Ultrastructural alterations in renal tissues of rabbits induced by diclofenac sodium (Voltaren)

Noory T. Taib, BSc, PhD, ***Bashir M. Jarrar,*** BSc, PhD, ***Mohammed M. Mubarak,*** BVSc, PhD.

ABSTRACT

Objective: Although diclofenac sodium (Voltaren) is one of the most frequently prescribed non-steroidal anti-inflammatory drugs (NSAIDs) worldwide for the treatment of inflammation and pain; data on the ultrastructural alterations in renal tissues due to its chronic exposure are limited. Therefore, the present study was designed to identify the ultrastructural renal alterations induced by diclofenac sodium.

Methods: The experiment was conducted at the animal house of the Department of Zoology, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia during the period from April 2003 to June 2003. A total of 30 male rabbits were exposed to intraperitoneal injection with a daily dose of diclofenac sodium (1.5 mg/kg body weight) for 70 days to investigate the resultant ultrastructural alterations in renal tissues.

Results: In comparison with the respective control rabbits, chronic exposure to therapeutic doses of diclofenac sodium produced significant ultrastructural renal alterations, which involved swelling and cristolysis of the mitochondria, marked dilatation of the endoplasmic reticulum, detachment of ribosomes, increased lysosomal structures, nuclear chromatin condensation in the tubular cells, thickening of the glomerular basement membranes, distention of glomerular capillaries, which showed lodgment of neutrophils, mesangial and endothelial cell proliferation in the glomeruli, swelling and fusion of the glomerular podocytes foot processes with focal obliteration of the filtration slits.

Conclusion: The obtained results indicate that chronic exposure to diclofenac sodium produces significant ultrastructural alterations in renal tissues.

Saudi Med J 2004; Vol. 25 (10): 1360-1365

Diclofenac sodium (Voltaren) is probably one of the most common nonsteroidal compounds with analgesic, anti-inflammatory, antirheumatic and antipyretic properties with inhibitory effect on prostaglandin biosynthesis. This drug is used as an initial therapy for pain conditions such as musculoskeletal, postoperative pain and acute attacks of gout and ureteric colic.[1] Diclofenac is metabolized in the liver to 4-hydroxy diclofenac and other hydroxylated form after glucoronidation and sulfation before being eliminated principally by urinary and biliary excretion.[2] This drug is claimed to be faster acting than Ibuprofen, longer acting than Paracetamol and as safe as Ibuprofen.[3,4] Voltaren is available in various pharmaceutical preparations including tablets, suppositories and oral drops. For adults, the recommended initial daily dose is 100-150 mg (divided into 2-3 doses) and in milder cases, as well as for long term therapy 75-100 mg daily is usually sufficient. The maximum daily dose ranges from 150-200 mg/day according to the severity of clinical signs. Children aged ≥1-year should be given 0.5-2 mg/kg body weight daily, in 2-3 divided doses and the daily dose can be raised to

From the Department of Zoology (Taib, Mubarak), College of Science, King Saud University, Riyadh and the Department of Clinical Laboratory (Jarrar), College of Science, King Saud University, Al-Jouf Branch, *Kingdom of Saudi Arabia.*

Received 21st February 2004. Accepted for publication in final form 4th May 2004.

Address correspondence and reprint request to: Prof. Noory T. Taib, Department of Zoology, College of Science, King Saud University, PO Box 2455, Riyadh 11451, *Kingdom of Saudi Arabia*. Tel. +966 (1) 4675781. Fax. +966 (1) 4678514. E-mail: ntaib2663@hotmail.com

a maximum of 3 mg/kg in divided doses. There are many side effects observed with the use of diclofenac sodium. It is not recommended for pediatric uses while its overdosing is potentially toxic. This drug causes a rare but potentially fetal hepatotoxicity that may be associated with the formation of reactive metabolites and has been reported to cause adverse hepatitis effects in certain individuals.[5,6] Diclofenac causes proliferation of bile ductules, hepatocellular degeneration, nonspecific hepatitis with portal and lobular activity. Also, it elevates markedly transaminases levels, decreases the glycogen content of the hepatocytes and impaires adenosine triphosphate (ATP) synthesis by the mitochondria with futile consumption of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH).[6,7] Diclofenac is also associated with bone marrow toxicity due to reactive acylglucuronide with rare acute immune hemolytic anemia.[8] Also, diclofenac has been reported to damage moderately the seminiferous tubules and to impair spermatogenesis.[9]

The effects of diclofenac on renal structure and function are worth attention. Some studies indicated the responsibility of this drug for the thickening of the glomerular basement membranes (GBMs) with mild focal tubular necrosis and intraluminal secretions in the proximal convoluted tubules.[3] Other less frequent alterations in renal tissues include interstitial nephritis, lipid peroxidation and papillary necrosis. Clinical signs such as hematuria and proteinuria associating the use of diclofenac and reflecting the resultant renal dysfunction were also reported.[3,10] Studies on the ultrastructural alterations in renal tissues due to chronic diclofenac sodium exposure are limited. With this objective, an ultrastructural study has been undertaken in renal tissues of adult rabbits killed at weekly intervals up to 70 days of diclofenac sodium treatment.

Methods. A total of 30 male rabbits at 3-months of age and weighing 950-1000 gm obtained from Tahsin Center, Riyadh, Kingdom of Saudi Arabia were used. Animals were kept in cages containing 2 animals each and maintained at the animal house of the Department of Zoology, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia during the period from April 2003 to June 2003.

Following a period of stabilization (7 days), diclofenac sodium (Proanalysis-Merck, Germany) was administered daily to the animals of the treatment group (n=20) via intraperitoneal route at the rate of 1.5 mg/kg body weight throughout the experimentation period (70 days). The control group (n=10) received sterile distilled water via the same route and all animals maintained on standard laboratory animal diet pellets ad libitum. Three animals (2 treated and one control) were killed by decapitation of the neck at weekly intervals during the 70 days of treatment with diclofenac sodium.

Small pieces of kidney tissues from each experimental rabbit of both groups were transferred immediately to a pool of cold (4ºC) cacodylate buffered (pH 7.2) glutaraldehyde fixative on a glass surface for 10-15 minutes to attain suitable hardness and then chopped into proper sized pieces (1 mm^3) and transferred to the same fixative for 2 hours. Tissues were washed in 3 changes, one of which was overnight, of 0.1 M cacodylate buffer (pH 7.2) at 4ºC. Tissue specimens were then post-fixed in 1% osmium tetroxide (O_5O_4) in cacodylate buffer for 2 hours at 4ºC, then washed in the same buffer. Subsequently, tissues were dehydrated in ascending grades of ethyl alcohol, cleared in 3 changes of propylene oxide, infiltrated gradually with resin and embedded in plastic capsules in fresh full strength Agar 100 epoxy resin before being cured at 60ºC for 2 days. For the purpose of tissue orientation, semithin sections (1 µm) were cut using glass knives by the aid of Jung ultramicrotome and stained with 1% toluidine blue. Ultrathin sections (700 A^o) were cut with a diamond knife on Leica ultramicrotome UCT (Leica, Germany), mounted on copper grids and double stained with uranyl acetate and lead citrate. Tissue sections were then examined and photographed under a transmission electron microscope (JEOL, JEM-100 CX) operating at 80 Kv.

Results. Renal tubular cells of the control rabbits revealed the normal ultrastructural morhology (**Figure 1**). The ultrastructural changes induced by diclofenac sodium in renal tissues of the treated rabbits were as follows:

(a) Renal tubular cells. Tubular lining cells including those of the convoulted and collecting tubules showed significant fine changes. However, the proximal convoluted ones were more affected. Cytoplasmic vacuolation, apical blebbing and attenuation or loss of the microvillar brush border were general findings. The ultrastructure of the affected renal tubular cells were as follows: (i) Mitochondrial alterations. The mitochondrial ultrastructural abnormalities were evident at 6 weeks and more of diclofenac treatment. There was a marked swelling of the mitochondria in the majority of the convoluted tubular cells (**Figure 2**). In the swollen mitochondria, cristae were disoriented, marginated and short in comparison with the control well-preserved parallel linear ones. Noticeable reduction of the matrical density and indistinction of the inner membranes were obvious in the swollen mitochondria. In many of the affected mitochondria, there was a loss of cristae structures

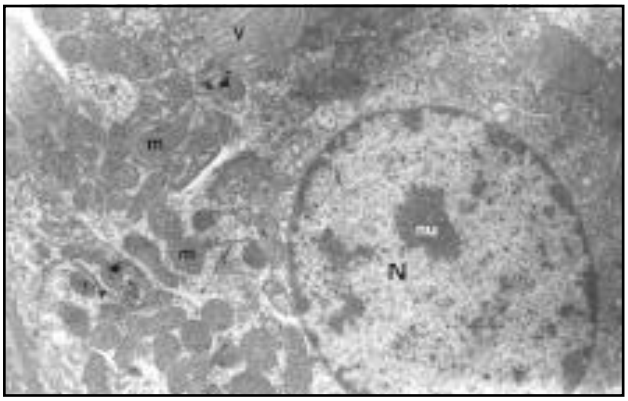

Figure 1 - Transmission electron micrograph of a renal proximal convoluted tubular cell of control rabbit received distilled water for 10 weeks. Note the normal-sized mitochondria (m) with intact cristae and homogeneous matrix. Nucleus (N) is of normal size and has prominent nucleolus (nu) and evenly distributed chromatin. Microvilli (V) are present on the apical cytoplasmic surface (x 10000).

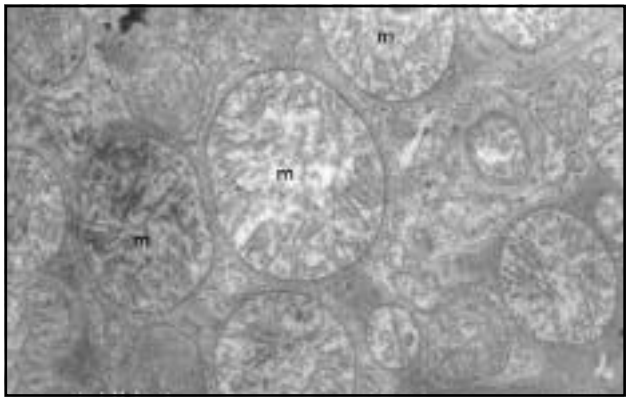

Figure 2 - Tubular convoluted cell containing markedly swollen mitochondria (m) which have disoriented and partially deteriorated cristae (*). Kidney of a rabbit treated with diclofenac sodium for 6 weeks (x 27000).

due to cristolysis and only matrical flocculent densities were detected. (ii) Endoplasmic reticulum alterations. The affected renal tubular cells showed dilatation of the endoplasmic reticulum. The dilated rough endoplasmic reticulum (RER) lost the characteristic parallel arrangement of their cisternae and disclosed partial detachment of the bound polyribosomes and accordingly had degranulated appearance. Occasional marked dilatation of endoplasmic reticulum (ER) was discerned and the dilated ER was filled with fine granular material (**Figure 3**). (iii) Lysosomes alterations. The number of lysosomal-related structures was generally increased within the renal tubular cells especially the proximal convoluted ones of the diclofenac-treated rabbits at 8 weeks and more of exposure (**Figure 4**). Also, some tubular cells contained irregular dense bodies of similar electron density and showed accumulation of lipid droplets. (iv) Nuclear alterations. The considerable number of nuclei in the convoluted and collecting tubular cells were condensed and their euchromatin was of higher density and heterochromatin was more clumped (**Figure 5**). The cytoplasm of tubular cells with these nuclear changes was of more density, which added to the darkness of the affected cells (dark tubular cells).

(b) Renal glomeruli. Segmental thickening of the GBMs was recognized in renal tissue of treated animals (**Figure 6**). These membranes were uniformly thickened but had no dense deposits. Lodgement of polymorphnuclear leukocytes (neutrophils) in the glomerular capillaries was also frequent. Crowdness of pleomorphic irregular nuclei in the affected glomeruli was the evidence for the proliferated mesangial and endothelial cells. Podocytes (visceral epithelium) revealed obvious swelling and vacuolation of their foot processes (**Figure 7**). Cytoplasm of these podocytes had many endocytotic vacuoles and their foot processes were frequently fused with a resultant focal oblitration of the filtration slits, which apparently decreased in number (**Figure 8**). Glomerular capillaries were greatly distended as evidenced by the lodged multiple raws of erythrocytes.

Discussion. Renal structures including tubular cells and glomeruli showed ultrastructural alterations due to diclofenac treatment. The fine changes involved the organelles such as mitochondria, RER, lysosomes and nuclei of renal tubular cells. In addition, significant alterations in the glomerular structures were noticed. The results of the present investigation clearly indicate that mitochondria are highly vulnerable to diclofenac as evidenced by the resultant noticeable mitochondrial swelling and cristolysis. Similar mitochondrial changes were reported in acute cell injury.[11] The alterations were most probably a reflection of a direct toxic damage due to chronic exposure to diclofenac, which may has the ability to bind with mitochondria. The desruction of the mitochondrial cristae might indicate special diclofenac affinity for mitochondrial membranes, which play a key role in the functional integrity of this organelle.[12,13] Regarding the mitochondrial swelling, toxins can interact directly with mitochondrial membranes and thus increasing its permeability.[14] Mitochondria behave as osmometers and their swelling indicate entry of water and solutes into mitochondrial matrix.[15] Diseases linked to defective mitochondrial function are also characterized by swollen mitochondria with distortion of their cristae.[16] Such altered mitochondria were described as toxic ones

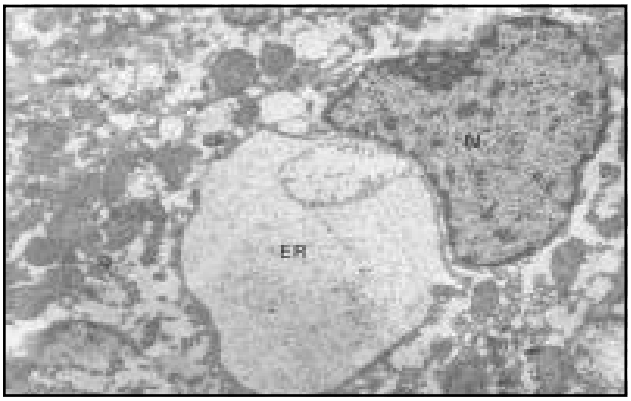

Figure 3 - Markedly dilated endoplasmic reticulum (ER) in the cytoplasm of a convoluted tubular cell. The dilated ER contains fine granular material and it obviously compresses the cell nucleus (N). Kidney of a rabbit treated with diclofenac sodium for 6 weeks (x 10000).

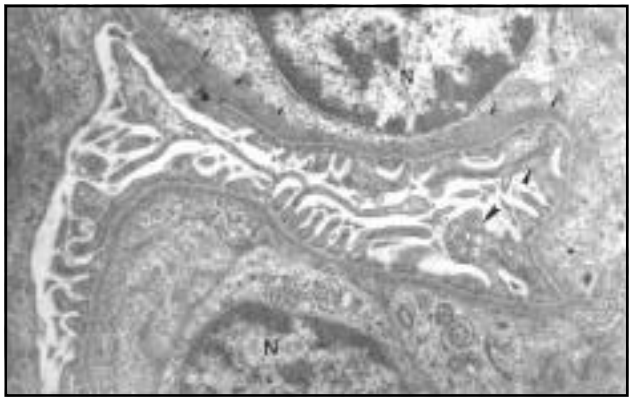

Figure 6 - Glomerulus showing segmental thickening of the GBMs (arrows). Note the swollen and fused foot processes of podocytes (arrowheads). Kidney of a rabbit treated with diclofenac sodium for 8 weeks (x 14000).

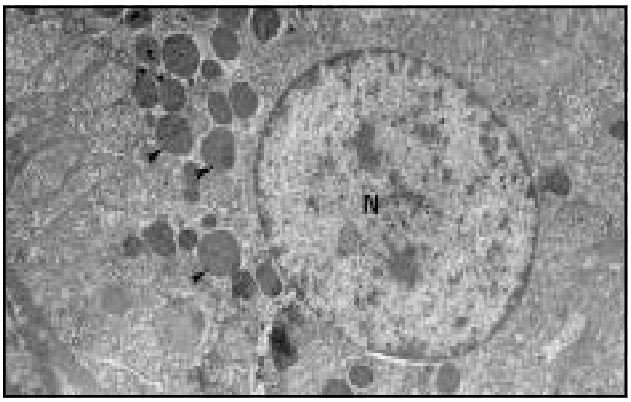

Figure 4 - Increased lysosomal-related structures (arrowheads) in a proximal convoluted tubular cell. Kidney of a rabbit received diclofenac sodium for 8 weeks (x 10000).

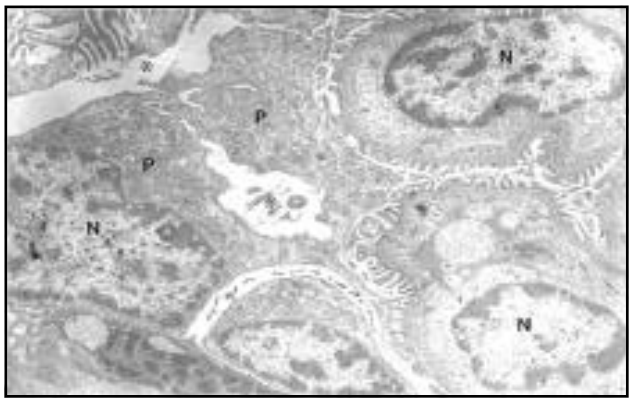

Figure 7 - Glomerulus showing evident swelling of the podocytes (P) associated with narrowing of the glomerular spaces (*). Organelles of podocytes are degenerated. Nuclei (N) of mesangial cells and capillary endothelial cells are pleomorphic. Kidney of a rabbit received diclofenac sodium for 8 weeks (x 8000).

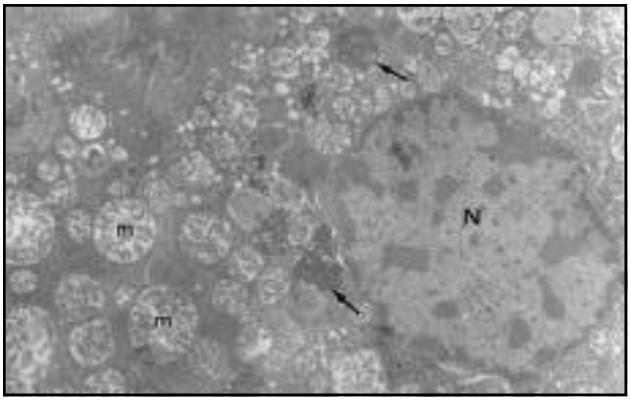

Figure 5 - Tubular cell showing distinctly condensed nucleus (N) and highly dense cytoplasm. Note the irregular nuclear membrane, dense euchromatin and the more clumped heterochromatin. There are dense amorphous bodies (arrows) deposited in cytoplasm. Mitochondria (m) disclose cristolysis. Kidney of a rabbit received diclofenac sodium for 8 weeks (x 10000).

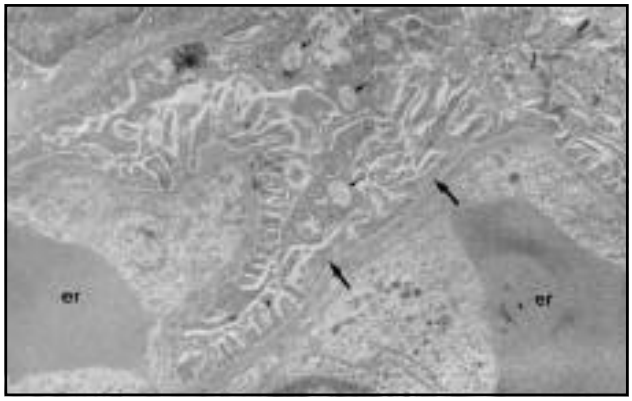

Figure 8 - Podocytes revealing endocytotic vesicles (small arrows) in their cytoplasm. Foot processes of podocytes are fused with a resultant focal obliteration of the filtration slits (large arrows) erythrocytes (er). Kidney of a rabbit treated with diclofenac sodium for 8 weeks (x 20000).

having compromised function of matrical enzymes concerned with the intermediary metabolism.[17] Since the electron transport takes place in mitochondrial cristae, the presently encountered cristolysis implies a fall of the total ATP level and consequently impaired energy-derived metabolic activity.[18,19] Respiration stimulation and inhibition of ATP synthesis together with calcium reflux induction in the mitochondria after diclofenac treatment were reported by some investigators.[6,20] The mitochondrial damage provoked by diclofenac in the present study indicates the capability of this drug to impair oxidative phosphorylation as a consequence to the inhibition of ATP synthesis. The dilated ER was most likely the smooth one, which usually manifests fluid accumulation and accentuates the vacuolation appearance of the cell cytoplasm.[21] It was reported that ER dilatation is one of the earliest consequences of cation and fluid influx into the cells.[12] Also, direct damage of ER membrane leads to leakage of potassium and water from cell sap into ER cisternae. Dilatation of SER may be related to its function in detoxification of certain drugs and toxins.[22] It was found that diclofenac inhibits cyclo-oxygenase, which decreases Na-K abundance in the thick ascending limb of Helene's loop.[23] This may explain the demonstrated ER dilatation as an early consequence of cations and fluid influx due to disturbance of Na^+ pump system. Concerning the observed detachment of ribosomes (degranulation) from the vesiculated RER, it is documented to impair protein synthesis through blocking of mRNA function.[24] The increased number of lysosomal-related structures might be considered as a structural evidence for stimulated autophagic activity, which acts as a removal mechanism of damaged organelles. Also, it reflects an increase in the synthesis of hydrolytic and detoxifying enzymes. It seems likely that there were intralysosomal lipids in the damaged renal tubular cells and the lysosomal membranes were closely apposed to the lipoid globules. Because of the encountered mitochondrial damage, lipid metabolism in the affected renal tubular cells is supposed to be defective with subsequent increase in the fatty acid pool and finally triglycerides (neutral lipids) accumulate in the tubular cell cytoplasm. The observed chromatin clumping in nuclei of the tubular cells of the treated rabbits is supposed to be an evidence of cell injury, which contributes to nuclear chromatin condensation. In the presence of mitochondrial damage and ATP deficiency, pH decreases due to lactate accumulation as a result of stimulated glycolysis.[3] The demonstrated shrunken dark tubular cells may represent a form of cellular degeneration or necrosis and also indicate the occurrence of apoptosis, which is considered as an expression of pathological programed cell death.[25] These dark cells contained electron dense amorphous bodies, which may represent accumulated pigment granules such as lipofuscin or iron pigments.[26] The encountered thickening of GBMs was most likely the result of the precipitation of drug-derived macromolecules on these membranes during renal filtration. This form of drug-induced membranous glomerulopathy is sometimes associated with production of neutrophil chemotactic factors. This may explain the frequent lodgement of neutrophils in the glomerular capillaries, which simulates the situation in renal anti-glomerular basement membrane disease.[27] The infiltrated neutrophils can induce renal glomerular damage through release of their lytic enzymes. Mesangial cell proliferation noticed here was presumably stimulated by the released factors at the site of renal damage[28] to replace the damaged mesangial cells. Mesangial cells play an important role in the processing of macromolecules and may represent the first specific site of glomerular damage.[29] Glomerular podocytes disclosed distortion, swelling and fusion of their foot processes associated with oblitration and decreased number of the filtration slits. These changes are possibly a sequel to a direct toxic damage at the level of glomerular filtration barrier. The changes indicate impaired glomerular function due to altration of the filtration slit pores.[30,31] In general, the mostly affected tubular cells were the proximal convoluted ones, which are known to be highly susceptible to nephrotoxic agents that may affect the cellular metabolic pathways.[32,33] The present ultrastructural data showed that the main targets of chronic diclofenac toxicity are the cytoplasmic organelles of the renal tubular epithelium and the various glomerular structures. Base on the present data, it is reasonable to raise a warning for patients treated chronically with voltaren considering the resultant renal tissue alterations.

Acknowledgment. We are grateful to the EM unit supervised by Dr. O. A. Al-Dokhi, Research Centre at College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia for the invaluable technical assistance. This research project (Zoo/1422/03) was supported by a research grant from the Research Center, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.

References

1. Morgan G. Beneficial effects of NSAIDS in the gastrointestinal tract. ***Eur J Gastroenterol Hepatol*** 1999; 11: 393-400.
2. Mascher H. Comparative metabolism of the nonsteroidal anti-inflammatory drug, diclofenac, in the rat, monkey and human. ***Drug Metab Dispos*** 1996; 24: 969-997.
3. Farag MM, Mikhail M, Shehata R, Abdel-Meguid E, Abdel-Tawab S. Assessment of gentamicin-induced nephrotoxicity in rats treated with low doses of ibuprofen and diclofenac sodium. ***Clin Sci Colch*** 1996; 91: 187-191.

4. Tawab S. Assessment of gentamicin-induced nephrotoxicity in rats treated with low doses of ibuprofen and diclofenac sodium. ***Clin Sci Colch*** 1996; 91: 187-191.
5. Bhogaraju A, Nazeer S, Al-Bafhdad Y, Rahman M, Wrestler F, Patel N. Diclofenac associated hepatitis. ***South Med J*** 1999; 92: 711-713.
6. Bort H, Huber R, Steinijans VW, Koch HJ, Wurrst W, Boyer DP. The enzymes. 3rd ed. New York (NY): Academic Press; 1991.
7. Gabry MS, Elewa FH, Ibrahim MA. Histochemical evaluation of the hepatic and intestinal damage associated with diclofenac administration. A light and electron microscopic study. ***Journal of Egyptian Society of Zoology (Histology, Histochemistry)*** 1999; 27: 117-153.
8. Bougie D, Johnson ST, Weitekamp LA, Aster RH. Sensitivity to a metabolite of diclofenac as a cause of acute immune hemolytic anemia. ***Blood*** 1997; 90: 407-413.
9. Steitiea FM, Ali SS, Muhamed SA. Morphological changes of the mouse testis under the influence of diclofenac sodium (Voltaren). A light and electron microscopic study. ***Egyptian Journal of Anatomy*** 1994; 7: 387-340.
10. Kim H, Yu M, Lin Y, Cousins MJ, Eckstein, RP, Jordan V et al. Renal dysfunction associated with the perioperative use of diclofenac. ***Anesth Analg*** 1999; 89: 999-1005.
11. Ades IZ, Cascarano J. Mitochondrial alterations in heart, kidney and liver of rats subjected to anemia hypoxia. ***Exp Mol Pathol*** 1979; 30: 49.
12. Aldridge WN. In: Maniloff JR, Coleman Jr, Miller MW, editors. Effects of Metals on Cells, Subcellular Elements and Mitochondria. Illinois (USA): Thomas Springfield; 1970. p. 255-271.
13. Racker E. Membranes of mitochondria and chloroplasts. New York (NY): Vanostrand-Reinhold Press; 1970.
14. King DW, Fenoglio CM, Leekowitch JH. General pathology. Principles and Dynamics. Philadelphia (PA): Lea & Febiger; 1983.
15. Hirakawa N, Heuser JE. Structural evidence that botulinum toxin blocks neuromuscular transmission by impairing the calcium influx that normally accompanies nerve depolarization. ***J Cell Biol*** 1981; 88: 160.
16. Joshi MS, Crouser ED, Julian MW, Schanbacher BL, Bauer JA. Digital imaging analysis for the study of endotoxin-induced mitochondrial ultrastructure injury. ***Anal Cell Pathol*** 2000; 21: 41-48.
17. Goyer RA, Rhyne BC. Toxic changes in mitochondrial membranes and mitochondrial function. New York (NY): Academic Press; 1975.
18. Aithal HN, Toback FG. Defective mitochondrial energy production during potassium depletion nephropathy. ***Lab Invest*** 1978; 39: 186.
19. Robinson JM. Gossypol-induced damage to mitochondria of transformed Sertoli cells. ***Am J Pathol*** 1986; 25: 484.
20. Morenzo-Sanchez R, Bravo C, Vasquez C, Ayala G, Silveira LH, Martinez-Lavin M. Inhibition and uncoupling of oxidative phosphorylation by nonsteroidal anti-inflammatory drugs: study in mitochondria, submitochondrial particles, cells and whole heart. ***Biochem Pharmacol*** 1999; 57: 743-752.
21. Ryffel B, Mihatsch MJ. Cyclosporin nephrotoxicity. ***Toxicol Pathol*** 1986; 4: 73-82.
22. Gartner LP, Hiatt JL. Color Atlas of Histology. 3rd ed. Philadelphia (PA): Lippincott Williams & Wilkins; 2000.
23. Turner R, Nielsen S, Knepper MA. Cyclooxygenase inhibitors increase Na-k-2cl cotransporter abundance in thick ascending limb of Henle's loop. ***Am J Physiol*** 1999; 277: 219-226.
24. Leduc EH. Sulfaguanidine protection of mouse liver from carbon-tetrachloride induced necrosis. ***Lab Invest*** 1973; 29: 186.
25. Wyllie AH, Kerr JFR, Currie AR. Cell death: The significance of apoptosis. ***Int Rev Cytol*** 1980; 68: 251-306.
26. Ikeda H, Tauchi H, Shimasaki H, Ueta N, Sato T. Age and organ differences in amount and distribution of autofluorescent granules in rats. ***Mech Ageing Dev*** 1985; 31: 139-146.
27. Couser WG, Baker PJ, Adler S. Complement mediation of immune glomerular injury. A new perspective. ***Kidney Int*** 1985; 28: 879-890.
28. Kashigarrian M. Mesangium and glomerular disease. ***Lab Invest*** 1985; 52: 569-571.
29. Johnson RJ, Garcia RL, Pritzl P, Alpers CE. Platelets mediate glomerular cell proliferation in immune complex nephritis induced by anti-mesangial cell antibodies in the rat. ***Am J Pathol*** 1990; B6: 369-374.
30. Kurihara H. The altered glomerular filtration slits seen in puromycin aminonucleoside nephrosis and protamine sulfate-treated rats contain the tight junction protein Zo-1. ***Am J Pathol*** 1992; 141: 805.
31. Venkatachalam MA, Rennke HG. The structure and molecular basis ofglomerular filtration. ***Circ Res*** 1978; 43: 337.
32. Bulger RE, Dobyan DC. Proliferative lesions found in rat kidneys after a single dose cisplatin. ***J Natl Cancer Inst*** 1984; 73: 1235-1242.
33. Rush GF. Cephaloridine-induced renal pathological and biochemical changes in female rabbits and isolated proximal tubules in suspension. ***Toxicol Pathol*** 1992; 20: 155.