Effect of Ethinylestradiol on *hsp70* Expression in Transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9

Yasir Hasan Siddique*, Gulshan Ara, Mohammad Afzal

Drosophila Transgenics Laboratory, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, U.P., 202002, India.

Summary

In the present study the effect of 0.25, 0.50, 1.0 and 2.0 µl/ml of ethinylestradiol was studied on 3rd instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9 for 6, 24 and 48 hrs of duration. The treatment of 0.25 µl/ml of ethinylestradiol did not induce significantly the activity of hsp70 as compared to control. The treatments of 0.5, 1.0 and 2.0 µl/ml of ethinylestradiol induced significant increase in the activity of *hsp70* for the different durations of exposure. The results of the present study suggest that the doses of 0.5, 1.0 and 2.0 µl/ml of ethinylestradiol are cytotoxic in the 3rd instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ)* Bg^9.

Keywords: *Drosophila melanogaster* (*hsp70 -lacZ*) Bg^9, ethinylestradiol, cytotoxicity, heat shock proteins.

Introduction

Estrogens are used for the cure of many types of sexual disorder and in oral contraceptive formulations [1]. There are sufficient evidences of the estrogens carcinogenicity and genotoxicity in various experimental models [2]. Ethinylestradiol, one of the common estrogens, is commonly used in oral contraceptives and in various other drugs formulations [1]. There are reports on the genotoxicity of ethinylestradiol in various experimental models [3-5].

All living organism under stressful condition responds by synthesizing heat shock proteins (HSPs) [6-7]. HSPs functions as molecular chaperons that prevent the cellular damage [8]. In the recent years, *hsp70* has been considered to be one of the candidate genes for predicting cytotoxicity against environmental chemicals [9-11]. In the present study, the toxicity of ethinylestradiol was investigated by *hsp70* expression in the larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9, for the different doses and hours of exposure.

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 [12]. In the said strain of flies, the transformation vector is inserted with a P-element, the line contain wild type *hsp70* sequence up to the *lacZ* fusion point. The flies and larvae were cultured on standard *Drosophila* food containing agar, corn meal, sugar, and yeast at 24^{o}C±1 [13].

Experimental Design

Ethinylestradiol was dissolved in dimethylsulphoxide and the 0.25, 0.50, 1.0 and 2.0 µl/ml of food concentrations were established. The third instar larvae were allowed to feed on them for different time intervals (6, 24 and 48 hr). Expression of *hsp70* gives the measure of cytotoxicity [14-15].

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

We followed the method as described by Nazir et al. [13]. Briefly, after washing, in phosphate buffer the larvae were taken in a micro centrifuge tube (20 larvae / tube, 5 replicates/group), permeabilized for l0 min by acetone, and incubated overnight at 37^{o}C in 600 µl of ONPG staining buffer. Following incubation, the reaction was stopped by adding 300 µl of Na_2CO_3. The extent of reaction was quantified by measuring the absorbance at 420 nm using systronics UV/VIS Spectrophotometer 118, India.

Positive and Negative Control

The healthy third instar larvae were placed on a petridish lined with moist filter paper and was given a temperature shock at 37±1^{o}C for 1 hr as positive control [13,16]. Dimethylsulphoxide at the dose of 2 µl/ml of food act as a negative control and the third instar larvae were allowed to feed for different durations.

Statistical analysis

Statistical analysis was carried out by student's t test using commercial software statistica Soft Inc (2007).

Results

The treatment of 0.25 µl/ml of ethinylestradiol did not show any significant increase in the β-galactosidase activity for various time intervals (Table 1) and the β-galactosidase activity was equivalent to that observed in the control. The treatment of 0.5 µl/ml of ethinylestradiol showed an increase in the β-galactosidase activity with an increase in exposure time (Table 1).

The increased activity of β-galactosidase at this concentration was significant ($P<0.005$), when compared with the control. The larvae fed on 1.0 and 2.0 µl/ml of ethinylestradiol also showed a significant increase in the activity of β-galactosidase for 6, 24 and 48 hrs of exposure compared to the control (Table 1). The negative control was associated with mean Optical Density values of 0.2432, 0.2506 and 0.2520, for 6, 24 and 48 hrs of exposure respectively (Table 1).

Table 1. β-galactosidase activity measured in transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae exposed to different concentrations of ethinylestradiol for various time intervals.

Treatments	**After 6 hr O.D (Mean±SE)**	**After 24 hr O.D (Mean±SE)**	**After 48 hr O.D (Mean±SE)**
Ethinylestradiol (µl/ml)			
0.25	0.2292 ± 0.0030	0.2291 ± 0.0005	0.2299 ± 0.0017
0.50	0.2766 ± 0.0008*	0.2864 ± 0.0014*	0.2950 ± 0.0011*
1.0	0.2928 ± 0.0031*	0.3018 ± 0.0040*	0.3196 ± 0.0051*
2.0	0.2920 ± 0.0004*	0.3132 ± 0.0035*	0.3944 ± 0.0014*
Control	0.2286 ± 0.0073	0.2196 ± 0.0073	0.2184 ± 0.0031
Negative control DMSO, 2µl/ml	0.2432 ± 0.0005	0.2506 ± 0.0026	0.2520 ± 0.0004

*$P<0.05$ compared to control

O.D. = Optical Density; SE = Standard Error; DMSO: Dimethylsulphoxide

Regression analysis was also performed for the dose, duration and β-galactosidase activity (Table 2).

Table 2. Regression analysis for the β-galactosidase activity in transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae exposed to different concentrations of ethinylestradiol for various time intervals.

S. No.	Duration	Regression equation	r	Standard error	P
1.	6 hr	Y = 0.23047+0.0845X	0.719	0.193	< 0.007
2.	24 hr	Y = 0.24671+0.03831X	0.794	0.238	< 0.009
3.	48 hr	Y = 0.2304+0.08454X	0.962	0.021	< 0.007

The treatment of 0.25, 0.50, 1.0 and 2.0 µl/ml of ethinylestradiol for 6 hr of exposure was associated with r value of 0.719 (Fig. 1).

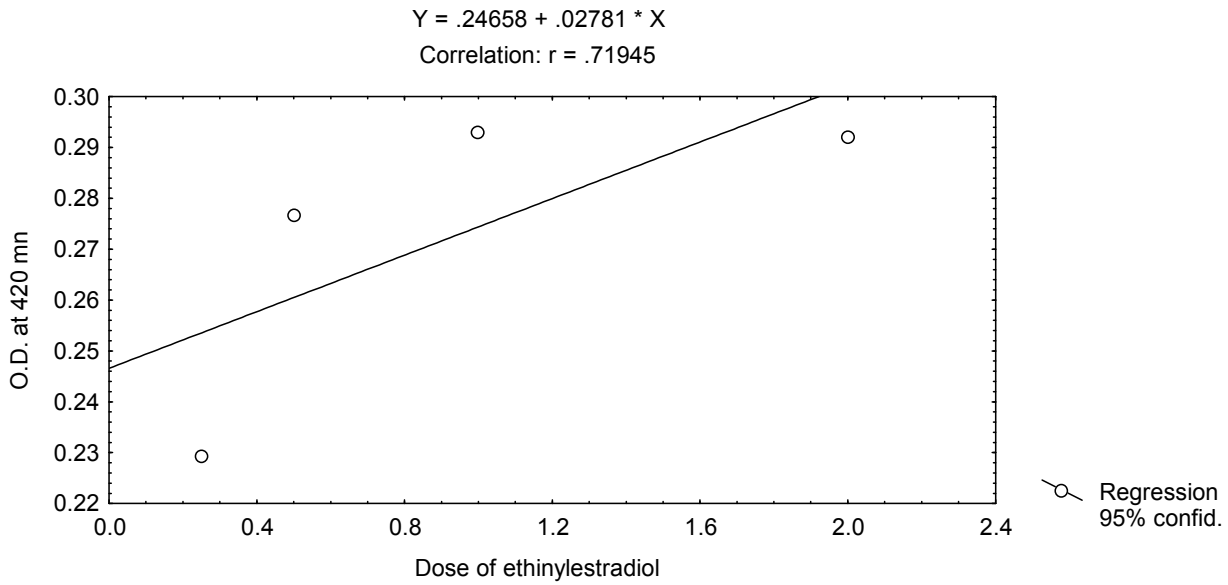

Fig.1. Regression analysis for the dose effect of ethinylestradiol for 6hr of exposure to transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae (dose of ethinylestradiol is in µl/ml)

Similarly, the treatments of 0.25, 0.50, 1.0 and 2.0 µl/ml of ethinylestradiol for 24 and 48 hrs of exposure were associated with r values of 0.794 and 0.962 respectively (Fig. 2 and 3).

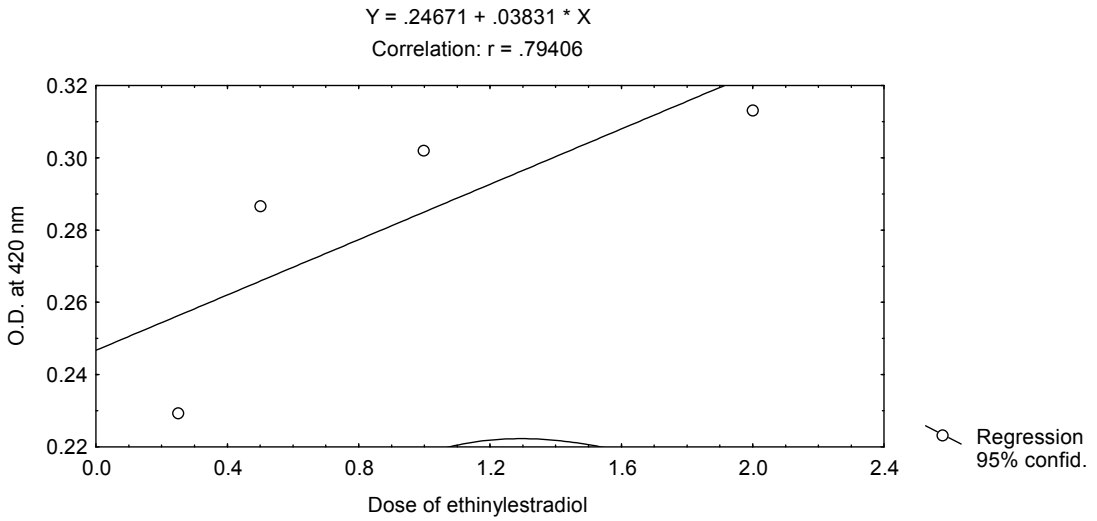

Fig.2. Regression analysis for the dose effect of ethinylestradiol for 24hr of exposure to transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae (dose of ethinylestradiol is in µl/ml)

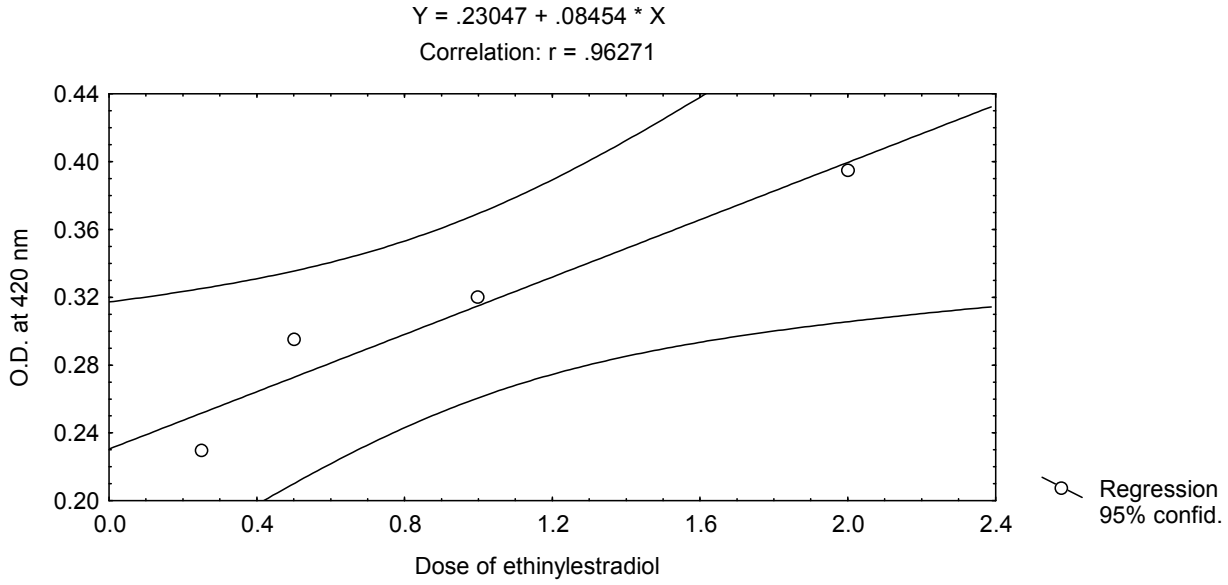

Fig. 3. Regression analysis for the dose effect of ethinylestradiol for 48hr of exposure to transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9 third instar larvae (dose of ethinylestradiol is in µl/ml)

Discussion

The results of the present study reveal that the ethinylestradiol was not potent to induce *hsp70* significantly (compared to control) as is evident by the β-galactosidase activity in third instar larvae of Bg^9 at 0.25 µl/ml of food. All other doses induced significant expression of *hsp70*. Expression of *hsp70* in the tissues was found to increase for the remaining doses with an increase in dietary concentrations. Although having protective roles in living systems, HSPs are being exploited by toxicologists [13-15, 17-18]. As being the effective biosensor to even a minor assault, now-a-days *hsp70* expression is considered to be an effective marker for toxicological evaluations [10]. Now-a-days the use of animals for toxicological evaluations has become the fundamental concern for scientists, not only because of protests from animal rights organizations but also because of difficulty in interpreting data due to intra species variation and exorbitant costs [10, 19]. This has led researches to encourage the use of alternative animals in toxicological evaluations [10]. *Drosophila* is a well established animal model for not only for geneticists but also for developmental and molecular biologists. In the past years a significant contribution has been made by successfully employing transgenic *D. melanogaster* as an alternative animal model for toxicological research [10-11, 14-16]. Although there is no comparative data but the studies by Hirsch et al. [20] indicates that fly and human have similar dose-response relationship with lead. In the present study a clear dose and duration response was observed (above 0.25 µl/ml of diet) on *hsp70* induction. Our earlier study with ethinylestradiol has shown that the metabolic activation and possible conversion of it to a reactive species is responsible for the genotoxicity [4]. HSPs are formed in response to stressors like LPO, DNA damage, osmotic imbalance, protein misfolding, membrane perturbation, metals, heat shock etc. [13]. A dose dependent increase in the activity β-galactosidase clearly demonstrates the dose dependent toxic effects of ethinylestradiol in transgenic *Drosophila melanogaster* (*hsp70-lacZ*) Bg^9, and strengthened the utility of *hsp70* expression as bioindicator of exposure to environmental chemicals.

Acknowledgements

The authors are grateful to the Chairman, Department of Zoology, Aligarh Muslim University, Aligarh for providing laboratory facilities. We are also thankful to Dr. D. Kar Chowdhuri, Scientist F & Head Embryotoxicology, IITR, Lucknow, UP, India for providing Bg^9 *Drosophila* strain.

References

1. Schwend TH, Lippman JS. Comparative review of recently introduced oral contraceptives containing norgestimate, desogestrel and gestodene and older oral contraceptives. In: Pavlik, E.J. *Estrogens, Progestins and their antagonists*, Birkhauser, Boston. 1996; 273-296.
2. IARC. Sex Hormones (II). IARC Monographs on the evaluation of carcinogenic risk of chemicals to humans. IARC, Lyon France, 1979; 21.
3. Siddique YH, Ara G, Beg T, Afzal M. Anticlastogenic effect of apigenin in human lymphocytes treated with ethinylestradiol. Fitoterapia 2010; 81: 590-594.
4. Siddique YH, Beg T, Afzal M. Genotoxic potential of ethinylestradiol in cultured mammalian cells. Chem Biol Interact 2005; 151: 133-141.
5. Hundal BS, Dhillon VS, Sidhu IS. Genotoxic potential of estrogens. Mutat Res 1997; 389: 173-181.
6. Nover L. Heat Shock response of eukaryotic cells. Berlin: Springe-Verlag, 1994.
7. Nover L.The heat shock response", Boca Raton, FL: CRC Press. 5-344, 1991.
8. Bennett AD, Waters MD. Applying biomarkers research. Environ Health Persp 2000; 108: 907-910.
9. Bierkens JGEA. Applications and pitfalls of stress proteins in biomonitoring, Toxicology 2000; 153: 61-72.
10. Mukhopadhyay I, Saxena DK, Chowdhuri DK. Hazardous effects of effluent from the chrome plating industry: 70kDa heat shock protein expression as a marker of cellular damage in transgenic *Drosophila melanogaster* (hsp70 lac Z). Environ Health Perspec 2003; 3: 1926-1932.
11. Mukhopadhyay I, Nazir A, Mahmood K, Saxena DK, Das M, Khanna SK, Chowdhuri DK. Toxicity of argemone oil: Effect on *hsp70* expression and tissue damage in transgenic *Drosophila melanogaster* (hsp70 lac Z) Bg^9. Cell Biol Toxicol 2002; 18: 1-11.
12. Lis JT, Simon JA, Sutton CA. New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an *hsp70-lacZ* hybrid gene. Cell 1983; 35: 403-413.

13. Nazir A, Mukhopadhyay I, Saxena DK, Siddiqui MS, Chowdhuri DK. Evaluation of toxic potential of captan: Induction of *hsp70* and tissue damage transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9. J Biochem Mol Toxicol 2003; 17: 98-107.

14. Chowdhuri DK, Saxena DK, Vishwanathan PN. Effect of hexachlorocyclohexane (HCH), its isomers, and metabolites on hsp70 expression in transgenic *Drosophila melanogaster*. Pesticide Biochem Physiol 1996; 63: 15-25.

15. Chowdhuri DK, Nazir A, Saxena DK. Effect of three chlorinatedpesticides on hsr ω sress gene in transgenic *Drosophila melanogaster*. J Biochem MolToxicol 2001; 15: 173-186.

16. Lakhotia SC, Singh AK. A novel set of heat shock polypeptides in Malpigian tubules of *Drosophila melanogaster*. J Genet 1989; 68: 129-138.

17. Guven K, Pomerai DI de. Differential expression of *hsp70* proteins in response to heat and cadmium in *Caenorhabditis elegans*. J Thermal Biol 1995; 20: 355-363.

18. K. Guven, J.A. Duce, D.I. de Pomerai, "Evaluation of a stress-inducible transgenic nematode strain for rapid aquatic toxicity testing," *Aquatic Toxicology,* Vol. 29, pp. 19-137, 1994.

19. Benford DJ, Hanley AB, Bottrill K, Oehlschlager S, Balls M, Brance F, Castegnara JJ, Descotes J, Hemminiky K, Lindsay D, Schilter B. Biomarkers as predictive tools in toxicity testing. The Report and Recommendations of ECVAM workshop 40, ATLA, 2000; 28:119-131, 2000.

20. Hirsch HV, Mercer J, Sambaziotis H, Huber M, Storke DT, Torno-Morley T, Hollocher K, Ghiradella H, Ruden DM. Behavioral effects of chronic exposure to low levels of lead in *Drosophila melanogaster*. Neurotoxicology 2003; 24: 435-442