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Original Research Paper

Modulatory Action of Vitamin C on the Genotoxicity of an Anti Cancer Drug (Etoposide) Tested in the Wing Spot of *Drosophila*

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ABSTRACT

This work deals with genotoxicity of an antineoplastic drug, etoposide and its modulation by Vitamin C in the somatic wingspot cells of *Drosophila*. Four sub-lethal doses of etoposide (0.5 mM, 0.25 mM, 0.1 mM, 0.05 mM) and two doses of Vitamin C (50 mM, 100 mM) were selected for the purpose. Etoposide was found to be genotoxic at all tested doses in this assay. It was found that Vitamin C (100 mM) caused significant reduction of wing spots when admitted before, along with or after treatment with any doses of etoposide. The possible causes of induction of wingspots and modulatory action of Vitamin C have been discussed.

INTRODUCTION

Man-made synthetic chemical compounds have become inseparable entities of modern human civilization. Many of them remain stable in the environment and become mutagenic and carcinogenic. Synthetic pharmaceutical compounds constitute one such group of chemicals, which, in addition to their therapeutic action may also act as mutagens, carcinogens and teratogens. Many of the antineoplastic drugs are reported to be genotoxic (Raj & Katz 1985, Fishbein 1987, Harbach et al. 1986, Yajima et al. 1989, Hoffman et al. 1995, Keohara et al. 1997, 1998, Gentile et al. 1998, Brooks et al. 2000, Haque et al. 2001, Blasiak et al. 2002, Buschini et al. 2003). Since the therapeutic benefits of these drugs outweigh their ill effects, discontinuation of their use has not become possible. Thus, attempts are being made to find out ways to modulate their genotoxic potential, so as to minimize the damages caused by them. One of the anti-mutagenic groups of compounds is vitamins, which are not only naturally occurring nutrients of man, but also have little adverse effects. In this study, data are presented on the genotoxicity of antineoplastic drug, etoposide and its modulation by Vitamin C tested in the somatic cells of wing spots of *Drosophila.*

MATERIALS AND METHODS

The second and third instar transheterozygous larvae of two bioactive strains of *Drosophila melanogaster*, ORR; *mwh/mwh* and ORR; *flr*^{3}/TM3 Ser were the test animals of this study. The test chemicals are an antineuoplastic drug, etoposide and Vitamin C. The method of Grant et al. (1984) was followed to study the wing spots induced by the test chemicals through somatic mutations in the wing primordia.

For treatment of larvae, the sub-lethal doses of etoposide used were 0.5 mM, 0.25mM, 0.1mM and 0.05mM and two doses of Vitamin C used were 100mM and 50 mM. For each dose of test

chemical, four treatment schedules were undertaken, i.e., positive control treatment, pre vitamin treatment, concurrent vitamin treatment and post vitamin treatment. The wings of the adult flies (80 wings from each treatment) eclosed from treated larvae were mounted on clean slides and different induced spots i.e., single (*mwh* or *flr*^{3}) or twin (*mwh/flr*^{3}) and small spot (1-2 affected cells) were separately scored. Following Graf et al.(1984), the clones were classified into different size classes like 1-2, 3-4, 5-8, 9-16, 17-32, 33-64, 65-128 and so on.

The statistical methods, the conditional binomial test with a multiple decision procedure, employed by the Frei & Wurgler (1988) were followed in this work for studying significance of data on frequency of wing spots induced by the drug and their modulation by Vitamin C.

RESULTS

The positive control series of experiments was conducted by treating the transheterozygous 2nd instar larvae with four sub-LD_{50} concentrations of etoposide (LD_{50} concentration was found to be 1mM) and two concentrations of Vitamin C. Different types of wing spots were induced by these four different sub-lethal doses of etoposide (0.5mM, 0.25mM, 0.1mM, 0.05mM) on treating the larvae for 48 hours and 72 hours separately (Tables 1, 2, 3). Out of these two sets of experiments, the 48 hours treatment was taken into consideration for further observation and evaluation (Table 2). When total spots induced by different concentrations and those of normal control were statistically evaluated, the wing spots data appeared positive for 0.5mM, 0.25mM and 0.1mM and inconclusive for 0.05mM.

When 2nd instar larvae were exposed to 0.05mM of etoposide, a total of 1676 spots (887 small singles, 596 large singles and 193 twin spots) were induced in 80 wings. The spots per wing frequencies of this positive control experiment were 20, 95, 11.09, 7.45 and 2.41 respectively. When the larvae were treated with 0.25 mM concentration, the 80 wings of eclosed adults exhibited 1470 spots which included 758 small singles, 563 large singles and 149 twin spots. The spots per wing were 565 small singles, 377 large singles and 89 twin spots with respective spots per wing frequencies of 7.06, 4.71 and 1.11. When these data were statistically compared the results were positive for small singles, large singles and total spots while the same for twin spots was inconclusive. To assess the effect of Vitamin C on the mutagenicity of etoposide, the 2nd and 3rd instar *mwh+/+flr*^{3} transheterozygous larvae were exposed to a particular concentration of Vitamin C (50mM or 100mM) before etoposide treatment, along with etoposide and after etoposide treatment. The wing spots induced in these Vitamin C treated experiments were found to be drastically reduced in comparison to the corresponding wing spots induced by etoposide only (in the positive control series) (Table 4).

On larval exposure to 100mM of ascorbic acid (vitamin C) before treatment with 0.5mM of etoposide, the 870 wings of enclosed adults contained 680 spots including 333 small singles, 265 large singles and 82 twin spots. The spots per wing frequencies were 4.21, 3.31, 1.02, and 8.5 for small singles, large singles, twin spots and total spots. When statistically analysed, the outcome for small single appeared weak and those for large singles, twin spots and total spots emerged as negative in comparison to those of positive control. On exposing the 3rd instar larvae to a mixture of 0.5mM of etoposide and 100mM of ascorbic acid, the total spots observed in the 80 wings of eclosed adults were 764 which included 387 small singles, 282 large singles and 95 twin spots. The spots per wing frequencies were 4.84, 3.52, 1.19 and 9.55 for small singles, large singles, twin spots and total spots respectively. When 0.5mM of etoposide exposed 3rd instar larvae were post treated with 100mM of ascorbic acid for 24 hours, the 80 wings of eclosing adults showed 909 spots comprising 442 small

singles, 334 large singles and 133 twin spots. The spots per wing frequencies were 5.52, 4.17, 1.66 and 13.36 for small singles, large singles, twin spots and total spots respectively.

When 2nd instar larvae were pretreated with ascorbic acid before their exposure to 0.25 mM of etoposide, the 80 wings of eclosing adults were found to contain 517 spots including 264 small singles, 212 large singles and 41 twin spots. The spots per wing frequencies were 3.3, 2.65, 0.51 and 6.46 for small singles, large singles, twin spots and total spots respectively (Table 4). Here again, while the statistical outcomes were weak for small singles, they were negative for each of large singles, twin spots and total spots. On treating the third instar larvae with 0.25 mM of etoposide and 100 mM of ascorbic acid concurrently, it was observed that there were a total of 580 spots in the 80 wings of eclosing adults which included 295 small singles, 227 large singles and 58 twin spots. The spots per wing frequencies of small singles, large singles, twin spots and total spots were 3.69, 2.84, 0.72 and 7.25 respectively (Table 4). When the larvae were post-treated with 100mM of ascorbic acid after their exposure to 0.25mM of etoposide, a total of 754 spots (9.42spots/wing) were found to be induced in the 80 wings of eclosing adults. The number of different categories of spots were 387 small singles, 286 large singles and 81 twin spots whose respective per wing frequencies were 4.84, 3.75, and 1.01.

The 80 wings of the adults eclosing from the larvae pretreated with 100 mM of ascorbic acid for 24 hours before treating them with 0.1mM of etoposide were found to contain 261 induced spots comprising 129 small singles, 103 large singles and 219 twins spots. The spots per wing frequerncies were 1.61, 1.29, 0.36 and 3.26 for small singles, large singles, twin spots and total spots respectively (Table 4). When third instar larvae were concurrently treated with 0.1mM of etoposide and 10mM of ascorbic acid mixed together, the 80 wings of eclosing adults exhibited 311 spots including 144 small singles, 131 large singles and 36 twin spots. The spots per wing frequencies were 1.8, 1.64, 0.45 and 3.89 for small singles, large singles, twin spots and total spots respectively (Table 4). When larvae exposed to 0.1mM etoposide were post-treated with 100 mM ascorbic acid, a total of 486 spots (6.07 spots/wings) were found to be induced in the 80 wings of adults eclosing from them. These included 191 small singles, 138 large singles and 57 twin spots whose respective per wing frequencies were 2.39, 1.72 and 0.71 (Table 4).

The wings of adults eclosing from 2nd instar larvae pretreated with 100 m of ascorbic acid before their exposure to 0.05 mM of etoposide have 68 singles, 41 large singles and 11 twin spots which together constituted 1230 spots. The spots per wing frequencies were 0.85, 0.51, 0.14 and 1.5 for small singles, large singles, twin spots and total spots respectively (Table 4). There were 145 induced spots including 79 small singles, 52 large singles and 14 twin spots in the 80 wings of the adults eclosing from 3rd instar larvae concurrently treated with a mixture of 0.05mM etoposide and 100mM ascorbic acid. The spots per wing frequencies were 0.99, 0.65, 0.17 and 1.88 for small singles, large singles, twin spots and total spots respectively (Table 4). On post-treating the larvae with 100mM ascorbic acid after their exposure to 0.05mM etoposide, 217 spots (2.71 spots/wing) were found to be induced in the 80 wings of adults eclosing from them. These spots included 103 small singles, 91 large singles and 23 twin spots with respective spots per wing frequencies of 1.29, 1.14 and 0.29.

DISCUSSION

Etoposide, the antineoplastic test chemical of this study was found to be genotoxic in the somatic wing spot cells of *Drosophila.* When transheterozygous larvae were treated with a potent mutagen, *mwh/flr*^{3} twin spots on the wings of eclosing adults developed due to an induction of mitotic recom-

Table 1: Wing spot data after larval exposure to Vitamin C.

Treatment Duration	Conc. of Vitamin C(mM)	No. of wings tested	Spots per wing (No. of spots) Stat. Diagn.* Small singles SS=1-2 M=2.0	Large singles LS > 2M=5.0	Twins (t) M=5.0	Total (T) M=2.0
72 hr and 48 hr	Control	160	0.43(68)	0.19(31)	0(0)	0.62(99)
	200	160	0.73(117)i	0.8(12)i	0(0)i	0.81(129)i
	100	160	0.63(101)-	0.05(8)-	0(0)i	0.68(109)-
	50	160	0.47(75)-	0.03(5)-	0(0)i	0.5(80)-

*Statistical diagnoses following Frei & Wurgler (1988) + = Positive, - = Negative, w = Weak, i = Inconclusive, m = Multiplication Factor. Probability levels:α =β=0.05. One sided statistical test.

Table 2: Wing spot data after larval exposure to etoposide (48 hr).

Treatment Duration	Conc. of Etoposide (μM)	No. of wings tested	Spots per wing (No. of spots) Stat. Diagn.* Small singles SS=1-2	Large singles LS > 2	Twins (t)	Total (T)
48 hrs	Control	160	0.64(103	0.03(5)	0(0)	0.68(108)
	0.5	80	11.09(887)+	7.45(596)+	2.41(193)+	20.95(1676)+
	0.25	80	9.47(758)+	7.04(563)+	1.86(149)+	18.38(1470)+
	0.1	80	7.06(565)+	4.71(377)+	1.11(89)+	12.89(1031)+
	0.05	80	2.59(207)+	1.89(151)+	0.46(37)i	4.94(395)i

*Statistical diagnoses following Frei & Wurgler (1988) + = Positive, - = Negative, w = Weak, i = Inconclusive, m = Multiplication Factor. Probability levels:α =β=0.05. One sided statistical test.

Table 3: Wing spot data after larval exposure to Etoposide (72 hr).

Treatment Duration	Conc. of Etoposide (μM)	No. of wings tested	Spots per wing (No. of spots) Stat. Diagn.* Small singles SS=1-2	Large singles LS > 2	Twins (t)	Total (T)
72 hrs	Control	160	0.64(103)	0.03(5)	0(0)	0.68(108)
	0.5	80	13.60(1088)+	8.61(689)+	2.81(225)+	25.02(2002)+
	0.25	80	12.39(991)+	8.43(674)+	2.45(196)+	23.26(1861)+
	0.1	80	8.61(689)+	6.47(519)+	1.45(116)+	16.55(1324)+
	0.05	80	4.05(324)+	2.94(235)+	0.84(67)+	7.83(626)+

*Statistical diagnoses following Frei & Wurgler (1988) + = Positive, - = Negative, w = Weak, i = Inconclusive, m = Multiplication Factor. Probability levels:α =β=0.05. One sided statistical test.

bination in the chromosome region between the *flr*^{3} locus and the centromere on the left arm of chromosome 3. Since higher doses of etoposide induce high frequencies of such spots, it could be presumed that this test chemical was recombinogenic in the wing primodial cells.

Single spots may arise from recombination between the *mwh/flr*^{3} loci in *mwh +/+flr*^{3} flies. In this study the wings from inversion-heterozygous flies carrying TM3 balancer were chosen for examination. The induction of spots originating from mitotic crossing over is suppressed by balancer mitotic chromosome heterozygosity. The treatment of 3rd instar larvae with etoposide led to increase in the induction of spot frequencies compared to negative control group. As spots in the balancer heterozygous flies were induced significantly, it may be presumed that not only recombination but also mutation contributes substantially to the induction of spots by etoposide in this wing spot assay.

From amongst different categories of spots induced by the test chemicals in the wings of adult eclosing from treated transheterozygous larvae, the frequencies of single spots with either *mwh/flr*^{3} phenotype were found to be highest in comparison to their spontaneous frequency in the negative control set. The single spot with either *mwh/flr*^{3} phenotype may originate following induction of gene mutations or gene conversions in their corresponding wild type loci (either *mwh* or i) (Graf et al. 1984). Single spots may also be formed due to induction of segmental aneuploidy via chromosome breakage (Haynie & Bryant 1977). Again, *mwh* spots may originate as a result of mitotic recombination induced in the chromosome region between *mwh* and *flr*^{3} loci on the left arm of 3rd chromosome. Etoposide was also found to induce higher frequencies of large spots and twin spots than those of large spots developing spontaneously. The statistical comparison of frequencies of single spots induced by etoposide with that of spontaneous ones (at all four test doses) produced positive outcomes . Thus, this study corroborates the findings of Torres et al. (1998) that etoposide can induce mutation and somatic recombination in *D. melanogaster*. Mutagenic action of etoposide has also been demonstrated in other test systems by many investigators in the recent past (Slavotinek et al. 1993, Noviello et al. 1994, Sjoblom et al. 1994, Racord et al. 1995, Fantini et al. 1998, Mosesso et al. 1998, Boos & Stopper 2000, Wang & Eastmond 2002, Snyder 2003, Wang et al. 2007).

There was a significant reduction in the frequencies of wing spots when the transheterozygous larvae were exposed to 100mM concentration of Vitamin C before or concurrently or after treatment with a particular concentration of etoposide. This was possibly due to the antimutagenic or antirecombinogenic effect of Vitamin C. It is believed that the antimutagenic action of Vitamin C (or

Table 4: Wing spot data after larval exposure to Etoposide and Vitamin C.

				Spots per wing (No. of spots) Stat. Diagn.*			
Modes of treatment	Conc. of Etoposide (μM)	Conc. of Vit. CμM	No. of wings tested	Small singles SS=1-2	Large singles LS > 2	Twins (t)	Total (T)
Etopo +ve control	0.5	100	80	11.09(887)	7.45(596)	2.41(193)	20.95(1676)
Pre Vit. C + Etopo	0.5	100	80	4.21(337) W	3.31(265) -	1.02(82) -	8.5(680) -
Etopo + Vit. C	0.5	100	80	4.84(387) W	3.52(282) -	1.19(95) -	9.55(764) -
Etopo + Post Vit. C	0.5	100	80	5.52(442) W	4.17(334) -	1.66(133) -	11.36(909) -
Etopo +ve control	0.25	100	80	9.47(758)	7.04(563)	1.86(149)	18.37(1470)
Pre Vit. C + Etopo	0.25	100	80	3.3(264) W	2.65(212) -	0.51(41) -	6.46(517) -
Etopo + Vit. C	0.25	100	80	3.69(295) W	2.84(227) -	0.72(58) -	7.25(580) -
Etopo + Post Vit. C	0.25	100	80	4.84(387) W	3.75(286) -	1.01(81) -	9.42(754) -
Etopo +ve control	0.1	100	80	7.06(565)	4.71(377)	1.11(89)	12.89(1031)
Pre Vit. C + Etopo	0.1	100	80	1.61(129)W	1.29(103) -	0.36(29) -	3.26(261) -
Etopo + Vit. C	0.1	100	80	1.8(144) W	1.64(131) -	0.45(36) -	3.89(311) -
Etopo + Post Vit. C	0.1	100	80	2.39(191)W	1.72(138) -	0.71(57) -	6.07(386) -
Etopo +ve control	0.05	100	80	2.59(207)	1.89(151)	0.46(37)	4.94(395)
Pre Vit. C + Etopo	0.05	100	80	0.85(68) W	0.51(41) -	0.14(11) -	1.5(120) -
Etopo + Vit. C	0.05	100	80	0.99(79) W	0.65(52) -	0.17(14) -	1.81(145) -
Etopo + Post Vit. C	0.05	100	80	1.29(103)W	1.14(91) -	0.29(23) -	2.71(217) -

*Statistical diagnoses following Frei & Wurgler (1988) + = Positive, - = Negative, w = Weak, i = Inconclusive, m = Multiplication Factor. Probability levels:$\alpha = \beta = 0.05$. One sided statistical test.

ascorbic acid) is connected with oxidative/reductive transmutation of ascorbic acid passing through the intermediate formation of monodehydroascorbic acid or, more likely its deionizing formation of a stable ascorbyl anion radical A with a high extent of unpaired electron delocalization' (Beiliski et al. 1981, Beiliski 1982 in Odin 1997). It has been repeatedly suggested that Vitamin C is an antioxidant that can trap O_2 and OH, inhibit free radical microsomal oxidation converting promutagens into mutagens and trap electrophilic species and compounds of an electrophilic nature (see Odin 1997). According to Odin (1997) Vitamin C seems not to be a very strong free radical scavenger or nucleophile, its action is probably mediated by oxiderivatives (*in vitro*) or some other bioantimutagens (*in vitro*). It increaseses the protective properties of an organism and reduces the genetic damages. The findings of this study suggest that Vitamin C can be used as a modulator of genotoxic effects of pharmaceutical compounds particularly that of cytotoxic antineoplastic drugs.

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