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Neuropathological Effect of Tributyltin on the Cerebral Ganglia of the Land Snail, *Eobania vermiculata*

^{1,2}Amina E. Essawy, ¹Sohier S. El-Sherief, ¹Ismail A. Sadek and ¹Ahmed A. Soffar

¹Department of Zoology, Faculty of Science, Alexandria University, Moharram Bey, Alexandria 2151, Egypt

²Department of Biology, Faculty of Applied Sciences for Girls, Umm Al-Qura University, Mekka, KSA

Corresponding Author: Dr. Amina Essawy, Department of Biology, Faculty of Applied Sciences for Girls, Umm Al-Qura University, Azizea, Mekka, KSA

ABSTRACT

The present study was designed to evaluate the neuropathological effect of Tributyltin (TBT) on the neurons of the cerebral ganglia in the land snail *E. vermiculata*. Sublethal dose ($1/5$ LD₅₀) was used and the experiments lasted for 4 weeks. Light-and electron microscopical examination of treated animals revealed severe histopathological and ultrastructural alterations in the cerebral ganglia. These alterations included hyperchromatic, pyknotic or highly shrunken nuclei, extreme indentation of plasma membrane, atrophy of the perikarya of some neurons, margination of nucleoli, fragmentation or dilation of rough endoplasmic reticulum, damage of mitochondria and vacuolation and destruction of cytoplasm. In addition, degenerated synaptic vesicles and increased number of autophagosomes and myelin figures were frequently observed.

Key words: Neuron, neurosecretion, organotin, snail, histopathology, ultrastructure

INTRODUCTION

Tributyltin (TBT) compounds are a subgroup of the trialkyl organotin family of compounds. They are the main active ingredients in biocides used to control a broad spectrum of organisms. Since the 1960s, tributyltin compounds have been in uses. Uses include wood treatment and preservation, antifouling of boats (in marine paints), antifungal action in textiles and industrial water systems, such as cooling tower and refrigeration water systems, wood pulp and paper mill systems and breweries.

There is special concern over the use of TBT because of its ecotoxicological effects in the aquatic environment on non-target organisms (ATSDR, 2003). People may be exposed to TBT compounds by eating contaminated food (ex., sea food from coastal water), drink contaminated water or from contact with household products that contain organotin compounds.

Ingestion of large doses of organotin compounds can lead to acute immunological, endocrine, renal, neural and reproductive damage in mammals (Karpiak *et al.*, 2001; Ogata *et al.*, 2001).

Neurotoxic effects of TBT compounds were reported in humans after inhalation, oral and dermal exposure to organotins (Feldman *et al.*, 1993; Lin *et al.*, 1998). In fishes, Fent and Meier (1992) mentioned a light microscopically detectable vacuolization of the brain of minnow (*Phoxinus phoxinus*) after Treatment with Bis (tri-n-butyltin) Oxide (TBTO). Vacuolization of fish brain caused by TBTO suggests similarities of organotin induced neuropathology in fish and mammals, since Aldridge (1992) registered intramyelin vacuoles as a specific reaction to triethyltin contamination in the brain of mammals.

Although, several reports have proved that the aquatic molluscs could be used as indicators for the TBT pollution, the availability of the land snails for such purpose remains incomplete and needs further work to be done. In Egypt, the land snail *Eobania vermiculata* (Müller) (Mollusca, Gastropoda: Stylommatophora: Helicidae) have become well known for most of the Egyptian farmers because of their serious damage to many economic crops. The central nervous system of *E. vermiculata* as is found throughout the pulmonate group consists of a ring of ganglia around the anterior part of the digestive tract. The dorsally situated nerve mass is composed of two cerebral ganglia connected by a short commissure. The ventrally placed nerve mass is, in turn, composed of two pedal ganglia and a visceral complex. This complex consists of two pleural, two parietal and one visceral ganglion. In addition to the complex nerve ring, there is a pair of small buccal ganglia situated on the dorsoposterior surface of the buccal mass and connected to the cerebral ganglia by cerebro-buccal connective (Essawy, 2001).

The paired cerebral ganglia commonly referred to as the brain are situated just dorsal to the origin of the oesophagus in the buccal mass and receive inputs, via peripheral nerves and connective nerves, from the sense organs and the distributed sensory cells of the entire body, but principally from the head region (Chase, 2000, 2001).

The use of aquatic gastropods as potential biomonitor for the trace metals was recommended (Abd-El-Gawad, 2009). However, the present work aimed to study the hazardous effects of TBT on the cerebral ganglia of *E. vermiculata* in an attempt to evaluate the ability of this land snail to be used as a biological indicator of TBT contamination.

MATERIALS AND METHODS

The experiment was conducted during 2008, at Department of Zoology, Faculty of Science, Alexandria University, Egypt. Adult herbivorous terrestrial snail, *Eobania vermiculata* (Müller) (Pulmonata: Stylommatophora: Helicidae) were collected from different gardens at Alexandria Governorate, Egypt, during spring and autumn seasons and transferred in plastic bags to the laboratory. They were kept in open air cages (40×30×30 cm, with 100 individuals per cage), allowed to feed on fresh leaves of lettuce and kept to acclimatize under laboratory conditions (26-30°C and 63-64 R.H) over two weeks prior to the experiment.

Tributyltin Chloride (TBTCl) used in the present study was obtained from Fluka Chemika, Switzerland. It has the molecular weight of 325.5 and its chemical (Empirical) formula is $C_{12}H_{27}ClSn$.

Experimental design: Preliminary experiments were carried out to determine the median lethal and sublethal doses of TBTCl (dissolved in dimethyl-sulfoxide DMSO) using topical application method. In order to study the toxic effect of TBT on the cerebral ganglia of *E. vermiculata*, a sublethal dose of TBT ($1/5$ LD₅₀) was used. The snails were divided into three groups, with 100 animals per group (ten animals in each box.). Few milliliters of water were added daily into each box to provide humidity for snail activity and treated as follows:

- Group I:** Snails of this group received no treatment and used as negative control animals
- Group II:** Snails of this group received a single dose of 30 µL of DMSO that is topically applied on the surface of the animals and used as positive control animals
- Group III:** Snails of this group received a single sublethal dose of TBT ($1/5$ LD₅₀) dissolved in 30 µL DMSO. This dose was topically applied on the surface of each animal

Dissection and tissue preparation: After four weeks of treatment, a definite number of snails from each group were randomly chosen and dissected for histological and ultrastructural studies.

Cerebral ganglia of unanaesthetized tested snails were dissected out with the help of a Zeiss binocular microscope and immediately dropped in the appropriate fixative.

For histological studies, cerebral ganglia were fixed in Bouin's solution or 10% neutral buffered formalin. Graded dehydration of the tissue was done by 70 to 100% alcohol in subsequent steps. Xylene was used as a clearing agent. The tissues were embedded in paraffin, sections were cut and stained with hematoxylin and eosin, other sections were stained with Mallory's trichrome stain.

For electron microscopical studies, the ganglia were fixed in formalin-glutaraldehyde fixative (4flg) in phosphate buffer. Specimens were then postfixed in 2% osO₄ in the same buffer at 4°C for 2 h. Samples were washed in the buffer and dehydrated at 4°C through a graded series of ethanol. Specimens were embedded in epon-araldite mixture. Lkb ultramicrotome was used to cut ultrathin sections which were picked upon 200 mesh naked copper grids and double stained with uranyl acetate and lead citrate. Scoping the grids was achieved by using jeol 100 cx tem.

RESULTS

Morphological and histological organization of the control cerebral ganglia in *E. vermiculata*: The two cerebral ganglia of *E. vermiculata* are symmetrical joined together by a cell-free short commissure and surrounded by a sheath of connective tissue called perineurium (Fig. 1a). Each ganglion is divided into three major regions called procerebrum, mesocerebrum and metacerebrum (Fig. 1b). Each region of cerebral ganglion consists of an outer perikaryal layer (cortex) containing nerve cell somata and an inner neuropile containing axonal processes (Fig. 1a, b). In addition, large number of glial cells was found between cell somata and in the neuropile.

The procerebral neurons are numerous and very small in size (about 5.5 µm in diameter). They have very little cytoplasm and large nuclei with a dense staining affinity (Fig. 1c). The mesocerebrum is characterized by possessing large-sized neurons with a mean diameter of about 30 µm. Their nuclei have a smooth ovoid form (mean diameter 19 µm) and contained small patches of heterochromatin and large number of nucleoli (Fig. 1d, e). Unlike the procerebrum and the mesocerebrum, the metacerebrum has no distinctive features. It contains neurons of variable sizes (7-25 µm in diameter) and the nuclei appeared rounded or ovoid in shape with a diameter ranging from 5-20 µm.

Histopathological changes of the cerebral ganglia in treated snails: Four weeks after treatment with $\frac{1}{5}$ LD₅₀ TBT (4.8 µg snail⁻¹), small-sized neurons of procerebrum were greatly affected as they displayed an obvious damage of their cell bodies (Fig. 2a). Also, the extracellular space was widened and the perineurium was disorganized. In the mesocerebrum, most of neurons appeared morphologically deformed with irregular nuclei and lytic chromatin (Fig. 2b). Some cells exhibited dissolved cytoplasm with large number of vacuoles and pyknotic or completely degenerated nuclei. A perinuclear halo of destructed cytoplasm appeared around the nuclei of some neurons. Disorganized and degenerated axons and neuropile and severe damage of the extracellular tissue is a common histopathological feature in this region. Concerning the metacerebrum, the perikarya of the neurons in this region showed loss of normal architecture and displayed multiple degenerative changes (Fig. 2c, d). Completely degenerated neurons with chromatolysis and dissolved cytoplasm with many vacuoles were noticed.

At the light microscopic level, no histopathological alterations were observed in the structure of cerebral ganglia of the snails treated with the solvent (DMSO). The neurons were more or less similar to those in group I (-ve control).

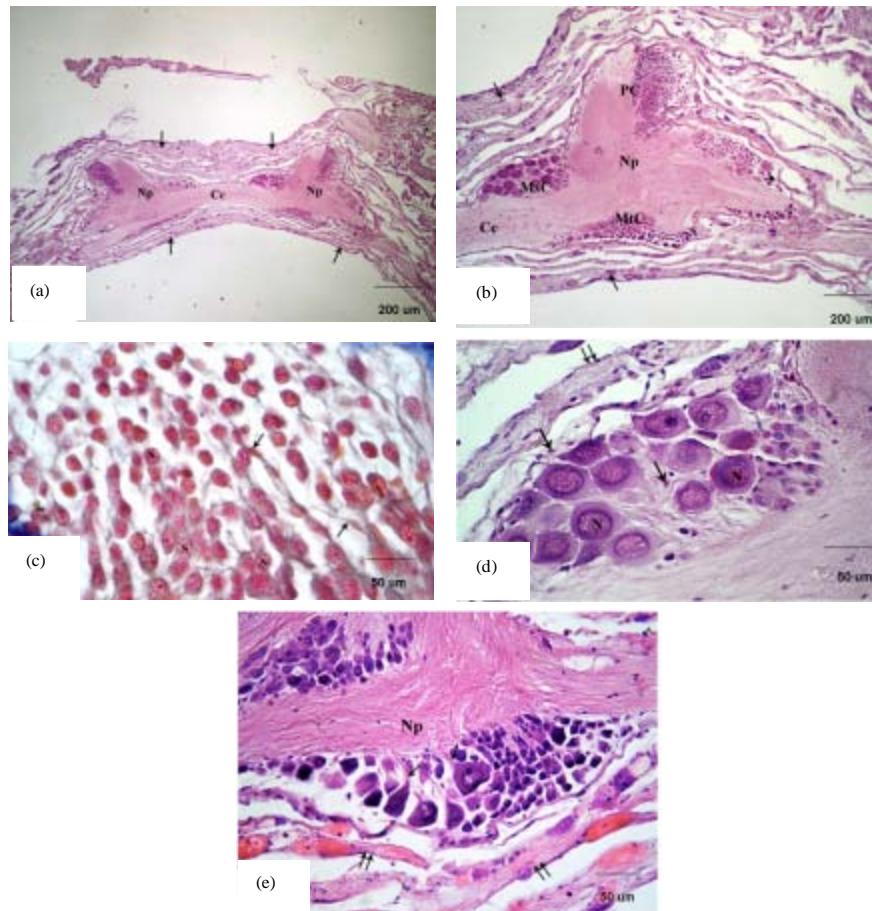


Fig. 1: Light micrographs of horizontal sections through the cerebral ganglia of *Eobania vermiculata*. (a) the two cerebral ganglia are connected by cerebral commissure and surrounded by a perineurium (arrows). (H and E) (x36), (b) higher magnification of figure (a) showing the right cerebral ganglion with its 3 regions; procerebrum, mesocerebrum and metacerebrum. (H and E) (x90), (c) a group of small-sized procerebral neurons. Arrows indicate neuroglial cells. (x900), (d) group of large-sized mesocerebral neurons with well defined nuclei and prominent nucleoli. Arrows pointed at neuroglial cells (H and E) (x360) and (e) metacerebrum containing neurons of variable sizes. Arrows indicate axons entering neuropile. (H and E) (x360). Cc: Cerebral commissure, MsC: Mesocerebrum MtC: Metacerebrum, N: Nucleus, Np: Neuropile, PC: Procerebrum

Ultrastructural pattern of cerebral ganglia in control snails: At the ultrastructural level, there are two types of procerebral neurons (Fig. 3a). The cells of the first type are neurosecretory cells mainly found at the boundary of the perikaryal layer and appeared elongated with a large eccentric nucleus and a relatively small amount of cytoplasm containing abundant electron-dense neurosecretory granules (Fig. 3b, c). The second type of cells was smaller in size with large nucleus and thin cytoplasm containing few cytoplasmic organelles. The nuclei contained a thin layer of heterochromatin along the nuclear envelope and few large blocks of heterochromatin scattered in the central area (Fig. 3d).

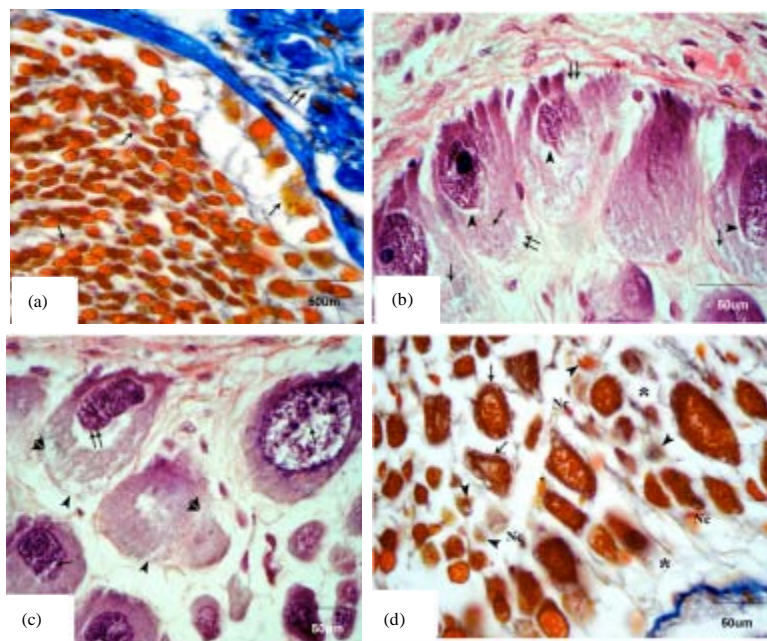


Fig. 2: Light micrographs showing parts of horizontal sections through cerebral ganglia of *Eobania vermiculata* after four weeks of treatment with TBT. (a) procerebrum with degenerated neurons (arrows) and disorganized perineurium (double arrows). (Mallory's trichrome) (x900), (b) a group of morphologically altered mesocerebral neurons having eccentric Nuclei (N) with peripherally located nucleoli, lysis of the cytoplasm (arrows) and destructed cell membrane (double arrows). arrowheads indicate perinuclear halo. (H and E) (x900), (c) a group of mesocerebral neurons showing karyolysis (arrows), shrinkage of nuclei (double arrows), dissolved cytoplasm (double arrowheads) and disrupted plasma membrane (arrowheads). (H and E) (x900) and (d) metacerebrum with damaged (arrows) and completely degenerated (arrowheads) neurons. Nc: degenerated neuroglia, *damaged extracellular tissue. (Mallory's trichrome) (x900)

Electron micrographs showed that, the mesocerebrum contained large-sized neurons with large centrally located nuclei containing one or more nucleoli and small patches of dispersed heterochromatin (Fig. 3e).

In the metacerebrum the neurons were variable in size and shape (Fig. 3f). They have polymorphic nuclei with dense heterochromatin strip along the nuclear envelope and some patches of heterochromatin dispersing all over the euchromatin. The cytoplasm contains numerous cisternae of rough endoplasmic reticulum, Golgi complex, free ribosomes and secretory granules (Fig. 3g).

Ultrastructurally, the neuropile in the cerebral ganglia of *E. vermiculata* is composed of a complex network of nerve fibers, some of which contain in addition to mitochondria, dark and light synaptic vesicles (Fig. 3h).

Ultrastructural pattern of cerebral ganglia in snails treated with TBT: Electron microscopic examination of the cerebral ganglia four weeks after topical application of TBT exhibited many interesting ultrastructural changes.

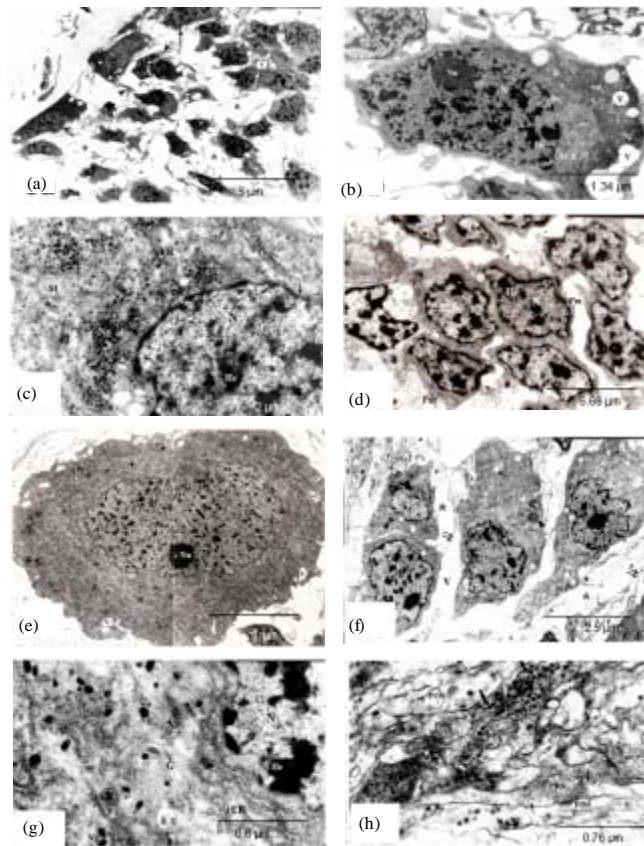


Fig. 3: Electron micrographs showing parts of horizontal sections of control cerebral ganglia of *Eobania vermiculata*. (a) A group of small procerebral neurons. Thin arrows indicate marginal neurosecretory cells, thick arrow points at perineurium, arrowheads indicate glial processes and asterisks point at extracellular space. (x4000), (b) magnified part of procerebrum showing cluster of neurons with large nucleus and abundant Euchromatin (Eu). (x10000), (c) magnified procerebral neurosecretory cell with large nucleus, peripherally located nucleolus and large number of neurosecretory granules. (x15000), (d) enlarged part of neurosecretory cell with part of the nucleus, Golgi complex, mitochondria, rough endoplasmic reticulum and secretory granules. Arrows indicate nuclear pores. (x26000), (e) a large sized mesocerebral neuron showing large nucleus with prominent nucleolus. (x3000), (f) four different shaped metacerebral neurons. Arrows point at fat droplets, arrowheads indicate dense bodies and double arrows show glial processes extending in the extracellular space (asterisks). (x8000), (g) magnified part of metacerebral neuron showing part of the nucleus and different cytoplasmic organellae. (x40000) and (h) part of the neuropile containing axon profiles packed with large electron-dense vesicles (thick arrows) and clear vesicles (thin arrows). Double arrows pointed at synaptic membranes (x26000). G: Gologic complex, Gc: He: Heterochromatin, Ly: Lysosomes, M: Mitochondria, N: Nucleus, Nc: Neuroglia cell, Nb: Nissle bodies, Ne: Nuclear envelop, Ng: Neurosecretory granules, Nu: Nucleolus, Pm: Plasma membrane, PRS: presynaptic zone, POS: postsynaptic zone. R: Ribosomes, rER: rough endoplasmic reticulum, V: Vacuoles

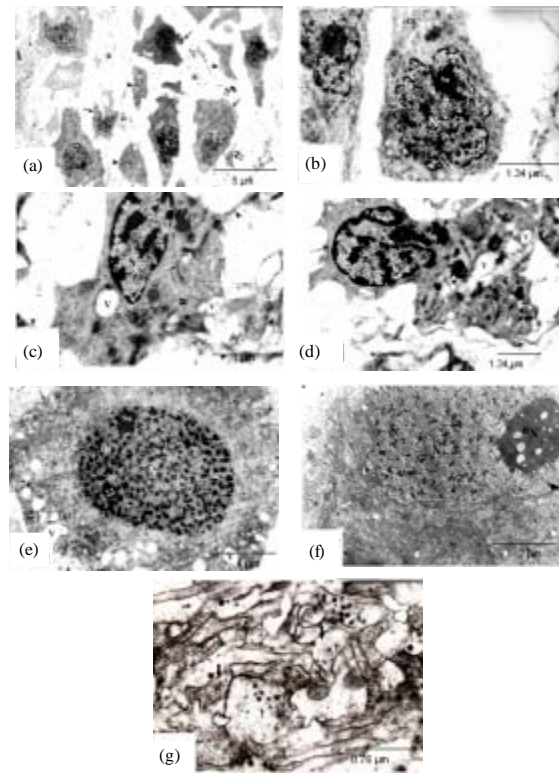


Fig. 4: Electron micrographs showing parts of horizontal sections of cerebral ganglia of snails treated with TBT. (a) a group of altered (arrows) and completely degenerated (headarrows) procerebral neurons. Note degenerated glial cells (double arrows) and destructed extracellular tissue (*) (x4000), (b) Two neurosecretory cells in the procerebrum with severe indentation of nuclear envelope (arrows), disorganized cisternae of rough endoplasmic reticulum and degenerated neurosecretory granules (arrowheads) (x15000), (c) a procerebral neuron with an eccentric nucleus, damaged mitochondria (arrows), cytoplasmic vacuoles, large dense lysosomes (double arrows) and long cytoplasmic processes(arrowhead) (x20000), (d) a procerebral neuron with peripherally located clefted nucleus, large number of lysosomes (arrowheads), short cisternae of degranulated rough endoplasmic reticulum (arrows) and damaged mitochondria (M) (x15000), (e) A metacerebral neuron showing nucleus with two peripherally located nucleoli, abnormal distribution of heterochromatin, vacuolated cytoplasm and destructed plasma membrane (arrow) (x5000), (f) magnified part of metacerebral neuron showing part of the nucleus with large nucleolus exhibiting segregation of fibrillar and granular components. Arrows indicate fragments of rough endoplasmic reticulum. (x10000) and (g) large number of axons (thick arrows) containing degenerated synaptic vesicles (thin arrows) and damaged mitochondria (M) (x26000). He: Heterochromatin,, M: Mitochondria, N: Nucleus, Nc: Neuroglia cell, Ne: Nuclear envelop, Ng: Neurosecretory granules, Nu: Nucleolus, rER: rough endoplasmic reticulum, V: Vacuoles

In procerebrum, the nuclei of neurosecretory cells appeared eccentric, hyperchromatic, pyknotic or highly shrunken with irregular contour and peripherally located nucleoli (Fig. 4a, b).

Alterations of the cytoplasm of these cells included destruction or loss of cytoplasmic texture, fragmentation or degeneration of rough endoplasmic reticulum, degenerated or small pleomorphic mitochondria with dense matrix. Moreover, neurons with irregular outline and eccentric oval or clefted nuclei were observed (Fig. 4c, d), The heterochromatin appeared associated with the inner nuclear membrane or in the form of scattered patches. The cytoplasm of these altered neurons appeared highly destructed containing fragmented rough endoplasmic reticulum, large number of free ribosomes, electron dense pleomorphic mitochondria and large number of vacuoles, dense lysosomes and long cytoplasmic processes.

Concerning the neurons in the metacerebrum, the nuclei appeared flat and pyknotic with small dense patches of heterochromatin, dilation of nuclear pores and peripherally located nucleoli with segregation of granular and fibrillar components (Fig. 4e, f). Moreover, the cytoplasm contained large number of vacuoles, damaged mitochondria and indistinct cytoplasmic organelles.

As the neurons in the mesocerebrum being extremely large, it was not easy to manipulate the ultrastructure of the whole cell, therefore, it was satisfactory to use the light microscopical preparations previously described rather than electron microscope for the neurons of this region.

Ultrastructural examination of neuropile in TBT-treated cerebral ganglia, revealed a marked damage of nerve fibers, degenerated clear and electron-dense synaptic vesicles and mitochondria with indistinct cristae (Fig. 4g).

No significant ultrastructural alterations were observed in neurons of cerebral ganglia of the snails treated with the solvent (DMSO).

DISCUSSION

Since Tributyltin (TBT) compounds are present in the environment, animal and human bodies may be exposed to several amounts of these chemicals via feeding or drinking. In this respect, the present investigation was designed to evaluate the toxic action of TBT on nervous system of the pulmonate snail *Eobania vermiculata* and to shed light on the possible use of this land snail as a bioindicator for environmental perturbation.

In the present study, light microscopical investigation demonstrated a diverse spectrum of cell body alterations of the neurons in the cerebral ganglia of TBT treated snails. All types of neurons and neuroglia were affected and the perineurium appears disorganized. In support to the present results, confocal microscopic study of immature rat hippocampus showed that TBT induce severe neuronal death in concentration and time-dependant manners (Mizuhashi *et al.*, 2000). Norberg *et al.* (1998) also reported that trimethyltin, an organotin compound with a similar chemical structure, seriously damaged the neurons in rat hippocampal slice cultures. The *in vitro* studies by Thompson *et al.* (1996) demonstrated that primary neuronal cell cultures are very sensitive to organotins, showing patterns of selective toxicity with respect to neuronal and glial cells. Moreover, Fent and Meier (1992) mentioned a light microscopically detectable vacuolization of the brain of minnow *Phoxinus phoxinus* after treatment with TBTO. Vacuolization of the fish brain caused by TBTO suggests similarities of organotin-induced neuropathology in fish and mammals, since Aldridge (1992) registered intra-myelin vacuoles as a specific reaction to triethyltin contamination in the brain of mammals.

Regarding the effect of TBT on the plasma membrane, some of the TBT-treated neurons possessed ruptured plasma membrane. Identical features were also represented by Mizuhashi *et al.* (2000) who revealed that the neurons of rat hippocampal slice culture treated with TBT for 24 h was died because of the severe damage of the cytomembrane. Moreover, similar

findings were observed by Rivera *et al.* (1992) who reported that TBT penetrates the plasma membrane only in the presence of endogenous O₂ and then induces morphological changes in erythrocytes and haemolysis. Therefore, TBT neurotoxicity may require endogenous H₂O₂ and O₂ rather than TBT itself producing harmful reactive oxygen species.

Dilation in the intercellular space was observed between neurons in the cerebral ganglia. These dilations probably reflected a dysfunction of ionic and osmotic balance of these cells (Cotran *et al.*, 1999).

The present study revealed impact of the TBT on the ultrastructure of neurons and neurosecretory cells in the cerebral ganglia of *E. vermiculata* and provided more precise information about the structural alterations induced in these cells. The nuclei of both ordinary neurons and neurosecretory cells revealed severe pathological changes. They appeared eccentric, hyperchromatic, pyknotic or highly shrunken with irregular contour and peripherally located nucleoli. Similar changes were reported in the cerebral ganglia neurosecretory cells of *Biomphalaria glabrata* treated with the herbicide Atrazine (Eissa and Omran, 2007) and in the neurons of buccal ganglia treated with methomyl and methiocarb (Essawy *et al.*, 2009).

In their study on the cytotoxicity of TBT, Mizuhashi *et al.* (2000) observed dead or damaged neurons with chromatin condensation which is one of the morphological characteristics of apoptosis. The authors reported that the TBT provokes apoptosis-like neuronal cell death which might be mediated by intracellular Ca²⁺ and free radical generation via non-NMDA receptor activation. Also Reader *et al.* (1999) demonstrated a role of Ca²⁺, protein kinase C and proteases in the induction of apoptosis in the hepatocytes of rainbow trout treated with TBT.

According to Mellwain and Hoke (2005), changes in the size and position of the nucleus and nucleolus could be attributed to the effect of the neurotoxin on the cytoskeleton of the affected neurons. Moreover, segregation of nucleolar components was noticed in some neurons of metacerebrum of *E. vermiculata* treated with TBT. Nucleolar segregation could be due to the inhibition of RNA synthesis resulting from a decrease in the activity of RNA polymerase which catalyzes the synthesis of RNA (Cmarko *et al.*, 2000).

The neurotoxic effect of TBT on the cerebral neurons also appears from the observations on the cytoplasmic organelles. The primary change was the fragmentation and degranulation of rough endoplasmic reticulum accompanied with an increased number of free ribosomes. Similar changes were described in the buccal ganglia of *Eobania vermiculata* treated with carbamate molluscicides (Essawy *et al.*, 2009). The degranulation and dilatation of rER are discussed as general changes of the cell in response to toxicants (Hamed *et al.*, 2007). Most of these reactions are attributed to membrane destabilization and increased membrane permeability to ions under the influence of toxicants, followed by osmotic effect and finally cell death.

On the other hand, the present results clearly indicated that, treatment with TBT induced damage and loss of the cristae of mitochondria in the perikarya of the neurons as well as in the axons of the neuropile. These alterations resemble those described in snails, slugs and vertebrates as cellular stress symptoms after intoxication (Heiba *et al.*, 2002; Prakash *et al.*, 2009; Rawi *et al.*, 2011).

In addition, the most obvious alterations observed in the present results were the presence of large dense lysosomes and autophagosomes in the cytoplasm of treated neurons. In *L. stagnalis*, lysosomes are prominent after experimental inactivation of neuronal secretory activity by incubation of cerebral ganglia in Vinca antitumor agents (Muller *et al.*, 1990). Autophagy is normally a cellular degradation pathway particularly important during development stages and under certain environmental stress conditions (Klionsky and Emr, 2000). This phenomenon may

be related to an autophagic degeneration of the cells or to a disruption of the regulatory mechanisms of autophagy described by Klionsky and Emr (2000).

The remarkable feature of the TBT-treated neurosecretory cells is the disappearance of neurosecretory granules and formation of large vacuoles in the cytoplasm that resulted in destruction of the cytoplasmic organelles. Vacuolation of neurosecretory cells was also noticed in the cerebral ganglia of *B. glabrata* treated with Atrazine (Eissa and Omran, 2007).

Degenerated synaptic vesicles, mitochondria and synaptic membranes were the most frequent changes in the neuropile of treated cerebral ganglia. Degradation of the synaptic vesicles could be attributed to the interruption of axonal transport which may promote degradation of synaptic terminals. Similarly, Tsunoda *et al.* (2006) showed that, TBT-induced modulations of neurotransmitters and their metabolites in discrete brain regions of mice.

From the above discussed results, it can be concluded that TBT is a neurotoxic compound and the land snail *E. vermiculata* can be used as a bioindicator for evaluating the pollution of terrestrial ecosystems by organic compounds.

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