

## Histological study of the effect of *Nigella sativa* on diabetic nephropathy in rats.

Dr. Abdul Karim Salim Mahood

Dept. of Anatomy, College of medicine, Tikrit University, Tikrit, Iraq.

### Abstract

Diabetes mellitus has been induced in Sprague Dawely rats, by injection of alloxan 75 mg/kg body weight, once daily for three successive days, The rats were randomly allotted into one of four experimental groups: A (control), B (diabetic untreated), C (diabetic treated with Glibenclamide) ; and d (diabetic treated with Glibenclamide and *Nigella sativa*), each group contain twenty four animals. Development of diabetes in three experimental groups was confirmed by measuring blood glucose levels in a tail vein blood samples. Rats with blood glucose levels of 250 mg/dl or higher were considered to be diabetic. The rats in NS treated groups were given NS extract (100 mg/kg body weight) once a day orally by using intra gastric intubation for 5 months, starting 2 days after alloxan injection. Treatment of NS reduced the glomerular size, thickening of capsular, glomerular and tubular basement membranes prevent the increased in the amounts of mesangial matrix and tubular dilatation as compared with diabetics untreated. We conclude that NS therapy causes renal morphologic and functional improvement after alloxan-induced diabetes in rats. We believe that further preclinical research into the utility of NS treatment may indicate its usefulness as a potential treatment in diabetic nephropathy.

### Introduction

Diabetic nephropathy is one of the most serious complications of diabetes and the most common cause of end-stage renal failure in the Western world. At present, diabetic kidney disease affects about 15 to 25% of type 1 diabetic patients<sup>1</sup> and 30 to 40% of patients with type 2 diabetes<sup>2,3</sup>. Diabetic nephropathy is characterized by specific renal morphological and functional alterations. Features of early diabetic renal changes are glomerular hyperfiltration, glomerular and renal hypertrophy, increased urinary albumin excretion (UAE), increased basement membrane thickness (BMT), and mesangial expansion with the accumulation of extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin. Advanced diabetic nephropathy is characterized by proteinuria, a decline in renal function, decreasing creatinine clearance (CrCl),

glomerulosclerosis, and interstitial fibrosis.

Black cumin or black seed (*Nigella sativa*) is the ancient traditional herbal medicine that has been used continuously in the Middle East and is well-known throughout the rest of the world<sup>4-7</sup>. The World Health Organization recommends black cumin as a herbal medicine<sup>8</sup> with anxiolytic effect<sup>9</sup> and it is used as an essential ingredient in Eastern medicine, including in Thai traditional medicine and Indian ayurvedic medicine. The active constituents of black cumin have been identified as thymoquinone, dithymoquinone, thymohydroquinone, and thymol<sup>10</sup>. Several studies have shown the various therapeutic actions of black cumin. It has activity against diabetes<sup>11,12</sup> radical scavenging activity<sup>13,14</sup>, prevents lipid peroxidation and increases the antioxidant defense system<sup>15</sup>. Renal dysfunction is a common complication in diabetes mellitus that is involved in oxidative stress

changes 16–18. The amelioration of renal hemodynamic and function changes in diabetics has been elucidated by supplementation with antioxidants. Black cumin was believed to be responsible for restoration in renal dysfunction in nephrotoxic rats through the antioxidant effect 19–21. However, knowledge in renal functions is still limited, especially in diabetes mellitus. Therefore, in the present study, the effect of black cumin oil (NS) on changes in renal dysfunction and renal hemodynamics was evaluated when administered in alloxan-induced diabetic rats 22,23.

### Material and methods

Laboratory animals: - Ninety six Sprague Dawley rats, weighing 250-275 gm at age of 5 to 6 months were used. Rats were housed in a well-ventilated room at 20 °C, had free access to food and water and they were under daily inspection. Seventy two rats acquired to be diabetic by injecting them with alloxan (75mg/kg/body weight) I.P. once daily for three successive days 24. A drop of blood was taken from injured tip of their tails before and after they have been diabetic. Blood and urine glucose was measured after three days of the last dose of alloxan by using Glucometer Elite and reagent strips (Glucometer Elite, Bayer A/S, Noergardsvej-32 DK-2800 Lyngby, Denmark). Diabetic rats with blood chloroform inhalation was used in sacrificing the rats, within the first two weeks, first month, second month, third month, fourth month and fifth month after measuring their blood sugar. Animals distributed randomly into four groups (24 rats in each group), as following:

Group A- Normal healthy rats

Group B- diabetic rats without treatment

Group C- diabetic rats treated with Glibenclamide only (0.0714 mg /kg.B.W. orally. Twice daily) 24.

Group D- Diabetic rats treated with both, Glibenclamide and *Nigella sativa* (100 mg/kg body weight) once a day orally (Supplied by the Allwerdy Pharmacy, with special permission from Iraqi Herbal Museum) by using intra

gastric intubation for 5 months starting 2 days after alloxan injection 25.

### Histological technique and sample taking

During the experimental period, the body weight and fasting blood glucose were monitored. Both the kidneys of all the rats were collected on the 14th day following treatment, and every month till the 5th month. The collected tissues were fixed in 10% formalin, dehydrated through graded alcohol series (30–100%), cleared in xylene and embedded in paraffin wax. Sections of 5- $\mu$ m thickness were made and stained with Haematoxylin and eosin. These stains were used as per standard histological procedures 26. Photomicrographs of the stained slides were taken using a light microscope (Leica, Germany) attached to a digital camera (Pixelink, Canada).

### Statistical analysis

Results were expressed as mean  $\pm$  SD. The differences between two mean values was analyzed by student t-test. Statistical significance was considered when  $p \leq 0.01$ .

### Results

The diabetic rats were found to have a decrease in the body weight with features of polyuria, hyperphagia and polydipsia as compared to the non-diabetic rats. At the end of the study, it was observed that the non-diabetic rats gained weight ( $39 \pm 5.22$  g) in comparison to the diabetic rats, which lost weight ( $42.2 \pm 9.50$  g). Prior to the sacrifice, the diabetic rats treated with NS showed an increase in weight ( $19.8 \pm 5.23$  g) (Table 1).

The entire dissected kidney in the end of the study in untreated diabetic rats weighed  $0.64 \pm 0.04$  g/100 g body weight in comparison to the kidneys of non-diabetic rats, which weighed  $0.45 \pm 0.01$  g/100 g body weight. Treatment with the NS extract showed a decrease in the kidney weight, which was observed to be  $0.42 \pm 0.03$  g/100 g body weight (Table 2).

### Glomerular diameter (table 3)

We also assessed the maximal glomerular diameter (GD) of the maximally hypertrophied glomerulus in each specimen. Max GD was identified in serial sections, and was calculated as the mean of two measurements, i.e. of the maximal diameter of the glomerulus and the maximal chord perpendicular to the maximal diameter, in glomeruli show both vascular and urinary poles. Glomerular hypertrophy was clear in diabetic animals without any treatment and in diabetic animals treated only with glibenclamide, which consider as a hypoglycemic agent. No glomerular hypertrophy was seen in diabetic animals treated with NS.

Interstitial fibrosis (table4)

Compared to normal control rats, the fibrosis score was significantly greater for all animals with diabetic nephropathy. The fibrosis score was significantly higher in diabetic untreated animals.

### Histopathological changes

Histological study of the normal kidney of the nondiabetic rats revealed normal glomerulus surrounded by the Bowman's capsule, proximal and distal convoluted tubules without any inflammatory changes (Fig:1&2). The kidneys of untreated diabetic rats showed degenerated glomeruli infiltrated by the Inflammatory cells and thickening of the basement membrane (Fig:3). The proximal convoluted tubule exhibited edematous changes with deposition of mucopolysaccharide and hyaline substances (Fig:4). All the necrotic changes observed in the proximal and distal convoluted tubules along with the deposits in the diabetic untreated animals and that treated with glibenclamide (Fig:5-11) were found to be absent in the diabetic rats treated with NS (Fig:12). The group that was treated with NS extract showed features of healing i.e. normal glomerulus, absence of inflammatory cells, normal basement membrane and capillaries, decrease in the mucopolysaccharide and hyaline deposit, respectively. The tissue necrosis was also observed to decrease in the group treated with NS.

(Figures13 and14).

### Discussion

Interestingly, the diabetic nephropathy has been considered an important cause of mortality and morbidity and many of the end stage renal failure results due to diabetic nephropathy<sup>27</sup>. streptozotocin and alloxan-induced diabetic rodents result in development of nephropathy similar to the early stage of human diabetic nephropathy<sup>28</sup>. In the diabetic animals, a significant increase in the kidney weight was observed. The result of this study is in accordance with the findings of earlier research studies 29-31. It has been described that the kidney enlargement in DM is attributed to certain factors like glucose over-utilization and subsequent enhancement in increase uptake, glycogen accumulation, lipogenesis and protein synthesis in the kidney tissue 32-34.

The NS extract administered to the diabetic rats successfully prevented the enlargement of the kidney. The histological study performed on the kidneys of diabetic rats showed damage to the glomerulus, thickened basement membrane and edematous renal convoluted tubules with increase in mucopolysaccharide deposits which were found to be absent in the diabetic kidneys treated with NS extract. A previous study had also reported similar histological findings 32-35. Admittedly, the research study was carried out in shorter duration and this might be insufficient for significant vascular changes in the kidney of the diabetic rats. The treated diabetic rats however showed healing features, which resembled that of a normal kidney. Advanced human diabetic nephropathy often exhibits arteriolar hyalinosis and tubular atrophy coupled with an increase in the interstitial volume 36. These features are largely absent in normal and treated animals with NS. At the more advanced age in other non treated animals lesions suggestive of arteriolar hyalinosis were noted. However, it has not been characterized whether both afferent and efferent arterioles are similarly affected. The tubular changes noted in rats diabetic kidney primarily consist of vacuolization

of tubular cells with evidence of tubular atrophy, tubulointerstitial fibrosis, and alterations of the medullary structure by light microscopy was discernable. An overall increase in renal collagen content has been reported also 37; however, comprehensive and sensitive studies of tubular and vascular damage have not yet been published.

#### Glomerular diameter

On the other hand, it has been well established by a great number of studies that glomerular hypertrophy plays crucial roles in the outcomes of kidney diseases in experimental models 38-41 and in humans 42-44. The presence of glomerular hypertrophy in minimal-change disease is an indicator of progression to focal segmental glomerular sclerosis 38. Glomerular hypertrophy and renal hypertrophy are also seen soon after the onset of type 1 diabetic nephropathy 39. Persistent renal hypertrophy precedes the development of microalbuminuria and a decline in GFR 40. Thus, the presence of glomerular hypertrophy has greater implications in regard to susceptibility to glomerular sclerosis and renal insufficiency. Measurements of glomerular size have been to be valuable to predict the progression of kidney diseases 45. Mechanisms of glomerular injury are thought to be responsible for the pathological process 46.

We hypothesize that extremely hypertrophied glomeruli imply the presence of glomerular stress and that sclerosed glomeruli indicate only past glomerular stress. On this hypothesis, it is not necessary to look for collapsing and sclerosing glomeruli but to focus on finding hypertrophied glomeruli in pathological prognostic prediction. We therefore measured the diameter of maximally hypertrophied glomeruli. In general, there is a tendency to consider the entire mechanism of glomerular hypertrophy to be compensatory reaction against a loss of renal function 47, 48. However, we consider it important to differentiate between morbid glomerular hypertrophy and physiological glomerular hypertrophy.

Compensatory glomerular hypertrophy is essentially an adaptive physiological reaction and is beneficial to a point 49 beyond which it results in glomerulosclerosis and loss of renal functional. Shea et al. 50 found that after subtotal nephrectomy in rats, the volume of the glomeruli of rats increased to ~2.5 to 3.0 times the volume of the control group. The results of these studies suggest the possible existence of a pathological threshold of glomerular size. We conclude that Max GD, which is a simple quantitative histological parameter, can be used as a prognostic indicator for disease progression.

#### Interstitial fibrosis

The importance of interstitial fibrosis in predicting the outcome of patients with diverse renal diseases including diabetic nephropathy has been well established by the numerous studies of Bohle and his colleagues 51. Consistent with these studies we have demonstrated that a higher interstitial fibrosis score clearly predicts a poor renal outcome

#### Conclusions

The results of the study showed that diabetic kidney changes may be protected by administration of NS extract. The NS extract possibly acts as an antioxidant thereby checking the oxidative damages to the microstructure of the kidney. Further studies maybe needed to corroborate additional findings.

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**Table 1 –Body weight in non-diabetic rats, untreated and treated diabetic rats**

| treated diabetic rats with G+NS | treated diabetic rats with G | untreated diabetic rats | non-diabetic rat | Period                |
|---------------------------------|------------------------------|-------------------------|------------------|-----------------------|
| 268.2±9.164                     | 256.25±8.770                 | 262±10.242              | 258±6.336        | Week2                 |
| 268.3±6.238                     | 258.7±6.599                  | 258.2±1.763             | 255±10.801       | 1 <sup>st</sup> month |
| 274.5±3.109                     | 261.2±3.274                  | 250.3±13.156            | 265±12.909       | 2 <sup>nd</sup> month |
| 275.2±3.593                     | 263.7±5.281                  | 238.6±13.661            | 278±3.559        | 3 <sup>rd</sup> month |
| 281±8.406                       | 260.3±4.500                  | 217.7±9.968             | 287.8±6.849      | 4 <sup>th</sup> month |
| 288±2.828                       | 262.6±6.263                  | 219.8±8.180             | 297.3±7.182      | 5 <sup>th</sup> month |

All data are expressed as mean±Sd. \*p<0.01 vs. the non-diabetic group.

**Table2 –Kidney weight in non-diabetic rats, untreated and treated diabetic rats**

| treated diabetic rats with G+NS | treated diabetic rats with G | untreated diabetic rats | non-diabetic rat | Parameter                        |
|---------------------------------|------------------------------|-------------------------|------------------|----------------------------------|
| 0.96±0.04                       | 1.05±0.07                    | 1.25±0.08               | 1.28±0.03        | Kidney weight [g]                |
| 0.42±0.03                       | 0.50±0.02                    | 0.64±0.04**             | 0.45±0.01        | Kidney weight / 100g body weight |

All data are expressed as mean±Sd. \*p<0.01 vs. the non-diabetic group

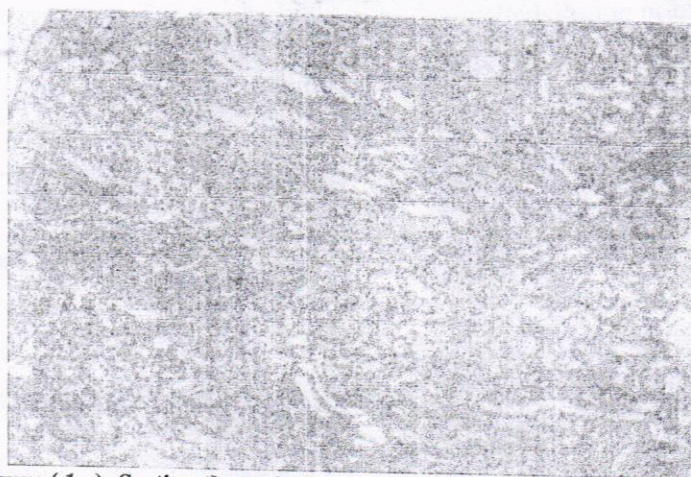
**Table 3 – Glomerular diameter in non-diabetic rats, untreated and treated diabetic rats**

| treated diabetic rats with G+NS | treated diabetic rats with G | untreated diabetic rats | non-diabetic rat | Period                |
|---------------------------------|------------------------------|-------------------------|------------------|-----------------------|
| 95.7±2.572                      | 96.2±2.077                   | 96.3±1.249              | 97.2±1.147       | Week2                 |
| 95.7±2.104                      | 96.0±1.928                   | 98.5±2.456              | 97.0±0.779       | 1 <sup>st</sup> month |
| 96±1.891                        | 98.4±1.643                   | 100.1±4.524             | 97.2±0.713       | 2 <sup>nd</sup> month |
| 97.1±1.496                      | 103.6±3.575**                | 109.1±9.050**           | 97.5±0.904       | 3 <sup>rd</sup> month |
| 98.11.410                       | 105.1±4.272**                | 113.1±4.645**           | 97.9±1.025       | 4 <sup>th</sup> month |
| 98±5.502                        | 107.2±11.391**               | 117.9±8.363**           | 97±3.036         | 5 <sup>th</sup> month |

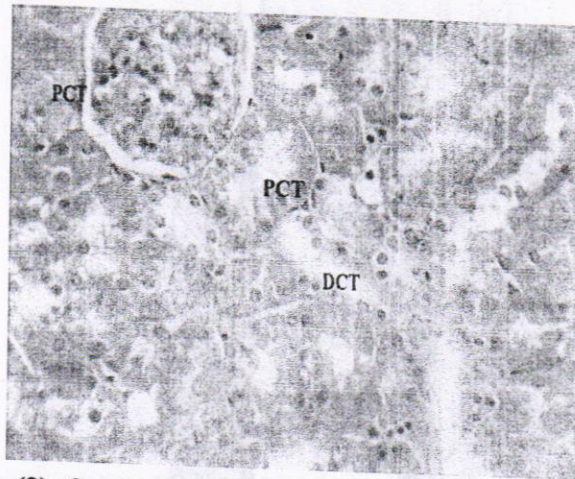
**Table 4** – Interstitial fibrosis in non-diabetic rats, untreated and treated diabetic rats

| treated diabetic rats with G+NS | treated diabetic rats with G | untreated diabetic rats | non-diabetic rat | period                |
|---------------------------------|------------------------------|-------------------------|------------------|-----------------------|
| 5.2±0.251                       | 5.0±0.146                    | 5.3±0.184               | 5.2±0.337        | Week2                 |
| 5.4±0.162                       | 5.7±0.351                    | 6.2±0.285**             | 5.2±0.360        | 1 <sup>st</sup> month |
| 5.±0.198**                      | 7.2±0.833**                  | 8.4±**0.186             | 5.3±0.457        | 2 <sup>nd</sup> month |
| 5.5±0.402                       | 11.6±0.299**                 | 17.2±**1.292            | 5.3±0.485        | 3 <sup>rd</sup> month |
| 6.9±0.852**                     | 15.65±3.248**                | 21.4±1.429**            | 5.395±0.474      | 4 <sup>th</sup> month |
| 7.25±0.910**                    | 16.75±3.209**                | 21.7±1.657**            | 5.39±0.462       | 5 <sup>th</sup> month |

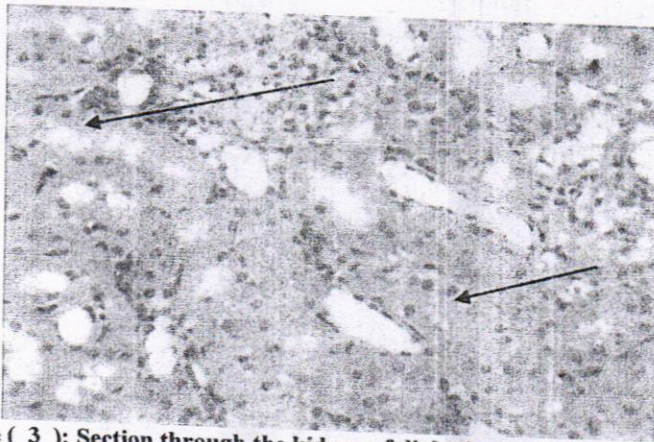
All data are expressed as mean±Sd. \* $p < 0.01$  vs. the non-diabetic group



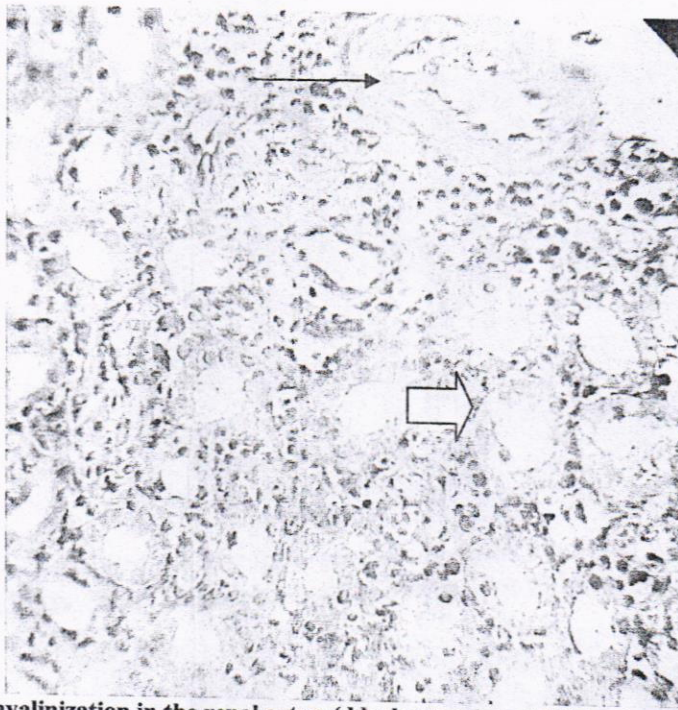
**Figure ( 1 )**: Section through the kidney of control rat showing normal histological structure of the cortex, H & E, 100 X.



**Figure (2):** Section through the kidney cortex of control rat showing normal glomerulus(arrow), and kidney tubules, H and E, &400X.



**Figure ( 3 ):** Section through the kidney of diabetic untreated rat showing high degree of degeneration of kidney tubule cells (  ), H and E,100X.



**Figure ( 4 ):** hyalinization in the renal artery( black arrow), and thickening in the basement membrane of the renal tubules(white arrow) of diabetic untreated rat, H & E, 400X.



**Figure ( 5):** Diabetic untreated kidney, showing loss and hyalinization of capillary surface(Arrow), which is closely associated with loss of glomerular function. H & E, 400 X.

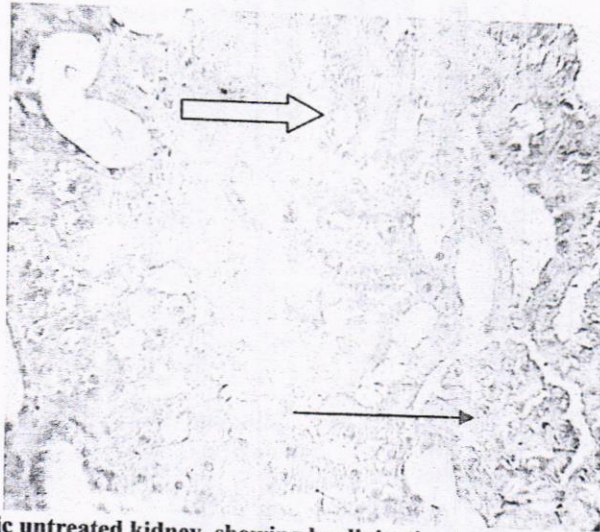


Figure (6): Diabetic untreated kidney, showing hyalinization in glomerulus(black arrow), and tubules(white arrow). H & E, 400 X.

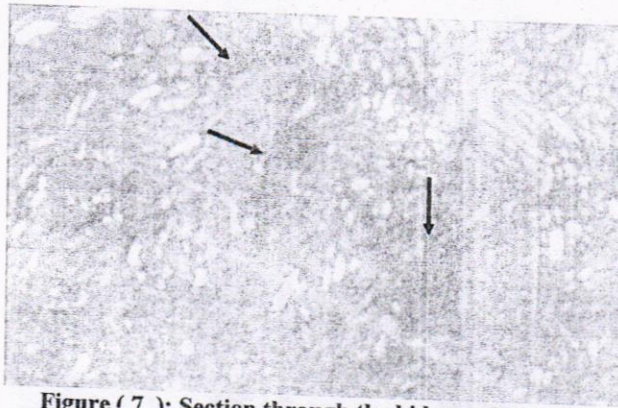


Figure ( 7 ): Section through the kidney of diabetic rat treated with glibenclamide showing the existence of inflammatory infiltration areas (arrow) and dilation of some tubules, H and E, 100 X.

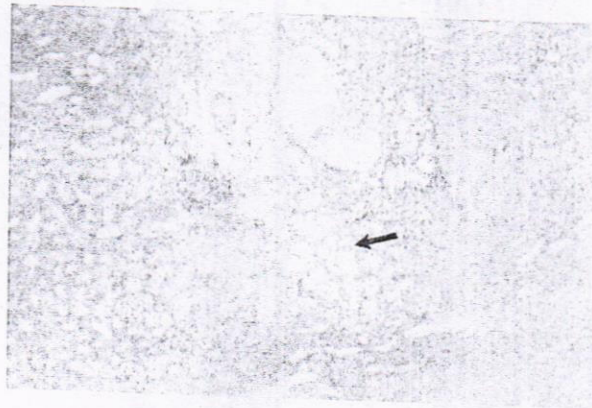


Figure (8): Section through the kidney of diabetic rat treated with glibenclamide showing, showing high degree of degeneration of kidney tubule cells ( arrow ), H and E, 100 X.

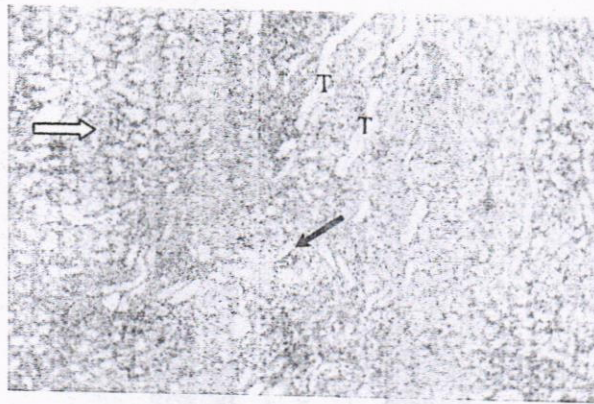


Figure (9): Section through the kidney of glibenclamide treated diabetic rat showing The necrotic area of the glomerulus( white arrow), dilation in tubules (T), and degeneration of most kidney tubule epithelial cells(black arrow) H and E, 100 X .

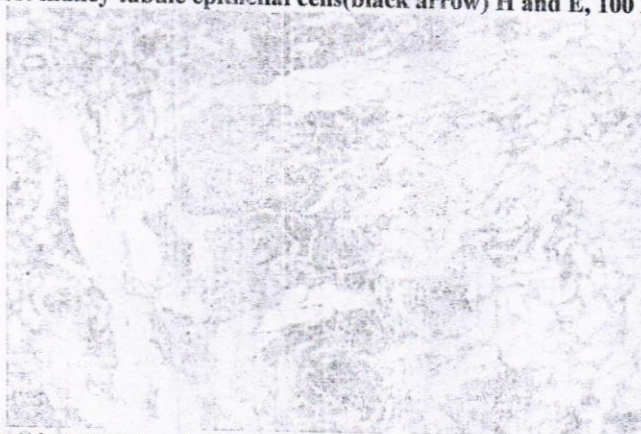


Figure (10 ): Dilation in the lumen of the kidney tubules in the kidney of diabetic untreated rat, H and E, 100 X.

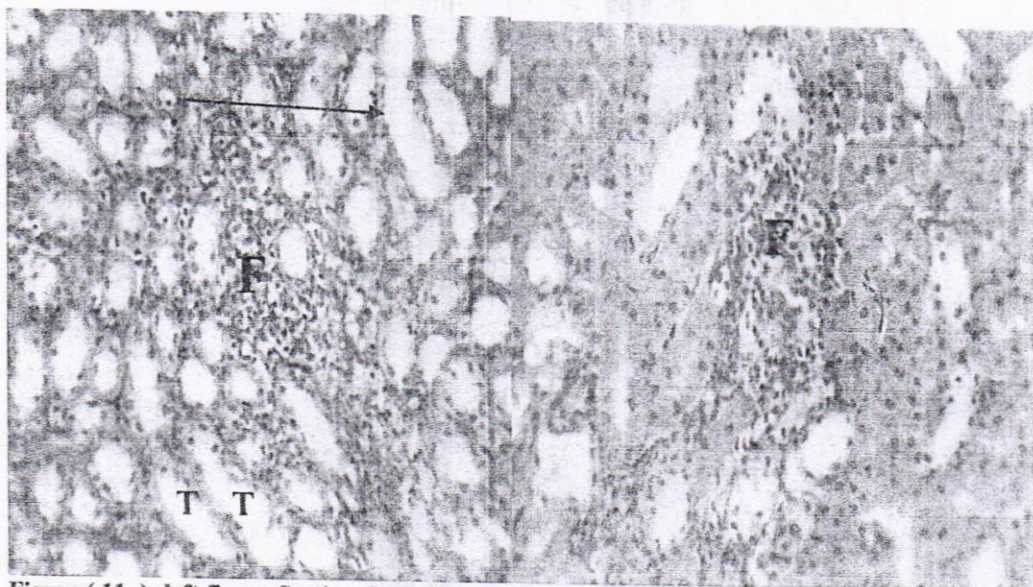


Figure ( 11 ): left figure-Section through the cortex of kidney in diabetic untreated rat, showing inflammatory cells (F), degeneration of kidney tubule cells (black arrow) and hypertrophy of some kidney tubules (T), , while the right figure is in the cortico-medullary region, H& E, 400 X .



Figure ( 12 ): Approximately normal appearance of cortical tubule in the kidney of diabetic rats treated with NS+ G, H and E, 100 X.

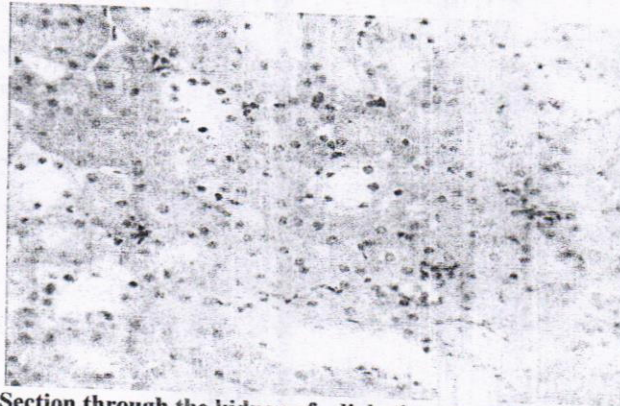


Figure ( 13 ): Section through the kidney of diabetic rats treated with NS+ G, showing approximately normalized cortical tubules, H and E, 400 X.



Figure ( 14 ): Section through the kidney of diabetic rats treated with NS+ G , showing normal appearance of cortical tubule cells ( arrow ) H and E, 400 X.