International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 12-01-2023, Accepted: 28-01-2023, Published: 14-02-2023
Volume 8, Issue 2, 2023, Page No. 76-84

Effect of Indian snake (*Naja naja*) venom on cognition and biochemical indices in *yellow* mutant of *Drosophila melanogaster* treated with N-Nitrosodiethylamine

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Abstract

The present study is based on the regulation of cognitive behaviour and biochemical function in N- Nitrosodiethylamine treated *yellow* mutant *Drosophila melanogaster* by snake venom (*Naja naja*). The flies were grouped into four divisions. (1) Control- *Yellow* mutant (2) *Yellow* mutant flies treated with 0.01% NDEA (3) *Yellow* mutant flies treated with 0.01% NDEA and 0.01% snake venom (4) *Yellow* mutant flies treated with 0.01% snake venom alone were distributed along with food medium for fourteen days. The behavioural changes were analysed after the experimental period. The behavioural assays including negative geotaxis, smell chemotaxis, phototaxis, taste chemotaxis, hygrotaxis and thermotaxis were performed on *yellow* mutant and NDEA-treated *yellow* mutant flies. When compared to the mutant control, the changes of its behaviour were noticed to be diversified in NDEA treated flies but seems to be normal in snake venom administered flies. Snake venom toxins (at appropriate concentrations) are used for the treatment of many pathological conditions. Snake venom has been reported to have anticancer, antioxidant, antihypertensive, antibacterial, anticoagulant and analgesic effects. The levels of non-enzymatic reactions such as thiobarbuturic acid reactive substance (TBARS), protein carbonyl, protein thiol and lipid peroxides were significantly elevated in NDEA treated flies than control *yellow* mutant flies and correspondingly tend to normalize in snake venom treated groups. Further, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidise (GPX) and reduced glutathione (GSH) were decreased in NDEA treated group and were significantly increased in snake venom treated groups. The proteins or peptides and enzymes isolated from snake venom may bind specifically to cancer cell membranes which may affect the migration and proliferation of cells.

Keywords: cognitive behaviour, snake venom, *yellow* mutant, peptides, cancer, antioxidants

Introduction

Drosophila melanogaster, the fruit fly is one of the most ideal organisms in pharmacological research especially in genetics and molecular biology. This is a very simple model organism, contains signaling pathways which controls cell growth and invasion as that of in mammals which have a conserved function in flies allowing their modulation into models that imitate the biology of tumors [1]. *D. melanogaster* has the ability to easily manipulate them genetically and 75% of genes have homologous in flies are responsible for human diseases [2]. In this study, we outline the basic behaviour changes during and after the treatment of NDEA and to identify the flies are how responsive in inducing uncontrolled growth and cancer development. We also studied the protective influence of small dose of snake venom under these conditions.

Drosophila has been a widely used vehicle to discover, elucidate and order components of pathways [3, 4]. Indeed, many of the known components of signaling pathways and other processes critical for human disease including cancer were first discovered in *Drosophila* screens [5]. The similarities between flies and humans are further supported by the fact that components of signal transduction pathways and the molecular mechanisms involved in specification, development, cell cycle regulation and human diseases were first identified in flies [6].

Yellow mutant flies are yellower than normal flies. They are defective in their *yellow* gene which is present in the X chromosome. Since *yellow* gene is required for the black pigment production, this yellow mutant flies cannot able to produce this pigment. The absence of a particular gene in *Drosophila* will result in a mutant embryo that does not develop a heart. *Yellow* (*y*) is the gene coding for melanin and could define the patterns of melanin on the skin of *D. melanogaster*. The yellow locus controls the melanotic pigment pattern of the cuticle of the adult fly and the pigmented mouth parts and denticle belts of the larval cuticle. Mutants that show a total loss of pigmentation from the cuticle (*y*-type) and mutants that show a mosaic pigment pattern, some regions of the cuticle being wild type and others yellow in color (*y2*-type). It is found that *yellow* gene have some neural function. The genetic changes in *yellow* gene are easily identified by the presence of unique yellow pigment in the cuticle of the adult flies and the mouth region of 3rd instar larva. In male flies, there is a disadvantage during mating when paired with wild type females due to a reduced level of locomotion [7]. *Y gene* have an effect on behaviour through the varied levels of neuroactive

catecholamines that are synthesized through dopa- like melanin [8].

D. melanogaster provides a model of choice for cancer analysis as the collection of sophisticated genetic manipulation techniques have been invaluable for dissecting signaling pathways that affect cell specification, differentiation and growth [3, 9]. *Drosophila's* genetic and genomic tools can be altered to construct sophisticated disease models for studying cancer and metastasis and for therapeutic development, while testing with mammalian models it is essential to get prior approval for human trials [10]. Any new drug can be tested in *Drosophila* much faster than the mammalian model and can also be used for high throughput screening methods as an alternative to cell culture [11].

Oxidative stress or excess reactive oxygen species (ROS) as an unavoidable result of aerobic respiration which is associated with disruption of cell signalling processes that control cell growth and cell death [12]. Excessive accumulation of ROS can damage DNA and proteins, disrupt important cell signalling pathways and ultimately leads to the disruption of cellular processes, contributing to aging and disease susceptibility of organisms [13]. *D. melanogaster* and mice have multifaceted effect on increasing tolerance to oxidative stress. Biochemical studies have linked superoxide dismutase (SOD) and catalase (CAT) as the basic mediators of ROS removal. Overexpression of both SOD and CAT transgenes result in improved oxidative stress tolerance and longevity in the genetic background of certain *Drosophila* [14, 15].

N-Nitrosodiethylamine (NDEA) is a group 2A carcinogen to all animal species. It acted upon the cytochrome P450 results in the formation of a carcinogen methyl diazonium ion. In general, administration of NDEA can affect the cytotoxicity, cell proliferation and DNA replication. An increase in cell proliferation is measured by increase in DNA replication which has been noticed in normal cells and changes in the organs were noticed during chronic NDEA administration [19]. Exposure of NDEA-initiated cells to a chemical that has tumor promoting activity which can promote tumor development by augmenting the proliferation of the initiated cells. At high doses the cell toxicity of NDEA might enhance the rate of restorative cell proliferation which could increase tumorogenicity. It has been found that cells initiated with NDEA are persistent and do not require the continued presence of NDEA to develop tumors. A single high dose or multiple low doses are sufficient to develop cancer [16].

Snakes use their own venom to make biological changes as the medicines do. Snake venom contains different bioactive proteins, enzymes, carbohydrates, minerals, polypeptides and proteins with low molecular weight with specific biological activities [17,18]. The cytotoxic effect of snake venom has been potentially used to destroy tumor cells. The deposition of fibrin around tumor could form a protective barrier and also prevent tumor progression [19]. Snake venom comprising of cystatin which decreases the tumor invasion and metastasis [20]. Snake venom compounds basically inhibits cell proliferation and promotes cell death[18, 20, 21].

The major issue identified with the cancer treatment is, after chemotherapeutics in the patients eventually gets resistance and do not response properly after initial treatment. This has led to the increased use of anticancer drugs produced from natural resources. Snake venoms have been used as a potential agent since it contains enormous biologically active compounds which has the cytotoxic effect that can be used to destroy tumor cells. Earlier, we have studied the behavioural abnormalities during the administration of NDEA and *Naja naja* venom in wild type flies [22]. The current study has been performed to identify the prevention of behavioural abnormalities and oxidative stress by the *Naja naja* venom during carcinogenesis by the action of N-nitrosodiethylamine in the *yellow* mutant flies.

Materials and methods

Yeast tablets, Benzaldehyde, agar, nepagin, propionic acid, sucrose, phosphate buffered saline, trichloroacetic acid, acetic acid, guanidine hydrochloride, thiobarbituric acid, n-butanol, dichromate, cumene hydroperoxide, ethylenediaminetetraacetic acid, hydrogen peroxide, glutathione reductase, Tris-hydrochloride, nicotinamide adenine dinucleotide phosphate and glutathione were purchased from Sigma-Aldrich, Bangalore, Karnataka, India. *Yellow* mutant flies of *Drosophila melanogaster* were obtained from the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, Telangana, India.

Venom source

Naja naja venom (500 mg) was acquired from the Irula Snake Catchers Co-operative Society, Mamallapuram, Tamil Nadu, India after obtaining proper approval. For experiments, venom was weighed and diluted in distilled water based on the requirement.

Experimental protocol

In the present study, *yellow* mutant flies of *D. melanogaster* were used. The mutant flies (received from Centre for Cellular and Molecular Biology (CCMB), Hyderabad) were retained at 20-25°C and cultured on standard in Light-Dark (12:12) condition. The experimental flies were divided into 4 groups, *yellow* mutant (group 1 – control, group 2 – NDEA (0.01%), group 3 – (snake venom + NDEA treated) and group 4 – snake venom (0.01%)) delivered through food medium for 21 days. Flies were grown in yeast, maize powder and sucrose diet with nepagin as anti-fungal agent at 21 ± 2°C in a 12:12 (light: dark) cycle. At the end of 21st day, flies (n = 30/group) were collected from all the four groups and were used for further analysis.

Haemolymph and tissue homogenate collection

In a 0.5ml eppendorf tube suitable holes were made and placed into 1.5ml eppendorf tube with removed lid. Approximately 30 number of *yellow* mutant flies were dissected by removing legs and wings. The tubes were centrifuged at 3500 rpm for 10 minutes and Haemolymph was collected from the bottom of 1.5 ml tube and was mixed with cold phosphate buffered saline. Head and intestine tissues were dissected from *yellow* mutant flies (n = 30). Using 0.1 M sodium phosphate buffer (pH 7.4) the respective tissue homogenates were prepared and then centrifuged at 5000 rpm for 10 minutes at 4°C and further used for biochemical assays.

Histopathology

For histopathological examination, the head and intestine were dissected from all the groups of *yellow* mutant flies of *Drosophila melanogaster*. The samples were kept for 48hrs in 10% neutral buffered formalin, dehydrated in ethyl

alcohol, washed in xylene, embedded in paraffin and stained with haematoxylin and eosin (H&E) and observed under the microscope (40x magnification) [23].

Behavioural assays to assess cognition in flies

The behavioural assays such as (a) negative geotaxis, (b) phototaxis, (c) smell chemotaxis, (d) taste chemotaxis, (e) thermotaxis and (f) hygrotaxis was carried out on *yellow* mutant flies and NDEA-treated *yellow* mutant flies by using the methods of Vang, Neckameyer and Bhatt [24,25] with minor changes.

Negative geotaxis

30 number of adult flies were held on a vertical glass column (15 × 1.5 cm) fixed to one side. By tapping the vial, the flies were trapped in the bottom of the column. Count the flies at the top of the row and the lowest flies separately after 1 minute. The results were given as the number of flies escaped at a minimum interval of 13 cm in 60 seconds (Figure 1a).

Phototaxis

In this study, two vials (15 × 1.5 cm) were connected to each other with clear tape and divided into three equal partitions (I, II and III, compartment I close to the light source). About 30 flies were placed in vials and sealed with cotton plugs. The flies were stored in the dark for 30 minutes. This validated the flies adaptation to the darkness. The flies were then introduced into a light source (emergency light, Philips, India) and held 5 cm away from the vial. (Figure 1b).

Smell chemotaxis

A powerful repellent benzaldehyde is used in this experiment and it was performed in the dark, 15 cm away from the light source which was visible during the experiment. Approximately 30 flies were placed in a vial (15 × 1 cm) and marked on three identical compartments (1, 2 and 3). The test tube (compartment 3 next to the cotton plug) was closed with a cotton plug soaked in 1 ml of 100 mM benzaldehyde. (Figure 1c).

Taste chemotaxis

In this study, we used sucrose, a non-volatile attractant. Approximately 30 flies were placed in test tube (18 cm × 1 cm) and divided into three equivalent sections. 1 ml of 0.1% sucrose was prepared and the cotton plug was dipped in it and was placed in a test tube. (Figure 1d).

Thermotaxis

Two vials (15 × 1.5 cm) were used in this study. The first vial was heated to a temperature of 45º C and was connected directly (at room temperature) to another vial with clear tape containing about 30 flies. The connected vials were divided into three equal zones (I, II and III – chamber III heating zone) (Figure 1e).

Hygrotaxis

1 ml of distilled water was placed in a vial (15 × 1.5 cm) sealed with a parafilm cover and was stored overnight. After 12 hours, about 30 flies were transferred to another vial (15 × 1.5 cm) and 2 vials were connected using clear tape. The connected vials were divided into three similar zones (I-humidification zone, II and III) (Figure 1f).

After 1 minute, count the flies present in each compartment and recorded the results as a percentage of flies. Repeated the tests on 3 separate sets of flies to calculate the mean ± Standard deviation for all the parameters.

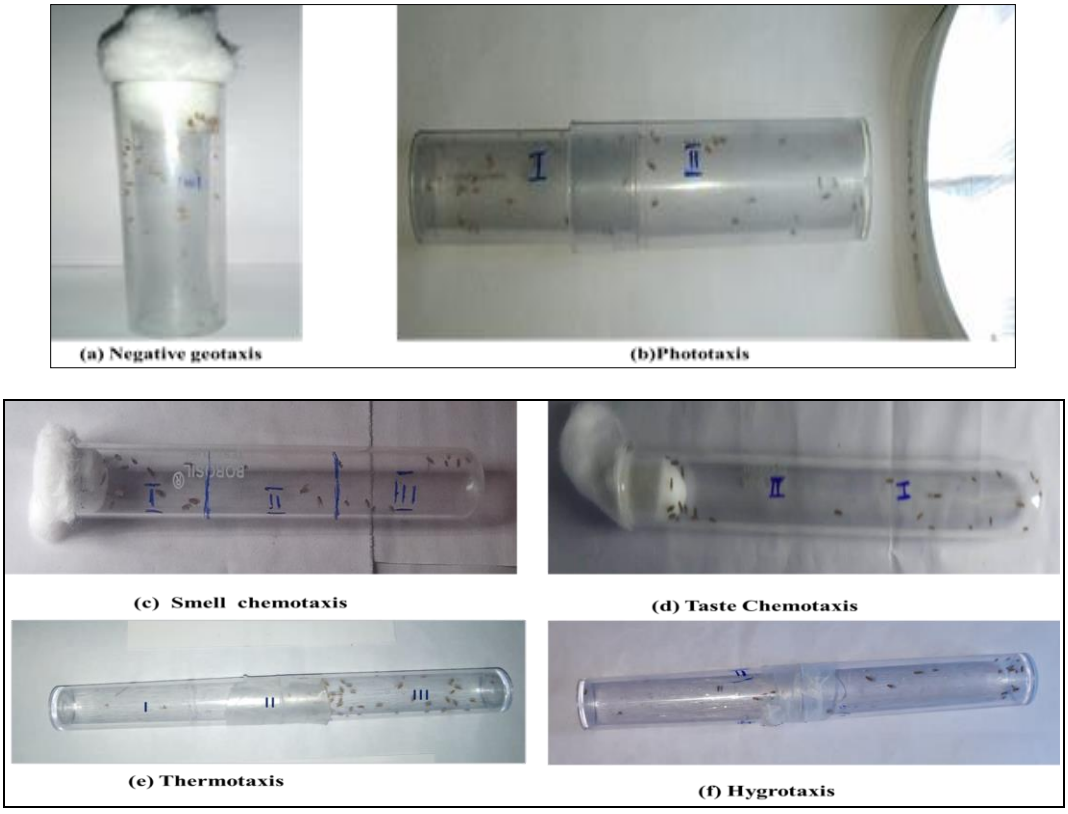

Fig 1: Behavioural assays in *yellow* mutant flies. Negative geotaxis (a), phototaxis (b), smell chemotaxis (c), taste chemotaxis (d), thermotaxis (e) and hygrotaxis (f).

Biochemical assays

Estimation of indices of redox homeostasis

Biochemical assays were first performed in *yellow* mutant *D. melanogaster* flies which were dissected to collect head and intestines from all the treated groups. To measure the carbonyl content of protein, the homogenated yellow mutant sample was split into two portions containing 1-2 mg of protein, then an equal amount of 2N HCl was added and incubated at 25 °C for 1 hour. After incubation, the mixture was precipitated with 10% TCA and centrifuged. The precipitate was mixed with a mixture of ethanol and ethyl acetate (1:1) and diluted with 1 mL of 6 M guanidine HCl and the difference in absorbance was measured at 360 nm. In TBARS (Niehaus & Samuelson method (1968)), malondialdehyde and other thiobarbituric acid-reactive substances (TBARS) are quantified by activity with acidic thiobarbituric acid (TBA) to produce a pink chromophore measured at 535nm. In addition, the thiol group of free proteins is determined by derivatization with 5, 5′ dithiobis (2-nitrobenzoic acid) (DTNB) in hemolymph/tissue homogenate of *yellow* mutant. The measurement depends on the appearance of the tinted mixture of thiolated ions which can be measured spectrophotometrically at 410 nm.

This lipid peroxidation assay was developed based on the reaction of the color developing reagent, N-methyl-2-phenylindole (R1) with malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) at 40°C. MDA or 4-HNE reacts with N-methyl-2-phenylindole to form a stable chromophore with an O.D. measured at 580 nm. Glutathione S transferase (GST) activity was assayed with tissue homogenate of *yellow* mutant by the Habig method using CDNB as a substrate, resulting in increased absorbance at 340 nm. Superoxide dismutase in erythrocytes and tissues of *yellow* mutant flies was determined by the kakkar method. After 90 seconds of incubation, the reaction was stopped by adding glacial acetic acid. After completion of the reaction, the color was extracted from the n-butanol layer and the absorbance at 520 nm was measured.

The activity of catalase in hemolymph/tissue homogenate of *yellow* mutant was investigated. 0.4 ml of hydrogen peroxide and 0.1 ml of tissue homogenate/hemolymph of the mutant flies were added with 0.9 ml phosphate buffer. Next 2.0 ml of dichromate-acetic acid mixture was added and the reaction was stopped after 15, 30, 45 and 60 s. The mixture was held in a boiling waterbath for 10 minutes, then cooled and the colour formation was measured at 610 nm. Specific activity was expressed as μmol of H_2 O_2 consumption / min/ mg protein for tissues or μmol. The Rotruck method was used to examine the GPx activity of red blood cells and tissues. After reacting a known amount of enzyme preparation with H2O2 in the presence of GSH for a period of time, the required GSH content was calculated. Reduced glutathione was estimated by the method of Ellman (1959) [26]. This procedure indicates that when 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to the sulfhydryl-containing compound, a yellow formation was observed.

Statistical analysis

Percentage of volume contribution was noted as mean ± SD. To analyse the data, the Statistical Package for Social Sciences (SPSS) version 17 (IBM Corporation, New York, NY, USA) was used which was assessed by one-way of analysis of variance (ANOVA). The test of homogeneity was employed to estimate the sample distribution of the data set.

Results

The histopathological observation of the head and intestine tissues of control and treated *yellow* mutant *D. melanogaster* are represented in Figure 2. Control tissues of head and intestine (Group-1), shows the normal architecture, granulated cytoplasm, small nuclei and nucleolus (Figure-2A and 2B). In NDEA treated group (Group-2), the figure shows loss of architecture, tumour cell is smaller than normal cells, large hyperchromatic nuclei (Figure-2C and 2D). Tumour island containing 'Keratin pearls' in its center, stratified epithelia migrating tumor cells with dysregulated proliferation, hyperplasia. In brain neoplastic overgrowth in tissues and in intestine tubule shaped structure encircling lumen. In NDEA+snake venom treated group (Group-3), the figure shows few neoplastically transformed cells and appear normal architecture (Figure-2E and 2F). In snake venom alone treated group (Group-4), the appearance was more or less similar to control that is few neoplastically transformed cells and maintaining near to normal architecture (Figure-2G and 2H).

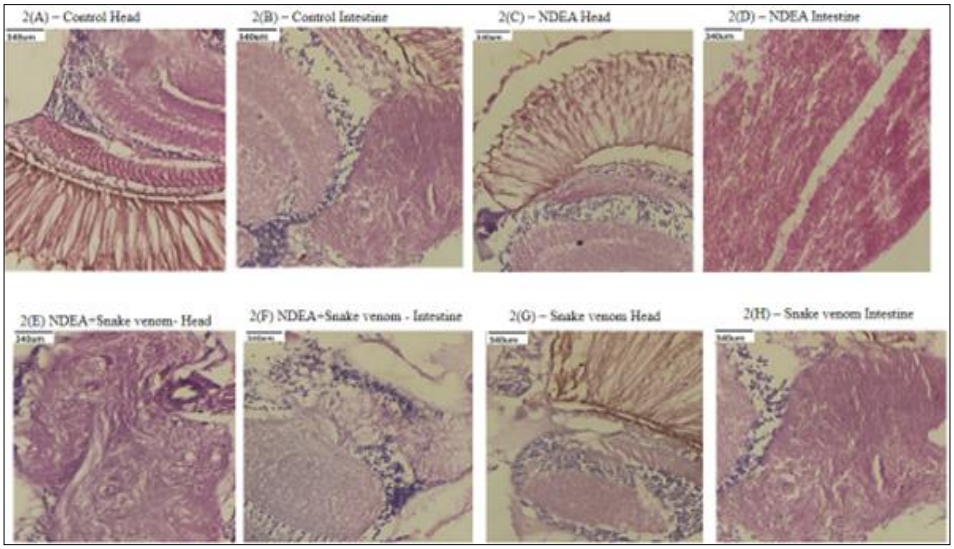

Fig 2: Histopathological observations in the head and intestine tissues of control and treated *D. melanogaster*.

Statistical analysis shows that the negative geotaxis value was decreased significantly when compared to wild type after NDEA treatment. In NDEA and snake venom treated group, the value was increased ($p>0.05$) when correlated to normal flies of *yellow* mutant. In snake venom alone treated (group IV), the value was almost the same level when related to the control (normal *yellow* mutant) group ($p>0.05$) Table 1.

Table: 1 Negative geotaxis in *Drosophila melanogaster*. Values with a * indicate significant variation at $p<0.05$ by Duncan's Multiple Range Test (DMRT)

Negative Geotaxis (%±SD) (*Yellow* mutant-control)	NDEA Treated	NDEA + snake venom	Snake venom treated
92.6±14.3	79.4±13.2*	94.2±15.7*	92.8±12.3*

Normally, the greatest number of *yellow* mutant flies tend to move nearer to the light source (compartment I), Although, this response was noticeably decreased ($p<0.05$) when related to the control i.e., normal *yellow* mutant. Likewise, a huge number of NDEA treated *yellow* mutant flies were observed in compartment II when related to the control group ($p<0.05$). When the *yellow* mutant flies were treated with NDEA + snake venom, the response was significantly elevated in 1st compartment ($p<0.05$) when compared with those of the NDEA treated group. A larger number of mutant control flies were recognised to diverge from the pungent smell of the chemical benzaldehyde from 1st compartment to 3rd compartment when correlated with NDEA treated flies ($p<0.05$, Table 2). Significantly increased locomotions were noticed in snake venom + NDEA treated flies to compartment III ($p<0.05$). Large number of mutant control flies were tend to go towards to the cotton-plug dipped with sugar solution (compartment I) when related to the mobility of NDEA treated flies towards compartment III $p<0.05$). The response of snake venom treated flies was closer to the movement of control flies ($p>0.05$, Table 2).

Table: 2 Behavioural assays in Yellow mutant D. melanogaster. Values not sharing a common superscript numerical vary significantly at $p<0.05$ by Duncan's Multiple Range Test (DMRT)

% of flies present in compartment / Behavioural assay	Compartment I (mean ± SD)	Compartment II (mean ± SD)	Compartment III (mean ±SD)
Phototaxis			
Yellow mutant (control)	83.2±3.1[1]	25.4±2.8[2]	7.8±1.6[1]
NDEA treated	88.3±4.6[2]	30.2±3.9[1]	11.2±3.3[2]
NDEA+ snake venom	87.4±4.2[1]	28.6±3.7[1]	10.6±2.8[1]
Snake venom alone	82.6±3.7[1]	25.2±2.7[1]	5.9±1.0[1]
Smell chemotaxis			
Yellow mutant (control)	23.8±4.5[2]	15.2±4.1[1]	92.6±2.7[1]
NDEA treated	19.3±2.2[2]	23.6±3.7[2]	72.8±3.6[2]
NDEA+ snake venom	14.6±1.4[1]	19.4±3.2[1]	84.3±3.1[1]
Snake venom alone	21.4±3.6[1]	14.8±4.8[1]	90.4±2.4[1]
Taste chemotaxis			
Yellow mutant(control)	82.2±12.4[1]	20.2±5.3[1]	11.3±4.3[1]
NDEA treated	68.7±7.6[2]	28.6±5.7[2]	19.4±7.8[2]
NDEA+ snake venom	81.5±6.3[1]	21.3±4.2[1]	13.6±5.7[1]
Snake venom alone	83.6±8.9[1]	19.7±5.0[1]	10.8±4.0[1]
Thermotaxis			
Yellow mutant(control)	78.2±10.8[1]	8.5±1.6[1]	1.8±0.7[1]
NDEA treated	65.3±6.9[2]	19.7±4.9[2]	9.4±3.4[2]
NDEA+ snake venom	79.8±6.4[1]	6.8±1.3[1]	2.3±1.6[1]
Snake venom alone	77.4±7.6[1]	7.6±1.2[1]	2.1±1.0[1]
Hygrotaxis			
Yellow mutant (control)	94.2±9.5[1]	24.8±6.3[1]	8.8±2.6[1]
NDEA treated	73.6±7.4[2]	28.3±5.7[2]	10.7±1.9[2]
NDEA+ snake venom	86.4±6.3[1]	23.6±5.9[1]	7.2±2.3[1]
Snake venom alone	91.8±8.7[1]	21.2±5.2[1]	8.2±2.4[1]

As far the thermotaxis assay, a large percentage of control flies move to diverge from the hot surface of 3rd compartment to 1st compartment when related to NDEA treated flies ($p<0.05$). Meanwhile, the trend shown averted in NDEA + snake venom treated flies compared to group II flies ($P<0.05$). At the same time an insignificant difference noticed in presence of snake venom alone treated flies in 3rd compartment when related to control flies. The hygrotaxis assay revealed an increased proportion of control flies related to NDEA treated flies which had moved towards the humid region in 1st compartment ($p<0.05$, Table 2). The percentage of NDEA +snake venom treated flies were considerably increased in 3rd compartment when related to NDEA treated flies ($p<0.05$). Invariably snake venom treated flies illustrated the behavioural activities of negative geotaxis, smell and taste chemotaxis, phototaxis, hygrotaxis and thermotaxis alike to those that were noticed in the control group ($p>0.05$).

The variations in the amount of lipid peroxidation products in normal-control and experimentally treated flies are given in Table-3. In NDEA administered flies TBARS, protein carbonyl and lipid peroxides were considerably increased ($p<0.05$) when compared to the normal-control. The range of TBARS, protein carbonyl and lipid hydro peroxidases were insignificantly decreased ($p>0.05$) after the execution of snake venom when related to the normal-control flies. The level of phase I cytochrome p450 (phase I enzyme) was noticeably reduced whereas the cytochrome p450 (phase I

enzyme) was significantly elevated in tumor bearing flies as compared to normal-control flies ($p<0.05$). Administration of snake venom to NDEA treated flies significantly decreased and raised phase II enzyme activities compared to group III. Table-3 illustrates the levels of enzymatic and non-enzymatic antioxidants such as SOD, CAT, GPX and GSH in head, intestine and hemolymph of normal-control and treated flies. A noticeable reduction ($p<0.05$) in the actions of enzymatic and non-enzymatic antioxidants in group II flies was noticed. In snake venom administered group the levels were noticeably increased ($p<0.05$) when compared to NDEA administered flies.

Table 3: Biochemical assays in *D. melanogaster*. Values not sharing a common superscript numerical vary significantly at $p<0.05$ by Duncan's Multiple Range Test (DMRT)

S. No	Biochemical Parameter	Group	Haemolymph	Head	Intestine
Redox homeostasis					
1.	Protein carbonyl (nmole/mg protein	*Yellow* mutant (control)	5.9±1.04[1]	3.9±0.86[1]	2.3±0.09[1]
		NDEA treated	7.8±2.15[2]	2.7±0.51[2]	1.6±0.07[2]
		NDEA +snake venom	5.2±0.07[1]	3.1±0.65[1]	1.5±0.05[1]
		Snake venom alone	5.6±1.00[1]	3.7±0.81[1]	2.1±0.05[1]
2.	Thiobarbituric acid reactive substances (TBARS) (nmole/mg protein)	*Yellow* mutant (control)	12.7±2.53[1]	8.4±1.31	7.2±1.74[1]
		NDEA treated	16.9±3.63[2]	5.4±0.81[2]	4.9±1.14[2]
		NDEA +snake venom	12.3±2.11[1]	7.8±1.21[1]	6.5±1.24[1]
		Snake venom alone	12.5±2.01[1]	7.9±1.13[1]	7.1±1.62[1]
3.	Protein thiol (mmol/mg protein)	*Yellow* mutant (control)	37.6±6.01 [1]	27.4±4.32 [1]	23.5±3.65 [1]
		NDEA treated	48.6±3.12 [2]	20.3±2.85 [2]	16.3±1.38 [2]
		NDEA +snake venom	37.2±5.08 [1]	25.9±2.58 [1]	23.1±2.98 [1]
		Snake venom alone	36.4±5.82[1]	26.3±3.81[1]	24.5±3.08 [1]
4.	Lipid peroxides (nmole/mg lipid)	*Yellow* mutant (control)	36.8±5.93 [1]	26.4±4.82 [1]	25.8±3.32 [1]
		NDEA treated	41.6±3.12 [2]	27.7±2.16 [2]	12.8±1.04 [2]
		NDEA +snake venom	32.8±2.08 [1]	25.3±2.84 [1]	21.5±1.77[1]
		Snake venom alone	36.0±2.60 [2]	25.5±2.15 [2]	20.6±2.14[2]
5.	Superoxide dismutase (SOD) (Unit[a] nmole/mg protein)	*Yellow* mutant (control)	12.1±2.01 [1]	11.9±1.21 [1]	6.5±1.24 [1]
		NDEA treated	10.5±1.68 [2]	9.12±2.7 [2]	8.0±1.06 [2]
		NDEA +snake venom	17.8±2.65 [1]	11.6±2.46 [1]	9.1±2.04 [1]
		Snake venom alone	18.6±3.02 [1]	8.2±1.83 [1]	10.6±2.43 [1]
6.	Catalase (CAT) (Unit[b] /min/mg protein)	*Yellow* mutant (control)	145.6±2.35 [1]	137.6±12.6 [1]	107.4±9.32 [1]
		NDEA treated	141.3±12.68 [2]	126.2±10.35[2]	87.4±4.32 [2]
		NDEA +snake venom	149.7±16.84 [1]	129.3±11.32 [1]	98.7±8.13 [1]
		Snake venom alone	144.2±20.45 [1]	136.3±12.02 [1]	110.6±10.52 [1]
7.	Glutathione-S-transferase (Unit/100mg protein)	*Yellow* mutant (control)	13.4±0.26 [1]	8.5±0.71 [1]	7.4±0.69 [1]
		NDEA treated	9.8±0.12[2]	6.3±0.61[2]	5.6±0.45[2]
		NDEA +snake venom	12.9±1.10 [1]	8.72±1.23 [1]	7.8±0.68 [1]
		Snake venom alone	13.1±1.27 [1]	7.6±1.74 [1]	12.8±1.72 [1]
8.	Glutathione peroxidase (GPx) (Unit[c]/mg protein)	*Yellow* mutant (control)	12.6±4.83 [1]	8.7±1.12 [1]	5.3±0.96 [1]
		NDEA treated	9.9±1.46 [2]	5.6±0.93 [2]	3.7±0.64 [2]
		NDEA +snake venom	11.3±4.15 [1]	6.1±1.62 [1]	11.8±1.20 [1]
		Snake venom alone	12.4±2.67 [1]	7.3±1.5 [1]	5.0±0.84 [1]
9.	Reduced glutathione (GSH)	*Yellow* mutant (control)	17.3±8.59 [1] (µl/ml)	11.2±2.42 [1] (µl/mg tissue)	10.6±1.32 [1](µl/mg tissue)
		NDEA treated	15.4±6.22 [2]	8.4±0.26 [2]	7.3±0.62 [2]
		NDEA +snake venom	16.4±7.41 [1]	10.8±1.46 [1]	9.81±1.24 [1]
		Snake venom alone	17.0±8.13 [1] (µl/ml)	11.0±1.89 [1] (µl/mg tissue)	10.2±1.27 [1] (µl/mg tissue)

Discussion

Histopathological studies clearly indicate, the tumorigenesis in brain and intestine tissues of *D. melanogaster*. After the administration of snake venom, the histopathological appearance tends to be reversed to near normal. NDEA when administered in mutant flies tend to cause oxidative stress, since it has been shown to cause cancer. The treatment with NDEA may affect cell proliferation, cell toxicity and rate of DNA replication. Cell toxicity after NDEA administration is reflected in decreased rate of cell proliferation [16]. Some of the behavioural abnormality observed in the *yellow* mutant flies could be due to oxidative stress during tumorigenesis. To improve the cognitive function by introducing snake venom (*Naja naja*) in NDEA treated *yellow* mutant *Drosophila*. Our findings demonstrate normalization of the behaviour in the mutant flies, ROS levels could be neutralized and carcinogenesis could also be inhibited by treating with snake venom.

Our results clearly show that behaviours (negative geotaxis, thermotaxis, odor and taste chemotaxis, phototaxis and humidity) change significantly during tumor development. Cognitive deficits are commonly reported in various types of cancer [27]. Our findings also added additional evidence that the fly carcinogenesis may have been affected the cognitive behaviour. Our results also shows that snake venom tends to reverse the harmful effects of NDEA and thus restore the behaviour of flies near to normal.

The development of new drugs in oncology is one of the most promising targets in the pharmaceutical industry. Consequently, the exploration for novel active ingredients for cancer treatment is one of the goals of biotechnology research. The development of new therapies specifically designed to target important genes and biological substances

were made by studying the measures which turns the normal cells into cancer cells [28]. Based on their mechanism of action, cell cycle-targeted antitumor drugs can be broadly divided into three categories. DNA damage, blocking synthesis of DNA and mitosis arrest [29].

Snake venom consists of different mixture of components includes peptides, proteins, enzymes, carbohydrates and minerals. Cytotoxicity of snake venoms is related to alterations in cellular metabolism with a major effect on tumor cells when compared with normal cells. Snake venom including cystatin reduces tumor infiltration and melanoma. Snake venom components can be definitely target cancer cells by uniting the constituents with nanoparticles [30]. Cobra venom factor is an important factor for the synthesis of immune complexes that are more specific to cancer cells [18]. At very low doses, cobra venom is more analgesic than morphine and may reduce cancer related intractable pain [17, 20]. The enzyme LAAO isolated from snake venoms induces apoptosis, changes in cell cycle processes and cytotoxicity and has promising potential for the development of new antitumor agents [31].

The various mechanisms behind the effects of snake venom L-amino acid oxidases such as production of hydrogen peroxide during enzymatic reactions, the activation of caspases and the interaction of LAAOs with membrane receptors [32]. Snake venom component basically inhibits cell proliferation and promotes cell death. It is also included that cancer cells thereby controlling tumour size such as direct toxicity and free radical production, inhibition of nucleic acid synthesis thereby suppressing cell proliferation, reduced expression and activity of matrix metalloproteinase, inhibition of integrins thereby cancer cells prevent migration and invasion and anti-angiogenesis. Compared to normal cells, the cytotoxicity of snake venom is associated with changes in cell metabolism that have a significant impact on tumor cells [18]. The genetic changes give rise to changes in expression, activation of regulatory proteins in the cells and affecting the signalling pathways which can change the response to regulatory stimuli and allow the unrestricted cell growth. The anti-cancer property of snake venom might be moderated by regulating various signalling pathways in various configurations.

Our results are also related to previous analysis in mammals, showing increased TBARS levels in yellow mutant flies treated with NDEA. This may be due to the extreme protection of ROS and the first outbreak associated with hypoxia [33]. *D. melanogaster* has a vast amount of antioxidant defense mechanisms to prevent the damage to reactive oxygen species (ROS) to cells. The antioxidant defence system plays an important role in blocking ROS and preventing lipid peroxidation and play a protective role in the development of carcinogens [34]. CAT and SOD are equivalent protection enzymes in contrast to ROS. This protection mechanism works through enzymes (including CAT, GPx, SOD, GST) and non-enzymatic compounds [35]. The range of non-enzymatic and enzymatic antioxidants range was reduced in NDEA-induced mutant flies.

In this study, the elevated levels of TBARS and lipid hydroperoxides in hemolymph and tissues such as the brain and intestines of *yellow* mutant flies may be due to free radical synthesis, membrane damage and cell destruction caused by NDEA. Improvements in lipid peroxidation have been observed in *yellow* mutant flies in which snake venom has been administered due to the enhanced activity of antioxidants. The antioxidant properties of polyphenolic compounds may maintain the resistance of flies to free radical damage from NDEA. Significant increases in GSH levels in *yellow* mutant flies treated with snake venom represent the venom's ability to prevent glutamate toxicity and maintain GSH levels by absorbing cysteine (cysteine is a precursor to GSH and an excellent source of thiol group). The rational mechanism by which snake venom caused its protective effect relied on the property of removing free radicals and maintaining the cellular integrity of cells in *D. melanogaster*.

Components of TBARS, protein carbonyls, protein thiols, and lipid peroxide (a product of extreme oxidative stress) were increased in hemolymph ($p>0.01$) but obviously decreased in the yellow mutant fly's head and intestine tissues. During tumorigenesis, administration of reactive oxygen species (ROS) levels is an important factor as elevated levels of ROS can damage the cells. Therefore, the tumour cells exhibit a mechanism of action, such as a peroxide removal system to maintain the ROS balance to identify the cells proliferative state. In addition, previous reports have shown that rapidly dividing tumour cells in the head and intestine tissue can take elevated levels of ROS [36]. Similarly, the results of this study showed that, ROS levels could be reduced in tumour cells, and ultimately leading to a reduction in the end-products of oxidative stress in these tissues. Similarly, the amount of antioxidants (CAT, SOD, GST, GSH and GPx) in tissues and hemolymph is constantly decreasing. This may be due to the accelerated utilisation of antioxidants by cancer cells [37]. Substances with strong antioxidant activity, such as ascorbic acid are known to prevent liver cancer. As mentioned above, snake venom is known for its antioxidant activity, such as removing toxins present in reactive oxygen species and reactive nitrogen species. By interacting with hydrogen peroxide, the snake venom-stimulating toxic pathway can form a network of antioxidant defense proteins by regulating oxidative stress [38]. Snake venom doses may reverse the oxidative effects of flies by reducing oxidative stress and thereby providing a significant regulation of most redox homeostasis indices. In particular, the markedly reduced lipid peroxide content in the hemolymph tissues of *D. melanogaster* administered with snake venom indicates a reduced lipid peroxidation content of the lipids.

Conclusion

The cytotoxic effects of snake venom have the potential to degrade or destroy cancer cells. The venom causes damage to cell membrane or interfere with the transport of substances or transduction of signals across the membranes. The present study indicates that the mutant flies exposed to NDEA (500 µM) for 21 days evidenced that elevation in oxidative stress markers along with a significant decrease in activities of antioxidant enzymes such as SOD, GST, GSH and GPx which suggested an increased generation of ROS and toxic aldehyde.

Acknowledgement

The authors thank the financial assistance of RUSA grant by Annamalai University (DRD/RUSA 2.0/R&I/Project Proposal/Field 2/5/2021 dt. 22.02.2022). The authors also thank UGC-SAP and DST-FIST grants sanctioned to Department of Biochemistry and Biotechnology, Annamalai University.

Funding support
The authors declare that there is no funding support for this study.

Conflict of interest
The authors declare that there is no conflict of interest.

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