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Effect of Paclobutrazol on neurocytes, its DNA content and lipid peroxidation mechanism of *Semperula maculata*

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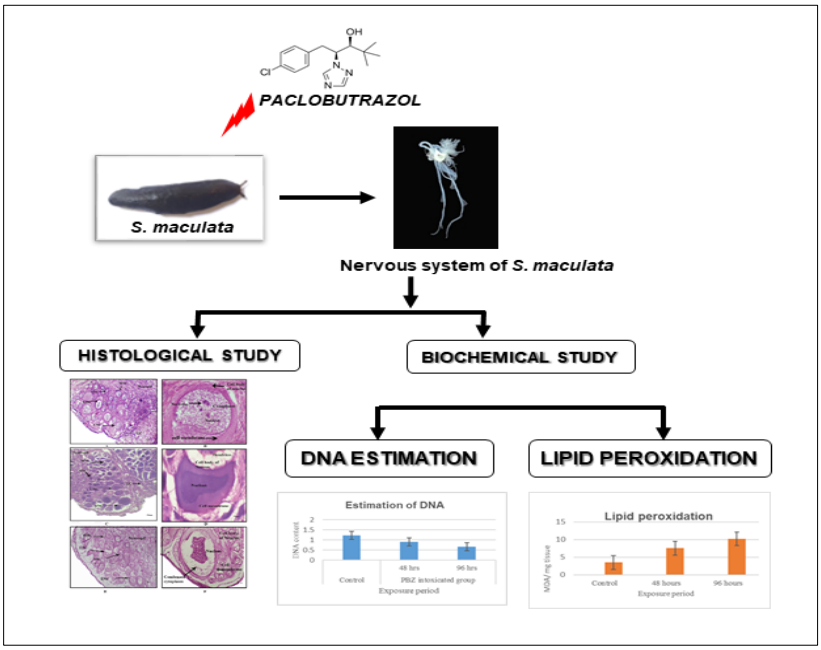
Abstract

Overall, for perfect co-ordination in bio- physical activities, under sensory mechanism and initiation of action potential, neurocytes with its network play vital role. Present investigation emphasized to observe the cytological alterations in the neuronal tissue of terrestrial slug *Semperula maculata* after intoxication of pre-determined LC_{50} dose of Paclobutrazol (PBZ). Experimental animals were exposed for the period of 48 hrs and 96 hrs. respectively to PBZ. Animals under experimental group showed histological and biochemical alterations in the neurocytes. Neurocytes were biochemically analyzed for to estimate DNA content after pre- determined dose induction of PBZ. Biochemically lipid peroxidation activity was assessed for to study oxidative stress from both control and experimental animals. Cytometric and histopathological symptoms were prominently seen in neuronal cells of experimental animals. Biochemically DNA content of targeted cells were declined. Lipid peroxidation activity was increased in neuronal cells, which indicates significant neurocytic damage leading to incoordination of physiological mechanism. Observed alterations were interpreted for the impact of PBZ on the secondary mechanism and biopotential of animal for servility.

Figureical abstract



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Keywords: Neurocytes; Histopathology; Paclobutrazol; *S. maculata.*

1. Introduction

Among invertebrates molluscs are second largest phylum after arthropods and maintain ecobiota [1]. Systematically phylum mollusca is subdivided into eight classes where, Gastropods scientifically reported as a largest class including 80 % of terrestrial and aquatic slugs and snails [2]. In general gastropods reported as indicator of pollution. [3] reported, molluscan nervous system have been studied as model nervous system to study the basic mechanism related to neuronal function and plasticity. For clear understanding of neuronal network, molluscs were preferred and recommended as scientific model. Neurons were well defined and segregated as per internal neurocytic parameters meant for excitation and conduction of stimulation [4,5].

Now a days in agriculture sector crop yield was increased by abuse of organic and inorganic chemicals such as pesticide, herbicide, phytoregulators etc. Over use of these chemicals contaminate terrestrial and aquatic ecosystem. From the agriculture point of view paclobutrazol is a trizol phytoregulator, showed anti gibberellin activity [6]. Paclobutrazol showed growth stimulating potential, which promotes photosynthesis and can be used for to increase the crop yield in some medicinal plants [7, 8]. From toxicity relation PBZ has negative impact in environment which have been reported by [9]. To study toxicology, histopathology is a widely used tool to evaluate the impact of environmental stressors on soil organisms [10].

Present work is aimed to study induced histopathology of neuronal tissue, estimation of DNA content and the activity of lipid peroxidation against predetermined LC_{50} of paclobutrazol from invertebrate experimental sensitive animal model as terrestrial slug *S. maculata*. The obtained results were interpreted for excitatory and stimulatory activity of invertebrate terrestrial molluscan slug *S. maculata***.**

2. Materials and methods

2.1. Slug collection and experimental condition

The gastropod molluscs, terrestrial slug – *Semperula maculata* was selected as experimental animal model (Figure 1). Live specimens were collected from Betel leaf farm- Panmala Arag- Bedag, Tal. Miraj, Dist. - Sangli, Maharashtra, India. Animals were kept in maintained laboratory condition for accumatization upto eight days before the experiment. Animals were fed with mulberry leaves (*Morus indica)*, tomato (*Solanum lycopersicum*) and cabbage (*Brassica oleracea)*. Daily water was sprinkled to maintain the moist condition, after acclimatization healthy adult animals were used for the study.

Animals for the experiments were used as per the approval of Maharashtra State Biodiversity Board, Nagpur (MSBB/ Desk-5/ Research/623/2023-24). For present investigation, Paclobutrazol (PBZ) 95% (Himedia) one of the synthetic phytoregulator selected as the toxicant. LC_{50} dose was determined by applying standard procedure of Probit analysis [11].

For experimentation, animals were divided into two groups. one as control and another as experimental group. The normal group was without any induction of toxicant. In experimental group the animals were exposed for the predetermined Mean Lethal Concentration LC_{50} value of PBZ 4243 ppm dose was prepared in distilled water and DMSO was used as solvent [12]. The animals were exposed upto 48 and 96 hrs. respectively. After completion of exposure period animals were keenly dissected for histological and biological observations. DNA content from nerve tissue was estimated and study was supported by lipid peroxidation analysis.

2.2. Histological study

Nervous tissue of control and experimental group was skilfully dissected and subjected for standard microtechnique. Sections of 4-5 µ were stained with standard Heamtoxylin- Eosin (HE) staining technique [13] to observe the histopathological changes in neurocytes. The sections were deparaffinised in xylene, hydrated in descending grades of ethanol followed by staining with Haematoxylin, washing was done in water then slides were kept in 1 % Eosin for counter staining. Finally stained sections were dehydrated in alcohol, cleared in xylene then mounted in DPX. Stained slides were examined under the compound microscope.

2.3. Tissue preparation for DNA estimation

DNA content of neurocytes were estimated by [14] in which 200 mg of nerve tissue was homogenized in ice cold distilled water. Homogenate was treated with 0.6 N Perchloric acid ($HClO_4$), and allowed to stand for 10 min at 0º C. Content was centrifuged at 2000 RPM, for hydrolyse RNA then discard the supernatant. Sediment was washed with 0.2N $HClO_4$ then add 4 ml of 0.3 N Potassium Hydroxide (KOH) and incubated for 60 min at 37°C. 1.2 N Perchloric acid was added and kept sample at 0°C for 10 min. Whole content was centrifuged and supernatant was discarded. 0.5 N Perchloric acid was added and incubated at 70ºC for 15 min in water bath and centrifuged again and finally supernatant was used for DNA estimation by treating it with Diphenylamine reagent. For standard DNA 1mg of calf thymus DNA in 1 ml of 0.5 N Perchloric acid was used. All samples were incubated at room temperature for 20 hrs. blue colour was developed and optical density was measured at 595 nm.

2.4. Lipid peroxidation

Neuronal tissue of experimental animal *S. maculata* was subjected for Lipid peroxidation activity by method [15]. Tissue homogenate 2 mg/ ml was prepared in chilled mortar and pestle by using 75 µM potassium phosphate buffer pH 7.04. The samples were centrifuged at 3000 RPM for 10 minutes. The MDA was reacted with thiobarbituric acid (TBA) and develops faint pink colour as indicator. MDA level was measured at 532 nm and concentration expressed in n mol MDA/mg wet tissue. Malondialdehyde (MDA) is end product of lipid peroxidation formed during the oxidation of poly unsaturated fatty acids. Thiobarbituric acid as reagent were used to detect lipid peroxidation.

2.5. Statistical analysis

All the data were presented as a mean value ± SD. One way ANOVA analysis of variance and Student's t-test was applied to determine the significant ($p < 0.05$) difference between treated and control group of slugs [16].

3. Results

3.1. Anatomy of nervous system of *S. maculata*

Anatomically nervous system in *S. maculata* was centralized type and located on ventral side of body. Overall length of system is 35 ± 5 mm in which length of ring is 10 ± 2 mm with diameter of 10± 2 mm. Morphologically it is ganglionic type, were all ganglions were located at the periphery of upper alimentary tract. Specifically in circum oesophageal part of digestive system (Figure2).

Figure 1 Terrestrial slug *Semperula maculata*

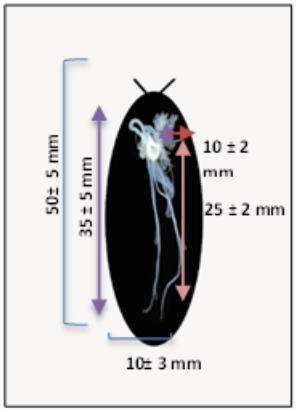


Figure 2 Location of nervous system of *S. maculata*

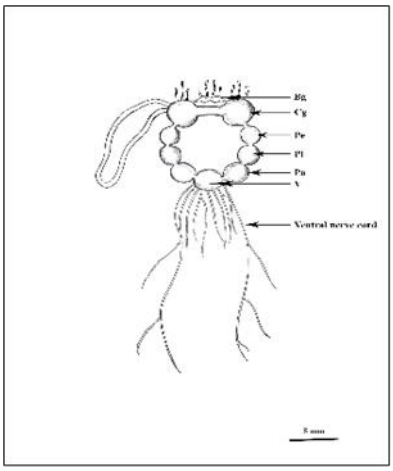


Figure 3 Schematic presentation of neuronal network of slug *Semperula maculata*

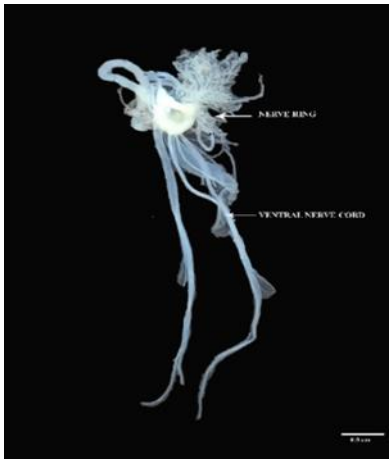


Figure 4 Neuronal network of slug *Semperula maculata*

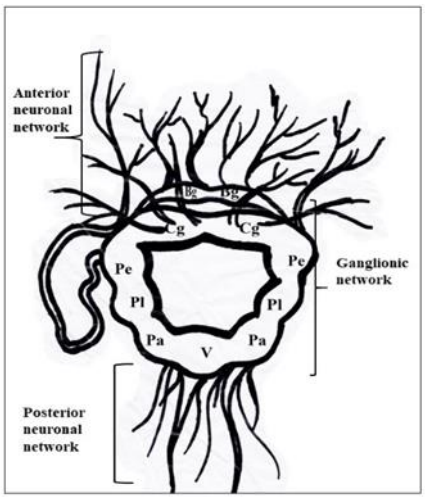


Figure 5 Schematic diagram of nerve ring of *S. maculata*

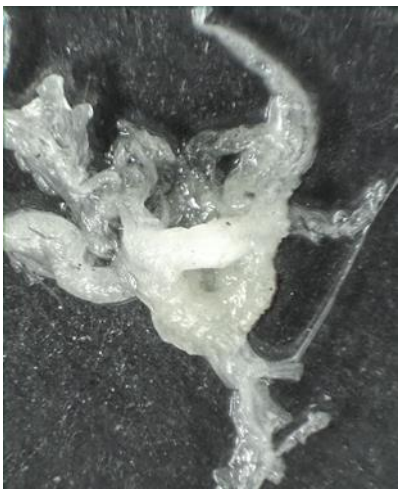


Figure 6 Magnified Ex- situ view of nerve ring of *S. maculata*

The nervous system of adult terrestrial slug *S. maculata* was consists of a nerve ring with nerve cord (Figure 3 and 4). As shown in (Fig 5 and 6), The nervous system of *S. maculata* consists of total 11 ganglions includes, a pair of buccal ganglia (95 µm), a pair of cerebral ganglia (210 µm) which are connected together by short commissure, a pair of plural ganglia (110 µm), a pair of pedal ganglia (135 µm), a pair of parietal ganglia (142 µm) and a single visceral ganglia (185 µm). All ganglia are covered with two layers of connective tissues outer layer which is loosely packed and inner layer is compactly packed. Ganglions contains nerve cells, glial cells and neuropil. A single neuron is consists of axon, axon hillock, nucleus, nucleoplasm and cell membrane.

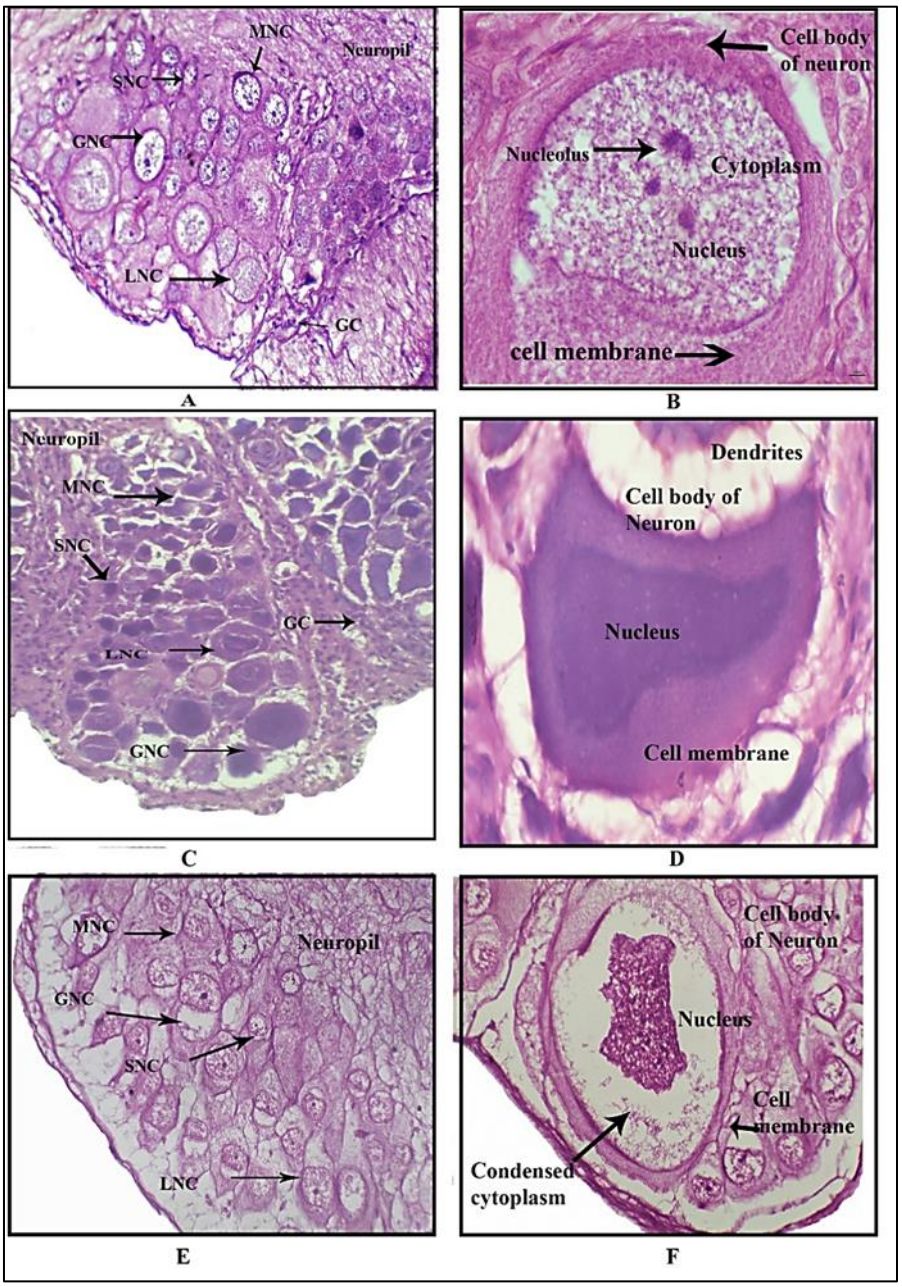
3.2. Histology of nerve ring of *S. maculata*

Histological section of nerve tissue showed the different types of neuronal cells and glial cells. Neuronal cells were arranged in the form of ganglion. Anatomically neuropil is space between neurons and glial cells, including dendrites, axons, synapses, glial processes and microvasculature [17]. According to the size, neuronal cells are of four types - Giant Neuronal Cells (GNC) are large size with diameter of 38 – 45 µm with prominent nucleus. Large Neuronal Cells (LNC) are of size of 27– 32 µm diameter, with nucleus. Medium Neuronal Cells (MNC) are round to oval in shape with diameter of 15 - 20 µm. Small Neuronal Cells (SNC) are oval cells with a prominent nucleus with diameter of 8 – 10 µm.

As shown in Figure 7 A Control section of nerve tissue showed that the neurons were arranged at the peripheral side of the ganglions and are compactly arranged. The body of neurons is called as perikaryon which is largely filled with slightly oval or rounded shaped nucleus with is surrounded by nuclear membrane. The nucleus is with granulated network of heterochromatin and thus correspond to nuclei. Glial cells are smaller than neuronal cells, with oval nucleus surrounded by cytoplasm widely distributed around the neuronal cells and in the neuropil. Under microscope the glial

cells were showed darkly stained nucleus and weakly stained cytoplasm. We found prominent differentiation between neuron and glial cells, glial cytoplasmic matrix was more electronically dense than neuronal matrix. Anatomically glial cells are supportive cells performing duties like neuroprotection and provision of sufficient amount of nutrition to neurons . Figure7 B showed the giant neuron at 1000X magnification with well distinguished, prominent nucleus with nucleoplasm.

3.3. PBZ exposure after 48 hrs. and 96 hrs. to the slugs



GNC – Giant neuronal Cells, LNC- Large neuronal Cell, MNC – Medium Neuronal Cells, SNC – Small Neuronal Cell and GC- glial cells;

Figure 7A - Control section of neurocytes from cerebral ganglion of nerve ring of *S. maculata* at 400 X**; Figure 7B-** Magnified neurocytes of cerebral ganglion at 1000 X**; Figure 7C -** PBZ intoxicated section of neurocytes after 48 hrs. of exposure from cerebral ganglion of nerve ring of slug at 400 **X; Figure 7 D** – Magnified view of PBZ intoxicated neurocytes after 48 hrs. at 1000 X magnification; **Figure 7E-** Section of PBZ intoxicated neurocytes from cerebral ganglion of nerve ring of slug after 96 hrs. of exposure at 400 X; **Figure 7 F-** Magnified view of PBZ intoxicated nerve cell after 96 hrs. of exposure at 1000 X magnification;

After 48 hrs. of PBZ exposure to the slugs, neuronal alterations was observed. In the histological sections the neuronal cells showed shrinkage, nucleus of the cells bursts as shown in Fig 7. C the GNC showed disturbed nucleus with

decreased neucloplasm. Shape of the neuron is gets changed dilated (Figure D). The cell diameter of GNC was 45- 47 µm, LNC was 30- 35 µm, Medium MNC with 18- 22 µm of diameter and SNC with 9- 11 µm.

After 96 hrs. of exposure the glial cells were distorted, cytoplasmic content was gets depleted, in large and medium neuronal cells the nucleus was burst leading to damage to the neuronal cells (Figure 7 E). neurons undergo for hypertropic condition, pyknotic nucleus was observed, cytoplasmic material gets condensed (Figure7 F). The cell diameter after 96 hrs. of exposure was changed as follows GNC was 48-50 µm, LNC was 35- 38 µm, MNC with 22- 24 µm of diameter and SNC with 12- 14 µm. Dimensional and morphological changes were more in 96 hrs. as compare to 48 hrs. of exposure of PBZ

3.4. DNA estimation from the nerve tissue of slug

Biochemical study was conducted to estimate the DNA content from both samples control and experimental i.e. Intoxicated with dose of LC_{50} of PBZ for 48 hrs. and 96 hrs. (Table No. 1). The results shows significantly decline in the content of DNA. Control group of *S. maculata* showed 1.236 ± 0.150 mg of DNA / 200 mg of nerve tissue. After the exposure of PBZ for 48 hrs. and 96 hrs. DNA content was depleted to 0.90 ± 0.50 mg of DNA / 200 mg of nerve tissue and 0.676 ± 0.037 mg of DNA / 200 mg of nerve tissue respectively.

Table 1 Effect of Paclobutrazol on the DNA content from nerve tissue (µg/mg wet weight of tissue) of the slug *S. maculata*

DNA estimation from nerve tissue of *Semperula maculata*		
Control (mg of DNA / 200 mg of nerve tissue)	After 48 hrs. of PBZ exposure (mg of DNA / 200 mg of nerve tissue)	After 96 hrs. of PBZ exposure (mg of DNA / 200 mg of nerve tissue)
1.236± 0.150	0.90± 0.50*	0.676 ± 0.037***

p< 0.05, p < 0.01, p < 0.001 and p >0.05 indicates as *, **, *** and non-significant respectively.

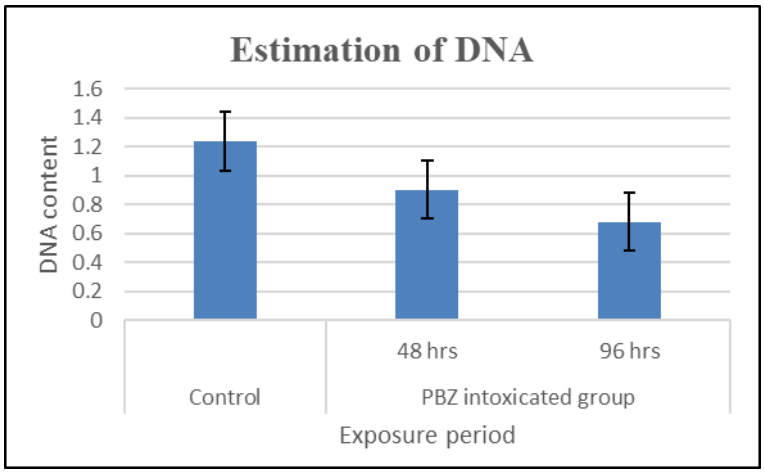


Figure 8 Comparative data of DNA estimation from the nerve tissue of *S maculata* for control, PBZ exposed for 48 hrs. and PBZ exposed for 96 hrs.

Figure No. 8 showed the comparative data of DNA estimation from nerve ring of *S. maculata*. Data showed as compare to control in experimental group of 48 hrs. and 96 hrs. DNA content from the cells was decreased respectively.

3.5. Lipid peroxidation

Lipid peroxidation for control and from 48 hrs. and 96 hrs. are as shown in Table no. 2. The lipid peroxidation value in control group of nervous tissue of terrestrial slug was 3.52 ± 0.003 n mol MDA/mg. In experimental group after 48 hrs. of PBZ exposure lipid peroxidation was gets increased 7.53± 0.15 n mol MDA/mg and lipid peroxidation after 96 hrs. of exposure of it is 10.22 ±0.25 n mol MDA/mg.

Table 2 Effect of Paclobutrazol on lipid peroxidation from nerve tissue (µg/mg wet weight of tissue) of the slug *S. maculata*

Lipid peroxidation in nerve tissue of Semperula maculata		
Control (n mol MDA/mg)	After 48 hrs. of PBZ exposure (n mol MDA/mg)	After 96 hrs. of PBZ exposure (n mol MDA/mg)
3.52± 0.003	7.63± 0.15 ***	10.22± 0.25 ***

p< 0.05, p < 0.01, p < 0.001 and p >0.05 indicates as *, **, *** and non-significant

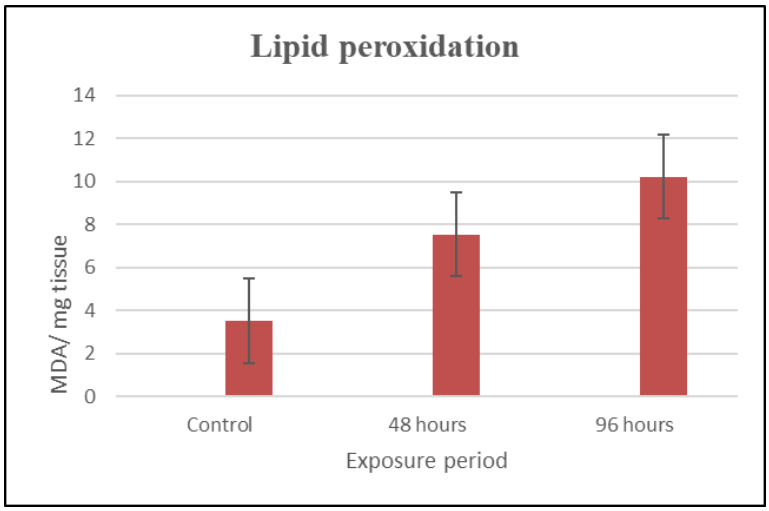


Figure 9 Comparative data of lipid peroxidation from the nerve tissue of *S maculata* for control, PBZ exposed for 48 hrs. and PBZ exposed for 96 hrs

Figure No. 9 showed the comparative data for the values of lipid peroxidation from control and experimental animal group.

4. Discussion

[18], emphasized morphometric and functional specificity gastropod nervous system as a powerful tool for understanding overall functional capabilities of neurons, nerve circuits, intracellular signalling pertaining to animal physiology and behaviour. [19] mentioned in their study, presence of massive and identifiable neurons in gastropods allows for stable intracellular interactions, making them ideal for research into neuronal circuits and neurobiology. [17] stated that neurons of gastropod having simple structure with a large soma on outside of ganglia and single axon that projects into interior of ganglia. [20] documented Glial cells, which functions as intra- neuronal partitions in the brain, which were widely distributed in invertebrates, including the tick *Boophilus microplus* [21,22] insects and gastropods [23, 24] and cephalopods [25, 26, 27]. [18] Documented, the gastropod nervous system were with glial cells, comprised of 50% or more the volume of nerve tissue.

[28, 29] reported that pond snail *L. stagnalis* could be an useful model in genetic and translational research for the study of molecular basis of human brain diseases and for development of new therapeutic strategies. [30] Observed the central nervous system of *L. luteola* composed of ganglia which were found paired located very close to each other and were differentiated in four types of neurosecretory cells. [31] observed the similarity in the CNS of *Megalobulimus oblongus* and *Helix pomatia*, consists of cerebral ganglia interconnected by means of cerebral pedal and cerebral plural connectives, pair of pedal, parietal and pleural ganglia and a single visceral ganglia similar type of neuronal network was observed in present study. [32] stated that, for discovery of drugs *Lymnea stagnalis* should be considered as an excellent model.

Reactive Oxygen Species (ROS) as a the biomarkers of oxidative stress, can reacts with all biological macromolecules including lipids and results in peroxidation of lipids [33]. Lipid peroxidation (LPO) activity reported as a primary indicator of oxidative damage and considered as significant factor in the toxicity study pertaining to various induced toxicants [34]. Elevated level of malondialdehyde (MDA) are considered proof of substantial damage to cells due to

elevated oxidation stress during the capture process [35]. MDA serves as a sign of lipid peroxidation, indicating a singnificant pro- oxidant threat that enables the formation of reactive oxygen species (ROS) and the related activation of antioxidant enzymes for a short period [36]. The formation of MDA has been widely studied and attributed to many organic and inorganic pollutants in various marine organisms worldwide, including mussels [37], oysters [38]. [39], observed the biochemical changes in nucleic acid content reduction in the level of DNA and RNA from the ovatestis of Freshwater Snail *Lymnaea acuminata* after intoxication of bait containing Eugenol (*Syzygium aromaticum*). [40] studied the effect of sulfentrazone a trizol herbicide on the *Eisenia fetida* they observe the histopathological alteration and DNA damage in the experimental animal. [41].observed, alteration in the glycoproteins content of neuronal cells from *S. maculata* after $HgCl_2$ and $CdCl_2$ intoxication at 24 hrs, 48 hrs. 72 hrs and 96 hrs. [42] investigate the toxic impact of $PbCl_2$ on gills and digestive glands of clam *Venus verrucosa* they found histological alterations and DNA damage with significant increase in the lipid peroxidation after short time exposure.

[43] interpreted that, prohexadione-calcium, a phytoregulator inhibits gibberellin metabolism similar to the PBZ, significantly decreases the population of *Cacopsylla pyricoloa* and *Aphis spireacola*. [44] observed the neurosecretory cells and labelled them on the basis of size of neurocytes- A- type which are giant cells , B- type large cells, C- type large cells , D- type medium cells , E- type small cells and F type very small cells from brain and optic ganglia from white shrimp, *Fenneropenaeus indicus.*

5. Conclusion

Under the toxicological investigation, number of cases considering vertebrates and invertebrates recommended as ideal scientific models for study, *Semperula maculata* is one of them. Present investigation was aimed for neurological, morphometric, biochemical and histological assessment. We induced predetermined PBZ dose and keenly observed excitation of animals after 48 hrs. and 96 hrs. of induction. Sensation is due to the neurocytes, were we found that PBZ intoxication leads to anatomical, morphometric and cell based differentiations such as hyper or hypotrophic shapes of cells, shrinkage, cellular damage and necrotic effect. These morphological damages are due to significantly declined DNA content after 48 hrs. and 96 hrs. of exposure respectively among experimental groups. To supports and strengthen our estimated data, we have carried lipid peroxidation activity from ganglionic tissue. We found that the lipid peroxidation was noticeably elevated which is prominent indication of cellular damage. As the period is increases, we observed that experimental animals were more sluggish proving that excessive induction of PBZ has enters and accumulate in the animal and disturbs neuronal components to reduce excitatory potential. Abuse of PBZ responsible for neuronal damage and may be lethal to the animals. There must be regulations for permissible doses of phytoregulators to avoid excessive damage to the ecosystem.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declared no conflict of interest.

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