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RESEARCH ARTICLE

PATHOLOGY

ULTRASTRUCTURAL AND HISTOPATHOLOGICAL STUDIES IN LEAD ACETATE INDUCED NEUROTOXICITY IN WISTAR ALBINO RATS AND ITS AMELIORATION WITH *OCIMUM SANCTUM* (OS) A LEAF EXTRACT

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ABSTRACT

Lead is a long – known poison of environment and industrial origin. Prolonged exposure damages cellular material and alters cellular genetics and produces oxidative damage. In the present study rats were treated with different doses of lead acetate (60 mgs and 30 mgs / Kg.b.wt. / 3days a week respectively) over a period of 12 weeks and acetyl cholinesterase activity, gross & histopathological changes, and ultrastructural changes were studied in brain. Decreased acetyl cholinesterase activity in lead treated animals was observed. Histopathologically dose dependent changes were observed in brain and revealed severe degenerative changes of neurons, submeningeal haemorrhages, demyelinating changes, capillary proliferation and necrotic nodules were observed in majority of higher doses of lead treated rats. Shrinkage and rounding and loss of purkinje cells in cerebellum were noticed. Ultrastructurally brain revealed swollen and vacuolated vascular endothelial cells, degenerated myelin sheath with interlaminar splitting, empty myelin sheath, decrease in mitochondrial density with degenerative changes, margination and clumping of chromatin in nucleus of lead fed groups. Where as in OS treated groups the similar changes were observed with mild intensiry. However, the altered acetyl cholinesterase activity, histopathological, and ultrastructural changes were dose dependent in the present investigation. . The present experiment suggests that the *Ocimum santum* exhibited significant protective effect on lead induced brain damage in rats.

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KEY WORDS

Neurotoxicity, lead acetate poisoning, *Ocimum sanctum*, rats

INTRODUCTION

Lead occurs in a variety of organic and inorganic compounds with a multitude of additional uses in the manufacture of protective paints for iron and steel, explosives, rodenticide, batteries etc. The manipulation of lead for these uses has caused lead contamination of air, dust and soil. It can be present in air in the vicinity of factories and on the highways. Lead levels in air, water and soil have been increasing during the last 10 years and it is considered to be one of the most important environmental pollutants of both urban and semi urban areas. Vegetables are polluted by lead from the air and a considerable amount of lead contamination is found in cereals and broad leafed vegetables. Because of its persistence in the environment, exposure to lead has become a major public health concern[1]. Lead damages cellular material and alters cellular genetics and produces oxidative damage. It causes increased production of free radicals and decreased availability of anti oxidant reserves to respond to the resultant damage. It also interrupts enzyme activation and competitively inhibits trace mineral absorption. Lead binds to sulfhydryl proteins (interrupting structural protein synthesis), alters calcium homeostasis and lowers the levels of available sulfhydryl antioxidant reserves in the body[2].

Several authors tried various ameliorating agents like Thiamine, vitamin E, selenium, zinc etc. There was a meager information was available regarding herbal products as ameliorating agent. Keeping in view, *Ocimum sanctum* was used as an ameliorating agent in present research. *Ocimum sanctum* (OS) commonly known as 'Tulasi' in Hindi is a medicinal plant commonly grown in India. The use of this herb has been reported in Indian traditional systems of Medicine and its modern applications are receiving wide spread attention day by day. Different parts of this plant have been claimed to be valuable in a wide spectrum of diseases. It has been observed that tulasi has antioxidant, antibiotic, antiatherogenic, immunomodulatory, anti-inflammatory, analgesic, antiulcer, chemopreventive and antipyretic properties[3].
The literature regarding histopathological and ultrastructural changes and AchE activity in brain was meager. Hence present work was undertaken to study the dose dependent impact of lead acetate on histopathological and ultrastructural changes and AchE activity in brain of wistar rats and also investigate role of *Ocimum sanctum* (OS) a leaf extract on lead induced neurotoxicity.

MATERIALS AND METHODS

(i) Procurement of experimental animals, lead acetate and Ocimum sanctum (OS)

Ale[check this please] Wister albino rats with body weight around 150g (procured from Laboratory Animal House facility, Hebbal, Bangalore) were utilized for the present experiment. Rats were acclimatized to the experimental conditions for one week, after acclimatization, animals were grouped and housed in standard poly propylene rat cages (three rats 1 cage) during the experiment. They were maintained at 25^0 $\pm 1^0$c and a 12:12 hour interval light / dark cycle through out the experimental period for 6 weeks by taking necessary precautions, standard

laboratory hygienic conditions by providing laboratory animal feed and water *adlibitum*. The approval of the institutional animal ethical committee was obtained prior to commencement of the experiment (Approval number Roc.no.813/Admn.III/07 dt: 06.06.2009). The Lead acetate ($(CCH_3 COO_2)_2$ Pb $3H_2O$, M.w = 379.33) with a laboratory reagent grade was procured from the Qualigens Fine chemicals, Bombay and *Ocimum sanctum* (OS) leaf extract from the Natural Remedies Pvt. Ltd. Bangalore, India.

Total of 216 healthy young male rats were randomly assigned to the control and treatment groups. Six groups of rats consisting of 36 rats in each group were used for the study. To know the LD_{50} value of lead acetate a separate study was conducted with 36 rats before starting of the experiment and according to that dose was calculated. Lead acetate and OS gavaged per orally by using distilled water as vehicle. The groups and the doses employed for the present study were shown below Six rats from each group were randomly sacrificed at every fortnight intervals after starting the experiment i.e. 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th} and 12^{th} weeks.

(ii) Experimental Trail

Groups	Number of Rats per group	Doses
Group I (D.W. Control)	36	0.2 ml of D.W
Group II	36	$1/10^{th}$ of LD_{50} (60mg of lead acetate /kg bwt / 3 days in a week)
Group III	36	$1/20^{th}$ of LD_{50} (30mg of lead acetate / kg bwt /3 days in a week)
Group IV	36	$1/10^{th}$ LD_{50} (60mg /kg bwt of lead acetate / 3 days a week and 400mg / kg bwt OS/3 days in a week)
Group V	36	$1/20^{th}$ of LD_{50} (30mg /kg bwt of lead acetate / 3 days a week) and 400mg OS / kg bwt /3 days in a week)
Group VI	36	400mg / kg bwt of OS / 3days in a week

(iii) Selection of dose Lead and Ocimum sanctum

The lead acetate dose was selected based on the pilot study OS dose was selected based on the observations of Manikandan *et al.*[4], who reported that 100, 200,300,400 mg /kg bwt of OS were administered to Wistar rats followed by intra peritoneal injections of DMBA (7, 12, Dimethyl Benz (a) Anthracene) (30mg /kg bwt) 90 minutes after the final dose of the extract. At this dose of DMBA induced the genotoxicity by formation of micronuclei. The animals pretreated with OS @ a concentration of 300mg and 400mg /kg bwt was significantly reduced micronuclei formation i.e. reduced genotoxic effect of DMBA. Hence the higher dose of OS i.e. 400mg /kg bwt was selected as dose.

(iv)Acetyl cholinesterase activity in Brain

At each sacrifice, pieces of brain from treated and control groups were collected and preserved immediately at -20^0C until use.

Sample preparation

Brain pieces were minced and homogenized in 0.05M ice cold phosphate buffer (pH 7.4) by using virtis homogenizer. The homogenate sample was used for estimation of acetyl

cholinesterase activity by using the method described [5].

(v) Gross and Histopathology

A detailed postmortem examination was conducted on all the sacrificed rats in all the experimental groups. The gross lesions were recorded and representative tissue pieces from brain were collected and preserved in 10% neutral buffered formalin for histopathological studies. Fixed tissues were processed by routine paraffin embedding technique. Sections of 5-6 microns thickness were cut and were stained with routine Haematoxylin and Eosin method (H&E)[6]. Special stains like Hortega's Silver carbonates staining, Thionin stain and Cajal's gold chloride stain were employed.

(vi) Electron microscopic examination

The specimens (subjected for TEM examinations) were rinsed in 0.1M phosphate buffer pH 7.2 (PB) to remove blood from the surface. Liver, kidney and brain tissues greater than 2 cm long were minced into smaller pieces of approximately 3 x 3 mm and were fixed in 3 percent glutaraldehyde, buffered with phosphate buffer for 3 hours. It was rinsed twice with phosphate buffer for 10 minutes per rinse. The tissues were then fixed in 2 percent aqueous osmium tetraoxide for 2hrs and rinsed in 3 changes of distilled water for 10 minutes. Each dehydration was accomplished by immersion in a graded series of ethanol solutions of 25, 50, 75, 95 and 100 percent. Infiltration with propylene oxide and embedding with increasing concentrations of propylene oxide followed by dehydration were carried. Thin sections (600nm) were obtained by use of Ultra microtome and were placed on a copper 200 – mesh grid. They were stained with uranyl acetate and lead citrate. Two blocks were prepared for each specimen and two grids/ blocks were observed. There after, four grids were observed for each specimen[7].

(vii) Statistical analysis

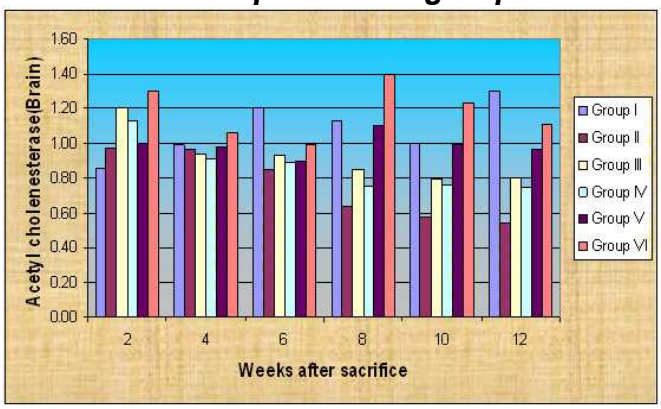
The results were analysed statistically by performing oneway ANOVA[8].

RESULTS

(i) Acetyl cholinesterase

Graph.1

Mean values of acetyl cholinesterase (brain) (µ mol of Ache /g of Wet wt) in animals of different experimental groups



The overall mean Acetyl cholinesterase values in Group I, II, III, IV, V and VI were 1.08, 0.76, 0.92, 0.86, 0.99 and 1.18 (µ mol of Ache/g of wet wt) respectively. There was a significant ($P<0.05$) decrease in Ache values in lead treated group of higher dose (Group II) and its corresponding OS ameliorated group (Group IV) when compared to control (Group I). A significant improvement was noticed in lower dose ameliorated group(GroupV).No significant difference was noticed among lead treated groups.

(ii) Pathology

In the present study gross lesions were prominently observed in the brain. Brain of the rats from lead acetate treated revealed severe congestion and hemorrhages throughout the experimental period.

Cerebrum

Microscopic examination of cerebral cortex revealed submeningeal hemorrhages with spongiosis (Fig.1), proliferation of capillaries (Fig. 2), swollen neurons with central chromatolysis (Fig.3), satellitosis and neuranophagia, focal areas of congestion during 2nd week of lead feeding in majority of animals. In addition extensive demyelinating changes with mild glial cell proliferation (Fig.4), atrophy of nerve cells and moderate capillary proliferation were noticed by the end of 4th week. During 6th to 8th week, congested cerebral blood vessels and choroid plexus, severe demyelinating changes, gliosis, and infiltration of MNC, edema, proliferation of endothelial cells of blood vessels and perivascular infiltration of MNC (Fig.5) were more evident in all treated animals. Extensive meningeal and cerebral hemorrhages, intense accumulation of astrocytes (gliosis) (Figs.6&7), arteritis (Fig.8) and at places necrotic nodules with proliferation of capillaries (Fig.9) were more prominent in majority animals by the end of 10 and 12 weeks.

In OS ameliorated group of higher dose showed similar lesions like group-II was observed up to 8 weeks of lead feeding. Later the changes were gradually reduced in its intensity and by the end of 12th week mild changes were noticed. Where as OS ameliorated group of lower dose showed similar lesions like group-III were observed with mild intensity, the ameliorating effect was more prominently observed from 2nd week onwards and the toxic changes were negligible by the end of 12th week.

Cerebellum

Histopathologically a dose dependent changes were observed in cerebellum such as Spongiosus in grey matter, focal loss and shrinkage of Purkinje cells (Fig.10) with central chromatolysis, capillary proliferation were more evident during 2nd to 8th week of lead feeding. In addition to above changes congestion and hemorrhages, rounding and tapering of Purkinje cells (Fig.11), demyelination and vacuolatary changes were conspicuous in majority of animals by 10th and 12th week.

In OS ameliorated group of higher dose showed similar lesions like group-II was observed up to 6 weeks of lead feeding. Later the changes were gradually reduced in its intensity and by the end of 12th week mild changes were noticed. Where as OS ameliorated group of lower dose showed similar lesions like group-III were observed with mild intensity, by the end of 12th week, the cerebellum comes to near normal appearance.

(iii) Ultrastructural examination of brain

Electronmicroscopic examination of brain revealed Swollen and vacuolated vascular endothelial cells, degenerated myelin sheath with interlaminar splitting, empty myelin sheath, decrease in mitochondrial

density with degenerative changes, margination and clumping of chromatin in nucleus of lead fed groups were noticed as dose dependent manner (Group II & III) (Fig.12,13 & 14). These changes were mild intensity in Os ameliorating groups (Group IV & V).

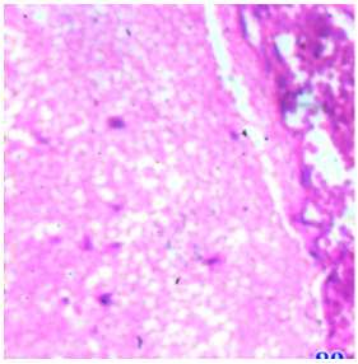


Figure.1
Cerebrum: Group II: Submeningeal hemorrhages with spongiosis. H & E: x 280.

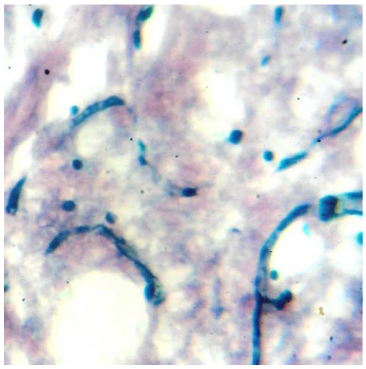


Figure.2
Cerebrum: Group II: Note capillary proliferation in cerebrum.Hortega's Silver carbonates staining: x 280.

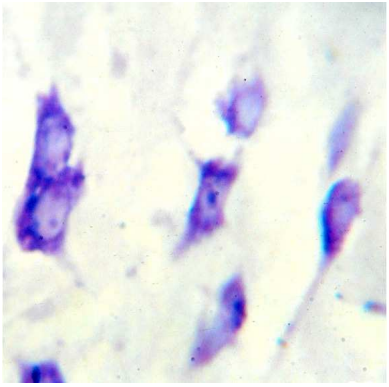


Figure.3
Cerebrum: Group II: Section showing swollen neurons with central chromatolysis. Thionin stain: x 700

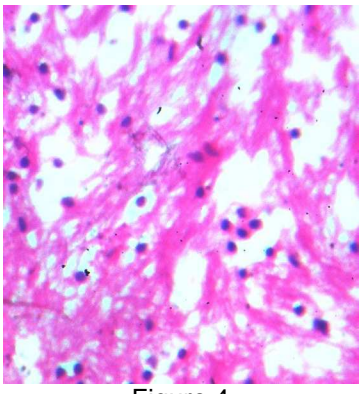


Figure.4
Cerebrum: Group II: Extensive demyelinating changes with mild glial cell proliferation. H & E: x 280

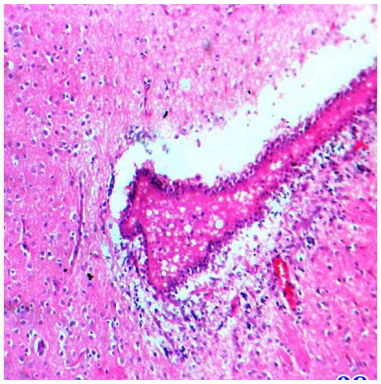


Figure.5
Cerebrum: Group II: Proliferation of endothelial cells of blood vessels.H &E: x 70.

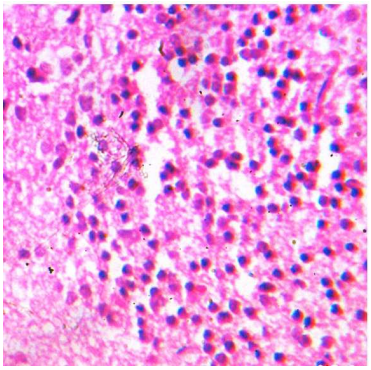


Figure.6
Cerebrum: Group II: Note intense accumulation of astrocytes(gliosis). H &E: x 280.

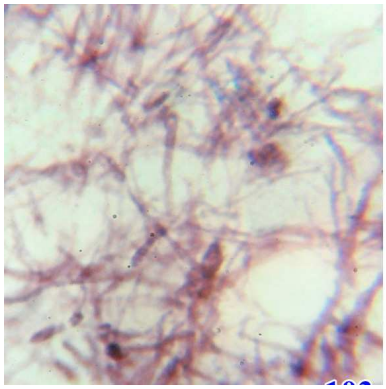


Figure.7
Cerebrum: Group II: Note gliosis. Hortega's Silver carbonate staining: x 280.Figure.8: Cerebrum: Group II: Section showing arteritis in cerebrum. H & E: x 280.

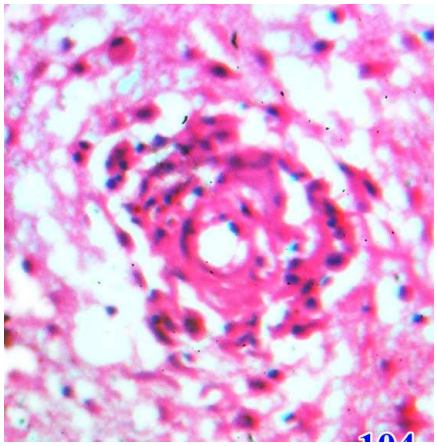


Fig.8

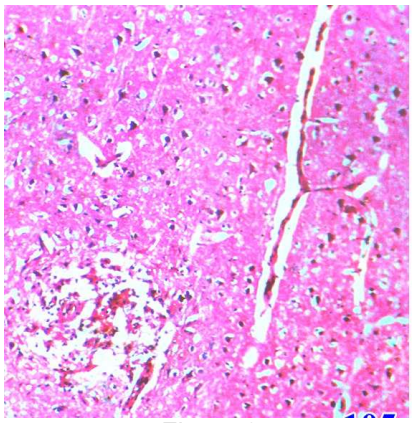


Figure.9
Cerebrum: Group II: Necrotic nodules with proliferation of capillaries in cerebrum. H & E: x 70.

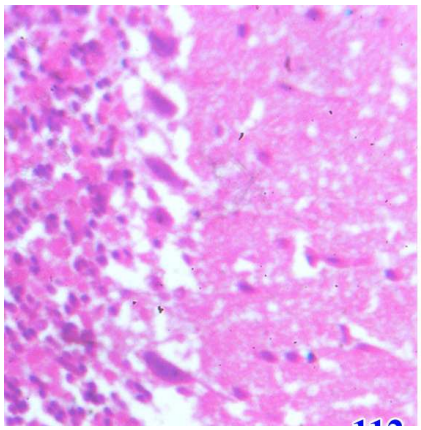


Figure.10
Cerebellum: Group II: Focal loss and shrinkage of purkinje cells in grey matter. H & E: x 280.

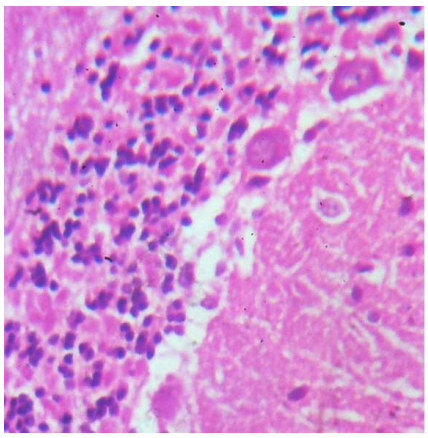


Figure.11
Cerebellum: Group II: Section showing rounding of purkinje cells in cerebellum. H & E: x 280.

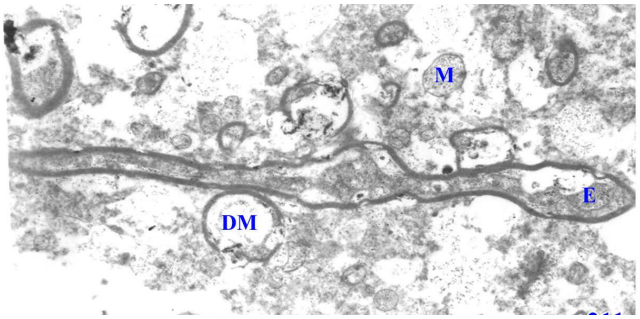


Figure.12
Brain: Transmission Electron Microscopy: Group II: Showing swollen and vacuolated endothelial cells (E), degenerated myelin sheath (DM) and decrease in mitochondrial density. Uranyl acetate: x 12740.

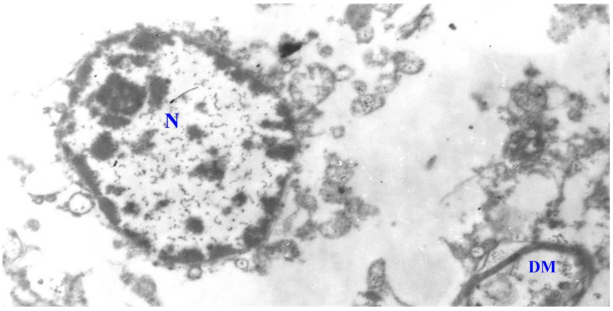


Figure.13
Brain: Transmission Electron Microscopy: Group II: Note margination and clumping of chromatin in nucleus and decrease in mitochondrial density. Uranyl acetate: x 9100.

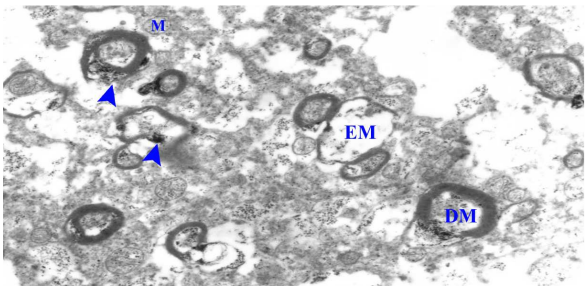


Figure.14
Brain: Transmission Electron Microscopy: Group II: Note degenerated myelin sheath (DM), empty myelin sheath (EM), interlaminar splitting (arrow heads) and decrease in mitochondrial density. Uranyl acetate: x 10920.

DISCUSSION

A significant (P<0.05) decrease in acetyl cholinesterase values was recorded in lead fed groups (Groups II and III) when compared to control rats. Where as in OS treated group of lower dose a significant improvement was noticed when compared to corresponding lead treated group. The present findings were in agreement with Reddy *et al.*[9] .In contrary increased values were observed by earlier author [10] in rat brain on giving 20mg / kg bwt / lead nitrate / I/P/ daily for 15 days. The changes might be due to neurotoxic effect of lead[9]. Lead alters certain membrane bound enzymes and leads to oxidative stress as lead crosses the blood brain barrier quite readily. Decreased acetyl cholinesterase values in lead toxicity indicate the reduction in cholinergic synapses in the brain[9] or alteration in Ach turnover rates and activities. Lead induced impairments in Ach / AchE system can be attributed to locomotor and cognitive dysfunction.

Dose and duration dependent changes were noticed in cerebrum and cerebellum of rats. Submeningeal hemorrhages, spongiosis, neuronal degeneration, perineuronal vacuolation, proliferation of capillaries, extensive demyelinated changes with few glial cell proliferation, atrophy of nerve cell, gliosis, edema, MNC infiltration and necrotic nodules were more prominent in lead treated groups. In cerebellum, spongiosus in grey matter of granular layer, shrinkage of purkinje cells, central chromatolysis and focal loss of purkinje cells were more evident. Rounding and tapering purkinje cells, demyelinating changes were conspicuous at 12th week. The authors [11-14] observed similar changes. The changes in present study might be due to neurotoxic effect of lead by altering certain membrane bound enzymes and might have led to oxidative stress[9] as lead crosses the blood brain barrier quite readily.

Swollen and vacuolated vascular endothelial cells, degenerated myelin sheath, empty myelin sheath, decrease in mitochondrial density with degenerative changes, magination and clumping of chromatin in nucleus and cytoplasmic vacuoles in the endothelial cells in brain were noticed in dose dependent manner. The similar findings were observed by earlier author[15] in brain. Mitochondria were highly susceptible to toxic injury of lead. The swollen mitochondria might be due to changes in osmolarity that leads to an influx of salts and water in to the inner mitochondrial membrane[16].

Ultrastructurally brain of lead treated groups revealed disorientation and fragmentation of

the mitochondrial cristae might indicate a special affinity for mitochondria membranes, which play a key role in the functional integrity of this organelle[17-18]. The marked ultra structural changes in the mitochondria due to lead intoxication suggest that cells with such a degree of mitochondrial injury are unable to perform efficient functions, especially oxidative phospharylation and ATP production[17].

The observations made in this study indicate that lead had many adverse effects on brain due to damage of endothelial cells of blood vessels and accumulation of lead in organs or soft tissue and cause oxidative damage in brain. *Ocimum* was given as 400 mg/ kg bwt / 3 days a week and at this dose *Ocimum* reduced the effects effectively in lower dose of lead treated group where as in higher lead dosed group the reduced effects were seen during final stages of experiment (8 to 12 weeks). Further research is needed to know molecular mechanism of neurotoxicity of lead poisoning and to establish the dose and molecular basis of the anti-toxic mechanism of *Ocimum* in experimental animals.

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