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In vivo genotoxicity evaluation of thiamethoxam using *Drosophila melanogaster*

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Abstract : Present research execution deals with genotoxicity evaluation of a systemic neonicotinoids insecticide, thiamethoxam by using larval salivary polytene chromosomes of *Drosophila melanogaster,* exposed to LC_{20} for 24 hours. To achieve specific target, third instar larvae were sacrificed for temporary squash preparation of polytene chromosomes, both in treated and control stocks. Structural alterations induced in salivary polytene chromosomes of exposed groups, were considered and were compared with natural population. The procured data indicated that the genotoxicity of concerned insecticide was co-related with enhanced frequency of chromosomal aberrations in treated stocks, in comparison to natural population with corresponding vales 32.3±8.25 and 18.33±5.57 respectively. Furthermore, it was discerned that treated stocks comparatively more structural malformation including intra chromosomal ,inter chromosomal and telomeric fusions, paracentric inversions, intrachromosomal and interchromosomal ectopic pairings, asynapses, translocations and breaks , were reported, whereas in control groups only inversions, fusions and ectopic pairings were reported, whereas in control groups only fusions, inversions and ectopic pairings were observed. Subsequently, procured data was analysed by student't' test, which indicated statically significant induced genotoxicity due to selected insecticide.
Key words: Genotoxicity evaluation, thiamethoxam, polytene chromosomes, *Drosophila melanogaster*.

Introduction and Experimental

In modern agricultural practices, the use of synthetic agro-chemicals has become unavoidable, to fulfil the increasing demand of food for continuous growing human population. Therefore, billion tons of synthetic chemicals are being sprayed on agricultural crops to control different categories of pests. Additionally, pesticides are predominately used to control the insects and other pathogenic organisms, which are related with the vector-borne diseases such as dengue fever, Zika virus and other arboviruses, malaria, Chikungunya, filariasis, West Nile virus, yellow fever, viral encephalitis and many other pathogenic infections, which pose threat to the health of human populations. Besides that, exposure of pesticide formulations to human, can occur during manufacture, transportation, application time, improper handling or by accidental. Such chemicals can find their way into the body via oral, dermal or respiratory routes. Generally, such chemicals are associated with chronic and acute effects, which include abdominal pain, dizziness, headache, nausea, vomiting, skin rash and impairment of vision, respiratory complications, memory and dermatological disorders, immune suppression, cancer, depression, neurological complications, miscarriages and birth defects[1-7]. As use of pesticide formulations has become unavoidable, therefore, it has become crucial to find out suitable exposure limits and genotoxic impact of various agro-

chemicals on living organisms, before intensive application of such chemicals on agricultural commodities. Numerous genotoxicity evaluation reports are available in scientific literature, specifying innovative techniques with appropriate biomarkers, on different test organisms. Similarly, polytene chromosomal aberrations analysis had been used by various investigators on different test organisms, where increased percentage frequency of various chromosomal aberrations in chemical exposed had been considered as basic criterion to measure genotoxicity of suspected chemicals[8-18]. Motivated by peculiar cognition, present research work had been executed to find out the genotoxic instinct of concerned insecticide on *Drosophila melanogaster*.

Thiamethoxam is selected for present research as only limited research investigations have been carried out in field of genotoxicity. Thiamethoxam, a neonicotinoids insecticide, is a systemic, broad spectrum insecticide applied to control aphids, whitefly, thrips, ricehoppers, ricebugs, mealybugs, white grubs, flea beetles, wireworms and ground beetles on field crops leafy and fruity vegetables, potatoes, rice, cotton, citrus, tobacco and soya beans, cereals, sugar beet, peas, sunflowers etc. The absorption of concerned insecticide takes place by stomach lining or by direct contact. The mode of action of the thiamethoxam insecticide includes interference in functionality of nicotinic acetylcholine receptors in the central nervous system, which ultimately resulted in paralyzes the muscles of the insects. The insect acetylcholine receptors are more sensitive than mammalian acetylcholine receptors, therefore the concerned chemical is more toxic to insects in comparison to mammals. According to Food and Agriculture Organization (FAO), thiamethoxam is moderately hazardous to human (WHO class III). Few other reports are also available in scientific literature that specify that the concerned chemical possess cytotoxic and cytoclastic consequences that affect genomic structural and functional integrity, in different organisms.

In present research execution, mutagenicity of thiamethoxam is assessed at sublethal concentration, a comparatively low exposure limit than actual field exposure concentration. To achieve present objective, percentage frequency of chromosomal aberrations in treated stock is compared with that of control population. Subsequently, procured data from pesticide exposed group is compared with control stock and statistical analysis was done, which indicated statistically significant induced genotoxicity.

Test Model : *Drosophila*, a holometabolous insect (2n=8) has been selected for present research execution , as it is easy to handle, short life span ,high fecundity rate, adaptability to laboratory conditions and presence of polytene chromosomes . To achieve the present target, the *Drosophila melanogaster*, Oregon strain was used, and the initial stock was received from Department of Biotechnology, Punjab Agriculture University, Ludhiana, which was maintained at 25+1 ^{0}C in BOD incubator.

Laboratory maintenance of test organism*:* Larvae and adult of *Oregon* strain of *Drosophila melanogaster* were reared in BOD incubator set at 25$\pm$ 1 ^{0}C. The culture medium for rearing was prepared by mixing agar, yeast, maize powder and brown sugar in cultural vials. The medium was kept hydrated for easy movement of larvae and kept away from dirt, direct sunlight and other heat sources.

Insecticide: Thiamethoxam, a neonicotinoid, broad spectrum, contact and systemic insecticide, is widely used against a variety of sucking pests of plants and animals. Thiamethoxam has molecular formula $C_8H_{10}ClN_5O_3S$ [12] (Figure.1) and CAS No. 153719-23-4. For present research execution, a 100 ml packet of this pesticide, available under trade name Pele (Bharat Insecticides Limited, India) was procured from market, and was used as such as the basic motive of present investigation was an evaluation of clastogenic and cytotoxic instrict of selected commercial formulation, which is really going to agro-fields.

Standardization of selected concentration and mode of exposure of test organism— For LC_{20} calculation, mortality of second instar larvae, exposed to serial dilution concentrations of 1% stock solution for 24 hours, was observed. Required concentration of serial dilution was prepared by adding aliquots of the stock solutions in culture medium. For each particular dose, three replicates of twenty larvae were kept simultaneously, with respective controls under controlled conditions of laboratory. In each experimental set, mortality of exposed larvae in treated group and in control groups, was monitored after 24 hours . Exact value of LC_{20} was calculated, on the basis of observed mortality of larvae in each set, by probit analysis, which correspond to value of 19.9 pl/ml for *Drosophila melanogaster*, as elucidated by Figure 2. The no mortality was observed in the control groups.

Methodology for temporary squash preparation: For temporary squash preparation, healthy and active third instar larvae were dissected in 0.67% saline taken in a cavity slide, and from thoracic region, two bilobed salivary glands were taken out (Figure 3). For proper staining of salivary glands , standardized technique was

followed , with required modifications[13-20]. The staining was carried out in 2% lacto-aceto-orecein stain. The chromosomal complements which were well spread and with proper banding pattern were photographed (Figure 4) and were scrutinized for various chromosomal aberrations in treated and control stocks.

Data Analysis: The obtained data of various structural malformation including fusions (Figure 5), inversions (Figure 6), ectopic pairings(Figure 7), asynapses (Figure 8), translocations and breaks, in treated and control stocks, was considered for analysis of mean, standard deviation and further statistical significance of data was analysed by carry out student 't' test.

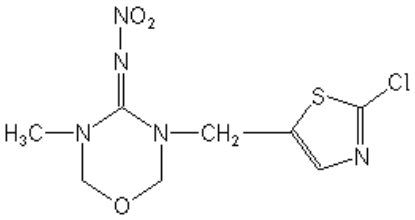

Figure 1: Chemical structure of thiamethoxam

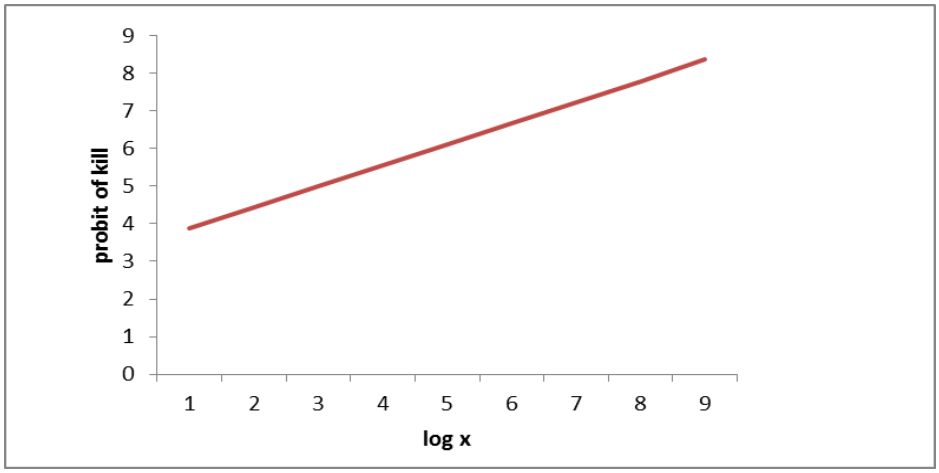

Figure 2: Regression line between concentration of thiamethoxam and probit of kill of larvae

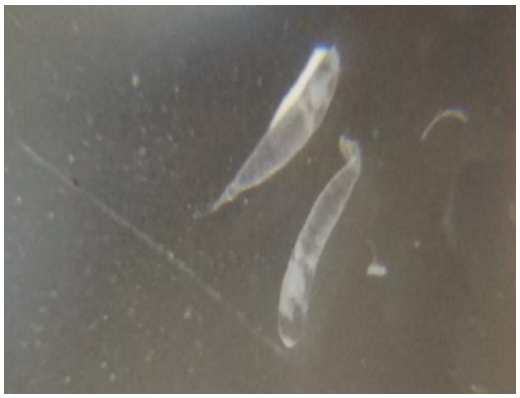

Figure 3: Salivary glands of *Drosophila Melanogaster*

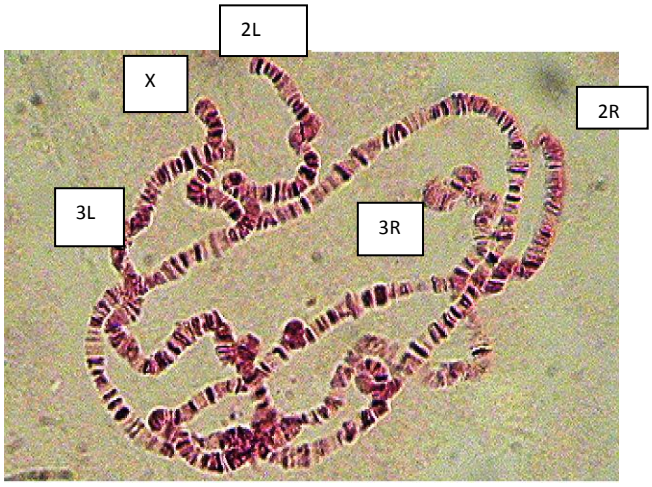

Figure 4: Normal Compliment in *Drosophila melanogaster*, with intact chromo-center and chromosomal arms radiation out from it

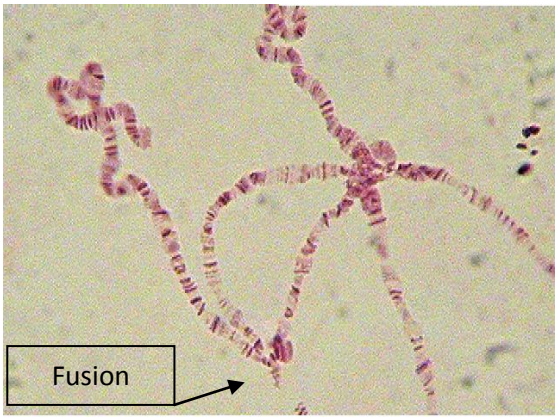

Figure 5: Paracentric chromosomal inversion in treated group

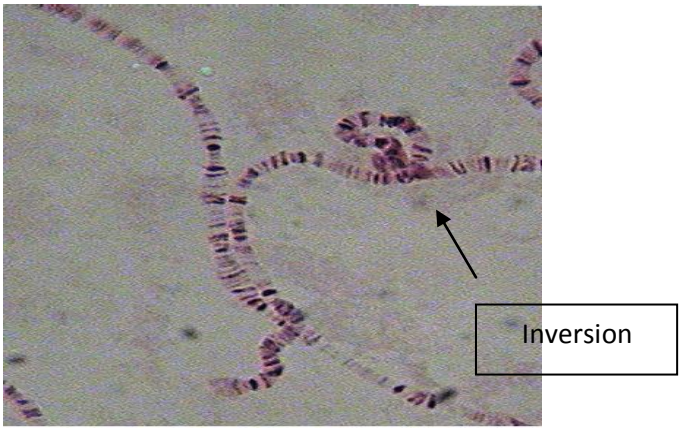

Figure 6: Chromosomal Asynapses in treated group

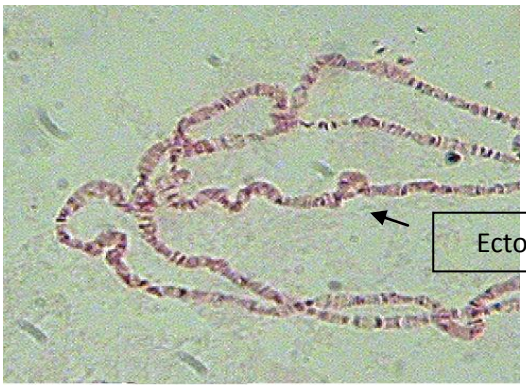

Figure 7: Chromosomal fusion in treated group

Figure 8: Ectopic pairing in chromosomes

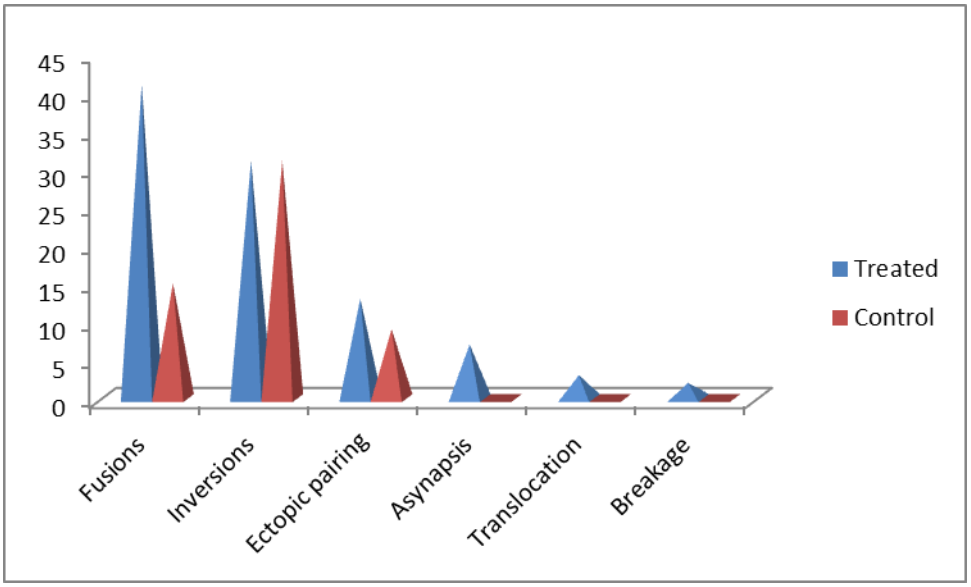

Figure 9: Number of various types of structural chromosomal abnormalities in treated and control stocks. Thiamethoxam was observed to induce maximum chromosomal fusions, which could be due to induced stickiness in genetic material. Asynapses , translocation and breaks were reported only in insecticide exposed sample only. More incidences of ectopic pairing was reported in treated groups in comparison to natural population.

Table 1: Statistical analysis of chromosomal aberrations in treated and control stocks.

Type	Fusions (Mean±S.D)	Inversions (Mean±S.D)	Ectopic pairing (Mean±S.D)	Asynapsis (Mean±S.D)	Translocation (Mean±S.D)	Breakage (Mean±S.D)	Total	t value
Treated	13.66±1.15	10.33±2.16	4.35±2.16	2.33±1.6340	1±0	0.66±1.15	32.3±8.25	3.5*
control	5±2.00	10.33±2.16	3±1.41	--	--	--	18.33±5.57	

Degree of freedom 4
S.D= Standard Deviation
*p>0.05

Results and Discussion

Genotoxicity evaluation study had been carried out in three different replicates, and it had been reported that in salivary polytene chromosomes of *Drosophila melanogaster* of control stock, total 56 chromosomal aberrations were present in form of paracentric chromosomal inversions, fusion due to sticky telomeric end formation, intrachromosomal and interchromosomal ectopic pairing, whereas, in larval salivary polytene chromosomes of pesticide exposed groups, total 97 structural chromosomal aberrations were reported, in the form of fusions, inversions, ectopic pairing, asynapsis, translocation and breaks. Furthermore, it was observed that selected insecticide possess maximum tendency to cause chromosomal fusion in salivary gland polytene chromosomes, followed by inversions, ectopic pairings, asynapses, translocations and breaks with corresponding values 42.26, 31.95, 13.40, 7.21, 3.09 and 2.06 while in control stocks only fusions, inversions and ectopic pairings was noticed. It was further observed that structural chromosomal aberration such as asynapses, translocations and breaks were only observed in treated sample, not even a single incidence of aforesaid aberrations was observed in control stock.

Salivary polytene chromosomes of *Drosophila melanogaster*, comprise four giant sized chromosomes ; one acrocentric chromosomes, two metacentric chromosomes and a dot like fourth chromosomes. All four chromosomes are connected at the centomeric position and form chromocenter, rich in heterochromatin. In temporary squash preparation with intact chromocenter, all four arms of polytene chromosome usually radiate out from it (Figure 4).

During present genotoxicity assessment study, pesticide concentration related enhanced percentage frequency of different structural chromosomal aberrations including fusions, inversions, ectopic pairing, asynapsis, translocation and breaks, in treated stocks, was observed, in comparison to control stocks. Available scientific literature indicated that various structural and numerical chromosomal malformations usually results due to abnormal interaction of different mutagens with genetic material and with other bio-molecules of living organisms. During present genotoxicity evaluation study, maximum incidences of chromosomal fusions were discerned in treated stocks. Furthermore, an elevated percentage frequency of intrachromosomal, interchromosomal and telomeric fusions was reported in treated stocks, in comparison to control stocks. Various research executions carried out so far concluded different responsible factors for occurrence of chromosomal fusion, as one of report on D*rosophila*, concluded that the responsible factor for chromosomal fusion is malfunctioning of certain the impaired proteins[21], whereas one another study indicated that chromatid fusion generally occur due to simultaneous breakage of chromatids or by the loss of telomere capping[22]. In addition to chromosomal fusions, comparative analysis of pesticide treated and control stocks indicated that the elevated frequency of inversions, and ectopic pairings. Numerous reports are procurable in scientific literature, which suggest that different environmental mutagens including drugs, preservatives, pesticides and radiations, generally induced various type of aberrations in the genetic material by causing chromatid breaks. Sometimes, the interstitially chromatid breaks rotate by 180^0 and gets reinserted in the same place, which ultimately resulted in inversion. One of the investigation carried out on spleen cells of mice indicated that, due to effects of mitomycin C, X-irradiations and methylene chloride, somatic intrachromosomal recombination (SICR) occurs that leads to genetic abnormalities in the effected individuals[23]. Furthermore, it was observed that inversions, in the salivary gland polytene chromosomes of *Chironomus riparius*, exposed to different concentration of copper, treated for 48 hrs, occur due to the breakage of chromosomal at weak points[24]. Similarly, in one of investigation, it was reported that various environmental pollutants induced chromosomal inversions in the polytene chromosomes of naturally inhabiting species of *Chironomus*[25], whereas, another study provided

general conclusion, that most of the somatic chromosome rearrangements occur more frequently in the specific regions of the chromosomes which are composed of repetitive DNA loci[26]. Moreover, reasons snapped from literature search pointed out, possible reasons for chromosomal inversions could be structural changes induced by various mutagens in different test models. In comparison to the elevated frequency of fusions, inversion, ectopic pairings, in pesticides exposed individuals suffered from lower frequency of chromosomal asynapses. During the present mutagenicity assessment study, asynapsis of the somatically paired homologues of polytene chromosomes, was also a peculiar chromosomal aberration in the insecticide treated individuals, whereas not even a single incidence of aforementioned chromosomal malformation was reported in control groups. Earlier, executed investigations, concluded asynapses aberration occur due to the denaturing of the binding proteins, due to abnormal interaction of exposed chemicals. In one of investigation, it was reported enhance percentage frequency of chromosomal asynapsis were more in polytene chromosomes of mosquito larvae treated with lead and mercury, than in their control stocks[27]. Similarly, few other reports concluded that the induction of asynapsis in the polytene chromosomes of *Glyptotendipes barbipes*, treated with semilethal concentration of lead, and concluded that, asynapsis occur due to polymorphism in those regions of the chromosomes which had structural heterozygosity[28-29]. During, the present set of investigations, asynapsis of the somatically paired homologues and segmental breaks of polytene chromosomes was also a predominant chromosomal aberrations, in the insecticide exposed individuals. In one of investigation , it was concluded that the reparative mechanism of breaks in *Drosophila melanogaster*, involved the function of nuclear antigens (PCNA) in the proliferating cells, which somehow related with the eukaryotic replication factors, essential for repair of DNA double-strand breaks (DSBs) and lesions arising due to the action of mutagens[30]. Few reports are also procurable in scientific literature related with harmful effects of selected insecticide; as Thiamathoxam was concluded to changed life expectancy and declined age-specific fecundity in insects[31], whereas, some studies specified that thiamethoxam possess inductive potential to affect structural integrity of hepatocytes and to cause liver tumor in mice[32-34]. The contact toxicity as well as mortality was observed in adult eye gnat[35]. Furthermore, the selected insecticide caused the impairment of olfactory learning and abnormal responsiveness to water in *Apis mellifera*[36] and blocked the normal process of oviposition as well as feeding in brown cocoa mired[37]. Moreover in a report, a significant increase in high affinity choline uptake and acetylcholine activity had been observed, in the brain of rats, exposed to the thiamethoxam[38] .

Similarly genotoxicity of thiamethoxam has proven during present investigation, which could be due to abnormal interaction of selected insecticide with genetic material or bio-molecules of test organism. Furthermore, intensive studies on mutagenicity has been recommended, before excessive commercial use of selected insecticide, additionally alternative of synthetic chemicals with required toxicity and comparatively low genotoxicity, should be developed and use of natural predators, for pest control should also be practised.

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