Global Journal of Pharmacology 8 (3): 369-377, 2014
ISSN 1992-0075

DOI: 10.5829/idosi.gjp.2014.8.3.83264

Histological and Ultrastructural Studies on the Effect of Diclofenac Sodium on the Renal Cortex of Fetuses of Albino Mice

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Abstract: The present study was carried out to evaluate the effect of the non-steroidal anti-inflammatory drug diclofenac sodium (DS) on the renal cortex of fetuses of albino mice from the histological and ultrastructural points of view. Twenty pregnant female mice were allocated into 2 groups (10 mice each). The first group served as control and each animal was injected intraperitoneally (i.p.) with the solvent of the drug, daily for 8 days during pregnancy from day 7 till day 14 of gestation (GDs 7-14). The second group is the treated group; each animal was injected (i.p.) daily with 1.5 mg/kg body weight of DS for 8 days (GDs 7-14). Histological examination of the renal cortex of maternally treated fetuses showed atrophic glomeruli with widened capsular spaces of the renal corpuscles. Renal convoluted tubular cells had a vacuolated cytoplasm and pyknotic nuclei. Some proximal tubules showed disruption of their apical brush borders. Also, the lumina of some proximal and distal tubules were occluded with hyaline casts. Electron microscopic examination of the renal cortex of fetuses maternally treated with DS revealed conspicuous alterations, represented by thickening of the capillary basement membrane in some glomeruli. The foot processes of podocytes were frequently fused thus obliterating the infiltration slits. The cells of the proximal convoluted tubules displayed partial destruction of the microvilli of the apical brush borders and degeneration of mitochondria. Besides, many vesicles and large vacuoles were observed near the basal part of the microvilli. The cells of the distal convoluted tubules showed marked thickening of their basement membranes and their mitochondria lost their cristae and appeared vacuolated.

Key words: Diclofenac Sodium · Fetuses · Histopathology · Mice · Renal Cortex · Ultrastructure

INTRODUCTION

Diclofenac (DCLF) is a non-steroidal anti-inflammatory drug (NSAID) that is widely used for the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis and acute muscle pain conditions. A generally accepted mechanism of action of NSAIDs is the inhibition of cyclooxygenases, the rate-limiting enzymes that catalyse the formation of prostaglandin precursors from arachidonic acid [1, 2]. Prostaglandins play a role in the control of cell proliferation and regulation of immune functions [3, 4]. However, in spite of such beneficial role of these drugs several reports or investigations were issued dealing with their toxic and pathological side effects on various body organs including the kidney. Toxic doses of DCLF can cause nephrotoxicity in humans and experimental animals [5]. Its clinical use is limited by the occurrence of DCLF-induced renal toxicity, which can manifest as acute kidney injury [6, 7]. Acute DCLF-induced nephrotoxicity is attributed to the blocking of cyclooxygenase (COX) and subsequent inhibition of prostaglandin synthesis by DCLF [8]. The use of NSAIDs during pregnancy has increased, in spite of the emerging findings: even short-term administration of NSAIDs during the late pregnancy period is correlated with a significant increase in risks of premature closure of ductus arteriosus [9]. It is worth of mention that selective COX-2 inhibitors, often prescribed during pregnancy are capable of crossing into placenta although these compounds are not used in the newborn [10]. Biochemical, histological and ultrastructural investigations regarding the effects of NSAIDs on the kidney of adult experimental animals have been extensively studied. However, there are few available data

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on the effect of diclofenac sodium (DS) on renal tissue of the developing kidney. The present study was designed to investigate the histological and ultrastructural alterations that may occur in the renal cortex of mice fetuses maternally treated with DS during pregnancy from day 7 to day 14 of gestation.

MATERIALS AND METHODS

Experimental Animals: The present investigation was carried out on mature albino mice of pure CD-1 strain with an average body weight of 20-30g obtained from the breeding unit of Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, A.R. Egypt. Females and males were housed separately in plastic cages, each cage contained two mice in order to avoid over crowding. Mice were fed on cubes consisting of crude proteins, minerals and fibres. Vitamins were added as fresh vegetables and the animals were provided with milk and tap water *ad libitum*. Pregnancy was achieved by housing one adult virgin female with one well marked fertile male overnight, from 5 pm until 9 am of the next day. Successful mating was indicated either by the presence of a vaginal plug or by the presence of spermatozoa in the vaginal smears according to the method suggested by Snell [11]. Females which give positive vaginal smears are considered pregnant and the day of detection was defined as the first day of pregnancy.

The Drug Used: The drug used in the present study is diclofenac sodium (Declophen; pharco pharmaceutical company, Egypt). The chosen dose of diclofenac sodium (DS) was nearly comparable to the effective therapeutic dose for the human being. The chosen dose of the drug for mice was calculated according to Paget and Barnes [12] and was estimated to be equivalent 1.5mg/Kg body weight.

Experimental Design: Twenty pregnant female mice were divided into two groups comprising 10 animals in each group. The first group is considered as the control group (A) and the second group (B) is the drug treated group and treatment of these groups was achieved in the following manner:

Group (A): Each pregnant female was injected intraperitoneally with 0.1ml distilled water (the solvent of the drug) daily for 8 days during pregnancy from day 7 till day 14 of gestation (GDs 7-14).

Group (B): Each pregnant mice was intraperitoneally injected with 1.5 mg/kg body weight of DS daily for 8 days during pregnancy (GDs 7-14).

Pregnant mice of both control and experimental groups were sacrificed on day 19 of pregnancy. They were dissected and their uteri were removed, placed in normal saline solution and the fetuses were taken out. For light microscopic examination small pieces of the kidney of fetuses of control and maternally treated animals were fixed for 24 hours in aqueous Bouin's fixative. The specimens were then dehydrated, cleared in terpineol and embedded in paraffin wax. Serial transverse sections of about 5 µm thickness were stained with haematoxylin and eosin, microscopically examined and photomicrographs were made as required.

For the electron microscopic studies, small pieces of the kidney were fixed in 2.5% glutaraldehyde for 4 hours and 2% paraformaldehyde in 0.1M cacodylate buffer (pH7.4). The samples were post-fixed in 2% buffered osmium tetroxide at 4°C for one hour. This was followed by dehydration in ascending series of ethyl alcohol for two changes, clearing in two changes of propylene oxide, 5 min each. Then, specimens were embedded in Epon-epoxy-resin. Semi-thin sections of 1µm thickness were stained with toluidine blue and examined, for general orientation under a bright field light microscope. Ultrathin sections were prepared, stained with uranyl acetate and lead citrat [13]. Sections were examined and photographed on a Joel 1200 EX 2 transmission electron microscope, at the Faculty of Science, Ain Shams University.

RESULTS

Histological and Histopathological Observations

Group A (The Control Group): The kidney of 19-day old control mice fetuses is differentiated into two regions; an outer cortex and an inner medulla. The cortical tissue consists of a number of renal or Malpighian corpuscles and convoluted tubules. The Malpighian corpuscle is formed of the Bowman's capsule and glomerulus (Fig. 1). The Bowman's capsule consists of two layers of simple squamous epithelium, an outer parietal layer and an inner visceral one, which are separated by a capsular space, the urinary space. The glomerulus is composed of tortuous capillary loops of the afferent and efferent arterioles, supported by the mesengial cells (Fig. 1).

The proximal convoluted tubules appear rounded, oval or elongated in cross section of the kidney. Each tubule is lined with a single layer of short columnar or

pyramidal cells with indistinct cell boundaries. Their granular cytoplasm stains deeply with eosin and possesses rounded basal nuclei. The free ends of these cells, near the lumen, are provided with a peculiar brush border as seen in Figure 1.

The distal convoluted tubules are lined with cuboidal cells that possess distinct cell boundaries and contain conspicuous spherical centrally located nuclei. The cytoplasm of these cells stains less intensely than that of the cells lining the proximal convoluted tubules. The lumen of the distal convoluted tubule is wider compared with that of the proximal convoluted tubule. In addition, the free ends of the cells of the distal convoluted tubule have no brush borders (Figs. 1&2).

Group B (DS Maternally Treated Fetuses): The kidney of 19-days old mice fetuses maternally treated with 1.5mg/kg body weight of DS during organogenesis exhibited signs of alterations in renal cortical tissues. These changes included the Malpighian corpuscles and the renal tubules. The glomeruli in the Malpighian corpuscles showed hypocellularity. Thus the renal corpuscles revealed an apparent shrinkage of the glomeruli and widening of the capsular (Urinary) space of the renal capsules (Fig. 3). The lining epithelial cells of some renal convoluted tubules displayed degenerative changes. These tubular cells had vacuolated cytoplasm and pyknotic nuclei (Fig.4). Other cells suffered from necrosis, lost their regular architecture and their nuclei displayed distinct features of pyknosis and karyolysis. Some proximal convoluted tubules showed loss of their brush borders. The lumina of few proximal and distal convoluted tubules were occluded with hyaline casts and cell debris (Figs. 3 & 4).

Ultrastructural Study

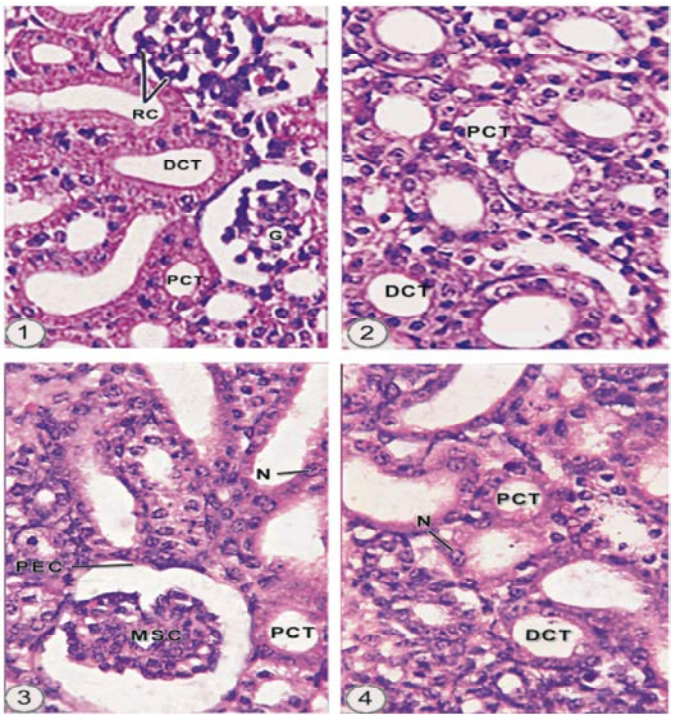
Group A (The Control Group): The electron micrographs of the renal cortex of the control mice fetuses revealed that the Malpighian corpuscle consists of two parts, a thin walled cup-like expansion, the "Bowman's capsule" and a lobulated tuft of capillaries, the glomerulus. Each glomerular capillary is bounded by an outer wall or basal lamina and an inner endothelial lining. The inner wall of the capsule (The visceral epithelium) is formed of podocytes. Each podocyte has several processes which give rise to numerous secondary processes known as "pedicles". These processes rest upon the basement membrane of the glomerulus leaving narrow slits between them called filtration slits (Figs. 5&6). The axial portion of the hilus of the glomerular capillaries has certain cells, known as intercapillary or mesangial cells. These cells are separated from the endothelial cells by an amorphous mesangial matrix.

The cells of the proximal convoluted tubules have an elaborate shape, well developed microvilli (Or brush border) along their lumina, an active endocytotic apparatus and many spherical or elongated mitochondria (Fig. 7). The nuclei of such cells are relatively large, mostly euchromatic with prominent nucleoli and always lying at the basal portion of the cells (Fig. 7). The distal convoluted tubules don't have a brush border, but a few microvilli appear at the apical membrane (Fig. 8). The mitochondria are relatively elongated and occupy the cytoplasmic compartment between ill distinct basal infoldings (Fig. 8). The nuclei are relatively large and their heterochromatin appears always adherent to the inner surface of the nuclear envelope.

Group B (DS Maternally Treated Fetuses): The electron micrographs of ultrathin sections of the renal cortex of 19-days old fetuses maternally treated with DS revealed ultrastructural changes of the renal cortical cells. A marked thickening of the capillary basement membrane, enclosing electron lucent area was observed in some glomeruli. The capillary lumen appeared completely obliterated and filled with blood cells (Fig. 9). The same figure exhibited the podocytes nuclei with clumping heterochromatin material. The foot processes of podocytes were frequently fused thus obliterating the infiltration slits. In addition swelling of some foot processes was also observed (Fig. 9).

The proximal convoluted tubules showed marked thickening of their basement membranes. In some parts of the tubules, the microvilli of the apical brush border of the lining cells were partially degenerated with aggregation of many vesicles and large vacuoles near the basal part of the microvilli. The mitochondria were swollen and their matrices were condensed so that their fine structures become obscure. The nucleus appeared with irregular nuclear envelope (Fig. 10).

The distal convoluted tubules appeared with marked thickening of their basement membranes. The mitochondria lost their cristae and appeared vacuolated so that they don't show any demarcation of their detailed fine structure (Fig. 11). The nuclear heterochromatins were aggregated on the inner surface of the nuclear envelope (Fig. 11).



Figs. 1&2: Photomicrographs of kidney sections of 19-days old control mouse fetus, showing the renal corpuscles (RC), glomeruli (G), the proximal convoluted tubules (PCT) as well as the distal convoluted tubules (DCT). X 400

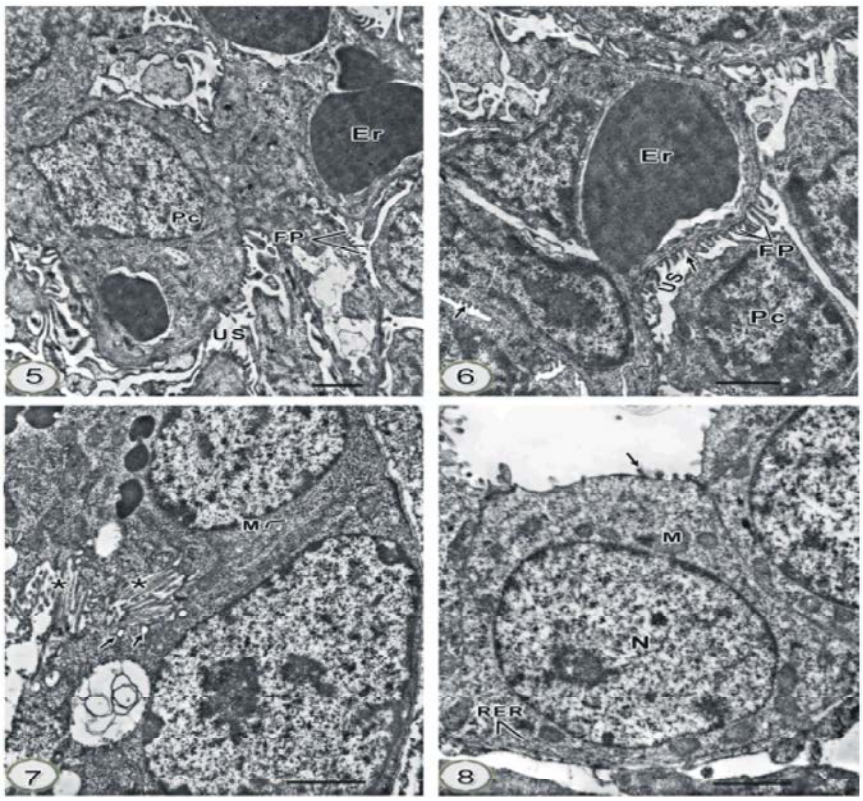
Figs. 3&4: Photomicrographs of kidney sections of 19-days old fetus maternally treated with 1.5mg/kg body weight of diclofenac sodium during GDs (7-14), showing degeneration of the parietal epithelial cells (PEC) of Bowman's capsules. The renal corpuscles show hypoplasia of the mesangial cells (MSC) of the glomerulus. The proximal (PCT) and distal (DCT) convoluted tubules show marked cellular vacuolation of the lining epithelial cells and the nuclei (N) show distinct karyolysis. X400

DISCUSSION

Diclofenac is of common use for certain medical purposes by both pregnant and lactating women; it was mentioned in medical reports to have certain side effects on body organs [14,15]. The present investigation was constructed to study the effect of diclofenac sodium on the histological and ultrastructural features of the renal cortex of maternally treated mice fetuses. The results of the present work clearly illustrated that the application of 1.5mg/kg body weight of diclofenac sodium to pregnant mice during gestation (GDs 7-14) had induced conspicuous consequences in the histological and the ultrastructural features of the renal cortex of fetuses of such treated animals. The major impairments comprised the Malpighian corpuscles and the renal convoluted tubules. The glomeruli exhibited hypocellularity, thus the renal corpuscles showed marked shrinkage of their glomeruli and widening of the capsular (Urinary) spaces of the renal corpuscles. The lining epithelial cells of some renal convoluted tubules displayed degenerative changes including vacuolated cytoplasm and pyknotic nuclei. The apical brush borders of some proximal tubules were disrupted and their lumina as well as the lumina of certain distal convoluted tubules were occluded with hyaline casts and cell debris.

During the last decades, an increasing number of reports and investigations have been published concerning the nephrotoxic effects of several drugs, including NSAIDs, in developing fetus.

Administration of the antibiotic gentamicin to pregnant rats during the period of gestation corresponding to early nephrogenesis in the fetus causes a reduction of the number of nephrons normally present at birth [16,17]. Prenatal exposure to cyclosporine A [18], impaired postnatal nephron differentiation in newborn rats [19] and induced 25% nephron deficit in newborn rabbits [20]. Marked devastations of the glomerular capillaries and narrowing of the urinary spaces were observed in the kidney of mice fetuses maternally treated with the



Figs. 5-8: Electron micrographs of kidney sections of 19-days old control mouse fetus

Fig. 5: Showing a part of the glomerulus capillaries which recognized by their content of erythrocytes (Er). The podocytes (Pc) are seen embracing the wall of the blood capillaries. Notice, part of urinary space (US) and foot processes (FP). X 10800

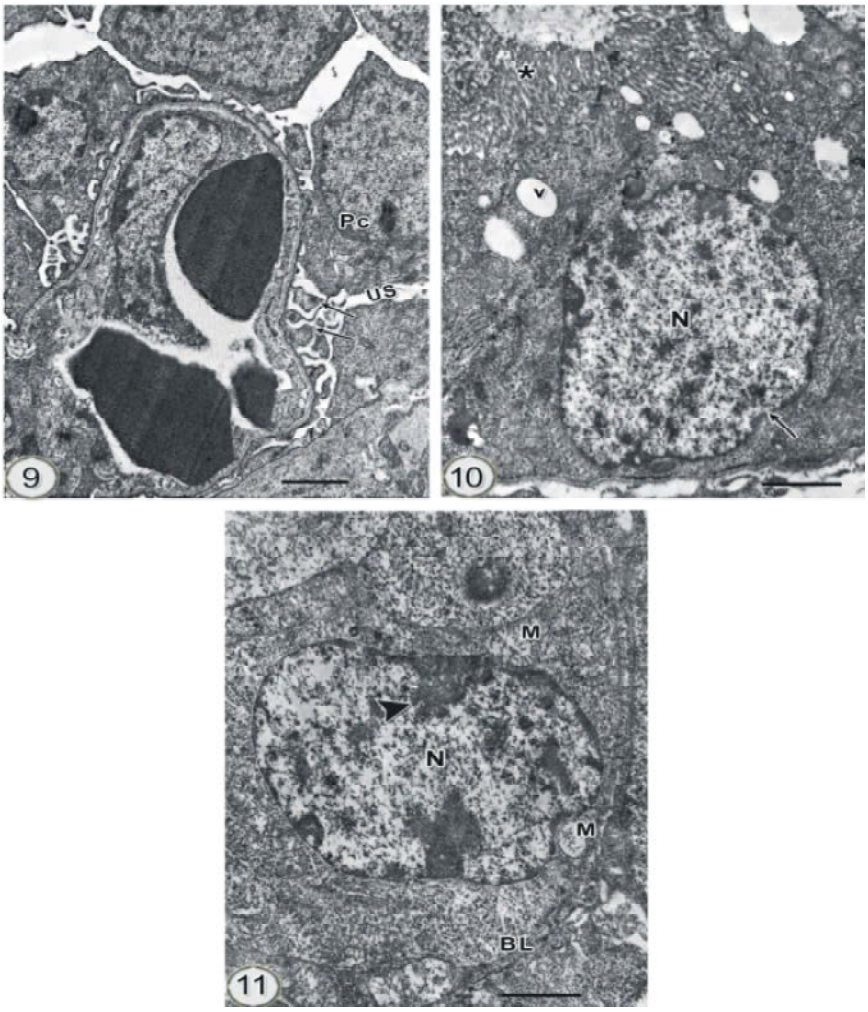
Fig. 6: Showing erythrocyte (Er), podocytes (Pc), part of urinary space (US) and foot processes (FP) which embracing the wall of the capillaries forming small pores known as the filtration slits (Arrows). X 13500

Fig. 7: Showing a proximal convoluted tubule cell has a lumen occupied by a multitude of densely backed microvilli (*). The cytoplasm contains some pinocytotic vesicles (Arrows), numerous mitochondria (M) and rough endoplasmic reticulum (RER). X16200

Fig. 8: Displaying distal convoluted tubule cells which possess a few small microvilli (Arrow). The cytoplasm contains a relatively large nucleus (N) and mitochondria (M). X 16200

anti-ulcer drug omeprazole [21]. The author added that the epithelial cells of the convoluted tubules were markedly degenerated. Drukker [22] reported that treatment with NSAIDs during pregnancy may affect renal structure and function in developing fetuses and newborns. On the same line, Espiridiao, [23] revealed some adverse effects in fetal kidneys of rats maternally treated with acetyl salicylic acid. Maternal and fetal renal tissue of rat were adversely affected post paracetamol treatment during gestation [24]. Treatment of pregnant subjects with NSAIDs during pregnancy can cause renal dysgenesis in neonates [25]. Extensive tubular necrosis was observed in fetal kidneys of mice maternally treated with retinoic acid [26]. Along the same line, Bao, [8] reported that adult mice exposed to nephrotoxic dose of diclofenac (200mg/kg) via oral gavage showed a typical pattern of acute tubular necrosis in the proximal and distal tubules, which was characterized by isometric vacuolization of the tubular epithelium, luminal ectasia, sloughing of tubular cells into the lumen, loss of brush borders, nuclear enlargement and pleomorphism. It is worth noting that, the chosen dose (1.5mg/kg b.wt.) used in the present work is nearly comparable to the effective therapeutic dose for human being.

The present ultrastructural observations showed apparent thickening of the capillary basement membrane enclosing electron lucent area and the capillary lumen appeared obliterated with blood cells. The obvious



Figs. 9-11: Electron micrographs of kidney sections of 19-days old mouse fetus maternally treated with 1.5mg/kg body weight of DS during GDs (7-14).

Fig. 9: Showing marked fusion of some foot processes (Arrows) and blood capillaries dilated and filled with blood corpuscles leaving narrow urinary space (US). Podocyte (Pc) processes are fused with swelling foot processes of the blood capillaries. X 13500

Fig. 10: Showing proximal convoluted tubule cells with disrupted microvilli (*) and increased apical vacuoles (V). Notice, the central nucleus (N) with irregular nuclear envelope (Arrow). X16200

Fig. 11: Showing distal convoluted tubule with thickening of tubular basal lamina (BL) and vacuolated mitochondria (M). The nucleus (N) with irregular nuclear envelope and heterochromatin condensed on the inner membrane of the nuclear envelope (arrowhead). X 16200

alterations in podocytes included their nuclei which appeared clumped with heterochromatin material and the swelling of their foot processes. The foot processes of some podocytes were frequently fused thus obliterating the infiltration slits. Local fusion of foot processes of visceral epithelial cells and loss of the regular endothelial lining of the basement membranes are recorded in subjects suffered from renal impairment after acute diclofenac over doses [27]. Fusion of podocytes foot processes and degenerative lesions in tubular epithelial cells were observed in fetal rat kidney exposed to adriamycin [28]. The authors added that this leads to damage in the glomerular filtration barriers. More recently, Abdel-Aziz and Mohamed [29] observed apparent widening of Bowman's space in glomeruli and marked disruption of foot processes, in offspring of rats maternally treated with monosodium glutamate.

In the current ultrastructural study severe degenerative changes in the mitochondria of the convoluted tubules of maternally treated fetuses were observed post diclofenac sodium treatment. In this respect, several studies have implicated mitochondrial damage in the toxic process initiated by various NSAIDs [30, 31]. Several reports suggest that mitochondria are a primary target of diclofenac [32-34]. Significantly, tubular cell mitochondria of rat renal cortex are a prime target of diclofenac [35]. Furthermore, diclofenac can induce the mitochondria permeability transition in isolated mitochondria from rat hepatocytes [36, 37] which may lead to the release of proapoptotic factors into the cytosol and nucleus and activate the cell death pathways.

The present histo- and cytopathological findings could be attributed to growth retardation caused by DS and these results agree with those reported by Sabry [38] in mice fetuses maternally treated with DS during organogenesis. Such retardation in growth may be reflected on the renal tissue of the fetus which appears to be functionally immature enough to excrete the drug or its metabolites rapidly as adult, so it may cause such pathological effects. Tuchmann-Duplessis [39] suggested that the functional immaturity of prenatal kidney limits the excretion of many drugs, therefore increasing the toxic effects. In the same direction, Merlet-Benichou, [40] reported that intrauterine growth retardation leads to a permanent nephron deficit. The assumption that inhibition of COX-2 by NSAIDs may be responsible for impaired renal development is supported by the observation that mice with genetic deletion of COX-2 exhibited morphological damages to the kidney, similar to those found in human neonates post NSAIDs [41, 42].

The duration of treatment, in the present investigation, extends from day 7 to day 14 of gestation. This period of pregnancy covering organogenesis and overlapping the first stages of renal development in mice fetuses. In mice, nephrogenesis is reported to start by day 11 of gestation. By 17 and 18 days the kidney is fully differentiated [43, 44]. During organogenesis, very low concentrations of toxic drugs may have devastating effects [45]. It is a period when drugs are known to have the greatest potential to cause malformations [46].

It seems logical that a drug having a renal effect in the mother and able to cross the placental barrier, would also have a renal effect in the fetus and/or in the neonate [47]. Siu, [48] confirmed that diclofenac crosses the human placenta readily during the first trimester. Therefore, possible toxic effects of DS were among the main causes of the histological changes observed in the present investigation in the fetal renal tissue as a result of diffusion and excretion of DS through the renal tissue.

Several mechanisms were reported to explain the action of NSAIDs on nephrogenesis. In this respect, it is worthy to note that [49] reported that kidney synthesizes several prostaglandins important in the regulation of renal function. Studies in rats have suggested that prostaglandins may play a role in renal development [50]. The nephrotoxic effects of NSAIDs are attributed to the block of prostaglandin synthesis [51]. Since disturbances in homeostasis of prostaglandins have been implicated in the teratogenesis of fetal malformations, NSAIDs are therefore a potential teratogen [48]. The aforementioned explanations could declare the renal damaging effect of DS reported in the current study.

The foregoing results showed the deleterious effect of DS on the renal cortex of the developing kidney of mice when given during organogenesis (GD 7-14). Since the drug is very commonly used, therefore, further investigations are needed to confirm these findings in humans.

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