . Moreover, literature review deciphers that 1- naphthol (a metabolite of carbaryl) has been explored to have inhibitory chemical properties for protein phosphatase, specifically required for entry into anaphase [23]. The present finding also demonstrated that the results of DLT was also in consistency with the discussion depicted here which suggested the positive correlation between carbaryl exposure and abnormal reproductive outcomes in exposed individuals.

As such, despite of ubiquitous application of this pesticide, available literature unambiguously suggestive of association of quite hazardous effects to human health particularly to the workers occupationally engaged in production and handling of this pesticide. Wyrobek *et al.* (1981) noticed that carbaryl exposed workers had significantly higher proportion of sperm head abnormalities which were related to genetic damage[24] whereas in another exploration, conclusion indicated that Xia *et al.* (2004) observed that carbaryl induced DNA fragmentation and numerical chromosomal aberrations of X, Y and chromosome 18 in the sperm cells of exposed workers which finally considered responsible for adverse effects on their reproductive system[25]. Decreased sperm motility and concentration were recorded by Meeker *et al.* (2004) in rat exposed to carbaryl which concluded that these effects were due to increased lipid peroxidation which reduced the intracellular level of glutathione[26].

5. Conclusion

The overall conclusion from, polytene chromosomal study is that carbaryl possess detrimental potential to effect integrity of genetic material hence possess tendency to induce divergent chromosomal configurations such as ectopic pairing, inversion, fusion, translocation, asynapsis and break in exposed individuals. Moreover intense efforts are required for further genotoxic evaluation of the pesticide on more intensive level before using such formulations on more wider scale. Considerable genotoxicity of synthetic pesticides , the non-chemical alternatives include cultural practices such as polyculture, crop rotation, use of biological products and agents including beneficial insects which are natural enemies of target pests should be encouraged .

6. Acknowledgment

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7. Conflict of Interest

None Declared

8. References

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