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Evaluation of the toxic potential of cefotaxime in the third instar larvae of transgenic *Drosophila melanogaster*

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ABSTRACT

The present study was carried out to evaluate the toxic potential of cefotaxime in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}. Cefotaxime at final concentration of 10, 20, 40, 60 and 80 μg/ml was mixed in the diet and the larvae were exposed to the selected doses for 6, 12, 24, 48 h. The *hsp70* expression, trypan blue exclusion test, *in situ* histochemical β-galactosidase activity, lipid peroxidation, total protein content, glutathione (GSH) content, glutathione-S-transferase (GST) activity, protein carbonyl content, caspase 3 and 9 activity, apoptotic index and comet assay were taken as parameters for the study. The larvae exposed to 40, 60 and 80 μg/ml for 12, 24 and 48 h showed a dose and duration dependent significant increase in the activity of β-galactosidase and lipid peroxidation but decrease in the total GSH content as compared to unexposed larvae. The decrease in protein content was observed in the larvae exposed to 40, 60 and 80 μg/ml of cefotaxime for 24 and 48 h. The larvae exposed to 40, 60 and 80 μg/ml of cefotaxime for 24 and 48 h showed a dose and duration dependent increase in the tissue damage, GST, caspase 3 and 9 activity, PC content, apoptosis and the DNA tail length (comet assay). The result suggests that the cefotaxime is toxic at 40, 60 and 80 μg/ml of doses for the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9}. Cefotaxime at 10 and 20 μg/ml was not toxic for any duration of exposure.

1. Introduction

Cefotaxime (CTX), a third generation broad spectrum cephalosporin is commonly used as perioperative anti-microbial prophylactic in orthotropic liver transplantation (OLT) [1]. It provides protection against wide range of bacteria [2]. It has been reported for the adverse effects in humans showing an increase in serum aspartate amino transferase level and the occurrence of pseudomembranous colitis [3]. It has been reported for extremely low toxicity profile with an intravenous LD_{50} values in the range of 9–10 g/kg [4]. The higher doses of cefotaxime (600 mg/kg/day) showed a significant mortality in neonatal and adult mice [5]. Antibiotics have been used in therapeutics for more than 70 years, though no rational standardized approaches have been defined for the treatment in humans [6]. Due to ethical reasons, the toxic evaluations cannot be performed directly on humans. The fruit fly *Drosophila melanogaster* has been extensively used in basic and applied research for various toxicological evaluations and human disorders [7–11]. Fly has advantage over mammalian model because of the ease of laboratory maintenance and genetic manipulations. As an alternative to higher animal, *Drosophila* has been well documented and recommended for toxicological evaluations. About 50% of the fly protein sequences have mammalian homologs and the results obtained can be extrapolated by using various mathematical models [12]. Heat shock proteins (HSPs) were initially reported to be expressed in response to heat but their expression is also triggered in response to diverse range of stress [13]. In the recent years, hsp70 has been considered to be one of the candidate genes for predicting cytotoxicity against environmental chemicals [14]. The present study was conducted to evaluate the toxic potential of cefotaxime using *hsp70* expression, tissue damage, X-gal staining, lipid peroxidation, protein content, apoptosis and comet assay as parameters in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9} at various doses and durations of exposure.

2. Materials and methods

2.1. Fly strain

A transgenic *D. melanogaster* line expressing bacterial β-galactosidase in response to stress was used in the present study [15].

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In the afore mentioned strain, the transformation vector is inserted with a P-element, i.e., the line contains wild type *hsp70* sequence up to lacZ fusion point. The flies and larvae were cultured on standard *Drosophila* food containing agar, corn meal, sugar and yeast at 24 ± 1 °C [16,17].

2.2. Experimental design

The final concentrations of cefotaxime, i.e., 10, 20, 40, 60 and 80 μg/ml were established in diet. The third instar larvae were allowed to feed for 6, 12, 24 and 48 h.

2.3. 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. [16]. After giving a wash of phosphate buffer, the larvae were placed in microcentrifuge tubes (20 larvae/tube; 5 replicates/group), permeabilized for 10 min by acetone and incubated overnight at 37 °C in 600 μl of ONPG buffer. 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 [18,19].

2.4. In situ histochemical β-galactosidase activity

The larvae (10 larvae/treatment; 5 replicates/group) were dissected out in Pole's salt solution (PSS) and X-gal staining was performed using the method as described by Chowdhuri et al. [18]. The larvae explants were fixed in 2.5% glutaraldehyde, washed in 50 mM sodium phosphate buffer (pH 8.0) and stained overnight in X-gal staining solution at 37 °C in the dark.

2.5. Trypan blue exclusion test

For studying the extent of tissue damage in larvae caused by the exposure to different dosages of cefotaxime dye exclusion test was performed [16,20]. Briefly, the internal tissues of larvae were explanted in a drop of PSS, washed in phosphate buffer saline (PBS), stained in trypan blue (0.2 mg/ml in PBS) for 30 min, washed thoroughly in PBS, and scored immediately for dark blue staining. About 50 larvae per treatment (10 larvae per 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 [20].

2.6. Preparation of larval homogenate

The larvae (10 larvae/dose; 5 replicates/group) were homogenized in 1 ml of cold homogenizing buffer (0.1 M phosphate buffer containing 0.15 M KCl; pH 7.4). The supernatant after centrifugation at 9000*g* was used for estimating lipid peroxidation, total protein, glutathione content, glutathione-S-transferase activity and protein carbonyl content.

2.6.1. Lipid peroxidation assay

Lipid peroxidation assay was performed as described earlier using 1,1,3,3-tetramethoxy propane as a standard [21,22].

2.6.2. Protein estimation

The protein content in all the treated as well as untreated groups were estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard [23].

2.6.3. Estimation of glutathione (GSH) content

The glutathione (GSH) content was estimated colorimetrically using Ellman's reagent (DTNB) according to the procedure described by [24]. The supernatant was precipitated with 4% sulphosalicylic acid (4%) in the ratio of 1:1. The samples were kept at 4 °C for 1 h and then subjected to centrifugation at 5000 rpm for 10 min at 4 °C. The assay mixture consisted of 550 μl of 0.1 M phosphate buffer, 100 μl of supernatant and 100 μl of DTNB. The OD was read at 412 nm and the results were expressed as μ moles of GSH/gram tissue.

2.6.4. Estimation of glutathione-S-transferase (GST) activity

The glutathione-S-transferase activity was determined by the method of Habig et al. [25]. The reaction mixture consist of 500 μl of 0.1 M phosphate buffer, 150 μl of 10 mM CDNB, 200 μl of 10 mM reduced glutathione and 50 μl of supernatant. The OD were take at 340 nm and the enzyme activity was expressed as μ moles of CDNB conjugates/min/mg protein.

2.6.5. Estimation of protein carbonyl content

The protein carbonyl content was estimated according to the protocol described by Hawkins et al. [26]. The larvae homogenate was diluted to a protein concentration of approx. 1 mg/ml. About 250 μl of each diluted homogenate was taken in Eppendorf centrifuge tubes separately. To it 250 μl of 10 mM 2,4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added, vortexed and kept in dark for 20 min. About 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 4 °C for 10 min at 9000 rpm. The supernatant was discarded and the pellet obtained was washed twice by ice cold ethanol:ethyl acetate (1:1). Finally the pellets were re-dissolved in 1 ml of 6 M guanidine hydrochloride and the absorbance was read at 370 nm.

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

For estimating the activities of caspase 3 and 9 the midguts of the larvae were collected in PBS.

The assay was performed according to the manufacturer protocol with some modification (Bio-Vision, CA, USA). The assay was based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) obtained after specific action of caspase-3 and caspase-9 on tetrapeptide substrates, DEVD-pNA and IETD-pNA, respectively. The assay mixture consisted of 50 μl of midgut cells homogenate and 50 μl of chilled cell lysis buffer incubated on ice for 10 min. After incubation, 50 μl of 2× reaction buffer (containing 10 mM DTT) with 200 μM substrate (DEVD-pNA for Drice, and IETD-pNA for Dronc) was added and incubated at 37 °C for 1.5 h. The reaction was quantified at 405 nm.

2.8. Assay to detect apoptosis

The apoptotic cells were analyzed by staining with ethidium bromide (EB) and acridine orange (AO). The midguts of the larvae were collected in PSS. The PSS was replaced by 300 μl of collagenase (0.5 mg/ml) and kept for 15 min at 25 °C. The collagenase was removed and the pellet was washed three times by PBS with gentle shaking [27]. Finally the pellet was suspended in 80 μl of PBS. About 25 μl of cell suspension was mixed with 2 μl of EB/AO dye. The staining dye was prepared by dissolving 100 μg/ml AO and 100 μg/ml EB in PBS. About 100 cells were scored per treatment (5 replicates/group) for estimating the apoptotic index and expressed in percent [28].