Chapter IV

NEUROSECRETION
INTRODUCTION

Neurotoxicity has been defined as “any adverse effect on the chemistry, structure or function of the nervous system, during development or at maturity, induced by chemical or physical influences” (Costa, 1998a). An adverse effect is “any treatment related change which interferes with normal function and compromises adaptation to the environment” (ECETOC, 1992). Thus, most morphological changes such as neuronopathy (a loss of neurons), axonopathy (a degeneration of the neuronal axon), myelinopathy (a loss of the glial cells surrounding the axon), or other gliopathies, would be considered adverse, even if structural and/or functional changes were mild or transitory. In addition, neurochemical changes, also in the absence of structural damage, should also be considered adverse, even if they are transient and reversible, as they lead to impaired function. The general definition of neurotoxicity also points out to a potential difference between the developing and the mature nervous system, to underscore the fact that developmental neurotoxicity is an important aspect of neurotoxicology. Neurotoxicity can also occur as a result of indirect effects, such as damage to hepatic or cardiovascular structures, or because of interference with the endocrine systems. Some chemicals may have multiple modes of action and may affect the nervous system both directly and indirectly. For example, some halogenated compounds may interact directly with brain cells, and also affect the development of the nervous system by altering thyroid hormone homeostasis (Costa and Giordano, 2007; Crofton, 2008).

There are approximately 200 chemicals which have been found to be neurotoxic in humans (Grandjean and Landrigan, 2006), and for many more there is at least some evidence of neurotoxicity deriving from animal studies.
Organophosphate and organochlorine pesticides are one of the main classes of insecticides, in use since the mid 1940s and are excellent insecticides can also exert significant adverse effects in non target species including humans.

Organochlorine and organophosphate pesticides are one of the main types of cholinesterase inhibiting pesticide. Acetylcholine is the neurotransmitter released by the nerve stimulation on postsynaptic cholinergic receptors in the nervous system. The action of acetylcholine neurotransmitter is terminated by the enzyme acetylcholinesterase. Ops inhibit the function of acetylcholinesterase which is irreversible. Walker et al., (2001) and, Costa, (2008) have reported that the main toxic effect is a cholinergic crisis, due to accumulation of acetylcholine in synapses because of inhibition of acetylcholinesterase (AChE). Since the principle site of action of Ops is the nervous system, it causes variety of toxic effects. These effects have been studied in many animals including human in laboratories as well as in their natural habitat, due to either accidental or intentional exposures (Mineau, 1991; Ecobichon and Joy, 1994). Acute exposure to Ops produces effects such as distress with salivation, lacrimation, urination, defecation and impairment of neuromuscular functions. Extremely high doses produce convulsions and death, due to interference with brainstem structures involved in respiration. Organophosphates induced delayed neuropathy (OPIDN) has been observed in several vulnerable species like birds and human etc. Johanson (1975) suggested that, acute exposure to some Ops creates an irreversible delayed neuromuscular effect seen mainly in the extremities leading to paralysis.

Organochlorine pesticides also act primarily on nervous system and cause hyper- excitability followed by tremors leading to severity and convulsions and finally the death. According to Sternburg and Kearns
(1952), DDT after acting on nerves, they release the toxic factor other than DDT, the autotoxin in the haemolymph. The autotoxin brings impairment of the nerve conduction. BHC is a nerve poison and creates paralysis leading to death. According to pharmacological data, it is a neurotoxic agent. It attacks the entire CNS and acts as an inhibitor of \( \text{Na}^+ \), \( \text{K}^+ \) and \( \text{Mg}^{2+} \), ATPase and blocks the activity of the sodium – potassium pump that maintains the ionic transport of these ions across the nerve membrane and impulse conduction.

Therefore there is need to study the effect of pesticide on neurosecretory cells and its subsequent recovery. Hence in the present investigation the freshwater bivalve *Parreysia cylindrica* was selected as model animal for the present study.

In bivalve molluscs, nervous system and hormonal apparatus are not separated and no endocrine glands have so far been encountered. Therefore, it is possible that hormonal activity is restricted to the nervous system itself. Thus, nervous system and hormonal system are unified structurally and functionally, which is supported by the fact that the secretory cells occur in ganglia of these molluscs. The nervous system plays a role in neurotransmission as well as in the synthesis and discharge of secretion. Neurons secrete both neurohumors and neurohormones. Studies on neurohumors have involved their chemical nature (Paparo, 1972; Stefano and Aiello, 1975), their control of ciliary activity (Aiello, 1962 and 1970; Paparo and Aiello, 1970) and their influence on oxygen consumption (Moore *et al*., 1961; Moore and Gosselin, 1962). Nassel (2002) studied the presence of neuropeptides in the nervous system of *Drosophila* and other insects and also studied their multiple roles as neuromodulators and neurohormones.

Scharrer (1935), first discovered neurosecretory cells in molluscs. He reported cells with secretory droplets in the cerebral and
visceral ganglia of *Aplysia limacina* and *Pleurobranchaea*. Since then several investigators have studied distribution of neurosecretory cells in different classes of mollusks. Molluscs show great variability in their nervous system, ranging from primitive arrangement in chiton to the complex mass of fused ganglia forming the “brain” of cephalopods. Pharmacological and physiological aspect of mollusca using effector organs has received considerable attention (Bayne, 1976). Perusal of literature reveals that the aspect of neurosecretion in bivalve molluscs has been reviewed by Gabe (1965, 1966), Lubet (1966, 1973), Martoja (1972) and Golding (1974). Gabe (1955) reported the presence of neurosecretory cells in 20 marine Lamellibranch mollusks. Relationship between neurosecretion and sexual cycle in *Mytilus* and *Chlamys* was described by Lubet (1955). Fahrmann (1961) described two types of neurosecretory cells in *Unio tumidus* in its cerebral, visceral and pedal ganglia, while Baranyi and Solanki (1963) observed three types of neurosecretory cell in the three ganglia of *Anodonta cygnea*. Antheunisse (1963) studied the neurosecretory cells in all three ganglia of *Dreissena polymorpha*. Nagabhushanum (1963) studied the neurosecretory cell in the oyster *Crassostrea virginica*. Boer (1965) and Bonichon-Laubier (1971) studied the neurosecretory cytology and cytochemistry of *Lymnea stagnalis* and subpendiculate lobe of *Pteroctopus tetracirrhus*. Yahata (1971) studied the neurosecretory cells in the cerebral ganglion of abalone, *Nordotis discus*. Neurosecretory cells in the nervous system of neptune whelk, *Neptunea arthritica* were studied by Yahata and Takahashi (1972). Evidence for the occurrence of a wide variety of neurotransmitters in different tissue of bivalve mollusks including the nerve ganglia, has been discussed from the functional point of view by Leak and Walkar (1980). Muley (1988) studied the neurosecretory cell in the mussel, *Modiolus demissus*. Thorat (1990) observed two types of cells, cell type I and cell
type II in the fresh water bivalves, *Lamillidens corrianus*. Waykar (1998) also observed two types of cells, cell type I and cell type II in the fresh water bivalves, *Parreysia cylindrica*. The development of the subject has been hampered by the presence of shell, very small ganglia in small bivalves, diffused distribution of NSCs, and by the ignorance of the chemical nature of the neurohormones.

Classic histological studies on a number of species demonstrated the presence of neurosecretory cells. Their number and location vary among different species. Neurosecretory cells are located in cerebral and visceral ganglia. Generally these cells are found in the dorsal caps of cerebral ganglia and the dorsal cell layer of visceral ganglia (Lubet, 1959 and 1965a; Nagabhushanam, 1963 and 1969; Nagabhushanam and Mane, 1973; Mane, 1986). The neurosecretory cells were found to be less numerous in the cerebral ganglia than in the visceral ganglia. The presence of neurosecretory cells in the pedal ganglia is controversial. However, Gabe (1955) and Lubet (1955a) concluded that they are absent in *Mytilus edulis* or *M. galloprovincialis*, but Umiji, (1969) in *Perna perna* and Nagabhushanam et al., (1972) in *Mytilus viridis*, have recorded their presence in the pedal ganglia. Yahata and Takahashi (1972) showed presence of four types of neurosecretory cells in the ganglia of neptune whelk, *Neptunea arthritica*. The neurosecretory cells have been reported to occur in all ganglia of freshwater mussels *Unio tumldus* (Fahrmann, 1961). *Dreissf;na polymorpha* (Antheunisse, 1963), *Lamellidens marginalis* and *L. corrianus* (Muley, 1985).

In most species, neurosecretory cells are small or medium-sized, with an approximate diameter of 20 µm. Neurosecretory perikarya are ovoid or pyriform. The general histological features are similar to those of plasmochrome cells, but neurosecretory perikarya can be distinguished by the marginal position of the Nissl bodies and the presence of
acidophilic secretions in the cytoplasm (Gabe, 1955; 1966). Different categories of neurosecretory cells have been distinguished, based on their size and morphology. In *M. edulis* and *Chlamys varia* some neurosecretory cells are pear-shaped, unipolar, and upto 25 µ, while others are small and multipolar (Lubet, 1959). Pear-shaped (type I) and oval-shaped (type II) neurosecretory cells were distinguished in *Crassostrea virginica* and *Meretry casta* (Nagabhushanam, 1963; 1969) and *Katelysia opima* (Nagabhushanam and Mane, 1973). Different categories of neurosecretory cells have also been reported in the freshwater mussel, *U. tumldus* (Fehrmann, 1961).

It is a well known fact that the neurosecretory cells control the physiological processes like reproduction and digestion. Exposure of animals to pesticides interferes with the normal functional processes and ultimately creates an imbalance in the normal system. Uncontrolled release of neurohormones after insecticidal treatment was studied in *Rhodnius prolixus* (Maddrell and Cacida, 1971; Maddrell and Reynold, 1972) and in *Periplaneta americana* (Granett and Leeling, 1972). The effects of pesticides like endrine and sumithion on the neurosecretory cells of *Periplaneta americana* were studied by Nanda (1974). Doherty and mastumara (1975) studied the effects of DDT on ATPase systems and the functions of the nerve cells in peripheral nervous system of the lobster, *Homarus americanus*. Elvin (1976) observed the effects of light and temperature on the neurosecretory cells of *Mytilus edulis*. The effect of stress factors such as temperature, salinity, starvation and dessication on neurosecretory cells have been studied by many workers (Lomte and Barhanpurkar, 1979; Hanumante *et al.*, 1979a, 1979b). Akarte *et al.*, 1982 studied the histopathological changes in the neurosecretory cells after insecticidal treatment in *Indonaia caeruleus* and *Lamellidens marginalis*. Muley (1988) studied the histopathological changes in the neurosecretory

Several investigators studied the neurosecretory cells and neurosecretion of various organisms. Arvy and Gabe (1962) studied the histochemistry of neurosecretory product of the pars intercerebralis of pterygote insects. Dogra and Tandan (1965) studied the ontogenic fate of the neurosecretory cells in the larval brain of *Sarcophaga ruficornis*. Tripathi (1977) studied the role of light on the neurosecretory activity of millipede *Gonoplectus malayus*. Othamann and Reddy (1985) studied the rhythmic changes in the activity of the neurosecretory cells in the

Aquatic pollutant causes oxidative stress in aquatic animals resulting from the redox cycling of pollution. It is known that xenobiotics metabolism causes continuous production of reactive oxygen species (ROS) even without pollution (Ahmad *et al.*, 2000). To cope with the continuous generation of ROS from normal aerobic metabolism, cells and tissues contain a series of cellular antioxidants with both enzymatic and non-enzymatic activities (Nordberg and Arner, 2001). Stressful conditions leads to the formation of excessive free radicals which are major internal threat to cellular homeostasis of aerobic organisms. Environmental stress has been demonstrated to cause an increase in the oxidative stress, an imbalance in the antioxidant status (Yildirim *et al.*, 2010).

The nervous system is especially vulnerable to reactive oxygen species (ROS)-mediated injury for the following reasons. (1) High oxygen consumption of the brain for high energy needs, that is, high O2 consumption, results in excessive ROS produced. (2) Neuronal membranes are rich in polyunsaturated fatty acids (PUFA), which are particularly vulnerable to free radical attack. (3) The ratio of membrane
surface area to cytoplasmic volume is high. (4) Specialized neuronal conduction and synaptic transmission activity depend on efficient membrane function. (5) Extended axonal morphology is prone to peripheral injury. (6) Neuronal anatomic network is vulnerable to disruptions. (7) The excitotoxic glutamate is the major effector that causes oxidative stress. (8) The high Ca$^{2+}$ traffic across neuronal membranes and interference of ion transport increase intracellular Ca$^{2+}$, often leading to oxidative stress. (9) Auto oxidation of neurotransmitters can generate O$_2$ and quinones that reduce glutathione. (10) Iron is formed throughout the brain, and brain damage readily releases iron ions capable of catalyzing free radical reactions. (11) Antioxidant defense mechanisms are modest, in particular, low levels of catalase, glutathione peroxidase, and vitamin E. (12) ROS directly downregulate proteins of tight junctions and indirectly activate matrix metalloproteinases (MMP) that contribute to open the blood–brain barrier. (13) Activated microglia produce ROS and cytokines in a perpetual process. (14) Cytochrome P450 produces ROS. (15) Loss of trophic support can activate NADPH oxidase, which increases ROS. (16) The presence of hemoglobin within the neural tissues secondary to spontaneous, iatrogenic, or traumatic causes is neurotoxic. Heme and iron are released and promote ROS. (17) Neuronal mitochondria generate O$_2$. (18) The interaction of NO with superoxide can be implicated also in neuronal degeneration. (19) Neuronal cells are nonreplicating and thus are sensitive to ROS (Friedman, 2011).

In bivalve molluscs, ROS are produced in response to exposure to xenobiotics (Winston et al., 1990; Mitchelmore et al., 1998), and changes in temperature and especially heat stress (Abele et al., 2002). Reactive oxygen species production is a common feature of many marine invertebrates (Abele and Puntarulo, 2004). Exposure of bivalves to
pollutants in tropical environments also results in an increase in oxidative stress (Angel et al., 1999).

Many pollutants are known to enhance the formation of reactive oxygen species (ROS) (Gomez-Mendikute, Cajaraville 2003). Both antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, and low molecular weight antioxidants, such as glutathione, ascorbate and α-tocopherol, belong to the cellular antioxidant system that counteracts the toxicity of reactive oxygen species (Santovito et al., 2005). Cellular antioxidant defence systems in biological systems are impaired when exposed to environmental pollutants, but the levels of antioxidants in living organisms can increase in order to restore the imbalance caused by oxidative damage. Antioxidants can decrease oxidative stress and toxic effect by direct scavenging of ROS or by inhibiting cellular damage through breaking the chain propagation reactions of lipid peroxidation (Niki, 1991; Irshad et al., 2002).

Vitamin-C (Ascorbic acid), water-soluble antioxidant is extremely powerful and has a multifunction. It helps to strengthen the immune system and it prevents some diseases, plays important roles in the brain, nervous system, and immune system; mobilizes iron in the body; prevents anaemia and has many benefits such as prevents several debilitating conditions and increases the body’s immunity (Len, 2009).

Ascorbic acid (AA) or vitamin C, is a potent antioxidant used as a therapeutic agent against many diseases (Balz, 2003) and in the prevention of the adverse effects of stress factors in laboratory animals and humans when the body’s ascorbic acid is exhausted (Altan et al., 2003; Tauler et al., 2003). It is reported to donate a free molecule of hydrogen that detoxifies the harmful reactive oxygen species generated by the body especially when the body’s natural anti-oxidants are overwhelmed and exhausted. AA is also known to potentiate gamma-
amino butyric acid (GABA) which reduces neurotransmission, including release of corticosteroids (Koshebekov, 1991; Altan et al., 2003).

Vitamin-C is essential for normal growth and has some properties that allow resisting oxidation (Ibiyo et al., 2007). Heo and Lee (2004) studied protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration. The mechanism of neurobehavioral toxicity of arsenic and monocrotophos and neuroprotective efficacy of curcumin and Bacopa monnieri was briefly studied by Yadav (2012).

Investigation regarding the impact of pesticides dicofol and dichlorovos on neurosecretory cells of cerebral ganglia of freshwater bivalve and recovery responses after toxicant withdrawal and with supplement of L-ascorbic acid is scanty. Therefore, the aim of the present study was to investigate whether the treatments of ascorbic acid would protect the changes in neurosecretory cells due to dicofol and dichlorovos in an experimental model, Parreysia cylindrica.
MATERIALS AND METHODS

Medium sized, healthy, fresh water bivalve, Parreysia cylindrica was collected from Girna Dam, 48 km away from Chalisgaon. Animals were brought in laboratory and were acclimatized for a week to tap water. The medium sized animals were selected for experiment. The animals were exposed to chronic concentration of dicofol (0.04023 ppm), LC$_{50/10}$ values of 96 hrs) and dichlorovos (0.09376 ppm) alone and along with 50 mg/l of L-ascorbic acid upto 21 days. Every day the solution was changed.

Experimental design:

Set – I

For experimental studies the animals were divided into five groups –

a) Group ‘A’ was maintained as control.

b) Group ‘B’ animals were exposed to chronic dose of dicofol (0.04023 ppm), LC$_{50/10}$ values of 96 hrs.) upto 21 days.

c) Group ‘C’ animals were exposed to chronic dose of dicofol (0.04023 ppm), along with 50 mg/l of L-ascorbic acid upto 21 days.

d) Group ‘D’ animals were exposed to chronic dose of dichlorovos (0.09376 ppm), LC$_{50/10}$ values of 96 hrs.) upto 21 days.

e) Group ‘E’ animals were exposed to chronic dose of dichlorovos (0.09376 ppm), along with 50 mg/l of L-ascorbic acid upto 21 days.

Experimental design for recovery studies -

Set – II

1) Group ‘B’ animals from set – I were divided into two groups for recovery studies.
i) Animals pre-exposed to chronic dose of dicofol (0.04023 ppm) were allowed to self cure normally in untreated fresh water up to 21 days.

ii) Animals pre-exposed to chronic dose of dicofol (0.04023 ppm) were allowed to cure in 50 mg/l of L-ascorbic acid added fresh water up to 21 days.

2) Group ‘D’ animals from set – I were divided into two groups for recovery studies.

iii) Animals pre-exposed to chronic dose of dichlorovos (0.09376 ppm) were allowed to self cure normally in untreated fresh water up to 21 days.

iv) Animals pre-exposed to chronic dose of dichlorovos (0.09376 ppm) were allowed to cure in 50 mg/l of L-ascorbic acid added fresh water up to 21 days.

During experimentation animals were fed on fresh water algae. After every 7\textsuperscript{th} and 21\textsuperscript{st} days of interval, animals from set-I and set-II were removed from the test medium and fixed in Bouin’s fluid for 24 hours. The cerebral ganglia of these bivalves were dissected out, washed and dehydrated in alcohol grades, cleared in toluene and embedded in paraffin wax (58-60\degree C).

Prepared blocks of tissues were cut at the thickness of 5\micron and stained with Mallory’s triple stain. Stained slides with serial sections were examined under light microscope for histopathological impact. The cerebral ganglia of bivalves from all groups i.e. control, exposed and recoveries were screened and data is presented [Photomicroplates - to --] for comparison.
OBSERVATIONS AND RESULTS

Cytological examinations of the sections of cerebral ganglia stained with Mallory’s triple stain revealed the presence of group of neurosecreatory cells which are cytologically different and larger than ordinary ganglion cells. The chromatin material of control NSCs was intact. They were provided with definite regular envelope and neuropile. Most of the large cells possess large nuclei and abundant cytoplasm, their perikarya and axons are filled with fine granules and showed moderate synthetic activity, which stained conspicuously. Histologically, two types of neurosecretory cells have been distinguished in the ganglia. The size, general shape of the cell body, presence or absence of vacuoles in the cytoplasm and staining properties of the secretory material were used as the basic criteria in distinguishing the type of the NSCs. Fig. a of plate I shows two types of neurosecretory cells in Fresh water bivalve, Parreysia cylindrica these are cell type I and cell type II.

Cell type I (A cell) or Pyriform cells:

These cells are pyriform in shape ranging from 15 to 18μ in length and 8 to 10 μ in width. The nucleus is round or oval measuring 4 to 8 μ in diameter, it may be either central or eccentric in position. Fine granule of secretory material is present in the cytoplasm. The nucleus generally contains a large nucleolus.

Cell type II (B cell) or Oval cells:

These cells are smaller than type I cells and are oval or round in shape, measuring about 9 to 11 μ in diameter. The nucleus is large vesicular and measure about 10 to 13 μ in diameter. Generally nucleus has got one large nucleolus, but 2 to 3 nucleoli are sometimes noticed.
Effect of dichlorovos intoxication on cerebral ganglia: (Fig. b and c of plate I)

When compared with control, dichlorovos exposed neurosecretory cells from cerebral ganglia showed some changes in pyriform and oval cells. After 7 days of exposure, both pyriform and oval cells were swelled and increase in size of cell, nucleus and nucleolus. Intensity of, synthetic activity was increased. At this stage the rate of transport must be very less compared to the rate of synthesis, which causes accumulation of secretory material in cells which appeared bulky. Along with these changes, the changes like, cell type I (Pyriform cell) showed vacuolization, nucleus become oval shape, clumping of chromatic material, neurosecretory material was clumped and accumulated in cytoplasm and also near the axon hillock and cell become bulky.

After 21 days of exposure, neurosecretory cells were elongated, and became smaller in size, nuclear and nucleolus size was also decreased, the chromatin material was condense, and showing lowering synthetic activity of pyriform and oval cells. The pyriform cell becomes narrow and elongated. Along with these changes, the pesticide stress caused undulation of cell wall; vacuolization and clumping of chromatin material and neuropile were observed. The perikarya showed vacuolization and condensation of secretory material.

In combined treatment of dichlorovos along with 50mg/liters of L-ascorbic acid histopathological disorders were relatively less compared to the animals exposed to chronic dose of dichlorovos alone. There were mild swelling and disruption of the neurosecretory cells with a few vacuolization. The neurosecretory cells, nucleus, nucleolus and chromatic material did not show damage like that of chronic dichlorovos treatment alone. The neurosecretory cells were more or less comparable to control neurosecretory cells. After 21 days of exposure, the
histopathological changes were more but relatively less as comparable to animal exposed to dichlorovos alone at 21 days (Fig. e of plate I). This indicates the protective and curative role of ascorbic acid in dichlorovos induced alterations in neurosecretory cells of bivalve, *Parreysia cylindirca*.

**Recovery study:**

Animals pretreated to chronic treatment of dichlorovos alone and were allowed to cure in untreated fresh water; the restoration of structure of neurosecretory cells was noted. In histological section of 7 days of recovery period (Fig. a of plate II), exhibited reduction in vacuolization and other cellular abnormalities of the neurosecretory cells. This was more evident in 21 days of histological section (Fig. b of plate II), but still slight swelling, shrinkage, vacuolization and abnormalities in cells, nucleus and nucleolus shape and size and other cellular abnormalities are found indicating it requires more time to recover.

When animals pretreated to chronic treatment to dichlorovos alone and were allowed to cure in 50mg/l of L-ascorbic acid medium, bivalve showed the restoration of normal structure of neurosecretory cells. In the histological section of 7 days of recovery period (Fig. c of plate II), shows slight shrinkage, slight degenerative changes of A and B type of neurosecretory cells. After 21 days of recovery period (Fig. d of plate II), histological section exhibit all most of all structural changes was recovered. The neurosecretory cells show normal shape and size of nucleus and nucleolus. This indicates the protective and curative role of ascorbic acid in dichlorovos induced alterations in neurosecretory cells of bivalve, *Parreysia cylindirca*.

**Effect of dicofol intoxication on cerebral ganglia:** (Fig. b and c of plate III)
Neurosecretory cells of cerebral ganglia showed many cytomorphic alterations in cell type I and II after dicofol stress as compared to control. Neurosecretory cells of cerebral ganglia were highly damaged when subjected to chronic treatment of dicofol. Pesticide stress caused undulation of cell envelope. Pyriform cells showed vacuolization, neurosecretory material was clumped and accumulated in cytoplasm and also near the axon hillock. After 7 days of chronic exposure, size of cell, nucleus and nucleolus was increased. This indicates enhanced in synthetic activity of the pyriform and oval cells. At this stage the rate of transport must be very less compared to the rate of synthesis, which causes accumulation of secretary material in cells which appear bulky.

After 21 days of exposure pyriform cells become larger, nucleus and nucleolus became smaller, clumping of chromatin material, and shrunken nuclei were also observed. The synthetic activity was lowered. Along with these changes, the changes like, undulation of cell wall, vacuolization, clumping of chromatin material and damage of cell envelope were observed.

In combined exposure to dicofol along with 50mg/l L-ascorbic acid treatment histopathological disorders were relatively less compared to the animals exposed to the chronic dose of dicofol alone. There was slight swelling of neurosecretory cells, less damaged chromatin material and vacuolization was also less affected. The histological structure of neurosecretory cells was more or less comparable to control NSCs at 7days of treatment (Fig. c of plate III).

After 21 days of exposure, the histopathological changes were more but relatively less as comparable to NSCs exposed to dicofol alone at 21 days (Fig. d of plate III). This indicates the protective and curative role of ascorbic acid in dicofol induced alterations in neurosecreory cells of bivalve, Parreysia cylindrica.
Recovery study:

Animals pretreated to chronic treatment of dicofol alone and were allowed to cure in untreated fresh water; the restoration of normal NSCs structure was noted. In histological section of 7 days of recovery period (Fig. a of plate IV), reduction in swelling, and vacuolization was noted. The hyperplasia and vacuolization were still present until after 7 days of post-exposure. At 21 days of recovery (Fig. b of plate IV), more restoration of NSCs structure was noted, but still some celler abnormalities are found indicating it requires more time to recover.

When animals pretreated to chronic treatment to dicofol alone and were allowed to cure in 50mg/l of L-ascorbic acid in fresh water, bivalve shows the restoration of normal structure of NSCs. In the histological section of 7 days of recovery (Fig. c of plate IV), shows normal shape and size of NSCs but still slight hyperplasia, damaged chromatin material and vacuolization were seen. At 21 days of recovery period (Fig. d of plate IV) more restoration of NSCs structure was observed. Neurosecretory cells showed normal shape and size. Overall study indicates protective and curative role of ascorbic acid in dicofol induced perturbations.

Thus during the present study it has been observed that in the initial stage of toxication (at 7 days of exposure) there was increased rate of synthesis of secretary material and its accumulation in cells, but as the exposure period was prolonged, a gradual release of secretary material was observed which become vigorous at further prolongation of exposure leading to complete drainage of secretary material from neurosecretory cells. It was observed that, in combined exposure to pesticides and 50mg/l ascorbic acid the cytomorphic alterations in both the cells of cerebral ganglia were relatively less as compared to the animals exposed to the chronic dose of pesticides individually. Obtained results also showed that in presence of ascorbic acids animals showed faster recovery.
with normal shape and size of NSCs than animals allow recovering in normal water.
Plate –I: Microphotographs of cerebral ganglia of bivalve, *Parreysia cylindrica* after chronic exposure to dichlorovos without and with ascorbic acid (X1000).

**Fig.a** - Microphotograph of cerebral ganglia from control bivalve, *Parreysia cylindrica*.

**Fig.b**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dicofol for 7 days.

**Fig.c**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dicofol for 21 days.

**Fig.d**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dicofol along with ascorbic acid for 7 days

**Fig.e** - Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dicofol along with ascorbic acid for 21 days

**Abbreviations:**

A - A cell

B - B cell

AX - Axon

N - Nucleus

NU - Nucleolus

NM - Neurosecretory material
Plate –II: Microphotographs of cerebral ganglia of bivalve, Parreysia cylindrica after chronic exposure to dichlorovos and its subsequent recovery (X1000).

Fig. a - Microphotograph of cerebral ganglia of bivalve, Parreysia cylindrica during recovery in normal water for 7 days.

Fig. b - Microphotograph of cerebral ganglia of bivalve, Parreysia cylindrica during recovery in normal water for 21 days.

Fig. c - Microphotograph of cerebral ganglia of bivalve, Parreysia cylindrica during recovery in ascorbic acid (50 mg/l) for 7 days.

Fig. d - Microphotograph of cerebral ganglia of bivalve, Parreysia cylindrica during recovery in ascorbic acid (50 mg/l) for 21 days.

Abbreviations:

A - A cell
B - B cell
N - Nucleus
NU - Nucleolus
NM - Neurosecretory material
PLATE-II

a

b

c
d
Plate –III: Microphotographs of cerebral ganglia of bivalve, *Parreysia cylindrica* after chronic exposure to dicofol without and with ascorbic acid (X1000).

**Fig.a**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dichlorovos for 7 days.

**Fig.b**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dichlorovos for 21 days.

**Fig.c**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dichlorovos along with ascorbic acid for 7 days.

**Fig.d**- Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* exposed to chronic dose of dichlorovos along with ascorbic acid for 21 days.

**Abbreviations:**

A - A cell  
B - B cell  
AX - Axon  
N - Nucleus  
NU - Nucleolus  
NM - Neurosecretory material
Plate –IV: Microphotographs of cerebral ganglia of bivalve, *Parreysia cylindrica* after chronic exposure to dicofol and its subsequent recovery (X1000).

**Fig.a** - Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* during recovery in normal water for 7 days.

**Fig.b** - Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* during recovery in normal water for 21 days.

**Fig.c** - Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* during recovery in ascorbic acid (50 mg/l) for 7 days.

**Fig.d** - Microphotograph of cerebral ganglia of bivalve, *Parreysia cylindrica* during recovery in ascorbic acid (50 mg/l) for 21 days.

**Abbreviations:**

A - A cell
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AX - Axon
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DISCUSSION

Pesticides have brought about the green revolution in the world and are being widely used to control agricultural pests and insects causing public health hazards. Problems and outbreaks are reported to occur among animals and human from insecticide toxicity, which usually occurs either from direct exposure to insecticides or indirectly from contaminated feeds or water by such chemicals. Prolonged exposure to insecticides causes chronic neurological syndrome, malignant tumors, immunosuppressive action and teratogenic effects in experimental animals (Nafstad et al., 1983; El-Rahman, 1988; Al-Qwari et al., 1999; Meeker et al., 2006). The pesticides are not only neurotoxic but also affect other systems and have shown a high degree of impact on metabolism by inhibiting enzymes like acetyl cholinesterase (O'Brien, 1967; Matsumura, 1975). Organophosphate (OPs) and the organochlorine group of pesticides are the recently developed pesticides used against many pests. The chemicals in these classes kill organisms by disrupting the brain and the nervous system.

Due to their toxicity, potential to bioaccumulate on fatty tissues and biomagnification through the food chain, exposure to these pesticides is a topic of huge concern (Otchere, 2005; Tomza et al., 2006). In fact, they have been directly related with deleterious health problems, including endocrine disruption, reproductive disorders, cardiovascular diseases, carcinogenicity and neurotoxicity (Amodio et al., 2012). The pesticides act by inhibiting the activity of cholinesterase enzymes which normally block the nerve transmissions after they are sent.

Acetylcholinesterase activity has been identified and biochemically characterised in many aquatic invertebrates (Fulton and Key, 2001). Bivalve and prosobranch molluscs in particular have high levels of AChE
activity in the haemolymph (Srivatsan, 1999). The role of acetylcholine in neurotransmission in mollusks has not yet been clearly defined (Heyer et al., 1973; Mercer and McGregor, 1982; Dauberschmidt et al., 1996) although AChE is present in both cholinergic and non-cholinergic neurons and in the central ganglia of the nervous system (Giller and Schwartz, 1971).

Cholinesterases are serine hydrolase enzymes and degrade the neurotransmitters in cholinergic synapses. The toxicity of pesticides, such as organophosphates and organochlorine is mainly caused by the inhibition of ChE activity of vertebrates and invertebrates. This inhibition leads to the accumulation of acetylcholine in the synaptic terminals and therefore to a change in the normal transmission of the nervous impulse. This interference may result in neurological manifestations, such as irritability, restlessness, muscular twitching, and convulsions that may end in the respiratory failure and death of the animal (WHO, 1986). Consequently, most studies describe the use of ChE levels as a biomarker of exposure and the effect of several pesticide compounds in aquatic species. However, distinct enzyme isoforms with different sensitivities towards anticholinergic contaminants may exist, depending on the species. These isoforms are usually divided into two broad classes: acetylcholinesterases (AChE) and butyrylcholinesterases (BChE), which are distinguished primarily based on substrate specificity (Sultatos, 2005).

The inhibition of AChE activity decreased the cellular metabolism, including deformities of cell membrane and disturbance of metabolic and nervous activity (Gill et al., 1990; Suresh et al., 1992). Also, the decreased AChE activity could lead to ionic refluxes and differential membrane permeability (Tolosa et al., 1996). Suresh et al. (1992) reported that the decrease in AChE activity could be due to the decrease
of the enzyme synthesis by the inhibitory nature of toxicant. Obviously, an impaired neurochemical mechanism could limit the muscular activity of the animal, including such vital functions as respiration, with lethal consequences (Gill et al., 1990).

The molluscan nervous system has been extensively studied by electrophysiologists, comparative neurologists, and recently cytologists are very much interested in the structure, formation, and secretion of neurosecretory substance in different animals (Bonga, 1970). The molluscan nervous system play important role in controlling growth and metamorphosis. The accumulation of toxicants at the cellular level is capable of interacting with many biological legends and interferes with different mechanisms (Gurd and Wilcox, 1956), some of which may affect to the nerve function (Scheinberg and Sternlieb, 1976). In the molluscan fauna, it is worthy to mention that in the freshwater snails nervous system has been proved to be sensitive to many toxic materials and cytotoxicants that may induce injurious consequences (Hernadi and Vehovszky, 1992; Boer et al., 1995; Wiemann et al., 1995).

In the present investigation, cell type I and cell type II neurosecretory cells from cerebral ganglia of the freshwater bivalve, *Parreysia cylindrica* were studied in relation to pesticide dicofol and dichlorovos stress and its subsequent recovery in normal water and 50mg/l of L-ascorbic acid added water. The obtained results are presented in fig. a to e of plates I to IV. The photomicrographs revealed that there was, elongation of pyriform cells enlargement of oval cells, vacuolization, acute cellular degeneration, clumping of chromatin material, undulation of cell envelope and damage of neuropile. The staining properties as well as neurosecretory activity of neurosecretory cells were also significantly altered.
Effects of various pollutants on the neurosecretory cells of different aquatic invertebrates have been studied by many workers. Gundevia and Ramamurthi (1972) have first of all reported various histopathological changes like vacuolization in the perikarya, undulation of the cell boundaries, and clumping of the chromatin material in the neurosecretory cells of *Hydrophilus olivaceous* when exposed to different pesticides like dimecron, diazinon and dieldrin. Nanda (1974) observed the effect of endrine and sumithion on the brain neurosecretory cell of *Periplaneta americana* and reported the impairment in both inter and intracellular structure of neurosecretory cells and also noted the various grades of disturbances in the compactness of neurosecretory elements and undulation in the periphery of cell boundaries along with the appearance of small to large number of vacuoles inside the perikarya.

Similar changes were also recorded by Hanumante *et al.*, (1979) in snail *Indopanorbis exustus* after exposure to different pesticides. Nagabushanam *et al.*, (1982) studied the impact of organophosphates on neurosecretory cells in the cerebral ganglia of fresh water prawn, *Caridina weberi* and reported different cytomorphic changes like vacuolization, degenerative changes in the neurosecretory cells and the neuropilar tissue, such as undulation of the cell boundaries, loss of compactness and necrosis. Utkar (1982) reported the toxic effect of copper sulphate on the neurosecretion and has reported a decrease in all number cell and all nuclear area, depletion of neurosecretory cells of the freshwater snail, *Viviparous bengalensis*. He also reported that effect of biogenic amines (reserpine and esperpine) on neurosecretory cells and observed that the size of both neurosecretory cells (A and B) was reduced. Sarojini and Mirajkar (1982) reported different histopathological changes like vacuolization in cytoplasm, undulation of cell wall and
clumping of chromatin material in the neuroprofile (brain) of fresh water prawn, *Macrobranchium kistnensis* when exposed to organophosphorus insecticide dimicron. Bodhankar (1984) observed that there was decrease in neurosecretory material in both A and B types of cells number of cell types and nuclear areas of these cells of the slug, *Laevicaulis alte* on exposure to five different molluscicides, viz. Malathion, hygro, Tiodon, Sevimol, and Copper sulphate. Bhaumik (1986) studied the impact of insecticides on neurosecretion in jute semilooper, *Anomis sabulifera*. Bhatlawande (1989) has reported similar phenomenon in the snail, *Cerastus moussonianus*. Thorat (1990) studied the impact of pesticides and heavy metals on the bivalve, *Parreysia corrugate* and reported different histopathological changes such as vacuolization, cellular degeneration, and alterations in cytoplasmic and nuclear areas and altered staining properties and neurosecretory activities of NSCs. Ahirrao and Khedkar (2012) also observed that there was an acute cellular degeneration, vacuolization and pronounced decrease in the cell number in cerebral ganglion of *Thiara lineata* on exposure to a carbamate pesticide, sevin.

In the present study, it was observed that at 7 days of exposure (the initial stage of poisoning) there was a gradual synthesis of secretory material but as the incubation period was prolonged (21 days of exposure) a gradual release of secretory material was started. This discharge became more vigorous and finally the entire secretory material got drained off. These results are in agreement of with the previous findings (Nanda, 1974; Nagabhushanam *et al.*, 1982; Mirajkar and Sarojini, 1985; Patil, 1986; Muley, 1988, Thorat, 1990 and Deshmukh, 1995). Similar results were reported by many investigators. Sabesan and Ramalingam (1969) reported the accelerated synthesis after short duration and release of
secretory material after long duration of the endosulfan intoxication in the median neurosecretory cells of *Odontopus varicornis*. Various histopathological changes were observed for the first time in the neurosecretory cells of *Hydrophilus olivaceous* after treating them with dimecron, diazion and dieldrin (Gundevia and Ramamurthi, 1972). In this case it was shown that, short exposure periods with these insecticides trigger the synthetic activity of the neurosecretory cell of brain. Sarojini and Mirajkar, (1982) reported that after acute exposure neurosecretory activity was increased while decreased after chronic exposure. The initiation of synthesis, its gradual acceleration and ultimately accumulation of secretory material by pesticides is indicative of the fact that the accelerated pace of synthesis may be an initial response to the emergency caused by the pesticide action.

In the present study it was observed that due to pesticide stress, size of cell, nucleus and nucleolus was changed. The functional status of the neurosecretory elements is linked with changes in the size of the nucleus and nucleolus and may be considered as the index of cell activity (Ortman, 1960; and Ghosh *et al.*, 1968). Thus the chromatin material in the nuclei of the neurosecretory cells, treated with pesticides, became so immobilized after clumping, that it was unable to act with other cellular constituents and it was possible that in this state the DNA content of such nuclei became quite diminished leading to a loss in production of an optimum amount of RNA. In this way the inhibition synthetic activity after prolonged incubation period may be assumed as a failure of the RNA synthetic machinery which inhibits the further synthesis of secretory material (Ghosh *et al.*, 1968).

The comparative study of alterations in the cytoarchitecture of NSCs due to stress of different types of pesticides revealed that all the
pesticides did not show the same effect on the neurosecretory cells because no pesticide is specific in its action. The pesticides show great variation in their toxicity and persistence (Moore, 1969).

Cooke (1977) suggested a working hypothesis. The pesticides like, lindane acts on neurosecretory cells and other neurons by rendering the plasma membrane very permeable to Ca++. The role of Ca++ ions is linked with the stimulus (Fingerman et al., 1977).

In the present study the pesticides toxicity can be explained by the hypothesis of Cooke (1977). Pesticides might be causing hormonal release due to excessive entry of Ca++ inside the NSCs. Thus it can be concluded that pesticides might be exerting their effects on the neurotransmitters which in turn give messages to neurosecretory cells for the release of neurohormones.

In present investigation it is observed that, in combined exposure to dicofol and dichlorovos along with 50mg/l L-ascorbic acid the histological alterations in neurosecretory cells of cerebral ganglia were relatively less as compared to the animals exposed to the chronic dose of dicofol and dichlorovos alone. This indicates the protective and curative role of ascorbic acid in dicofol and dichlorovos induced alterations in neurosecreory cells of bivalve, Parreysia cylindrica. Pesticides are known to cause oxidative stress. Oxidative stress results from an imbalance in the production of reactive oxygen species (ROS) and the ability of the cell to scavenge them. Generation of reactive oxygen species (ROS) is inevitable for aerobic organisms and, in healthy cells, occurs at a controlled rate. Under conditions of oxidative stress, ROS production is dramatically increased, resulting in subsequent alteration of membrane lipids, proteins, and nucleic acids. Oxidative damage of these biomolecules is associated with aging as well as a variety of pathological
events, including atherosclerosis, carcinogenesis, ischemia reperfusion injury, and neurodegenerative disorders. Neural tissue may be particularly susceptible to oxidative damage because it receives a disproportionately large percentage of oxygen and it has a high concentration of polyunsaturated fatty acids which are highly prone to oxidation (Muller 1994).

Vitamin C or L-ascorbic acid or L-ascorbate is an essential nutrient for humans and certain other animal species. Vitamin C is a cofactor for several enzymes involved in the biosynthesis of collagen, carnitine and neurotransmitters (Burri and Jacob, 1997; Tsao, 1997). Vitamin C prevents free radical damage in the lungs and may even help to protect the central nervous system from such damage. In living organisms ascorbate acts as an antioxidant by protecting the body against oxidative stress. Free radicals are continually produced in the body as the result of normal metabolic processes and interaction with environmental stimuli. Under physiological conditions, a wide range of antioxidant defenses protects against adverse effects of free radical production in vivo (Halliwell and Gutteridge, 1989). Oxidative stress results from an imbalance between radical production and reduced activity of antioxidant defenses or both these phenomena. Insecticides causes release of tissue damaging reactive oxygen species (ROS) balance between radical production and protective antioxidant defense (Halliwell and Gutteridge, 1990; Signorini et al., 2002).

Protective effects of quercetin and vitamin C against oxidative stress-induced neurodegeneration were studied by (Heo and Lee, 2004). Yadav (2012) reported the mechanism of neurobehavioral toxicity of arsenic and monocrotophos and neuroprotective efficacy of curcumin and *Bacopa monnieri* briefly.
In present study, the bivalves pre-exposed to chronic concentration of pesticides dicofol and dichlorovos showed fast recovery in the structure of neurosecretory cells of cerebral ganglia in presence of 50 mg/l ascorbic acid than those allowed to cure in normal water. Thus it indicates the protective and curative properties of ascorbic acid against the pesticide damage. Pandit and Bhattacharya (2013) studied the effect of mercuric chloride on Nucleus Lateralis Tuberis (NLT) in *Heteropneustes fossilis* and their recovery by spirulina and chlorella. The authors observed that neurons in the hypothalamic nuclei (NLT) of *H. fossilis* had potential inhibitory effect of Hg on neurosecretion and when fish (mercuric chloride pretreated) fed on spirulina and chlorella separately, showed recovery responses in damaged cells of hypothalamic nuclei (NLT). The recovery responses might be due to the effective role of the contents (i.e. antioxidants) present in spirulina and chlorella.