Protective and Therapeutic Effect of Boerhaavia Diffusa L. on Fluoride-Induced Ultrastructural Changes in Kidney of Rats

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Abstract:
The present study evaluated the possible protective effects of Boerhaavia diffusa L. against the toxicity of sodium fluoride in kidney of rats. Rats were divided into eight groups. Group I received 1ml double distilled water/kg b.w./day orally daily by a gastric tube for 40 days. Group II and Group III were treated with sodium fluoride 300 and 600 mg/kg b.w./day. Group IV positive control were orally administrated with 500mg/kg b.w./day of leaf extract of Boerhaavia diffusa L. for 20 days. Group V and VI were pretreated with 500 mg/kg b.w./day of leaf extract of Boerhaavia diffusa L. for 20 days followed by sodium fluoride treatment 300 and 600 mg /kg b.w./day. Group VII and VIII were post-treated with 500mg/kg b.w. /day of leaf extract of Boerhaavia diffusa L. for 20 days after sodium fluoride treated 300 and 600 mg /kg b.w./day. All groups were allowed free access to standard rat chow and water through the period of experiment. The electron microscopic, ultrastructural features of fluoride nephrotoxicity were degeneration of the tubular epithelial cells, reduction of cellular and nuclear sizes, nuclear membrane blebbing, irregular nucleus, massive loss of most cytoplasmic organelles, large number of lysosomes, swollen and damaged mitochondria, mitochondrial vacuolation, myeloid bodies, thickened cell basement membrane, and loss of microvilli, degeneration filtration barrier, constriction, and necrosis of podocyte memberane. The post-treatment with Boerhaavia diffusa L. leaf extract revealed that the kidney was better preserved when compared with group II and III. Improvement was also noticed in groups pre-treated with Boerhaavia diffusa L. leaf extract but not the same degree like post-treated group. In the current study, however, the post-treatment scored effectiveness more than pre-treatment.

Keywords: Albino rats, Boerhaavia diffusa L., Nephrotoxicity, Sodium fluoride, Transmission electron microscopy.

Introduction:
Excessive fluoride intake over a long period of time result in a serious public health problem called fluorosis, which is characterized by dental mottling and skeletal manifestations in the form of crippling deformities, osteoporosis, and osteosclerosis [1]. The kidneys perform the most excretory processes in the human body, hence become the prime target organ for various circulating toxins that may cause nephrotoxicity. Kidney is one of the target organs attacked by excessive amounts of fluoride. Abnormal function, metabolism, and histopathological changes have been observed in this organ with fluoride toxicity [2]. Many studies have shown that elevated concentrations of fluoride can occur in kidney as it has a major route for removal of fluoride from the body [3]. Fluoride-induced nephrotoxic changes in the glomeruli and tubules of experimental animals have been reported earlier [4].

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*Boerhaavia diffusa* L. (*Nyctaginaceae*) is an important medicinal plant much used in Ayurveda, Unani medicines, and other traditional medicines in many parts of the world. The plant is commonly known as *punarnava* in Ayurveda because the top of plant dies during hot summer and put forth fresh shoots after rains and is believed to be a rejuvenator [5]. Pharmacological studies have demonstrated that *Boerhaavia diffusa* L. exhibits a wide range of properties such as diuretic, antiurathiatic, antioxidant, and anti diabetic activity. Flavonoids and other antioxidant constituent of this medicinal plant have been reported to inhibit xenobiotic induced nephrotoxicity in experimental animal models due to their potent anti-oxidant effects [6]. The aim of the present manuscript was to elucidate the nephroprotective role of leaf extract of *Boerhaavia diffusa* L. against sodium fluoride induced nephrotoxicity in experimental model.

**Materials and Methods:****

**Animals:** Young Wistar albino rats, weighing between 100-200gm were housed in polypropylene cages with stainless grill tops and fed with standard rat pellet diet (Hindustan lever limited, India) and water was given *ad libitum*. Animals were maintained at a constant room temperature of 20-22°C and 60% humidity. The experiments were performed under the approval of the animal ethical committee of Punjabi University, Patiala (Animal Maintenance and Registration No. 107/99/ CPCSEA /2014-23).

**Experimental design:** Rats were allowed a 2-week acclimatization period and then they were divided randomly into eight groups (6 rats each). Group I received 1 ml double distilled water/kg b.w./day orally daily by a gastric tube for 40 days. Group II and Group III were treated with sodium fluoride 300 and 600 mg/kg b.w./day for 40 days. Group IV antidote control group was orally administrated with 500mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L. for 20 days. Group V and VI were pretreated with 500 mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L. for 20 days followed by sodium fluoride in the concentration of 300 and 600 mg /kg b.w./day for 40 days. Group VII and VIII were post-treated with 500 mg/kg b.w./day *Boerhaavia diffusa* L. leaf extract for 20 days after sodium fluoride in the concentration of 300 and 600 mg /kg b.w./day for 40 days. The control and experimental animals were sacrificed under ether anesthesia. The kidney tissue was dissected out, was had in normal saline and processed for ultrastructural examination. The leaf extract of *Boerhaavia diffusa* L. was prepared by the method of Narendrikhanan et al. [7].

**Preparation for transmission electron microscopy:**

The kidney tissues were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 4-6 hours at 4°C by the method of Karnovsky [8]. The tissues were subsequently postfixed in 1% osmium tetraoxide for 1 hour, dehydrated in acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK) cut with an ultramicrotome (Leica Ultracut UC7, Austria), stained with aqueous toluidine blue. The ultra-thin sections with 8% uranyl acetate of Venable and Coggeshall [9]. All the studies were performed under a Tecnai G2 20 high resolution transmission electron microscope (Fei Company, The Netherlands) at an operating voltage 200 kV. A CCD camera (Megaview III, Fei Company) using TIA software attached to the microscope digitally acquired images. The primary magnification of the Electron Microscopic examination was X1100, X1550, X2100, X2550 for renal tubules and renal glomeruli.

**Results**

**Electronic microscopic finding of the kidney**

The kidney in control rat consisted of tubules and renal corpuscle with normal appearance.

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**Figure 1:** Electron micrograph of ultrathin section of kidney of control rat showing basement membrane (bm), microvillar brush border (mb), large oval nucleus (N) and numerous elongated mitochondria (M). X 1100

**Figure 2:** Magnified view of ultrathin section of kidney of control rat showing basement membrane (bm), microvillar brush border (mb), numerous rounded and elongated mitochondria (M). X2100

**Figure 3:** Magnified view of ultrathin section of kidney of control rat showing numerous elongated and rounded mitochondria (M) with highly dense matrix and closely packed cristae. X2550.
The renal tubules has epithelial cells rests on a well-developed less electron dense thick basement membrane. The electron dense cytoplasm contained large oval nucleus with a distinct peripheral nucleolus, margined heterochromatin surrounded by nuclear membrane (Fig.1). The well-developed interlacing microvillar brush border was visible (Fig.2). Numerous elongated and rounded mitochondria were seen dispersed in the supranuclear and basal cytoplasm (Fig.3).

In control rat, renal corpuscle showed glomerular capillary loops with lumen containing red blood cells and mesangial cell with its nucleus and electron dense cytoplasm. The podocyte exhibited highly indented nucleus and electron dense cytoplasm. The foot processes of the podocytes were regularly arranged on capillary surface and were separated from the podocyte and their primary cell processes by a clear subpodocyte urinary space. The endothelial cell lined the capillary wall from inside and its nucleus was situated towards the mesangial matrix. Its cytoplasm was adhered to the glomerular basement membrane (Fig.4).

In rats treated with 300 mg/kg b.w./day sodium fluoride for 40 days, renal tubular epithelial cells revealed large irregular nucleus, vacuolated cytoplasm and few lysosomes. The mitochondrial vacuolation and moderate numbers of damaged and disfigured mitochondria were observed around the nucleus and in the basal cytoplasm (Fig. 5). The extrusion of the lysosomes, cellular debris and large packages of cytoplasmic material into the tubular lumen were prominent. Many mitochondria within extruded cytoplasmic packages appeared to be still morphologically intact (Fig.6). The apoptotic cells were visualized through dilation of the nuclear envelope forming membrane blabbing (Fig.7). The glomerular basement membrane was thickened, wrinkled and contain granular debris. There was extensive focal fusion of foot processes and distorted fenestrated epithelium (Fig. 8).

![Figure 4](image1.png)
![Figure 5](image2.png)
![Figure 6](image3.png)
![Figure 7](image4.png)

**Figure 4**: Electron micrograph of ultrathin section of kidney of control rat showing glomerular capillary loop (CL) containing red blood cell (RBC), the podocyte (P) with highly indented nucleus(N), foot processes of the podocyte (P) are regularly arranged, mesangial cell (mc), endothelial cell (ec) and urinary space (S) are also visible. X1550

**Figure 5**: Electron micrograph of ultrathin section of kidney treated with 300 mg/kg b.w./day of NaF showing large irregular nucleus (N), numerous lysosomes (Ly), irregularly arranged swollen mitochondria and damaged mitochondria (M), mitochondrial vacuolation (mv) and destructed microvillar brush border (mb). X1100

**Figure 6**: Electron micrograph of ultrathin section of kidney treated with 300 mg/kg /b.w./day of NaF showing irregular nucleus (N), numerous lysosomes (Ly), lumen of tubule fill with cellular debris and damaged mitochondria (M). X1550

**Figure 7**: Electron micrograph of ultrathin section of kidney treated with 300mg/kg b.w./ day of NaF showing nuclear membrane blebbing. X2550
Figure 8: Electron micrograph of ultrathin section of kidney treated with 300 mg/kg b.w./day of NaF showing wrinkled basement membrane (bm) and fused foot processes (FP). X2100

Figure 9: Electron micrograph of ultrathin section of kidney treated with 600 mg/kg b.w./day of NaF showing large irregular nucleus (N), numerous lysosomes (Ly), irregularly arranged swollen mitochondria (M), mitochondrial vacuolation (mv) and destructed microvillar brush border (mb). X1100

Figure 10: Electron micrograph of ultrathin section of kidney treated with 600 mg/kg b.w./day of NaF showing irregular nucleus (N) and damaged mitochondria (M). X2550

Figure 11: Electron micrograph of ultrathin section of kidney treated with 600 mg/kg b.w./day of NaF showing thick basement membrane (bm), membranous whirlung finger print like appearance. X2500

The ultrastructural changes were much more pronounced in rats treated with 600 mg/kg b.w./day sodium fluoride for 40 days. The renal tubular epithelial cells showed irregular nucleus, swollen and damaged mitochondria and loss of apical interlacing microvillar brush border (Fig.9). The irregular nucleus and scanty numbers of mitochondria were observed severely damaged with partially deteriorated cristae or with flocculent dense matrix. In many of the affected mitochondria, there was a loss of cristae structures due to cristolysis and only metrical flocculent densities were detected (Fig.10). The extensive proliferation and swirling of basal infoldings was found in renal tubules (Fig.11). The unicentric and multicentric myeloid bodies were visible (Fig.12).

Figure 12: Magnified view of an electron micrograph of ultrathin section of kidney treated with 600 mg/kg b.w./day of NaF showing membranous whirlung finger print like appearance. X2500.

Figure 13: Electron micrograph of ultrathin section of kidney treated with 600 mg/kg b.w./day of NaF showing fused or fragmented foot processes (FP) and injured podocytes (P). X2100
The glomerular capillary wall lined by fenestrated endothelium was severely damaged. From the outer surface of the glomerular capillary wall, the podocytes were severely injured, losing their nuclei and most of their cytoplasmic organelles. Very few foot processes appeared normal projecting through urinary space but others showed fragmentation or fusion and some of them completely lost (Fig.13).

In rats treated with leaf extract of *Boerhaavia diffusa* L. (500 mg/kg b.w./day), the renal tubular epithelial cells were lined by cuboidal cells having intense cytoplasm due to high content of organelles. The nucleus was spherical and centrally located. Patches of heterochromatin were observed at the nuclear envelope. Numerous elongated mitochondria was filled the perinuclear and subnuclear cytoplasm with highly dense matrix and closely parallel cristae. The apical cell membrane was occupied by numerous microvilli (Fig.14).

The renal corpuscles showed glomerular basement membrane and podocytes surrounded the capillaries. Each capillary loop was lined by endothelial cells. Podocytes give rise to primary processes which in turn form numerous secondary foot processes or pedicels that rest on thin basal lamina. Each podocyte contained large nucleus and abundant cytoplasm. The pedicels were separated by split pores having split membranes. The filtration barrier was consisted of capillary endothelial inner layer, thin glomerular basement membrane and podocyte layer (Fig.15).

In rats pre-treated with leaf extract of *Boerhaavia diffusa* L.(500mg/kg b.w./day) for 20 days followed by 300 mg/kg b.w./day of sodium fluoride for 40 days, showed wrinkled and distortion of cell basement membrane. The rounded nucleus, numerous basal arranged mitochondria and well-developed apical interlacing microvillar brush border were similar to control (Fig.16). Another degree of degeneration of tubular epithelial cell basement were recorded as irregular (Fig.17). The podocytes showed a normal structure and an orderly arranged and resembled those of control rat. Foot processes seems to be thinner and longer. The capillary lumen exhibited marked reduction of basement membrane thickening as compared to sodium fluoride treated rat (Fig.18).

**Figure 14:** Electron micrograph of ultrathin section of kidney 500mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L. showing thick basement membrane (bm), numerous rounded and elongated mitochondria (M), large rounded nucleus (N) and microvillar brush border (mb). X1550

**Figure 15:** Electron micrograph of ultrathin section of kidney 500mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L. showing glomerular basement membrane (GBM), podocytes (P), foot processes (FP), capillary loop (CL) and red blood cells (RBCs). X1550

In rats pre-treated with leaf extract of *Boerhaavia diffusa* L.(500mg/kg b.w./day) for 20 days followed by 300 mg/kg b.w./day of sodium fluoride for 40 days, showed wrinkled and distortion of cell basement membrane. The rounded nucleus, numerous basal arranged mitochondria and well-developed apical interlacing microvillar brush border were similar to control (Fig.16). Another degree of degeneration of tubular epithelial cell basement were recorded as irregular (Fig.17). The podocytes showed a normal structure and an orderly arranged and resembled those of control rat. Foot processes seems to be thinner and longer. The capillary lumen exhibited marked reduction of basement membrane thickening as compared to sodium fluoride treated rat (Fig.18).

**Figure 16:** Electron micrograph of ultrathin section of kidney pre-treated with 500/ kg b.w./day leaf extract of *Boerhaavia diffusa* L. before 300 mg/kg b.w. of NaF treated rat showing irregular thickening of basement membrane (bm), rounded or elongated mitochondria (M) in cytoplasmic vacuole, rounded nucleus (N) and well developed microvilli brush border (mb). X830

**Figure 17:** Magnified view of an electron micrograph of ultrathin section of kidney pre-treated with 500/ kg b.w./day of leaf extract of *Boerhaavia diffusa* L. before 300 mg/kg b.w. /day of NaF showing irregular thickening of basement membrane (bm), rounded or elongated mitochondria (M) in cytoplasmic vacuole, rounded nucleus (N), well developed microvilli brush border (mb). X1550
In rats pre-treated with leaf extract of *Boerhaavia diffusa* L. (500 mg/kg b.w./day) for 20 days followed by 600 mg/kg b.w./day of sodium fluoride for 40 days, remaining architecture of renal tubule retained their normal appearance except for loss of microvilli (Fig. 19). The renal glomeruli has glomerular basement membrane that contained less fused processes as compared to 600 mg/kg b.w./day sodium fluoride treated alone group (Fig. 20).

Figure 18: Electron micrograph of ultrathin section of kidney pre-treated with 500/ kg b.w./day of leaf extract of *Boerhaavia diffusa* L before 300 mg/kg b.w./day of NaF showing glomerular basement membrane (GBM), Podocytes (P), foot processes (FP), capillary loop (CL) and red blood cells (RBC). X 1550

Figure 19: Electron micrograph of ultrathin section of kidney pre-treated with 500/ kg b.w./day of leaf extract of *Boerhaavia diffusa* L before 600 mg/kg b.w./day of NaF showing thickening of basement membrane (bm), preservation of rounded or elongated mitochondria (M), rounded nucleus (N) and loss of microvilli brush border (mb). X 830

Figure 20: Electron micrograph of ultrathin section of kidney pre-treated with 500/ kg b.w./day of leaf extract of *Boerhaavia diffusa* L before 600 mg/kg b.w./day of NaF showing glomerular basement membrane (GBM), podocytes (P), foot processes (FP), and fragmented parts (F). X 2100

Figure 21: Electron micrograph of ultrathin section of kidney of rat treated with 300 mg/kg b.w./day of NaF for 40 days followed by 500 mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L for 20 days showing thickening of basement membrane (bm), preservation of rounded or elongated mitochondria (M), rounded nucleus (N) and microvilli brush border (mb). X 830

Figure 22: Electron micrograph of ultrathin section of kidney of rat treated with 300 mg/kg b.w./day of NaF for 40 days followed by 500 mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L for 20 days showing glomerular basement membrane (GBM), podocytes (P) and foot processes (FP). X 830

Figure 23: Electron micrograph of ultrathin section of kidney of rat treated with 600 mg/kg b.w./day of NaF for 40 days followed by 500 mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L for 20 days showing thickening of basement membrane (bm), preservation of rounded or elongated mitochondria (M), rounded nucleus (N) and loss of microvilli brush border (mb). X 1550
In rats post-treated with leaf extract of *Boerhaavia diffusa* L. (500mg/kg b.w./day) for 20 days after 300 mg/kg b.w./day of sodium fluoride for 40 days, showed preservation of cell architecture containing intact basement membrane, enlarged round and elongated mitochondria, round nucleus and well-developed interlacing microvilli brush border. The less number of cytoplasmic vacuoles and lysosomes were observed as compared to sodium fluoride treated group (Fig.21). The glomeruli has viable podocytes and foot processes (Fig.22).

![Figure 24: Electron micrograph of ultrathin section of kidney of rat treated with 600 mg/kg b.w. /day of NaF for 40 days followed by 500mg/kg b.w./day of leaf extract of *Boerhaavia diffusa* L. for 20 days showing glomerular basement membrane (GBM),less fused foot processes (FP) and podocytes (P). X1550](image)

In rats post-treated with leaf extract of *Boerhaavia diffusa* L.(500mg/kg, b.w./day) for 20 days after 600 mg/kg b.w./day of sodium fluoride for 40 days, showed intact basement membrane, dense clusters of rounded or enlarged mitochondria and loss of microvilli brush border (Fig.23). The glomerular basement membrane with less numbers of fused foot processes or no fragmented parts was seen as compared to group III (Fig.24).

**Discussion:**

In this study, the ultrastructural renal abnormalities were an immediate response to acute fluoride exposure with signs of cellular injury. Both glomeruli and tubules were sensitive to acute fluoride toxicity. The glomerular ultrastructure changes included loss of normal organization of fenestrae, widening of some pedicels and loss of the homogenous appearance of basement membranes displaying thickening in many areas. These findings are in accord with previous fluorosis reports [10,11] which stated that glomeruli showed increased glomerular basement membrane thickening and irregular foot processes on acute sodium fluoride exposure. In addition, similar changes have also been noted in kidneys of experimental animals upon exposure to other trace metals such as cadmium by Moneim and Said [16], and Moneim [17]. In this work, renal tubular cells exhibited gradual loss of their microvilli with time of exposure. This was in same line of Condron *et al.* [18] who stated that, cadmium could reduce the surface density of microvillus membrane of convoluted tubules per unit cell volume to 19 % in cadmium contaminated rats. Another outstanding abnormality seen in the tubular cells was the numerous lysosomes seen in animal group II and III. The functions of lysosomes organelle is known to be concerned with the segregation and degradation of substances taken up by cells from the environment as well as of cytoplasmic constituents. At any rate, it is possible that the observed increase in this organelle is connected with the sequestration and excretion of fluoride given. The increase in lysosomes may be a result of the attempt to digest the toxic substance, and this was considered a general manifestation of injury. The sequestration of damaged organelles in lysosomes is a mechanism of cellular repair and follow all types of sublethal injury [19].

Another important abnormality seen in the renal tubular cells was appearance of more vesicular and swollen mitochondria. These results were in agreement with previous fluorosis reports in rats [20].

In the present study, ultrastructural examination of the kidney of rats treated with sodium fluoride showed increased number of lysosomes, vacuolated cytoplasm, mitochondrial vacuolation, presence of tubular casts, swollen mitochondria, damage and disfigured mitochondria, irregular nucleus, unicentric and multicentric myeloid bodies, whirling of the basal infoldings, nuclear condensation with nuclear membrane bleb formation and loss of microvilli brush border. These findings were in agreement with...
previous fluorosis reports concerning mice, [15] who reported cytoplasmic vacuolation, distorted foot processes and thick glomerular basement membrane. Similar changes also agree with the findings of Chang [21] who recorded the effect of chronic exposure of halothane on the kidney of rats and reported extensive proliferation and swirling of the basal infoldings in renal tubules. Haughton et al.[22] demonstrated the effect of gentamycin on kidney of rat and reported swollen mitochondria, unicentric and multicentric myeloid bodies. Neto et al. [23] studied protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide and observed numerous vacuolization and loss of microvilli. Adewole and Ojewole [24] reported molecular and ultrastructural changes in the proximal tubules of Wistar rats treated with streptozotocin and artocarpus communis forst (Moraaceae) root bark extract and found extensive fusion of foot processes, thickening of glomerular basement membrane. Abdel-Moneim and Said [16] assessed the acute effect of cadmium treatment on the kidney of rats and reported loss of brush border, swollen mitochondria and increased number of lysosomes. Al-Kahtani [14] evaluated renal damage mediated by oxidative stress in mice treated with aluminium chloride: protective effects of taurine and reported mitochondria with loss of cristae structure due to cristolysis, foot processes lost leaves their fragmented parts. El-Gerbed [25] determined protective effect of lycopene on deltamethrin-induced histological and ultrastructural changes in kidney tissues of rats and reported swelling in mitochondria with loss of cristae with an increase in number of lysosomes, numerous vacuoles, and disappearance of brush border.

In the current study, there was a considerable amelioration in kidney cortex damaging effect of sodium fluoride when leaf extract of Boerhaavia diffusa L. given concurrently with sodium fluoride. At the electron microscope level, this amelioration was in form of less noticeable cytoplasmic vacuolation and distortion in the glomerular and tubular cells, lacking evidence of major morphological injury, reduction in the inflammatory cell infiltration and preservation of the micro architecture were also observed. These ultrastructural findings were in the same line with the study that compared cadmium intake with Nigella sativa [26,27].

Conclusion:
The present investigation demonstrated that sodium fluoride treatment induced renal damage and pre, post-treatment with leaf extract of Boerhaavia diffusa L. provided protective effect against this sodium fluoride-induced nephrotoxicity in rats.

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