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Protective effect of capsaicin against methyl methanesulphonate induced toxicity in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}

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[ABSTRACT] Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) is the main component in hot peppers, including red chili peppers, jalapenos, and habanero, belonging to the genus *Capsicum.* Capsaicin is a potent antioxidant that interferes with free radical activities. In the present study, the possible protective effect of capsaicin was studied against methyl methanesulphonate (MMS) induced toxicity in third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}. The third instar was allowed to feed on the diet having different doses of capsaicin and MMS separately and in combination. The results suggested that the exposure of third instar larvae to the diet having MMS alone showed significant hsp70 expression as well as tissue DNA and oxidative damage, whereas the larvae feed on the diet having MMS and capsaicin showed a decrease in the toxic effects for 48-h of exposure. In conclusion, capsaicin showed a dose-dependent decrease in the toxic effects induced by MMS in the third instar larvae of transgenic *Drosophila melanogaster.*

[KEY WORDS] Capsaicin; Methyl methanesulphonate; *Drosophila melanogaster;* Toxicity

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Introduction

Methyl methanesulphonate (MMS) is a well-known alkylating agent and carcinogen, having neoplastic as well as mutagenic properties [1-2]. Its toxic effects have been well documented in various experimental models like *Drosophila*, fish, Chinese Hamster Ovary (CHO) cells, cultured human lymphocytes, and rats [3-7]. MMS has the property to change both guanine (to 7-methyl guanine) and adenine (to 3-methyl adenine) to allow the mispairing of bases and replication blocks, respectively [8]. Originally, this action is believed to cause directly double stranded DNA breaks, because homologous recombination deficient cells are particularly vulnerable to the exposure of MMS [9]. MMS is known to cause somatic and sex linked mutation in *Drosophila* [10]. It is also reported to form protein adduct apart from DNA adduct as it methylates the N-terminus of valine and histidine residues in protein and is thus classified as super clastogen [11]. It also induce sister chromatid exchanges in human peripheral blood lymphocytes [6, 12-13].

Capsaicin is the principle ingredients in chili peppers belonging to the genus *Capsicum*. *Capsicum annum* fruits contained 1.27% capsaicinoids and 0.03% capsaicin [14]. It acts as an animal repellent and can be use as an insecticide [15]. It is also used in various creams and ointments [16] and has been found to reduce prostate cancer [17] and pain in rheumatoid arthritis [18] and delay the loss of the climbing ability of the transgenic flies expressing human alpha synuclein (h-αS) [19]. In the present study, an attempt was made to study the effect of capsaicin against MMS-induced toxicity in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}.

Materials and Methods

Fly strain

A transgenic *D. melanogaster* line expressing bacterial β-galactosidase in response to stress was used in the present study [20]. In the aforementioned strain, the transformation vector was inserted with a P-element, *i.e.,* the line contained wild type *hsp70* sequence up to lacZ fusion point. The flies and larvae were cultured at 24 ± 1 °C with standard *Droso-*

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phila food containing agar, corn meal, sugar, and yeast [21-22].

Experimental design

The final concentrations of capsaicin were 20, 40, and 80 μmol·L^{-1} alone and in combination with MMS (0.50 μL·mL^{-1}) were established in diet. The third instar larvae were allowed to feed on it for 48 h.

Soluble O-nitrophenyl-β-D-galactopyranoside (ONPG assay)

The expression of *hsp70* was quantified by performing soluble *O*-nitrophenyl-*β*-D galactopyranoside (ONPG) assay as described by Nazir *et al*. [21]. After being washed with phosphate buffer, the larvae were placed in microcentrifuge tubes (20 larvae/tube, 5 replicates/group), permeabilized by acetone for 10 min, and incubated in 600 μL of ONPG buffer at 37 °C overnight. After incubation for the desired duration, the reaction was stopped by adding 300 μL of Na_2CO_3 and the extent of the reaction was quantified by measuring absorbance at 420 nm [23-24].

In situ histochemical β-galactosidase activity

This assay was performed according to the method described by Chowdhuri *et al*. [23]. The larvae (20 larvae/treatment; 5 replicates/group) were dissected out in Pole's salt solution (PSS) and X-gal staining was performed. The larvae explants were fixed in 2.5% glutaraldehyde, washed with 50 mmol·L^{-1} sodium phosphate buffer (pH 8.0), and then stained with X-gal staining solution at 37 °C overnight in the dark.

Trypan blue exclusion test

The extent of tissue damage in larvae caused by the exposure of MMS alone and along with capsaicin was assayed by dye exclusion test [21, 25]. In this method, the internal tissues of larvae were explanted in PSS, washed with phosphate buffer saline (PBS), stained with trypan blue (0.2 mg·mL^{-1} in PBS) for 30 min, washed thoroughly with PBS, and scored immediately for dark blue staining. About 100 larvae per treatment (20 larvae/dose; 5 replicates/group) were scored for the trypan blue staining on an average composite index per larvae: no color = 0; any blue = 1; darkly stained = 2; large patches of darkly stained cells = 3; or complete staining of most cells in the tissue = 4 [25].

Preparation of larval homogenate

The larvae (50 larvae/dose; 5 replicates/group) were homogenized in 1 mL of cold homogenizing buffer (0.1 mol·L^{-1} Phosphate buffer containing 0.15 mol·L^{-1} KCl; pH 7.4). After centrifugation at 9 000 *g,* the supernatant was used for estimating lipid peroxidation, glutathione content, glutathione-S-transferase, catalase, protein carbonyl content, and acetylcholinesterase activity.

Lipid peroxidation assay

LPO was measured according to the method described by Ohkawa *et al*. [26]. The reaction mixture was consisted of 5 μL of 10 mmol·L^{-1} butyl-hydroxytoluene (BHT), 200 μL of 0.67% thiobarbituric acid, 600 μL of 1% *O*-phosphoric acid, 105 μL of distilled water, and 90 μL of supernatant. The resultant mixture was incubated at 90 ºC for 45 min and the OD was measured at 535 nm. The results were expressed as μmol of TBARS formed/h/g tissue.

Estimation of glutathione (GSH) content

The glutathione (GSH) content was estimated colorimetrically using Ellman's reagent (DTNB), according to the procedure described by Jollow *et al*. [27]. The supernatant was precipitated with 4% sulphosalicyclic acid (4%) in the ratio of 1 : 1. The samples were kept at 4 ºC for 1 h and then subjected to centrifugation at 4 500 *g,* at 4 ºC for 10 min. The assay mixture was consisted of 550 μL of 0.1 mol·L^{-1} phosphate buffer, 100 μL of supernatant and 100 μL of DTNB. The OD was read at 412 nm and the results were expressed as μmoles of GSH/g tissue.

Estimation of glutathione-S-transferase (GST) activity

The glutathione-S-transferase activity was determined using the method of Habig *et al*. [28]. The reaction mixture was consisted of 500 μL of 0.1 mol·L^{-1} phosphate buffer, 150 μL of 10 mmol·L^{-1} CDNB, 200 μL of 10 mmol·L^{-1} reduced glutathione and 50 μL of supernatant. The OD was taken at 340 nm and the enzyme activity was expressed as μmoles of CDNB conjugates/min/mg protein.

Catalase activity

The activity of catalase was estimated using the method of Beers and Sizer [29]. About 50 μL of homogenate was mixed with 438 μL of 0.1 mol·L^{-1} phosphate buffer and then 250 μL of 0.5 mol·L^{-1} H_2O_2 was added and the change in OD was read at 240 nm. The activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

Estimation of Protein carbonyl content

The protein carbonyl content was estimated according to the protocol described by Hawkins *et al*. [30]. The homogenate was diluted to a protein concentration of approximately 1 mg·mL^{-1}. About 250 μL of each diluted homogenate was placed into an eppendorf centrifuge separately, to which 250 μL of 10 mmol·L^{-1} 2, 4-dinitrophenyl hydrazine (dissolved in 2.5 mol·L^{-1} HCl) was added, vortexed and kept in dark for 20 min. Then 125 μL of 50% (*W/V*) trichloroacetic acid (TCA) was added, mixed thoroughly, and incubated at −20 °C for 15 min. The tubes were then centrifuged at 8 000 *g,* 4 °C for 10 min. The supernatant was discarded and the pellet obtained was washed twice with ice-cold ethanol : ethyl acetate (1 : 1). Finally, the pellets were re-dissolved in 1 mL of 6 mol·L^{-1} guanidine hydrochloride and the absorbance was read at 370 nm.

Determination of acetylcholinesterase (AChE) activity

AChE activity was estimated according to the method of Ellman *et al*. [31]. The reaction mixture was consisted of 650 μL of 0.1 mol·L^{-1} phosphate buffer (pH 7.4), 100 μL of 10 mmol·L^{-1} 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), and 100 μL of supernatant. The content was mixed thoroughly and the absorbance was measured at 412 nm. After having a stable absorbance value, 10 μL of 0.075 mol·L^{-1} acetylthiocholine was added and the change in absorbance per minute was recorded to calculate the enzyme activity.

Assay for caspase-3 (Drice) and caspase-9 (Dronc) activities

For estimating the activities of caspases 3 and 9, the