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RESEARCH ARTICLE

NEPHROTOXICITY OF 5-FLUOROURACIL IN MALE MICE AND THE PROBABLE PROTECTIVE ROLE OF VITAMIN C: HISTOLOGICAL AND ULTRASTRUCTURAL STUDIES

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ABSTRACT

The present study investigated the probable protective role of vitamin C in alleviating 5-fluorouracil (5-FU)-induced nephrotoxicity in male mice (*Mus musculus*). Thirty male CD-1 mice were equally divided into three groups: Group I (the control group), received 0.9% NaCl; group II, received intraperitoneally (i.p.) 80 mg 5-FU/kg body weight (b.wt)/day for four weeks; group III, received (i.p.) 80 mg 5-FU plus 12 mg vitamin C/kg b.wt/day for four weeks. Animals of all groups were killed at the end of the experiment, and the renal tissue samples were taken and processed for light and electron microscopical examinations. Light microscopic observations revealed that administration of 5-FU caused erosion of the parietal cells of Bowman's capsules, widening of the urinary spaces, and disruption of the glomerular capillaries and hemorrhage. The cells of the proximal and distal tubules exhibited vacuolar degeneration and coagulative necrosis. The nuclei of these cells manifested pyknosis and karyolysis. Ultrastructural examination revealed damage of the parietal epithelium of Bowman's capsules with fusion and destruction of the foot processes of the podocytes. The cells of the proximal tubules displayed destruction of the microvilli constituting the brush borders and degeneration of the mitochondria. The distal tubules displayed destruction of both the basal infoldings and the mitochondria with fragmentation of the rough endoplasmic reticulum. Histological and ultrastructural results revealed that the treatment with vitamin C simultaneously with 5-FU led to apparent protection of the renal tissue. This might suggest that vitamin C has a protective potential against 5-FU-induced nephrotoxicity.

INTRODUCTION

5-Fluorouracil (5-FU) is a pyrimidine analogue that blocks the action of thymidylate synthase, and thus stops the production of DNA in normal and tumor cells. Chemotherapy for various cancers including the liver carcinoma and other solid tumors (breast, colorectal, and head and neck carcinomas) extensively used 5-FU[1,2]. Although it generates acceptable

antitumor outcome, severe toxicity arise from its use[3]. 5-FU causes nephrotoxicity[4]. Furthermore, 5-FU-induced alterations in the liver functions with increases in the activities of blood aminotransferases and the level of lipid peroxidation in the liver tissues, indicating hepatic damage[1,4,5]. The 5-FU is mainly eliminated by liver metabolism; only few percentages of 5-FU are eliminated by renal excretion. Dihydro-pyrimidine dehydrogenese (DPD) is the initial and rate-limiting enzyme in 5-FU catabolism, which occurs mainly in the liver. Therefore, it is expected that there is no reduction in 5-FU dose in patients with liver dysfunction[6]. The 5-FU causes generation of extensive reactive oxygen species (ROS) and reduces the efficiency of the cellular antioxidant defense system, which may induce oxidative liver and renal injuries[4].

In the past decades, world gave attention towards the probable protective role of some natural products against chemotherapy-induced nephrotoxicity[7]. Rashid *et al*.[8] found that 5-FU-induced renal toxicity in Wister rats *via* targeting oxidative stress and inflammation. In addition, they reported that the bee propolis is able to alleviate the 5-FU-induced renal damage including the necrosis in the tubular epithelium and glomerular congestion in Wistar rats. Antioxidants are substances that can prevent or slow damage the ROS-induced cell injury[9]. Vitamin C is one of the anti-oxidants that limit the injury produced by drugs[10]. It is an essential nutrient that acts as a non-enzymatic antioxidant in cystol[10]. Vitamin C is a water soluble vitamin with strong antioxidant and anti-inflammatory potential, which provides cellular protection against various toxic insults[11-13]. Some studies indicated that this vitamin is effective in preventing the oxidative renal damage and stress[10,14]. Therefore, the aim of the present study is to investigate the effect of 5-FU on the kidney structure of adult male mice, and to evaluate the probable role of vitamin C in minimizing the renotoxicity induced by this drug.

MATERIAL AND METHODS

Experimental animals

Thirty adult male albino mice of CD-1 strain obtained from Theodor Bilharz Research Institute (Imbaba, Giza, Egypt) were used in the current study. They were three months old and of an average weight of 25-30g. The experiments were performed according to the Guide for the Care and Use of Laboratory Animals of the Faculty of Education, Ain Shams University. The animals were fed *ad libitum* with a standard diet. They were kept in cages and acclima-tized in the laboratory for four weeks prior to experimentation.

Drugs

The 5-FU ampules (250 mg/ampule; EBEWE Pharma Ges. m.b.H. NFg. KG, Unterach, Austria) were purchased from the local pharmacy. The vitamin C ampules (1000 mg per ampule) were obtained from Memphis Pharmaceutical & Chemical Industry (Cairo, Egypt).

Experimental design

The mice were divided into three groups comprising 10 animals in each group. The first group served as the control group and received normal saline solution. The animal of the second group were daily injected intra-peritoneally (i.p.) for four weeks with 80 mg/kg body weight (b.wt) of 5-FU. The animals of the third group were i.p. administrated 12 mg/kg b.wt of vitamin C simultaneously with the dose of 5-FU daily for four weeks. Twenty-four hours after the last dose(s), the animals were killed after anesthetized with light diethyl ether.

Microscopic sections preparation

For light microscopic preparations, small pieces of the kidney were immediately fixed in alcoholic Bouin's solution for 24 hours that gives the best results among other fixatives used in our preliminary studies. Sections of 5 μm in thickness were stained with hematoxylin and eosin[15], microscopically examined, and photo-graphed. For the electron microscopic preparations, kidneys were cut into small

pieces and fixed in 2.5% glutaraldehyde for 4 hours, and 2% paraformaldehyde in 0.1 mol cacodylate buffer (pH: 7.4) for 15 minutes (three times). The samples were post-fixed in buffered solution of 1% osmium tetroxide at 4°C for 1-5 hours. This was followed by dehydration in ascending grads of ethyl alcohol, clearing in propylene oxide for two changes, 5 minutes each, and embedded in EPON epoxy resin. Semi-thin sections of 1 μm thickness were stained with toluidine blue and investigated under a bright field light microscope. Ultrathin sections were cut, mounted on formvar-coated grids, and stained with uranyl acetate and lead citrate[16]. Sections were examined and photographed on a Joel transmission electron microscope (JEOL Inc., Peabody, MA, USA) at acceleration voltage of 60-80 kv.

RESULTS

Light microscope examination

Group I (control group)

The kidney is a tubular gland consisting of a large number of renal tubules, each formed of two main parts; the nephron and the collecting tubules. The nephron is the structural and functional unit of the kidney. It is made up of two main portions, the renal or Malpighian corpuscles and the urini-ferous tubules. The renal corpuscle consists of a tuft of blood capillaries "the glomer-ulus" surrounded by the Bowman's capsule, which consists of double-walled epithelial layers. The internal leaflet of the capsule enveloping the glomerular capillaries is visceral layer of Bowman's capsule. The space between the two leaflets is the urinary space. The uriniferous tubule includes two parts, the proximal tubule with a relatively small lumen lined with low columnar epithelium and supported with luminal brush border, while the distal part possesses a wider lumen with a cuboidal epithelium (Figure 1).

Group II (5-FU-treated group)

Examination of the kidneys of mice after administration of 5-FU (80 mg/kg b.wt) revealed variable degrees of pathological alterations of many glomeruli; they were shrunken and destructed (Figures 2a and c). Contractions of glomerular tufts, which were packed with red blood corpuscles, in addition to the widening of the urinary space were clearly obvious. Focal area of necrosis invaded by lymphocytes was detected (Figure 2c). The lining epithelial cells of the proximal convoluted tubules showed marked cloudy swelling (swelling of cells and granularity of the cytoplasm due to water accumulation) with cellular vacuolation (Figure 2b). Erosion of brush borders of some proximal convoluted tubules were also detected in Figures "2a and b". Edematous appearance (hydropic degeneration) of the cells of proximal convoluted tubules was observed (Figures 2b and c). The nuclei of some deteriorated cells manifested pyknosis, while a few of these nuclei were markedly karyolyzed (Figure 2b). The blood vessels appeared congested with thickening of its endothelial lining (Figure 2d). Hemorrhagic areas were noticed in between renal tubules (Figures 2e and f).

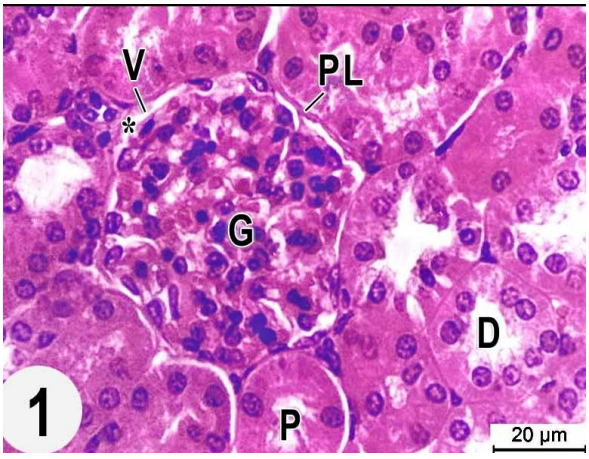


Figure 1: Photomicrograph of a section of kidney of a control mouse, illustrating a part of the cortical region containing a renal corpuscle that consists of the Bowman's capsule enclosing the glomerulus (G). The Bowman's capsule is formed of the parietal (PL) and visceral (V) layers separated by the urinary space (*). Portions of the proximal (P) and the distal (D) convoluted tubules are also illustrated.

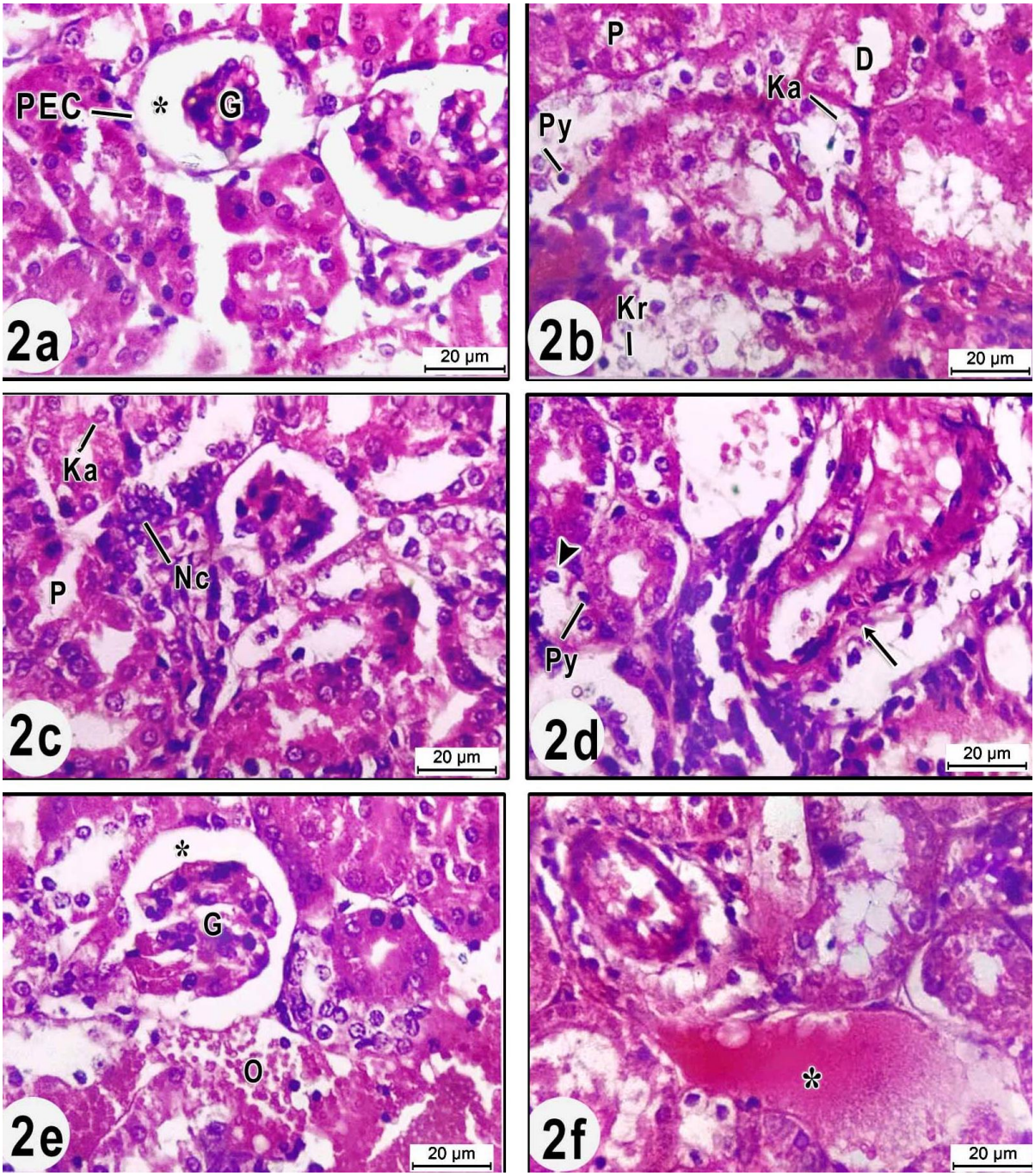


Figure 2: Photomicrographs of kidney sections of mice treated for four weeks with 5-fluorouracil showing (**a**) atrophied and shrunken glomeruli (G) with widening of the urinary space (*) and degeneration of the parietal epithelial cells (PEC) of Bowman's capsules, (**b**) marked cellular vacuolation of the lining epithelial cells of the proximal (P) and distal (D) convoluted tubules, distinct nuclear pyknosis (Py), karyorrhexis (Kr) and karyolysis (Ka), as well as erosion of brush border of proximal convoluted tubule were noted, (**c**) focal area of necrosis (Nc) with lymphocytic infiltration, erosion of brush border of proximal convoluted tubules (P), and karyolyzed nuclei (Ka), (**d**) congested blood vessel with thickened endothelial lining (arrow) and cytoplasmic vacuolation (arrowhead) of epithelial cells with pyknotic nuclei (Py), (**e**) shrinkage in glomerulus (G) with widening of the urinary space (*), in addition to hemorrhagic edema (O), and **(f)** congested blood vessels with hemolyzed blood cells (*).

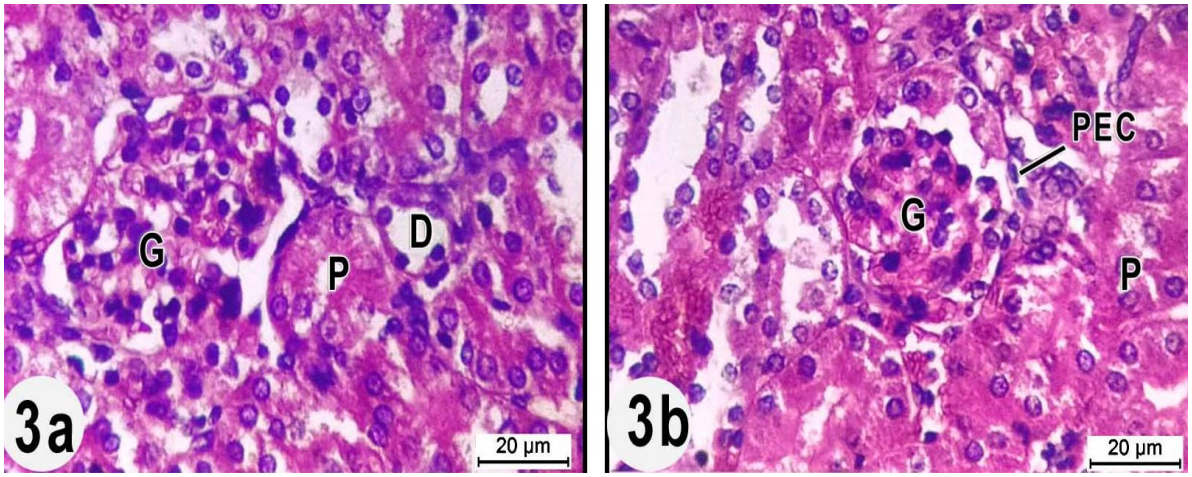


Figure 3: Photomicrographs of kidney section of mice treated simultaneously with 5-fluorouracil and vitamin C for four weeks showing (**a**) tuft adhesion of glomerulus (G) and many recovered proximal (P) and distal (D) renal tubules, while others revealed swollen lining epithelium and (**b**) glomerular capillaries (G) content (tuft and mesangial cells) with relatively normal parietal epithelial cells (PEC) of Bowman's capsules and some recovered proximal convoluted tubules (P).

Group III (5-FU + vitamin C-treated group)

The histological structure of the kidney of most mice of this group revealed mild renal damage when compared with animals given 5-FU only. Bowman's capsules were intact, the glomeruli displayed normal built-up except for mild congestion (Figure 3a). In addition, some glomeruli were swollen and displayed tuft adhesion. The epithelial cells of renal tubules were almost healthy, except some showing pyknotic nuclei (Figures 3a and b).

Ultrastructural examination

Group I (control group)

Ultrastructural examination of the kidney of the control group showed glomerular capillary bounded by an outer wall (basal lamina) and an inner endothelial lining. The inner wall is called the visceral layer, formed of specialized cells called 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 (Figures 4a and b). The axial portion in the hilus of the glomerular capillaries has certain cells, known as intercapillary or mesangial cells with darkly-stained often lobulated nuclei, being thus distinguishable from the endothelial cells. These cells are separated from the endothelial cells by an amorphous mesangial matrix (Figure 4b).

The proximal convoluted tubules are distinguished by their narrow lumina. The cells of the proximal convoluted tubules have an elaborate shape, well-developed microvilli along their lumina, an active endocytotic apparatus, and many spherical or elongated mitochondria (Figure 4d). The nuclei of such cells are relatively large, mostly euchromatic with prominent nucleoli and always lying at the basal portions of the cells (Figure 4c). The distal convoluted tubules have larger luminal diameters. The cells have a few microvilli at apical membrane. The mitochondria are elongated and occupy the cytoplasmic compartment between the basal infoldings (Figure 4e). The nuclei are relatively large and their heterochromatin appears always adherent to the inner surface of the nuclear envelope. They are located at the apical part of the cytoplasm (Figure 4e).

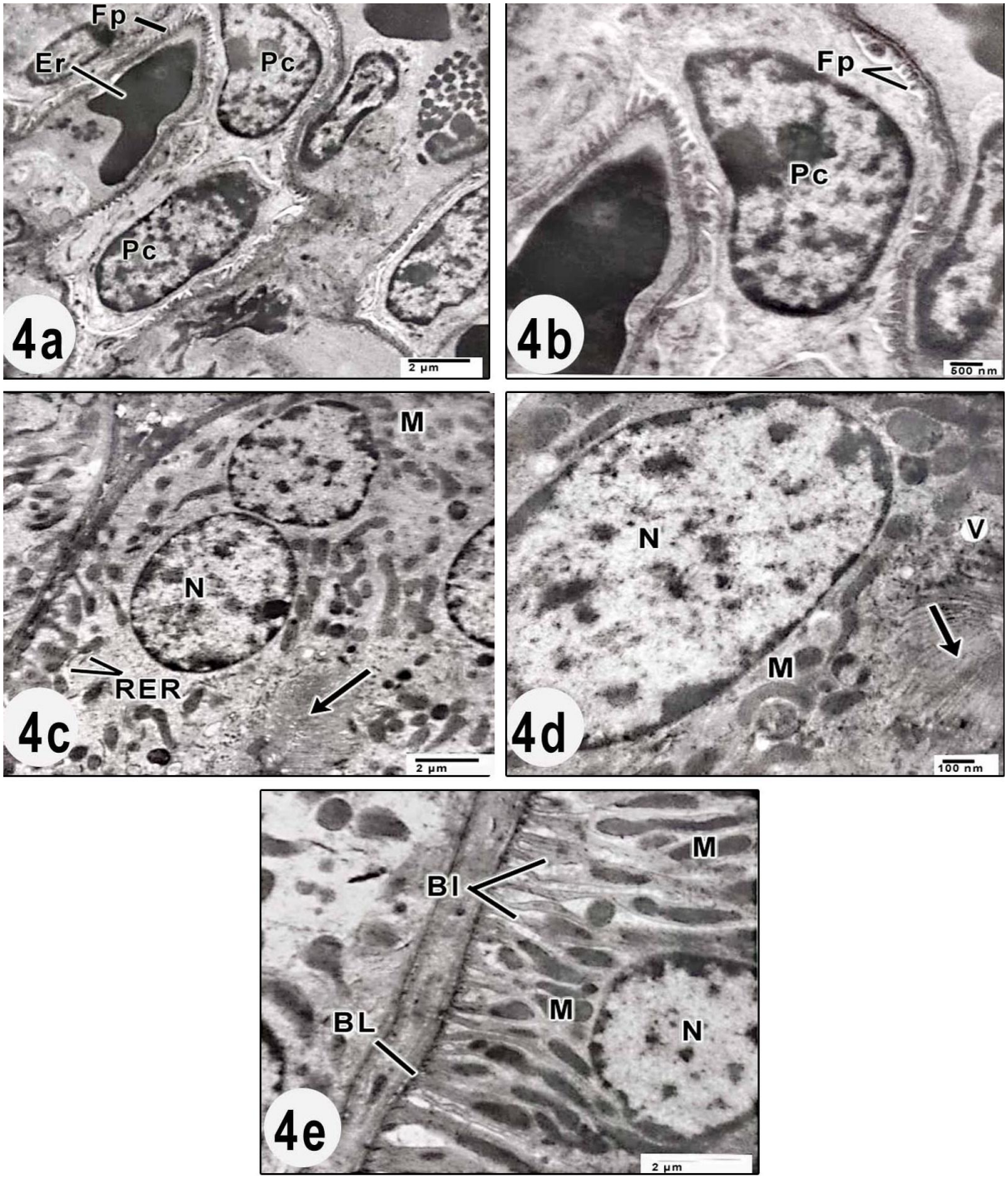


Figure 4: Electron micrographs of sections of the renal cortex of control mouse showing (**a**) the visceral epithelium of Bowman's capsule made up of podocytes (Pc) with extended foot processes (FP), and the erythrocytes (Er) was also noted, (**b**) high magnification of the previous Figure illustrating the podocyte (Pc) with extended foot processes (FP), (**c**) part of the proximal convoluted tubule has a lumen occupied by microvilli (arrow), the cytoplasm contains numerous elongated mitochondria (M), few stacks of rough endoplasmic reticulum (RER), and relatively large nucleus (N), (**d**) high magnification of the proximal convoluted tubule illustrating the microvilli (arrow), mitochondria (M), apical vacuole (V), and large nucleus (N), and (**e**) displaying part of distal convoluted tubule cell with basal lamina (BL) and well defined basal infoldings (BI), large number of mitochondria (M), and spherical nucleus (N) with normal chromatin distribution.

Group II (5-FU-treated group)

The electron micrographs of ultrathin sections of the kidneys of 5-FU-treated mice revealed damage of the parietal epithelium of Bowman's capsules, fusion and destruction of the foot processes of podocytes. The nuclei of many endothelial cells of the glomerular capillaries became pyknotic (Figures 5a and b). 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, the mitochondria were swollen and their matrices were condensed so that their fine structure became obscure. The increase of lysosomal numbers was also observed. The nuclei showed signs of pyknosis (nuclear shrinkage, since the chromatin material condensed into shrunken mass) as shown in Figures "5c and d".

The distal convoluted tubules became shrunken with marked thickening of their basement membranes. The basal infoldings became destructed, and the mitochondria were swollen and their matrices became condensed so that they did not show any demarcation of their detailed fine structure. These cells showed also marked fragmentation of rough endoplasmic reticulum and pyknotic nuclei (Figure 5e).

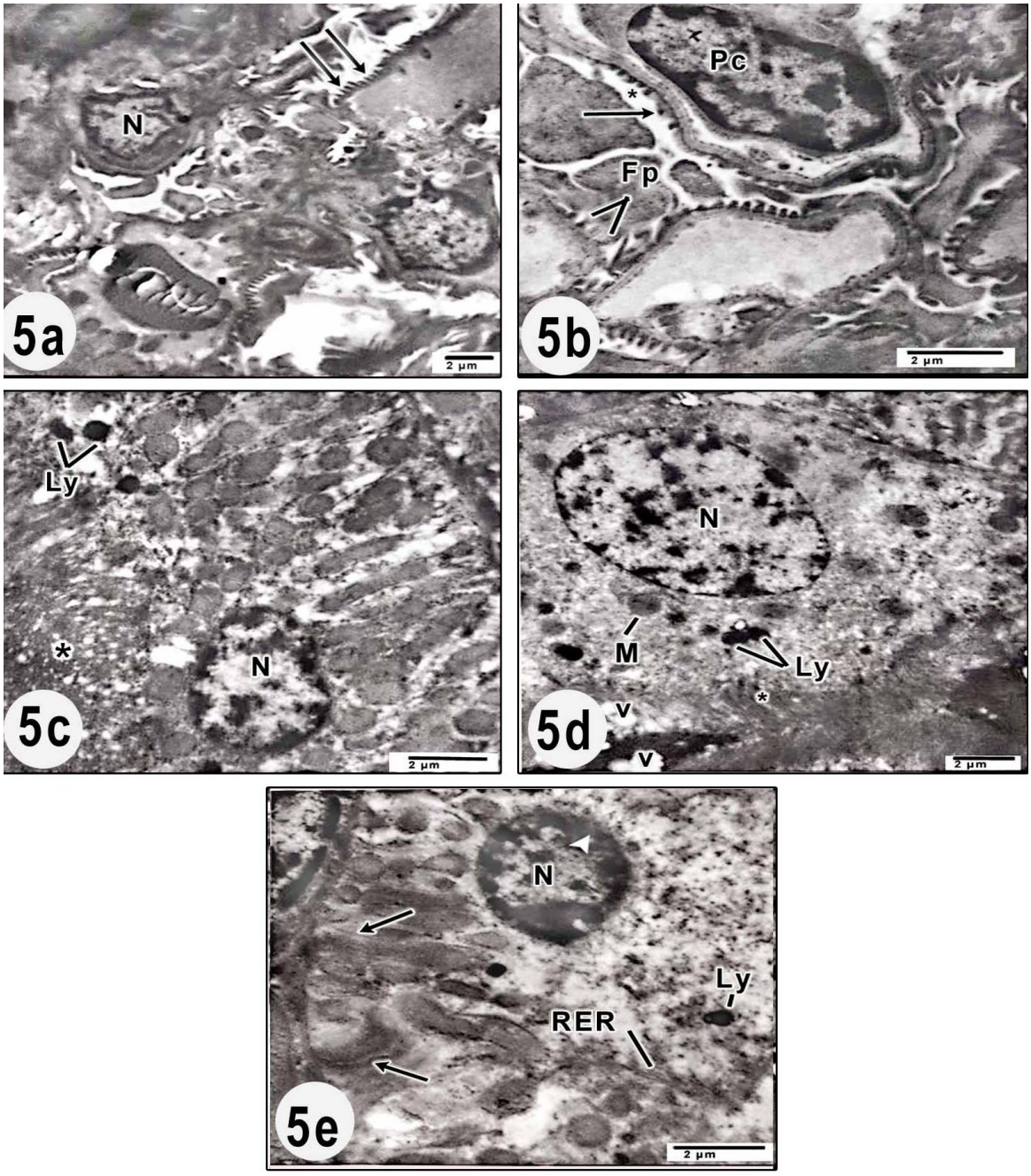
Group III (5-FU + vitamin C-treated group)

Moreover, examination of ultrathin sections of the kidney of this group showed that the general architecture of the renal tissue was partially improved as compared with that of the Group II. The capillary basement membrane acquired a uniform thickness and normal capillary endothelium. However, the urinary spaces were slightly narrowed. The foot processes had a somewhat normal picture (Figures 6a and b). The ultrastructure of the proximal convoluted tubules restored the normal appearance of their apical microvilli, in spite of the presence of a few vesicular materials inside the lumen and a slight increase in the number of lysosomes. The nuclei showed a distinct nuclear envelope and the nucleoplasm retained its normal picture (Figure 6c). The distal convoluted tubules displayed nearly normal appearance of both the basal infoldings and the mitochondria. In addition, the nuclei of these cells appeared approximately normal in shape (Figure 6d).

DISCUSSION

Kidney is very sensitive to the adverse effects of drugs and chemicals. In this regard, Walker and Duggin[17] stated that even low concentrations of any chemical or its metabolites could generate a certain degree of nephrotoxicity. The present study was done to observe the conspicuous alteration occur in the renal tissue after 5-FU administration and to detect the potential protective role of vitamin C against these lesions. Possible mechanisms of 5-FU-induced nephrotoxicity are through DNA and non-DNA damage a long with the production of ROS, reactive nitrogen species (RNS), and a variety of inflammatory responses[8]. Thus, chemicals with anti-inflammatory/antioxidant properties and minimal side effects, which could be incorporated as dietary agents may serve as potential therapeutic agents for the treatment of chemotherapy-induced organ toxicity, especially 5-FU, and are worthy of detailed investigation[18].

The present study showed that 5-FU-induced marked histopathological changes in mice kidneys and these alterations were represented in both the Malpighian corpuscles and renal tubules. The most devastation signs in the renal corpuscles were tuft adhesion and marked congestion of the glomerular capillaries. Besides, other corpuscles were shrunken. The kidney tubules were also markedly affected as indicated by swelling, detachment from each other and inter-tubular invasion by inflammatory cells. Besides, some epithelial cells were necrotic. Similar findings were recorded by Rashid *et al*.[8] who concluded that 5-FU caused significant structural damage in renal tissue.



Figures 5: Electron micrographs of the renal cortex sections of mice treated for four weeks with 5-fluorouracil showing (**a**) damage of the parietal epithelium of Bowman's capsule, fusion and destruction of the foot processes of podocytes (arrows), and the nucleus is surrounded by irregular nuclear envelope (N), (**b**) part of the glomerulus with widening of urinary spaces (*), the podocytes (Pc) had fusion and swelling foot processes (Fp) of the blood capillaries (arrow) on some spaces, (**c**) a part of proximal convoluted tubule with apical microvilli (*) constituting a brush border, the nucleus (N) with heterochromatin condensed on the inner membrane of nuclear envelope, and electron dense lysosome (Ly) was present, (**d**) part of the proximal convoluted tubule cell with disrupted microvilli (*), numerous of apical vacuoles (V), devastated mitochondria (M), electron dense lysosomes (Ly), and large nucleus (N), and (**e**) the distal convoluted tubule cell with marked destruction of the basal infoldings (arrows) and fragmented rough endoplasmic reticulum (RER), in addition to the presences of electron dense lysosomes (Ly) and small pyknotic nucleus (N) with a dense peripheral rim of chromatin (arrowhead).

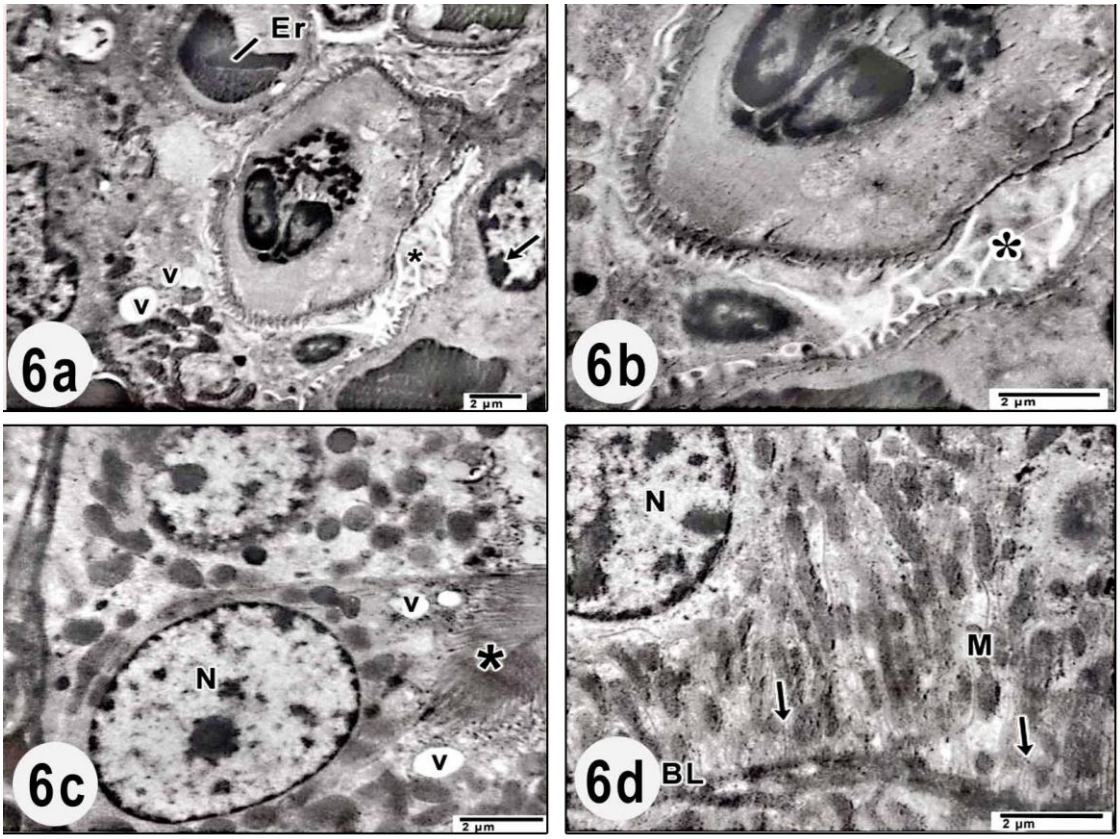


Figure 6: Electron micrographs of the renal cortex sections of mice treated simultaneously with 5-fluorouracil and vitamin C for four weeks showing (**a**) vacuolated cytoplasm of the parietal epithelium of Bowman's capsule, fusion of some foot processes of podocytes (*), and condensed of chromatin on the nuclear envelope (arrow), in addition to erythrocytes (Er) and some vacuoles (V), (**b**) magnified part from the previous Figure illustrating fusion of some foot processes of the podocytes (*), (**c**) the proximal convoluted tubule with well-developed microvilli (*) forming the brush border, a large central basal nucleus (N), and vacuoles (V) of endocytotic apparatus, and (**d**) the distal convoluted tubule with well-developed basal infoldings (arrows) resting on the basal lamina (BL), elongated mitochondria (M), and central nucleus (N).

Schetz *et al*.[19] concluded that most drug induced nephrotoxicity exert toxic effects by one or more common pathological mechanisms, including altered intra-glomerular hemodynamic, tubular cell toxicity, inflammation, and crystal nephron-pathy. The present investigation revealed that most glomeruli and tubules appeared necrotic with extravasations of blood around the cortical tissue. These results agree with those obtained by Yousef and Aboelwafa[20] who reported a destruction of the normal renal structure by 5-FU, in addition to ultrastructural alterations represented by thickened and irregular glomerular basement membranes, con-gested glomerular capillaries, mesangial cells hyperplasia, and distorted podocyte's processes. Holdass *et al*.[21] explained that the congestion and extravasation of blood in the kidney could be referred to the weakness of the renal parenchymal tissue as a result of the degenerative changes. Necrosis may be due to either a severe degeneration[22] or to metabolic disturbances[23].

The ultrastructural study demonstrated thickening and disruption of the basement membrane of the renal corpuscles. This in turn affects the mechanism of ultrafiltration, leading to an increase in immune reaction in

the basement membrane, induction of local fusion of foot processes of visceral epithelial cells, and loss of the regular endothelial lining of the basement membrane, which are similar to those occurring in proteinuria[24]. The fusion of foot processes in certain areas together with the thickening of basement membrane indicates that renal dysfunction is expected. This finding confirms what has been reported by other authors[20,25]. The ultrastructural changes in 5-FU-treated mice were characteristically situated in the proximal portion of the renal tubules. These lesions included destruction of the microvilli constituting the brush border, degeneration of the epithelial cells, disorganization and distortion of the mitochondria, and increased the number of lysosomes. Other studies proved that 5-FU-induced ultrastructural alterations in the glomerular capillaries[8,20]. In addition, the proximal and distal convoluted tubules of 5-FU-treated group exhibited thickened basement membranes, destructed apical microvilli, and loss of basal infoldings of their epithelial cells. Moreover, 5-FU caused impaired mitochondrial function and mitochondrial damage, which play a pathogenic role in events leading to cell death. The conspicuous alterations in the renal tissue observed in our study agree with those reported by Ali[4] who found that 5-FU-induced increase in the renal injury.

The present study revealed that the treatment with vitamin C markedly ameliorated some of histopathological features manifested in kidney of mice treated with 5-FU. Some vitamins including vitamin C was found to be effectively protecting from chemically-induced oxidative renal damage in animals[26,27]. Antioxidants are necessary for preventing the formation of free radicals and the inhibition of deleterious action of reactive oxygen species that damages lipids, DNA, and protein. Although, the authors found no reports on the role of vitamin C as a protective agent against 5-FU-induced nephrotoxicity, however other studies revealed that some vitamins could be protective against chemotherapy-induced acute renal failure in rodents[26,28]. Other article suggested the role of vitamin C in reducing the oxidative stress induced renal failure through attenuating lipid peroxidation and preventing the loss of membrane permeability and dysfunction of the cellular proteins in kidney tissues[29]. In addition, Yousef[30] reported the protective role of vitamin C against tamoxfen-induced renotoxicity in mice.

In conclusion, the current study found that the 5-FU caused renotoxicity in mice at the histological and ultrastructural levels, which was partially modulated by vitamin C administration. Therefore, vitamin C supplementation may protect patients treated with 5-FU from its nephrotoxicity.

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السُمية الكُلوية لعقار فلورويوراسيل في ذكور الفئران والدور الوقائي المحتمل لفيتامين ج: دراسات نسيجية وتركيبية دقيقة

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أجريت هذه الدراسة على ذكور الفئران المهقاء "*Mus musculus*" البالغة، وقد هدفت إلى تحديد الدور الوقائي المحتمل لفيتامين ج كمضاد قوي للأكسدة في التخفيف من السُمية الكُلوية لعقار 5-فلورويوراسيل. تم توزيع الفئران بالتساوي إلى 3 مجموعات. اعتبرت المجموعة الأولى كمجموعة ضابطة حيث عوملت بمحلول كلوريد الصوديوم (0.9%)، بينما عوملت المجموعة الثانية بالحقن داخل التجويف البريتونى بعقار 5-فلورويوراسيل (80 مجم/كجم وزن الجسم) يوميا ولمدة 4 أسابيع؛ أما المجموعة الثالثة فعوملت بالحقن داخل التجويف البريتونى بعقار 5- فلورويوراسيل (80 مجم/كجم وزن الجسم) وبفيتامين ج (12 مجم/كجم من وزن الجسم) يوميا ولمدة أربعة أسابيع. في نهاية التجربة تم ذبح الحيوانات وأخذ عينات من نسيج الكُلي وإعدادها للفحص المجهري الضوئي والإلكتروني. كشف الفحص بالمجهر الضوئى أن تناول عقار 5- فلورويوراسيل قد تسبب فى مظاهر سُمية كُلوية تمثلت في تآكل الخلايا الجدارية لمحافظ بومان واتساع الحيزات البولية، وكذلك تمزق الشعيرات الدموية الكبيبية ونزيف دموي. علاوة على الانتفاخ السحابي للخلايا المبطنة للأنيبيبات الكُلوية الملتفة القريبة والملتفة البعيدة، وأيضا انكماش ودكنة أنويه تلك الخلايا وتحللها. كما أوضح الفحص بالمجهر الإلكتروني تهدم بالخلايا الجدارية لمحافظ بومان، واتحاد للزوائد القدمية مع بعضها البعض، وتكسر الخملات الدقيقة المكونة للحافة الفرجونية للخلايا الطلائية المبطنه للأنيبيبات القريبة، وتهدم واضح فى الميتوكوندريا. كما أظهرت الدراسة تدميرًا ملحوظا بالأنيبيبات الملتفة البعيدة لكل من الثنيات القاعدية للأغشية الخلوية المبطنة، وكذلك تهدم للميتوكوندريا في بعض المناطق، مع تفتت ملحوظ للشبكة الإندوبلازمية الخشنة. وكشفت النتائج النسيجية والتركيبية الدقيقة أن معاملة ذكور الفئران بفيتامين ج في الوقت نفسه مع عقار 5- فلورويوراسيل أدى إلى الوقاية من السُمية الكُلوية المُستحثة بهذا العقار.