Original Research Article

Effect of spironolactone on diabetic nephropathy in albino rats: ultrastructural and immunohistochemical study

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ABSTRACT

BACKGROUND: Diabetic nephropathy (DN) has become one of the most common causes of end stage renal disease (ESRD). Hyperglycemia induces oxidative stress in renal tubular epithelial cells that initiate tubulointerstitial fibrosis, which is a characteristic feature of diabetic nephropathy that becomes progressively complicated by renal failure. Aim: To assess the effect of spironolactone (SPL) on WT-1 protein expression and ultrastructural changes associated with the progression of experimental diabetic nephropathy (DN).

METHODS: Forty female albino rats were divided into five groups. Group I (control group), Group II (untreated diabetic rats), Group III (insulin-treated diabetic rats), Group IV (spironolactone-treated diabetic rats) and Group V (insulin and spironolactone-treated diabetic rats). At the 4th and 8th weeks, 4 rats from each group were sacrificed and renal tissue and blood samples were obtained. The rats were anaesthetized using ether inhalation. Each kidney was longitudinally divided and processed for immunohistochemical analysis with rabbit polyclonal anti-WT-1 Antibody and electron microscopic examination.

RESULTS: Treatment of STZ-induced diabetic rats with insulin and spironolactone (Group V) showed improvement in renal corpuscles as well as their capsular space, the basement membrane became normal with preserved minor and major processes and subpodocytic space, most of the proximal convoluted tubules retained their brush border, and their cells showed normal euchromatic nuclei and scattered mitochondria with apical microvilli, which is similar to the findings of the control group. Quantitative analyses showed significant increase in area of fibrosis and focal thickening of the glomerular basement membrane in non-SPL treated groups. There was a marked decrease in proteinuria compared to other treated groups. The results were better after 8 weeks compared to those after 4 weeks.

CONCLUSIONS: The administration of SPL significantly prevented the extent of interstitial fibrosis in the diabetic kidney.

Keywords: Spironolactone, WT-1 protein, Podocytes, Glomerulofibrosis, Diabetic nephropathy

INTRODUCTION

Diabetic nephropathy (DN) has become one of the most common causes of end stage renal disease (ESRD).¹ Hyperglycemia induces oxidative stress in renal tubular epithelial cells that initiate tubulointerstitial fibrosis, which is a characteristic feature of diabetic nephropathy that becomes progressively complicated by renal failure.² Numerous studies have shown that the loss of podocytes...
with mesangial expansion and tubulointerstitial fibrosis are the earliest manifestations of glomerular structural damage and are the main determinants of the clinical course of DN. Evidence has suggested a key role of podocytes in development of proteinuria and progression of glomerular dysfunction. Unlike other glomerular cells, podocytes have a limited ability to replicate, thus, their loss inevitably leads to the development of glomerulosclerosis.

Morphometric studies in patients with diabetes of short duration show progressive depletion of podocytes. Podocytes are lost in the stage of glomerulonephritis. These findings suggest that the loss of podocytes precedes diabetic glomerulosclerosis and ultimately the deterioration of renal function.

Some authors have shown that aldosterone infusion in rats causes loss of podocytes and mineralocorticoid receptor blockade, i.e. spironolactone prevents this phenomenon. However, the effect of aldosterone blockade on podocyte loss at an early stage of DN remains uncertain.

The Wilms’ tumor 1 gene (WT-1 protein) is normally expressed in the developing genitourinary tract, heart, spleen and adrenal glands. WT-1 staining is nuclear, not cytoplasmic. WT-1 protein used as a podocyte marker to evaluate podocyte loss.

The renin-angiotensin-aldosterone system (RAAS) and its main effector, angiotensin II (Ang. II), have a central role in the structural remodeling of the diabetic kidney progressing to glomerulosclerosis. For a long time, it was believed that inhibition of RAAS with angiotensin- converting enzyme (ACE) inhibitors block the production of aldosterone. However, it has been described that blocking the RAAS by ACE inhibitors does not necessarily produce a sustained aldosterone level decrease, but aldosterone increases progressively over time (known as ‘aldosterone escape’). 

Similarly, several clinical studies have shown that spironolactone (SPL), an aldosterone blocker, improves proteinuria in patients with DN even in those where treatment with ACE inhibitors have not been effective.

Matrix metalloproteinase (MMPs) are zinc-dependent endopeptidases with a major role in remodeling of the extracellular matrix and can be activated by reactive oxygen species (ROS). These alterations were attenuated by spironolactone which has antioxidant effects and reduced MMPs activity.

The prevention and treatment of diabetic nephropathy in the early stages, and the slowing down of diabetic nephropathy progression are among the most importance topics for several ongoing research studies.

This study aimed to assess the effect of spironolactone (SPL) on the loss of podocytes by WT-1 protein expression and ultrastructural changes associated with the progression of experimental DN.

METHODS

Animal model

Forty albino rats aged 6 weeks, weighing between 140 and 200 gm from the Mansoura Faculty of Pharmacy were used in this study. The animals were housed in cages at room temperature (22–25°C) in a photoperiod of 14-h light/10-h dark/day, with specific pathogen-free conditions. Rats were maintained on an ad libitum standard laboratory balanced commercial diet, and water. All experiments were performed according to the ethical considerations, animal care protocols and laboratory guidelines of the Faculty of Medicine, Mansoura University, Egypt.

Induction of diabetes mellitus

The animal model of diabetes was created using streptozotocin (STZ) that was purchased from Sigma (St. Louis, MO, USA). STZ was injected intraperitoneally at a dose of 40 mg/kg body weight under light ether anesthesia after 12 hours of fasting. Diabetes mellitus was confirmed by measuring blood glucose (Accu_check Active blood glucose meter, Roche diagnostic, Germany) on day 3 after the first injection of STZ. Rats were confirmed to be diabetic when fasting blood glucose (FBG) was >13.9 mMol (250 mg/dl) for 2 consecutive days. STZ selectively destroys the pancreatic β-cells, which causes the inhibition of synthesis and release of insulin leading to the onset of diabetic mellitus.

Groups: The rats were randomly divided into 5 groups:

- Group I (control, non-diabetic group): Eight rats received a single intra-peritoneal (IP) injection of 0.9 % NaCl (normal saline) (pH: 7.4) and olive oil daily in equal volume as the experimental groups throughout the duration of the study (4 and 8 weeks).
- Group II (Untreated diabetic group): Eight diabetic animals did not receive any treatment throughout the duration of the study.
- Group III (Insulin-treated diabetic group): Eight diabetic animals received subcutaneous injection of 2 U/Kg/day of an insulin mixture (Egyptian Drug Trading Company) in 2 divided doses at 8 am and 8pm throughout the duration of the study (4 and 8 weeks). The dose was modified to maintain a blood glucose level of approximately 170-200 mg/dl.
- Group IV (Spironolactone-treated diabetic group): Eight diabetic rats (only proven diabetic cases) received SPL in doses of 50 mg/kg/day by intragastric administration throughout the duration of the study.
• Group V (SPL-and insulin-treated diabetic group): Eight diabetic rats (only proven diabetic cases) received SPL and insulin in similar doses to previous groups throughout the duration of the study.

Animal sacrifice and specimen collection

At the assigned times (4 and 8 weeks), 4 rats from each group provided blood samples. The rats were anaesthetized using ether inhalation. Each kidney was weighed and was longitudinally divided and processed for immunohistochemical and electron microscopic examination.

Techniques used

Histological Techniques

A. Histopathological evaluation of renal fibrosis

Masson's trichrome stain was used for the assessment and calculation of area and percentage area of fibrosis. Collagen and the other extracellular matrix components (ECM) were also evaluated in Masson's Trichrome stained slides. Areas of fibrosis appear as blue and parenchyma as red.

B. Immunohistochemistry (IHC)

Paraffin sections of formalin-fixed tissue were stained immunohistochemically with rabbit polyclonal Anti-rat WT-1 antibody (Thermo Scientific: PA516879). The antibodies were diluted in phosphate-buffered saline plus 0.1% non-fat dried milk and applied at 4°C for 12 hours. The primary antibody dilution was 1:100 for the WT-1 antibody. Tissue was incubated with biotinylated antimouse secondary antibody in a species-specific manner for rats (R and D system. MAB002). The label was peroxidase conjugated streptavidin. Color development was performed with diaminobenzidine peroxidase substrate (D-4293, Sigma Chemical Co.). Sections were counter stained for 2 minutes in hematoxylin.

C. Transmission electron microscopic study (TEM)

The specimens (1 mm² thickness) of renal cortex were fixed in a mixture of 2.5% gluteraldehyde and 2.5% paraformaldehyde, placed in phosphate buffer for 24 hours, post-fixed in 1% osmium tetroxide, dehydrated and embedded in resin. The specimens were trimmed, followed by sectioning into semi-thin and ultrathin sections. The ultrathin sections were transferred to copper grids for staining with lead citrate and uranyl acetate. The sections were examined by a transmission electron microscope at the electron microscope unit in Mansoura University using a Zeiss EM 100 S transmission electron microscope at 60 KV to detect the ultrastructure changes together with changes in the glomerular basement membrane thickness.

Biochemical assay

A. Blood glucose level

For better visualization of the lateral tail vein, the animal was warmed for 5-10 minutes, and the collection site was wiped with 70% ethanol prior to blood collection. Blood was gently milked from the tail. One droplet of blood was placed on a glucose test strip and read using a glucometer. Direct pressure was applied to achieve hemostasis.

B. Protein in 24-hours urine

A plastic metabolic cage with a wire mesh floor was placed above a fraction collector, the times of voiding were recorded, and each individual voiding was collected.

C. Microalbuminuria

The level of albumin protein produced by microalbuminuria was detected by special albumin-specific urine dipsticks. A microalbumin urine test determines the presence of the albumin in urine. If a properly functioning body, albumin is not normally present in urine because it is retained in the bloodstream by the kidneys. Microalbuminuria was diagnosed from a 24-hour urine collection (between 30–300 mg/24 hours) or, more commonly, from elevated concentrations in a spot sample (30 to 300 mg/L).

Computer assisted digital image analysis (digital morphometric study)

Slides were photographed using Tucsen 5.0 MB digital cameras (ISH 500) installed on an Olympus® microscope CX21. The resulting images were analyzed on an Intel® Core I3®-based computer using IS capture software. Five slides from each case were prepared, and five random fields from each slide were analyzed.

Statistical analysis

Data were tabulated, coded, and then analyzed using the SPSS computer program (Statistical Package for Social Science) version 17.0. Descriptive data were calculated in the form of means and standard deviations (±SD). The significance of differences between groups was tested using ANOVA (analysis of variance) or numerical (parametric) data. A p-value <0.05 was considered statistically significant.
RESULTS

Histopathological results

Histopathological examination of the kidney specimens of the healthy control group after Masson’s trichrome stain, showed the presence of a minimal amount of fibrous tissue (Figure 1A). On the other hand, the renal cortex of Groups II, III and IV revealed an obvious increase in the amount of collagen deposition around the glomeruli, in the interstitium and between the tubules (Figures 1B and 1C). Comparing with the previous results; insulin and spironolactone treated group (Group V) for 8 weeks showed little amount fibrous tissue around renal corpuscles with retraction of fibrosis (Figure 11).

Quantitative analyses showed significant increase in area of fibrosis by approximately 3.7 fold in Groups II, III and IV versus control group which were highly significant in Group II (8 weeks). Whereas, insulin and spironolactone treated group (Group V) for 8 weeks showed little significant difference in calculated area of fibrosis compared with control healthy group (n=5-8; p<0.05), (Figure 9).

Immunohistochemical results

Furthermore, immunohistochemical stain of the renal cortex of the control rats (Group I) showed strong positive WT-1 expression in the glomeruli (Figure 2A). In Group II, the renal showed moderate reaction in the density of podocytes expressing WT-1 in the glomeruli (Figure 2B). After two months, the renal cortex of the same group showed marked reduction in the density of podocytes expressing WT-1 in the glomeruli (Figure 2C).

In Groups III and IV, the renal cortex showed mild reduction (Figure 2F and 2D) and moderate reduction (Figure 2G and 2E) in the density of podocytes expressing WT-1 in the glomeruli after one and two months respectively.

After one and two months in Group V, the renal cortex showed, strong positive WT-1 expression in the glomeruli and preserved density of podocytes (Figures 2H, I).

Statistical analysis related to the thickness of the basement membrane of all groups showed significant increase in the thickness of the membrane in the animals of Group (II) after two months (Figure 10).

In Group II, the podocytes number significantly decreased. In Groups III and IV, the podocytes number significantly decreased compared to that of Group I and V, but was significantly increased compared to Group II. In Group V, the podocyte number significantly increased compared to that of all the experimental groups, but was significantly decreased compared to that of Group I. (Figure 5).

Electron microscopic results

The transmission electron microscopic (TEM) study of the control group showed a normal renal cortex in terms of...
of glomerular capillaries, podocytes and their processes in the subpodocytic space (Figure 3A) and cells of the proximal convoluted tubule (Figure 4A).

In Group II, the renal cortex, showed marked thickened glomerular basement membrane surrounded by podocytes with irregular nuclear membrane, disrupted minor processes and wide subpodocytic space and a cytoplasm with many vacuoles (Figures 4B, 3B and 3C). The proximal convoluted tubule showed an elongated nucleus and disarranged basal mitochondria (Figure 4C).

Group III, the renal cortex showed focal thickening of the glomerular basement membrane and widening of the subpodocytic space and podocytes with irregular nuclear membranes after one and two months (Figure 4D and 4E). The proximal convoluted tubule cells showed vacuoles in the cytoplasm, elongated nuclei, lysosomes and disarranged basal mitochondria and basal mitochondria and disrupted microvilli after two months (Figures 3D and 3E).
1000). I: (Group V) STZ injected rats received insulin and spironolactone for 2 month showing cells of PCT with euchromatic nuclei (N), scattered mitochondria (M), apical microvilli (MV), lysosomes (L) and nearly normal basement membrane (arrow) (X 1000). (Uranyl acetate and Lead citrate X).

In Group IV, the experimental rats showed nearly normal renal cortex (Figure 3F and 3G), whereas the cells of the proximal convoluted tubule showed cytoplasmic vacuolations, thickened basement membrane and elongated nuclei especially in second month (Figure 4F and 4G).

Figure 5: Changes in WT-1 protein expression in the podocytes of the adult rats of the control and experimental groups.

Fig. 6: Changes in the blood glucose level of the adult rats of the control and experimental groups

Figure 7: Changes in the proteinuria of the adult rats of the control and experimental groups.

In Group V, the one month experimental rats had cell lining of the proximal convoluted tubules showed some cytoplasmic vacuolations and scattered mitochondria (Figure 4H), but had a nearly normal glomerular basement membrane thickness and preserved minor and

Figure 8: Changes in the microalbuminuria of the adult rats of the control and experimental groups.

Figure 9: Area of fibrosis of the adult rats of the control and experimental groups (Masson’s trichrome stain).

Figure 10: Changes in the thickness of basement membrane of the adult rats of the control and experimental groups.
major processes and subpodocytic space (Figure 3H). In two month experimental rats, the cells lining the proximal convoluted tubule and some podocytes appeared normal. (Figure 4I and 3I)

**Biochemical assay results**

**A. Blood glucose level**

The blood glucose in Group II was significantly increased. In Group III, blood glucose significantly increased compared to that of Group I and V, but was significantly decreased compared to that of Group II and IV. In Group IV, blood glucose significantly increased compared to that of Group I, III, and V but was significantly decreased compared to Group II. In Group V, blood glucose was significantly decreased compared to that of all the experimental groups (Figure 6).

**B. Protein in 24-hour urine**

In Group II, there was proteinuria significantly increased. The proteinuria in Group III was increased significantly compared to that of Group I and V, but was significantly lower than that of Group II in the 2nd month. However, there was an insignificant difference compared to that of Group IV. Group IV had proteinuria increased significantly compared to that of the control group and Group V, and decreased significantly in relation to that of Group II in the 2nd month. In Group V, proteinuria was significantly decreased compared to those of other treated groups in the 2nd month. In comparison to Group I, there was an insignificant increase in both months. (Figure 7)

**C. Microalbuminuria**

In Group II, microalbuminuria was significantly increased in the second month. The microalbuminuria in Group III showed a significant increase compared to that of Group I, but was significantly lower than that of group II and III. In Group IV, there was microalbuminuria that increased significantly in second month compared to that of Group I and III, but was significantly lower than that of Group II. In Group V, there was microalbuminuria that was decreased significantly compared to all the experimental groups. (Figure 8)

**DISCUSSION**

Hyperglycemia induces oxidative stress in renal tubular epithelial cells and initiates tubulointerstitial fibrosis, a characteristic feature of diabetic nephropathy, which then progressively results in renal failure.2

Hyperglycemia, independent of aldosterone levels, induces podocyte injury through Mineralocorticoid (MR)-mediated ROS production. MR blockade inhibits hyperglycemia-induced podocyte injury, focusing on the involvement of ROS production, in diabetic rats and cultured podocytes.21

Statistical results of this study showed significant increase in area of fibrosis in Groups II, III and IV which were highly significant in Group II (8 weeks). These results were confirmed by a review article that discussed glomerulosclerosis in diabetic nephropathy. The most common matrix proteins detected in diabetic glomerulosclerosis were collagen types I, III, and IV and fibronectin. These accumulate due to increased synthesis by mesangial cells and reduced degradation by mesangial matrix metalloproteinases. The mesangial cell, podocytes and endothelial cells appear to play important roles in the evolution of diabetic glomerulosclerosis.22 However, more recent reports have indicated that isolated podocyte damage and loss leads to glomerulosclerosis and that podocyte loss appears to be an early event in diabetic nephropathy.23,24

A recent study indicates that, the podocytes of wild-type mice, insulin activation of the insulin receptor results in physiologic remodeling of the actin cytoskeleton and preservation of cell function and survival. In podocytes of mice lacking the insulin receptor, insulin signaling is abolished and results in the obliteration of foot processes, thickening of the glomerular basement membrane, and cell malfunction or death, leading to proteinuria.25 Aldosterone acts as a renal injury mediator through inflammation induction, fibrosis and necrosis in the kidney tissue. Aldosterone induced Type IV collagen formation which is a component of glomerular basement membrane and mesangial matrix. SPL reduces the progression of renal fibrosis.26 Increased intrarenal aldosterone is associated with a marked increase in the synthesis of TGF-β1 mRNA and selective blocking of aldosterone reduced collagen synthesis in kidneys. Fujisawa et al were the first to report the ability of SPL to attenuate renal fibrosis by inhibiting the expression of TGF-β1 mRNA in the diabetic rats.27

Several transcription factors have been described to be associated with podocyte differentiation, including WT-1, POD1, maf-1, and Lamx1.28 The glomeruli were subjected to immunohistochemistry for WT-1 expression and showed decreases in the glomeruli WT-1 expression of rats in the STZ group for one month (Group II) compared to the control rats. This result was in agreement with some authors who stated that the reduction in podocyte number and density per glomerulus has been linked to the development of proteinuria and the progression of diabetic nephropathy (DN).3 More importantly, in the developing glomerulus, WT-1 expression is restricted in the differentiating and mature podocyte from the capillary loop onward, before nestin is expressed.29 It is possible that WT-1 may be involved in transcriptional regulation of nestin expression in the podocytes. This result was in agreement with other results that stated various intracellular signaling pathways implicated in the pathogenic pathway of DN, including oxidative stress, NO production, angiogenesis, nestin and α-SMA expression and ECM accumulation.29

An electron microscopic examination of the renal cortex of the same group revealed thickened glomerular basement membranes surrounded by podocytes with disrupted minor processes, whereas the cell lining of the proximal convoluted tubules showed disturbed microvilli, vacuolations in the cytoplasm and degenerated mitochondria with shaded apical cells and large casts in their lumen. These findings were consistent with that of other scientists who explained that the increase in glomerular basement membrane thickness in DN is due to increase in collagen type IV deposition and impairment of excess extracellular matrix degradation. Additionally, injury of podocytes and degeneration of their minor processes may be due to oxidative stress, which increases intracellular ROS that mediates apoptosis of podocytes. They also reported that cells of the PCT die and slough into the tubular lumen and contribute to cast formation and vacuolations in the cytoplasm of the cell lining.30

In the 2nd month in Group II, the renal cortex showed more immunohistochemistry and ultrastructural pathological changes than those after the first month of induction of DM. Others indicated that there is a long time effect of diabetes on the kidneys of the rats, as diabetic nephropathy develops in rats 3 weeks after induction of diabetes.31 Treatment of STZ-induced diabetic rats with insulin only (Group III) showed less deteriorated immunohistochemistry and ultrastructural changes than that of the diabetic group (Group II) and the STZ-induced diabetic group that received spironolactone (Group IV), but more deterioration than the STZ-induced diabetic group that received insulin and SPL (Group V) as some glomeruli show improvement and some proximal convoluted tubules showed preserved brush border with few vacuolations and lysosomes in the cytoplasm. There was a decrease in proteinuria. There was improvement in the 1st month than in the 2nd month. These results were in consistent with those who stated that, continuous subcutaneous insulin therapy improves but does not normalize the hyperglycemia, as insulin administration delays progression of diabetic nephropathy, arrests proteinuria and glomerular basement membrane thickening in diabetic rats with reducing tubular epithelial cells apoptosis.37

Group IV, in the 1st month, demonstrated that the renal cortex of experimental diabetic rats showed apoptosis of glomerular cells and necrosis of proximal tubular cells, but these changes were less noticed than in those of STZ-induced diabetic rats. Compared to the control rats and the STZ group, the analysis of cells with WT-1 positive glomeruli showed no reduction in the SPL group. These results were in agreement with other results that demonstrated the SPL is capable of preventing the loss of expression of WT-1 in podocyte induced by diabetes by reducing oxidative stress. However, these changes in the 2nd month were more deteriorated than those in the 1st month as there was no improvement in renal tissue after two months of administration of spironolactone because its role is preventive.32

Other authors have stated that administration of an aldosterone receptor blocker, SPL, might rescue podocyte adhesion injury by decreasing mechanical stretch, which was attributed to alteration of glomerular hemodynamics. This process may be one of the possible mechanisms of SPL’s protective effects on podocyte adhesion.33

CONCLUSION

In the present study, treatment of STZ-induced diabetic rats with insulin and spironolactone (Group V) showed improvement in renal corpuscles as its capsular space and basement membrane became near normal similar to control rats, whereas, most of the proximal convoluted tubules retained their brush border and their cells showed normal euchromatic nuclei and scattered mitochondria with apical microvilli, more or less similar to normal rats. There was a marked decrease in proteinuria compared to other treated groups. This improvement was clearer in the 2nd month than the 1st month. By the end of this study, we concluded that the administration of SPL significantly prevents interstitial fibrosis in the diabetic kidney.

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