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

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**KEY WORDS**

L. terrestris
Acrylamide
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Locomotion

**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, Ca2+/Mg2+ 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 Ca2+ and 5' Nucleotidase were found to be affected at 7.0 mg/kg bw of ACR. Mg2+ 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 toxics by Swedish National Food Administration as out significant amount of this neurotoxin is present in heated and high carbohydrate food items such as potato chips and crisps, coffee and bread4 meats5 and olives6 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 terrestris* 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.
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 20°C 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.19 Briefly, sexually mature *L. terrestris* with developed clitellum was placed in petri dish containing Lumbricus growth medium (Buss-beding -6.0 gm, walnut meal- 1.5 gm, agar- 1.25 g and peanut oil -0.2099 cc in 100 ml) at 20°C 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 ganglia**

Sexually mature worms weighing between 3–6 g were used for the study. The earthworms were anesthetized by the use of phos-plate buffer saline. The operations were carried at 4°C. Cocoonas were housed in 14 × 12 × 5 plastic containers with wet buss-beding. Using the tap water, the moisture content was maintained as 25% (fortnightly). Cocoonas were hand- sent weekly from initial stocks, their weight and size were measured and incubated at 20°C until hatching. Hatchings were kept in same size plastic vessels with fed buss-beding and soil: the vessels were maintained in the same temperate (20°C). 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.

**Biochemical assays**

**Superoxide dismutase (SOD)**

SOD activity was determined based on the method by Misra and Fridovich.13 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-H2O2 complex II to release active CAT enzyme. After 30 min, the tubes were brought back to RT and 10 µl of Triton X–100 was added. In a cuvette containing 200 µl of phosphate buffer, 50 µl of 0.066 M H2O2 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) assay method of Anderson14 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 nicotine adenine dinucleotide phosphate (NADPH) (pH-7.5) and 2.0 ml of 5', 5’ dithiobis –2-nitrobenzoic 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.18 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 30 s in a spectrophotometer. The activity of the enzyme was calculated using the molar extinction coefficient of NADPH (ε250 = 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.19 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 used for further analysis. The resulting homogenate was centrifuged at 1000 rpm for 25 min at 4°C. The supernatant was transferred in to centrifuge tubes and stored at –80°C.
and glutathione reductase. The reaction was started by adding 100 µl of 7.5 mM of H₂O₂ 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 GPrx was calculated from the molar extinction coefficient of NADPH (ε₃₄₀ = 6220) and the results were expressed in terms of nmol hydrogen peroxide degraded min⁻¹ mg⁻¹ protein.

**Glutathione S-transferase (GST)**

Glutathione S-transferase activity was determined by using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate according to the method of Habig et al. 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, dinitrophenylglutathione (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 method described by Ohkawa et al. 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°C. The sample was then added and incubated for 10 min at 37°C. About 2500 µl of 65% HCL was mixed well. The tubes were allowed to stand at room temperature for an additional 3 hours. About 2500 µl of 4 mM MgCl₂, 200 µl of 1 mM EDTA and 200 µl of 3 mM Tris-ATP. The reaction was then allowed to proceed for 20 minutes 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.

**Protein carbonyl oxidation (PCO)**

Protein carbonyl oxidation was measured according to the procedure of Levine et al. Briefly, 100 µl of 10 mmol/dodecylphosphate (DDP) 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 MAD per mg of protein.

**Ascorbic acid (Vitamin C)**

Ascorbic acid was determined by the method of Omaye et al. Briefly, 500 µl of tissue homogenate was mixed thoroughly with the 1500 µl of 5% TCA and centrifuged 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. The 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 MgCl₂, 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.

**Mg²⁺ ATPase activity**

The activity of Mg²⁺ ATPase was measurement using modified method of Warren et al. 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 4 mM MgCl₂, 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.

**Ca²⁺ ATPase activity**

The activity of Ca²⁺ ATPase was measured by the modified method of Warren et al. 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 CaCl₂ 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. 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 MgSO₄, 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 using sodium hydrogen phosphate (Na₂H₂PO₄). The enzymes activity was expressed as nanomoles of Pi liberated/mg protein/min.

**Protein estimation**

Protein was measured according to Lowry et al 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. Worms were placed in shallow...
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 observations27 the burrowing behaviors of earthworms were observed in two different environments: in cylinder consisting of (50 cm x 35 cm) 40 cm filled with 2 mm soil and in cylinder consisting of (50 cm x 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 Datta28 with minor modifications. Reconstructed trajectories provided estimation for the locomotory activity in Lumbricus terrestris. Reconstructed trajectories (50 cm x 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 LC50 values were found to be 17.5 mg/kg body weight after 7 days. Survived worms also had reduced body weight and pathological features which were recorded including rigidity, coiling, ataxia, constriction and swelling of the body of L. terrestris.

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 GSGS 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 terrestris 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, Mg2+/Ca2+ 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.

container filled with water (21–22°C). The worms were placed in flat bottomed (23 x 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, on the video recorder allowed single –frame analysis of locomotor 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.
Table 1: Biochemical response of *Lumbricus terrestris* cerebral ganglions exposed to different concentrations of acrylamide

| Biochemical measurements | Range of concentrations of ACR (mg/kg body weight) | 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 (nmolmg⁻¹ proteinmin⁻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 (nmolmg⁻¹ proteinmin⁻¹) | | 1.33 ± 0.16 | 1.21 ± 0.016 ns | 1.45 ± 0.23 ns | 1.40 ± 0.21 ns | 3.26 ± 0.02*** | 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*** |

Results 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 within 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

| Specific activity of ATPase (µmol Pi/hr/mg protein) | Range of concentrations of ACR (mg/kg body weight) | 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*** |
| Mg²⁺ 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*** |
| Ca²⁺-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 terrestris* 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 within 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.

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 BDE²⁹,³⁰ in soil and thus an increased health risk (WHO 2004) subsequently.³¹ The oxidative stress and reduction in thiol components were reported in *Eudrilus eugeniae*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-triazine³³ resulted
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), 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/day 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. 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 cell followed by four folds of reduction was at highest dosages.

### 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) | 0.0 mg | 3.5 mg | 7.0 mg | 10.5 mg | 14.0 mg | 17.0 mg |
|---------------------------------------------------|-------|-------|-------|--------|--------|--------|
| Earthworm Behaviors                               |       |       |       |        |        |        |
| 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 *** | 32.11 ± 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 ± 1.21 ns | 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.

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; however the current study had not shown change in the level of vitamin E (not shown). Except the activity of Mg2+ ATPase, other enzymes (Na+/K+ ATPaseCa2+ ATPase, and 5’ Nucleotidase) showed significant reduction well from 7.0 mg/kg bw of ACR while the former did show significant only at double the concentration of 14.0 mg/kg bw) When the activities of Na+/K+ ATPase and Ca2+ 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 Ca2+ ATPase, it is more specifically related to the activity of Ca2+ ATPase. Mg2+ 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
Behavior of earthworm on ACR neurotoxicity

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 magnesium ATPases 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. 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 Ca²⁺ 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. The oxidant/antioxidant status of the worms did show significant changes beyond the concentration of 7.0 mg/kg bw of ACR leading to an imbalance is exacerbating loss of ionic imbalance. 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 anelids.

Conclusion

The study echoed that the status of glutathione family, activities of Ca²⁺ 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 Parkinson disease (PD).

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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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