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Original Article

Protective Effect of Caffeine on Ethyl Methanesulfonate-Induced Wing Primordial Cells of *Drosophila Melanogaster*

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ABSTRACT

Objectives: The antimutagenic effect of caffeine is evaluated against ethyl methanesulfonate (EMS)-induced mutation rate in Drosophila. **Materials and Methods:** The mutation rate is evaluated using wing mosaic assay. In transheterozygous larvae, multiple wing hair (mwh 0.3-3) and flare (flr 3-38.8) genes were used as markers of the extent of mutagenicity. **Results:** The results at 0.5 and 1.0 mM EMS concentration at both 48 ± 4 and 72 ± 4 h have shown consistent increase in mutation rate, which was being measured as frequency of clone formation per 105 cells. Toxicity of caffeine at 5 mM concentration was parallel to that of distilled water alone. At 0.5 mM EMS concentration at 42 ± 4 and 72 ± 4 h, Drosophila larvae mutation rate was significantly increased. Although caffeine prevented mutation rate in all pre, post, and combined treatment, it was more significant in pretreatment experiments where it was found to be effective in reducing the genotoxicity of EMS. However, the concentration of caffeine as recommended in dietary allowance did not induce the frequency of mutant clones in somatic mutation and recombination test (SMART) recorded. **Conclusion:** This study shows that caffeine significantly reduced the genotoxicity induced by EMS. However, the limitation in completely abolishing genotoxicity induced by EMS as observed at the dietary allowance of caffeine makes it interesting for further in-depth study. Further studies on the molecular mechanism of antigenotoxic effect of caffeine will also be interesting.

Key words: Antimutagenicity, caffeine, drosophila melanogaster, ethyl methanesulfonate

INTRODUCTION

The consumption of antimutagens has been suggested as an effective preventive measure for possible occurrence of deleterious effects resulting from exposure to number of mutagenic and carcinogenic agents in environment.[1-3]

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Many of the compounds with known antimutagenic, antcarcinogenic, and antioxidant properties are naturally present in fruits, vegetables, spices, coffee, tea, and so forth.[1,4] Hence natural products can be perceived as potential source of inhibitors of mutagenesis and carcinogenesis caused by environmental substances. Thereby gradual intake of such natural products could be protective to animals in disguise.

Coffee is the most widely consumed natural beverage by the people around the world. It contains caffeine as major bioactive constituent along with caffeic acid, chlorogenic acid, kahweol palmiate, and cafestol palmiate as trace amounts. Caffeine acts as neurostimulator and exerts protective effect against genotoxic/carcinogenic activity

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of environmental chemicals in *in vitro* and *in vivo* assay system.[1,5-8] These elaborate findings indicate caffeine is a chemopreventive drug against mutagens and carcinogens.

Several studies have been reported during recent years on genotoxic and antigenotoxic properties of caffeine. It acts as double-edged sword, as an antigenotoxic,[9,10] antioxidant,[9,11,12] and genotoxic molecule.[13] Notwithstanding the aforementioned reports, somatic mutation and recombination test (SMART) has been assumed as the most effective way to assess the antigenotoxicity of natural compounds. There are no reports on antigenotoxicity of pure caffeine (CAF) in multiple wing hair (mwh) and flr3 Drosophila larvae barring a lone report being published by Abrahm[14] on coffee powder using Drosophila larvae. Therefore, we made an attempt to evaluate the antimutagenicity of pure caffeine in Drosophila larvae. Hence, this study may be regarded as an important step forward toward understanding the protective effect of caffeine in different mode of treatments against ethyl methanesulfonate (EMS)-induced mutation in Drosophila larvae.

MATERIALS AND METHODS

Chemicals

EMS (CAS No. 62-50.0) was purchased from Sigma Co., St. Louis, USA, sodium chloride, gum arabic, glycerol, and chloral hydrate from Himedia Chemicals, Mumbai, India. Distilled water served as a negative control and 0.1 mM EMS was used as a positive control.

Strains

Two *Drosophila melanogaster* strains were used: The mwhs strain with genetic constitution mwh/mwh and the flare strain with genetic constitution flr3/In (3LR) TM3, Bds. The transheterozygous larvae were obtained by crossing ORR: Mwh/mwh males and ORR: Flr3/TM3 females and were obtained from Agarkar Institute, Pune. The more detailed information on the genetic symbols and descriptions can be found in the work of Lindsley and Zimm.[15] The tests were performed as described in Graf *et al*.[16]

Drosophila SMART test

The SMART was essentially performed as described by Graf *et al*.[16] For this assay, the following cross of *D. melanogaster* flies was used: *ORR (1)*; *ORR (2)*; *flr3/In (3LR) TM3, Bds* virgin females were crossed with *mwh* males (flies that were kindly provided by Agarkar Institute, Pune). The first strain is characterized by constitutively high cytochrome P-450 activity. The markers *mwh* and *flr3* (misshapen, flare-like hairs) are recessive wing-hair mutations located on the third chromosome at 0.3 and 38.8, respectively. This test is able to detect a wide spectrum of genetic alterations including point mutations, deletions, unbalanced half-translocation and mitotic recombination, chromosomal loss, and non-disjunction as described in Graf *et al*.[16]

Transheterozygous larvae were obtained by parental crosses between *flr3* virgin females and *mwh* males. Eggs were collected from this cross during 8-h period in culture bottles containing fresh standard Drosophila medium (wheat powder, jaggery, agar agar, propionic acid, and water cooked). After 72 h, third instar larvae were floated off with tap water and transferred to plastic vials containing 1.5 g of Drosopila instant medium rehydrated with 9 ml of freshly prepared test solutions (mutagens, mutagens plus extracts, distilled water, and EMS used at positive control at 0.1 mM). For each treatment group in a total of 4000 larvae, 200 in each vial were used. The larvae were fed on this medium until pupation of the surviving larvae. All the experiments were carried out at 24 ± 1°C and at ~60% relative humidity.

Preparation and analysis of wings

The crossing procedure is distinguished phenotypically based on the TM3 and Bds marker. Marker-heterozygous flies (mwh/flr3) and balancer-heterozygous (mwh/TM3, Bds) genotypes were mounted on slides with Faure's solutions (30 g gum arabic, 30 ml glycerol, 50 g chloral hydrate, and 50 ml distilled water). Both the dorsal and ventral surfaces of the wings were analyzed under a microscope at 400× magnification for the presence of clones of cells showing malformed wing hairs, i.e. occurrence of small single spots consisting of one or two *mwh* cells, large single spots consisting of three or more cells, and twin spots consisting of adjacent mwh and *flr3* cells.[16] Single spots can be produced by somatic point mutation, chromosome aberration, deletion, or mitotic recombination; twin spots originate exclusively from mitotic recombination. To determine the recombinogenic activity, the frequencies of mwh clones on the marker-heterozygous wings are compared with the frequencies of mwh clones on the balancer-heterozygous wings. The difference in mwh clone frequency is a direct measure of the proportion of recombination.[17]

Statistical analysis

For the statistical assessment of genotoxicity, the frequencies of each type of spot per fly were compared pairwise with the corresponding negative control; for the antigenotoxicity of amifostine, the frequencies of each type of spot per fly were compared pairwise with the corresponding dose of 8 μg/ml fotemustine. A multiple-decision procedure was used to decide whether a result is positive, weakly positive, inconclusive, or negative.[18,19] For the statistical calculations, the conditional binominal test according to Kastenbaum and Bowman[20] was used with $P = 0.05$

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significance levels. The frequency of clone formation was calculated.[19,21] Based on clone induction frequencies per 105 cells, the recombinogenic activity was calculated as follows: Mutation frequencies (FM) = frequencies clones mwh/TM3 flies/frequencies clones mwh/flr3 flies; recombination frequencies (FR) = 1 − FM. Frequencies of total spots (FT) = total spots in mwh/flr3 flies spots/number of flies; mutation = FT × FM; recombination = FT × FR.[22,23] Based on the control-corrected spot frequencies per 105 cells, the percentage of amifostine inhibition was calculated as follows: (fotemustine alone − amifostine plus fotemustine/fotemustine alone) × 100[14].

RESULTS AND DISCUSSION

Many chemoprotective agents are found in the dietary material,[10] and these dietary sources containing phytochemicals have a positive bearing effect on ill health. Caffeine is one such phytochemicals that may reduce mutagenicity caused by mutagens in different ways: (1) competition with the nucleophilic sites on DNA for an electrophilic mutagen, (2) inhibition of promutagen bioactivation by blocking oxidation process, and (3) reaction with the electrophilic metabolites of a promutagen. Mechanisms one and three might be involved when direct acting mutagens like EMS interacts with DNA. Caffeine along with minor constituents like caffeic acid, chlorogenic acid, kahweol palimiate, and cafestol palmiate present in coffee may possibly play a crucial role in preventing the deleterious interaction between DNA and EMS. Caffeine can also block the binding of activated carcinogens to DNA, thus reducing the formation of DNA adducts.[24] The present investigation revealed that all the three doses of EMS had significantly increased the number of small, large, twin, and total spots tested either at both in 48 or 72 h larvae as compared with distilled water group [Table 1, Figure 1]. Based on these results, we have chosen 0.5 mM of EMS to conduct experiment for 48 ± 4 and 72 ± 4 h larvae. Caffeine was administered in pre, post, and combined treatment doses at 0.5 mM to EMS-treated Drosophila. All the three ways of caffeine treatment have inhibited the effect of EMS. It is notable that pretreatment of caffeine in both 48 ± 4 and 72 ± 4 h had significantly decreased number of twin spots, total spots, and frequency of clones per 10^5 cells compared with distilled water along with post and combined treatment doses [Table 2].

Our reports have confirmed the previous reports on *in vivo* antigenotoxicity of coffee in Drosophila and mice.[4-6,14] However, the concentration of caffeine is of critical importance because high doses of caffeine induce apoptosis and low concentration can act as antioxidant.[25-27] Furthermore, this study has demonstrated the suitability of non-mammalian *in vivo* assay for obtaining qualitative and quantitative data on antigenotoxic compounds. Our results were interesting when investigated through different ways in which the caffeine could interfere *in vivo* on the effect of genotoxic agent.

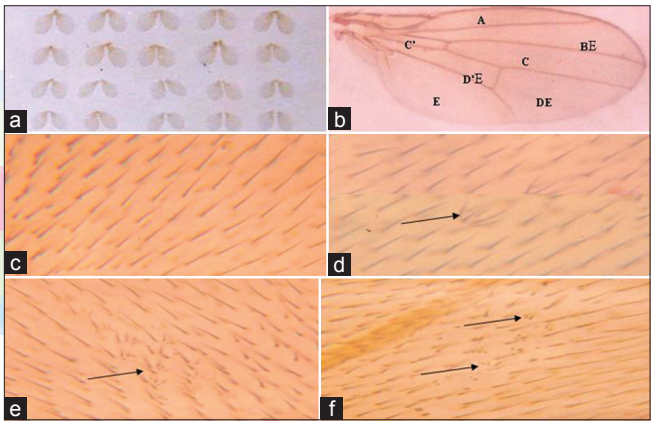


Figure 1: Effects of EMS on wings cells of *Drosophila melanogaster*. (a) Mounted wings, (b) Wing showing region a-e for scoring spots, (c) Normal trichomes of the wing, (d) *Mwh* spot with two-cell affected, (e) *Mwh* spots with more than two cells affected, and (f) Twin spot

Table 1: Comparison of wing spots with caffeine and different doses of EMS at 48±4 and 72±4 h in Drosophila larva

	Spots										Frequency of clone formation per 10^5 cells
	Small single		Large single		Twins		Total mwh		Total		
	N	Fr	*N*	Fr	*N*	Fr	*N*	Fr	*N*	Fr	
48±4 h Drosophila larva											
Distilled water	16	0.20	1	0.01	1	0.01	18	0.22	19	0.23	0.92
Caffeine 5mM	17	0.21	2	0.02	0	0.00	19	0.23	19	0.23	0.97
EMS 0.5 mM	27	0.33	15	0.18	9	0.11	42	0.52	51	0.63	2.15*
EMS 1.0 mM	42	0.52	21	0.26	14	0.18	63	0.78	77	0.96	3.22*
EMS 1.5 mM	46	0.58	23	0.28	16	0.20	69	0.86	85	1.06	3.53*
72±4 h Drosophila larva (mM)											
Caffeine 5	16	0.20	1	0.01	2	0.02	17	0.21	19	0.23	0.87
EMS 0.5	25	0.31	13	0.16	9	0.11	38	0.47	47	0.58	1.94*
EMS 1.0	40	0.50	21	0.26	13	0.16	61	0.76	74	0.92	3.12*
EMS 1.5	46	0.57	22	0.27	16	0.20	66	0.85	82	1.02	3.38*

EMS = Ethyl methanesulfonate, *N* = Number of wings (80 wings in all cases), Fr = Frequency of clone formation per 105 cells; clones/wings/24,400. *P<0.05, **P<0.01

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Table 2: Effect of pre, post, and combined treatment of caffeine against EMS-induced mutation rate at 42±4 and 72±4 h in Drosophila larvae

	Spots										Frequency of clone formation per 10^5 cells
	Small single		Large single		Twins		Total mwh		Total		
	N	Fr	*N*	Fr	*N*	Fr	*N*	Fr	*N*	Fr	
48±4 hrs Drosophila larva											
Distilled water	16	0.20	1	0.01	1	0.01	18	0.22	19	0.23	0.92
Caffeine 5 mM	17	0.21	2	0.02	0	0.00	19	0.23	19	0.23	0.97
EMS 0.5 mM	27	0.33	15	0.18	9	0.11	42	0.52	51	0.63	2.15
Pretreatment of CAF	19	0.23	2	0.02	2	0.02	21	0.26	23	0.28	1.07*
Post treatment of CAF	25	0.31	6	0.07	8	0.10	31	0.38	39	0.48	1.50**
Combined treatment of CAF	31	0.38	10	0.12	7	0.87	41	0.51	48	0.60	2.10
72±4 hrs Drosophila larva											
EMS 0.5 mM	25	0.31	13	0.16	9	0.11	38	0.47	47	0.58	1.94
Pre treatment of CAF	18	0.22	4	0.05	5	0.06	22	0.27	27	0.33	1.12*
Post treatment of CAF	27	0.33	5	0.06	3	0.03	32	0.40	35	0.43	1.63**
Combined treatment of CAF	30	0.37	7	0.08	6	0.07	37	0.46	43	0.53	1.89

EMS = Ethyl methanesulfonate, *N* = Number of wings (80 wings in all cases), Fr = Frequency of clone formation per 105 cells; clones/wings/24, 400. *P<0.05, **P<0.01

CONCLUSION

Pretreatment of caffeine significantly reduces the frequency of clones as compared with post and combined treatment in both 48 ± 4 and 72 ± 4 h larvae against EMS. These results suggest that the caffeine has antigenotoxic factors. As detected in the DNA repair test it is also involved in the antirecombinogenic activity.

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