Protective role of curcumin against the toxic effects of cyclophosphamide in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg^9*

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Abstract

In the present study the effects of curcumin was studied against the toxic effects induced by 0.025 and 0.050 μL/ml of cyclophosphamide (CP) in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg^9* using *hsp70* expression and dye exclusion test as a parameter. The exposure of the third instar larvae to 0.025 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin results in the dose dependent significant decrease in the *hsp70* expression and tissue damage for 12,24 and 48 h of duration. Similar results were obtained with the exposure of third instar larvae to 0.050 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin. The selected doses of curcumin *i.e.* 1, 5 and 10 μg/mL were not toxic but reduced significantly the expression of *hsp70* and tissue damage induced by CP. The results of the present study suggest that the curcumin has a protective role against the toxic effects of CP in the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70*-lacZ)Bg^9.

Introduction

Cyclophosphamide (CP) is an alkylating agent.[1] It is used as a chemotherapeutic agent to treat various forms of leukemia,[2] tumors,[3] rheumatoid arthritis,[4] and Wegner's granulomatosis.[5] All living organisms under stressful condition respond by synthesizing heat shock proteins (HSPs).[6,7] In the recent years, *hsp70* has been considered to be one of the candidate genes for predicting the cytotoxicity against environmental chemicals.[8-10] Curcumin is the active ingredient of tumeric plant (*Curcuma longa* Linn., Zingiberaceae).[11] It has been reported to possess antimutagenic, anticarcinogenic, antigenotoxic, antioxidant, antitumor, anti-inflammatory properties in different tests systems.[12-16] Now-a-days the use of animals in toxicological/pharmacological research and testing has become an important issue for both science and ethics. As a result the emphasis has been given to the use of alternative to mammals in testing, research and education.[17] The European Centre for the validation of Alternative Methods (EVCAM) has recommended the use of *Drosophila* as an alternative model for scientific studies.[18] In our earlier study the effect of CP was studied for the *hsp70* expression at 0.0025, 0.025, 0.050, 0.10 and 1.0 μL/mL in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg^9* and was found to increase *hsp70* expression significantly as compared to the untreated at 0.025, 0.050 and 0.10 μL/mL.[19] In the present study an attempt has been made to validate this model for the evaluation of the natural plant products for their protective action. In the present study the effect of curcumin was studied against the toxic effects of CP in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg^9*.

Materials and Methods

Fly strain

A transgenic *Drosophila melanogaster* line that expresses bacterial β-galactosidase as a response to stress was used in the present study.[20] In the said strain of fly the transformation vector is inserted with a P-element, the line contains wild type *hsp70* sequence up to the lacZ fusion point. The flies and larvae were cultured on standard *Drosophila* food containing agar, cornmeal, sugar, and yeast at 24±1°C.[20]

Experimental design

CP at 0.025 and 0.050 μL/mL of food concentration alone and with 1, 5, 10 μg/mL of curcumin were established. The third instar larvae were allowed to feed on them for different durations (12, 24 and 48 h).

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

The expression of *hsp70* gives the measure of cytotoxicity.[21] Briefly, after washing the larvae in phosphate buffer, the larvae were taken in micro centrifuge tube (20 larvae/tube; 5 replicates/group), permeabilized for 10 min in acetone and incubated overnight at 37°C in 600 μL of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 μL Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm.[20]

Trypan blue exclusion test

Trypan blue, a vital dye fails to exclude blue staining in dead or dying cells and gives a reliable measure of tissue damage. The extent of the tissue damage in larvae by the exposure of different concentrations of CP alone and in combination with different doses of curcumin (1, 5 and 10 μg/mL) was assayed by a dye exclusion test.[22] Briefly, the internal tissues of larvae were explanted in a drop of phosphate buffer (PB), rotated in trypan blue stain for 30 min, washed thoroughly in PB and scored immediately for dark blue staining. Total 50 larvae per treatment (10 larvae per dose; 5 replicates per group) were scored for the trypan blue staining on an average composite index per larvae; no color, 0; any blue 1; darkly stained nuclei, 2; large patches of darkly stained cells, 3; or complete staining of most cells in the tissue, 4.[22]

Statistical analysis

Statistical analysis was carried out by one way analysis of variance (ANOVA) using commercial Software Statistica Soft Inc (2007).

Results

The results of the present study reveals that the exposure of 0.025 and 0.050 μL/mL of CP to the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg^9* for the duration of 12, 24 and 48 h showed an increase in the expression of *hsp70* (Table 1). The exposure of the third instar larvae to 0.025 μL/mL of CP

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along with 1, 5 and 10 μL/mL of curcumin for 12 h results in the reduction of the expression of *hsp70* (Table 1). Similar results were obtained for 24 and 48 h of exposure (Table 1). Similarly, the exposure of third instar larvae to 0.050 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin for 12, 24 and 48 h respectively, results in the reduction in the expression of *hsp70*. The regression analysis was also performed to study the dose effects of curcumin on the *hsp70* expression induced by 0.025 and 0.050 μL/mL of CP for the exposure to different durations (Table 2). The exposure of third instar larvae for 12 h to 0.025 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin was associated with the β-coefficient of -0.97 (F=15.446) (Table 2). For 24 and 48 h of exposures the β-coefficient values were -1.0 (F=177.96) and -1.0 (F=2375.11), respectively (Table 2). The reduction in the value of β-coefficient demonstrates the reduction in the β-galactosidase activity. The exposure of third instar larvae to 0.050 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin was associated with the β-coefficient values of -0.98 (F=23.24), -0.99 (F=79.48) and -0.98 (F=22.73) for 12, 24 and 48 h of exposure, respectively (Table 2). Trypan blue staining was performed to study the tissue damage induced by 0.025 and 0.050 μL/mL of CP alone and in combination with different doses of curcumin (1, 5 and 10 μg/mL) for different durations of exposure. About 95% of the larvae of untreated were negative to trypan blue staining even after 48 h except for light staining in the head region (Figure 1A). The target tissues were similar in the both treatments *i.e.* 0.025 and 0.050 μL/mL of CP only the intensity of staining was different. The exposure of 0.025 and 0.050 μL/mL of CP for 12 h results in the tissue damage of 90% of larvae in the mid gut, hind gut, malpighian tubules and brain ganglia (Figure 1B). The exposure of 0.025 and 0.050 μL/mL of CP for 24 h results tissue damage in the brain ganglia, salivary glands, gastric caecae, mid gut, hind gut and malpighian tubules of about 95% of the larvae (Figure 1C). The exposure of 0.025 and 0.050 μL/mL of CP to 48 h results in the tissue damage of about 95% of the larvae in brain ganglia, salivary glands, gastric caecae, proventriculus fore gut, mid gut, hind gut and malpighian tubules (Figure 1D). The exposure of larvae to 0.025 and 0.050 μL/mL of CP along with various doses of curcumin results in the reduction of the tissue damage as the dose of curcumin was increased. The exposure of larvae to 0.050 μL/mL of CP along with 1, 5 and 10 μg/mL of curcumin for 48 h results in the reduction of tissue damage of about 75% of larvae.

Discussion

The results of the present study reveal that the curcumin is potent in reducing the toxic effects induced by CP. *D. melanogaster* is a well established model for the study of antigenotoxic effects of different compounds and mixtures due to its well documented genetics and developmental biology.[23-26] *D. melanogaster* is also capable activating promutagens and procarcinogens.[27] The metabolite of CP is phosphoramide mustard.[28] It forms DNA cross links between and within DNA strands of guanine N-7 positions that heads to the cell death.[29] The higher doses of CP are associated with cytotoxicity.[28] Curcumin has a protective role against various mutagenic agents but the higher doses of curcumin are also cytotoxic.[11] In the present study the curcumin reduced the toxic effects of CP. The study of the anticancer drugs is of special significant due to the possibility that they may induce secondary tumors in cancer patients.[30] In several studies, the flavonoids have been found to be very protective against chemotherapy toxicity.[31] According to National Toxicological Programme guidelines development and validation of alternative models is necessary to get reliable and sensitive results. For traditional toxicological studies a shift for the use of animal models has been taken place to alternative models and in silico approaches to avoid animal experimentation.[32] *Drosophila* has a lot of similarities with human genome and is easy to handle, culture and ethical problems are less with this model. *Drosophila* as a model in pharmaceutical research is time efficient and cost effective in comparison to rodents. In future *Drosophila* will be used to detect adverse drug reactions. It will be helpful in reducing time and cost in the field of drug development process.[33] Mid gut tissues of insects have the highest concentration of cytochrome species and high microso-

Table 1. β-galactosidase activity measured in transgenic *Drosophila melanogaster (hsp70-lacZ)Bg[9]* third instar larvae exposed to different concentrations of cyclophosphamide and curcumin for various durations.

Treatments	O.D. (Mean±SE) after 12 h	O.D. (Mean±SE) after 24 h	O.D. (Mean±SE) after 48 h
CP (μL/mL)			
0.025	0.2483±0.0111*	0.2684±0.0132*	0.2793±0.0164*
0.050	0.2553±0.0122*	0.2783±0.0149*	0.2868±0.0183*
CP (μL/mL) + Curcumin (μg/mL)			
0.025+1	0.2301±0.0096*°	0.2493±0.0131*°	0.2541±0.0114*°
0.025+5	0.2283±0.0092°	0.2411±0.0103*°	0.2443±0.0101*°
0.025+10	0.2219±0.0086°	0.2332±0.0086*°	0.2329±0.093*°
0.050+1	0.2311±0.0087*°	0.2539±0.0116*°	0.2634±0.0128*°
0.050+5	0.2250±0.0073°	0.2421±0.0109*°	0.2501±0.0106*°
0.050+10	0.2213±0.0070°	0.2321±0.0067*°	0.2415±0.0109*°
Curcumin (μg/mL)			
1	0.2138±0.0098	0.2234±0.0068	0.2243±0.0057
5	0.2212±0.0053	0.2273±0.0078	0.2238±0.0063
10	0.2219±0.0074	0.2293±0.0089	0.2268±0.0082
Control	0.2122±0.0083	0.2133±0.0043	0.2136±0.0058

CP, Cyclophosphamide; OD, optical density; SE, standard error. *Significant at P<0.005 compared to untreated; °Significant at P<0.005 compared to CP treatment.

Table 2. Regression analysis for the dose effect of curcumin along with 0.025 and 0.050 μL/mL of cyclophosphamide for various duration of exposure.

S. No.	Treatments CP (μl/mL)	Duration (h)	Regression equation	r-value	β-coefficient	SE	P-value	F-value
1.	0.025	12	Y=0.23171-0.0009X	-0.9691	-0.97	0.00152	<0.0042	15.44
2.	0.025	24	Y=0.25070-0.0018X	-0.9972	-1.0	0.000864	<0.0022	177.96
3.	0.025	48	Y=0.25631-0.0024X	-0.9998	-1.0	0.00030	<0.0008	2375.11
4.	0.050	12	Y=0.23153-0.0011X	-0.9792	-0.98	0.00144	<0.0040	23.24
5.	0.050	24	Y=0.25553-0.0024X	-0.9938	-0.99	0.00174	<0.0044	79.48
6.	0.050	48	Y=0.26432-0.0023X	-0.9787	-0.98	0.00317	<0.0076	22.73

CP, cyclophosphamide; SE, standard error.

mal oxidase activity.[34] In the present study the damage is first observed in the mid gut tissues, probably the metabolic activation of CP takes place in the mid gut. The addition of curcumin results in the reduction of *hsp70* expression and tissue damage. This may be due to the possible prevention of metabolic activation of cyclophosphamide by curcumin.[35] The lower doses of chemotherapeutic agents when combined with selected antioxidants, could be used to obtain the same killing power as higher doses of the agent. Unlike, using higher dose of chemotherapeutic agents the enhanced efficiency mixture would be expected to reduce significantly for the complications associated to reduce significantly for the complications associated with chemotherapeutic agents.[36] The results of the present study showed that the selected doses of curcumin reduce the toxic effects of CP, and also supports the promotion of the use of alternative to higher laboratory animals such as mice/rats for the initial screening of the chemical agents for their possible toxic or protective effects.

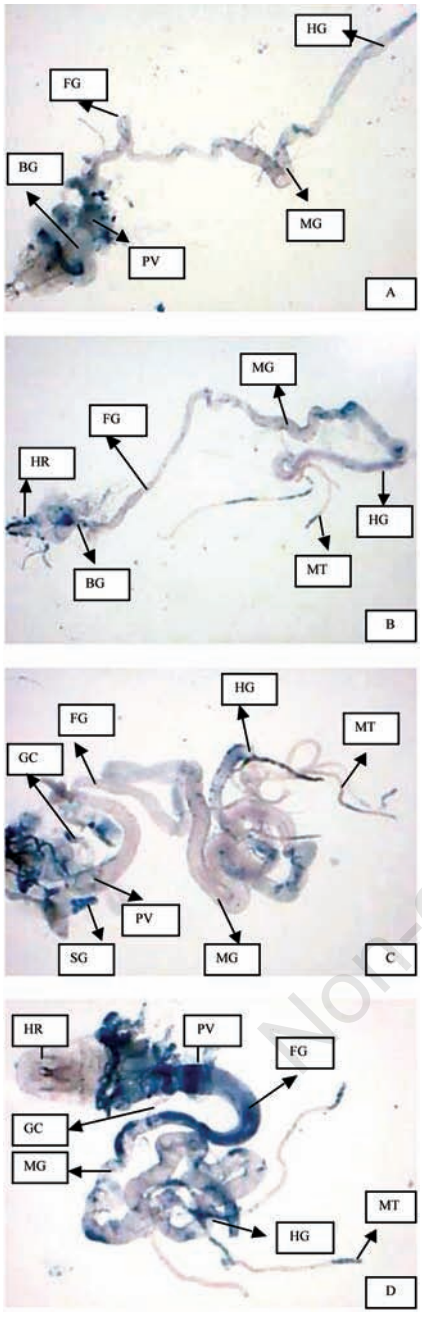

Figure 1. A) to D) show the Trypan blue staining in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*[9] exposed to 0.050 µl/mL of cyclophosphamide for 12 h (B), 24 h (C), 48 h (D) and control (A). HR, head region; BG, brain ganglia; PV, proventriculus; SG, salivary gland; GC, gastric caecae; FG, fore gut; MG, mid gut; HG, hind gut; MT, malpighian tubule.

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