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DETERMINATION OF THE NEPHROPROTECTOR ACTION OF A NEW DERIVATIVE OF THEOPHILINE ON THE MODEL OF ACUTE TOXIC RENAL INJURY BY ETHYLENE GLYCOL

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Abstract

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Introduction

The prevalence and constant increase in the incidence of kidney disease remains an urgent medical and social problem both in Ukraine and abroad. According to statistics from the Ministry of Health of Ukraine in 2020 [20], kidney and urinary tract diseases affect more than 10% of the adult population of the world, and incidence rate is constantly growing. High mortality rate in acute renal failure, it frequent combination with multisystemic pathology [5, 6] and insufficiently effective treatment raises the issue of improving pharmacotherapy and finding new approaches to solving this problem with the inclusion of nosological, ethiological, pathogenetic and systemic components. Given the above, the search for new effective and safe diuretics with nephroprotective activity remains one of the current problems of modern experimental pharmacology [9, 11, 14-16]. It is known that xanthine derivatives inhibit the phosphodiesterase, increase the content of intracellular cAMP, are antagonists of adenosine receptors on cell membranes, are involved in the regulation of many processes and have a variety of pharmacological effects [10, 11]. The anti-inflammatory action of xanthine derivatives, the restoration of the blood rheology and the effect on vascular tone are important links in the prevention of complications of renal disease. The results of studies of a number of derivatives of 7,8-disubstituted theophylline [8, 9-11] indicate that among them are promising nephroprotectors. Given that, new 7-n-methylbenzyl, 8-hydrazine-substituted theophylline derivatives were synthesized at Zaporizhzhya State Medical University of the Ministry of Health of Ukraine. The study of their pharmacological activity revealed promising substances that can affect renal function in experimental pathology. The compound bromobenzylidenehydrazinoteophylline (code name "benophylline") was selected for the study of nephroprotective effect among these substances.

The aim of the study – study of the nephroprotective effect of benophylline in experimental renal injure with ethylene glycol in rats.

Methods

A new derivative of 7,8-disubstituted theophylline synthesized at the Department of Biological Chemistry and Laboratory Diagnostics of the Zaporizhzhya State Medical University was used as the object of research. The structure of the compound was confirmed by modern physicochemical methods of elemental analysis, IR-, UV-spectroscopy ("Specord OR - 75" in potassium bromide tablets), PMR-spectroscopy ("Bruker AC 300") and counter synthesis. The purity of the synthesized substance was controlled by the method of thin-layer chromatography. The identity of the compound was confirmed on Silufol UV-254 plates in different solvent systems. Determination of melting point was carried out in accordance with the requirements of the State Pharmacopoeia of Ukraine [18, 19].

Benophylline is a white loose, odorless, bitter-tasting crystalline powder, soluble in dimethylformamide, dimethyl sulfoxide and sparingly soluble in water, stable in storage, and non-hygroscopic.

The nephroprotective effect of benophylline was studied on an experimental model of acute renal failure (ARF) induced by ethylene glycol, in accordance with the methodological recommendations of the SPhU of Ukraine [21]. To study the nephroprotective activity of the leader compound on the model of ethylene glycol-induced ARF experimental animals were randomly allotted into four groups: I – IC (n=6); II – CP (animals with ARF; n=6); III – animals with ARF treated with benophylline at a dose of 35 mg/kg (ED50); IV – nimals with ARF treated with reference drug chophytol at a dose of 70 mg/kg. The test compound and chophytol were administered intragastrically in the form of aqueous solutions stabilized with tween-80, in a volume of 2 ml per animal. Animals from the CP group received an equivalent volume of water. Chophytol was chosen as a reference drug because, according to the instructions for medical use, the complex of biologically active substances in its content has diuretic, nephroprotective, antioxidant effect, improves metabolism and reduces urea level in the blood. Ethylene glycol intoxication was induced by subcutaneous administration of ethylene glycol (qualification "high grade") at a dose of 6 ml/kg. The test compound and the reference drug were

administered to the animals intragastrically in the treatment and prevention regimen for three days before the induction of ARF (the last dose – 40 minutes before modeling the pathology), and 5 days after the induction the ARF. The effectiveness of the new compound and chophytol was determined by GFR on the first day of ARF, by biochemical parameters of urine and serum (content of urea, creatinine, uric acid, total protein, sodium ions, potassium, etc.).

In the study of the histological structure of the kidneys, tissue samples were fixed in 10% formalin solution, dehydrated in alcohols with increasing concentration, and embedded in paraffin. Sections were stained with hematoxylin and eosin [13]. Microscopic study of preparations was performed using the microscope Granum. Microphotography of images were carried using digital video camera Granum DSM 310. Photos was processed on a computer Pentium 2,4 GHz using Toup View. Morphological study of the kidneys structure was carried out on the basis of the Central Scientific-Research Laboratory (CSRL) NUPh with the advice of senior scientist researcher of CSRL, Candidate of Biological Science Laryanovska Yu. B.

All data expressed as Mean ± SEM and data entered and analyzed using statistical package «Statistica 8.0». Student's t-test (pairwise comparisons) or one-way analysis of variance ANOVA were used to determine intergroup differences in the case of normal sample distribution. Paired Wilcoxon T-test used to determine difference between groups [7]. Values of $P < 0.05$ was considered as statistically significant.

Results and Discussion

Acute renal injure (ARI) is a potentially reversible syndrome characterized by rapid development and impaired homeostatic renal function. Clinical manifestations of ARI are manifested by disturbances of water-electrolyte metabolism and acid-base balance, increased inflammatory processes, azotemia with the development of uremia, pathological lesions of almost all organs and systems. One of the most harmful nephrotoxins is ethylene glycol. The toxic effect of ethylene glycol on the renal parenchyma is due to the influence of its metabolites (glycolic, glyoxylic and oxalic acids), which cause acute renal failure (ARF). Evidence of ethylene glycol-induced ARF includes macrohematuria, oligo- or anuria, hypercreatininemia, hyperproteinuria, intensification of lipid peroxidation and symptoms of CNS damage (ataxia, coma) [17]. Approximately 50% of ARI cases are fatal in the absence or untimeliness appropriate treatment. In our research macrohematuria occurred in animals of the CP group after 30 minutes after injection of ethylene glycol. Ataxia, areflexia, lateral position of animals, and coma were observed after 3 hours. During the first 12 hours after nephrotoxin injection all animals from the CP group died (table 1). Treatment with benophylline from the first 12 hours of acute pathology statistically significant decreased mortality. At the end of the experiment (5 days of observations), the highest survival was recorded – 50%. Mortality of the animals treated with the reference drug chophytol at the end of the experiment was 67%. This indicator was significantly higher than the CP group, but chophytol was inferior to benophylline. Improving the survival of animals proves the presence of nephroprotective properties in the test compound.

The oliguric stage of ethylene glycol-induced ARF is characterized by a decrease in diuresis of more than 2 times, which is associated with a decrease in GFR by almost 5 times and a significant decrease in tubular reabsorption by 1% compared to the same rate of intact rats. The results of the study of the functional state of the kidneys are shown in Fig. 1-6.

The development of pathology is accompanied by a significant decrease in the ability of the kidneys to excrete nitrogen-containing metabolic products, which leads to hyperemitrogenemia and uremia (Fig. 4-5). The level of creatinine and urea in the serum of rats increased by 1.3 and 5.5 times, respectively, and urinary excretion significantly decreased by 1.5 and 2.3 times, respectively, compared with animals from the intact control group. The degree of renal damage is also indicated by a sharp decrease in urea clearance by 21.5 times compared to control (Fig. 3). The progression of ethylene glycol-induced ARF is indicated by the development of proteinuria, which even in conditions of significant reduction of spontaneous urination is characterized by

significantly greater loss of protein in the urine (by 3.2 times) while significantly reducing this indicator by 20% in serum compared to intact animals (Fig. 6).

The administration of benophylline led to the statistically significant normalization of impaired renal function in all the above indicators, except for the level of protein in the urine, which tended to decrease (Fig. 6). Significant recovery of diuresis in animals with ethylene glycol-induced ARF treatedt with benophylline is characterized by its increase by 2.3 times compared to the level in the pathology group and even by 10.7% relative to the level of IC. However, in this respect, benophylline is insignificantly (8%) inferior to the reference drug chophytol (Fig. 1). This dynamics of diuresis in animals treated with benophylline is associated with an increase in GFR by 5.4 times and a significant normalization of tubular reabsorption, which is only 0.14% below normal compared to ethylene glycol-induced ARF. These indicators show the normalizing effect of benophylline on the nitrogen-releasing function of the kidneys. Hyponitrogenemic activity of benophylline is characterized by a significant decrease in the concentration of creatinine and serum urea by 1.2 and 6.5 times with a simultaneous increase in their excretion in the urine by 1.4 and 1.9 times, respectively, compared with the CP group. The informative indicator in the experiment was the urea clearance, which was significantly normalized in animals treated with benophylline (Fig. 3).

This indicator was slightly lower than in the IC group, but increased by 18 times compared with ethylene glycol-induced ARF. The total hyponitrogenemic activity of benophylline in all studied indicators also exceeds the similar effect of chophytol: the creatinine content by an average of 4-12%, the dynamics of changes in urea levels – 1.5-2.6 times, and the urea clearance – 4 times compared with reference drug. An important criterion for the nephroprotective effect of benophylline can also be considered its ability to prevent protein loss in the urine as one of the main signs of renal failure. Benophyllin normalized the content of total protein in serum almost to the level of IC (66.4±1,10 g/l) (P≤0,05). At the same time, animals treated with chophytol showed only a slight tendency to increase the level of serum protein, which remained almost at the level of CP (57.8±1.80 g/l). The decrease in protein content in the urine of animals treated with benophylline and chophytol had a tendency – by 26.1% and 8.7%, respectively. Thus, according to the results of experimental analysis of the effect of benophylline on renal function in rats, the experiment showed that the test compound against the background of acute toxic renal injure by ethylene glycol significantly normalizes all studied renal parameters and showed a higher nephroprotective effect than the reference drug chophytol.

To confirm the nephroprotective properties of benophylline, the next stage of the research was a histological study of its effect on morphological changes in the kidneys in acute renal failure induced by ethylene glycol. The histological structure of the kidneys was studied by light microscopy. Microtome sections were stained with hematoxylin and eosin. The obtained digital data were processed using nonparametric methods.

Macroscopic observations. In animals from the IC group, it was found that the kidneys are normal in size, the capsule is removed without difficulty. The surface of the organs is smooth, the color is reddish-brown. In the section, the pattern of the tissue is clear, the dark cortical substance of the kidney is separated from the medullar one. Numerous glomeruli, which are located with normal density, can be seen in the cortical substance. The nephrothelium is not changed, the nuclei are clear. Interorganic blood vessels and stroma without significant changes. In rats from the CP group, a whole range of pathological changes of renal tubular cells was observed – from dystrophy (swelling and vacuolation of the cytoplasm) to the decay of individual capillary segments. The kidneys are enlarged, the capsule is tense and immediately slips out of the organ during the incision. The surface of the kidney is smooth, pale gray. On the cut section in most animals, the pattern of the layers is smoothed, the cortical layer is swollen, its color is paler than in the medullar layer. In animals of the third group (ARF + benophylline, 35 mg/kg), the kidneys were similar to those in the intact control in terms of size, color, capsule state, and the nature of the patterns of the layers. In the majority of rats of the fourth group treated with the reference drug Chophytol at a dose of 70 mg/kg, the kidneys were

slightly enlarged in size, had a grayish tinge, a pattern of tissue on the cut section.

Renal microscopy showed that intact animals on histological sections of the kidneys preserved all structural elements: nephrons, vascular and stromal components (Fig. 11-14).

A clear division into cortex and medullar substance is determined. In the cortex the nuclei are clear, numerous glomeruli of nephrons, moderately variable in size, and a spherical shape with a slightly rough surface. The data of morphological studies correspond with the results of functional ones both on the confirmation of the development of ARF and on the protective effect of the investigated substance on nephrocytes. The cortical and medullar layers are clearly observed on the micropreparations of the kidneys of intact rats. Renal glomeruli are normal in size and density. In the vascular component of the glomeruli, the pattern of capillary loops is expressed, their blood filling and cellular saturation are within the norm. The lumen of the glomerular capsule is moderate and free. The tubules of the cortical substance are tightly packed. Perikaryons of cells are compact, nuclei are monomorphic. Nephrocytes of the proximal part of the tubules of the nephrons are moderately high, the lumen of the tubules is sufficient, which indicates the production of a normal amount of urofiltrate in the glomerulus. The epithelium of the distal part of the nephron tubules is smaller in size, the part of the cells was characterized by moderate loosening of the apical ends. The lumen of the tubules is somewhat narrower, in some the so-called cylinders were visualized – small compacted "clumps" of coagulated protein. The epithelium of Henle's loops, straight tubules of the medullar layer and collecting ducts is normal. Microcirculatory processes and interstitial state of the cortical and medullar layers without features. Changes in the morphological state of the kidneys in rats from the CP group can be characterized as dystrophic changes in the parenchyma and stroma, moderate dyscirculatory disorders (Fig. 7-10). Changes in the parenchyma were manifested by the diversity of the state of the renal glomeruli and nephron tubules. Thus, part of the vascular glomeruli was shrunken, the pattern of capillary loops is incomprehensible. In other vascular glomeruli (most of them) the pattern of loops is clearer, capillaries are extremely full-blooded, red blood cells are in a state of stasis. In some glomeruli of nephrons, the decay of individual capillary segments was observed. The endothelium of the glomerular capsule. and the mesangium (an amorphous intercellular substance that forms the central part of the glomerulus) was often swollen, but the lumen of the capsules was free. In the tubular system the proximal tubules of nephrons were characterized by the greatest state variety The lumen of some was not visualized, the tubules were destructured, the pattern of this section was destroyed. In the second tubules the lumen was very markedly expanded (tubuloectasis), often with the accumulation of eosinophilic amorphous protein masses (which often clog the tubule), flattened nephrocytes (Fig. 9). Damage zones interspersed with "unchanged" zones – the pattern of tubules is preserved, but in a significant number of tubules (both proximal and distal part of nephrons) the apical membrane of nephrocytes is excessively loosened or ruined. Sometimes nephrocytes in the tubules are exfoliated. The nuclei of epithelial cells were often found in the state of karyopycnosis, karyorexia, karyolysis, and edema (Fig. 10). The changes observed in the collecting tubules and straight tubules of the medullar layer are similar to the above, but less pronounced. Their lumen is preserved, partially filled with protein masses, the cytoplasm of nephrocytes is sometimes vacuolated, around the nuclei there are rarefaction zones (Fig. 9). In all rats of this group in the interstitium of the kidneys there was a stagnation in the venous sector with foci of small hemorrhages (Fig. 10), numerous lymphocytic-cellular infiltrates of different sizes, partial destruction of the tubules located nearby (Fig. 8). After preventive and therapeutic administration of benophylline on the background of pathology modeling in the renal parenchyma, 62.5% of rats showed a significant decrease in dystrophic and dcirculatory disorders. Among the renal glomeruli, glomeruli with a distinct pattern of capillary loops, much smaller than in control animals. Blood-filling of vessels – red blood cells in capillaries are centrally located, the lumen of capillaries is visible, which testifies to restoration of a blood flow in them (fig. 12-13). Zones with unchanged structure of convoluted tubules have

noticeably increased, especially in the outer parts of the cortical layer. Only the increased disintegration of the apical parts of nephrocytes and the presence of cylinders in the lumen of the tubules of the juxtamedullary nephrons were preserved (Fig. 12). The straight tubules of the medullar layer and the collecting ducts were practically unchanged (Fig. 13). In the remaining 37.5% of rats, after the introduction of benophylline, the characteristic changes in the renal glomeruli and convoluted tubules of the nephrons were more pronounced and varied, but less than in the control pathology. In contrast to CP, in different areas of the nephron tubules to one degree or another in all rats treated with benophylline, there were noticeable signs of epithelial regeneration – the nuclei of nephrocytes clearly fluctuated in size, the number of cells in the wall of the tubules increased. In all rats of the group, the lymphocytic-cellular infiltration of interstitium, signs of venous stasis and stroma edema were markedly reduced or practically absent. After administration of the reference drug Chophytol in 44.44% of rats, the state of the renal glomeruli and tubular system practically corresponded to the norm. In another 22.22% of animals, the state of the vast majority of renal glomeruli was normal, but the lumen of a fairly significant portion of the proximal and distal tubules, as well as parts of some stright tubules was significantly expanded, in some protein masses were present. Apical ends of nephrocytes of convoluted tubules are considerably opened. In 33.34% of rats of this group, more pronounced fluctuations in the state of vascular glomeruli, more pronounced tubuloectasis of convoluted tubules, occlusion by protein masses of the lumen of part of the tubules, and more pronounced fluctuations were observed. Dyscirculatory disorders in all rats were minimal. Lymphocytic infiltration of the interstitium was of a secondary nature and was determined in the majority of animals. Signs of regeneration have been observed in a number of tubules.

Conclusions:

1. Benophylline has shown clear nephroprotective properties on the model of ethylene glycol-induced ARD. The survival rate of animals in the first 12 hours treated with benophylline was by 17% higher than in animals eated with chophytol. The preventive and therapeutic administration of benophylline increased diuresis by 2.3 times relative to CP ($p < 0.05$) and had a normalizing effect on nitrogen-excretory renal function in animal, namely: reduced serum urea and protein in the urine, increased creatinine excretion and urea clearance by 6.5; 1.4; 3.2 and 18 times, respectively, relative to CP ($p<0,05$). The increase in urea clearance and the ability to resolve proteinuria revealed an advantage of benophylline over chophytol by 3.6 times and 1.2 times, respectively ($p<0,05$).
2. A study of the histological structure of the kidneys in animals with ethylene glycol-induced nephropathy showed that the preventive and therapeutic administration of benophylline helped to improve the state of the organ in all rats. At the same time, in 62.5% of rats the morphological state of the kidneys was close to the state of IC group, and in 37.5% of animals there was a marked decrease in dystrophic and dyscirculatory disorders, manifestations of inflammation, signs of nephrocyte regeneration were found.

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Table 1. Mortality of rats with ethylene glycol-induced ARF (n=6)

Group	Day 1	Day 2	Day 3	Day 4	Day 5
	died/ survived	died/ survived	died/ survived	died/ survived	died/ survived
Intact control	0/6	0/6	0/6	0/6	0/6
Control pathology	6/6 (100%)	–	–	–	–
ARF+Benophylline, 35 mg/kg	2/4 (33%)*	3/3 (50%)*	3/3 (50%)*	3/3 (50%)*	3/3 (50%)*
ARF+Chophytol, 70 mg/kg	3/3 (50%)*	4/2 (67%)*	4/2 (67%)*	4/2 (67%)*	4/2 (67%)*

Note:
1. in the numerator – the number of died animals;
2. in the denominator – the total number in the group;
3. in parentheses - % of died animals;
4. * - P < 0.05 versus control group.

Figure 1. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Diuresis per day, ml)

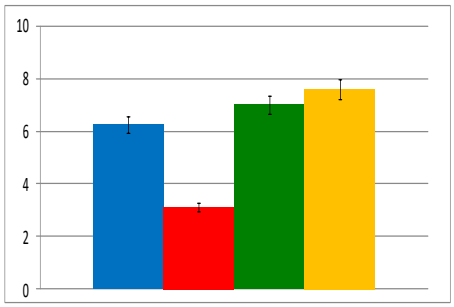

Figure 2. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Creatinine excretion, μM/2h)

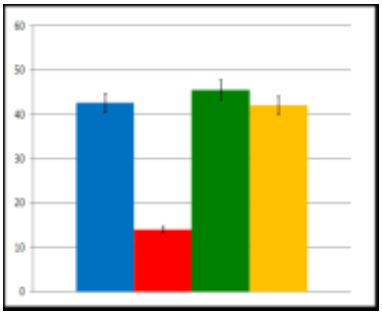

Figure 3. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Urea clearance, mmol/min)

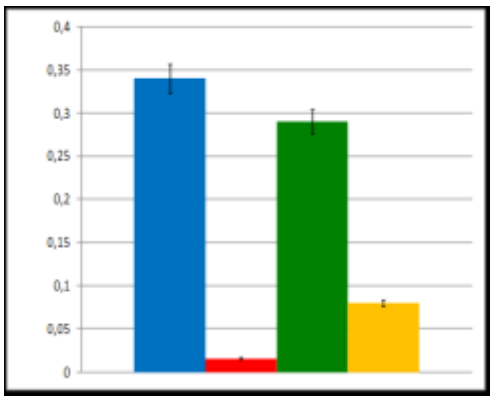

Figure 4. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Urea in serum, mmol/l)

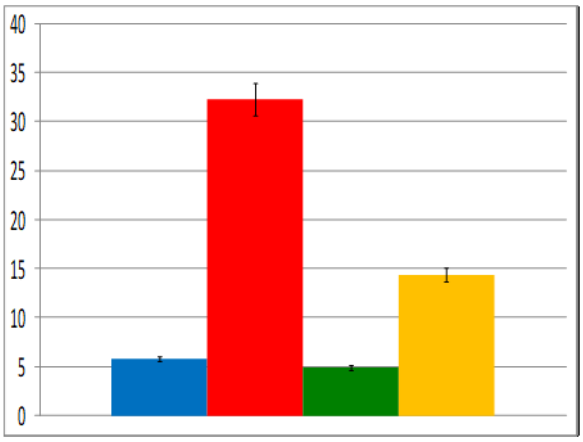

Figure 5. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Serum creatinine, µmol/l)

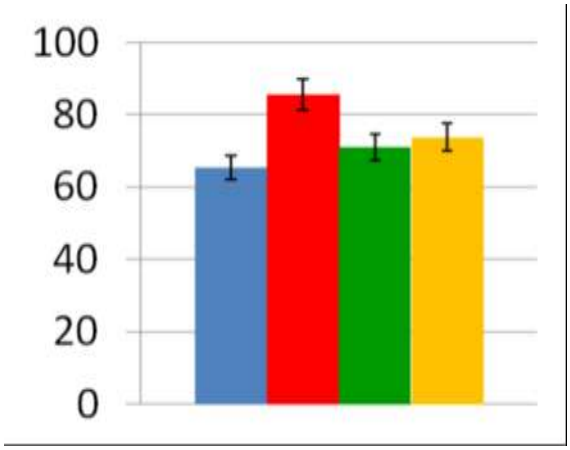

Figure 6. Effect of benophylline and chophytol on renal function in rats with ethylene glycol-induced nephropathy (Total protein in urine, g/l)

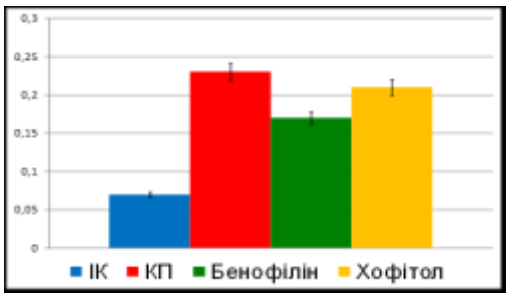

Figure 7. Shrinkage of glomeruli x400 (Hematoxylin-eosin)

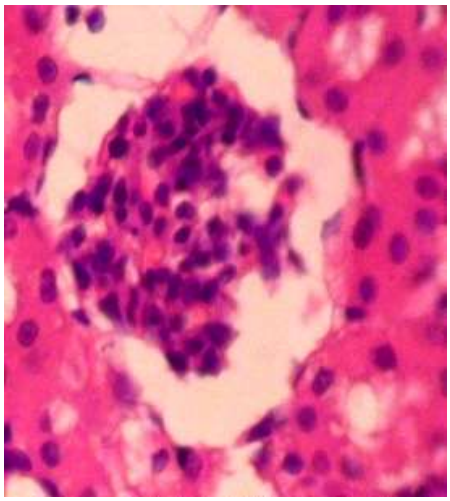

Figure 8. Destruction of tubules x 200 (Hematoxylin-eosin)

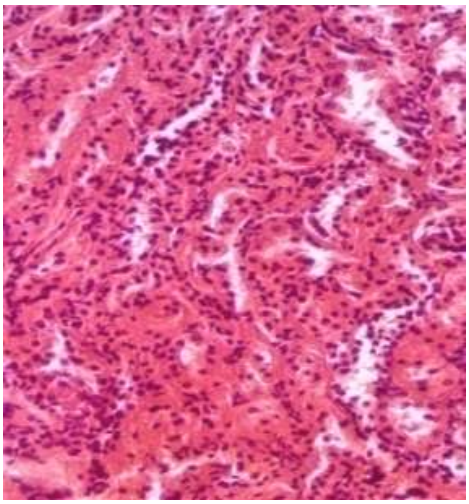

Figure 9. Occlusion of tubules x 250 (Hematoxylin-eosin)

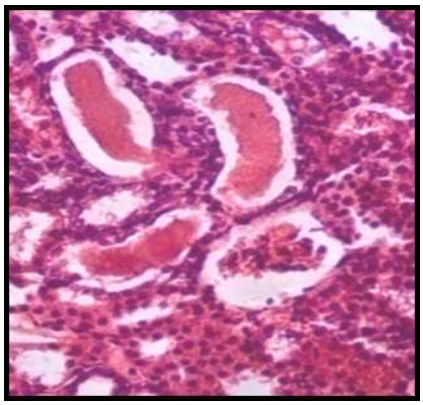

Figure 10. Destruction of tubules by infiltrate cells x250 (Hematoxylin-eosin)

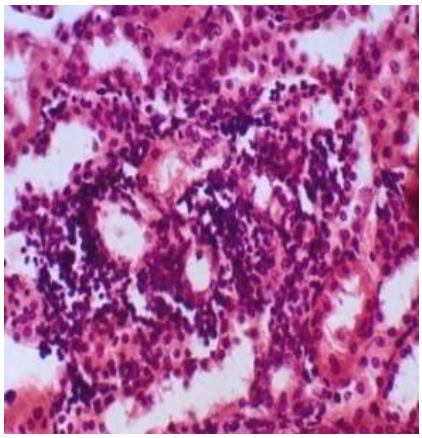

Figure 11. Restoration of the structure of the renal glomerulus x 400

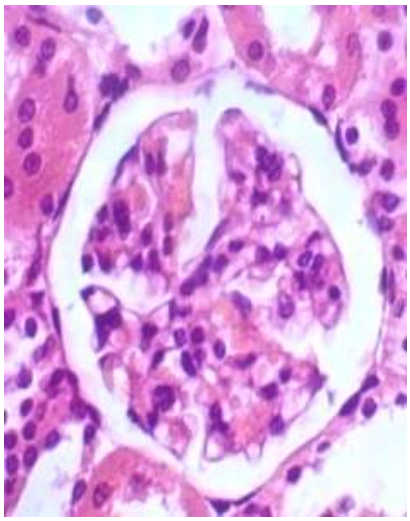

Figure 12. Unchanged convoluted tubules of cortical substance with fluff of nephrocytes x 200

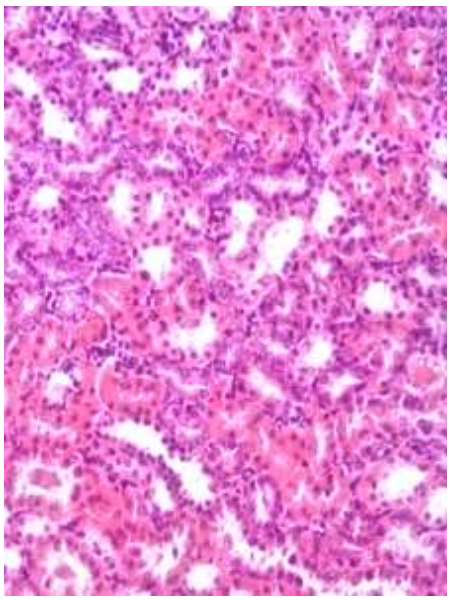

Figure 13. Unchanged convoluted tubules of the outer parts of the cortex x 200

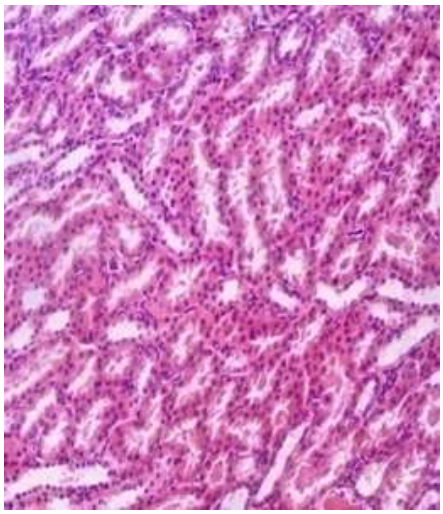

Figure 14. Moderate tubuloectasia of the tubules, the presence of protein masses in the lumen x100