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Antimutagenic Effects of Ascorbic Acid against the Genotoxicity of Dimethyl Sulphate in Drosophila

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Abstract:

The present work involves the study of the genotoxicity of an alkylating agent, dimethyl sulphte, and the possible protective efficacy of vitamin C against the toxicity of this chemical in the somatic cells (wing primordial) of *Drosophila melanogaster* following chronic larval exposures. For the wing mosaic assay two high bioactive strains of *Drosophila melanogaster*, viz., ORR; *mwh/mwh* and ORR*; flr*^{3}/TM3, *Ser* were used. Third instar larvae of *flr*^{3}/TM3, Ser and *mwh* homozygous male cross were exposed to the LD_{50} and lower doses of dimethyl sulphate (DMS) to find out its genotoxicity. Concurrent negative control was run simultaneously in each experiment, where the larvae were exposed to the solvent (distilled water). All the doses of DMS were found to be clearly genotoxic in Drosophila wing mosaic assay in comparison to the control. The frequencies of induction of small and large single spots were positive. With respect to the frequency of twin spots, the statistical outcome was positive at the highest dose and inconclusive at the three lower doses. When the larvae were concurrently treated with the mixture of 300mM of ascorbic acid and the different doses of dimethyl sulphate, except for the outcomes being weak for the small singles at 100mM and 50mM of DMS, the same for these spots at the two lower doses as well as for other types of spots at all the doses were negative compared to the DMS alone. In the present experiments the results clearly indicate antimutagenic and/or anticarcinogenic action of ascorbic acid against the mutagenicity of dimethyl sulphate irrespective of the mechanisms involved.

Keywords: Drosophila, Dimethyl sulphate, ascorbic acid, antimutagenicity, somatic cells

1.0 Introduction:

Different chemical substances used for human welfare and development in agriculture, industries, mining, as drugs, detergents, food additives and so on are constantly imported into our environment, thereby, leading to its pollution. Many of them are toxic and thus affect the flora and fauna. Some chemicals even alter the genotype of organisms leading to mutations. Mutations may lead to foetal death (when lethal), congenital abnormalities, heritable genetic diseases, mental retardation, senility, cancer etc. In the somatic cells mutation lead to cell death, premature ageing and cell malignancy. The conventional animal cancer tests are expensive and tedious, the use of short term mutagenicity tests for detection of possible induction of carcinogenicity by chemical substances have been advocated by Fearon and Vogelstein (1990). Alkylating agents are potent carcinogens and do not require metabolic activation. They have the affinity for alkylating the oxygens of nucleic acids. However, dimethyl sulphate primarily alkylate ring nitrogens (Singer, 1975) and used for the conversion of active hydrogen compounds to their corresponding methyl derivatives, produces 7-methyl-guanine in nucleic acids on chemical reaction (Reiner and Zamenhof,1957). DMS has been tested in many systems and has been reported to be genotoxic (Newbold *et al*., 1980; Teo *et al*., 1983; Nishi *et al*., 1984; Mohn and Van Zeeland, 1985; Braun *et al*., 1986).

In the past years several attempts have been made to reduce the toxicity of certain xenobiotics through the use of vitamins (Khan and Sinha, 1993; Hoda and Sinha, 1993). Thus antimutagens reduce the frequency of spontaneous or induced mutations (Waters *et al*., 1990), prevent mutagen formation, intercept mutagen by enzymes and neutralize mutagenic lesions and utilize mechanisms which enhance induction of error free repair and block error-prone DNA repair (Hartman and Shankel, 1990).The different groups of antimutagens include vitamins, fatty acids, plant derivatives and sulfahydryl compounds. The role of vitamin A, vitamin C and vitamin E as antimutagens, anticlastogens and anticarcinogens have been evaluated in different test systems *in vivo* (Bose and

Sinha, 1991; Sinha and Bose, 1992). Vitamin C, ascorbic acid, is one of the most effective aquous phase antioxidants in human plasma (Frei *et al*., 1989). It contributes to the natural antioxidant effects in cells (Ames, 1983). In higher mammals this is not synthesized, though in lower mammals like rats it is synthesized in the liver from D glucose by the uronic acid pathway. Depending upon whether it is in oxidized or reduced state, it is biologically act as a pro-oxidant or an oxygen radical scavenger at low concentrations (Weitberg and Weitzman, 1985). It has been reported to be antimutagenic and anticarcinogenic in different systems including man (Block, 1991; Minnuni *et al*., 1992). It is reported that the antimutagenic and anticarcinogenic activity of this vitamin is primarily due to the scavenging of mutagenic and carcinogenic precursors (Shamberger, 1984). Since it also exhibits genotoxic effect in certain systems (Norkus *et a*l., 1983; Shamberger, 1984), an appropriate dose of vitamin C would be necessary.

Most of the studies on antimutagenicity have been conducted in *in vitro* systems. However, in order to obtain conclusive results on the antimutagenic and anticlastogenic properties of vitamin C, it becomes necessary to undertake such studies in eukaryotic *in vivo* systems. The fruit fly, *Drosophila melanogaster*, with its well studied genetics and available genetic markers has been used as a viable alternative to the mammalian system for antimutagenic studies. In this system a wide array of genetic changes ranging from point mutation to recombination can be easily detected (Graf *et a*l., 1983, 1984; Wurgler *et al.*, 1984; Tripathy *et al*., 1990).The Somatic Mutation and Recombination Test (SMART) can detect a wide range of genetic end points like point mutations, deletions, mitotic recombinations and gene conversions (Graf *et al.*, 1984; Wurgler and Vogel, 1986; Vogel and Zijlstra, 1987).The basis of the test involves the exposure of imaginal disc cells of the larva to a mutagen so that the genetically altered cells, if survive and proliferates will be expressed as a mutant clone(mosaic spot) on an otherwise normal body surface (wing of the adult flies). The wing recessive markers (*mwh* and *flr3*) are autonomously expressed in adult wings, approximately 24,400 target cells can be screened per adult wing and the mutant cells can be expressed as single cells and can be easily scored (Garcia-Bellido and Dapena, 1974; Graf *et al*., 1989, 1992). Since this clastogenic agent has not been tested for its genotoxicity in the somatic cells of Drosophila and no studies have been undertaken on the antimutagenicity of vitamin C against the mutagenicity of this compound, the authors were inclined to study the same using the wing mosaic assay.

2.0 Materials and Methods

2.1 Chemicals used:

Dimethyl sulphate (DMS, CAS No. 77-78-1), also known as methylsulphate, was obtained from S.D. Fine- Chem Limited with 99% purity. Vitamin C (L-ascorbic acid) used in these experiments was obtained from LOBA-Chemic Pvt. Ltd., Mumbai, with 99% purity. Both the chemicals were water soluble and prepared freshly before use. Distilled water was used as a negative control.

2.2 Stocks used:

Two high bioactive strains of *Drosophila melanogaster*, viz., ORR; *mwh/mwh* and ORR; flr^3/TM3, *Ser* were used. These stocks carry chromosome 1 and 2 obtained from a DDT-resistant Oregon R® line (Dapkus and Merrell, 1977). These stocks have high constitutive level of cytochrome P-450. As a result, they have high metabolic activity. The marker flr^3 (flare) is a recessive autosomal marker on the left arm of chromosome 3 (3-38.8) and is expressed as a short, thick and misshaped wing hair. Since the marker is zygotic lethal in homozygous condition, it is kept on a balancer chromosome TM3. The allele *mwh* (multiple wing hairs) is also a recessive autosomal mutation located on the tip of chromosome 3 (3-0.3). In homozygous condition it is expressed as multiple wing hairs in contrast to a single trichome per wing cells of the wild type fly. For details about the markers please refer Lindsley and Zimm (1992), Frolich and Wurgler (1989) and Graf and Singer (1992).

2.3 Treatment Procedure:

For standard (ST) cross virgin females from the ORR; flr^3/TM3, *ser* stock were mated with ORR, *mwh/mwh* males. Eggs were collected from the above cross for a period of 8h on standard Drosophila food (corn powder, molasses, agar agar and propionic acid cooked together). After 72 ± 4h, counted from the middle of the egg collection period, i.e., when the larvae were in the 3rd instar stage (Graf *et al.*, 1984), they were washed out from the medium in 20% NaCL solution. Larvae, in batches of approximately 100 per batch were transferred into glass vials containing 1.5gm instant food (Carolina Biological Supply Co., USA), rehydrated with different concentrations of dimethyl sulphate or ascorbic acid

or both and distilled water as negative control, for the rest of the larval life. All the experiments were performed at 25 ± 1ºC and at approximately 60% relative humidity. The LD_{50} for DMS was noted as 100mM for 3rd instar larvae. The larvae were thus exposed to LD_{50} and lower doses to study its genotoxic effects. For concurrent treatment 3rd instar *mwh* +/+ *flr*^{3}transheterozygous larvae were exposed to different concentrations of DMS and 300mM of ascorbic acid in different vials.

2.4 Microscopic Examination of Wings

The wings of adult flies, on eclosion, were stored in 70% ethanol and subsequently the wings of the flies were detached and mounted in Faure's solution (Gum Arabic, glycerol, chloral hydrate and water). The mounted slides were charged with metal cubes which allowed the wings to dry up and harden without crumpling them. Both dorsal and ventral surfaces of the mounted wings were screened under a compound microscope at 400X magnification. Only the distal wing compartments were screened (Garcia Bellido *et al*., 1976). The size and type of each spot (i. e., whether small or large and whether *mwh* and *flr*^{3} single or *mwh*/*flr*^{3} twin) was recorded for statistical analyses. The mutant clones were classified into three types i) small singles: Clones with one or two mutant cells ii) large singles: containing 3 or more cells and iii) twin: twin spots, containing adjacent mwh and flr3 cells (Graf *et al*., 1984).

2.5 Statistical Analysis:

The frequencies of different types of spots were statistically evaluated on the basis of a multiple decision procedure (Frei and Wurgler, 1988) to determine whether the result was positive, weakly positive, inconclusive or negative. Since small single spots(s = 1-2) and total spots (T) have high spontaneous frequencies, m (multiplication factor) was fixed at a value of 2. On the other hand, since the large single spots(S>2) and twin spots (t) have low spontaneous frequencies, m was fixed at 5. The data were evaluated at 5% level of significance.

3.0 Results and Discussion:

In the present experiments, 3rd instar larvae, transheterozygous for the recessive genetic markers *mwh* and *flr*^{3} were given chronic exposures to different doses of DMS (100mM, 50mM, 25mM and 10mM). In each experiment a concurrent control was run simultaneously. The experiments were repeated and since there was no significant statistical difference between the data of repeated experiments, they were pooled and summarized in the Table 1. Under our test condition all the concentrations of DMS are highly genotoxic in the SMART test of *Drosophila melanogaster*. On statistical evaluation, the induction frequencies for all types of spots were positive at the highest tested dose (100mM), compared to the corresponding control data. At the three other test doses, the frequencies of single spots (both small and large) as well as the total spots were significantly positive while the same for twin spots was inconclusive.

3.1 Modifying Effects of Ascorbic Acid on the Genotoxicity of DMS:

To find out the antimutagenic effects of ascorbic acid, if any, against the genotoxic effects of DMS, a dose of 300mM of the vitamin was taken as the test dose since this dose did not induce wing spots in statistically higher frequencies following treatment of *mwh*+/+ *flr*^{3} transheterozygous larvae (data not shown). In the present experiments, third instar larvae were given concurrent treatment with 300mM of ascorbic acid along with different concentrations of DMS for 48h. Each experiment was repeated and, since there was no statistical difference between spot frequencies in the two repeated experiments, the data were pooled and are summarized in Table 2.

When the larvae were concurrently treated with 100mM and 300mM of ascorbic acid, a total of 759 spots were induced as against 932 spots when the larvae were treated with 100mM DMS alone. Under the effect of ascorbic acid the spot frequencies was decreased by 36.9%, 4.76% and 18.56% for large singles, twin spots and total spots respectively. But the incidences of small single spots were increased in comparison to DMS (100mM) alone. When the data were statistically evaluated there was a significant reduction in the frequencies of large single, twin spots and total spots, though for the small singles the outcome was weak. Third instar larvae treated with 50mM of DMS contained a total of 496 spots. However, a co- treatment with 300mM of the vitamin and DMS (50mM) the total number of spots was reduced to 237. The spot frequencies are reduced by 19.2%, 64.09%, 55.88%and 52.21% in small single, large single, twin spot and total spots respectively. Here, too, the statistical analyses outcomes were negative with respect to the frequencies of induction of large singles, twin and total spots and weak for small singles.

Table 1: Data on the wing mosaic assay after larval exposures to Dimethyl sulphate (DMS)

Treatment duration	Concentration (mM)	No. of wings tested	Spots per wing (No. of spots) Stat. diagn.*			
			Small singles (S =1- 2) m = 2.0	Large singles (S > 2) m = 5.0	Twins (t) m = 5.0	Total (T) m = 2.0
48 h	Control	80	0.25 (20)	0.13 (10)	0.00 (0)	0.38 (30)
	100	80	2.96 (237) +	8.16 (653) +	0.52 (42) +	11.65 (932) +
	50	80	1.56 (125) +	4.21 (337) +	0.42(34) i	6.20 (496) +
	25	80	1.30 (104) +	2.22 (178) +	0.26 (21) i	3.78 (303) +
	10	80	1.20 (96) +	1.79 (143) +	0.20 (16) i	3.19 (255) +

* Statistical diagnoses following Frei and Wurgler (1988). + = positive,i= inconclusive, m = multiplication factor. Probability levels: α = β = 0.05. One sided statistical tests.

Table 2: Data on the wing mosaic assay after larval exposures to Dimethyl sulphate (DMS) and Ascorbic acid (AA)

Mode of treatment	Conc.of DMS (mM)	Conc. of AA (mM)	No. of wings tested	Spots per wing (No. of spots) Stat. diagn.*			
				Small singles (S =1- 2) m = 2.0	Large singles (S > 2) m = 5.0	Twins (t) m = 5.0	Total (T) m = 2.0
-ve control	-	-	80	0.25 (20)	0.13 (10)	0.00 (0)	0.38 (30)
DMS (+ve control)	100	-	80	2.96 (237) +	8.16 (653) +	0.52 (42) +	11.65 (932) +
DMS + AA	100	300	80	3.83(307)w	5.15(412)-	0.5(40)-	9.49(759)-
DMS (+ve control)	50	-	80	1.56 (125) +	4.21 (337) +	0.43(34) i	6.20 (496) +
DMS + AA	50	300	80	1.26(101)w	1.51(121)-	0.19(15)-	2.96(237)-
DMS (+ve control)	25	-	80	1.30 (104) +	2.22 (178) +	0.26 (21) i	3.78 (303) +
DMS + AA	25	300	80	0.58(46)-	1.85(148)-	0.22(18)-	2.65(212)-
DMS (+ve control)	10	-	80	1.20 (96) +	1.79 (143) +	0.20 (16) i	3.19 (255) +
DMS + AA	10	300	80	0.40(32)-	0.84(67)-	0.06(5)-	1.30(104)-

* Statistical diagnoses following Frei and Wurgler (1988).w = weak positive, - = negative, + = positive, i= inconclusive, m = multiplication factor. Probability levels: α = β = 0.05. One sided statistical tests.

In another experiment, where larvae were exposed to a mixture of 25mM of DMS and 300mM of ascorbic acid for 48h, ascorbic acid reduced the incidence of spots by 55.76%, 16.85%, 14.28% and 30.03% in small singles, large singles, twin spots and total spots respectively. The statistical outcomes were negative for all types of spots when compared to the positive control (25mM DMS). This demonstrated a modifying effect of ascorbic acid against the genotoxicity of DMS. In a similar experiment was conducted where the larvae were exposed to a mixture of 10mM of DMS and 300mM of ascorbic acid the spot frequencies were reduced by 66.6%, 53.14%, 68.75% and 59.21% in small singles, large singles, twin spots and total spots respectively. The statistical outcomes were negative for all categories of spots indicating a modifying effect of ascorbic acid.

Dimethyl sulphate is an active alkylating agent and commercially produced for more than half a century. When administered through subcutaneous or intramuscular route, local sarcomas are induced in rats (IARC, 1974).Genotoxicity of the compound have been studied in different test systems. In *Salmonella typhimurium*, forward mutation was induced by DMS, even in the absence of any activation system (Skopek and Thilly, 1983). In the *E.coli* spot test and forward mutation test, DMS gave positive results (Quillardet *et al*., 1985). In *Saccharomyces cerevisiae* it induced homozygosis and reverse mutation (Pavlov and Khromov-Borisov, 1981) and recombination (Zimmermann *et al*., 1984). In animal cell DMS induced strand breaks *in vitro* (Sina *et al*., 1983). In the Chinese hamster cells, this compound was reported to induce chromosome aberrations and sister-chromatid exchanges *in vitro* (Teo *et al*., 1983; Nishi *et al*., 1984).

In this study, DMS has induced both small and large single spots containing either *mwh* or *flr*^{3} cells in large frequencies. Single spots in the adult wings are a result of either gene mutation or gene conversion in their corresponding wild type loci (Graf *et al.,* 1984), whereas, the origin of the single spots with *mwh* phenotype could be due to segmental aneuploidy (Haynie and Bryant, 1977), non-disjunction (Graf *et al.*, 1984) and mitotic recombinations (Garcia-Bellido and Dapena, 1974) in the chromosome. Thus it is concluded that DMS could be a potent mutagen/clastogen/recombinogen or aneuploidogen in the wing-disc cells of *Drosophila melanogaster.* Twin spots with *mwh* and *flr*^{3} subclones are formed on the wings of adults due to the induction of mitotic recombination in the chromosome region between the *flr*^{3} locus and the centromere in the left arm of chromosome 3 of the wing primordial cells of Drosophila larvae carrying the markers *mwh* and *flr*^{3} in a trans-position following exposures to a chemical mutagen. According to Ramel and Magnusson (1979), twin spots may also develop due to the induction of double chromosome nondisjunction in the wing primordial cells following exposures to a mutagen. The possibility of origin of twin spots in this manner, although rare, cannot entirely be disregarded as DMS is reported to be aneuploidogenic in other systems (Sharma *et al.,* 1980). In the present experiments, the frequencies of twin spots, although very high at different doses of treatment, is positively only at the highest tested dose and inconclusive at lower doses. Thus, although it is difficult to comprehend the exact mechanism involved in the process of induction of wing spots, it is concluded that DMS is genotoxic in the wing disc cells following chronic larval exposures.

Studies on the area of antimutagenesis and anticarcinogenesis are primarily devoted to detect anti risk factors in human through several *in vitro* and *in vivo* short term tests. There are evidences to show the antimutagenic effect of ascorbic acid against the mutagenicity of a wide range of chemical substances in different test systems (Munkres, 1979, Dion *et al*., 1982; Sinha and Bose, 1992; Khan and Sinha, 1996). Many hypotheses have been put forwarded to explain the beneficial effects of ascorbic acid. According to Bhattacharjee and Pal (1982), it may shield the genetic material inside a living cell from a mutagenic agent. It can also interact with the mutagenic agent itself and prevent it from inducing mutagenesis. Jamaluddin *et al*.(1981) have shown a direct interaction of ascorbic acid with adenine and guanine. Kaya (2003) has reported the antimutagenic effect of ascorbic acid against the mutagenicity of alkylating agents like ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS) and N-nitroso N-ethylurea (ENU) in Drosophila SMART assay. Ascorbic acid reduced the genotoxicity of potassium dichromate but did not show any antigenotoxic effect on the genotoxicity of 4-nitroquinoline and significantly increased the frequency of mutant clones in Drosophila wing spot test (Kaya *et al*., 2002). Turkez (2011) have demonstrated a protective role of ascorbic acid against titanium dioxide indued genotoxicity. Co treatment of ascorbic acid with titanium dioxide decreased the rate of sister chromatid exchange, micronucleus and DNA damage in comparison to titanium dioxide treated cultures alone. Another study showed that orange juice, lemon juice and their active component like hesperidin and limonene are able to protect DNA against hydrogen peroxide in somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Fernández-Bedmar et al., 2011). Similarly, β-carotene (BC), pro-vitamin A, is an efficient antioxidant, exerted protection against the genotoxic action of the chemotherapeutic free-radical generator doxorubicin in wing spot test of *Drosophila melanogaster* (Dias et al., 2009).It has been also reported that drug consisting of selenium and vitamins A, C and E (selenium-ACE) significantly reduced the percentage of chromosomal aberrations

induced by Beryllium Chloride ($BeCl_2$) in somatic and germ cells of mice (Fahmy et al., 2008).

In the present experiment, it has been noted that DMS is a potent genotoxic agent in the somatic cells of *Drosophila melanogaster*. On exposure of larvae concurrently to a mixture of DMS and ascorbic acid there was a slight increase in the frequency of small single spots of the highest tested dose of DMS. The frequencies of other types of spots were however lower than their frequencies in the corresponding positive control. A reduction in the frequencies of twin spots following concurrent treatment indicates that ascorbic acid is anti recombinogenic. A reduction in the frequencies of single spots due to the action of ascorbic acid may be due to the fact that ascorbic acid is an electron donor which react with superoxides, peroxides and hydroxyl ions, nullifying their action in the genetic material inside the living cell. In other words, it has shielded the genetic material from the genotoxic effects of the alkylating agent. Further, it might have acted as scavenger of electrophilic ions as a result of which the frequency of genetic damage due to the action DMS might have been reduced.

4.0 Conclusions:

Based on present experimental condition and result, all doses of DMS (100mM, 50mM, 25mM, and 10mM) are clearly genotoxic/mutagenic in wing mosaic assay of *Drosophila melanogaster*. Ascorbic acid (300mM) exerts a protective effect against the genotoxic action of the all doses of DMS, as tested using somatic mutation and recombination test (SMART) in *Drosophila melanogaster.* Drosophila wing mosaic assay is a suitable assay system for studying the mutagenic effects of wide variety of chemicals to which human beings are exposed intentionally, unintentionally and occupationally, moreover, the results are of interest to investigate the underlying mechanisms involved in the antimutagenic effect of ascorbic acid.

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