# Cerebral ganglionic variations and movement behaviors of *Lumbricus terrestris* on exposure to neurotoxin

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**KEY WORDS**

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**ABSTRACT**

**Background:** Invertebrate worms serve as models for understanding the features of neurological functions. Acrylamide (ACR), the well-known neurotoxin, is a water-soluble chemical widely used in various industrial and laboratory processes. ACR is also found in food items which are cooked under high temperature.
**Purpose:** The study attempts to assess the neuropathological changes in cerebral ganglions along with the locomotion and neuronal behavior of *Lumbricus terrestris* on ACR intoxication.
**Methods:** The dosage of acrylamide induced neurotoxicity ranged from 0–17.5 mg/kg body weight for 7 days. The time/dose dependent changes in the oxidant and antioxidant status, activities of $Na^+/K^+$ATPase, $Ca^{2+}/Mg^{2+}$ ATPase and 5′ Nucleotidase were assessed along with the locomotor behavioral analysis.
**Result:** The activities of super oxidase dismutase and catalases were not altered appreciably. However, the glutathione family, lipid peroxide, protein carbonyl content and vitamin C did show significant variations ($p<0.001$) in a dose-dependent manner, depicting more of oxidative stress, when compared to control worms. The activities of $Na^+/K^+$ ATPase was significantly affected ($p<0.001$) at 3.5 mg/kg bw itself while those of both $Ca^{2+}$ and 5′ Nucleotidase were found to be affected at 7.0 mg/kg bw of ACR. $Mg^{2+}$ ATPase showed significant reduction ($p<0.001$) in its activity only at 10.5 mg/kg bw of ACR. These dose dependent biochemical variations observed were found to be linked with the behavior of the worms as evident from the latency of movement in a dose-dependent manner which is less pronounced at 7.0 mg and more pronounced at 17.5 mg/kg bw of ACR.
**Conclusion:** The study suggests that ACR disrupts GSSS/GSH balance and perturbs ionic homeostasis in worms and thus affect the motor function highlighting their (GSH-ions) interrelationship in influencing neuromuscular activity. These simple analyses implicate that the cerebral ganglionic variations in the worms may be useful to appreciate the pathology of the neurological diseases (provided sophisticated analyses are employed) especially which involve movement dysfunction, where the brain tissue samples from the affected human patients are scarce.

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## Introduction

Earthworm *Lumbricus terrestris* (Annelida, Oligochaeta) is a favorite model in neuroscience and behavioral studies because of the known ventral nerve cord connections of the CNS and the peripheral nervous system (PNS). In earthworm, cerebral ganglions function as a simple brain which is located above pharynx and is connected to the first ventral ganglion the removal of which would result in uncontrolled movement of the worm. Neuronal and reflex pathways control the coordinated movements and behavior of the worms. Paired ganglion (group of nerve cells) is connected to a nerve cord and pair of nerves in each segment carries the signal to the brain. Some nerve fibers are motor in function and some are sensory in function.[1,2]

Behavior of an organism is exhibited following multiple levels of integration: it is the manifestation of alterations in the communication between neurons, integrity of neuronal circuit and their morphology. Behavior is generally considered a sensitive indicator of neuronal function.[3] ACR is used in construction sites to avoid the seepage of water during the processes of drilling and mining. It is also used in the manufacture of flocculates. In 2002, ACR has been categorized as food-borne toxicants by Swedish National Food Administration as out significant amounts of this neurotoxin is present in heated and high carbohydrate food items such as potato chips and crisps, coffee and bread[4] meats[5] and olives[6] also were reported to contain ACR. The hypothetical molecular mechanism of ACR on neuronal function is its interaction with signaling components and affecting the concentration of neurotransmitters which eventually cause damage to both neural and glial cell functions.[7] Reports indicate that morphological changes such as distal axonal swelling, accumulation of neurofilaments and degenerative cells occur in the nervous system on ACR intoxication.[8] ACR is an irritant and a potent neurotoxin used in various neurological investigations which had used model such as *Eisenia fetida*.[9] Currently there exists no report on this species of *Lumbricus terrristeris* with regard to its response in locomotion and cerebral ganglionic features on impact by neurotoxins. The study attempts to assess the neuropathological changes in cerebral ganglions along with the locomotion and neuronal behavior of *Lumbricus terrestris* on ACR intoxication.

## Methods

### *Chemicals*

Acrylamide (ACR) of analytical grade (purity >97%) was purchased from the sigma-Aldrich company (St. Louis, MO USA). Stock solution was prepared at a concentration of 1 M for which 3.554 g of ACR was weighed and dissolved in 50 ml of water.

### *Animals*

Earthworm *L. terrestris* was used as model system in the study due to wide occurrence and easy accessibility to the laboratory. Seventy two adult, sexually mature *Lumbricus* were obtained

from Chennai, (Tamil Nadu, India).Prior to the experiment, thaw worms were housed in 14 × 12 × 5 plastic containers with wet buss-bedding. Using the tap water, the moisture content was maintained as 25% (fortnightly). Cocoons were hand-sorted weekly from initial stocks, their weight and size were measured and incubated at 20ºC until hatching. Hatchings were kept in same size plastic vessels with fed buss-bedding and soil: the vessels were maintained in the same temperate (20ºC). Weights of the worms as well as their sexual maturation were monitored periodically and the latter was confirmed by fully developed clitellum. All experiments were performed using sexually mature earthworms weighing 3–6 g, 12–14 cm with well –developed clitellum. Study has been conducted as per guidelines that comes under the purview of ethical committee.

*Acrylamide neurotoxicity*

*Lumbricus terrestris* were kept for several weeks in their parental medium.25 ml of the earthworm medium were taken in petri dishes (size 20 × 25 cm) to which different concentrations of acrylamide (0–17.5 mg/kg/bw) were added. Earthworms were weighed and introduced into each of the Petri dish. Worms were acclimatized to the temperature at 20ºC for 7 days in a room with a 24-hour light-dark cycle.

*Earthworm survival and growth rate*

The weight of earthworms was determined for 7–22 days on exposure to different concentrations of acrylamide (0–17.5 mg/bw/days) and compared with the control (seven replicates of 7 worms per sample). Survival analysis was done on exposure to 3.5–17.5 mg of concentrations ACR for 7 to 22 days.

*Growth and Culture of Lumbricus terrestris*

Growth and culture maintained as reported previously in Cooper and Baculi.[10] Briefly, sexually mature *L. terrestris* with developed clitellum was placed in petri dish containing Lumbricus growth medium (Buss-bedding -6.0 gm, walnut meal- 1.5 gm, agar- 1.25 g and peanut oil -0.2099 cc in 100 ml) at 20ºC and then transferred in to medium with concentration of ACR (0–0.75 mg/kg body weight day). For each assay seven worms were used for 7 days.

*Dissection of Lumbricus terrestris cerebral ganglions*

Sexually mature worms weighing between 3–6 g were used for the study. The earthworms were anesthetized using a 0.2% solution of chlorobutanol in water. The worms were anesthetized (not with water) solution for about 10–15 minutes, until the muscles turned flaccid. When their skin turns moist the worms were washed and dissected. All of the solutions used for the dissection were sterilized with filtration or autoclave before use. The cerebral ganglions were excised from the upper lateral lines of setae from point near three quarters of the length and were separated from ventral blood vessel. The cerebral ganglion were excised gently with both super and sub ganglion. Dissected cerebral ganglions were kept no longer then 1 hr in ice cold phosphate buffer saline. The operations were carried at 4ºC.

*Sample preparation (Cerebral ganglions)*

Tissue preparation was done as previously described.[11] The cerebral ganglions were harvested and homogenized in calcium free- Lumbricus balanced salt solution (LBSS; 1.5 mM Nacl, 4.8 mM Kcl,1.1 mM $MgSo_4$, 0.4 mM $KH_2Po_4$, 0.3 mM $Na_2HPo_4$, 4.2 mM $NaHCO_3$ and adjusted to pH-7.3 with HCl) and used for further analysis. The resulting homogenate was centrifuged at 1000 rpm for 25 min at 4ºC. The supernatant was transferred in to centrifuge tubes and stored at –80ºC.

*Biochemical assays*

*Superoxide dismutase (SOD)*

SOD activity was determined based on the method by Misra and Fridovich.[12] The reaction mixture contained 100 μl of the homogenate was added to 880 μl of carbonate buffer of 0.05 M, (pH-10.2), 100 μl of 0.1 mM EDTA. 20 μl of 30 mM epinephrine of 0.05% acidic acid were added to the mixture, and incubated for 4 minutes. The absorbance was read at 480 nm using a spectrophotometer. One unit was defined as the amount of enzyme that resulted in 50% inhibition of epinephrine auto –oxidation.

*Catalase (CAT)*

CAT activity was determined by the method of Aebi.[13] The reaction mixture contained 100 μl of tissue homogenate was added to equal proportion of absolute alcohol and incubated for 30 minutes in ice bath for degradation of the inactive CAT-$H_2O_2$ complex II to release active CAT enzyme. After 30 min, the tubes were brought back to RT and 10 μl of Triton ×–100 was added. In a cuvette containing 200 μl of phosphate buffer, 50 μl of 0.066 M $H_2O_2$ in phosphate buffer were added and the decrease in absorbance was read at 240 nm for 30 s. A molar absorptivity of 43.6 M $cm^{-1}$ was used to determine CAT activity. One unit of which is equal to the units of hydrogen peroxide degraded per minute per mg of protein.

*Reduced and oxidized glutathione (GSH and GSSG)*

Levels of reduced and oxidation glutathione were measured by the DTNB-GSSG reductase (GR) assays method of Anderson[14] with a few modifications. The assay buffer containing 1000 μl of 125 mM sodium phosphate, 100 μl of the homogenate, 50 μl of 6.3 mM disodium EDTA, 50 μl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) (pH-7.5) and 2.0 ml of 5, 5′ dithiobis –2-nitobenzoic acid (DTNB) solution (6 mM DTNB in assay buffer without NADPH) were added. One unit of GSH reuctase (GR) was added to the assay mixture to convert GSSG in to GSH. The optical density (OD/min) was observed for 3 min at 412 nm in a spectrophotometer. The values were expressed as μg GSH $mg^{-1}$ protein and μg GSSG $mg^{-1}$ protein of respectively.

*Glutathione Reductase (GR)*

GR activity was measured following the method of Kenji.[15] The reaction mixture contained 1000 μl of 50 mM phosphate buffer, 100 μl of tissue homogenate, 500 μl of 1 mM EDTA, 200 μl of 0.1 mM NADPH and 500 μl of 1 mM GSSG was made up to 3 ml with distilled water. The change in optical density was read at 320 nm for 30s in a spectrophotometer. The activity of the enzyme was calculated using the molar extinction coefficient of NADPH ($£_{340}$ = 6.22 × 102 $M^{-1}$ $cm^{-1}$) and result were expressed in terms of units NADPH oxidized $min^{-1}$ $mg^{-1}$ protein.

*Glutathione Peroxidase (Gpx)*

Gpx activity was measured according to the method of Clair and Chow.[16] The assay mixture contained 1000 μl of coupling reagent of 2 mM disodium EDTA, 50 μl of 1 mM sodium azide, 100 μl of tissue homogenate, 50 μl of 1 mM GSH, 100 μl of 0.2 mM NADP, 2000 μl of 75 mM of Phosphate buffer (pH-7.0)

and glutathione reductase. The reaction was started by adding 100 μl of 7.5 mM of $H_2O_2$ and the conversion of NADPH to NADP was monitored by a continuous decoding of the change of absorbance at 340 nm for 5 min in a spectrophotometer. The activity of GPx was calculated from the molar extinction coefficient of NADPH ($£_{340}$ = 6220) and the results were expressed in terms of nmol hydrogen peroxide degraded $min^{-1}$ $mg^{-1}$ protein.

*Glutathioine –S-trasferase (GST)*

Glutathioine –S-trasferase activity was determined by using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al.[17] The assay mixture contained 2900 μl of 100 mM phosphate buffer, 100 μl of 1 mM GSH, 100 μl of 1 mM CDNB in ethanol and 100 μl of tissue extract. The formation of adduct of CDNB, S-2, 4, dinitrophenylglutothine (DNPG) was monitored by measuring the rate of absorbance at 340 nm in a spectrophotometer. One unit of GST (U) was calculated from the extinction coefficient (9.6 $mM^{-1}$ $cm^{-1}$) (GSH-CDNB adduct) and expressed as unit DNPG produced $min^{-1}$ $mg^{-1}$ protein.

*Lipid peroxidation (LPO)*

Lipid peroxidation assay was done by measuring thiobarbituric acid reactive substance (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the methods described by Ohkawa et al.[18] Briefly, 100 μl of sample was taken and 50 μl 8.1% sodium dodecyl sulphate (SDS) was added and incubated for 10 min at RT.370 μl of 20% acetic acid pH-3.5 and 375 μl of 0.8% TBA were added and then placed in the boiling water bath for 60 min. The sample was then allowed to cool and 1.25 ml of butanol: pyridine (15:1 v/v) mixture was added, vortexed and centrifuged at 1000 rpm for 5 min. Absorbance of the colored layer was measured at 532 nm in a spectrophotometer and the concentration was expressed in terms of μmoles MAD per mg of protein.

*Protein carbonyl oxidation (PCO)*

Protein carbonyl oxidation was measured according to the procedure of Levine et al.[19] Briefly, 100 μl of 10 mmoldintrophenylhydrazine (DPNH) in 2.5 M HCl was added to the tissue and incubated in the dark for 60 min at RT. This was followed by vortex mixing; 500 μl of 20% trichloro acetic acid was added and subsequently washed thrice with ethanol: ethyl acetate (1:1 v/v) mixture. Precipitation protein was then re-dissolved in 1000 μl of 6 M guanidine hydrochloride in 20 mM phosphate buffer (pH-6.5). Insoluble substances were removed by centrifugation and absorbance of the supernatant was read at 370 nm in a spectrophotometer. An extinction coefficient of 22,000 $M^{-1}cm^{-1}$ was used to determine the protein carbonyl content which was expressed as μmoles $mg^{-1}$ protein.

*Ascorbic acid (Vitamin C)*

Ascorbic acid was determined by the method Omaye et al.[20] Briefly, 500 μl of tissue homogenate was mixed thoroughly with the 1500 μl of 5% TCA and centrifuged for 20 min at 3500 rpm.To 100 μl of sample, 0.5 μl of DTC reagent was added and mixed well. The tubes were allowed to stand at room temperature for an additional 3 hours. About 2500 μl of 65% HCL was added and allowed to stand for 30 min. A set of standards containing 10–15 μg of ascorbic acid were taken and processed similarly along with a blank, containing 500 μl 5% TCA. The color developed absorbance was read at 530 nm in a spectrophotometer. The values were expressed as μg/mg tissues.

*$Na^+/K^+$-ATPase activity*

The activity of $Na^+/K^+$ ATPase was determined by the method of Slack et al.[21] $Na^+/K^+$ ATPase assay mixture contained 100 μl of sample, 100 μl of 3 mM Tris –HCl buffer (pH-7.4), 200 μl of 20 mM KCl, 200 μl of 120 mM NaCl, 200 μl of 4 mM $MgCl_2$, 200 μl of 1 mM EDTA and 200 μl of 3 mM Tris-ATP. The reaction was started by the addition of 3 mM Tris-ATP 200 μl and stopped after an incubation period of 20 min at 37°C, by addition of 1000 μl in 10% TCA followed by centrifugation. The reaction rate was determined by measurement of the absorbance at 340 nm.

*$Mg^{2+}$ ATPase activity*

The activity of $Mg^{2+}$ ATPase was measurement using modified method of Warren et al.[22] $Mg^{2+}$ assay mixture contained 100 μl of sample, 1000 μl of 50 mM Tris –HCl buffer (pH-7.4), 200 μl of 20 mM KCl, 200 μl of 120 mM NaCl, 200 μl of 4 mM $MgCl_2$, 200 μl of 1 mM EDTA, and 200 μl of 3 mM Tris-ATP. The reaction rate was determined by measurement of the absorbance at 340 nm nm for 37°C. The reaction was initiated by addition of ATP, allowed to proceed for 20 minutes at 37°C, and terminated by addition of 1000 μl in 10% TCA, followed by centrifugation. The reaction rate was determined by measurement of the absorbance at 340 nm.

*$Ca^{2+}$ ATPase activity*

The activity of $Ca^{2+}$ ATPase was measured by the modified method of Warren et al.[22] The assay mixture contained 100 μl of sample, 1000 μl of 50 mM Tris –HCl buffer (pH-7.4), 200 μl of 20 mM KCl, 200 μl of 120 mM NaCl, 200 μl of 1 mM EDTA, 200 μl of 3 mM Tris-ATP. The reaction was started by addition of 200 μl of 3 mM $CaCl_2$ to the reaction mixture. The reaction was arrested by the addition 1000 μl of 10% TCA to the incubation mixture. The reaction rate was determined by measurement of the absorbance at 340 nm.

*5′ Nucleotididase activity*

5′ Nucleotidase was assayed by the method of Jonathan et al.[23] The assay containing 100 μl of sample, 1000 μl of 50 mM Tris –HCl buffer (pH-7.4), 100 μl of 20 mM KCl, 100 μl of 50 mM $MgSO_4$, 100 200 μl of 1 mM EDTA, 200 μl of 3 mM Tris- 5′AMP. The reaction was started by the addition of 5′ AMP and stopped after an incubation period of 20 min at 37°C, by addition of 10% TCA, followed by centrifugation. The ATPase and 5′ nucleotidase activity was expressed in nanomoles of Pi liberated/mg protein/min.

*Inorganic phosphate estimation*

The inorganic phosphate was estimated by the method of Taussky and Shorr[24] using sodium hydrogen phosphate ($Na_2H_2PO_4$). The enzymes activity was expressed as nanomoles of Pi liberated/mg protein/min.

*Protein estimation*

Protein was measured according to Lowry et al[25] using bovine serum albumin (BSA) as the standard.

*Behavior studies of earthworm*

*Swimming behavior*

Swimming pattern of earthworm assessed using the method by Drewes and Fourtner.[26] Worms were placed in shallow

container filled with water (21–22°C). The worms were placed in flat –bottomed (23 × 23 cm) container, each containing about the 1500 ml water. Touch stimuli were delivered with the tip of a hand held probe. The probe tip consisted of thin rubber band loop (1.5 mm thickness for worms). Several characteristic swim helical, distances and velocity were done respectively to study the behavior of the worms. Touch stimuli were delivered at 10 min interval to minimize habituation and optimize consistency in evoked responses. Behavior response was recorded on videotape using a color video camera attached to a tripod. The camera lens was directly above the worm and shutter speed was adjusted to $1/500_s$ on the video recorder allowed single –frame analysis of locomotors movements. Swim distances was expressed as a percentage of resting body length and measured by determining the net translocation of the worm head relative to a fixed point in its container. Swim velocity were determined by measuring reward progress of the helical wave along the worms body axis during two consecutive video frames.

*Burrowing behavior*

The 2- dimensional terraria consist of two glass sheets (30 cm x 42 cm) 3 mm apart filled by 2 mm sieved soil in which earthworm movement and behavior can be observed. Earthworm trajectories were then reconstructed as the most probably movements between two observations[27] the burrowing behaviors of earthworms were observed in two different environments: in cylinder consisting of (50 cm × 35 cm) 40 cm filled with 2 mm soil and in cylinder consisting of (50 cm × 35 cm) 40 cm filled with 2 mm Lumbricus growth medium. Several characteristics such as burrow length, burrow depth, rate of burrow reuse and distance covered were recorded with video camera in order to study the behavior of the earthworms (burrowing and movements in existing burrows). The time taken for the movement of each worm to burrow were recorded at 45 min. Burrow length, depth, and distance behaviors were expressed in cm.

*Locomotory behavior*

Locomotory behavior was assessed using the methods of Datta[28] with minor modifications. Reconstructed trajectories provided estimation for the locomotory activity in *Lumbricus terrestris*. Reconstructed trajectories (50 cm × 3 mm) were noted on a transparent tube with soil. Worms located outside the reconstructed trajectories, approximately 40 cm from the goal area. The experiment was performed on all of the experimental worms (n = 7). The time taken by earthworms to burrow entirely into the soil was recorded for 45 min. Locomotory behavior was expressed as movements in cm.

*Statistical analysis*

The results were expressed as mean ± SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests and other data were evaluated using Graph pad PRISM software Vision 5. A p values were $*P<0.05$; $**P<0.01$; $***P<0.001$ as significant.

**Results**

*Acrylamide neurotoxicity*

Mortality of the worms increased with time of expose to different concentrations of acrylamide (fig.1) and $LC_{50}$ values werefound to be 17.5 mg/kg body weight after 7 days. Survived worms also had reduced body weight and pathological features which were recorded includerigidity, coiling, ataxia, constriction and swelling of the body of *L. terestries*.

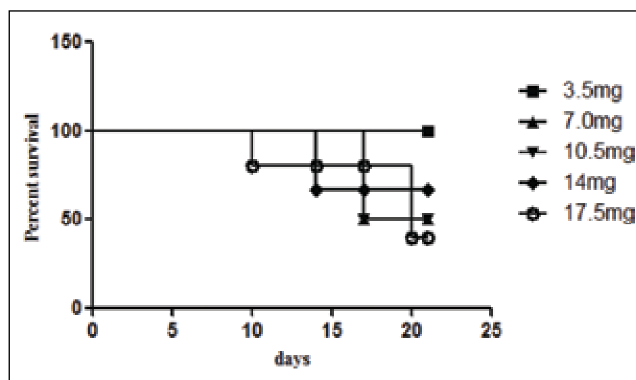


**Fig. 1:** Survival curves at 4 weeks for *L. terrestris* exposed to different concentrations of ACR ($LC_{50}$ -17.5 mg/kg body weight). Time course of mortality were occurred the following ACR exposures. Worms were placed in one of five concentrations (3.5, 7.0, 10.5, 14, 17.5 mg; n = 7 per concentration) and mortality was checked daily.

*Effect of ACR on the activities of enzymic antioxidants and the levels of non-enzymic antioxidants*

The status of oxidant/antioxidant in earthworms on ACR exposure is presented in Table 1. The result shows that SOD and CAT activities are not significantly influenced by acrylamide at all of the doses of 0–17.5 mg/kg bw/days. Significant changes were observed in the contents of GSH and GSSG and their ratio (GSSG/GSH) were significantly affected at the dose of 7.0 mg/kg bw/day. GSSG/GSH ratio showed a dose-dependent increase ($p<0.001$). The activities of GR, GST and Gpx showed a significant reduction after 7 days with increase in concentration of ACR with respect to control. The table also shows that the concentrations of MDA and PCO in *Lumbricus terresteris* were significantly increased ($p<0.001$) on exposure to different concentrations of ACR along with the significantly decreased levels of vitamin C ($p<0.001$).

Effect of acrylamide on ATPase activities and 5' Nucleotide activities.

Exposure to different concentrations of acrylamide resulted in a significant reduction ($p<0.001$) in the activities of $Na^+/K^+$- ATPase, $Mg^{2+}/Ca^{2+}$ ATPase and 5' Nucleotidase (Table 2).

*Behavior studies*

In animals exposed to different concentrations of ACR for 7 days (Table 3) swimming behavior was affected by three folds when compared to untreated worms. The burrowing behavior such as burrowing length, the rate of burrow reuse and the distances travelled were significantly shorter when compared controls (Table 3) and were found to be concentration dependent. Locomotion of earthworm was significantly affected on the different concentrations of ACR/kg body (Table 3) with three fold of changes observed on 17.5 mg/kg body weight.

**Table 1: Biochemical response of *Lumbricus terrestris* cerebral ganglions exposed to different concentrations of acrylamide**

| Range of concentrations of ACR (mg/kg body weight) | | | | | | |
|---|---|---|---|---|---|---|
| Biochemical measurements | 0.0 mg | 3.5 mg | 7.0 mg | 10.5 mg | 14.0 mg | 17.5 mg |
| SOD activity (U $mg^{-1}$ protein) | 5.22 ± 0.08 | 5.31 ± 0.05 ns | 5.15 ± 0.16 ns | 5.10 ± 0.07 ns | 5.05 ± 0.03 ns | 5.04 ± 0.05 ns |
| CAT activity (U $mg^{-1}$ protein) | 13.95 ± 0.05 | 13.91 ± 0.06 ns | 13.92 ± 0.02 ns | 13.91 ± 0.02 ns | 13.91 ± 0.02 ns | 13.89 ± 0.02 ns |
| GR activity (nmol$mg^{-1}$ protein$min^{-1}$) | 6.43 ± 0.03 | 6.40 ± 0.10 ns | 5.82 ± 0.06 *** | 5.24 ± 0.04*** | 4.85 ± 0.02*** | 4.24 ± 0.02*** |
| GST activity (nmol$mg^{-1}$ protein $min^{-1}$) | 1.33 ± 0.16 | 1.21 ± 0.016 ns | 1.45 ± 0.23 ns | 1.40 ± 0.21 ns | 3.26 ± 0.03*** | 0.28 ± 0.02*** |
| GPx activity (U $mg^{-1}$ protein) | 4.49 ± 0.07 | 4.37 ± 0.16 ns | 3.93 ± 0.04 ns | 2.58 ± 0.06*** | 1.70 ± 0.16*** | 0.95 ± 0.02*** |
| GSH (μg $mg^{-1}$ protein) | 4.86 ± 0.02 | 4.75 ± 0.02 ns | 4.22 ± 0.01** | 3.98 ± 0.01*** | 3.26 ± 0.03*** | 1.85 ± 0.02*** |
| GSSG (μg $mg^{-1}$ protein) | 1.28 ± 0.01 | 1.26 ± 0.001 ns | 1.28 ± 0.002 ns | 2.17 ± 0.02*** | 2.45 ± 0.03*** | 2.97 ± 0.01*** |
| GSH/GSSG ratio | 3.79 ± 0.01 | 3.76 ± 0.02 ns | 3.29 ± 0.01*** | 1.83 ± 0.01*** | 1.33 ± 0.05*** | 0.62 ± 0.09*** |
| Vitamin C (μg $mg^{-1}$protein) | 9.85 ± 0.06 | 9.64 ± 0.57 ns | 8.70 ± 0.25 ns | 8.81 ± 0.21 ns | 7.78 ± 0.80** | 6.15 ± 0.02*** |
| Lipid per oxidation (μmol/$mg^{-1}$ protein) | 1.56 ± 0.24 | 1.76 ± 0.07 ns | 1.89 ± 0.006* | 2.64 ± 0.14*** | 2.65 ± 0.10*** | 2.80 ± 0.07*** |
| Protein oxidation (μmol/$mg^{-1}$ protein) | 2.55 ± 0.29 | 2.76 ± 0.03 ns | 2.91 ± 0.04* | 3.62 ± 0.04*** | 4.30 ± 0.21*** | 5.45 ± 0.09*** |

wResults are expressed as mean ± standard error. SOD, Superoxide dismutase; CAT, Catalase; GR, Glutathione reductase; GST, Glutathione –S-transferase; GPx, Glutathione peroxidase; GSH, Reduced glutathione; GSSG, Oxidation glutathione; GSH/GSSG, Reduced glutathione/Oxidation glutathione. All values indicate mean ± SD of seven replicates. Significance was tested statistically by one- way analysis of variance and Dunnett's multiple comparison tests with in the same exposure duration between different concentrations of ACR. NS = none statistically significant; All *asterisks* statistically significant $*P<0.05$; $**P<0.01$; $***P<0.001$ as compared to control values.

**Table 2: The activities of ATPase on the cerebral ganglions of *Lumbricus terrestris* exposed to different concentrations of acrylamide**

| Range of concentrations of ACR (mg/kg body weight) | | | | | | |
|---|---|---|---|---|---|---|
| Specific activity of ATPase (μmol Pi/hr/mg protein) | 0.0 mg | 3.5 mg | 7.0 mg | 10.5 mg | 14.0 mg | 17.5 mg |
| $Na^+/K^+$ATPase activity | 218.27 ± 0.42 | 198.01 ± 0.03*** | 123.41 ± 0.43*** | 97.76 ± 0.67*** | 97.76 ± 0.67*** | 63.42 ± 0.46*** |
| $Mg^{2+}$-ATPase activity | 88.37 ± 1.07 | 87.35 ± 1.16 ns | 87.57 ± 1.11 ns | 72.00 ± 0.97** | 63.28 ± 0.65*** | 55.81 ± 0.51*** |
| $Ca^{2+}$-ATPase activity | 150.07 ± 0.18 | 148.29 ± 0.93 ns | 117.07 ± 0.28*** | 86.62 ± 0.69*** | 4.29 ± 0.58*** | 55.88 ± 0.39*** |
| 5′ Nucleotides activity | 94.23 ± 0.27 | 94.04 ± 0.37 ns | 74.21 ± 0.11** | 57.10 ± 0.30*** | 42.47 ± 0.89*** | 33.91 ± 0.11*** |

Results are expressed as mean ± standard error (n = 7). ATP- ases activities of *Lumbricus terrestr* is cerebral ganglions exposed to different concentrations of acrylamide for 7 days. All values indicate mean ± SD of seven replicates. Significance was tested statistically by one- way analysis of variance and Dunnett's multiple comparison tests with in the same exposure duration between different concentrations of ACR. NS = none statistically significant; All *asterisks* $*P<0.05$; $**P<0.01$; $***P<0.001$ as compared to control values.

## Discussion

Biochemical variations of earthworms are regarded as early warning indicative biomarkers of insult in endocrine and nervous system. Studies using earthworms had shown toxic effects of BDE[29,30] in soil and thus an increased health risk (WHO 2004) subsequently.[31] The oxidative stress and reduction in thiol components were reported in *Eudriluseugeniae*[32] stressing the significance of impact of oxidant/antioxidant imbalance. The oxidant burden and the antioxidant depletion observed in the current study were well manifested in the symptoms such as rigidity, coiling, ataxia, constriction in the worms (Fig. 2). In a study using *Eisenia fetida*, exposure to hexanitrohexaazaisowurtzitane (CL-20) and hexahyro-1, 3, 5-trinitro-1, 3, 5-triazine[33] resulted

**Table 3: Behavior studies of *Lumbricus terrestris* exposure to different dose concentrations and time dependent for 7 days**

| Range of concentrations of ACR (mg/kg body weight) | | | | | | |
|---|---|---|---|---|---|---|
| Earthworm Behaviors | 0.0 mg | 3.5 mg | 7.0 mg | 10.5 mg | 14.0 mg | 17.0 mg |
| **Swimming** | | | | | | |
| Number of waves per swim episode | 9.16 ± 0.30 | 9.13 ± 0.15 ns | 7.80 ± 0.10*** | 6.73 ± 0.20*** | 3.30 ± 0.36 *** | 1.80 ± 0.08*** |
| Swim distance (% of body length) | 90.61 ± 1.40 | 88.84 ± 2.09 ns | 72.4 ± 1.00*** | 64.93 ± 2.92*** | 42.69 ± 2.69*** | 31.21 ± 1.33*** |
| Swim velocity (mm/s) | 71.66 ± 1.34 | 70.46 ± 1.80 ns | 68.96 ± 0.94*** | 51.15 ± 0.68*** | 38.10 ± 0.33*** | 29.35 ± 0.36*** |
| **Burrowing in soil** | | | | | | |
| Burrowing depth (cm) | 6.21 ± 0.20 | 6.21 ± 0.18 ns | 5.56 ± 0.11*** | 4.44 ± 0.02*** | 3.16 ± 0.02*** | 2.35 ± 0.22*** |
| Burrowing length (cm) | 9.85 ± 0.45 | 9.36 ± 0.30 ns | 9.10 ± 0.15* | 8.10 ± 0.20*** | 6.76 ± 0.15*** | 3.50 ± 0.26*** |
| Burrowing reuse | 6.18 ± 0.079 | 6.16 ± 0.073 ns | 5.28 ± 0.37*** | 4.43 ± 0.14 *** | 2.81 ± 0.14 *** | 1.54 ± 0.03*** |
| Burrowing distance covered (cm) | 10.57 ± 0.12 | 10.57 ± 0.06 ns | 8.33 ± 0.11 *** | 7.44 ± 0.05*** | 5.37 ± 0.28*** | 4.19 ± 0.11*** |
| **Burrowing in medium** | | | | | | |
| Burrowing depth (cm) | 7.45 ± 0.07 | 7.38 ± 0.05 ns | 6.43 ± 0.10*** | 4.35 ± 0.15 *** | 3.55 ± 0.29 *** | 2.36 ± 0.19*** |
| Burrowing length (cm) | 18.36 ± 0.30 | 18.26 ± 0.20 ns | 16.30 ± 0.08*** | 14.26 ± 0.12*** | 11.40 ± 0.20*** | 9.36 ± 0.45*** |
| Burrowing reuse | 7.11 ± 0.07 | 7.10 ± 0.07 ns | 6.35 ± 0.12*** | 5.33 ± 0.10*** | 4.14 ± 0.10*** | 3.25 ± 0.15*** |
| Burrowing distance covered (cm) | 13.83 ± 0.23 | 13.61 ± 0.44 ns | 10.54 ± 0.20*** | 9.30 ± 0.39*** | 7.44 ± 0.31*** | 5.43 ± 0.39*** |
| **Locomotion** | | | | | | |
| Forward movement (cm) | 30.83 ± 1.10 | 28.80 ± 1.21ns | 24.36 ± 2.77*** | 19.33 ± 0.37*** | 15.86 ± 0.47 *** | 9.56 ± 0.25*** |

Behavioural changes in worms exposed to different concentrations of ACR for 7 days. All values indicate mean ± SD of seven replicates. Significance was tested statistically by one- way analysis of variance and Dunnett's multiple comparison tests with in the same exposure duration between different concentrations of ACR. NS = none statistically significant; All *asterisks* statistically *$P<0.05$; **$P<0.01$; ***$P<0.001$ as compared to control values.

in abnormal symptoms similar to the current study. In another study using this model (*Eisenia fetida*), increased levels of MDA and PCO were reported on exposure to decarbromodiphenyl ether (BDE-209),[34] supporting the current observations in *L. terrestris* on ACR exposure. The current study did not show significant variations in the activities of SOD and CAT in all concentrations of ACR; whereas the level of MDA and PCO were significantly increased in cerebral ganglions on the ACR dose of 7.0 mg/kg bw/days exhibiting its neurotoxic effect. The GSH and GSSG dependent family also show non-significant changes up to 7.0 mg/kg bw after which only there was a significant reduction ($p<0.001$) in the levels. Supporting this observation a report had shown an increased level of GSSG on exposure to lead in *Lampito mauritii* for 7 days.[35] Generally, GST and GPx activation would result in reduction in the pools of GSH along with replenishment of GSH by its re-synthesis. At 7.0 mg of ACR exposure there was a decreased level of GSH along with the ratio of educed to oxidized glutathione, an indicative of redox status of cell followed by four folds of reduction was at highest dosages. This scenario of moderate change in low significant changes in higher doses was found to be correlated with the locomotive behaviour of the worms (Table 3). Glutathione is best characterized as neuromodulator of central nervous system and as neurotransmitter the deficiency of which is implicated in the pathology of neurodegenerative diseases. The reduced levels and the GSSG production are linked to the loss of neurons.[36,37] thus, the observed impaired GSH/GSSG ratio in the cerebral ganglions would have led to the dysfunction of motor neurons which reflects in the altered locomotion and behavior of ACR expos worms when compared to those of control ones.

Vitamin C was the significantly decreased at 10.5–17.5 mg/kg body weight. In body fluids of *E. fetida*, rich content of antioxidant vitamins has been reported;[38] however the current study had not shown change in the level of vitamin E (not shown). Except the activity of $Mg^{2+}$ ATPase, other enzymes ($Na^+/K^+$ ATPase$Ca^{2+}$ ATPase, and 5′ Nucleotidase) showed significant reduction well from 7.0 mg/kg bw of ACR while the former did show significance only at double the concentration of 14.0 mg/kg bw) When the activities of $Na^+/K^+$ ATPase and $Ca^{2+}$ ATPase were analyzed, only the activity of $Na^+/K^+$ ATPase was significantly affected ($p<0.001$) on 3.5 mg/kg bw of ACR itself (Table 2). Movement of worms were observed to be significantly affected at 7.0 mg/kg bw of ACR while the dose of 3.5 mg/kg bw had registered non-significance. Thus, though the movement of worms changes in accordance with the activities of $Na^+/K^+$ ATPase and $Ca^{2+}$ ATPase, it is more specifically related to the activity of $Ca^{2+}$ ATPase. $Mg^{2+}$ ATPase on the other hand may not be involved in controlling the movement to the extent as controlled by other assessed ATPases for unknown reasons. The maintenance of electrochemical equilibrium, gradient, processing and transmission of nerve impulses are mediated by ion pumps. The co-ordinated

**Fig. 2:** Neurotoxicity manifested as the morphological symptoms which were observed in worms exposed to ACR and untreated worms.

action of ionic ATPases *viz* sodium potassium, calcium and megnesium ATPases[39–41] maintains the function of neuronal system by preserving the synaptic plasticity. Thus ACR challenges cerebral ganglions by causing altered GSH/GSSSG along with the variations in the activities of ATPase, (fig. 3) culminating in behavioural changes by virtue of the loss synaptic integrity.

Neuronal function could be best assessed by spontaneous locomotor activity which reflects the proper integration of the neurons.[41] The dosage of 7.0–17.5 kg bw ACR is anticipated to cause perturbation in the membrane potential owing to its impact on $Na^+/K^+$ ATPase and $Ca^{2+}$ ATPases along with severe oxidative stress (Table 2). This had resulted in 2–3 fold changes in the behavior of the worm with an intense impact in burrowing reuse (Table 3). There may be a neuronal membrane depolarization in a reversible manner and an increased level of free calcium which could be either or due to its release from the organelle or inhibition on its reuptake.[42] The oxidant/antioxidant status of the worms did show significant changes beyond the concentration of 7.0 mg/kg bw of ACR leading toan imbalance is exacerbating loss of ionic imbalance. It is assumed that the oxidative stress and membrane potential have a role in the sensory modulation of locomotion movements in the annelids. Thus the loss of biochemical integrity of cerebral ganglions and membrane potential induced changes are interrelated which would drastically affect the movement of the worms. In this study of acrylamide exposed worms, we show a dose dependent effect of ACR on the activities of ATPases and suggest that a perturbation of ionic environment could alter the sensory and motor functions of annelids.

## Conclusion

The study echoed that the status of glutathione family, activities of $Ca^{2+}$ and $Na^+/K^+$ ATPase and thus the ionic potential variations are interrelated which, on disturbance by any insult such as ACR, could have significant impact on motor neuronal function and locomotion of worms. This relationship in worms might be exploited to study the pathology of neurological disease where the availability of the sample is scarce as in Parkinsion disease (PD).

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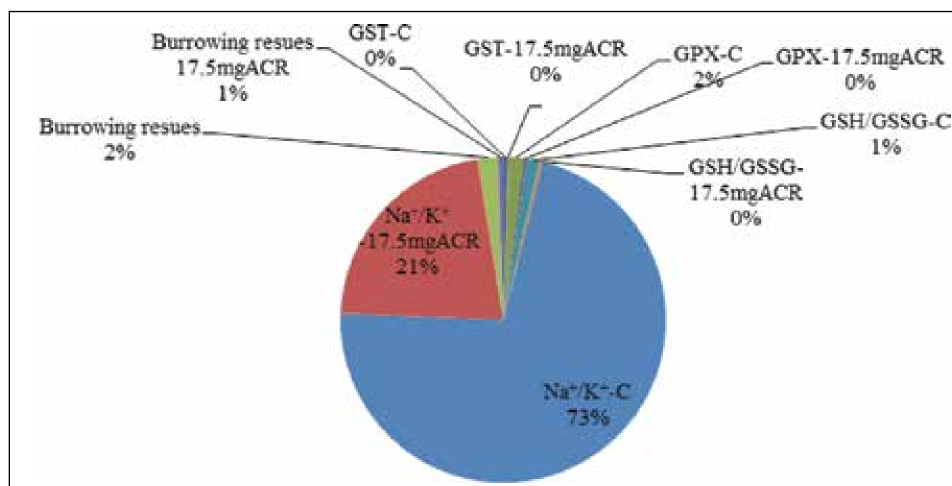


ACR- Acrylamide, C-Control, GST-Glutathione –S-transferase, GPx-Glutathione peroxidase and GSH/GSSG - Reduced glutathione/Oxidized glutathione. Worms were exposed to different concentration of ACR for 7 day. The result showed that diagrammatically representation of glutathione family, $Na^+/K^+$ ATPase activities and burrowing reuses behavior. The acrylamide effects of enzymes and movement in *Lumbricus terrestris.*

**Fig. 3:** Status of glutathione family, $Na^+/K^+$ ATPase activities and burrowing reuses.

### Authorship Contribution

**Arambakkam J Vanisree:** Conceived the work, designed, analysed the results and wrote the major part of the manuscript. **Mamangam Subaraja:** Executed the bench work and also involved in manuscript preparation.

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### References

1. Drewes CD and Brinkhurst RO. Giant fibers and rapid escape reflexes in newly hatched aquatic oligochaetes, *Lumbriculu svariegatus* (Family Lumbriculidae). Invertebrate Reproduction and Development. 1990; 17: 91–95.
2. Kladt N, Hanslik U, Heinzel HG. Teaching basic neurophysiology using intact Earthworms. The Journal of Undergraduate Neuroscience Education. 2010; 9(1): A20–A35.
3. Kulig, BM. Comprehensive neurotoxicity assessment. Environ Health Perspect. 1996; 104(2): 317–22.
4. Friedman, M. Chemistry, biochemistry, and safety of acrylamide. A review. J. Agric. Food Chem 2003; 51: 4504–4526.
5. LoPachin RM. The change view of acrylamide neurotoxicity. Neurotoxicology. 2004; 25: 617–630.
6. Schweden weisen Acrylamid in Lebensmitteln nach. [Swedes find acrylamide in foodstuffs]. 2002; Berlin: bgvv.
7. Amrein TM, Andres L, Escher F, et al. Occurrence of acrylamide in selected foods and mitigation options. Food Addit Contam. 2007; 24(1): 13–25.
8. LoPachin RM. Acrylamide neurotoxicity: Neurological, morphological, and molecular endpoints in animal models. Friedman. M and Mottram D, Editor. In Chemistry and Safety of Acrylamide in Food. New York: 2000; 21–37.
9. Guo F, Zhang Q, Sun Z, Ma F. Toxicity effects of polyacrylamide to earthworm (*Eiseniaf etida*). Transactions of the Chinese Society of Agricultural Engineering. 2012; 28: 224–229.
10. Cooper EL, Baculi BS. Degenerative changes in the annelid, *Lumbricus terrestris*. Journal of Gerontology. 1968; 23–375.
11. Townsend D, Maitin V, Cisneros I, et al. Evaluation of potential redox modulatory and chemotherapeutic effects of a proprietary bioactive silicate alka-V6/ alkahydroxy (AVAH). International Journal of Applied Research in Natural Products. 2011; 3(4): 5–18.
12. Misra HP, Fridovich I. The role of superoxide anion in the auto oxidation of epinephrine and a simple assay for superoxide dismutase. Journal of Biological Chemistry. 1972; 247: 3170–3175.
13. Aebi H. Catalase. Methods in Enzymology. 1984; 105: 121–126.
14. Anderson ME. Glutathione. In: Neville, A.P., Frank, J.K. (Eds.), Free Radicals: A Practical Approach. New York, 1996; pp. 213–225.
15. Kenji A. Glutathione reductase. In: Gutteridge, JMC, Taniguchi N, Editor. Experimental protocols for reactive oxygen and nitrogen Species. New York: 1999; pp. 81–82.
16. Clair DKS, Chow CK. Glutathione peroxidase: activity and steady Staten level of mRNA. In: Neville AP, Frank JK, Editor. Free Radicals: A Practical Approach. New York,1996; pp. 227–238.
17. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry. 1974; 249: 7130–7139.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxidation in animals and tissues by thiobarbituric acid test. Analytical Biochemistry. 1979; 95:351–358.
19. Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods in Enzymology. 1994; 233: 346–357.
20. Omaye S, Turnball T, Sauberlich H. Selected methods for the determination and ascorbic acid in animal's cell, tissue and fluids. Methods of enzymology. 1979; 62: 1–11.
21. Slack JR, Anderson BH, and Day WA. Biochemistry and Biophysiolgy. 1973; Acta 232; 547–559.
22. Warren GB, Toon PA, Birdsall NJM, Lee AG, and Metcalfe, JC. Reconstitution of calcium pumps using membrane components. Proc Natl Acad Sci USA. 1974; 71: 622–626.
23. Jonathan RS, Arch and Eric A, Newsholme. Activities and some properties of 5′-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. Biochem. J. 1978; 174: 965–977.
24. Taussky, HH, Shorr E. A microcolorimetric method for the determination of inorganic phosphorus. J Biol Chem. 1952; 202(2): 675–85.
25. Lowry OH, Rose rough NJ, Farr AL, Randall. 1951. Protein measurement with the Folin -phenol reagent. J of Biol Chem. 1951; 193: 265–275.

26. Drewes CD and Fourtner CR. Helical swimming in a freshwater oligochaete. Biol. Bull. 1993; 185: 1–9.
27. Capowiez Y. Difference in burrowing behaviour andspatial interaction between the two earthworm species *Aporrectodea nocturna* and *Allolobophora chlorotica*. Biology and Fertility of Soils. 2000; 30: 341–346.
28. Datta LG. Learning in the earthworm, Lumbricus terrestris. Am. J. Psycho. 1962; 75: 531–553.
29. Zhu SZ, Liu M, Tian SY, et al. Bioaccumulation and single and joint toxicities of penta-BDE and cadmium to earthworms (*Eisenia fetida*) exposed to spiked soils. Sci China Chem. 2010; 53(5): 1025–1032.
30. Leung AOW, Luksemburg WJ, Wong AS, et al. Spatial distribution of polybrominateddiphenyl ethers and polychlorinated dibenzo-p-dioxins and dibenzofurans in soil and combusted residue at Guiyu, an electronic waste recycling site in southeast China. Environ Sci Technol. 2007; 41: 2730–2737.
31. World Health Organization (WHO) Environmental Health Criteria 162: Brominated Diphenyl Ethers. International Program on Chemical Safety. 2004; Geneva, Switzerland.
32. RavikiranT, Aruna HT. Antioxidant enzyme activities and markers of oxidative stress in the life cycle of earthworm, *Eudrilus eugeniae*. Italian Journal of Zoology. 2010; 77(2): 144–148.
33. Ping G, Laura S, Inouye, et al. Comparative neurotoxicity of two energetic compounds, hexanitrohexaazaisowurtzitane and hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine, in the earthworm *Eisenia fetida*. Environmental toxicology and Chemistry. 2007; pp. 954–959.
34. Xie X, Yingxing Wu, Mengying Z, et al. Hydroxyl radical generation and oxidative stress in earthworms (*Eisenia fetida*) exposed to decabromodiphenyl ether (BDE-209). Ecotoxicology. 2011; 20: 993–999.
35. Maity S, Roy S, Chaudhury S, et al. Antioxidant responses of the earthworm Lampito mauritii exposed to Pb and Zn contaminated soil. Environ Pollut. 2008; 151(1): 1–7.
36. Aoyama K, Watabe M, Nakaki T. Regulation of neuronal glutathione synthesis. J. Pharmacol. Sci. 2008; 108: 227–238.
37. Dringen R. Metabolism and functions of glutathione in brain. Prog. Neurobiol. 2000; 62: 649–671.
38. Zhenjun S, Xianchun L, Lihui S, et al. Earthworm as a potential protein resource. Ecology of Food and Nutrition. 1997; 36: pp. 221–236.
39. Scheiner-Bobis G. The sodium pump. Its molecular properties and mechanics of ion transport. Eur. J. Biochem. 2002; 269: 2424–2433.
40. Lichtstein D, Samuelov S, Bourrit A. Characterization of the stimulation of neuronal $Na(^{+})$, $K(^{+})$-ATPaseactivty by low concentrations of ouabain. Neurochem. Int. 1985; 7: 709–715.
41. Albers RW, Siegel GJ. Membrane transport. In: Brady ST, Siegel GJ, Albers RW, Price D (Eds.). Basic Neurochemistry: Principles of Molecular, Cellular and Medical Neurobiology, $8^{th}$ edn. Massachusetts, USA: Elsevier Academic Press. 2012; p. 41–62.
42. Gong P, Guan X, Pirooznia M, et al. Gene expression analysis of CL-20-induced reversible neurotoxicity reveals $GABA_A$ receptors as potential target in the Earthworm *Eisenia fetida*. Environ Sci Technol. 2012; 46(2): 1223–1232.