Original Article

Validation of 1-methyl-2-phenylindole method for estimating lipid peroxidation in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}

Abstract

Background: A method using 1-methyl-2-phenylindole was developed for the estimation of lipid peroxidation in third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9}. The method is specific for the estimation of malonaldehyde. **Materials and Methods:** The larvae were exposed to 0.0025, 0.025, 0.050, and 0.100 µl/ml of cyclophosphamide for 24 and 48 h. The homogenate was prepared of the larvae tissue explant and the absorbance was noted at 586 nm. **Results:** A significant dose-dependent increase in the mean absorbance values was observed for both 24 and 48 h of exposure as compared to the untreated group. **Conclusions:** On the basis of results obtained, it is suggested that the present method is more precise, accurate, and robust for the estimation of lipid peroxidation in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ)Bg*^{9}.

Key words: 1-methyl-2-phenylindole, *Drosophila melanogaster (hsp70-lacZ) Bg*^{9}, lipid peroxidation

Yasir Hasan Siddique, Smita Jyoti, Falaq Naz, Mohammad Afzal

Drosophila Transgenic Laboratory, Section of Genetics, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Address for Correspondence:
Dr. Yasir Hasan Siddique, Department of Zoology, Faculty of Life Sciences, Aligarh Muslim University, Aligarh - 202 002, Uttar Pradesh, India.
E-mail: yasir_hasansiddique@rediffmail.com

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INTRODUCTION

Lipid peroxidation assay (LPA) is used as a biomarker of oxidative stress. It uses the estimation of malondialdehyde (MDA) resulting from a series of chain reactions involving the peroxidation of biological membranes.[1] MDA reacts with DNA and forms adducts.[2] Thiobarbituric acid reactive substances (TBARs) assay is a method used commonly to estimate the MDA production.[3] Estimation of MDA production using the chromogenic agent 1-methyl-2-phenylindole was done successfully in cultured human peripheral blood lymphocytes.[4] MDA and 4-hydroxyl-2-noneal (HNE) are the two most prominent lipid peroxidative products.[2,3] In the present method, 4-hydrononenal gives negligible reaction as compared to MDA, which reacts with two molecules of 1-methyl-2-phenylindole to yield a stable chromophore having an intense maximal absorbance at 586 nm.[5] Hence, an attempt has been made in the present study to validate this method for the estimation of lipid peroxidation in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9}. The larvae were exposed to various doses of cyclophosphamide (CP), an alkylating agent, for 24 and 48 h of duration.

MATERIALS AND METHODS

Chemicals

The chemicals used and their suppliers were as follows:
1-methyl-2-phenylindole (Sigma, USA), 1, 1, 3, 3, tetramethoxypropane (Sigma, USA), acetonitrile (SRL, India), Tris buffer (SRL, India), methanol (Qualigens, India), and HCl (Qualigens, India).

Fly strain

A transgenic *D. melanogaster* line that expresses bacterial β-galactosidase as a response to stress was used in the present study. In the said strain of flies, the transformation vector is inserted with a β-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, maize powder, sugar, and yeast at 24°C ±1.[6]

Experimental design

CP concentrations of 0.0025, 0.025, 0.050, 0.100 µl/ml of food were established and the third instar larvae were allowed to feed on them for 24 and 48 h. The LPA was performed after 24 and 48 h of the exposure of CP to the larvae.

Preparation of buffers

Reagent 1 (R1) was prepared by dissolving 0.064 g of 1-methyl-2-phenylindole into 30 ml of acetonitrile to which 10 ml of methanol was added to bring the volume to 40 ml. The 37% HCl prepared served as the reagent R2.

Preparation of standard

The standard (S2) was prepared by dissolving 16.5 µl of 1, 1, 3, 3-tetramethoxy propane in 10 ml of 20 mM Tris HCl (0.242 g of Tris HCl in 100 ml H_2O DW). The solution S2 was diluted to 1:100 in H_2O (DW), i.e. 20 µl of S2 was added to 2 ml of H_2O. The final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µM of S2 were prepared according to the method of Siddique *et al.*[4] The tubes were vortexed after adding 300 µl of R2 and incubated at 45°C for 40 min. After incubation, the tubes were cooled in ice and centrifuged at 15,000 *g* for 10 min. The readings were noted at 586 nm, and the standard was prepared.

Preparation of larvae homogenate and estimation of lipid peroxidation

The larvae (explants) were taken [five larvae per tube; three replicates per treatment in 1.5 ml Tris HCl buffer (ice cold, pH 7.4)] and the homogenate was prepared while keeping the tubes in melting ice. The homogenate was centrifuged at 3000 *g* for 20 min. 100 µl of the supernatant, 650 µl of R1, 100 µl of distilled water, and 150 µl of R2 were taken in the microcentrifuge tubes and vortexed. The tubes were incubated at 45°C for 45 min. The tubes were then cooled in melting ice and the readings were noted at 586 nm.

Statistical analysis

The statistical analysis was done using Statistica Soft Inc, India. The Student's *t*-test was applied to observe the significant differences between treatment and untreated groups. Regression analysis was performed using Statistica Soft Inc.

RESULTS

Figure 1 shows the standard curve for MDA estimation. The regression analysis for the standard shows the β-coefficient of 0.99958 ($P < 0.7689$) [Figure 2]. Table 1 and Figure 3 show the mean absorbance value after 24 h of the exposure of CP to larvae. The exposure of 0.0025, 0.025, 0.050, and 0.100 µl/ml of CP was associated with the mean absorbance values of 0.0440 ± 0.0015, 0.0750 ± 0.0015, 0.0956 ± 0.0012, and 0.1300 ± 0.0011, respectively. The untreated group was associated with the mean absorbance value of 0.0080 ± 0.0005 [Table 1, Figure 3]. Similarly, the exposure of larvae to 0.0025, 0.025, 0.050, and 0.100 µl/ml of CP for 48 h was associated with mean value of 0.05600 ± 0.0017, 0.0930 ± 0.0005, 0.1263 ± 0.0020, and 0.1536 ± 0.0046, respectively [Figure 3, Table 1]. The regression analysis was also performed for the treated groups. The value

Table 1: Lipid peroxidation assay (LPA) performed on the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bgs*[9] exposed to different doses of cyclophosphamide

Treatments cyclophosphamide (µl/ml)	After 24 h OD (Mean±SE)	After 48 h OD (Mean±SE)
0.0025	0.0440±0.0015[a]	0.0560±0.0017[a]
0.025	0.0750±0.0015[a]	0.0930±0.0005[a]
0.050	0.0956±0.0012[a]	0.1263±0.0020[a]
0.100	0.1300±0.0011[a]	0.1536±0.0046[a]
Untreated	0.0080±0.0005	0.0080±0.0006

[a]$P<0.01$, Significant with respect to untreated

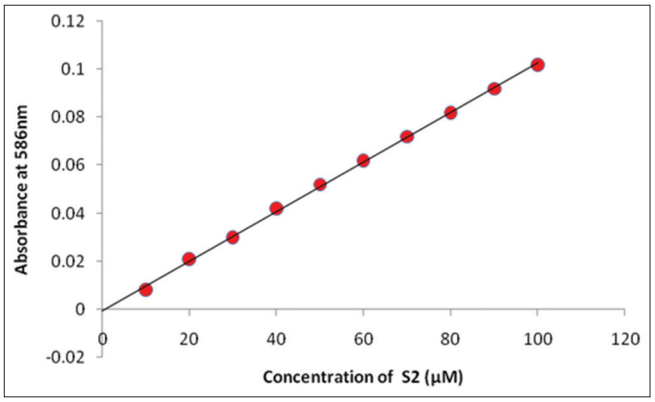

Figure 1: Standard graph for the estimation of lipid peroxidation

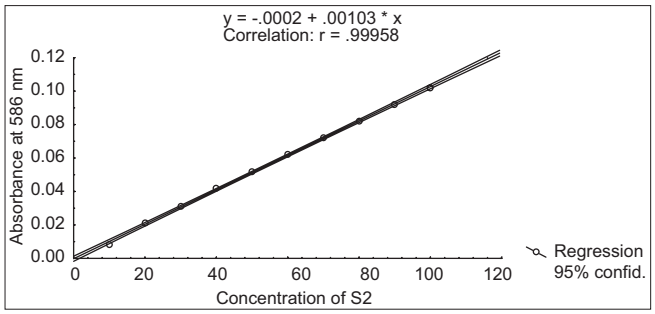

Figure 2: Regression analysis for the standard graph

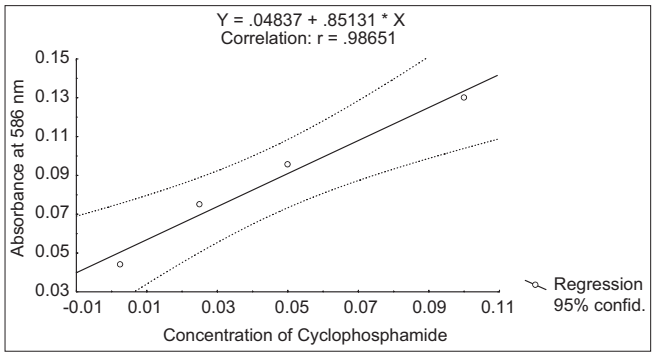

Figure 4: Regression analysis for the dose effect of cyclophosphamide on lipid peroxidation for the *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} third instar larvae exposed for 24 h

of β-coefficient (β = 0.98651; $P < 0.135$) for 24 h of exposure [Figure 4] clearly shows the concentration effect. The value of β-coefficient for 48 h (β = 0.96413; $P < 0.02745$) [Figure 5] demonstrates the dose as well as the duration effect of CP exposure to third instar larvae of transgenic *D. melanogaster (hsp70-lacZ) Bg*^{9}.

DISCUSSION

CP is an alkylating agent widely used to treat various types of malignant and non-malignant disorders.[7] The use of CP has limitations as it causes damage to the normal tissues and disrupts various biological and physiological processes.[8] CP has been reported to increase lipid peroxidation by increasing the oxidative stress.[9] The results obtained in the present study clearly demonstrate the effect of CP on lipid peroxidation and also validate the 1-methyl-2-phenylindole method for the estimation of lipid peroxidation. According to the National Toxicological Program's guidelines for development and validation of alternative models, it is necessary to obtain reliable and sensitive results. For traditional toxicological studies, a shift has taken place from the use of mammalian models to alternative models.[10] *Drosophila* as a model in toxicological evaluations is time- and cost-effective in comparison to rodents. The European

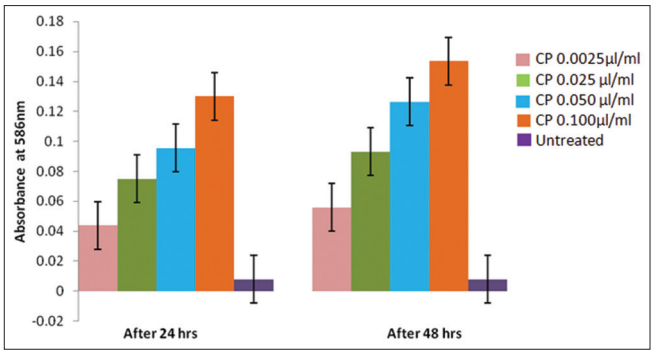

Figure 3: Lipid peroxidation in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} after the exposure of various doses of cyclophosphamide for 24 and 48 h of exposure

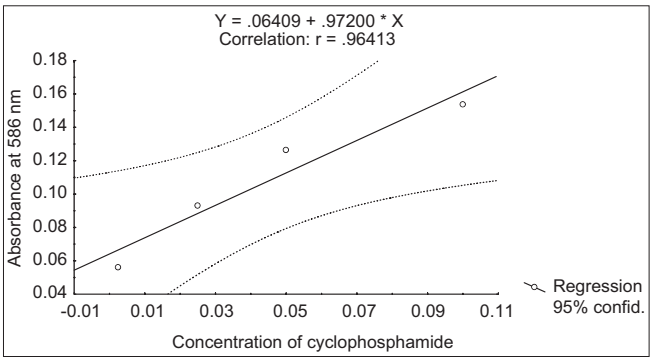

Figure 5: Regression analysis for the dose effect of cyclophosphamide on lipid peroxidation for the *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} third instar larvae exposed for 48 h

Centre for the Validation of Alternative Methods (EVCAM) has recommended the use of *Drosophila* as an alternative model for scientific studies.[11] The CP is converted into 4-hydroxycyclophosphamide by mixed function of oxidase enzymes in liver. Hydroxycyclophosphamide exists in equilibrium with its tautomer, aldophosphoramide. The major portion of the aldophosphoramide is oxidized by the enzyme aldehyde dehydrogenase (ALDH) to carboxyphosphoramide, but the minor portion is converted into phosphoramide mustard and acrolein.[1,8] The toxic effects has been attributed to the phosphoramide mustard which forms DNA crosslinks.[8] However, in one report, the depletion of glutathione has been suggested for the enhancement in lipid peroxidation by CP in rats.[12] Our earlier studies in transgenic *D. melanogaster (hsp70-lacZ) Bg*^{9} third instar larvae suggest the cytotoxic effects.[13,14] More damage was observed in the midgut tissue of the larvae which has high microsomal oxidase activity.[15] Therefore, it seems that the metabolites of CP are responsible for the enhancement of lipid peroxidation. The results suggest that this assay is specific for the measurement of MDA in the presence of hydrochloric acid[5] and is recommended for the estimation of lipid

peroxidation in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ) Bg*^{9}.

CONCLUSION

1-methyl-2-phenylindole method for the estimation of lipid peroxidation was found to be precise, accurate, and linear. Satisfactory results were obtained from this method. The most interesting feature of this method is its specificity for the measurement of MDA. The method is recommended for the estimation of lipid peroxidation in the third instar larvae of transgenic *D. melanogaster (hsp70-lacZ) Bg*^{9}.

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