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Original Article

Effect of L-ascorbic Acid on the *hsp70* Expression and Tissue Damage in the Third Instar Larvae of Transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9}

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ABSTRACT

All living organisms respond to various physical or chemical stressors by the induction of heat shock protein (HSP). The present study was performed on transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^{9} in which the transformation vector is inserted with a P-element, the line contains wild-type *hsp70* sequence up to the *lacZ* fusion point. The effect of L-ascorbic acid on the *hsp70* expression and tissue damage was studied at the doses of 1, 2, 4, and 8 × 10^{-4} g/ml in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}. The larvae were exposed to different doses of L-ascorbic acid for 24 and 48 hours. A dose-dependent significant increase in the *hsp70* expression was observed at 2, 4, and 8 × 10^{-4} g/ml of L-ascorbic acid for both 24 and 48 hours. The tissue damage was observed only in the 48 hours of exposure and mostly only in the salivary glands of the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}. The present study also validates and supports the use of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} for the toxicological evaluations.

Key words: *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^{9}, *hsp70*, L-ascorbic acid, trypan blue

INTRODUCTION

Stress response and anti-oxidant defense system comprising stress proteins and anti-oxidants respectively are the primary protective responses that are highly conserved components of cellular stress responses found in all phyla ranging from bacteria to man.[1] The highly conserved gene among the stress genes family is the *hsp70* and was first induced in *Drosophila*.[2] *Drosophila* has a well-defined genetics and molecular biology and hence, has been chosen as an experimental model.

The stress genes respond to thermal and several other environmental assaults and are collectively known as heat shock response. Due to their responsiveness to diverse form of stress, the stress proteins are nowadays used in biomonitoring and environmental toxicology.[3] The direct involvement of *hsp70* in defense, repair, or detoxification machinery of the cell makes it a direct and specific indicator of cellular stress.[4]

Vitamin C or L-ascorbic acid is an essential nutrient not only for humans, but for certain animal species.[5] Its ionic form ascorbate acts as an anti-oxidant by protecting the body against oxidative stress.[5] Ascorbate reacts with oxidants of the reactive oxygen species such as the hydroxyl radical (OH), a product of hydrogen peroxide. L-ascorbate is a strong reducing agent and is oxidized to L-dehydroascorbate. L-dehydroascorbate can be reduced back to the active L-ascorbate in the animals body by enzymes such as glutathione.[6]

The studies on heat shock protein (HSP) response have demonstrated the mechanisms of protection against various

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environmental stressors among animals.[7] Various factors that induce the HSP induction are also involved in the generation of reactive oxygen metabolites (ROM). As a result, the oxidative stress has been proposed as a key factor that mediates HSP response.[8,9] In the present study, the relationship between dietary supplementation of L-ascorbic acid and *hsp70* expression was carried out on the third instar larvae of transgenic *Drosophila melanogaster* (*hsp70-lacZ*) *Bg*^{9}.

MATERIALS AND METHODS

Fly strain

A transgenic *D. melanogaster* line that expresses bacterial beta-galactosidase as a response to stress was used in the present study.[10] The fly has a transformation vector inserted with P-element and the flies contain 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.[11,12]

Experimental design

L-ascorbic acid concentrations of 1, 2, 4, and 8 × 10^{-4} g/ml in the *Drosophila* diet were established. The third instar larvae were allowed to feed on them for 24 and 48 hours. The *hsp70* expression was quantified by soluble O-nitrop henyl-β-D-galactopyranoside assay and the tissue damage was evaluated by trypan blue exclusion assay separately, after 24 and 48 hours of exposure.

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

The expression of *hsp70* gives the measure of cytotoxicity.[13,14] We followed the method as described by Nazir *et al.*[11] The larvae were washed in the phosphate buffer (PB) and were put in a microcentrifuge (20 larvae per tube; 5 replicates per group), permeabilized for 10 minutes by acetone, and incubated overnight at 37°C in 600 µl of ONPG staining buffer. After incubation, the reaction was stopped by adding 300 µl of Na_2CO_3. The extent of the reaction was quantified by measuring the absorbance at 420 nm.

Trypan blue exclusion test

The extent of the tissue damage in larvae caused by the L-ascorbic acid was evaluated by a dye exclusion test.[11,15] Briefly, the internal tissues of larvae were explanted in a drop of phosphate buffer, rotated in trypan blue stain for 30 minutes, washed thoroughly in PB, and scored immediately for dark blue staining. A total of 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.[15]

Statistical analysis

Statistical analysis was carried out by Student's *t*-test using commercial software statistical Soft Inc. The regression analysis was also performed to see the dose effect on *hsp70* expression.

RESULTS

The results of the present study reveal that the exposure of the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} to different doses of L-ascorbic acid, i.e., 2, 4, and 8 × 10^{-4} g/ml for 24 and 48 hours increase the expression of *hsp70* significantly, as compared to the untreated [Table 1; Figure 1]. The expression was higher for 48 hours of exposure and dose dependent [Table 1; Figure 1]. However, the dose of 1 × 10^{-4} g/ml of L-ascorbic acid was not effective in inducing the expression of *hsp70* even after the 48 hours of exposure [Table 1; Figure 1]. The regression analysis was performed to study the dose effect of L-ascorbic acid on the expression of *hsp70* in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*)*Bg*^{9} for 24 and 48 hours of exposure [Table 2; Figures 2 and 3]. The exposure of 2, 4, and 8 × 10^{-4} g/ml of L-ascorbic acid for 24 hours was associated with the β-coefficient of

Table 1: β-Galactosidase activity measured in transgenic *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} third larvae exposed to different doses of ascorbic acid for different time intervals

Treatments ascorbic acid (g/ml)	After 24 hours OD (mean±SE)	After 48 hours OD (mean±SE)
1×10^{-4}	0.0550±0.0020	0.0586±0.0028
2×10^{-4}	0.0863±0.0170*	0.1056±0.0039*
4×10^{-4}	0.1056±0.0786*	0.1206±0.0040*
8×10^{-4}	0.1115±0.0115*	0.1820±0.0040*
Untreated	0.0510±0.0043*	0.0570±0.0036*

*Significant at *P*<0.05 compared to untreated. OD: Optical density, SE: Standard error

Table 2: Regression analysis for the β-galactosidase activity in the third instar larvae of transgenic *Drosophila melanogaster (hsp70-lacZ)Bg*^{9} to study the dose effect of ascorbic acid for 24 and 48 hours of exposure

Duration (hours)	Regression equation	*r*-value	β-coefficient	S.E	*P* value	*F*-value
24	*Y*=0.8335+0.00381*X*	0.883270	0.883	0.01070	0.0813	3.549
48	*Y*=0.07490+0.01311*X*	0.98920	0.989	0.0102	0.0868	45.535

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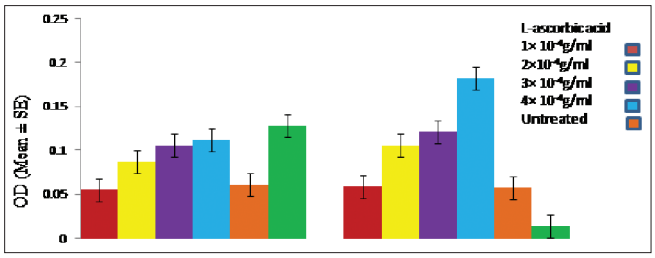


Figure 1: β-galactosidase activity observed after the exposure of L-ascorbic acid to the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}

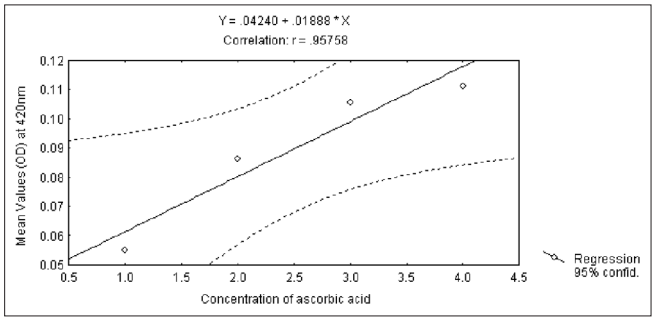


Figure 2: Regression analysis for the exposure of third instar larvae for 24 hours to different doses of ascorbic acid (×10^{-4} g/ml)

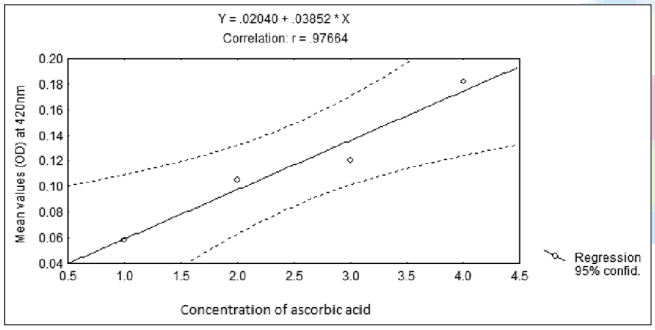


Figure 3: Regression analysis for the exposure of third instar larvae for 48 hours to different doses of ascorbic acid (×10^{-4} g/ml)

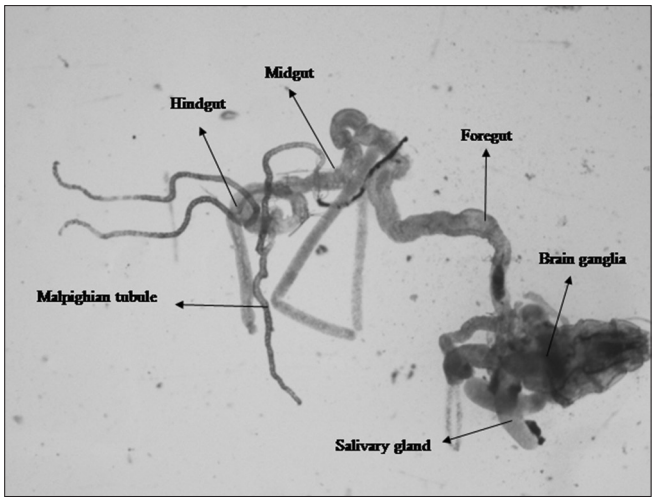


Figure 4: Trypan blue staining in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} (Untreated, after 48 hours)

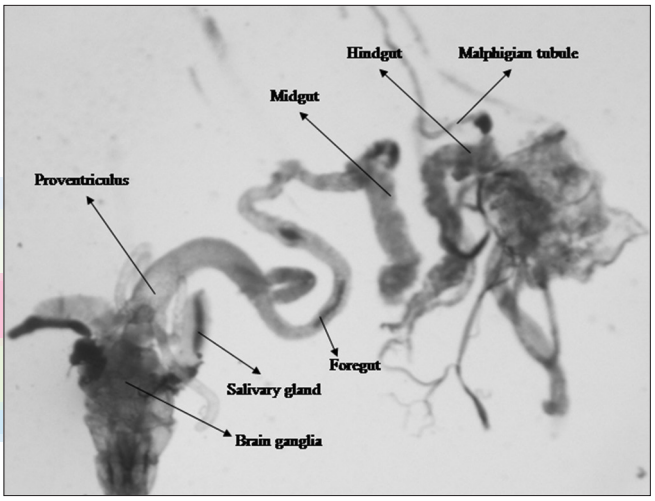


Figure 5: Trypan blue staining in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} exposed to 1 × 10^{-4} g/ml of ascorbic acid (after 48 hours)

0.883 (F = 3.549) [Table 2; Figure 2]. The exposure of 2, 4, and 8 × 10^{-4} g/ml of L-ascorbic acid for 48 hours h was associated with the β-coefficient of 0.989 (F = 45.53) [Table 2; Figure 3]. The values of β-coefficient for both durations of exposure clearly show the dose effect on *hsp70* expression. For 48 hours of exposure, the β-coefficient value is higher as compare to 24 hours of exposure, which demonstrates the duration effect. Trypan blue staining was performed to study the tissue damage induced by the exposure of L-ascorbic acid in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}. About 95% of the larvae were negative to trypan blue staining for all the doses of L-ascorbic acid studied for 24 hours of exposure. For 48 hours of exposure, about 85% of the larvae showed the damage in the salivary glands only and the intensity of damage was higher at higher doses of exposure. Figures 4-8 show trypan blue staining for the control and those exposed to 1, 2, 4, and 8 × 10^{-4} g/ml of L-ascorbic acid for 48 hours.

DISCUSSION

L-ascorbic acid can also act as a pro-oxidant besides acting as an anti-oxidant.[16] It has also been reported to reduce transition metals during the conversion of ascorbate to dehydroascorbate *in vitro* and generates superoxide and reactive oxygen species.[17] However, the possibility of free transition elements *in vivo* is negligible and hence, the possibility of the L-ascorbic acid acting as a pro-oxidant is also very low.[16] The LD50 of ascorbic acid in rats is 11.9 g/kg of body weight, the excess of ascorbic acid is not absorbed but is excreted in urine.[18] The mechanism of toxicity is not known, but the effect may be due to ascorbate high concentration.[19] *Hsp70* expression is a useful marker of cellular injury. It is express at very low level constitutively in human cells, but its expression increases in response to various physiological stressors.[20] The supplementation of ascorbic acid in the diet has been reported to reduce

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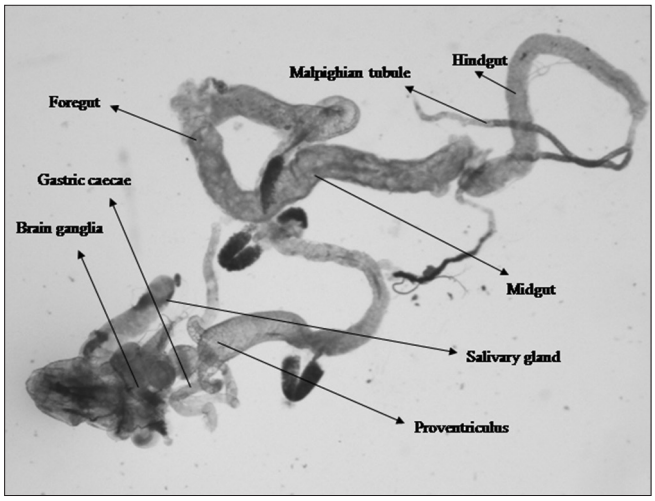


Figure 6: Trypan blue staining in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} exposed to 2 × 10^{-4} g/ml of ascorbic acid (after 48 hours)

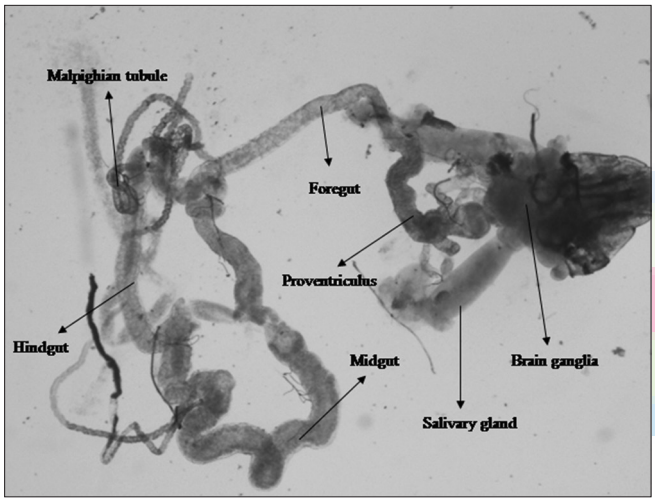


Figure 7: Trypan blue staining in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} exposed to 4 × 10^{-4} g/ml of ascorbic acid (after 48 hours)

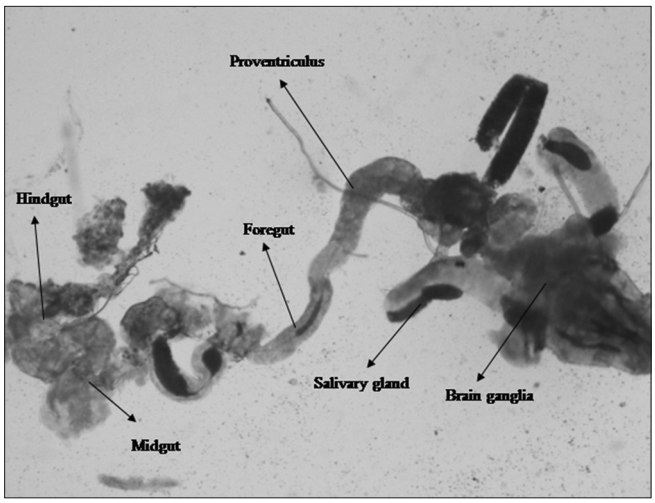


Figure 8: Trypan blue staining in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} exposed to 8 × 10^{-4} g/ml of ascorbic acid after (48 hours)

the expression of *hsp70*.[21] However, the higher doses may be toxic. As the reports on the toxic evaluations of L-ascorbic acid are warranted, the effects of various doses of ascorbic acid were studied in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}. At 1 × 10^{-4} g/ml of L-ascorbic acid, no significant increase in the *hsp70* expression was observed. The tissue damage by the exposure of L-ascorbic acid was observed at 8 × 10^{-4} g/ml dose only after 48 hours of exposure. A dose-dependent increase in the activity of β-galactosidase clearly demonstrates the dose-dependent cytotoxic effects of L-ascorbic acid in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}.

The National Toxicological Programme has suggested the development and validation of alternative models for the toxicological evaluations. For traditional toxicological studies, a shift has taken place from the use of mammalian models to alternative models such as *Drosophila*, Zebrafish, *C. elegans*.[22] The European centre for the validation of alternative method (EVCAM) has recommended the use of *Drosophila* as an alternative model for scientific studies.[23,24] The results in the present study suggest the cytotoxic potential of ascorbic acid at 2, 4, and 8 × 10^{-4} g/ml by increasing the expression of *hsp70* in the third instar larvae of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9}. Ascorbic acid supplementation has also been reported to increase DNA damage in the lymphocytes of humans.[19,25] High levels of 8-oxoadenine in DNA was observed after the supplementation of ascorbic acid with 500 mg per day.[25] Other studies have also shown oxidative DNA damage in cultured and isolated human lymphocytes in the presence of ascorbic acid.[26-28] The nature of ascorbic acid as a pro-oxidant is a debatable issue and has been very carefully worked out by Carr and Frei.[29] The presence of hydrogen peroxide also induce the oxidation of L-ascorbic acid and thus resulting it as a pro-oxidant.[30] The X-gal staining and the estimation of oxidative stress could have added more information to the study, but due to the limitation of our laboratory these tests have not been performed in the present study. The highest dose selected in our present study, i.e., 8 × 10^{-4} g/ml of diet also did not show any tissue damage in the third instar larvae of transgenic *D. melanogaster* (*hsp70-LacZ*) *Bg*^{9} except in the salivary glands. The reason for this damage is not known at present but it will be the part of our future study. Hence, it is concluded that the expression of *hsp70* on exposure to the environmental agents is a good indicator of non-target toxicity. The present study also validates and supports the use of transgenic *D. melanogaster* (*hsp70-lacZ*) *Bg*^{9} for the toxicological evaluations.

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