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Abstract: The antimutagenic effect of ascorbic acid (vitamin C) was investigated by using the Drosophila wing spot test. In this assay, 3-day-old transheterozygous larvae for the multiple wing hair (mwh, 3-0.3) and flare (flr, 3-38.8) genes were treated with 3 direct acting mutagens: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS) and N-nitroso N-ethylurea (ENU). The results obtained from 3 reference mutagens were clearly genotoxic in the Drosophila wing somatic mutation and recombination test. The calculated concentration of ascorbic acid as recommended dietary allowance (RDA) did not induce the frequency of the mutant clones recorded. When co-treatment experiments with ascorbic acid were carried out, ascorbic acid was effective in reducing the genotoxicity of the 3 direct acting mutagens.

Key Words: Drosophila, ascorbic acid, antigenotoxicity, somatic cells

Askorbik Asitin Üç Alkilleyici Ajanın Mutajenik Dozuna Anti-Genotoksik Etkisi

Özet: Bu çalışmada askorbik asitin antimutajenik etkisi Drozofila kanat benek testi kullanılarak araştırıldı. Bu çalışmada, "multiple wing hairs" (mwh, 3-0,3) ve "flare" (flr, 3-38,8) genleri bakımından 3-günlük transheterozigot larvalara doğrudan etkileyen mutajenler; etil metansülfonat (EMS), metil metansülfonat (MMS) ve N-nitrozo N-etilüre (ENU) uygulaması yapıldı. Drozofila Kanat Somatik Mutasyon ve Rekombinasyon Testinde elde edilen sonuçlar bu üç mutajenin açıkça genotoksik etkiye sahip olduğunu gösterdi. Askorbik asitin günlük alınması tavsiye edilen miktarına (RDA) göre hesaplanan konsantrasyon mutant klon frekansını indüklemedi. Askorbik asitle mutajenler birlikte uygulandığında, askorbik asit bu üç mutajenin genotoksik etkisinin azalmasında etkili oldu.

Anahtar Sözcükler: Drozofila, askorbik asit, antigenotoksisite, somatic hücreler

Introduction

The expression of mutation associated with genotoxic effects is the result of the interplay between risk factors and the responses of the host organism. These processes can be influenced by dietary agents such as micronutrients. Approximately 40 micronutrients (the vitamins, essential minerals and other compounds required in small amounts for normal metabolism) are required in the diet (1). Many epidemiological and experimental studies indicate that diets rich in antioxidant micronutrients may reduce the risk of certain types of cancer and mutation (2-6). Diet may well be a key factor in determining genomic stability since it impacts on all relevant pathways i.e. exposure to dietary carcinogens, activation/detoxification of carcinogens, DNA repair, DNA synthesis and apoptosis (7). Many environmental and chemical mutagens cause genetic damage (8-13) resulting in reduced egg and milk production (14,15), reductions in body weight (16) and sperm shape abnormalities (17) in animals. Many micronutrients act as substrate and/or co-factors in key DNA maintenance reactions, and the exact concentration of these in the cell may be critical.

Vitamins are very important as micronutrients in protecting macromolecules. One important micronutrient is ascorbic acid, which functions as a factor in several metabolic reactions such as post-translational modification, carnitine and noradrenalin synthesis, and tyrosine catabolism (18).

Various studies have been performed during recent years to investigate the genotoxic or antigenotoxic properties of ascorbic acid, as summarized in several detailed reviews (19-21). Most of the reported data indicate that ascorbic acid is antimutagenic in both in vitro and in vivo tests, i.e. in different eukaryotic tissues (22)

in human lymphocytes (23), in the *Allium* micronucleus test (24) and also in *Drosophila* assays (25). Although antioxidant protection is assumed for ascorbic acid against oxidative damage, under different conditions ascorbic acid seems to have co-genotoxic activity instead of the normal antigenotoxic action. Thus, it can promote the generation of oxygen radical-induced sister-chromatid exchanges in cultured mammalian cells (26), as well as some other types of genetic damage (22, 27). This would indicate that more experimentation is required to understand the antimutagenic and comutagenic action of ascorbic acid.

To increase our knowledge of the antigenotoxic action of ascorbic acid, we performed co-treatments with 3 direct acting mutagens ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS) and N-nitroso N-ethylurea (ENU). The ascorbic acid concentration (17 mM) was calculated as recommended dietary allowance (RDA) (28). We chose the *Drosophila* somatic mutation and recombination test (SMART) due to the many advantages that this eukaryotic organism has shown in in vivo genotoxicity testing studies (29). Thus, the wing variant of the SMART assay has proved to be a good tool to detect a broad range of genetic alterations in a fast and inexpensive way (30, 31)

Materials and Methods

Chemicals

The mutagens used in this study were EMS, MMS, ENU and L-ascorbik acid supplied by Sigma Chemical Co. (St. Louis, USA) Once the genotoxicity of the 3 selected direct acting mutagens was known, only one dose for each chemical tested was used in a co-treatment experiment with a 17 mM concentration of ascorbic acid. All the chemicals were prepared just before use. Distilled water was used as a negative control.

Strains

The principles and basic procedures for the *Drosophila* wing spot test have been described by Graf et al. (30). The transheterozygous larvae were obtained by crossing *mwh*/*mwh* males and *flr*^{3}/TM3, *Bd*^{6} virgin females. For detailed information on the genetic symbols and markers see Lindsley and Zimm (32).

Experimental Procedure

Eggs from the cross between *mwh* males and *flr*^{3} virgin females were collected over 8-h periods, and 72 ± 4 h later the resulting larvae were floated off with tap water and recollected. After that, larvae were transferred into plastic vials containing 4.5 g of Drosophila instant medium (Carolina Biological Supply, Burlington, NC, USA) re-hydrated with 9 ml of the respective test solutions (mutagen, ascorbic acid, mutagen + ascorbic acid, and distilled water for the negative controls). All experiments were performed at 25 ± 1 °C and at approximately 60 % relative humidity.

The emerging flies were collected and stored in 70 % ethanol. Afterwards their wings were removed, mounted in Faure´s solution on microscope slides and inspected under 400x magnification for the presence of clones of cells showing malformed wing hairs. The mutant clones were classified into 3 types: (1) small single spots, containing 1 or 2 cells; (2) large single spots, containing 3 or more cells; and (3) twin spots, containing adjacent *mwh* and *flr*^{3} cells (30). A total of 80 wings were examined for each concentration.

Statistical analysis

For the evaluation of the induced effects, the frequencies of spots per wing were analyzed with a computer programe based on a multiple decision procedure proposed by Frei and Würgler (33). This method tests 2 alternative hypotheses: (i) the mutation frequency in the treated group is no higher than the mutation frequency in the control group; and (ii) the frequency in the treated group is no less than *m* times as high as the observed spontaneous mutation frequency in the control. For the statistical calculations the conditional binomial test, according to Kastenbaum and Bowman (34), was used with 5 % significance levels.

Results

The results obtained regarding the mutagenic and antimutagenic effects evaluation of ascorbic acid are shown in Tables 1-3

The results obtained with EMS are indicated in Table 1. Under our test conditions EMS was shown to be clearly genotoxic are increased the frequency of the 3 types of spots recorded. The results obtained from the negative control and optimum concentration of ascorbic acid did not differ from each other. Although 0.5 mM concentrations of EMS increased the mutation frequency

Table 1. Genotoxicity of EMS and the effect of co-treatment with ascorbic acid (AA).

Compound conc. (mM)	No. of wings	Small single spots (m 2)			Large single spots m 5)			Twin spots (m 5)			Total mwh spots (m 2)			Total spots (m 2)			Frequency of clone formation per 10^5 cells
	(N)	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Dist. Water	80	18	(0.23)		2	(0.03)		1	(0.01)		21	(0.26)		22	(0.27)		1.127
17 mM Ascorbic. Acid	80	18	(0.23)	i	1	(0.01)	-	1	(0.01)	i	20	(0.25)	-	20	(0.25)	-	1.025
0.5 mM EMS	80	40	(0.50)	+	13	(0.16)	+	7	(0.09)	+	55	(0.69)	+	60	(0.75)	+	2.218
17 mM AA + EMS	80	23	(0.29)	i	5	(0.06)	i	4	(0.05)	i	30	(0.37)	i	32	(0.40)	i	1.537

conc., concentration; No., number of clones; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988): + positive; - negative; i, inconclusive; m multiplication factor; probability levels $\alpha = \beta = 0.05$.

Table 2. Genotoxicity of MMS and the effect of co-treatment with ascorbic acid (AA).

Compound conc. (mM)	No. of wings	Small single spots (m 2)			Large single spots m 5)			Twin spots (m 5)			Total mwh spots (m 2)			Total spots (m 2)			Frequency of clone formation per 10^5 cells
	(N)	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Dist. Water	80	18	(0.23)		2	(0.03)		1	(0.01)		21	(0.26)		22	(0.27)		1.127
17 mM Ascorbic. Acid 0.1 Mm	80	18	(0.23)	i	1	(0.01)	-	1	(0.01)	i	20	(0.25)	-	20	(0.25)	-	1.025
0.1 Mm MMS	80	37	(0.46)	+	9	(0.11)	+	5	(0.06)	+	49	(0.61)	+	51	(0.64)	+	2.510
17 mM AA + MMS	80	20	(0.25)	i	3	(0.04)	i	0	(0.00)	-	23	(0.29)	i	23	(0.29)	i	1.178

conc., concentration; No., number of clones; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988): + positive; - negative; i, inconclusive; m multiplication factor; probability levels $\alpha = \beta = 0.05$.

Table 3. Genotoxicity of ENU and effect the of co-treatment with ascorbic acid (AA).

Compound conc. (mM)	No. of wings	Small single spots (m 2)			Large single spots m 5)			Twin spots (m 5)			Total mwh spots (m 2)			Total spots (m 2)			Frequency of clone formation per 10^5 cells
	(N)	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Dist. Water	80	18	(0.23)		2	(0.03)		1	(0.01)		21	(0.26)		22	(0.27)		1.127
17 mM Ascorbic. Acid 0.01 mM	80	18	(0.23)	i	1	(0.01)	-	1	(0.01)	i	20	(0.25)	-	20	(0.25)	-	1.025
0.01 Mm ENU	80	38	(0.48)	+	10	(0.13)	+	6	(0.08)	+	50	(0.63)	+	54	(0.68)	+	2.561
17 mM AA+ENU	80	9	(0.11)	-	5	(0.06)	i	1	(0.01)	i	15	(0.19)	-	15	(0.19)	-	0.768

conc., concentration; No., number of clones; Fr., frequency; D., statistical diagnosis according to Frei and Würgler (1988): + positive; - negative; i, inconclusive; m multiplication factor; probability levels $\alpha = \beta = 0.05$.

in 5 categories, co-treatments of EMS with 17 mM concentrations of ascorbic acid drastically reduced the frequency of mutant clones induced by EMS towards the control value. Under the effect of ascorbic acid, the mutation rate decreased to 42.5 %, 61.5 %, 42.9 %, 45.5 %, 46,7 % for small single spots, large single spots, twin spots, mwh spots, and total spots respectively (Figure).

Under our experimental conditions 0.1 mM MMS is shown to be clearly genotoxic Table 2. Nevertheless, the results from co-treatment of MMS with the concentrations of ascorbic acid shown indicate that the mutation rates are not statistically different from the negative control in all categories. Ascorbic acid decreased the mutation rate to 45.9 %- 66.7 %.

In our experiment, a 0.01 mM concentration of ENU was shown to be clearly genotoxic (Table 3), inducing significant increases in the frequency of all the types of mutant clones recorded and the frequency of clone formation. Our results in the co-treatment experiments show that ascorbic acid reduced the genotoxicity of ENU in all types of mutant clones.

Discussion

The antimutagenic activity of ascorbic acid has been extensively studied using both in vivo and in vitro systems, but there are not enough data on its antimutagenic effects in in vivo systems. The present study was designed to determine the effect of ascorbic acid on the mutagenicity of direct-acting alkylating agents (EMS, MMS and ENU) that are mutagenic both in vivo and in vitro.

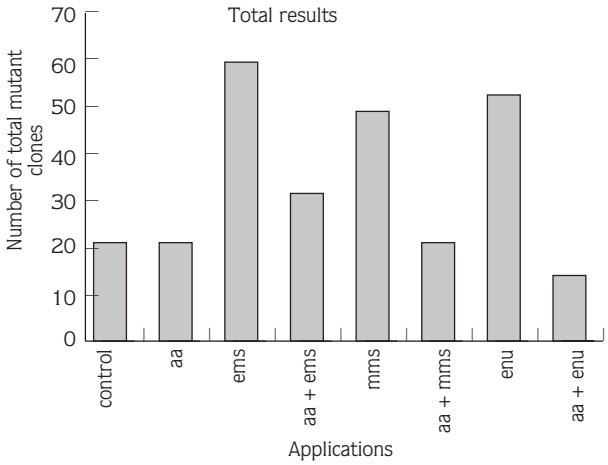

Figure 1. The antimutagenic effect of ascorbic acid (aa) against 3 alkylating agents (EMS, MMS, ENU).

Ascorbic acid is readily absorbed when the quantities ingested are small, but limited intestinal absorption occurs when excess amounts of ascorbic acid are ingested. Bioavailability of ascorbic acid in food is limited, but 80-90 % appears to be absorbed. Ascorbic acid is widely distributed throughout the tissues, both in animals capable of synthesizing ascorbic acid as well as in those dependent on an adequate dietary amount of vitamin C (35).

Antioxidants may reduce mutation induction by chemicals in different ways: (1) competition with the nucleophilic sites on DNA for an elecrophilic mutagen; (2) inhibition of promutagen bioactivation by blocking oxidation processes; (3) reaction with the electrophilic metabolite(s) of a promutagen (36). Mechanisms 2 and 3 may be involved when promutagens need activation whereas mechanism 1 rather concerns direct acting mutagens such as EMS, MMS and ENU. As a physiological molecule, ascorbic acid contributes to the natural antioxidant defense in cells (37). It inhibits the mutagenic activity of several N-nitroso compounds and other carcinogens such as benzo(a)pyrene and aflatoxine B1(38-40). Findings by Duthie et. al. (41) that both hprt mutant frequency and lymphocyte plating efficiency were weakly inversely associated with plasma ascorbic acid levels suggest that ascorbic acid may be protective against mutation at the hprt locus. It is also able to protect against in vivo nitrosation products of methyl urea in combination with sodium nitrite in the *Drosophila* wing SMART (25). The majority of previous studies shown the radical scavenging effects of ascorbic acid protect the cell from mutation mediated by radicals, but the results in this paper showed that its protective effects work not only against the reactive oxygen species, but also against direct acting mutagens.

Under physiological conditions ascorbic acid effectively competes with the alkylation of cellular nucleophilic sites such as those on DNA, RNA and proteins (42), thus masking these sites from binding by the mutagens. Therefore, the antimutagenic action of ascorbic acid against EMS, MMS and ENU may be related to its ability to block the covalent binding of tested direct alkylating agents to cellular DNA. The results presented indicate that EMS, MMS and ENU showed substantial mutagenicity

responses in *Drosophila* SMART assay. The results observed from EMS, MMS and ENU were compatible with our previous data (43). Treatment of larvae with 72 ± 4 h of mutagenic doses of EMS, MMS and ENU in combination with 17 mM of ascorbic acid supplementation clearly reduced the mutation frequencies as shown in the Figure and Table 1-3.

Finally, and from the results reported here, it should be noted that the *Drosophila* wing SMART is suitable for detecting the antimutagenic effects of some chemicals. Moreover, our results are of interest when investigating the different ways in which ascorbic acid can interfere in vivo with the mechanism of genotoxic agents.

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