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Mercuric Chloride Induced Neuropathology and Physiological Responses in Slug *Semperula maculata*

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ABSTRACT

The present study was designed to investigate the neuropathological effect of mercuric chloride in relation to physiological response on terrestrial slug *Semperula maculata* using topical application technique (contact action). Lethal concentration (predetermined LC_{50} value-107 ppm) of $HgCl_2$ was used and experiment lasted for 4 days. Histopathological and ultrastructural alterations in the cerebral ganglia were more obvious after exposure of $HgCl_2$ by topical application technique. The alteration includes shrinkage of perikarya of neurons, increased cytoplasmic basophilia and extreme indentation of the plasma membrane. In addition, the multiple karyosomes, eccentric and irregular nuclear envelope and its effect on physiological responses were recorded in the study.

Key words: *Semperula maculata*, $HgCl_2$, Cerebral ganglia, physiological response

INTRODUCTION

Metals are extremely toxic and ubiquitous in natural environment and were produced by an expanding variety of anthropogenic sources such as industrial activities, traffic, smelting, combustion of fuels and certain agricultural practices, suggesting an increasingly important role for pollution (Uyer *et al*., 2009). These pollutants have been known to possess adverse effects capable of degrading the ecological integrity of terrestrial environment and represent a potential threat to human health (Nyholm *et al*., 1995; Jarup, 2003). Metal pollution is very important in an advanced technological society. At present iron, copper, cadmium, zinc and mercury level is ten times higher in specific sites than their normal occurrence in nature (Simkiss, 1984). These cations are get accumulated in body of invertebrates until they reach concentration several thousands times higher than those in the environment (Couqhtrey and Martin, 1977; Ireland, 1979) Particularly, molluscs can accumulates higher concentration of metal ions than other group of invertebrates (Beady and Eaves, 1983).

Mercury is one of the most toxic metals. It causes protein denaturation in the tissue like foot, digestive tract and nervous system of terrestrial and aquatic invertebrate. Several authors (Nuenberg, 1984; Kiffiny and Clement, 1993) have been reported that the molluscan species are good indicators for monitoring heavy metal pollution. Abnormally high environmental concentration of heavy metal affects numerous biological processes involved in the development and maintenance of molluscan population such as feeding, growth, reproduction, general physiological activities and also maturity of animals (Nuenberg, 1984). Terrestrial snails and slugs are now considered as sentinels in ecological risk assessment of metal pollution (De Vaufleury and Pihan, 1983).

In general nervous system play important role in the control of all physiological processes like feeding, locomotion, mucus secretion, reproduction etc. It is worthy to mention that, in the terrestrial snail and slug nervous system has been proved to be sensitive to any of the toxic material and cytotoxins that may induce injurious consequences in the body (Hernadi *et al*., 1992; Boer *et al*., 1995; Wiemann *et al*., 1996). Although, a large amount of information exists relating neuropathology and its effect on physiological or behavioral responses of terrestrial molluscs against environmental heavy metal pollution (Scott and Major, 1972; Calabrese *et al*., 1977). Information concerning in this kind of responses related to terrestrial molluscs is poor and scanty.

In this study, we have described variations in physiological and behavioral responses occurred due to neurophysiological alteration in experimental slug *S. maculata* against predetermined (LC_{50}) lethal concentration of mercuric chloride doses.

MATERIALS AND METHODS

Animals and chemicals: Adult herbivores, terrestrial slug *Semperula maculata* (molluscan, pulmonate, stylommatophoran) weighted about 3-4 g were collected from the different gardens (Panmalas) of Dist. Sangli, Maharashtra, (India) during winter and rainy seasons (Fig. 1). Animal were carried by plastic container to the laboratory and were kept in plastic cages (100 individuals per cage), allowed to feed on fresh and clean leaves of *Brassica oleracea* (cabbage) and kept to acclimatize under laboratory condition (26-30°C) over one week prior to the experiment. For the experiment water miscible heavy metal mercuric chlorides ($HgCl_2$) was used. The stalk solution of mercury chloride was prepared by dissolving 0.107 mg content of mercury chloride in to the 1000 mL distilled water.

Experimental design: The toxicity of heavy metal Mercuric chloride against slug *S. maculata* was evaluated using Topical application method (Contact action) (Rorke *et al*., 1974; Radawan *et al*., 2008). The topical application method was selected to determine the different concentration that affects the slug. Lethality in this case was expressed as the LC_{50} value. Further slugs were exposed to predetermine LC_{50} Value (107 ppm) for the behavioral and neuropathological study at different exposure period 24, 48, 72 and 96 h. From each set slugs were sacrificed for neuronal study. Separated out cerebral ganglia from nervous complex and fixed in Bouins solution. A graded

Fig. 1: Terrestrial slug *Semperula maculata*

dehydration of tissue was carried out by 30-100% of alcohol in subsequent steps. Xylene was used as clearing agent. Further tissue was embedded in paraffin and sections were cut at 4-5 µ. Stained with standard Hematoxylene-Eosin (H-E) and Mallory's triple (M-T) staining technique to observe the histological alteration in the neuronal cells.

Slug feeding experiment: The slugs were selected by considering average size of (4-5 cm length, 1-1.5 cm width) and weight (3-4 g) was kept in to the plastic troughs. Animal were starved for 6 days prior to experiment in order to minimize physiological differences and provided acclimation to laboratory condition. Metal intoxication was among average weight 100 g of total animals. Mean temperature during assays was 22°C and relative humidity was maintained by using moist soil. Daily, animals and troughs were cleaned and water was spread by using spray machine. During experiment slug feed by green, fresh and clean leaves of Mulberry. Predetermined LC_{50} concentration of $HgCl_2$ was applied to the animal by Topical application method. Finally food consumption was calculated for the duration of 24, 48, 72 and 96 h of exposure period and compared with control. For the growth determination, slug was analyzed using the total weight and growth coefficient for each duration of exposure, respectively.

Statistical analysis: For LC_{50} of mercuric chloride to Pulmonate slug *S. maculata* were calculated using probit analysis method (Finney, 1971).

RESULT

Histological organization of cerebral ganglia in *S. maculata*: In Stylommatopharan, the paired cerebral ganglia were found coupled by a short commissure and consist of procerebrum, mesocerebrum and metacerebrum. The neuronal cells occur at periphery region of cerebral ganglion in *S. maculata* while neurophile (fibrous mass) found centrally located. The neurophile composed

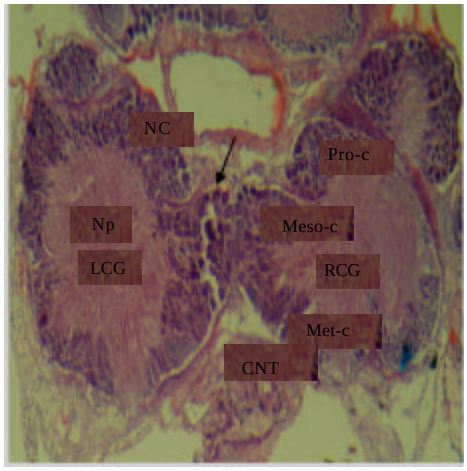

Fig. 2: Cross section of cerebral ganglia in slug *S. maculata* stained with H and E-technique x100, NC: Neuronal cells, NP: Neurophile, LCG: Left cerebral ganglia , RCG: Right cerebral ganglia procerebrum (Pro-c), Mesocerebrum (Mws-c), Metacerebrum (Met-c), CNT: Connective tissue, A: Axonal process

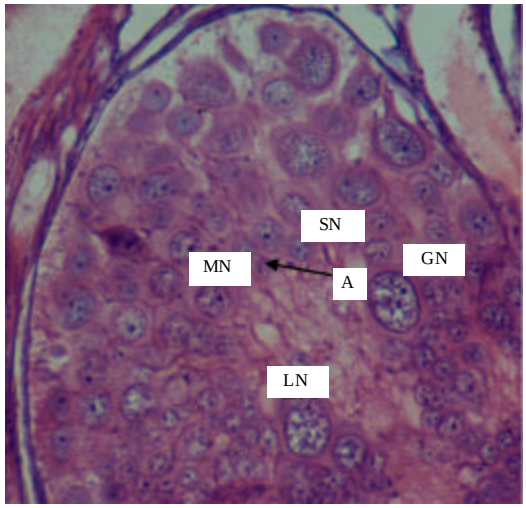

Fig. 3: Neuronal cells with different sizes and axonal processes (arrow)HE-technique ×400, GN: Giant neuronal cell, LN: Large neuronal cell, MN: Medium neuronal cell, SN: Small neuronal cell, A: Axonal process

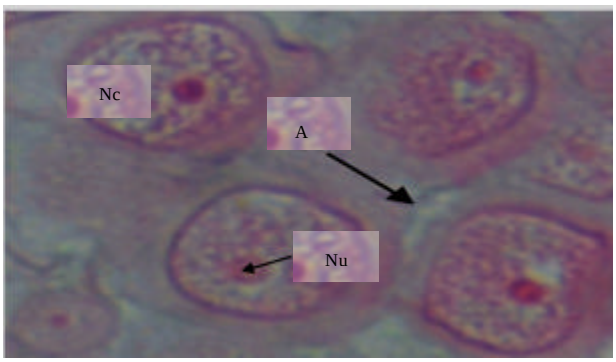

Fig. 4: Enlarged neuronal cells in cerebral ganglia of *S. maculata*, H-E tech X630. Nc: Neuronal cell, Nu: Nucleus, A: Axonal process

of few glial cells and large number of nerve axons that run in different direction (Fig. 2). The neurons are classified into four main types, giant, large, medium and small sized cells. In theses cells, cytoplasm stained pink and nucleus blue by Hematoxylene eosin staining technique (Fig. 3). Wijdenes and Runham (1976) observed five types of cells in some Pulmonate *D. reticularum* and *A. horrensis* and ten types of cells in Molluscan species *Helix aspersa* related to cerebral ganglionic structure. The neuronal cells are generally oval with well defined nucleus in *S. maculata*. Staining intensity in Mallory's triple showed dark red nucleus and cytoplasm with orange-red texture (Fig. 4).

Histopathology of cerebral ganglia in treated slug *S. maculata*: At the light microscopic level, histopathological changes were observed in the cerebral ganglia of slug *Semperula maculata* treated with mercuric chloride ($HgCl_2$).

Twenty four hours exposure of $HgCl_2$: After treatment with topical application of mercuric chloride, giant neurons were exhibited multinuclear showing eccentric and lysis of chromatin. On the other hand, large and medium sized neurons showed highly shrunken and vacuolated cytoplasm. The cytoplasms of many small neurons were greatly dissolved (Fig. 5).

Forty eight hours exposure of $HgCl_2$: After exposure of 48 h neuronal cells showed the lysis of perineurium and severe pyknosis. In addition to this we found disorganized and degenerated nerve fibers in the neurophile. The giant neurons showed a shrunken appearance with poor nuclear membrane integrity. The nuclei appeared with irregular "Crenated" shape rather than oval morphology. Large and medium sized neurons displayed shrinkage of the cell bodies along with migration of deformative nuclei. Small sized neurons exhibited an obvious lysis of cytoplasm and eccentric nuclei (Fig. 6).

Seventy two hours exposure of $HgCl_2$: After exposure of 72 h we observed that, the giant neurons become swollen and showed dark staining with eccentric karyosomes. The counters of cell membrane and nuclear envelope are irregular along with darkly stained heavily dissolved

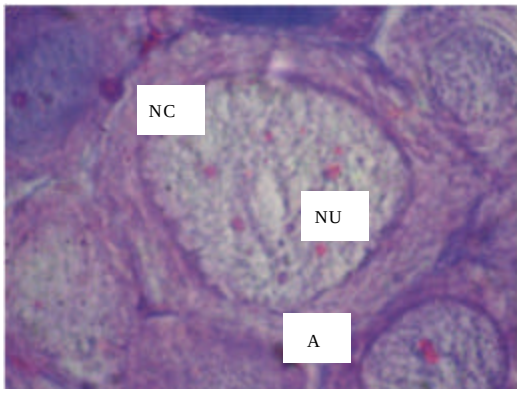

Fig. 5: Mercury chloride induced neuronal cells in the cerebral ganglia of *S. maculata*, MT tech X1100. Nc: Neuronal cell, Nu: Nucleus, A: Axonal process

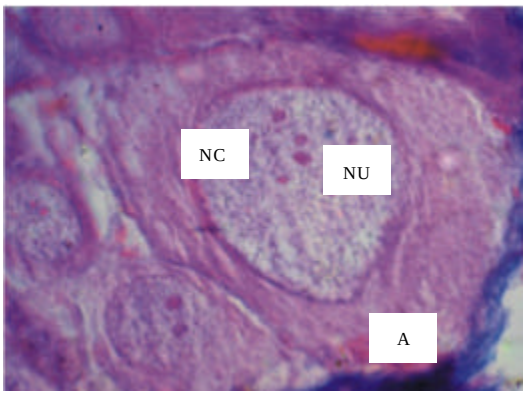

Fig. 6: Mercury chloride induced neuronal cells in the cerebral ganglia of *S. maculata*, MT tech X1100. Nc: Neuronal cell, Nu: Nucleus, A: Axonal process

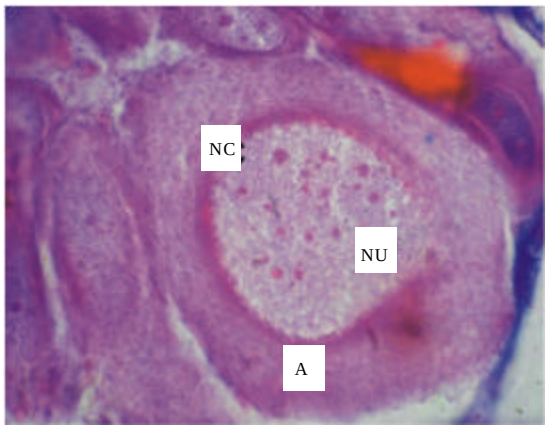

Fig. 7: Mercury chloride induced neuronal cells in the cerebral ganglia of *S. maculata*, MT tech X1100. Nc: Neuronal cell, Nu: Nucleus, A: Axonal process

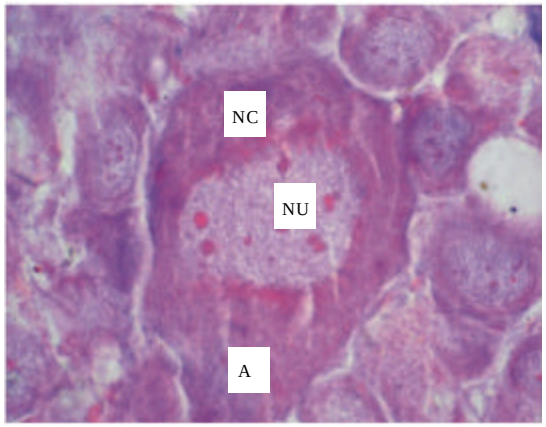

Fig. 8: Mercury chloride induced neuronal cells in the cerebral ganglia of *S. maculata*, MT tech X1100. Nc: Neuronal cell, Nu: Nucleus, A: Axonal process

perineurium. Other giant neurons showed disruption of both cell and nuclear membrane with migrated nuclei. Some medium and small sized neurons were irregular counters (Fig. 7).

Ninety six hours exposure of $HgCl_2$: After 96 h exposure with $HgCl_2$, the perikarya of neuronal cells showed darkly stained intensity. The giant neurons were in a shrunken and swollen appearance with eccentric multinuclear cells. In addition, completely degeneration of axonal process with lysis of nerve fiber in the neurophile. The large and medium sized nerve showed extreme shrinkage and some completely degeneration of cell cytoplasm. Several neurons were appeared fully disturbed and surrounding extracellular space found grossly swollen (Fig. 8).

Physiological response of slug *S. maculata* against mercury chloride: Physiological activities like feeding, locomotion, reproduction and growth of organism is always under the control of nervous system. Any neuronal disturbance greatly affects physiological activities like feeding and growth etc.

Table 1: Effect of $HgCl_2$ on the growth of *Semperula maculata* by contact action exposed up to 96 h

LC_{50} of $HgCl_2$ (107.4 ppm)

Time of exposure	Start of experiment mean weight (g)	$HgCl_2$ treated mean weight of slug (g)	G.C	G.I (%)
24 h	3.333	2.593	777.9	12.60
48 h		2.389	716.7	19.48
72 h		2.037	611.1	31.34
96 h		1.893	567.9	36.19

G.C: Growth coefficient = Mean weight of treatment×100/mean weight of the starting experiment, G.I: Growth inhibition = Mean weight of control- mean weight of treatment×100/ mean weight of control

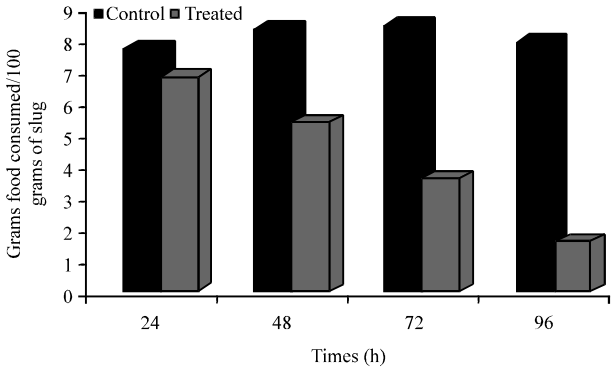

Fig. 9: Food consumption rate of slug *Semperula maculata* against $HgCl_2$

Feeding activities: Effect of $HgCl_2$ after exposure period 24, 48, 72 and 96 h to *S. maculata* showed decreased food consumption from 7.90 mg food consumption /100 g of slug (control group) to the 1.58 g food consumption /100 g of slug (treated). The data reveled that, food consumption rate was decreased as per increasing time of exposure. Reduced food consumption was noticeable after the 48, 72 and 96 h of exposure for predetermined LC_{50} (107.4 ppm) of $HgCl_2$ by topical application method. This was illustrated graphically (Fig. 9).

Growth response: Effect of $HgCl_2$ on the growth (mean weight) of *S. maculata* was determined. The growth of slug found depends upon time of exposure and concentration of metal. For slug predetermined LC_{50} was given by the contact action. Growth was decreased with slightly significant difference as compared to the control during the 24 h of exposure. We found that growth of slug was significantly inhibited/ reduced as per exposure time of 48, 72 and 96 h. This was presented in Table 1.

DISCUSSION

Up to the present investigation, the mode of action of heavy metal in the central nervous system of gastropods has predominantly been studied at a histological and ultrastructure level. The current study has been performed to show the changes in general physiological activities like feeding and growth occurred due to the histopathological alteration in the neurons of cerebral ganglia of slug *S. maculata* treated with mercuric chloride.

Our observation revealed that, a cerebral ganglion contains several neurons of viable size (Giant, large, medium and small). Similarly, many investigators have mentioned size differences in neurons of various Pulmonate species (Steffens, 1980; Kruatrachae *et al*., 1993; Essawy, 2001).

In the study, light microscopic investigation of cerebral ganglia in exposed slug demonstrated a diverse spectrum of cell alteration. Neurons of all size were greatly affected, while giant neurons, their cytoplasm and perikarya were the most affected elements of the cerebral tissue and displayed obvious alteration after toxicity of mercuric chloride by contact action. Giant neurons showed great shrinkage of perikarya, increased cytoplasmic basophilia, eccentric karyosomes envelope, etc. Dilation in the intracellular space also observed in the giant neurons of treated slug. This dilation probably reflected a dysfunction of ionic and osmotic balance of these cells (Cortan *et al.*, 1999).

The karyosomes showed eccentric, highly shrunken with irregular nuclear envelope. Karyolysis of the nucleus may be sign of cell response to the toxication of heavy metal. This is in agreement with the finding of Baryne (1985), who described karyolysis as a late reaction to intoxication of metal in vertebrate and invertebrates with regard to nuclear size modifications. It is known that, larger nucleus corresponds to higher cellular activities and vice versa (Hildedrand, 1980). The shrinkage of all sized neurons with eccentric nuclei was observed, to support this Mcllwain and Hoke (2005), observed shrinkage of cell bodies with eccentric nuclei and crenations of nuclear envelope could be attributed to the effect of the two carbonate molluscicides on the cytoskeleton of affected neurons. In case of small neurons highly vacuolated cytoplasm were found. Similar type of observation of Moussa and Banhawy (1984), who reported a distinctive cytoplasmic vacuolations in the locust, *Schistocerca gregaria* after insecticide treatment.

Several studies of effect of metals and other contaminants on the physiology of organism had lead to the development of several toxicity tests that can be used as tools for environmental assessments (Handy and Depledge, 1999). The neuronal system play important role in the physiological activates like feeding and growth of body and also to locomotion. In molluscan species, neuronal secretion like gastrin, cholecystokinin (CCk8) and acetyl cholinesterase are take part in the feeding and growth responses. The neuronal alteration inhibits these secretions when treated with the mercuric chloride resulting in changed feeding behavior of slug. Wedgwood and Bailey (1988) observed that, neurotoxic action of carbamates was due to the inhibition of acetyl cholinesterase (AchE) activity and the accumulation of acetylcholine (Ach) at synaptic junctions that results in the alteration in growth, locomotion and feeding behavior. In another study, neuronal secretion like gastrin, cholecystokinin (CCK8) may be involved in some integrated sensory functions, such as feeding related behavior and neurohormonal communication etc. (Vigna *et al.*, 1984; Gesser and Larsson, 1990; Sonetti and Fasolo, 1990).

Normal growth of slug was under the control of neuronal secretion. In this study we have observed that, neuronal alteration inhibits the growth of slug. Similarly, Gondan (1983) observed that, carbomate such as methiocarb were found to cause loss of muscle tones in terrestrial gastropods and exhibit neurotoxic effects on the nerve controlling the locomotion, growth and feeding behavior (Wright and Williams, 1980; Bailey, 1989).

Finally it could be concluded that, the alteration in the structure of cerebral ganglia of slug treated with mercuric chloride may possibly affect other biological perturbations like feeding Growth, locomotion etc.

Further studies need to be throwing a light on the exact action of metal on the aspects of slug *S. maculata.*

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