Histological and Ultrastructure Study of Toxic Effect of Sodium Fluoride on the Renal Cortex of Adult Albino Rats and the Possible Role of Calcium Therapy

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ABSTRACT

Aim of the Study: to evaluate the toxic effects of sodium fluoride on renal cortex of adult albino rat and to investigate the possible protective effects of calcium during fluoride exposure, if it is protective or not?

Material and Methods: 18 adult albino rats were used for this study. These animals were equally divided into three groups as follows: Control group: animals were received tap water and balanced diet, NaF treated group: animals were administrated sodium fluoride (NaF) at dose, (30 mg /kg/day) and NaF and Ca treated group: animals were treated with NaF (the same dose as mentioned before) and calcium chloride (CaCl) (20 mg /kg/day) orally by gastric feeding tube for 6 weeks. At the end of the experimental period, the rats were sacrificed and the kidney was rapidly removed and was utilized for light and electron microscopic examinations. Also blood samples from each group were collected for performing renal function tests by estimations the levels of serum urea and creatinine (mg/dl) and analyzed statistically using ANOVA (analysis of variance) test. The Results: by light microscope, in the NaF treated group, there was significant morphological damage to the renal cortex by exposure to NaF including; different forms of glomerular degeneration (marked lobulation, marked hypertrophy or shrinkage with expanded Bowman’s capsule) and massive degeneration of renal convoluted tubules with their epithelium (marked dilatation of some tubular lumens, sloughing of tubular epithelial cells, cell swelling, vacuolar degeneration and lysis of their cytoplasm with loss of their organelles). However, in the NaF and Ca treated group, Calcium failed to restore the renal damage induced by NaF exposure and still there were extensive degenerative changes on the renal cortex. However, by electron microscope, the most important morphological features of fluoride nephrotoxicity were a degeneration of kidney's filtration barrier, constriction and congestion of Glomerular capillaries, Mesangial hypercellularity, Mesangial matrix expansion and necrosis of podocyte. There also were different degree of degeneration of the tubular epithelial cells ranging from moderate to massive cellular destruction (reduction of the cellular and nuclear sizes, apoptosis, complete loss of the microvillar brush border, massive loss of most of cytoplasmic organelles and distortion of the cell basement membrane). However, the NaF and Ca treated group showed more excessive degenerative changes in Glomerulus more than that of NaF treated group manifested by marked swelling of the whole endothelial lining of the Glomerular capillary wall and injurious appearance of the podocyte with effacement of their foot processes, while, showed similar degenerative changes in proximal epithelial cells like that observed in NaF treated group. The Biochemical results showed significant difference between the three groups (P<0.05). Serum urea and creatinine levels were increased in NaF treated group (31.4±8.2 and 0.8±0.2 respectively), as compared to the control group (16.4±6.4 and 0.6±0.2 respectively). While in NaF and Ca treated group, the levels of Serum urea and creatinine were more greatly increased (52.6±11.6 and 1.4±0.3 respectively), if compared to the two previous groups. Conclusion: This work showed that sodium fluoride administration induced morphological degenerative changes and impairment on the renal cortex. Calcium administration not only failed to restore renal damage induced by NaF exposure but also enhanced the fluoride-induced toxicity.

Key words: kidney, fluoride, calcium

INTRODUCTION:

Fluoride is an essential trace element for human beings and animals. Fluoride intake can be either by ingestion or inhalation. Fluoride is abundant in the environment and exists only in combination with other elements as fluoride compounds, which are constituents of minerals in rocks and soil.
Sources of fluoride include natural fluoride in foodstuffs and water. Fluoride is also found in many common household products, including toothpaste, glass-etching, chrome-cleaning agents and insecticide (Chinoy and Sequeira, 1998; Wang et al., 2000 and Guney et al. 2007). A fluoride low concentration have a remarkable prophylactic influence on the dental system by inhibiting the dental caries and confers protective effects against mineral dissolution, with important implications for animals and humans demineralizing diseases (Machoy, 1991 and Warren and Levy, 2003). On the other hand, excessive fluoride intake over a long period of time may result in a serious public health problem called fluorosis, which is characterized by dental mottling and skeletal manifestations such as crippling deformities, osteoporosis, osteosclerosis and restricted movements of joints (Reddy et al., 2003 and Bailey et al. 2006), muscular weakness, convulsions followed by respiratory and cardiac failure and death (Korkmaz, 2000), and apoptosis in human epithelial lung cells (Thrane et al., 2001). It is now recognized that fluoride also affects cells from soft tissues, i.e., renal, endothelial, gonadal, and neurological cells [Barbiera et al. 2010].

The kidney is a well recognized organ to be affected for its histopathological and functional responses to excessive amounts of fluoride. Many studies have shown that elevated concentrations of fluoride can occur in the kidney as it has a major route in removal of fluoride from the body (Hodge and Smith, 1965; Ehrnebo and Ekstrand, 1986; Whitford, 1996; Shashi et al., 2002 and Inkielewicz and Krechniak, 2003). Fluoride nephrotoxicity causes pathological changes in the glomeruli and in the proximal, distal, and collecting tubules of experimental animals (Bouaziz et al. 2007).

Chinoy and Sharma (2000) stated that by the cessation of NaF-treatment was not conducive to bring a complete recovery from fluoride toxicity. However, the administration of ascorbic acid (AA) and calcium phosphate (Ca) to NaF-treated mice revealed significant recovery from fluoride toxicity. In contrast, Borke and Whitford (1999) suggested that chronic Fluoride ingestion may affect Ca++ homeostasis and decreases Ca++ uptake by rat kidney membranes.

The aim of this study was to evaluate toxic effects of fluoride on renal cortex of albino rat by using histological and ultrastructural study and to investigate the possible protective effects of calcium supplementation during fluoride exposure, if it is protective or not?

**MATERIAL AND METHODS:**

**Chemicals:** All chemicals as sodium fluoride (NaF) and calcium chloride (CaCl) were obtained from sigma chemical company.

**Experimental Animals:** 18 adult albino rats, 60-day-old (weighing 250-300g) were obtained from laboratory animal farm, Faculty of Veterinary medicine, Zagazig University. The animals were kept under standard laboratory conditions at 21± 2 °C, fed with balanced diet and water ad-libitum and exposed to 12h light / 12 h dark cycle for one week prior to the start of the experiments.

These animals were equally divided into three groups, every group contained 6 animals and was used as follows: Control group: animals were received tap water and balanced diet. NaF treated group: animals were administrated sodium fluoride (NaF) orally by gastric feeding tube at dose, (30 mg /kg/day) for 6 weeks. NaF and Ca treated group (n=6): animals were treated with sodium fluoride (NaF) (the same dose as mentioned before) and calcium chloride.
(CaCl) (20 mg /kg/day) at the same time by gastric feeding tube for 6 weeks. Dose selection of NaF and CaCl was based on the published studies (Das et al. 2006 and Guney et al. 2007). At the end of the experimental period, the rats were sacrificed and the kidney was rapidly removed and dissected out carefully, blotted of blood, and was utilized for light and electron microscopic examinations.

**Biochemical assays:** after sacrificing the rats, blood samples from each group were collected for renal function tests by estimations the levels of serum urea and creatinine (mg/dl).

**Preparation for Light microscopy:**
The kidney specimens from each experimental group were fixed in buffered neutral formalin. After routine histological laboratory procedures, tissues were blocked in paraffin and sections of 5 μm were cut and stained with haematoxylin and eosin and then examined in the Department of histology, Faculty of Medicine, Zagazig University.

**Preparation for Transmission electron microscopy:**
Small pieces from fresh kidney specimens, (about one cubic millimeter in size) from each experimental group were immediately fixed in 2.5 % glutaraldehyde in phosphate buffer (pH 7.2) for 24 hours. The specimens were then washed thoroughly in the buffer and post-fixed in 1% cold osmium tetroxide for one hour, dehydrated in graded alcohol, cleared in acetone and embedded in Epon. Semi-thin sections about 1 μm in thickness were stained with 1% toluidine blue. These sections were used to select regions for electron microscopy. Ultra-thin sections about 100 nm in thickness were cut from the selected regions, mounted on copper grids stained with uranyl acetate and lead citrate, for electron microscopic examination in faculty of science Ein-Shams University.

**Statistical analyses:** Data were analyzed using SPSS 17.0 for windows. Significance was calculated using ANOVA (analysis of variance) test. P<0.05 was considered statistically significant.

**RESULTS:**

1-Biochemical results showed significant difference between the three groups (P<0.05). Serum urea and creatinine levels were increased in NaF treated group (31.4±8.2 and 0.8±0.2 respectively), as compared to their levels in the control group (16.4±6.4 and 0.6±0.2 respectively). While in NaF and Ca treated group, the levels of Serum urea and creatinine were more greatly increased (52.6±11.6 and 1.4±0.3 respectively), if compared to the two previous groups (Table 1 and Figure 1).

Table (1): shows the comparison between mean values of the three studied groups regarding the levels of serum urea and creatinine using ANOVA test.

<table>
<thead>
<tr>
<th>Parameters (serum)</th>
<th>Control Mean ±SD (range)</th>
<th>NaF Mean ±SD (range)</th>
<th>NaF+Ca Mean ±SD (range)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea(mg/dl)</td>
<td>16.4±6.4 (9.25-24.75)</td>
<td>31.4±8.2 (24.25-42.75)</td>
<td>52.6±11.6 (39.25-67.5)</td>
<td>16.363</td>
<td>0.001*</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.6±0.2 (0.41-0.96)</td>
<td>0.8±0.2 (0.65-1.14)</td>
<td>1.4±0.3 (1.05-1.8)</td>
<td>9.52</td>
<td>0.006*</td>
</tr>
</tbody>
</table>
Figure (1): shows the comparison between mean values of the three studied groups regarding serum urea and creatinine levels.

2-Light microscopy:
A normal architecture of renal cortex was observed in the control animals. The glomeruli appeared with normal morphology formed of a tuft of glomerular capillaries containing normal erythrocytes. It was surrounded by normal epithelial-lined uniferous tubule (Bowman’s capsule) except at its vascular pole. Bowman’s capsule possessed visceral and parietal layers of Squamus epithelium with a lumen in between. The visceral layer was applied closely to the surface of glomerular capillaries. The proximal convoluted tubules appeared with narrow lumen and lined by truncated pyramidal eosinophillic cytoplasm. The distal convoluted tubules were demonstrated with cubical epithelium lining a normally wide lumen (Figure 2).

In the NaF treated group, there was significant morphological damage to the renal cortex. The glomeruli were observed exhibiting different forms of degeneration. Some glomeruli appeared markedly lobulated whereas others exhibited marked hypertrophy, with mesangial hypercellularity and markedly congested glomerular capillaries. The Bowman’s capsule revealed some dilatation in some areas and an adhesion between visceral and parietal layers at other sites (Figure 3). Other glomeruli appeared shrunken with a moderately congested capillary loops and an expanded Bowman’s capsule. Extensive interstitial hemorrhage was well demonstrated in the peritubular and perivascular areas. The vascular capillaries were enlarged, congested and engorged with blood. Some renal convoluted tubules were manifested either damaged with sloughing of their tubular epithelial cells or exhibiting abnormal marked dilatation of their lumens (Figure 4). Moreover, there was also damage to the architecture of the tubular epithelia of proximal convoluted tubules which showed vacuolar degeneration of their cytoplasm giving them cloudy swelling appearance. Some tubular epithelial cells exhibited cell swelling with lysis of their cytoplasm and their organelles. The nuclei were pushed towards the basement membrane whereas others appeared pyknotic (Figure 5). The interstitial tissue, in some perivascular areas, showed marked infiltration by mononuclear inflammatory cells. The capillary blood vessels appeared with marked thickening of their wall (Figure 6).

In the NaF and Ca treated group, there were still extensive degenerative changes on the renal cortex. Some glomeruli appeared shrunken, moderately congested with more expanded Bowman’s capsule. Others were seen moderately lobulated and hypertrophied with narrow Bowman’s capsule and moderately congested glomerular capillaries. Some renal convoluted tubules were regarded damaged with some sloughing of tubular epithelial cells inside their lumen while, others showed marked dilated lumen. The tubular epithelia of proximal convoluted tubules exhibited cell swelling with lysis of their cytoplasm and loss of their organelles. Extensive Interstitial hemorrhage was well
demonstrated in the peritubular areas (Figures 7, 8).

3-Transmission electron microscopy:

In the control group, the Glomerulus comprised a network of Neighboring Glomerular capillary loops with patent lumen containing red blood cells. The normal Glomerulus comprised three types of cells. The first cell was the Mesangial cell which was a specialized cell containing elongated nucleus and electron dense cytoplasm. It was embeded in the Mesangial matrix and located inside the Glomerular capillary loops. The second cell type was the podocyte which was a highly modified epithelial cell, resided between the adjacent capillary loops and laid in intimate contact with more than one capillary loop. The podocyte was consisted of a cell body which gave rise to primary processes that extent toward the capillaries to which they affixed by numerous small foot processes. These secondary processes (pedicles) completely enveloped the wall of the Glomerular capillary from outside (Figure 9). The foot processes of the podocyte were regularly arranged on the capillary surface, interdigitating with those of neighboring podocytes, and were separated from the podocyte and their primary cell processes by a clear subpodocyte space, mostly called the urinary space. In general, cell bodies and major processes of podocytes were not directly connected to the capillary surface but hanged freely in the urinary space, fixed to the underlying capillaries only via attachment of their foot processes to the capillary surface (Figure 10). The podocyte exhibited highly indented nucleus and electron dense cytoplasm. Its cytoplasm contained elongated and rounded mitochondria and rough endoplasmic reticulum. Some podocytes gave long primary processes which in turn sent large foot processes encircling the capillary wall or interdigitated with the foot processes of other podocytes (Figures 10, 11). The third cell type is the endothelial cell that lined the capillary wall from inside and protruded by its nucleus and the covering cytoplasm into the lumen of capillary loop. Its nucleus was situated towards the Mesangial matrix. Its cytoplasm was demonstrated adhered to the Glomerular basement membrane (GBM) (Figure 10).

The Glomerular capillary wall consisted of three layers (trilaminar in structure) with less electron dense central zone surrounded on either side by more electron dense layers. The Glomerular basement membrane (GBM) was covered by the pedicles (foot processes) of podocytes from outside, and lined by well developed fenestrated endothelium (displaying pores) from inside. The foot processes were regularly arranged (in an orderly fashion), covered by glycocalyx and protruded into the clear wide urinary space. Narrow clefts (filtration slits) appeared between adjacent foot processes and were bridged by thin slit diaphragm (membrane) connecting the adjacent foot processes and forming part of the filtration barrier (Figure 12).

Therefore, these capillary walls comprised the kidney's filtration barrier. The glomerular filter barrier consisted of three components: porous endothelium, glomerular basement membrane, and podocyte foot processes with the interposed slit diaphragm.

As regard the proximal tubules of renal cortex, the apical cytoplasm of the proximal tubular epithelial cell showed a well-developed interlacing microvillar brush border. Electron dense cytoplasm was observed containing large oval nucleus with a distinct peripheral nucleolus, marginated heterochromatin and surrounded by nuclear membrane. Numerous rounded and elongated mitochondria were seen dispersed in the

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supranuclear and basal cytoplasm. Few apical endocytic vacuoles were present in the apical cytoplasm. Few lysosomes like bodies were observed as small membrane bound dense bodies in the supra-nuclear region of the cell. The cell rested on a well-developed less electron dense thick basement membrane (Figure 13).

The NaF treated group: the kidney specimens showed a morphological impairment including the constriction and congestion of Glomerular capillaries. The podocytes revealed small, less indented nucleus and electron lucent cytoplasm. Some podocytes appeared with disintegrated nuclei, covered the capillary surface and separated from them by a very narrow urinary space. Some primary podocyte processes were seen broadened increasing the interstitial space between the capillary loops and showed degenerative features such as accumulation of electron dense deposits in its cytoplasm. The endothelial cells of Glomerular capillary appeared swollen and their cytoplasm expanded into the capillary lumen making it narrower. Large Mesangial cell was demonstrated containing large and small membrane bound vesicular materials engulfed by its nucleus (Figure 14).

The Mesangial cells increased in number with expansion of Mesangial matrix between them. Some Glomerular capillaries were more dilated, congested, engorged with blood and contained large intraluminal vacuoles. The Glomerular basement membrane showed local thickening in some areas and local corrugations or irregularities in other parts (Figure 15). The Glomerular capillary wall was formed of electron dense layer losing its trilaminar structure and its lining fenestrated endothelium was severely damaged. The inner surface of the glomerular basement membrane showed focal nodular projections with marked distortion of podocyte foot processes on its outer surface. Very few processes appeared normal projecting through a very narrow urinary space but others showed fragmentation or fusion and some of them completely lost (Figure 16).

Moreover, the fluoride administration induced various degrees of damage to the architecture of renal tubules especially the proximal convoluted tubules.

In the proximal tubular epithelial cell of moderate degeneration, the apical cell membrane of the same cell revealed Shrinkage of microvillar brush border in one part with complete loss of it in the other part. The apical cytoplasm revealed lysis and diffuse vacuolar degeneration displacing the heterochromatic nucleus (containing irregular clumps of heterochromatin) towards the cell basement membrane. Moderate numbers of damaged and disfigured mitochondria were observed around the nucleus and in the basal cytoplasm. The basement membrane appeared thin and electron dense (Figure 17). Another degree of degeneration of the tubular epithelial cells was recorded as a massive cellular destruction. These finding were including; reduction of the cellular and nuclear sizes, complete loss of the microvillar brush border of the apical cell membrane, massive loss of most of cytoplasmic organelles, and distortion of the cell basement membrane. The scanty numbers of mitochondria were observed severely damaged with partially deteriorated cristae or with flocculent dense matrix. Some of cells revealed necrosis (apoptosis) exhibiting a very small spherical shrunk pycnotic nucleus with condensed chromatin. Other cells showed disintegrated nucleus with loss of its nuclear membrane (Figures 18, 19).

The ultrastructural results of this work showed more excessive degenerative changes in Glomerulus by administration of Ca if compared to NaF treated group.

In NaF and Ca treated group, the endothelial cells of Glomerular capillary were swollen
with marked swelling of the whole endothelial lining of the Glomerular capillary wall. Their nuclei were large and situated towards the Mesangial matrix. Their cytoplasm was separated from the Glomerular basement membrane by the swollen endothelial lining (Figure 20). On the other hand from outside the Glomerular capillary wall, the podocytes were severely injured, losing their nuclei and most of their cytoplasmic organelles. The basal cytoplasm of the podocyte and their primary processes fused with their foot processes forming a continuous band of cytoplasm and became adherent to the thick glomerular basement membrane with complete disappearance of urinary space. This appearance denoted the effacement (spreading out) of the foot processes, where the foot processes may no longer be seen. The apical cytoplasm exhibited microvillar projections. The Glomerular capillary wall was seen engorged with blood and lined by swollen endothelial cell (Figure 21).

From figures 20, 21, the marked swelling of the whole endothelial lining of the Glomerular capillary wall and the foot processes effacement denoted massive destruction of the glomerular filter barrier.

As regard to the proximal epithelial cells in NaF and Ca treated group, the results of this work showed similar degenerative changes in them like that observed in NaF treated group. The apical cell membrane of the proximal tubular epithelial cell revealed complete loss of its microvillar brush border. Its cytoplasm appeared electron lucent and revealed lysis of most of its organelles. The spherical nucleus contained clumps of heterochromatin with marginated heterochromatin. Very few numbers of normal mitochondria were seen in the basal cytoplasm. Edematous mitochondria were also demonstrated with destructed cristae replaced by translucent dense matrix.

Fragments of degenerated mitochondria were also regarded. The cell basement membrane appeared thin and distorted (Figure 22)

Figure (2): A photomicrograph of a section from normal (control) rat kidney. The glomeruli (G) exhibit a tuft of glomerular capillaries surrounded by normal Bowman’s capsule (asterisk) except at its vascular pole (vp). Bowman’s capsule exhibits visceral (arrow) and parietal (arrowhead) layers with a lumen in between (L). Notice also the proximal convoluted tubules (pct) with their narrow lumens and the distal convoluted tubules (dct) with their wide lumens. (H&E x400)
tubules with sloughing of their tubular epithelial cells (arrowhead). Other tubules show marked dilated lumen (arrows). Extensive Interstitial hemorrhage (H) is well demonstrated in the peritubular and perivascular areas. A vascular capillary (vc) is seen large, congested and engorged with blood. (H&E x400)

Figure (5): A photomicrograph of a section from Naf treated rat kidney showing cloudy swelling of some tubular epithelial cells (black arrows). Others reveal cell swelling with lysis of their cytoplasm and loss of their organelles (black arrowheads). Notice the nuclei (white arrow) are pushed towards the basement membrane and others appear pyknotic (white arrowhead). Notice also the presence of Interstitial hemorrhage (H) is well demonstrated in some peritubular areas. (H&E x400)
**Figure (6):** A photomicrograph of a section from Naf treated rat kidney showing marked inflammatory cells (asterisk) infiltrated in the perivascular areas. There is marked thickening the capillary wall (arrowhead) and widening (arrow) of some renal convoluted tubules. (H&E x400)

**Figure (7):** A photomicrograph of a section from NaF and Ca treated rat kidney showing shrunken glomerulus (G), and widely expanded Bowman’s capsule (asterisk). Some renal convoluted tubules appear damaged with some sloughing of tubular epithelial cells (arrowhead) inside their lumen. The tubular epithelia exhibit cell swelling with lysis of their cytoplasm and loss of their organelles (arrows). Notice Interstitial hemorrhage (H) in the peritubular areas. (H&E x400)

**Figure (8):** A photomicrograph of a section from NaF and Ca treated rat kidney showing lobulated and hypertrophied glomeruli (G), narrow Bowman’s capsule (arrowhead) and moderately congested glomerular capillaries (white arrow). Some renal tubules show marked dilated lumen (black arrows). (H&E x400)
**Figure (9):** An electron micrograph of a section from normal (control) rat kidney showing Glomerular capillary loops (CL) containing red blood cells (Rbc's). The Mesangial cell (Mc) with its elongated nucleus (N) and electron dense cytoplasm is seen embedded in the Mesangial matrix (Mm) inside the Glomerular capillary loops (CL). The podocyte (p) resides between the adjacent capillary loops having a cell body (P), primary cell processes (pp) and foot processes (fp) enveloping the Glomerular capillary loops. (X 6480)

**Figure (10):** An electron micrograph of a section from normal (control) rat kidney showing the podocyte (p) with highly indented nucleus (N1) electron dense cytoplasm. The foot processes of the podocyte (fp) are regularly arranged, or interdigitating, (arrow) and are separated from the podocyte (P) and their primary cell processes (pp) by a clear urinary space (S). Notice the endothelial cell (En) lining the capillary loop (CL) and its nucleus (N2) is situated towards the Mesangial matrix (Mm). Its cytoplasm is seen adhered to the Glomerular basement membrane (GBM). (X 8100)

**Figure (11):** An electron micrograph of a section from normal (control) rat kidney showing the podocyte (P1) containing highly indented nucleus (N), elongated and rounded mitochondria (M) and rER. It is separated from the capillary loop surface (CL) and the foot processes (fps) by a clear urinary space (S). Notice the lower podocyte (P2) has long primary process (pp) which sends large foot process (fp) encircling the capillary wall or interdigitates with other foot processes (arrow). (X8100)
Glomerular capillary. The wall of Glomerular capillary loop (CL) appears trilaminar in structure with a less electron dense central zone surrounded on either side by more electron dense layers. Glomerular basement membrane (GBM) is decorated by foot processes (fp) of podocytes from outside, and lined by fenestrated endothelium (fe) from inside. The foot processes are covered by glycocalyx (g) and protrude into a clear wide urinary space (S). Narrow clefts known as filtration slits (fs) appear between adjacent foot processes and are bridged by slit diaphragms (d). Red blood cells=Rbc’s. Pores of fenestrated endothelium=pr. (X32,400)

Figure (12): An electron micrograph of a section from normal (control) rats kidney showing higher magnification of portion of microvillar brush border (mb). The cytoplasm is electron dense and contains large oval nucleus (N) with a distinct peripheral nucleolus (n) and margined heterochromatin (h). Numerous rounded and elongated mitochondria (M) are seen dispersed in the cytoplasm. Few apical endocytic vacuoles are present (AV) in the apical cytoplasm. Few lysosomes like bodies (Ly) are observed. The cell rests on a well-developed, less electron dense thick basement membrane (bm).

(x 10,800)

Figure (13): An electron micrograph of a section from normal (control) rat kidney showing the epithelial cell of the Proximal Convoluted Tubules. The apical cytoplasm shows a well-developed interlacing
Figure (14): An electron micrograph of a section from Naf treated rat kidney showing portion of Glomerulus with some constricted Glomerular capillaries (CL) (white arrow) and others congested (arrowhead). The podocytes (P₁) are small, with less indented nucleus (N) and electron lucent cytoplasm. Other podocytes (P₂) appear with disintegrated nucleus (asterisk) and separated from the capillary surface by a very narrow urinary space (S). Some primary podocyte processes (pp) are seen broadened between the capillary loops (CL). Some of them show an accumulation of electron dense deposits (black arrows). The endothelial cell (En) of Glomerular capillary appears swollen with expanded cytoplasm (cy). Large Mesangial cell (Mc) is seen containing membrane bounded vesicular materials (vs) engulfed by its nucleus. (X 6480)

Figure (15): An electron micrograph of a section from Naf treated rat kidney showing portion of Glomerulus with an increase in the number of the Mesangial cells (Mc) and expansion of Mesangial matrix (Mm) between cells. Some Glomerular capillaries (CL) are dilated, congested and contain large intraluminal vacuoles (V). The Glomerular basement membrane (GBM) displays local thickening (white arrow) in some areas and local corrugations or irregularities (black arrow) in other parts. (X 8100)
Figure (16): An electron micrograph of a section from Naf treated rat kidney showing portion of the Glomerular basement membrane (GBM) which appears thickened, corrugated losing its trilaminar structure. Its inner surface shows focal nodular projections (white arrows). The lining fenestrated endothelium (fe) is severely damaged. The regular organization of podocyte foot processes (fp) is distorted. A few number of normal foot processes (fp) are seen protruded through a very narrow urinary space (S) with complete loss of them (white arrowheads) in their vicinity. Notice the fused foot processes (black arrow) and their fragmented parts (black arrowhead). (X 32,400)

Figure (17): An electron micrograph of a section from Naf treated rat kidney. Apical cell membrane of the proximal tubular epithelial cell is covered by short microvillar brush border (mb) in some areas with complete lose of them in other place (arrow). The apical cytoplasm reveals lysis and diffuse vacuolar degeneration (asterisk) with pushing of the heterochromatic nucleus (N) towards the cell basement membrane (bm). Moderate numbers of damaged and disfigured mitochondria (M) are seen around the nucleus (N) and in the basal cytoplasm. The basement membrane (bm) appears thin and electron dense. (x 10,800)

Figure (18): An electron micrograph of a section from Naf treated rat kidney. The microvillar brush border of the proximal convoluted tubular epithelial cell is completely lost (arrow). The epithelial cell displays small size and exhibits small spherical basal euchromatic nucleus (N) with nuclear membrane (nm) about to disintegrate. The cytoplasm appears electron lucent with massive loss of most of its organelles. Damaged mitochondria (M) are seen with partially deteriorated cristae and other with flocculent dense matrix (M1). The cell basement membrane (bm) appears distorted. L = lumen (x 10,800)
**Figure (19):** An electron micrograph of a section from Naf treated rat kidney showing a number of tubular epithelial cells lining proximal convoluted tubule projecting into lumen (L). The cells display different degrees of degeneration. Some of them undergo necrosis exhibiting small size and very small spherical shrunken pyknotic nucleus (N) with condensed chromatin. Other cell shows disintegrated nucleus (arrowhead) with loss of its nuclear membrane. The apical cell membranes of all tubular epithelial cells distinct complete loss of the microvillar brush border (arrow). The apical cytoplasm of some cells reveals vacuolar degeneration (asterisk) with massive loss of most of its organelles. (x5000)

**Figure (20):** An electron micrograph of a section from Naf and Ca treated rat kidney showing some Glomerular capillaries. The endothelial cell (En) of Glomerular capillary is swollen with marked swelling (white arrows) of endothelial lining of the Glomerular capillary wall. It has large nucleus (N) situated towards the Mesangial matrix (Mm). Its cytoplasm becomes separated from the Glomerular basement membrane (GBM) by swollen endothelium. (X 8100)

**Figure (21):** An electron micrograph of a section from Naf and Ca treated rat kidney. The podocyte (p) appears lacking its nucleus and most of its cytoplasmic organelles. The cytoplasm of its cell body (p) and of their processes (pp) fuse with their foot processes forming a continuous band adherent to the thick glomerular basement membrane (GBM) with complete disappearance of urinary space. Its apical cell membrane exhibits microvillar projections (arrows). Notice the Glomerular capillary wall is seen congested with red blood cells (Rbc's) and lined by swollen endothelial cell (En). Notice also few Mitochondria (M) in the cytoplasm of the podocyte (p). (x8100)
Figure (22): An electron micrograph of a section from NaF and Ca treated rat kidney.

DISCUSSION:

Fluoride is an essential trace element for the body. After excessive exposure, it provokes damage and diseases (Monsour and Kruger, 1985 and Bouaziz, et al., 2007).

Indeed the kidney may be exposed to high concentrations of fluoride during the normal process of excretion and is therefore a site for fluoride mediated toxicity (Ichikawa et al., 1994 and Inkielewicz and Krechniak, 2003). Furthermore, the kidney has a very active oxidative metabolism that results in the production of reactive oxygen species (ROS). Oxidant injury is now recognized as playing a key role in the induction of experimental renal diseases (Ichikawa et al., 1994 ; Haugen and Nath, 1999 and Ailani, 2009).

Previous works indicated that fluoride treatment can induce functional changes, metabolic disorders, and histopathological modifications in kidney of rabbit (Shashi et al. 2002) and mice during pregnancy and lactating period (Bouaziz et al., 2007).

The apical cell membrane of the proximal tubular epithelial cell reveals complete loss of microvillar brush border (arrow). Its cytoplasm appears electron lucent and reveals lysis of most of its organelles. The spherical nucleus (N) contains clumps of heterochromatin and margined heterochromatin. Few numbers of normal mitochondria (M) are seen in the basal cytoplasm. Damaged mitochondria (M₁) are also demonstrated edematous with destructed cristae replaced by translucent dense matrix. Fragments of degenerated mitochondria (M₂) are also seen. The cell basement membrane (bm) appears thin and distorted. (x 10,800)

The histological results of this study showed that the administration of sodium fluoride treatment to adult albino rats led to extensive degenerative changes on the renal cortex (if compared to normal control rats) including different forms of glomerular degeneration (marked lobulation, marked hypertrophy or shrinkage with expanded Bowman’s capsule). The results of this work are compatible with those of Bouaziz et al., (2007) who had demonstrated that, in kidneys of adult mice and their suckling pups treated with NaF showed hypertrophy, and atrophy of glomeruli.

The present study observed massive degree of degeneration of renal convoluted tubules and their cellular epithelia such as; marked dilatation of some tubular lumens, sloughing of tubular epithelial cells, cell swelling, vacuolar degeneration and lysis of their cytoplasm with loss of their organelles. All these changes were signals of extensive cellular necrosis. These finding have been detected in renal cortex by some authors in rabbits exposed to high doses of NaF (Shashi et al., 2002) and in mice exposed to aluminum chloride (AlCl₃) (AL Kahtani, 2010).
In this study, the light microscopy of NaF treated group revealed marked thickening of capillary wall, extensive interstitial hemorrhage and infiltration of mononuclear inflammatory cells in some peritubular and perivascular areas. These findings were in agreement with the results of previous literature (Karaoz et al., 2004; Bouaziz et al., 2007 and AL Kahtani, 2010) where they claimed these changes were signs of interstitial nephritis.

Many investigations have searched for a correlation between NaF administration and oxidative damage in the body tissues. It was reported that fluoride can disturb the metabolism of proteins, and interfere with the metabolism of carbohydrate, lipid, and nucleic acids (Saralakumari and Ramakrishna Rao, 1991 and Liu et al., 2003). Other studies have shown that fluoride can induce Oxidative damage to cellular components by free radicals and other reactive oxygen species (ROS) (Shivarajashankara et al., 2001a,b and Hassan and Abdel-Aziz, 2010). Also, lipid, protein, and DNA damage was observed when these oxidizing agents were in excess or when antioxidant defense mechanisms were impaired or altered (Guan et al., 2000 and Hassan and Abdel-Aziz, 2010). Increased generation of reactive oxygen species (ROS) and lipid peroxidation had been shown to mediate the toxic effects of fluoride on visceral organs (Rzeuski et al., 1998). Reports describing fluoride induced oxidative stress during the stages of life have been carried out on rats (Wang et al., 2000) on humans after weaning (Shivarajashankara et al., 2001b), and on rabbits (Reddy et al., 2003).

From the ultrastructural point of view of this work, the most important morphological features of impairment induced by fluoride administration were a degeneration of kidney's filtration barrier. These features included; sever damage of the fenestrated endothelium, local thickening and corrugations of GBM, distortion of regular organization of podocyte foot processes covering the surface of the GBM (fragmented or fused or completely lost). These morphological changes would contribute partially to disturbed glomerular hemodynamics. Also, these ultrastructural findings can explain why the serum urea and creatinine levels were significantly increased (P<0.05) in NaF treated group (31.4±8.2 and 0.8±0.2 respectively) as compared to their levels in the control group (16.4±6.4 and 0.6±0.2 respectively).

Moreover, the focal nodular projections on the inner surface of basement membrane were also demonstrated. This may indicate that thickening of the basement membrane occurred on the side that facing the endothelium, and in turn this also may explain why the fenestrated endothelium was severely damaged. Similar changes have also been noted in kidneys of experimental animals upon exposure to other trace metals such as cadmium (Abdel-Moneim and Said, 2007) and mercury (Abdel-Moneim, 2009). In addition these observations are similar to the data reported previously in mice exposed to aluminum chloride (AlCl₃) (Kutlubay et al., 2007 and AL Kahtani, 2010).

Other ultrastructural changes of sodium fluoride nephrotoxicity in this study were also observed in the previous literature (Willinger et al 1995 and AL Kahtani, 2010). These changes included mesangial hypercellularity, mesangial matrix expansion, broadening of some primary podocyte processes with accumulation of electron dense deposits in their cytoplasm, constriction and congestion of Glomerular capillaries, containing large intraluminal vacuoles.
The transmission electron microscopy of the fluoride-treated groups of this work recorded different degree of degeneration of the tubular epithelial cells ranging from moderate to massive cellular destruction (reduction of the cellular and nuclear sizes, apoptosis, complete loss of the microvillar brush border, massive loss of most of cytoplasmic organelles especially mitochondria and distortion of the cell basement membrane). These findings were in agreement with previous fluorosis reports concerning rabbits (Shashi et al., 2002), mice, (Kour and Singh, 1980) and young pigs (Zhan et al., 2006). In addition, similar changes have also been noted in kidneys of experimental animals upon exposure to other trace metals such as lead (Jarrar, 2001) to aluminum chloride (AlCl₃) (Kutlubay et al., 2007 and AL Kahtani, 2010). The important ultrastructural alterations revealing cell damage may represent the severe oxidative stress level in NaF administered rats in the present study. Many works have concluded that fluoride induces apoptosis by elevating oxidative stress-induced lipid peroxidation, can alter glutathione levels, often resulting in the excessive production of ROS at the mitochondrial level, leading to the damage of cellular components. It is known that excessive ROS production leads to macromolecule oxidation, resulting in free radical attack of membrane phospholipids with resulting membrane damage via induction of lipid peroxidation, mitochondrial membrane depolarization (causing mitochondrial dysfunction) and apoptosis (Anuradha, et al., 2001; Xu et al., 2002; Ortiz et al., 2003; Flora, et al., 2009 and Karube, et al., 2009). In addition, Barbier, et al., (2010) confirmed that fluoride induces apoptotic cell death through the modification of gene expression and protein activity by disturbing signaling messages through multiple mechanisms.

Moreover, fluoride induced inhibition of enzymes involved in intracellular energy production such as ATPase or enolase. The degree of nephrotoxicity correlates loosely with maximal serum fluoride levels, but can probably be modulated by further factors like intrarenal in situ formation of fluoride, urinary pH and flow, and especially, the presence of other nephrotoxins. (Bosch 1996)

To protect cells from the damage caused by free radicals and related reactants, organism has evolved several defense mechanisms. The oxidants that are not scavenged by antioxidant defense system attack cellular components producing useless molecular debris and sometimes cell death. The antioxidant enzymes represent a first line of defense against toxic reactants by metabolizing them to innocuous byproducts (Rodriguez et al., 2004). When cells are exposed to oxidative stress they increase the activity and expression of antioxidant enzymes as a compensatory mechanism to better protect them from the damage. Moderate levels of toxic reactants induce rises in antioxidant enzymes while very high levels of reactants reduce enzyme activities as a result of damage of the molecular machinery that is required to induce these enzymes (Gechev et al., 2002; Wei and Lee, 2002 and Ikediobi et al., 2004).

The present study aimed to investigate the possible protective effects of calcium supplementation during fluoride exposure; if it is protective or not? To our knowledge there is no previous study about the ultrastructure effect of calcium on the renal tissue damage induced by sodium fluoride. The ultrastructural results of this work showed more excessive degenerative changes in Glomerulus in NaF and Ca treated group, if compared to NaF treated group. These excessive degenerative changes included; marked swelling of the
whole endothelial lining of the Glomerular capillary wall and the foot processes effacement of the podocytes. These findings denoted the massive destruction of the glomerular filter barrier in NaF and Ca treated group was more than NaF treated group. This ultrastructural results also explained why the serum urea and creatinine levels were more significantly increased (P<0.05) in NaF and Ca treated group (52.6±11.6 and 1.4±0.3 respectively) as compared to their levels in NaF treated groups (31.4±8.2 and 0.8±0.2 respectively).

In addition, in NaF and Ca treated group, the injurious appearance of the podocyte manifested by loss of its nucleus and most of its cytoplasmic organelles, apical microvillar projections and effacement (spreading out) of their foot processes). These degenerative changes of podocytes were typically seen in models of glomerular hypertension and hyperfiltration including subtotal renal ablation (Tenschert, 1995). In these models, the podocytes were unable to maintain their normal cell shape but change in its appearance. These changes comprise cell hypertrophy, foot process effacement, cell body attenuation, apical microvillous transformation (Kriz et al., 1995; Shirato, 1996; Kriz et al., 1999; Kerjaschki, 2001 and Pavenstädt et al., 2003).

The relation between fluoride and calcium has been suggested since 1948 (Peters et al., 1948). All over the years, more works have evidenced that fluoride could alters calcium homeostasis in human population and that calcium also plays an important role in a wide range of cellular alterations induced by fluoride (Ba et al., 2010). Calcium plays a key role in signaling mechanisms triggered by external or internal stimuli and regulates a variety of cellular processes (Clapham, 1995). Thus, calcium simultaneously represents an integrative signal and a central convergence point of many distinct signaling pathways in all cell types (Berridge, et al., 2000). Cellular responses to changes in calcium concentrations are modulated by a tight regulation of the spatial and temporal occurrence of calcium variation and the intensity of the amplitude of such changes (Knot, et al., 2005). Plasmatic and organelle membranes separate compartments that have different free calcium concentrations, and the mechanisms that regulate the release and recovery of calcium are diverse and complex. These mechanisms include calcium channels, calcium-binding proteins, calcium-sequestering organelles (ER), sodium/calcium exchangers and calcium pumps. Effects of fluoride on calcium homeostasis and intracellular quantity have been described, although the mechanism and pathways have not been sufficiently explained. It has been suggested that fluoride increases calcium retention by some tissues (redistribution process), as evidenced by hypocalcemia along with diminished urinary excretion and augmented intestinal absorption (Das and Susheela, 1993). Previous study suggested that chronic, high fluoride ingestion producing high plasma fluoride levels may occur in humans and may affect Ca++ homeostasis. This occurs by increasing the turnover or breakdown or reduction in calcium transport across the renal tubule ER and plasma membrane, as well as to a reduction of the amount of calcium pump proteins in isolated kidney membranes (Borke and Whitford, 1999). In addition, excessive reactive oxygen species (ROS) production as superoxide ion, hydrogen peroxide, and hydroxyl radical leads to enhancing lipid peroxidation, DNA damage, and altered calcium and sulfhydryl homeostasis occur (Stohs and Bagchi, 1995). The recent results have shown the relevance of the mitochondria, Golgi and peroxisomes as calcium stores. It is important to recognize that the action of fluoride on these organelles would also
impair calcium homeostasis (Davies and Terhzaz 2009 and Barbier et al., 2010). Moreover, many works have reported the role of intracellular calcium content in fluoride-induced apoptosis as a direct target of toxicity or an indirect consequence of altered cellular processes (Murao, et al., 2000 and Kubota et al., 2005) and the increase of $[\text{Ca}^{2+}]$ probably plays a key role on the mechanism of renal injury in fluorosis as well (Xu, et al., 2007).

On other hand, the previous significant recovery from fluoride toxicity on the reproductive functions and fertility of male mice and rats (Chinoy, 1994; Chinoy and Sharma, 2000, Das, et al. 2006 and Sarkar, 2006) or the significant reduction in serum fluoride and SOD and increase in urinary fluoride by the supplementation of calcium phosphate (Ca) to NaF-treated group (Susheela and Bhatnagar 2002 and Sharma, 2008) was be effective only if coadministred with Vitamin D or Vitamin C or both together.

Interestingly, both inhibitory and stimulatory effects of fluoride on the calcium pump have been shown in the cardiac sarcoplasmic reticulum. It was explained that the dissimilar responses were due to differential susceptibility of the conformational state of the calcium pump (Narayanan, et al 1991). A lot of experiments still needed to be performed to account for possible protective role of Calcium on flouride-induced toxicity.

**Conclusion:**
The results of this work showed that fluoride administration can induce morphological degenerative changes and impairment on renal cortex. Calcium not only failed to restore renal damage induced by NaF exposure but also enhanced the flouride-induced toxicity.

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الدراسة النسيجية والتركيب: فائق الدقة لتأثير السمي لصوديوم الفلوريد على القشرة الكلوية في الجرذان البيضاء
البالغين والحمية الممكنة بالعلاج بالكالسيوم
حنان السيد طلقي مختار
قسم التشريح والاجنة. كلية الطب. جامعة الزقازيق

هذى البحث: كان هدف هذه الدراسة هو تقديم التأثير السامي لصوديوم الفلوريد على قشرة الكلى من الجرذان البيضاء البالغين والتحقق في أثار النمطية الممكنة للكالسيوم أثناء التعرض للفلوريد إذا ما كانت وقائية أم لا؟

المواضيع والطرق المستخدمة: تم الحصول على 18 من الجرذان البيضاء البالغين من مزرعة الحيوانات المخبرية، كلية الطب البيطرية، جامعة الزقازيق. قسمت هذه الحيوانات بتساوي إلى ثلاث مجموعات (6 جرذان لكل مجموعة) على النحو التالي:

1- المجموعة الأولى: استخدمت كمجموعة طبية ضابطة و استقبلت ماء ونظام غذائي متوافن
2- المجموعة الثانية: تم إعطائها صوديوم الفلوريد فقط بواسطة أنوية تغذية في المعدة بجرعة 30 مجم/كم موم لمدة سنتين سابقين
3- المجموعة الثالثة: تم إعطائها صوديوم الفلوريد بنف الجرعة السابقة وهي 30 مجم/كم موم مع كالسيوم الكالسيوم
بجرعة 20 مجم/كم موم في نفس الوقت بواسطة أنوية تغذية في المعدة لمدة سنة سابقة.

في نهاية الفحص التجربة، تم إزالة الكلى بسرعه وتحضيرها للفحص الاتى.

أ- الفحص بالمجهز الضئي: كلية الطبل قسم اليميولوجي جامعة الزقازيق باستخدام صبغة الهيماتوكسيلين والأوانيين.
ب- في تخليق التركيب فائق الدقة باستخدام المجهز الإلكتروني بكلية العلم جامعة عين شمس وكلية الطب قسم اليميولوجي جامعة الزقازيق.

كما تم جمع عينات الدم من كل مجموعة لدائم اختبارات وظائف الكلى عن طريق تقديرات مستويات البوريا والكيراتينين في مصل الدم (مجم / ديسيلتر) وتحليلها إحصائياً باستخدام اختبار ANOVA (تحليل التباين) (P<0.05) اعتبرت ذات دلالة إحصائية.

النتائج:

启示 المجهز الضئي: في المجموعة التي قمت بالعلاج بصوديوم الفلوريد كان هناك نتائج مفوقة كبير في القشرة الكلوية عند التعرض لصوديوم الفلوريد بما في ذلك; أشكال مشكلة من ضمور الكبيبي (نقص، وتسخيم ملحوظ أو إمكان مع زعوس كستويا (باتمان) ودرجة هالة من تلك الأوقات المتوفرة الكلوية وخلايا الظهارية المبطنة لها (تسع ملحوظ في تكبير بعض الأوقات، نزع الخلايا الظهارية المبطنة تعلم القلوب في نفس الوقت وخلط الكليات. في نهاية الفحص، للكثيري، الرسومية ضعية معا الكالسيوم.

استعداد الضرور الكلوي الناجم عن التعرض لصوديوم الفلوريد، فلا تزال هناك تغيرات تنكسية واسعة على القشرة الكلوية في المجموعة التي قمت بالعلاج بصوديوم الفلوريد والكالسيوم مع ما قورنت بالجموعة التي قمت بالعلاج بصوديوم الفلوريد فقط.

ولكن من خلال الفحص بالمجهز الإلكتروني كان من أهم التغييرات الناجمة عن التعرض لصوديوم الفلوريد هي تدهور حاجز الترشيح الكبيبي، وانخفاض الضغط الشبيه الردودية للكبيبي وزيادة عد الكبيرة مسار حلاح، وتسخيم ملحوظ كبيبي (تقصص، وتسخيم ملحوظ فبتوكسيلين، وتتكرر خلايا رجلة. بالإضافة إلى وجود درجات مختلفة من تدهور الخلايا الظهارية المبطنة للخلايا المانفة كلية (العوما، والأنفية، تتكسر بال فيما تؤثر المحتوى الصغير على الخلايا المقطعة، وفقدان كامل للجزيئات الصغيرة المحتوية من غشاء الخلايا الفسي، خسائر فادحة في معظم الغشاءات المتوازية، وتشوه الغشاءات الفضية المكسية. ولكن أظهرت المجموعة التي قمت بالعلاج بصوديوم الفلوريد والكالسيوم تغيرات تنكسية مفرطة في الكبيبي بدرجة أكبر من المجموعة المذكورة، بوجود خط الظهارية وفقدان الكلية. "ورمن ملحوظ لبطانة جدار الشعيرات الدموية للكبيبي بالعوما بالإضافة إلى وجود دفع جلي للخلايا ج حال مع طمس أدقهم بينما أظهرت التغيرات تنكسية مماثلة في الخلايا الظهارية المبطنة للخلايا المانفة الكلوية مثل تلك التي وجدت في المجموعة التي قمت بالعلاج بصوديوم الفلوريد فقط، وأظهرت النتائج البيوبيثولوجيا فرق كبير بين المجموعات الثلاث (P<0.05).

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تم زيادة مستويات البوتاسيوم والكليتين في مصل دم المجموعة المعالجة بصوديوم الفلوريد والكالسيوم معا بدرجة أكبر بكثير (6.2 ± 0.8 على التوالي) في حين

8.2 و 0.8 ± 0.2 على التوالي) بالمقارنة مع المجموعة الضابطة (4.6 ± 0.4 و 0.6 ± 0.2 على التوالي) إذا قورنت بالمجموعتين السابقتين.

الخلاصة:

أظهرت نتائج هذا العمل أن التعرض للفلوريد ينجم عنه تحرر التغيرات التنكسية المورفولوجية مع تدهور للقشرة الكلوية. الكالسيوم فشل ليس فقط لإعادة الضرر الكلوي الناجم عن التعرض بصوديوم الفلوريد ولكن أيضا قد يعزز السمية التي يسببها الفلوريد.