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Effect of Diabetes on the Rat Renal Cortex and the Possible Protective Role of Vitamins C and E.

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ABSTRACT

Background: This work aims to study the histopathological changes induced by type 2 diabetes on the structure of the rat renal cortex and to throw more light on the possible protective role of combined vitamin C & E upon diabetic renal affection using light, electron microscopic and biochemical studies **Methods:** 40 healthy adult male albino rats were classified into 4 main groups: Negative Control group (Group 1): included 10 rats fed on standard diet. Positive control group (Group 2): included 10 rats fed on standard diet and were given intraperitoneal citrate buffer only. Experimental diabetic group (Group 3): included 10 animals were given streptozotocin (STZ) at a dose of 40 mg/kg body weight dissolved in citrate buffer. The possible protected or prophylactic group (Group 4): included 10 Rats fed with VCE (Vitamin C and vitamin E) supplemented diet for 15 days prior to induction of diabetes. At the time of sacrifice, serum fasting blood glucose, urea and creatinine levels were measured in all groups. The kidneys were removed and processed for light and electron microscope examination. **Results:** Light microscopic and electron microscopic examination of both control groups demonstrated the normal structure of the kidney. Examination of the diabetic rats revealed many histopathological changes including congestion, distortion and shrinkage of glomeruli with widening of Bowman's spaces. Focal areas of complete tubular damage with interstitial hemorrhage and cellular infiltration also appeared. Examination of the protected animals showed normal glomeruli and tubules. **Conclusion:** We concluded that type 2 diabetes resulted in different histological changes in the renal cortex and these changes may be attenuated with prophylactic VCE administration.

Keywords: Kidney, Diabetic Nephropathy, Vitamin C&E, Electron Microscope.

INTRODUCTION

The kidney is a vital organ which has a considerable cellular complexity and functional diversity. Its main function is to eliminate nitrogenous waste products and to maintain the composition, volume, pressure of the blood and the density of our bones.[1]

Diabetes mellitus (DM) is a metabolic condition which results from inability of the pancreas to produce adequate amounts of insulin, or insensitivity of the cells of the body to insulin effects, thus resulting in unusually elevated blood glucose levels (hyperglycaemia).[2,3]

Insulin resistance and chronic hyperglycaemia lead To various long-term problems and complications such as autonomic neuropathy, peripheral neuropathy and nephropathy.[2,4] Diabetic kidney disease (DKD) is defined as functional, structural and clinical alterations of the kidneys caused by diabetes.[5]

Vitamin C, also, known as ascorbic acid and ascorbate, is a basic compound that belongs to the group of water-soluble vitamins.[6] Vitamin C can be produced by most animals and plants from D-glucose and D-galactose, whereas it is not produced in humans due to the lack of L-gulonolactone oxidase enzyme; and therefore, it should be taken externally.[7] The biological importance of vitamin C is that it is used as an antioxidant. Vitamin C is also a compound that plays an important role in collagen synthesis.[7]

Vitamin E is a lipid-soluble chain-breaking antioxidant which protects especially biological membranes from lipid peroxidation.[8] Recently; it was reported that vitamin E prevents elevated lipid peroxidation in sciatic nerve of the streptozotocin (STZ)-diabetic rats.[9] The level of Vitamin C and E in plasma and renal tissues is significantly reduced

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in diabetic patients.[10-12] Decrease in Vitamin C causes hyperlipidemia and hypertension.[13,14] Some studies showed that Vitamins C and E are important to prevent or alleviate the complications of diabetes mellitus. Vitamins C and E can be used as antioxidants separately or in combination. Both vitamins act synergistically.[15-17]

This work aims to study the histopathological effects of diabetes type 2 on the normal structure of the rat renal cortex and to throw more light on the possible protective role of combined vitamin C & E using light, electron microscopy and biochemical studies.

MATERIALS AND METHODS

40 healthy adult male albino rats (150-220 gm) were obtained from the animal house of Faculty of Medicine of Zagazig University. All animal care and handling procedures conformed to Guidelines set of Faculty of Veterinary medicine, Zagazig University. The rats were kept with normal room temperature and were exposed to alternate cycles of 12 h light and darkness for 7 days to prepare them for the experiment.

Chemicals: Streptozotocin (STZ): obtained from Cairo Company Lab. for Chemical and Medical Trading, Zagazig, Egypt. Vitamin C (ascorbic acid): obtained from Sigma Chemical Co. at Cairo, Egypt. Vitamin E (di-α-tocopheryl acetate): obtained from Cairo Company Lab. for Chemical and Medical Trading, Zagazig, Egypt. Preparation of VCE supplemented and unsupplemented food compositions: They were homogenized using a mixer and pellets were prepared in laboratory by heating below 45°C for 2 days.[18]

The VCE supplemented diet contained a combination of one g vitamin C (ascorbic acid) and 600 mg vitamin E (di-α-tocopheryl acetate)/kg food.[16]

Group design: Animals were classified into 4 main groups: Negative Control group (Group 1): included 10 rats fed on standard diet consisted of 53% carbohydrates in the form of sucrose sugar, 20% protein in the form of casine, 6% fat in the form of butter and 21% water.[19] Positive control group (Group 2): included 10 rats fed on standard diet and were given intra peritoneal citrate buffer only (0.1 M, pH = 4.5).[18] Experimental diabetic group (Group 3): included 10 rats fed on standard diet and given single dose of STZ intraperitoneally at a dose of 40 mg/kg body weight dissolved in citrate buffer.[16] Serum levels of fasting blood glucose were measured, using a one-touch basic plus glucometer to all rats 2 weeks after the injection of STZ to confirm diabetes, and the glucose levels starting from 150 mg/dl and higher were considered diabetic.[20] The possible protected or prophylactic group (Group 4) :Included 10 rats. The animals were given Vitamin C and E supplemented diet for 15 days before induction of diabetes. The Vitamin C and E supplemented diet contained a combination of ascorbic acid (1 g) and di-α-tocopheryl acetate (600 mg) per kg of feed.[18]

Seriological study: Fasting blood sugar level was measured for all animals 6 weeks after injection of streptozotocin (STZ). At the time of sacrifice, animals were anaesthetized with 50 mg/kg sodium pentobarbital intraperitoneal. Blood samples were drawn by capillary tube from retro-oribital venous plexus and serum fasting blood glucose level was measured in all groups. Also Serum levels of urea and creatinine were determined.

Light microscopy: The fresh specimens of the renal cortex from each group were taken and fixed in 10% neutral buffered formalin for seven to twenty two hours and prepared for routine histological laboratory procedures. Then the specimens were blocked in paraffin and sections of 1-5µm thick were cut and stained with: A: Hematoxylin and eosin stain for the kidney's general histological structure. B: Mallory's trichrome stain for identification of collagen fibers. C: Periodic Acid Schiff's reaction (PAS) for identification of carbohydrates.[21-23] Light Microscopic examination: Stained slides are examined using Binuclear CX31 Olympus Microscope

Electron microscopy: The specimens of the renal cortex from all groups were fixed in buffered glutraldehyde solution at pH 7.4 from 2 to 24 hours in a refrigerator at 4C˚. After 2 rinses in cacodylate buffer solution for a period of 15 minutes for each, the specimens were post-fixed in 1% buffered osmium tetroxide for 1 to 2 hours at room temperature, and then prepared through several steps for staining.[24]

Image analysis and morphometry: Sections stained with haematoxlyin and eosin and Mallory trichrome stains were morphometrically analyzed by using image analyzer computer system. Data were obtained using Leica Qwin 500 Image Analyzer Computer System (England) at Anatomy Department, Faculty of Medicine, Cairo University.

Statistical analysis: The parameters for all groups including serum levels of urea and creatinine and fasting blood glucose levels after 6 weeks were expressed as mean ± standard deviation (X±SD). The data were subjected to SPSS program. Statistically-significant difference was determined by one way analysis of variance (ANOVA), followed by post hook test, for comparison between different groups.[25]

RESULTS

The control group

Light microscopy: Examination of H&E sections showed prominent Bowman's space. Normal apparance of the Proximal and distal convoluted

tubules. The blood vessels appeared normal. (PAS) stained sections showed; prominent tubular basement membrane in the proximal and distal convoluted tubules. Well-developed PAS +ve brush borders along their lumina. Bowman's capsules appeared as thin regular PAS +ve membrane.

Electron microscopy: The proximal tubular cells contained large rounded nuclei with prominent nucleoli and peripheral chromatin condensation. They also, contained long rod-like mitochondria situated mainly in the basal part of the cells, and oriented parallel to the cell axis and perpendicular to the regular basement membrane. The densely packed microvilli in a sieve like pattern forming the brush border were, also, seen along the tubular lumen [Figure 1]. Similarly, the epithelial cells lining distal convoluted tubules showed rounded euchromatic nuclei, less numerous mitochondria. The cells rested on a thin regular basement membrane. The brush border of the distal tubular cells showed few short microvilli [Figure 2]. The podocytes could be seen with their long primary and many short secondary foot processes. Bowman's spaces appeared normal and clear of cellular debris [Figure 3]. The filtration barrier is composed of three layers: Fenestrated endothelium of the glomerular capillaries, the fused basal lamina of the endothelial cells and, fenestrations between podocytes secondary foot processes [Figure 4].

The Diabetic group

Light microscopy: Examination of H&E stained sections showed distorted, bilobed and shrunken glomerulus. Its Bowman´s space was wide. There were homogenous acidophilic material and area of cellular infiltration with inflammatory cells in the interstitium. Area of complete renal damage with loss of renal parenchyma was, also, seen. Mallory's trichrome stained sections revealed increase in the collagen fibers in the glomeruli and in the interstitum around the renal tubules and blood vessels. PAS stained sections revealed strong positive PAS reaction of thickened basement membrane of parietal layer of Bowman's capsule, in the glomerulus and thickened basement membrane of renal tubules. But PAS reaction was absent at the brush borders of tubular cells.

Electron microscopy: The proximal tubular cells appeared with dark heterochromatic nuclei, multiple swollen vacuolated mitochondria and some electron dense lysosomes. The cells rested on a thick irregular basement membrane [Figure 5]. The distal tubular cells showed swollen vaculated mitochondria of variable size and shape all over the cytoplasm and dark nuclei with chromatin clumps. Some showed small dark blebbed apoptotic nuclei. The brush border of the cells contained short distorted microvilli. They were resting on a thick irregular basement membrane. The interstitium showed infiltration with some lymphocytic inflammatory cells and blood capillaries with hemorrhage. The podocyte appeared with electron dense irregular nucleus and fused foot processes. The Bowman's space showed cellular debris and inflammatory cell [Figure 6]. The filtration barrier was formed of three layers: The abnormal non-fenestrated (fused) endothelium of the glomerular capillaries, the thick fused basal lamina of the endothelial cells, and fused podocytes secondary foot processes. Areas of nodular thickenings of glomerular basement membrane were also seen [Figure 7].

The Possible protected group

Light microscopy: Examination of H&E stained sections showed normal glomerulus surrounded by Bowman´s capsule with the space in between. The proximal and distal convoluted tubules were normal. The macula densa and the urinary pole (UP) were also observed. Mallory's trichrome stained sections revealed few interstitial collagen fibers around the renal corpuscles and tubules and among the glomerular capillaries.

Electron microscopy: The proximal tubular cells revealed closely packed microvilli. Its sieve like appearance was restorted. The numerous mitochondria restorted its normal shapes. The cells were rested on regular basement membrane and contained rounded euchromatic nuclei, few cytoplasmic vacuoles and some lysosomes [Figure 8]. Podocytes appeared normal with long primary and multiple short secondary foot processes [Figure 9]. The filtration barrier was composed of three layers: Fenestrated endothelium of the glomerular capillaries, the fused basal lamina of the endothelial cells and the fenestrations between podocytes secondary foot processes. There were small areas still had fusion between the secondary foot processes [Figure 10].

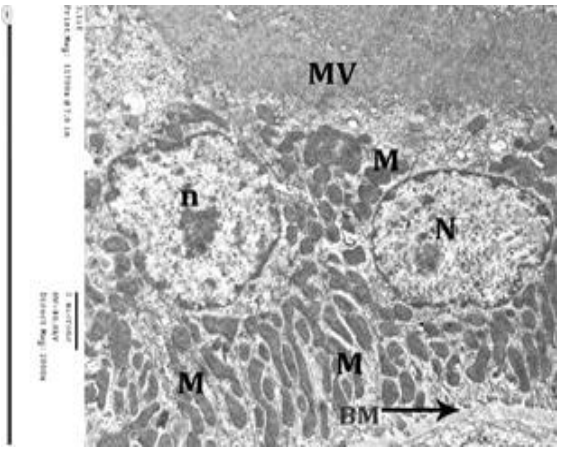

Figure 1: EM of the control rat renal cortex showing: proximal tubular cells with apical microvilli (MV), numerous mitochondria (M), rounded euchromatic nuclei (N) and prominent nucleolus (n) ,X2000.

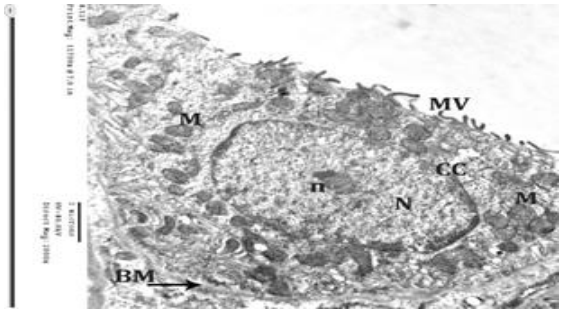

Figure 2: EM of the control rat renal cortex showing a distal tubular cell with rounded euchromatic nucleus (N), prominent nucleolus (n), peripheral chromatin condensation (CC) and some mitochondria (M). Few short microvilli (MV) are seen at the brush border of the cell, X2000.

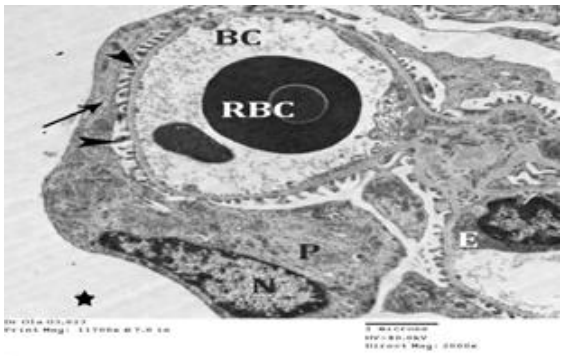

Figure 3: EM of the control rat renal cortex showing: a glomerular blood capillary (BC) lined with endothelial cell (E) and contains red blood cell (RBC).The podocyte (P) appear with its nucleus (N) and long primary foot process (arrow). There are multiple small secondary foot processes (arrow heads), X2000.

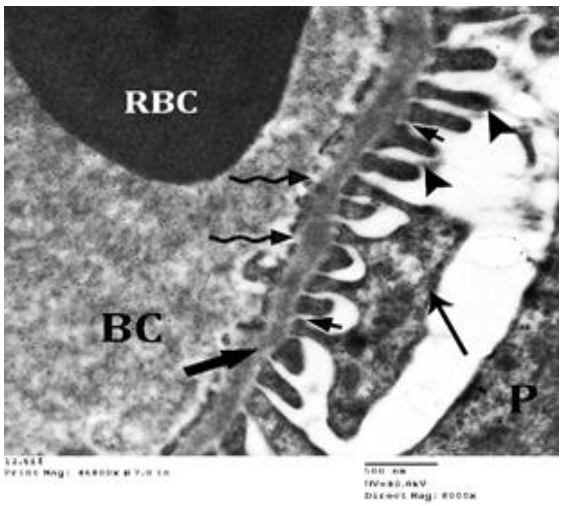

Figure 4: EM of a filtration barrier in a control rat renal cortex showing: the fenestration between endothelium of the glomerular capillaries (zigzag arrows), the fused basal lamina of the endothelial cells (thick arrow) and podocyte secondary foot processes (arrow heads) with fenestrations (short arrows) between them. Notice primary foot process (long arrow) of the podocyte (P), X8000.

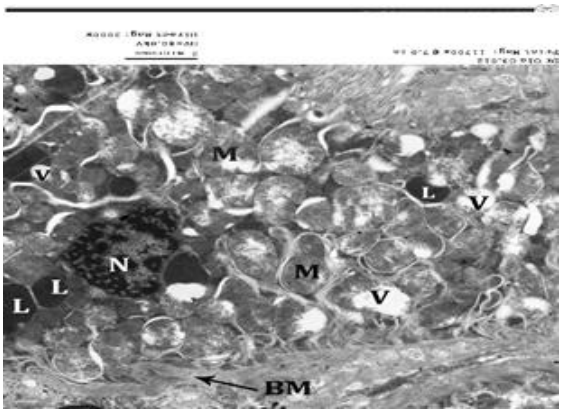

Figure 5: EM of the diabetic rat renal cortex of showing: A proximal tubular cell with a dark irregular heterochromatic nucleus (N), numerous swollen vaculated mitochondria (M), vacuoles (V) and electron dense lysosomes (L) in the cytoplasm, X2000.

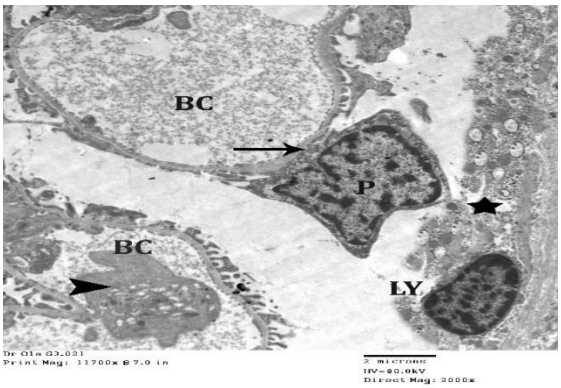

Figure 6: EM of the renal cortex of a diabetic adult male albino rat showing glomerular blood capillaries (BC), one of them containing homogenous material (arrow head). Podocyte (P) with irregular nucleus and fused foot processes (arrow) are seen. The Bowman's space (star) contains cellular debris and inflammatory cell (LY), X2000.

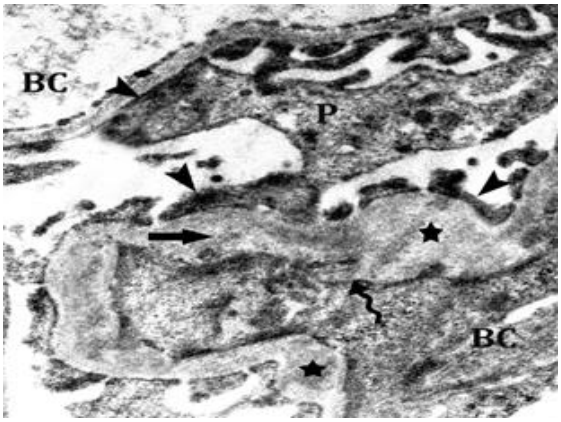

Figure 7: EM of a filtration barrier in a diabetic rat renal cortex showing: the non fenestrated endothelium (zigzag arrow) of the glomerular capillaries (BC), the thick fused basal lamina of the endothelial cells (thick arrow) and fused podocytes secondary foot processes (arrow heads), areas of nodular thickening (stars) of the glomerular basement membrane, X8000.

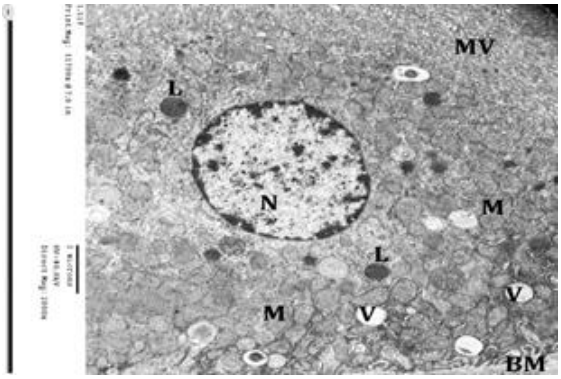

Figure 8: EM of the possible protected rat renal cortex showing: proximal tubular cell with rounded euchromatic nucleus (N), closely packed luminal microvilli (MV), numerous normal mitochondria (M) and some lysosomes (L). Few cytoplasmic vacuoles (V) are also seen, X2000.

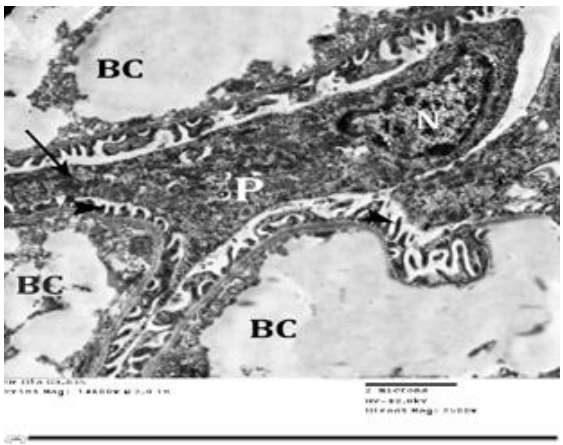

Figure 9: EM of the possible protected rat renal cortex showing: the glomerular blood capillaries (BC) surrounded by podocyte (P). The long primary (long arrow), short primary processes (arrow heads) and its nucleus (N) are also seen, X2500.

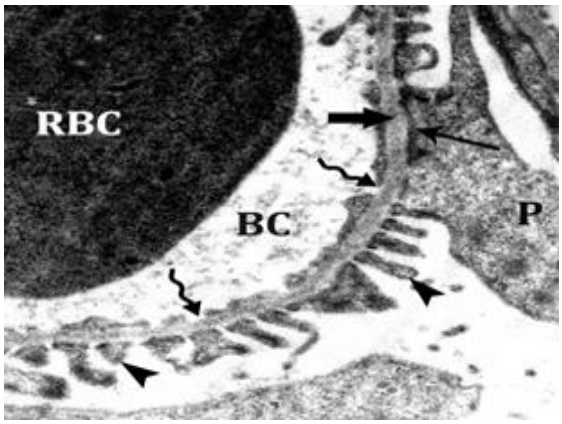

Figure 10: EM of the possible protected rat renal cortex showing: the glomerular blood capillaries (BC) containing red blood cells (RBC).A filtration barrier showing the fenestrations between endothelium of the glomerular capillaries (zigzag arrows), the fused basal lamina of the endothelial cells (thick arrow) and podocyte secondary foot processes (arrow heads). Notice part of fused foot processes (long arrow) of the podocyte (P), X8000.

Table 1: Diameter of glomeruli (µm) in the groups of study.

Parameter	Negative control	positive control	Diabetic	Possible	F	P
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD		
diameter of glomerulus (µm)	119.96 ± 20.3	119.5 ± 19.6	62.9 ± 19.1	114.1 ± 9.5	26.8	0.000 **

One way ANOVA test, there is highly significant difference between groups

DISCUSSION

Low doses of streptozotocin (STZ) resulted in a mild impairment of insulin secretion due to an oxidative stress produced in the pancreas, and a single strand break in pancreatic islets DNA which is similar to the feature of the stage of type 2 diabetes.26 In the current study, a single dose of STZ was used for induction of type 2 diabetes. There was a significant rise of fasting blood sugar in the diabetic group (group 3) as compared to the control groups. This was in agreement with Kumar et al., who reported hyperglycemia in type 2diabetes.[27] In this study, diabetic rats of group 3 showed several alterations in the basic kidney architecture. Some renal tubules were dilated while others were completely damaged and others contained vacuolated cytoplasm. This variability in tubular affection can be explained by the patchy way of affection in type 2 diabetic nephropathy as reported by Ackermann et al.[28] This was explained by Brownlee who proposed that the tubular cell susceptibility to glucose- induced toxicity is determined by its expression of glucose uptake mechanisms and by the ability of these cells to down regulate glucose uptake in the setting of hyperglycemia.[29]

This was in agreement with the electron microscopic results of this work, concerning the multiple vacuolization of the tubular cells referring to the increased pinocytotic activity as a trial of the cell to overcome hyperglycemia by increasing reabsorption. The electron dense bodies, which are supposed to be lysosomes, are considered an expected finding by which the cells can get rid of the excess reabsorped metabolites. These findings were in accordance with Yabuki who had the opinion that the electron dense bodies contained acid phosphatase-positive matrix confirming an association between them and lysosomes.[30]

Reactive oxygen species (ROS) are molecules with one or more unpaired electrons. A moderate amount of these radicals are formed endogenously as a result of physiological metabolic reactions.[31] ROS production develops a series of enzymes able to disarm them; superoxide dismutase, catalase, and

glutathione peroxidase enzymes.[32] Also referred the diabetic tubular complications to oxidative stress induced by excessively produced reactive oxygen species (ROS).

In the current study, ultra structurally, apoptotic cells appeared with small dark blebbed nuclei and swollen mitochondria. These findings can be explained by Seven et al., who clarified that the high glucose level in diabetes induced oxidative stress that plays an important role in tubular damage as losing control of ROS is very harmful and all the constituents of the cell can be targets for these molecules.[33]

PAS stained sections of the diabetic animals (group 3) revealed absent PAS reaction at the brush borders of the tubular cells indicating disturbed carbohydrate chemical composition. Also there is strong reaction of thick tubular basement membrane and thick glomerular basement membrane. This is in agreement with other authors who detected thickening of the glomerular basement membrane in diabetes and stated that diffuse thickening of the glomerular basement membrane depends on the severity of the disease.[34,35]

While Mallory trichrome´s stained sections of diabetic animals (group 3) revealed increased collagen fibers inside the glomeruli and in the interstitum around the renal tubules. This was in accordance with the results of anthers, who found in their research also that collagen fibers were clustered in the areas between the tubules of diabetic rats.[18]

Other alterations in the basic kidney architecture appeared. Most of the renal glomeruli appeared shrunken with increased collagen fibers inside them. This could be explained in this study as deposited collagen fibers in the mesangium around the tubules and around the glomerular capillaries, interfered with their function causing their shrinkage (sclerosis). This was in agreement with Venkatesh et al., who added that the deposited collagen fibers within the renal corpuscles (glomeruli) were derived from plasma through exudation or to a little extent from local production by mesangial cells.[36] The majority of past studies on DN have concentrated on the glomerular lesions.[37]

In the current study, examination of ultra-thin stained sections from diabetic group (group 3) showed hemorrhage in the interstitium. This was in agreement with other researchers who reported that the combined hyperlipidemia and hypertensive atherosclerosis forced exudation from the blood vessels through the glomerular basement membrane (GBM) leading to atherosclerosis in diabetic renal vasculature. They also depended on the immunofluorescence microscopy to reveal deposition of albumin, immunoglobulins, fibrin, and other plasma proteins along the glomerular basement membrane (GBM) in a linear pattern.[38] Examination of the interstitium in diabetic group also revealed cellular infiltration by inflammatory cells. Regarding inflammatory cells, the presence of leukocytic infiltration in diabetic renal tissue was previously reported.[39,40] Added that these immune cells may participate in the vascular injury encountered in diabetic nephropathy and could be considered as a crucial step in disease progression.

In the current work, in the diabetic group (group3) the podocytes appeared with irregular nuclei and fused foot processes resting on a diffusely thickened GBM. There were areas of nodular thickenings of their basement membranes. These findings could be explained previously; the podocytes are sensitive to injury which is characterized by foot process fusion and disappearance, a reduction in size and negative charge, and finally, detachment from the GBM and excretion in the urine.[41] And also by disruption of podocytes as actin cytoskeleton connection to apical integral membrane molecules.[42]

The arrested progression of type 2 diabetic nephropathy observed in possible protected group (group 4) could be attributed to use of vitamins C and E. Examination of the possible protected animals of group 4 insured this point of view, as it revealed that vitamin C&E extract significantly succeeded to keep the serum urea and creatinine levels within normal level. Additionally, vitamin C&E extract showed a significant anti hyperglycemic activity.

This was in agreement with the reports of positive relation that demonstrated between high plasma vitamin C level and reduction in complications of diabetes.[43] Vitamin C plays a central role in the antioxidant protective system, protecting all lipids undergoing oxidation and diminishing the number of apoptotic cells.[44-46] Furthermore, vitamin C regenerates the oxidized vitamin E.[14] These biochemical results were supported by the histological examination of the same group as it showed normally apparent glomeruli. Few collagen fibers were found among the glomerular capillaries. Ultra structurally, the proximal tubular cells appeared with closely packed microvilli, large pale nuclei, and numerous mitochondria. The Podocytes with their euchromatic nuclei and foot processes were seen resting on a thin glomerular basement membrane with fenestrated endothelium of glomerular capillaries.

CONCLUSION

The present study demonstrated that type 2 diabetes resulted in different histological changes in the renal cortex in the form of glomerulosclerosis and tubule interstitial fibrosis; it may put diabetics at risk for an end stage renal failure. On the other hand, changes in the renal structure were attenuated

with prophylactic VCE which helped alleviation of the renal degeneration by protecting the glomerular structures from oxidative injury. So concomitant administration of Vitamins C and E would be more effective in preventing the complications of diabetes.

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