Articles

The effects of diethylstilbestrol administration on rat kidney

Ultrastructural study

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ABSTRACT

الأهداف: تقييم التغيرات النسيجية والتركيبية التي يمكن أن يسببها عقار داي ايثيلستيلبيسترول على أنسجة الكلى من خلال دراسة نسيجية ومناعية وتركيبية.

الطريقة: أجريت هذه الدراسة في قسم التشريح، كلية الطب، جامعة الملك عبدالعزيز، جدة، المملكة العربية السعودية تمت في الفترة من ديسمبر 2011م إلى ديسمبر 2012م. حوالي 30 جرذاً ذكراً بالغاً تم تقسيمهم إلى 3 مجموعات (10 جرذان لكل مجموعة)، المجموعة الأولى هي الضابطة، والمجموعة الثانية تلقت عقار داي ايثيلستيلبيسترول بجرعة 60 ميكروجرام لكل كجم من وزن الجرذ في اليوم في 0.1 مل من زيت الذرة لمدة 20 يوماً، بينما المجموعة الثالثة تلقت عقار داي ايثيلستيلبيسترول بجرعة 60 ميكروجرام لكل كجم من وزن الجرذ في اليوم في 0.1 مل من زيت الذرة لمدة 50 يوماً عن طريق الفم. تم دراسة أنسجة الكلى بالمجهر الضوئي والمجهر الإلكتروني. وقد أجريت الدراسة في قسم التشريح، كلية الطب، جامعة الملك عبد العزيز.

النتائج: أن تعاطى عقار داي ايثيلستيلبيسترول لمدة 50 يوماً تسبب في تغييرات ملحوظة في الأنسجة التركيبية للكلى وظهر تلف الأنابيب الكلوية. وعلاوة على ذلك، كانت هناك زيادة كبيرة في الأجسام المناعية. وكانت كل هذه التغيرات أقل وضوحاً بعد 20 يوماً من العلاج.

خاتمة: أظهرت مركبات الاستروجين الاصطناعية آثار ضارة على أنسجة الكلى وتغيير في تركيبها وهذه التغييرات تعتمد على مدة التعاطي.

Objectives: To assess the histological and ultrastructural changes that can be induced by diethylstilbestrol (DES) on renal tissues using histological, immunohistochemical, and ultrastructural methods.

Methods: Thirty adult male Wistar rats were divided into 3 groups (10 rats each): Group 1 - control; Group 2 - received DES at a dose of 60 µg/kg/day, dissolved in 0.1 ml corn oil for 20 days; and Group 3 - received the same dose of DES for 50 days by oral gavage. The renal tissues were studied histologically, immunohistochemically (using an anti-BCL2-associated X protein [BAX protein] antibody), and ultrastructurally. This study was carried out at the Anatomy Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia between December 2011 and December 2012.

Results: The DES administration for 50 days caused noticeable degeneration, and alteration of the morphology of the renal tissues in the form of damaged renal tubules with loss of the brush border of the proximal convoluted tubules and increased cellularity of the glomeruli. In addition, there was a significant increase in BAX protein expression based on immunoreactivity, and in renal tubules, as well as glomerular cells. These changes were less obvious after 20 days of treatment.

Conclusion: Non-steroidal, synthetic estrogens showed harmful effects on the renal tissues and altered their morphology with an increased number of apoptotic cells, and these changes were duration dependent.

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The female sex hormones estrogen and progesterone are used in most contraception strategies. Estrogen replacement therapy is also a prevalent treatment in postmenopausal women to relieve climacteric pain.[1] Likewise, estrogen has been used for protection against osteoporosis.[2] Furthermore, estrogen plays a defensive role to alleviate dangerous causes of cardiac illness.[3] To avert the upsurge of endometrial cancer associated with estrogen treatment, consecutive progesterone use for 12 days monthly is preferred.[4] Moreover, numerous morphological alterations occur in different tissues with the use of estrogen components.[5] Management with hormone replacement therapy has been proposed to be associated with functional turbulence of the kidney.[6] Contraceptives drugs yield electrolyte imbalances and raise the uric acid level.[7] Estrogen leads to congestion in the kidney, lymphocytic grouping, interstitial hemorrhage, and cystic expansion of the renal tubules.[8] Diethylstilbestrol (DES), a non-steroidal synthetic estrogen was first produced in 1938,[9] and is the first synthetic estrogen.[10] The DES has occasionally been prescribed for the treatment of advanced breast and prostate cancer.[11] The DES resembles natural estrogens but can cause cancer in humans.[12] Previous research on the influence of DES in intrauterine development highlighted a decline in the serum levels of progesterone and estrogen in different animals.[13] The DES has a negative influence on contractions of the uterus and in placental detachment during delivery. This intrauterine lethal role leads to an eventual decrease in the progeny number.[14] The influence of DES on rats has been studied throughout the post-natal time.[15] The DES can cause uterine, vaginal, cervical, ovarian, and lymphoid tissue tumors in mice,[16] vaginal, testicular, and renal tumors in hamsters,[17] and hepatic, vaginal, and breast tumors in rats.[18] Diethylstilbestrol causes noticeable physiological and biochemical variations and inherited disorders, as well as carcinogenic consequences, predominantly in rat's kidney tissues.[19] The DES has been widely used as an anabolic mediator in household animals, although its use has been prohibited in most countries worldwide, subsequent to proof that quantities beneath residual levels cause toxicity and hereditary malformations.[20] However, there are regrettably no preemptive procedures available in many developing countries for local manufacture.[21] Programmed cell death (apoptosis) occurs when cells commit suicide for the sake of the whole tissue, and many extrinsic factors influence this process, such as drugs and toxins.[22] The BCL2-associated X protein (BAX protein) can be identified using immunohistochemical methods, and is a marker of apoptosis, and detection of BAX protein expression can be used to evaluate the localization and intensity of physiological and pathological apoptosis.[23] To assess the histological and ultrastructural changes that can be induced by DES on the renal tissues using histological, immunohistochemical, and ultrastructural methods, we studied the ultrastructural changes that occur in association with microscopic changes and increased apoptosis in the glomerulus caused by DES. We describe the effects on the glomerular membrane, urinary space, and tubular structures of the renal cortical tissues in rats.

Methods.

This present study was carried out between December 2011 and December 2012 in the Anatomy Department, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

Animals. Thirty male Wistar rats, weighing 200-250 grams, aged 8 weeks were divided into 3 groups (each with 10 animals) obtained from the Experimental Animal Laboratory, King Abdulaziz University. Adequate measures were taken to minimize pain or discomfort. The rats were bred and fed under specified pathogen-free standard laboratory conditions. The rats were placed in individual cages and acclimated for 7 days in a controlled environment at a temperature of 22±3°C, and a 12-hour cycle of light/dark, and a relative humidity of 55-60%, with tap water and commercial rat pellets available ad libitum.

Experimental procedure. The study consisted of 3 groups (10 rats in each group). Group 1 (control) received 0.1 ml corn oil without DES by oral gavage. Group 2 received DES (purity >99%, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 60 µg/kg/day,[24] dissolved in 0.1 ml corn oil for 20 days by oral gavage. Group 3 received DES, 60 µg/kg/day for 50 days by oral gavage.[23,24]All experimental procedures were carried out according to the current laws and regulations of the Faculty of Medicine, King Abdulaziz University on the care and handling of experimental animals, which conformed to the National Institutes of Health (NIH) Guidelines for Care, and Use of Animals in Research.

Histological evaluation. Renal samples were dissected, fixed in 10% neutral-buffered formalin, dehydrated in ascending grades of alcohol, and imbedded in paraffin wax. Paraffin sections of 5 µm thickness were then prepared and stained with hematoxylin and eosin (H&E) for routine histological inspection.[25,26] The sections were viewed and photographed under a light microscope (Olympus BX53, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Co.).

Immunohistochemistry using an anti-BAX antibody. Overexpression of BAX accelerated apoptotic cell death. Immunohistochemical detection of BAX and determination of the expression level were achieved

using a mouse monoclonal anti-BAX antibody and avidin-biotin complex (ABC) staining as described by the manufacturer. Sections were cut into 4 μm and then fixed in a 65°C oven for one hour. Trilogy (Cell Marque, CA, USA. cat# 920p-06) is a product that combines the 3 pretreatment steps: deparaffinization, rehydration, and antigen unmasking. Using this product enhances standardization of the pretreatment procedure, thereby producing more consistent and reliable results. The slides were placed in a Coplin jar filled with 200 ml of trilogy working solution, and the jar was securely positioned in an autoclave.[26] The autoclave was adjusted so that temperature reached 120°C and maintained this temperature for 15 minutes, after which the pressure was released and the Coplin jar was removed to allow the slides to cool for 30 minutes. Sections were then washed and immersed in tris-buffered saline (TBS) to adjust the pH, and this step was repeated between each step of the immunohistochemistry procedure. The endogenous peroxidase activity was determined by immersing the slides in 3% hydrogen peroxide for 10 minutes. The power stain 1.0 poly horseradish peroxidase (HRP) diaminobenzidine (DAB) kit (Cat# 54-0017, Genemed Biotechnologies, CA, USA) was used to visualize any antigen-antibody (AA) reaction in the tissues (for qualitative detection of antigen). The concentrated primary antibody (BAX cat # orb4655, Biorbyt, Cambridge, United Kingdom) was diluted 1:500, and 2-3 drops were applied. The slides were then incubated in the humidity chamber overnight at 4°C. Subsequently, polyclonal HRP-linked antibody conjugates were applied to each slide and incubated for 20 minutes. The DAB chromogen was prepared, and 2-3 drops were applied on each slide and incubated for 2 minutes. The DAB stain was rinsed off, counterstaining was performed with Mayer hematoxylin, and a cover slip was attached before the slides were examined under a light microscope. The brown areas were considered positive.[27]

Electron microscopy. Renal samples were cut into small pieces (1 mm^3) and fixed in 2.5% glutaraldehyde (pH 7.4) in phosphate buffer for 2 hours at room temperature. Post-fixation was performed in the same phosphate buffer containing 1% osmium tetroxide (OsO_4). Tissues were dehydrated in graded ethanol solutions, transferred to propylene oxide, and finally embedded in Epon 812. Semithin sections (1 μm thick) were cut using a glass knife and stained with toluidine blue. Ultrathin sections were obtained using LKB ultratome (Ultratome NOVA, LKB 2188, Bromma, Sweden) and spread on copper grids. The sections were stained by uranyl acetate followed by lead citrate and examined at 80 kV with a JEM transmission electron microscope (JEM-2000EX; JEOL, Tokyo, Japan).[28]

Results. *Effects of DES on general rat's health (mortality and clinical observations).* Death was not observed in any experimental group during the administration period (20-50 days). In comparison to the control group, tested groups revealed no drug-related changes in the clinical signs, such as external appearance, mentality, behavior, and activities per day.

Light microscopy. Histological picture of the kidney (H&E stain). The morphology of the cortex of the kidney contained both proximal and distal convoluted tubules, and the renal corpuscles consisted of a glomerular tuft of capillaries and Bowman's capsule. The visceral layer of the Bowman's capsule enclosed the glomerular capillaries with modified epithelial cells called podocytes. The parietal layer consisted of a thin layer of simple squamous epithelium forming the outer limit of the renal corpuscle. The space between the visceral layer and the parietal layer of the renal corpuscle is the capsular (urinary) space. The proximal convoluted tubules were abundant, with a small lumen and lined with cuboidal cells that were not well delineated. The cells contained an eosinophilic cytoplasm and rounded nuclei, as well as an apical brush border consisting of microvilli. The distal convoluted tubules were fewer, exhibited larger lumina, and were lined with smaller cuboidal cells with dark-stained nuclei. The cells contained a less intensely stained eosinophilic cytoplasm and no brush border (**Figure 1A**).

A microscopic study of the kidney of the animals that had received DES for 20 days revealed some glomeruli and appeared nearly healthy, with or without an increase in glomerular cellularity, others showed mild focal glomerular atrophy with a relative narrowing of the urinary space. Some glomeruli showed a nearly obliterated Bowman's space, minimal interstitial hemorrhage, and slight thickness of both the glomerular and tubular basement membrane in comparison with the control specimen; some tubules had a nearly obliterated lumen (**Figure 1B**).

The animals that received DES for 50 days showed a notable diffuse glomerular atrophy with apparent widening of the urinary space, obvious interstitial hemorrhage, and noticeable increase in the thickness of the glomerular and tubular basement membranes compared with the control, the animals also showed disseminated tubular cellular hydropic degeneration (**Figures 1C & 1D**).

Histochemical picture of the kidney (toluidine blue). Further examination of the specimens using toluidine blue-stained semithin sections, which permit much greater resolution at high magnification, revealed normal tubular epithelial cell morphology, similar to what was observed by H&E staining (**Figure 2A**). A section of the kidney of a rat receiving DES for 20 days showed a mild

increase in the cellularity of the glomeruli, especially the mesangial cells, and a moderate thickening of the glomerular basement membrane compared with the control. The urinary space contained a proteinaceous material compressing the capillary tuft. Most of the tubular epithelium, having numerous vacuoles and the lumen, contained a proteinaceous material similar to that observed in the urinary space of the glomeruli (**Figure 2B**). Meanwhile, a section of the kidney of a rat receiving DES for 50 days showed a marked increase in the cellularity of the glomeruli, with obvious thickening of the glomerular basement membrane, and a noticeable degeneration of the tubular epithelium (**Figure 2C**). In addition, the basement membrane of the capillary tuft of the glomeruli, as well as the mesangial matrix contained a markedly increased filling of the glomeruli and numerous deeply stained particles in the degenerated tubular epithelium (**Figure 2D**).

Immunohistochemical picture of the kidney (anti-BAX antibody). An immunohistochemical study using the anti-BAX antibody showed a duration-dependent increase in the intensity of the staining of the glomerular, cortical, and tubular epithelial cells in the group receiving DES for 50 days (**Figure 3C**), in comparison with the group receiving DES for 20 days, which showed mild BAX expression in the glomerular and tubular cells (**Figure 3B**). In contrast, the glomerular and tubular epithelial cells in control rats exhibited no, or minimal BAX-specific staining (**Figure 3A**).

Ultrastructure of the kidney of the control group. A more detailed ultrastructure study of the glomerulus showed the association of a podocyte (modified epithelial cell) with glomerular capillaries in the renal corpuscle of the kidney. The podocyte had a protrusion that extended from the podocyte cytoplasm to the surrounding capillary wall. Moreover, the proximal convoluted tubule was lined by cuboidal or low columnar cells with closely packed apical microvilli, which formed a brush border responsible for reabsorption, and numerous mitochondria concentrated at the basolateral surface to support energy requirements. The apical regions of the cells contained pinocytotic vesicles, which reflected the uptake of proteins that evaded the filtration barrier in the renal corpuscle and entered the filtrate. Furthermore, the distal convoluted tubule was lined by cuboidal and columnar cells with a few, short microvilli. These cells have basally located nuclei and tightly interdigitated lateral walls. Many mitochondria were present in the cytoplasm for energy production, and basal enfolding (basal plasma membrane infolding) had occurred due to corrugation of the cell membrane in the basal region of the cell (**Figures 4A - 4D**). The ultrastructure image of the kidney of the group treated with DES for 20 days showed mild affection of the renal corpuscles and the proximal convoluted tubules. On the other hand, the distal convoluted tubules were nearly healthy (**Figures 5A - 5D**). In **Figures 6A - 6D** the ultrastructure image of the kidney of the group treated with DES for 40 days showed dramatic changes of the components of the cortex. The podocytes were hypertrophied that obliterate urinary spaces and compressed the capillary tuft. Congestion of the capillaries were noticed with basement membrane thickening and mitochondria were swollen with loss of cristae and numerous vacuoles.

Discussion. Numerous research projects have dealt with the effects of exogenous estrogen on renal tissues. The effects of exogenous estrogen on renal tissues have remained unclear at the ultrastructural and apoptotic levels. The present work discusses the ultrastructural changes of the glomeruli and proximal and distal convoluted tubules following the treatment of adult rats with DES and changes that occur during the course of the administration. Early signs of the morphological changes were constantly detected in the basement membrane of the proximal tubules and the glomeruli. Meanwhile, the distal tubules showed minimal changes or appeared nearly healthy.

The DES has been used as non-natural synthetic estrogen but has different metabolism than natural estrogens. In previous research efforts, 4'-hydroxypropiophenone produced in metabolism of DES was considered responsible for carcinogenic effects.[27,29] During the prenatal period, DES-treated rats exhibited a conclusive decrease in the progeny number.[27,28] Furthermore, during the postnatal period, DES-treated rats showed toxic outcomes in the tissues of the kidneys rather than carcinogenic effects.[29] A previous study on the effects of DES treatment on rat kidneys established an increased cell number, cell size, and tissue-water retention, leading to a clear broadening of the cortical tissue.[30]

Previous findings indicated that the morphological changes of the renal tissues that accompany DES administration are not due to the presumed carcinogenic effect of DES. These findings contradicted the observations by other researchers that supported a hypothesis that DES had a precancerous role because catechols of the catechol cycle were created in response to the carcinogenic effects of DES reactivation.[31] Moreover, histological alterations, especially in the glomeruli and proximal convoluted tubules, support the hypothesis that these changes are related to the long duration of the DES administration in the compared groups.[32] Different degrees of narrowing of the urinary space, accompanied by apparent damage of the filtration membrane and glomerular capillary dilatation, are due to the long duration of DES administration, and

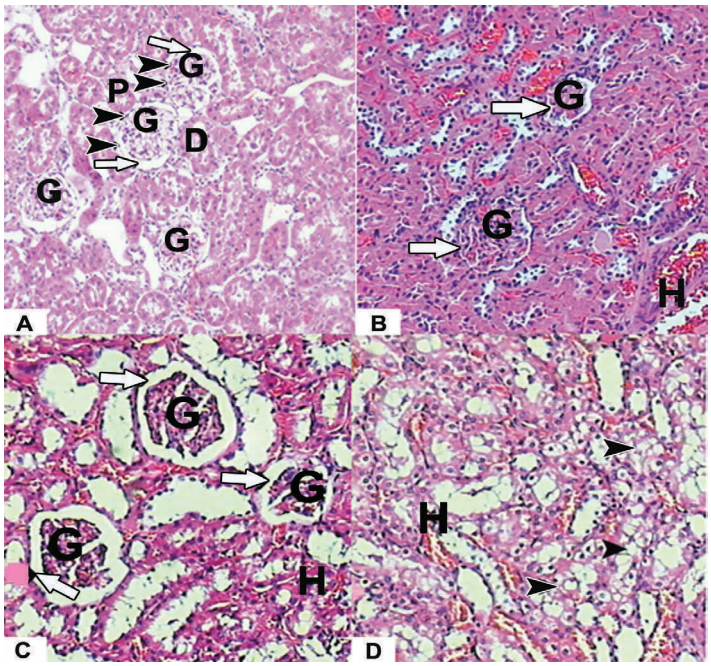

Figure 1 - Photomicrograph of a section of: **A**) control kidney cortex showing the Bowman's capsule where the parietal layer of the Bowman's capsule (arrowhead) is lined with simple squamous epithelium and where the glomerulus (G) is separated from the capsule by the Bowman's space (arrow), as well as the normal proximal (P) and distal (D) convoluted tubules (H&E × 100); **B**) kidney of a rat that received DES for 20 days showing focal glomerular atrophy (upper glomerulus) and increased glomerular cellularity (G) with a narrow or nearly obliterated Bowman's (urinary) space (arrow) and interstitial hemorrhage (H). Some tubules had a nearly obliterated lumen (H&E × 100); **C**) kidney of a rat that received DES for 50 days showing diffuse glomerular atrophy (G) with a relatively wide Bowman's (urinary) space (arrow) and H (H&E × 100); **D**) rat that received DES for 50 days showing tubular cellular hydropic degeneration (arrowhead) and H (H&E × 100). DES - diethylstilbestrol, H & E - Hematoxylin & Eosin stain

this agrees with the sodium and water imbalance and changes in the filtration system found by Markowitz.[32] In contrast, earlier efforts focused on the effects of different doses of DES on the proximal convoluted tubules in hamsters, showing visible proliferation of tubular cells, and these changes are comparable to the dosage of the treatment.[33] With increasing doses, the effects increased, and cancer cells appeared either singly, or in groups.[34] These observations agreed with the current outcomes, suggesting that the morphological changes are duration dependent. In addition, it is well noted that the distal convoluted tubules revealed sporadic or minor histological changes.

The morphological alterations varied from mild to moderate, and clear changes were observed during the treatment. Kidneys treated with DES in the present study showed changes, such as an obliterated or narrowed urinary space, increased glomerular cellularity, and hypertrophy of the tubular cells resulting in obliteration of the lumen of the renal tubules. These outcomes agreed with those of Al-Ani et al,[35] on the effects of oral contraceptives on the mice kidney. Al-Ani et al[35] showed increased glomerular cellularity and ascribed this finding to the proliferation of mesangial cells and assumed that the alterations in the mesangial cells were due to metabolic activity caused by toxic effects of long-duration intake of oral contraceptives. In addition, hypertrophy of the tubular cells was observed by Kuhl,[36] who reported that angiotensinogen increased in proportion to estrogen therapy, and led to an excessive angiotensin II (Ang II) manufacture. Furthermore, Kobori et al[37] stated that Ang II contributes to renal tubular changes, comprising cellular hypertrophy and oxidative stress. Additionally, Kobori et al[37] postulated that Ang I receptors participate in tubular cell hypertrophy.

In the current study, the PAS reaction disclosed mild to moderate thickening of the capsular and tubular basement membranes, which became more evident during the course of the treatment. This agreed with Al-Ani et al,[35] who noticed thickening of the basement membrane of the glomeruli with visible electron-dense deposits in rats that had received oral contraceptives, suggesting that this is an outcome of the proliferation of mesangial cells, resulting in the obliteration of the capillary lumen and glomerulosclerosis. In the present work, after treatment with DES for 50 days, excessive collagenous materials were noticed around the renal tubules and corpuscles. These results may be due to the increase in renal Ang II and are consistent with the work

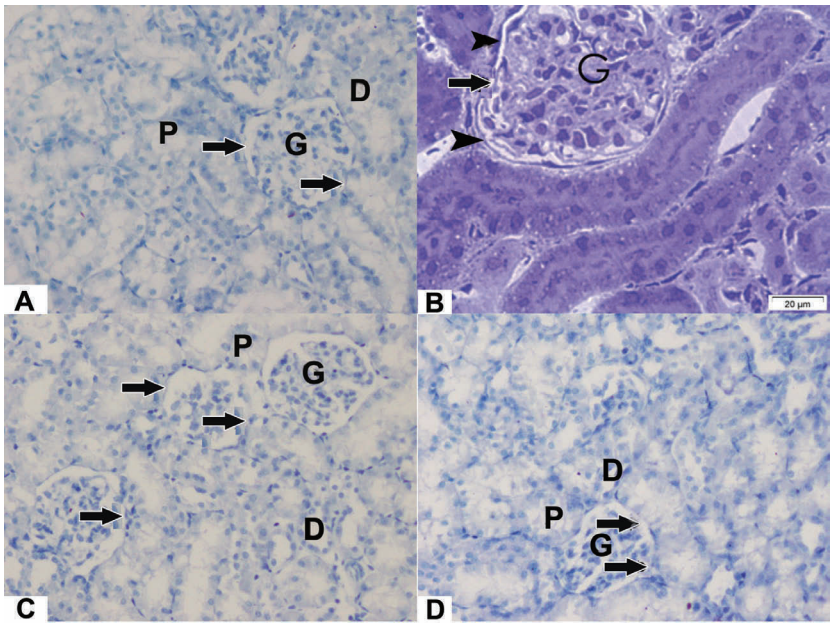

Figure 2 - Photomicrograph of a section of a kidney of: **A**) a control rat showing glomeruli (G) formed in the glomerular capsule, urinary space (arrow), capillary tuft, mesangial cell, and mesangial matrix. The proximal convoluted tubule (P) is lined by high cuboidal or columnar epithelium and has large vesicular nuclei and a small lumen, with microvilli protruding into the lumen. The interstitium is formed by small vasculature and fibroblasts. The distal convoluted tubules (D) have wide lumens, an apical nucleus, and basal striation (toluidine blue × 200); **B**) rat receiving diethylstilbestrol (DES) for 20 days showing an increased cellularity of G, especially of the mesangial cells, and thickening of the glomerular basement membrane (arrowhead). The urinary space (arrow) contains a proteinaceous material compressing the capillary tuft. Most of the tubular epithelium has numerous vacuoles, and the lumen contains a proteinaceous material similar to that observed in the urinary space of the glomeruli (toluidine blue × 400); **C**) rat receiving DES for 50 days showing an increased cellularity of G, with thickening of the glomerular basement membrane (arrow) and noticeable degeneration of the tubular epithelium with widening of the urinary space (toluidine blue × 200); **D**) rat receiving DES for 50 days showing the basement membrane (arrow) of the capillary tuft of G, as well as the mesangial matrix that increases the filling of the glomeruli and the presence of numerous deeply stained particles in the degenerated tubular epithelium (toluidine blue × 200).

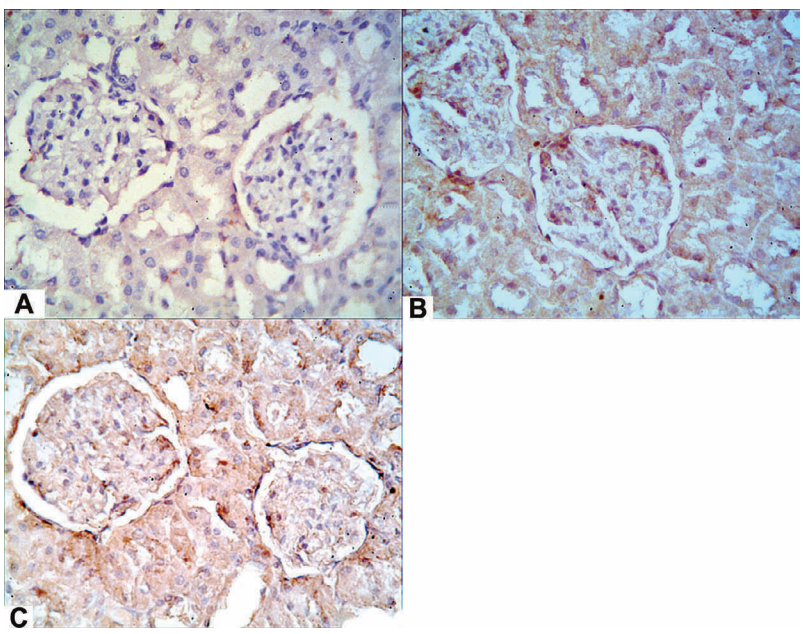

Figure 3 - Photomicrograph of a section of a kidney of: **A**) control rat showing minimal BCL2-associated X (BAX) expression in the glomerular and tubular cells (BAX expression × 200); **B**) rat receiving diethylstilbestrol (DES) for 20 days showing mild BAX expression in the glomerular, cortical, and tubular epithelial cells (BAX expression × 200); **C**) rat receiving DES for 50 days, showing increased BAX expression in the glomerular, cortical, and tubular epithelial cells (BAX expression × 200).

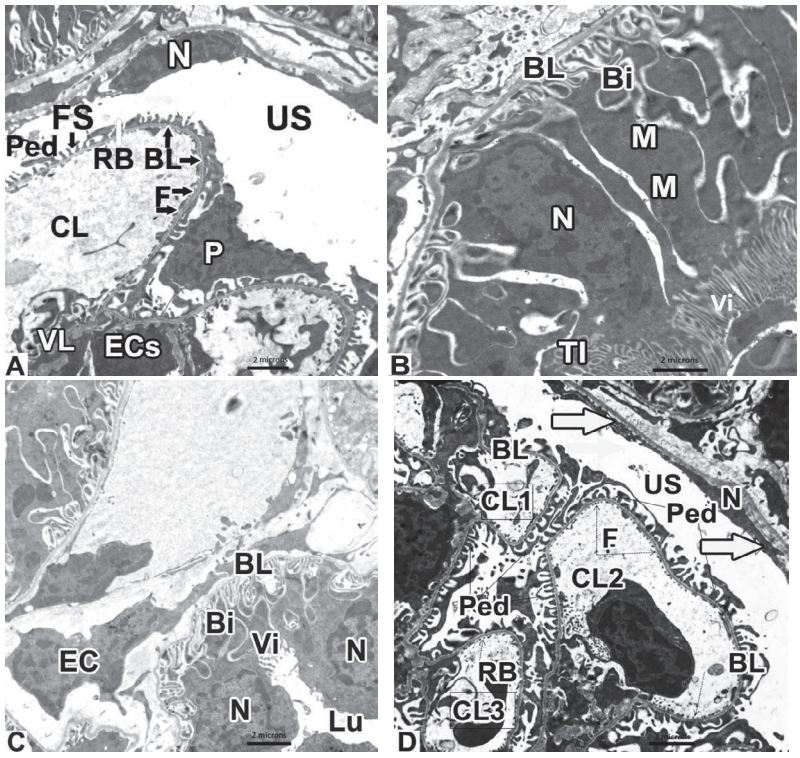

Figure 4 - Electron micrograph of a section of the renal cortex of a control rat showing: **A**) thin Bowman's capsule, the parietal layer of the epithelium flat with an elongated nucleus (N), a wide urinary space (US), and a visceral layer of the epithelium (podocyte) (P) having primary processes and foot process (podocyte foot process) (Ped) interdigitation with the adjacent nearby Ped along the basal lamina (BL) covering the capillary tuft (CL) and the space between the adjacent PEDs (filtration slits [FS]). Note that the CL is dilated and contains proteinaceous material in the renal terminal barrier (RB) formed by endothelial cells (ECs), BL, and Ped. Part of the EC of the tuff is flat, thin, and shows fenestrations (open pores) (F). Also note the nucleus of the visceral layer of the Bowman's capsule (VL) (microscopic magnification × 4800); **B**) proximal convoluted tubule with simple low columnar epithelium lining a thin basement membrane. The epithelial cell has a large number of invaginated membranes from the base toward the surface (basal infolding [Bi] of the basement membrane [BL]), and varies greatly in height. The N is euchromatic indented, oval or spherical, and is surrounded by a large cytoplasm filled with organelles including pleomorphic mitochondria (M). The cell surface contains closely packed microvilli (Vi) (brush border), lining the lumen with short, narrow, tubular invaginations (TI) of the cell membranes, present between the bases of the adjacent microvilli (microscopic magnification × 5800); **C**) distal convoluted tubule and an interstitial capillary dilated and filled with plasma; the endothelial lining (EC) is very thin and flat. The interstitium contains fibroblast cells. The tubular epithelium lies on a very thin BL, and the cells of low cuboidal have a large oval N with few very short Vi. From the BL, numerous membranes project into the cytoplasm of the cell (BI of the basement membrane). Note the tubular lumen (Lu) (microscopic magnification × 4800); **D**) clusters of glomerular capillaries (CL: 1, 2, & 3). Notice that the outer parietal epithelial layer N of the Bowman's capsule is flat and elongated (white arrows) and separated from the glomerular capillaries (CL: 1, 2, & 3) by US. The Ped (black arrow), with normal appearance, rested on a uniform BL of the very thin fenestrated glomerular endothelium (F) (dotted arrows). The capillary tuft contained plasma, red blood cells, and lymphoid cells. The mesangial cells contained a large N with scanty cytoplasm, and the mesangial matrix was scanty (microscopic magnification × 4800).

of Tsui,[38] who found that the disturbance in the renin-angiotensin system and Ang II might be an important mechanism in the development of interstitial fibrosis and glomerulosclerosis. Similarly, Chen et al[39] reported that Ang II stimulated the proliferation and biosynthesis of type I collagen in cultured murine mesangial cells. Moreover, Yamamoto et al[40] stated that the increase in intra-renal Ang II activity occurs in parallel with the severity of fibrotic renal damage. Baillargeon et al[41] reported that estrogen treatment induces hepatic manufacture of angiotensinogen, resulting in an increased level of Ang II. Moreover, Lubianca et al[42] stated that angiotensinogen concentrations increase 2- to 3-folds in reaction to estrogens in pre-menopausal women using oral contraceptives. This is also agreed with Ahmed et al,[43] who demonstrated higher concentrations of angiotensinogen, Ang II, and aldosterone in oral contraceptive users. Ultrastructural results emphasizing morphological changes were demonstrated by the inability to demonstrate fenestrae related to the endothelial cells of the glomerular capillaries, the basement membrane exhibiting undulation and thickening in some areas, and exfoliation of the podocytes.[44,45] It has been observed that hormonal management causes many histological alterations in the renal tissues.[46] These alterations could be explained by the existence of sex hormones that cause augmentation of the smooth endoplasmic

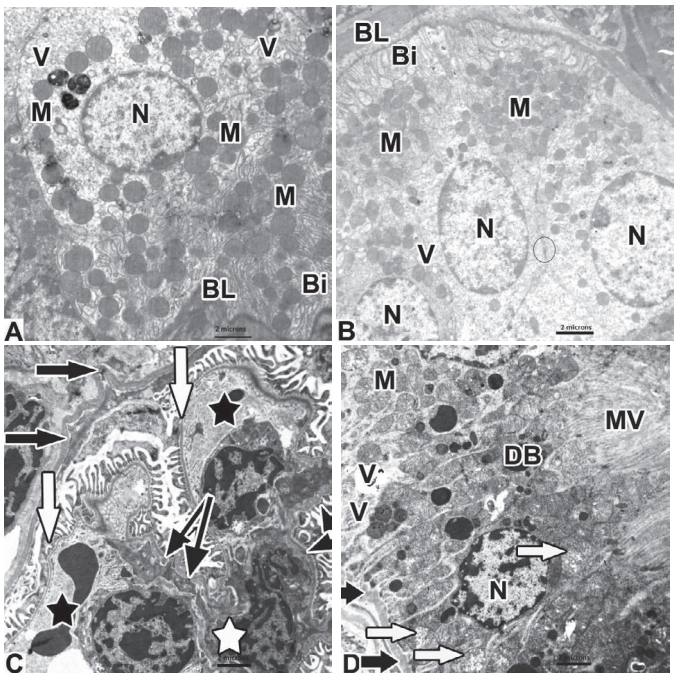

Figure 5 - Electron micrograph of a section of the renal cortex of rats treated with diethylstilbestrol (DES) for 20 days showing: **A**) epithelial cell of a distal convoluted tubule with numerous rounded or oval mitochondria (M) with apparent cristae, spherical vesicular nucleus (N), and numerous lipofuscin pigments and vacuoles (V). The surface microvilli are short and nearly absent. The basal lamina (BL) of the tubular epithelium is greatly thickened. Note the nearby basal infolding (Bi) of the basement lamina (microscopic magnification × 4800); **B**) tubular epithelium swollen and having spherical or oval N, abundant numerous pleomorphic M that vary in shape and size, with apparent healthy cristae, and with an outer part close to the thickened BL with basal infolding comprising an invaginated membrane from the outer basement membrane (Bi). Note the presence of V in the cytoplasm and intercellular junctions that link membranes of adjacent cells (circle) (microscopic magnification × 4800); **C**) section of the renal corpuscle showing an irregular thickened partial layer (thick black arrows). Dilated capillaries with accumulation of moderate electron dense pretentious or fibrinous material (black stars) and mononuclear cells (lymphocytes). The basement membrane of the glomerular capillaries showed focal irregular thickenings or humps (thin black arrows). Focal fusion of the podocytes footlet could be also seen (thick white arrow). Between the glomerular capillaries, deposition of electron dense mesangial cells and matrix are observed (white star) (microscopic magnification × 4800); D) part of a convoluted proximal tubule revealing intact apical microvilli (MV), abundant elongated pleomorphic M, numerous electron dense bodies (DB), and few vacuoles (V). Some mitochondria appeared swollen with prominent cristae (white arrows), and the tubular basal lamina was thick and irregular (black arrows). The N showed increased peripheral chromatin (microscopic magnification × 4800).

reticulum and mitochondrial swelling, as well as cellular granularity, or incompletely related to estrogen anabolic effects.[46,47]

The present ultrastructural findings closely agreed with Al-Ani et al,[35] who described overall dispersed pyknotic nuclei in the field in the proximal convoluted tubules, as well as different degrees of thickened basement membranes, ranging from mild to marked, and clear dilatation of the intracytoplasmic folding; the mitochondria between these foldings had electron-dense structures.[35] The structures located in the basal region of the proximal convoluted tubules are important for the function of the sodium pump; thus, morphological changes that occur in this region and in the glomeruli appear to be significant.[44,45] Oral contraceptives cause numerous histological changes in adult female albino rats, including renal tubular degeneration, congestion of renal blood vessels, and infiltration of inflammatory leukocytes.[35] Other authors ascribed that estrogen had stimulatory action on the renal cells, leading to renal cell proliferation.[48] Some histological results showed that DES is toxic to renal tissues.[49] These findings are consistent with the presented outcomes; DES promotes particular alterations in the renal morphology. Apoptosis is a key tool for regulating the cell number and development in diversified tissues and organs. This biological process is crucial in regeneration and aging, as well as the removal of deleterious and vain cells from the body.[50] Apoptosis initiation represents a cornerstone in the regulation of the number of renal cells in healthy and affected kidneys and the renewal of the cells.[51] The BAX protein is an apoptosis-inducing member of the Bcl-2 protein family that is localized to mitochondria, the mitochondrial permeability transition pore complex, the mitochondrial outer membrane, the endoplasmic reticulum membrane, and the cytoplasm. The BAX protein encourages apoptosis by augmenting cell predisposition to apoptotic stimuli.[52] The proapoptotic molecule BAX is required to initiate the mitochondrial pathway of apoptosis.[53] Increased expression of the

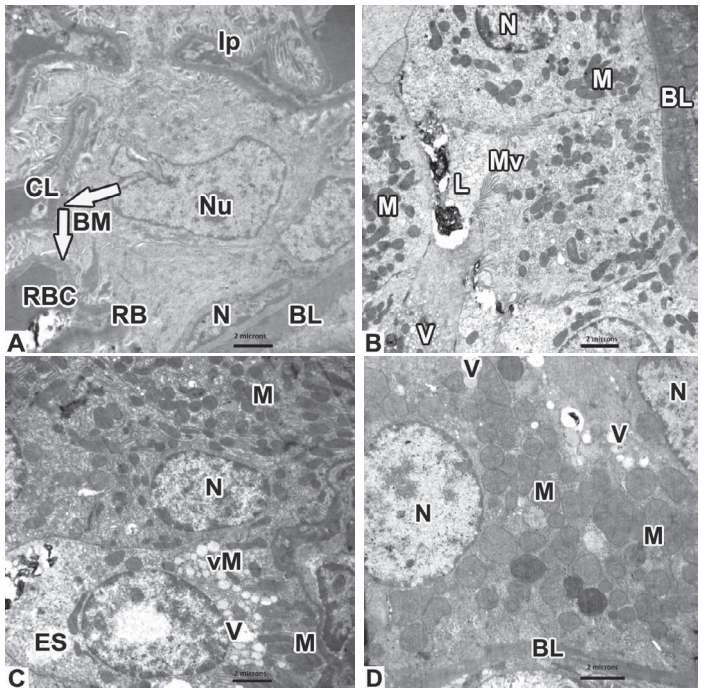

Figure 6 - Electron micrograph of a section of the renal cortex of diethylstilbestrol (DES) treated rats (for 40 days) showing: **A**) great thickening of the layer of the basal lamina (BL) of the Bowman's capsule, and the parietal epithelial cells are flat with an elongated nucleus (N). The visceral layer of the glomeruli (podocyte) is hypertrophied, obliterating, and occupying nearly all of the urinary spaces and compressing the capillary tuft (CL) with disrupted nuclear chromatin (Nu). Congestion of the capillaries by red blood cells with apparent thickening of the basement membrane (BM). Note the interlocking of the pedicles (Ip) with slit-pores in-between and in the blood renal barrier (filtration membrane) (RB) (microscopic magnification × 4800); **B**) distal convoluted tubule with great thickening of the BL. The epithelial cell has numerous elongated mitochondria (M) varying in shape, size, and oval vesicular euchromatic N. The surface contains very short and narrow microvilli (Mv), and the lumen of the tubule (L) contains casts. Note the presence of cytoplasmic vacuoles (V) (microscopic magnification × 4800); **C**) evidence of fat degeneration manifested by the presence of numerous rounded empty spaces (ES), swollen M along with loss of cristae (vM), and fatty changes in the cytoplasm and numerous V, other M are numerous, pleomorphic, and nearly healthy. Notice that other cells have a normal ultrastructure, and the BL is partially thickened (microscopic magnification × 4800); **D**) tubular epithelium with the presence of a large number of pleomorphic M of variable shape and size. Some M have preserved cristae and others have become more electron-dense with absence of cristae. In addition, numerous V could be seen. The N of the cell is euchromatic, large, rounded, or oval and vesicular, the BL is partially thickened. Note numerous electron-dense rounded bodies, variable in size, which seem to contain lipids (microscopic magnification × 5800).

BAX protein indicates that the mitochondrial pathway of apoptosis has started. The presence of apoptosis could be confirmed by microscopy in the current work. Previous work on apoptosis in embryos showed an increase in BCL-2 and BAX protein expression using immunohistochemical methods.[54]

In mammalian cells, 2 main apoptotic signal transmission pathways were described: external and internal (mitochondrial) pathways. The internal pathway is in response to factors causing DNA damage, and the process occurs mostly in the mitochondria, most often in connection with proapoptotic proteins from the BCL-2 group, including the BAX protein.[55] The upsurge in BAX protein expression noticed in the current study using the immunohistochemical method suggested that the apoptotic signal was in the mitochondrial pathway and emphasized the induction of apoptosis. Apoptosis occurred in parallel to proliferation in the control group; however, apoptosis was noticeably reduced in the experimental group receiving DES for 20 days. Meanwhile, a high level of apoptotic cells in the experimental group that received DES for 50 days was revealed by intensive expression of the BAX protein. In contrast, previous research on hepatocyte regeneration after hepatectomy described a decrease in BAX protein expression in the liver.[57,58] The previous research findings contradict the findings of the current study, in which an increase in the BAX protein expression compared with the control group suggests an increase in renal cells apoptosis.[57,58]

This study is limited as we used a few number of animals, therefore, it is recommended to use large number of animals to facilitate the statistical analysis and to add more morphometric results as stereology techniques.

Further studies are encouraged in this field to support or reject the hypothesis that functional alterations produced by DES on the renal tissues may progress to cancer formation, that is, that DES has precancerous properties especially with increasing treatment duration.

The DES effects are duration-dependent based on the current study. Meanwhile, same changes may occur by increasing the dose, thus, investigations are encouraged to clarify the relationship between the morphological changes and diverse modes of administration and different doses of DES.

In conclusion, natural estrogen is secreted periodically from the ovary and is used in contraceptive management; synthetic estrogen is given on a cyclic basis. Based on the present study, continuous artificial synthetic estrogen administration may lead to thickening of the basement membrane of proximal convoluted tubules, narrowing or widening the glomerular space, leading to either focal or diffuse degeneration of the glomerulus. Therefore, it is highly advised that synthetic estrogen treatment be administered on a cyclic basis.

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