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PATHOLOGY

LEAD ACETATE INDUCED NEPHROTOXICITY IN WISTAR ALBINO RATS. A PATHOLOGICAL, IMMUNOHISTOCHEMICAL AND ULTRASRUCTURAL STUDIES

K.SUJATHA*, CH.SRILATHA, Y.ANJANEYULU[1], AND P.AMARAVATHI

Department of Pathology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, INDIA

[1.] Associate Professor, Department of Pathology College of Veterinary Science, SVVU, Hyderabad.

K.SUJATHA

Department of Pathology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, INDIA

ABSTRACT

Lead is a long – known poison of environment and industrial origin. Prolonged exposure damage 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 biochemical, oxidative stress, gross & histopathological changes, histochemistry, immunohistochemistry and ultrastructural changes were studied in kidney. Increased serum creatinine levels and decreased antioxidant enzymes (CAT, SOD & GPx) activity in lead treated animals. Histopathologically dose dependent changes were observed in kidneys and revealed severe degenerative changes, intertubular haemorrhages, atrophied and cystic glomeruli were observed in majority of higher doses of lead treated rats. Alkaline phosphatase activity was more in the treated groups of kidney. The number of apoptotic bodies in kidney diffusely increased in PCT epithelium against BAX and BCl2 markers. However, the altered creatinine and enzymatic, histopathological, histochemical, apoptotic bodies and ultrastrucural changes are dose dependent in the present investigation.

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KEY WORDS

Lead toxicity, Kidney, Oxidative stress, histopathology, histochemistry, Apoptosis.

INTRODUCTION

Lead is the most important poison to the farm animals. For farm animals significant pollution is likely to occur from lead mining, painted and metallic lead in storage batteries, licking paints / puttyans from rubbish dumps. Areas near lead industrial establishments may be enriched by aerial deposition as a consequence, soil pollution as well as quality deterioration of edible portions of vegetation can be produced due to metal enrichment. Animals by eating this vegetation can accumulate enough lead to produce clinical signs of lead poisoning. Young animals are usually poisoned when they lick painted pens, troughs etc because of allatrophagia. Chronic lead poisoning commonly seen in young children from sucking lead paint or lead toys, in workers engaged in painting and petroleum industries.

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[1]. Kidneys play a major role in the excretion of lead from the body[2] and higher content of lead has been estimated in renal tissue than in liver and brain of the lead intoxicated animals[3]. However, information available on changes in kidney function in lead toxicity in animals is meager. The present investigation was carried out to study nephrotoxicity of lead acetate rats.

MATERIALS AND METHODS

Studies were conducted on healthy adult male Wistar rats weighing more than 150 g. All the rats were housed comfortably in standard rat cages at 25^{o} ±1^{0} C and a 12:12 hour interval light / dark cycle throughout the experimental period of 12 weeks and provided *ad libitum* feed and water. The approval of the institutional animal ethics committee permission was obtained prior to commencement of the experiment. After 10 days of acclimatization the animals were randomly divided into six groups. Lead acetate was given orally after mixing in double distilled water to rats at the dose rate of 1/10 LD_{50} (60mg/ Kg b.wt / 3 days a week), 1/20 LD_{50} and (30 mg/ Kg b.wt / 3 days a week) respectively to the groups II and III. In addition to lead acetate, Group I was kept as control. Rats from each group were randomly sacrificed at fortnight intervals after starting the experiment *i.e.*, 2nd , 4th, 6th , 8th , 10th and 12th weeks necropsy done and kidneys were collected for oxidative stress, histopathological, histochemical, immunohistochemical and ultrastructural examination. For histopathological study kidney pieces were preserved in 10% neutral buffered formalin and processed by routine paraffin embedding method and stained with haematoxyline and eosin[4].

(i) Biochemical assay

Blood was collected from all groups at each sacrifice directly in to the sterile test tube and allowed to clot. The serum was collected and stored at 4^{0}C i.e. in a refrigerator until use and was used for the estimation of creatinine (Kamineni life sciences Pvt. Ltd) using kit.

(ii) Oxidative stress

At each sacrifice, tissue pieces of kidneys were collected and stored at –20^{0}C until use.

Tissue preparation:

Tissue pieces of each organ minced separately and homogenized in 0.05M ice cold phosphate buffer (pH 7.4) by using a virtis homogenizer to make 10% homogenate. The homogenate was mixed with 10% trichloroacetic acid in the ratio of 1:1, centrifuged at 15,000rpm for 60min at 4^0C and the supernatant obtained and was used for estimation of super oxide dismutase[5], catalase[6] and glutathione peroxidase[7] in Liver, kidney and testis of all animals in all groups.

(iii) Histochemistry

Pieces of kidney from both experimental and control groups were collected in to chilled neutral buffered formalin. Frozen sections were taken and the alkaline phosphatase activity in kidney was demonstrated[8]

(iv) Immunohistochemical staining.

Bcl2 / BAx markers were used to know the apoptosis in kidneys of both treated and non treated groups. For these immunohistochemical studies, the primary and secondary antibodies were procured from BioGenex Company.

Procedure:

Paraffin sections were cut at 3-4 u thickness and kept at 58^0 C for 2hrs.Deparaffinized through xylene -15 minutes for 2 changes and 2 alcohol dips to remove xylene. Washed under running tap water for 10min. Rinsed in distilled water for 5min.Kept in the citrate buffer for 20min (10min at medium power and 10min at high powering the micro oven).Cooled to the room temperature, kept in the distilled water for 5min and slides were kept in PBS for 5min.The slides were kept in the humid chamber and in the peroxidase block solution for 30min (To block the endogenous peroxidase).Washed in PBS for 5min x 3 changes. The power block solution was put on tissue section for 15min. Primary antibodies (BCl_2 / BAX) were added on the sections of liver and kidney and slides were kept in room temperature for over night. Washed in PBS for 5 min x 3 changes. Added super enhancer solution and the slides were kept in PBS for 5 min X 3 changes. Added secondary antibody with HRP (BCl_2/ BAX) for 30 min. Washed in PBS for 5 min x 3 changes. DAB colouring reagent was prepared by adding one drop of DAB in 1ml of substrate. The sections were kept in the colouring reagent for 5-8 min.Washed in PBS for 2 min and in tap water for 2 min. stained with Harris haematoxylin for 1min. Washed in tap water for 5 min, dried and mounted in DPX. **(Results:** Positive reaction was indicated by brown color development in the site and other sites were stained by Haematoxylin)

(v) 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[9].

RESULTS

1. Serum creatinine and oxidative damage:

Statistically, a significant ($P<0.05$) dose dependent increase in creatinine values and reduced antioxidant enzymes (catalase, superoxide dismutase and glutathione reductase) values was recorded in lead treated animals (Group II & III) when compared to control Mean and SE values of serum creatinine and different antioxidant enzyme levels of animals of different experimental groups were shown in Table I.

2. Histopathological changes:

Kidneys sections of the animals in group II & III revealed mild to moderate intertubular hemorrhages and extensive degenerative changes in tubular epithelial cells, focal inter tubular mononuclear infiltration, congested glomeruli, atrophy of glomerulus in focal areas and hyalinized and thickened blood vessels by the end of 2nd week in all animals. Animals exposed to lead for 4 to 8 weeks revealed glomerular endothelial cell vacuolation (Fig.1), atrophy of glomerular tuft (Fig.2), karyomegaly and hyper chromatic nuclei in tubular epithelial cells (Fig.3), hyperplasia of renal tubular epithelium (Fig.4) and desquamated renal tubular epithelium in focal areas in addition to above changes. In some areas, renal tubules showed mild vesicular fatty change in tubular epithelial cells and pockets of hemorrhages in cortical region (Fig.5), mild fibroblast proliferation in between tubules and severe desquamation of renal epithelial cells with lysis of tubules were noticed by the end of 10th week. In addition atrophied and cystic glomeruli (Fig.6), severe hemorrhages in cortex and medulla, proteinacious casts in tubules (Fig.7) severe necrotic changes and desquamated renal epithelial cells leaving basement membrane of tubules and severe intertubular mononuclear cell infiltration were more conspicuous in many animals by the end of 12th week as dose dependant manner.

Fig. 1. Kidney: Group II
Section showing glomerular endothelial cell vacuolation. H & E: x 280.

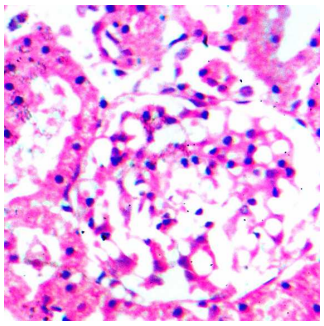


Fig. 2. Kidney: Group II
Note atrophy of glomerulous. H & E: x 70.

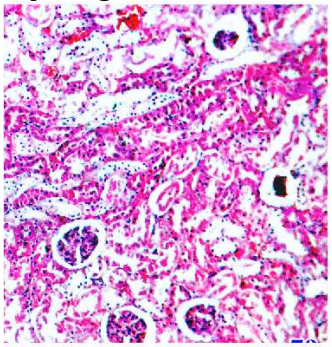


Fig. 3. Kidney: Group II
Note karyomegaly and hyperchromatic nuclei in tubular epithelial cells. H & E: x 700.

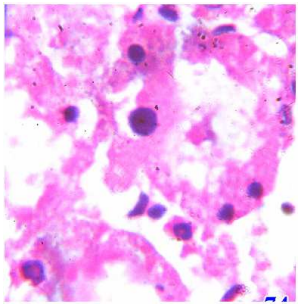


Fig. 4. Kidney: Group II
Note hyperplasia of renal tubular epithelium H & E: x 280.

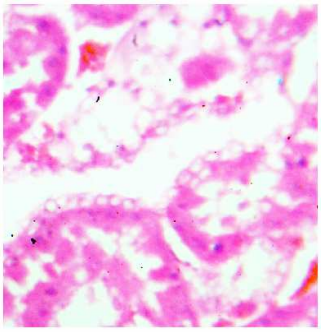


Fig. 5. Kidney: Group II
Section showing pockets of hemorrhages. H & E: x 70.

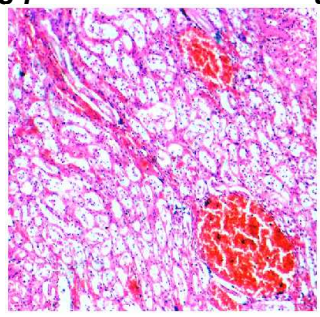


Fig. 6. Kidney: Group II
Atrophied and cystic glomereli H & E: x 280.

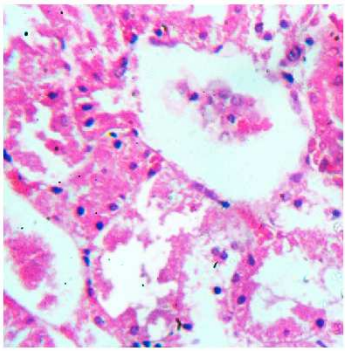
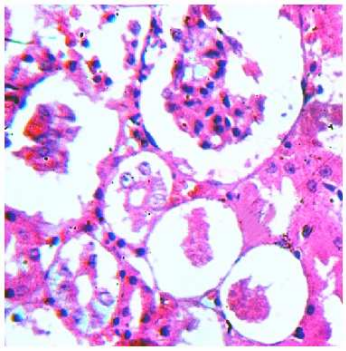


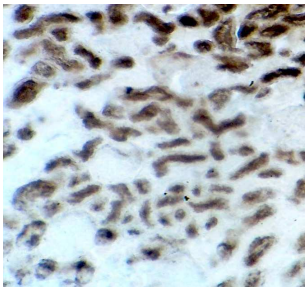
Fig. 7. Kidney: Group II
Proteinacious casts in renal tubules (arrows). H & E: x 280.



3. Histochemistry:

Alkaline phosphatase activity was detected in kidney by using Gomari method. In sections where brownish to black color develops indicate enzyme activity in the organ. In presented study, more intense reaction was observed in epithelial cells and basement membrane of peripheral proximal convoluted tubules of kidney (Fig.8) as dose dependent manner (Group II).

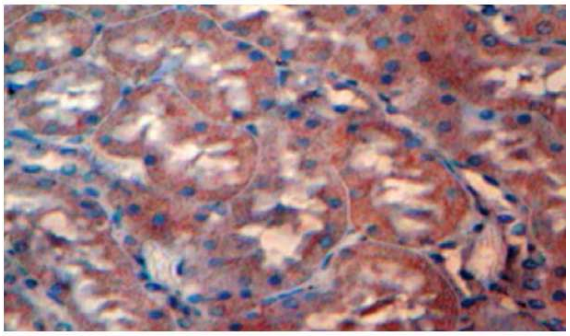
Fig.8.Kidney: Alkaline phosphatase: Group II:
Note intense reaction in epithelial cells and basement membrane of proximal convoluted tubules. Gomori stain: x 70.



4. Immunohistochemistry:

Immunohistochemisty was done for the detection of apoptotic bodies in kidney by using the monoclonal antibodies against BAX and BCl_2 antigens, which is expressed during apoptosis and site where the brown color develops indicates the presence of antigen. The BAX (or) BCl_2 antigen was detected in epithelial cells of proximal convoluted tubules (Fig. 9). Staining intensity was more pronounced in lead treated groups (Group II and III) as dose dependent manner.

Fig.9.Kidney.Immunohistochemistry:Group II
Increased reaction in epithelial cells of proximal convoluted tubules. BCl_2 Marker: x 70.



5. Ultra structural Studies:

Ultrastucturally kidneys of group II animals revealed reduced number of mitochondria in proximal convoluted tubules (PCT) when compared to control. Swollen mitochondria with rupture of the outer membrane and vacuolation of the inner compartment were conspicuous. Some of the damaged epithelial cells revealed complete distortion of cristae of mitochondria. Disruption, fragmentation and granulation of the cristae were most evident in lead treated group of higher dose. Numerous fragmented rough endoplasmic reticulum was observed in renal epithelium of PCT. Increased number of lysosomal related structures with in the epithelial lining cells in PCT and margination and clumping of nuclear chromatin were noticed in higher dose of lead treated group (Group II). Intranuclear electron dense lead inclusions of different sizes in PCT epithelial cells were noticed (Fig.10). The increased number of swollen and round mitochondria, increased number of lysosomes, fragmented endoplasmic reticulum was observed in group III (lower dose of lead treated group) animals.

DISCUSSION

An alteration in serum urea, uric acid and creatinine was observed in lead acetate administrated albino rats[10]. When lead acetate was given cross bred calves, it showed decreased levels in serum urea and creatinine level[11]. Increased SOD, CAT and GPX activity in renal cortex and medulla of rats was noticed during oral administration of lead acetate[12]. Lead is considered as a toxic metal, which reflects on human health. The earlier authors observed chronic lead exposure of residential and commercial painters shows decreased CAT, SOD, GPx and lipid peroxidation[13.] In the present study administration of lead acetate showed a dose dependant increase in the serum creatinine and reduction in antioxidant enzymes activity(CAT,SOD and GPx) (Table I). These changes could lead to the generation of reactive oxygen species (ROS) (or) by reducing the antioxidant cell defense systems by depleting glutathione (or) by inhibiting sulfhydryl dependent enzymes or by interfering with some essential metals (copper) needed for antioxidant enzyme activities[14] and by increasing cell susceptibility to oxidative attack by altering the membrane integrity and faulty acid composition[12.]

Table -1

Mean and S.E values of serum creatinine and antioxidant enzymes levels in animals of different experimental groups

	Group I	Group II	Group III
Serum creatinine**(mg/dl)**	2.67^{e} ±0.12	5.39^{a} ±0.60	4.03^{bc} ±0.37
Catalase (µg / g of protein)	126.43^{a} ±1.51	103.72^{e} ±4.11	114.25^{c} ±2.47
SOD(mg / g. of protein)	99.63^{bcde} ±0.40	93.51^{f} ±1.36	94.81^{def} ±1.13
GPx(µg / mg of protein)	28.23^{b} ±0.0.21	23.56^{f} ±0.95	25.05^{def} ±0.81

Mean and SE values with different superscripts differ significantly (P < 0.05)
ANOVA
S.E – Standard error

In present investigation by exposing the rat to the lead acetate several pathological changes were observed in kidney of rats at different doses (group II and III). Severe pathological changes were observed in higher dose rats. The kidney shows various pathological changes like thickened and congested blood vessels, pockets of intertubular hemorrhages (Fig.5), intertubular MNC infiltration, congested and atrophied glomeruli (Fig. 2), hyperchromatic nuclei (Fig. 3), hyperplasia of tubular epithelium (Fig.4), at places desquamated tubular epithelial cells. Proteinaceous casts in tubules (Fig.7) were more conspicuous in lead treated groups as a dose dependent manner. Earlier workers also mentioned histopathological changes caused by oral feeding of cross bred calves[15] and histopathologically congestion and degenerative changes in the liver, lung, kidney, brain and intestine and feeding of lead acetate causes severe congestion of intertubular blood vessels in renal cortex and degenerative changes in tubular epithelial cells[16]. Kidney is the one of the major target organ of inorganic lead. Lead was usually regarded as a protoplasmic poison. It binds sulfhydryl groups of essential enzymes of cellular metabolism. In tissues lead was found to be localized in the cytoplasm of capillary endothelium. The damage to other kinds of cells was attributed to the endothelial damage and circulatory deficiency[17].

The present study did not reveal any inclusion bodies in kidney of toxic groups (II & III). Earlier authors did not find any inclusion bodies in any of the buffaloes and cattle poisoned with lead[18] and the acid fast inclusion bodies were not always present and were not specific for lead poisoning[19].

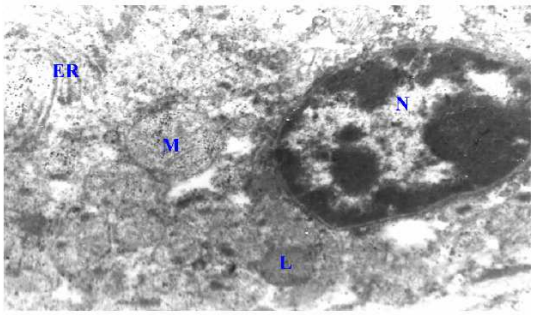
Alkaline phosphatase activity was found in kidney In the present study enzyme activity was more in tubular epithelial cells and basement membrane of proximal convoluted tubules of kidney (Fig,8) of lead treated groups in a dose dependent manner. The activity of alkaline phosphatase activity in the kidneys of both bovine fetuses and calves and a weak activity was noticed in the uriniferous tubules of fetuses and in calves. More activity at brush border of the epithelial cells of the proximal convoluted tubule and no change were found in glomerulus and distal convoluted tubule[20,21] observations made in this investigation might be due to damage of cell membrane of cells due to accumulation of lead or crossing of lead in the basement membrane of tubules.

In Present investigation the number of apoptotic bodies was increased in lead treated groups when compared to control. The number of apoptotic bodies in kidney diffusely increased in PCT epithelium against BAX and BCl2 markers (Fig.9). A significantly increased apoptotic bodies in 7-8 weeks old male Wistar rats were observed after intra-peritoneal injection of CCl_4[22]and oral administration of lead cause significant increase in P_{53} and BAX expressions in mice model [23]. It was strongly suggest that lead may induce oxidative stress and changes the expression of apoptosis related proteins in mouse liver.

In the present investigation Ultrastructurally, kidneys of lead treated groups revealed swollen mitochondria with rupture of the outer membrane and vacuolation of inner compartment, complete distortion of cristae of mitochondria in damaged epithelial cells, fragmentation of mitochondria, fragmented endoplasmic reticulum, increased number of lysosomes,clumping of nuclear chromatin and Intranuclear irregular shaped electron dense lead inclusions of different sizes in PCT epithelial cells (Fig.10) were noticed in dose dependent manner[24]. The earlier author[25] fed the four week old male broiler chicks with lead acetate studied electron microscopy of peripheral RBC and kidneys. Various stages of mitosis in erythrocytes and irregular shaped, electron dense inclusions in nuclei of epithelial cells of kidney were observed. When administration of lead acetate in to mice model ultrastructurally revealed that constant intranuclear inclusions in the cytoplasm of renal and endothelial cells[26,27].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[29].The disorientation and fragmentation of the mitochondria cristae might indicate a special affinity for mitochondria membranes, which play a key role in the functional integrity of this organelle[28,29] . 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[28]

Fig.10.Kidney: Transmission Electron Microscopy: Group II
Note swollen mitochondria (M), increased number of lysosomes (L) and margination of nuclear chromatin (N) and intranuclear lead inclusions (arrow).Uranyl acetate: x 10920.



CONCLUSIONS

In the present investigation revealed that different doses of lead acetate can cause a dose dependant kidney toxicity. Lead induces oxidative damage by reducing anti defense mechanism at cellular level and it causes increased apoptotic bodies in proximal convoluted tubules and due to accumulation lead in the cells cause severe damage to mitochondria and the cells unable to perform normal functions may lead to oxidative stress In the present experimental study revealed that lead is a powerful nephrotoxic and damage was more pronounced at cellular level in dose dependent manner.

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