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***In vivo* and *in vitro* genotoxicity analysis of silver nitrate.**

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Abstract

Silver nitrate is an inorganic compound which is toxic and corrosive. This heavy metal, when overdosed, leads to skin disease, blindness, and organ damage. The present study investigated the genotoxicity of silver nitrate in *Drosophila melanogaster* and human peripheral blood lymphocytes. Canton flies were exposed to 0.1M, 0.01M, and 0.001M of silver nitrate. Phenotypic analysis revealed discoloration of head and thorax in the treated flies and their progeny. The DNA from both parent and F1 was subject to Fragmentation assay to study the damages induced by the heavy metal, and the analysis showed significant shearing with fragmentation in the parent DNA. However, the F1 DNA depicted only shearing. To understand the type of mutation induced, Wing Somatic Mutation and Recombination test (SMART) was performed using trans-heterozygous larvae of *mwh/flr3* cross-over, exposed to the different concentrations of silver nitrate. Analysis of wings obtained from the emerging flies revealed spot formation characteristic of both the recessive markers. *In vitro* analysis

by Chromosomal Aberration (CA) assay was performed by exposing human peripheral blood lymphocytes to varying concentrations of silver nitrate for 24 hours and 48 hours. CA assay demonstrated absence of aberrations in the chromosomes of peripheral blood lymphocytes after 24 hours and 48 hours of exposure. **KEY WORDS:** *In vitro*, *in vivo*, silver nitrate, chromosomal aberration, fragmentation, genotoxicity.

Introduction

D. melanogaster is one of the most extensively studied organisms for genetic research as about 75% of recognizable human disease genes have been matched with them (1,2). They have been used as experimental models to study the role of genes for several diseases such as neurodegenerative disease, cancer, diabetes, and many more (3). The advantages of using these fruit flies as model organisms are that they are small and easily grown as they have a short generation time of 10 days and have high breeding efficiency (about 100 eggs/day) (4).

Genotoxicity is the property of a substance which makes it harmful to the genetic information of an organism. Substances which exhibit genotoxicity are known as genotoxins. These genotoxins can be carcinogens, mutagens, or teratogens. There are several ways by which genotoxicity can affect the genetic information. One such common mechanism of action is by forming chemical bonds between the genotoxin and the molecules which carry the genetic information, such as DNA or RNA, therefore affecting its integrity. Genotoxins can be of any type – chemical compounds and radiation. Examples of such chemical compounds include silver nitrate, benzaldehyde, ethyl methane sulfonate, and so on. Therefore, it is necessary to determine the genotoxic level of every chemical compound (5).

Silver nitrate, a salt of silver, is an inorganic compound which is generally used for the prevention of gonococcal ophthalmia neonatorum, cauterization (burning) of wounds and sluggish ulcers, removal of granulation tissue and warts, and aseptic prophylaxis of burns. Initially silver nitrate was used in newborns where a drop of the silver nitrate solution is applied in the eyes of the baby to clear gonococcal infection. But excess of the same can cause blindness and other infections such as blue-grey stain on skin (skin pigmentation), ulcerations, diarrhoea, shock, coma, convulsions, and methemoglobinemia. Fatal dose of silver nitrate is as low as 2 g. The mechanism of action of silver nitrate is by coagulating cellular proteins to form an eschar. Eschar is dead tissue that sheds from a healthy skin (5, 6).

The Wing Somatic Mutation and Recombination test (SMART) is a gold-standard technique, employed for the purpose of assessing the degree of genotoxicity, that is, exhibited by the mechanism of induction of loss of heterozygosity (LOH) resulting from incidents like gene mutation, chromosome break, and chromosomal rearrangement. In transheterozygous *mwh+ / flr3* flies, multiple wing hairs (*mwh*) and flare (*flr3*) are employed as wing- cell recessive markers in SMART. A mutation in the mitotic cells of wing disc gives rise to a clone of *mwh* and *flr3* cells. This expresses as spots on the wings of adult fly. The spots may appear singly or as twins. Single spots indicate the occurrence of a point mutation, a chromosomal alteration, or mitotic recombination. Twin spots indicate the occurrence of mitotic recombination (7).

Chromosomal aberrations result from changes in the structure or number of chromosome. The damage that occurs in a cell gives rise to abnormalities in chromosome. These defects are assessed by a technique called Chromosomal aberration (CA) assay (8). CA assay is often performed on human peripheral blood lymphocytes. As lymphocytes are in the G₀ (resting) stage of cell cycle, they are stimulated to divide by an antigen like Phytohemagglutinin (PHA). A spindle inhibitor, Colcemid, is added at the end of 46.5 hour to arrest the cells in the metaphase of first mitosis. The

culture is then harvested after 48 hours of incubation. In the present study, the blood culture is exposed to silver nitrate for 24 hours and 48 hours (9,10).

Materials and Methods

Exposure of Canton flies and DNA fragmentation assay

Flies were bred in the ratio of 1:3 (males:females) and were cultured in corn meal agar and incubated at 25°C during the day and 19°C at night. Test concentrations of silver nitrate solution of 0.1M, 0.01M, and 0.001M were prepared. Instant food (4-24 formula plain – Carolina Biologicals) was mixed with the above concentrations of silver nitrate, and the food was allowed to set for 3 hours. About 30 males were isolated added to the above and exposed for 24 hours and 48 hours at 25°C. After 48 hours, the surviving male flies were transferred (by etherization) to fresh corn meal medium, and 90 virgin female flies were added to each of the vials to check the breeding efficiency. All the vials were maintained in duplicates. The control used had instant food mixed with sterile distilled water. DNA extraction from both treated and control flies was performed by Phenol Chloroform method (PCI), and the quality of the DNA was checked using Nanodrop. Good quality DNA was run on 3% agarose gel for DNA fragmentation assay to assess the damage induced.

Wing spot assay

Wing Spot assay was performed by exposing 60 *mwh/flr³* larvae to the test concentrations of silver nitrate. The larvae were monitored till the emergence, and the emerged flies were dissected for their wings and analysis was performed on the wings for spots.

Chromosomal aberration assay

For Chromosomal aberrations (CA) assay, culture was set up for exposure to silver nitrate for 24 hours and 48 hours. The procedure involves 48 hours culture setup and harvesting at 47th hour. Two sets of culture were set up by adding 8 ml of RPMI-1640 and 2 ml of Fetal Bovine serum (FBS) to 1 ml of human peripheral blood. 400 µl of phytohemagglutinin was added to all the culture vials. To the first set of cultures, test concentrations of silver nitrate were added along with a control culture and incubated for 46 hours. The second set of cultures was incubated for 24 hours after which the test concentrations were added to the respective vials along with a control and incubated further till 46 hours. At the end of 46 hours, 10 µl of Colcemid was added to all the vials and incubated for 1 hour. At the 47th hour, all the cultures were harvested by adding 8 ml of hypotonic solution (KCl prewarmed to 37°C) and incubated at 37°C for 20 minutes. The contents were then centrifuged at 1000 rpm for 10 minutes, and the pellet was resuspended in 8 ml of prechilled Carnoy's fixative (methanol : acetic acid – 3:1) and incubated overnight at 4°C. The cultures were again centrifuged and the pellet was suspended in a small amount of the fixative and casted on slide held at an angle of 45°. The slides were then allowed to dry and observed under the microscope for any chromosomal aberrations.

Results

Phenotypic Changes

Adult male flies exposed to test concentrations of silver nitrate survived for 24 hours and 48 hours. To check the breeding efficiency, 90 female flies were added to each vial. The flies bred well.

The females laid their eggs in the untreated medium. The bottles were observed carefully each day for the emergence of adult flies. The adult flies emerged 19 days after the eggs were laid. The progeny were screened for pupal lethality, adult emergence, and adult mutant phenotypes. The F1 observed under the microscope showed discoloration of head and thorax as shown in Figure 1. Survival rates of both exposed flies and the F1 progeny were 100% in all the test concentrations of silver nitrate (Table 1).



Figure 1. Phenotypic changes observed following exposure to silver nitrate. Picture shows discoloration of head and thorax observed in flies (parent and F1). This change was seen at all concentrations.

Table 1. Results of phenotypic changes observed in exposed flies and F1 progeny.

Experiment	Concentration of Silver Nitrate	Survival Rate	Changes Observed
1	0.1M	100%	Discoloration of head and thorax
2	0.01M	100%	Discoloration of head and thorax
3	0.001M	100%	Discoloration of head and thorax
F1 analysis	All three concentrations	100%	Discoloration of head and thorax

Table 2. Results of wing spot assay.

Concentration of Silver Nitrate (μ L)	<i>flr3</i>	<i>mwh</i>	<i>mwh/flr3</i>	Observations
Control	-	-	-	Absence of spots
0.1M	+	+	+	Presence of large single spots- <i>flr3</i> ; cluster of multiple trichomes per cell- <i>mwh</i>
0.01M	+	+	+	Single large spots- <i>flr3</i> ; multiple trichomes and 2 hair per cell- <i>mwh</i>
0.001M	+	+	-	Multiple trichomes per cell- <i>mwh</i> ; multiple single spots- <i>flr3</i>

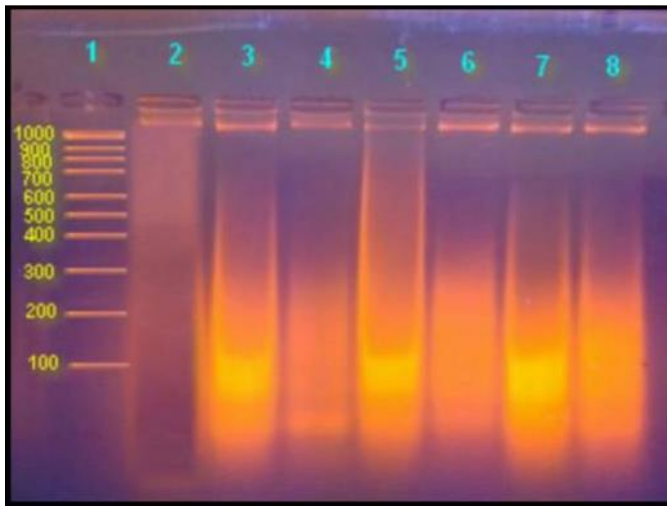


Figure 2. Agarose gel electrophoresis showing DNA fragmentation of Parent DNA samples. Description: Well 1 – 100 BP Ladder; 2 - Control; 3,4 – 0.1M silver nitrate for 24 hrs and 48 hrs; 5,6 – 0.01M silver nitrate for 24 hrs and 48 hrs; 7,8 – 0.001M silver nitrate for 24 hrs and 48 hrs.

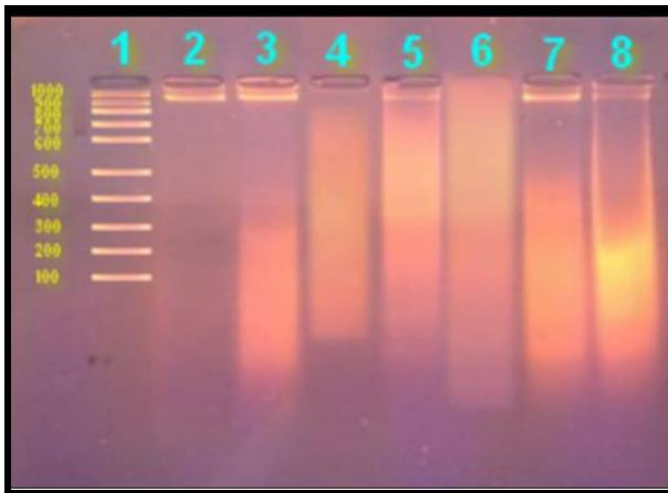


Figure 3. Agarose gel electrophoresis showing DNA fragmentation of F1 DNA samples. Description: Well 1 – 100 BP Ladder; 2 - Control; 3,4 – 0.1M silver nitrate for 24 hrs and 48 hrs; 5,6 – 0.01M silver nitrate for 24 hrs and 48 hrs; 7,8 – 0.001M silver nitrate for 24 hrs and 48 hrs.

DNA Fragmentation assay

DNA was isolated from Control, exposed flies, and F1 progeny by Phenol chloroform method, and the quality of the DNA was checked using Nanodrop. The Nanodrop results showed that the DNA was found to be of good quality. Good quality DNA was subjected to Fragmentation assay on 3% agarose gel to detect the damage. The results obtained were documented (Figures 2 and 3). Distinct shearing with fragments was observed in all the concentrations (after 24 and 48 hours exposure) of exposed sample (Parent), whereas DNA from the F1 sample showed patterns of shearing.

Wing Spot Assay

Analysis of patterns on the dissected wings of both control and exposed flies emerged from the trans-heterozygous larvae of the *mwh/flr3* cross-over clearly showed the genotoxic potential of silver nitrate (Table 2). No spots were observed in the control wing. In the wings of the exposed flies, spot formation was clearly seen. Single large spots indicative of *flr3* marker and multiple trichomes with two hair cells specific to *mwh* marker were both present (Figure 4).

Chromosomal Aberrations (CA) assay

The *in vitro* chromosomal aberrations assay performed on human peripheral blood lymphocytes demonstrated absence of any aberrations in all the concentrations (Figure 5).

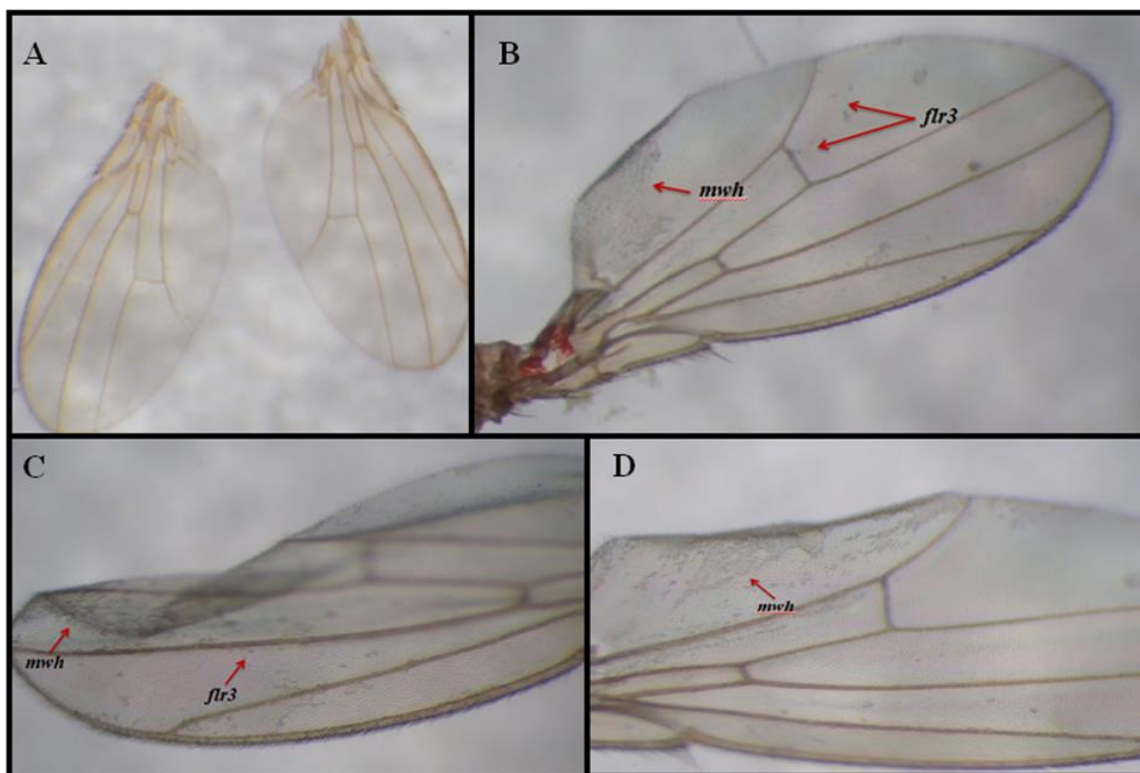


Figure 4. Wing spot assay analysis after exposure to silver nitrate. A - Control showing absence of spots; B - 0.1M - presence of large single spots - *flr3*; cluster of multiple trichomes per cell - *mwh*; C - 0.01M - single large spots-*flr3*; multiple trichomes and two hair per cell-*mwh*; D - 0.001M - multiple trichomes per cell-*mwh*; multiple single spots-*flr3*.

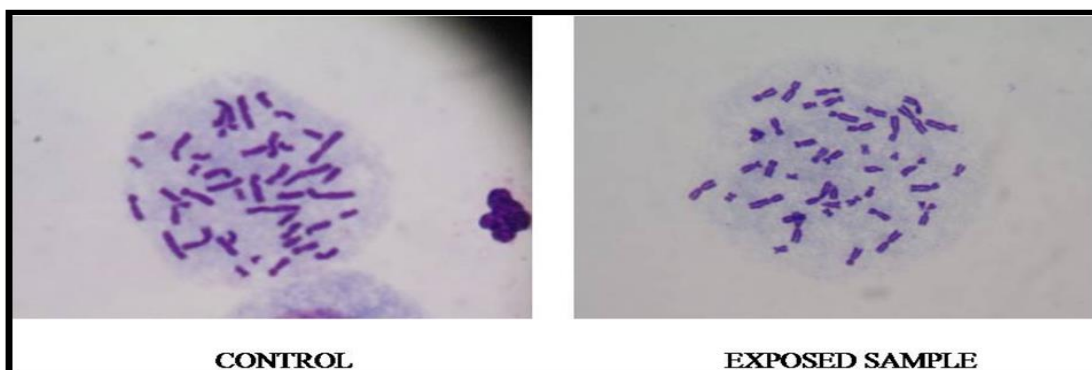


Figure 5. Chromosomal aberration assay. The picture shows chromosomes of control blood sample and blood sample exposed to test concentrations of silver nitrate. The results observed were the same in all the three concentrations.

Discussion

The present study evaluated the genotoxicity of three concentrations of Silver nitrate using *in vitro* and *in vivo* methods. In the *in vitro* chromosome aberration assay performed on human peripheral blood lymphocytes, no significant chromosomal aberrations were observed in all the three concentrations indicating that all the concentrations were not genotoxic at the cytogenetic level. However, the *in vivo* assessment of the same concentrations on *Drosophila melanogaster* was performed by evaluating the phenotypic changes and employing DNA fragmentation assay on the DNA isolated from the exposed flies and F1 generation.

The phenotypic changes observed in the exposed flies included mild discoloration of the head and thorax in all the three concentrations. Also, DNA isolated from both exposed and F1 was quantified by nanodrop and run on 3% agarose. The results of the parent DNA revealed significant shearing and defined fragments in all the three concentrations at both 24 and 48 hours of exposure. However, the DNA obtained from F1 demonstrated only shearing, and no fragments were observed. Shearing is indicative of extensive damage and hence both parent and F1 population have faced pronounced genotoxicity on exposure to the above said concentrations of silver nitrate. Once again the Wing spot assay performed on the third instar trans-heterozygous larvae of the *mwh/flr3* cross-over showed and confirmed the recombinogenic action of the heavy metal. Single large spots indicative of *flr3* marker and multiple trichomes with two hair cells specific to *mwh* marker were both present. In comparison with the control wing, the extent of damage induced in the exposed larvae was well understood. From the *in vivo* studies conducted it was evident that silver nitrate was genotoxic on both wild type and mutant flies.

Understanding the mechanism of genotoxicity and type of genetic damage is, however, beyond the scope of this study, and specific tools have to be employed to evaluate the same. The results of the *in vitro* and *in vivo* are not consistent, indicating the profound expression of genotoxicity at *in vivo* stages, and *in vitro* tests may be expanded on cell lines to validate their genotoxic effect. Further higher and lower concentrations of silver nitrate may be evaluated to arrive at the threshold concentration at which genotoxicity is defined and below which no genotoxicity is observed.

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