

Experimental Rat Model of Diabetic Nephropathy: RAGE Detection and Effect of Spironolactone Monotherapy

Malak Nabil¹; Manar Raafat¹; Emam Waked¹; Afkar Badawy² and Mohamed Salah³
Nephrology¹; Pathology² and Veterinary³ Departments, Theodor Bilharz Research Institute, Imbaba, Giza.

ABSTRACT

Background: The glycation process results in formation of advanced glycation end products (AGEs), which accumulate in different organs at an accelerated rate in diabetes, resulting in alteration of both structure and function. This effect is via the receptor for AGEs (RAGE), which is a signaling receptor leading to profibrotic reactions. The renin angiotensin aldosterone system (RAAS) is activated in diabetic nephropathy (DN) and leads to more renal damage. This is inhibited by angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) and mineralocorticoid receptor blockers (MRBs). **Aim of the study:** to show the monotherapeutic effect of spironolactone in diabetic nephropathy and to detect RAGE. **Method:** Diabetes was induced in rats by streptozotocin. Three weeks after, spironolactone (SPL) was given for 4 weeks. Then, control, diabetic and treated rats were sacrificed. **Results:** The results of blood chemistry at the end of 4 weeks showed statistical increase in serum sodium, potassium and urea with no effect on serum creatinine or blood glucose. Kidney pathological injuries were attenuated by SPL also, RAGE deposition compared to the diabetics. The study showed RAGE deposition in the experimental DN and confirmed the beneficial effects of MRB in DN. **Keywords:** Diabetic nephropathy, receptor for AGEs (RAGE), the renin angiotensin aldosterone system (RAAS), spironolactone (SPL).

INTRODUCTION

Advanced glycation end products (AGEs) have been known as the major factor contributing to the pathogenesis of diabetic complications,¹ and the receptor for advanced glycation end products (RAGE) dependent mechanisms are likely to be responsible.²

RAGE is composed of a single hydrophobic transmembrane – spanning domain, a highly charged cytosolic tail and an extracellular region.³ During embryonic development, RAGE is highly expressed in a constitutive manner.⁴ In adults, it is expressed in a regulated manner meaning that expression can be induced in situations, where there is accumulation of ligands and inflammatory mediators.⁵

In fact, RAGE is found on numerous immune cells that perpetuate the immune response as neutrophils, T and B lymphocytes, monocytes, macrophages and dendritic cells.⁶ RAGE is the most studied receptor for AGEs, however, many were identified as macrophage scavenger receptor, CD36, LOX-1 and others.⁷ It is not a scavenger of AGEs, but a signaling receptor involved in many inflammatory diseases as atherosclerosis⁸,

Alzheimer's disease⁹, arthritis¹⁰ and sepsis¹¹, by its ability to engage many ligands as nuclear factor κ B (NF- κ B), MAP kinases and adhesion molecules.¹²

AGEs are formed due to hyperglycemia, through a reaction between sugars and the free amino group of proteins, lipids and nucleic acids¹³, to form irreversible crosslinks. The glycation process (Maillard reaction) is divided into 3 stages: the early reactions resulting in formation of a Schiff base and Amadori products, the rearrangements of these chemical groups and the final reaction forming the classical Millard browning products or the AGEs.¹⁴ They are formed over periods of months on long lived cellular proteins. CML (N Carboxy- methyl lysine), pentosidine and FFI (fluoryl- furanyl- imidazole) are examples of studied AGEs, which accumulate in the skin, lens and vascular tissue at an accelerated rate in diabetes.¹⁵

DN is characterized by the accumulation of extracellular matrix (ECM) proteins in the mesangium and tubulointerstitium. AGEs modification results in alteration of both structure and function.¹⁶ This is a direct effect via

RAGE involving activation of the JAK/ STAT signal transcription pathway¹⁷, leading to induction of profibrotic cytokines as transforming growth factor β (TGF β), platelet derived growth factor (PDGF) and connective tissue growth factor (CTGF).¹⁸ The action of the later can be prevented by aminoguanidine.¹⁹ Expression and activity of degradative matrix metalloproteinases is also reduced.²⁰

The disruption of the RAGE gene was found to ameliorate diabetic nephropathy. The extent of attenuation was proportional to the RAGE gene dosage.²¹ Administration of soluble RAGE (sRAGE) or neutralizing RAGE antibodies was reported to block the early events of diabetic kidney changes.²² RAGE should be a target for therapeutic intervention. However, as said before, RAGE plays a vital role in normal physiology and prolonged RAGE blockade may have undesirable effects.²¹

The renin angiotensin aldosterone system (RAAS) has a key role in regulation of salt and water balance, and cardiovascular and renal hemostasis in DN. Excess activation of the RAAS results in progressive renal damage: constriction of renal arterioles leading to increase peripheral and renal resistance and increase glomerulocapillary pressure leading to proteinuria and proliferation of mesangial cells and activation of proinflammatory and profibrotic pathways. This is via NADPH oxidase and mitogen-activated protein kinase C (MAP kinase) dependent pathways.²³ However, a link between oxidative stress and the production of AGEs is difficult to establish *invivo*²⁴ and failure of antioxidant therapy to prevent end organ injury in the diabetic cohort of the Heart Outcomes Prevention Evaluation (HOPE) study casted some doubt on the pathological significance of oxidative stress in progressive diabetic renal injury.²⁵

The previous cascade is inhibited by angiotensin converting enzyme inhibitor (ACEIs), angiotensin receptor blockers (ARBs) and mineralocorticoid receptor blockers (MRB).²⁶ Adding mineralocorticoid receptor antagonists (MRA) to ACEIs or ARBs results in reduction of albuminuria suggesting a potential long term renoprotective effect, in spite of the drawback of hyperkalemia, particularly in

patients with renal insufficiency which limited their use in DN.²⁷ There may be an initial acute fall in GFR that predicted a later prolonged beneficial effect on decline in renal failure.²⁸ Novel approaches to mitigate the risk of hyperkalemia in patients treated with MRA in combination with ACEIs or ARBs are currently in development.²⁹

The KDOQI guidelines recommend temporary discontinuation of the RAAS blockers in patients with GFR < 60ml / min / 1.73 m² who are seriously ill as by dehydration or diarrhea that increase the risk of acute kidney injury.³⁰ Low sodium diet in the experimental animals can lead to renal failure and hypotension and increased renin levels. This does not occur if animals fed with high sodium diet.³¹

The morbidity and mortality of patients with DN receiving RAAS blockade remains high due to systemic and local RAAS cascades, genetic diversity in the RAAS. Novel genomic, proteomic or metabolomics techniques might be valuable tools to identify specific biomarkers that help to identify those patients that are more likely to benefit from therapy.³²

Aim of the study:

Detection of the monotherapeutic effect of spironolactone in the treatment and pathophysiology of the diabetic nephropathy.

Methods:

All animal experiments were conducted in accordance to guidelines for animal research.³³

Our study consisted of 35 male Wistar rats, weighing about 250 gm, who were given free access of water and regular laboratory chow. They were categorized as follows: 5 rats served as controls, 5 were excluded because were not becoming diabetics, 5 diabetics kept without SPL treatment, 18 diabetics were given SPL and 2 died during the experiment. Diabetes was induced by single intraperitoneal injection of streptozotocin (STZ) dose of 60 mg/kg body weight (Sigma St, Louis, MO, USA) in citrate buffer.³⁴ They were kept in the laboratory for 3 weeks during which blood glucose was measured from the tail vein using a portable glucose analyzer.

The level of serum glucose considered normal in rats from 50-135 mg%. Above these figures rats can be considered diabetics.³⁵ Proteinuria was measured by Comber

3 test strips (Pasteur Laboratory tests) and quantified as follows: + (< 30 mg%); ++ (30-300 mg%) and +++ (> 300 mg%). 3 weeks after becoming diabetics and after being sure of the presence of proteinuria, 18 rats were given spironolactone (SPL) 50mgm/kg/day by oral gavage for 4 weeks. The dose was selected and adopted from a previous study.³⁶ At the end of this period, blood samples of all groups were collected for blood chemistry estimation and kidneys removed for the pathological examination.

Histology methods :

Histology

Kidneys of the control, diabetic and treated rats were dissected out and fixed in 10 % buffered formalin. Tissues were processed to get paraffin blocks from which tissue sections of 4 micro meter thickness were sectioned and mounted on glass slides coated with 3-aminopropyltriethoxy silane (Sigma). Sections were stained with hematoxylin and eosin stain according to the method of **Bacncroft and Gamble**⁵² and Masson's trichrome stain according to the method of Carson⁵³ for the histopathological evaluation and collagen fibers.

Immunohistochemistry

Paraffin sections pretreated with PT link for antigen retrieval then immunohistochemically stained using Automatic Immunostainer (Dako) by mouse monoclonal antibody against RAGE (A11) (Santa Cruz Biotechnology, Inc), with DAB as chromogen and hematoxylin as counter stain.

Statistical analysis :

Results are expressed as mean \pm standard deviation (SD) or number (%). Comparison between mean values of the different variables was measured pre and post-treatment within the same group was performed using paired T-test. SPSS computer program (version 16 windows) was used for data analysis. *p* value less than or equal to 0.05 was considered significant and < 0.01 was considered highly significant.

RESULTS

Rats that have received STZ became diabetics with a frequency of ~ 85%. Those who were not diabetic were excluded from the study (5 rats). Diabetes was associated with reduced

body weight when compared to the beginning of the experiment.

Histopathological results :

Diabetic animals revealed variable grades of diabetic glomerulosclerosis features, some glomeruli were expanded and obliterated by glomerular tuft with increased mesangial matrix and thickening of glomerular basement membrane. Also, increased collagen fibres were observed. Diabetic glomerular changes were less evident in the treated animals (Fig.1).

Immunohistochemical results:

Control animals showed expression of RAGE in vascular endothelial lining and in some tubules specially those adjacent to glomeruli. In the diabetic animals number of blood vessels and tubules expressing RAGE increased and some glomeruli showed delicate linear RAGE expression in their capillary tuft. RAGE expression still seen in the treated animals in blood vessels and tubules but, with lesser extent in comparison with its expression in the diabetic animals and randomly seen in the glomeruli (Fig.2).

DISCUSSION

The present study investigated the nephroprotective effect of the mineralocorticoid receptor blocker, spironolactone, on experimental DN in rats. 85% of STZ treated rats developed hyperglycemia. These results are in agreement with those of **Volker et al.** and **Ji et al.** who concluded that about 80% of STZ treated rats developed stable hyperglycemia.^{37,38}

Rats lost weight when became diabetics as compared to controls despite normal or increased appetite. The reduction was less after SPL treatment. These is in agreement with **Mooradian**.³⁹ Also **Banki et al** found that diabetic rats had nearly 25% lower body weight than controls and this was prevented by SPL. In the same study, SPL reduced to some extent the blood glucose level of diabetic animals, as in this study. this could be explained by the inhibition of aldosterone induced insulin resistance.⁵⁴

Serum creatinine remained normal. However **Stefan et al.** observed increased serum creatinine in the diabetic rats 3 months after induction of diabetes, but our study was relatively of shorter duration⁴⁰. Normal serum

creatinine concentration can be obtained even when the GFR has dropped considerably, so it is an insensitive indicator of early renal impairment.⁴¹

There was a significant increase in BUN post-treatment, this may be because of dehydration of the rats, due to the diabetic state. Blood urea nitrogen is not used as an indicator of progression of renal function, as it is influenced by protein metabolism and state of dehydration.⁴²

We did find elevated K levels in the SPL treated group. Many trials found that the incidence of significant hyperkalemia was relatively low.⁴³ However, in the study of **Monemi et al**⁵⁵, serum K increased significantly in diabetic patients receiving SPL alone.

Aldosterone system blockade decreased proteinuria and even podocyte injury⁴⁴, under hyperglycemic conditions. Many trials used aldosterone receptor blockers in proteinuric diabetic patients.⁴⁵ The range of proteinuria reduction was 19-58%. Aldosterone antagonists were more effective when added to angiotensin converting enzyme inhibitors (ACEIs) more than angiotensin receptor blockers (ARBs). However, there was a small risk of acute GFR drop and hyperkalemia.⁴⁶

Hyperaldosteronism has been noted as a component of clinical chronic renal insufficiency of various etiologies including DN.⁴⁷ **Hene et al.** observed that the level of serum aldosterone increased several folds as creatinine clearance fell below 50% of normal⁴⁸.

The earliest morphologic abnormalities in DN are a thickening of the glomerular basement membrane, increased collagen fibres, expansion of the mesangium and accumulation of the extracellular matrix. With time, matrix accumulation becomes diffuse and evident as glomerulosclerosis on renal biopsy.⁴⁹ This was seen in our study and RAGE deposition was marked in the diabetics. The role of AGE deposition in the production of diabetic complications is well known,¹ and diabetic RAGE-null mutant mice did not display kidney features of DN.⁵⁰

After SPL, diabetic glomerular changes became less evident. The same applied to immunohistochemistry, RAGE expression was

attenuated in comparison to diabetics. This may improve the kidney function. **Banki et al., 2012** also found that aldosterone antagonists ameliorated all metabolic and renal parameters of STZ induced diabetic rats, prevented weight loss and attenuated the structural lesions of DN⁵¹.

In the diabetic rat model, spironolactone treatment was associated with decreased urinary albumin excretion and decreased glomerulosclerosis despite no change in blood pressure. It reduced intrinsic renal ACE and aldosterone synthase gene expression and decreased markers of inflammation in urine and renal tissue of the diabetic rats.³⁶ In spite of lack of quantitative proteinuria estimation, our results showed overall decrease in proteinuria.

Finally AGEs and RAGE play an active role in the development and progression of DN. Prophylactic and therapeutic strategies focusing on RAGE and its ligand axis will be of importance in conquering diabetic kidney injuries. RAAS blockade using ACEIs and ARBs is the corner stone of treatment of diabetic renal disease. Alternative strategies include renin inhibition and aldosterone blockade. Single agent interventions do not fully block the RAAS and dual blockade might be of greater benefit, but more clinical trials are needed to confirm these suggestions⁴⁶, and therapies that target multiple pathways might be indeed more successful than those that target one alone.

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Table 1: Comparison between mean values of blood chemistry in the control, diabetic and treated groups.

Variables	Control	Diabetic	Diabetic treated	p-value
Weight (gm)	250.8±22.170	216.7±10.4 ^a	229.3±12.8	<0.01
K (mq/L)	3.8±0.05	4.1±0.3 ^b	4.7±0.07 ^b	<0.01
Na (mq/L)	137±1.99	137.8±2.8	140.1±4.7	NS
BUN (mg/dl)	17.1±1.56	19.2±3.8	32.4±7.6 ^b	<0.01
S.Creatinine (mg/dL)	0.6±0.56	0.70±0.2	0.6±0.2	NS
S.Glucose (mg/dl)	80±5.27	189.3±42.40 ^b	160.9±53.4 ^b	<0.01
% change (weight)		-15.71%	-9.37%	

Data are expressed as mean ± SD.

p> 0.05= not significant; **p< 0.01= highly significant

K and blood urea nitrogen showed a statistically significant increase post treatment. Serum creatinine and sodium showed a small insignificant change. Serum creatinine was normal in both groups. Blood glucose significantly decreased with SPL treatment. Diabetes was associated with significant body weight decrease (-15.7%) when compared to the beginning of the experiment. This reduction fell to (-9.3%) after SPL treatment.

Table 2: Proteinuria in the diabetic, and spironolactone - treated groups.

	Diabetic rats	Diabetic rats treated with spironolactone
+	7 (38.8%)	11 (61.1%)
++	8 (44.4%)	7 (38.9%)
+++	3 (16.6%)	0 (0%)

Data are expressed as number (%).

As regards proteinuria, there was an overall decrease.

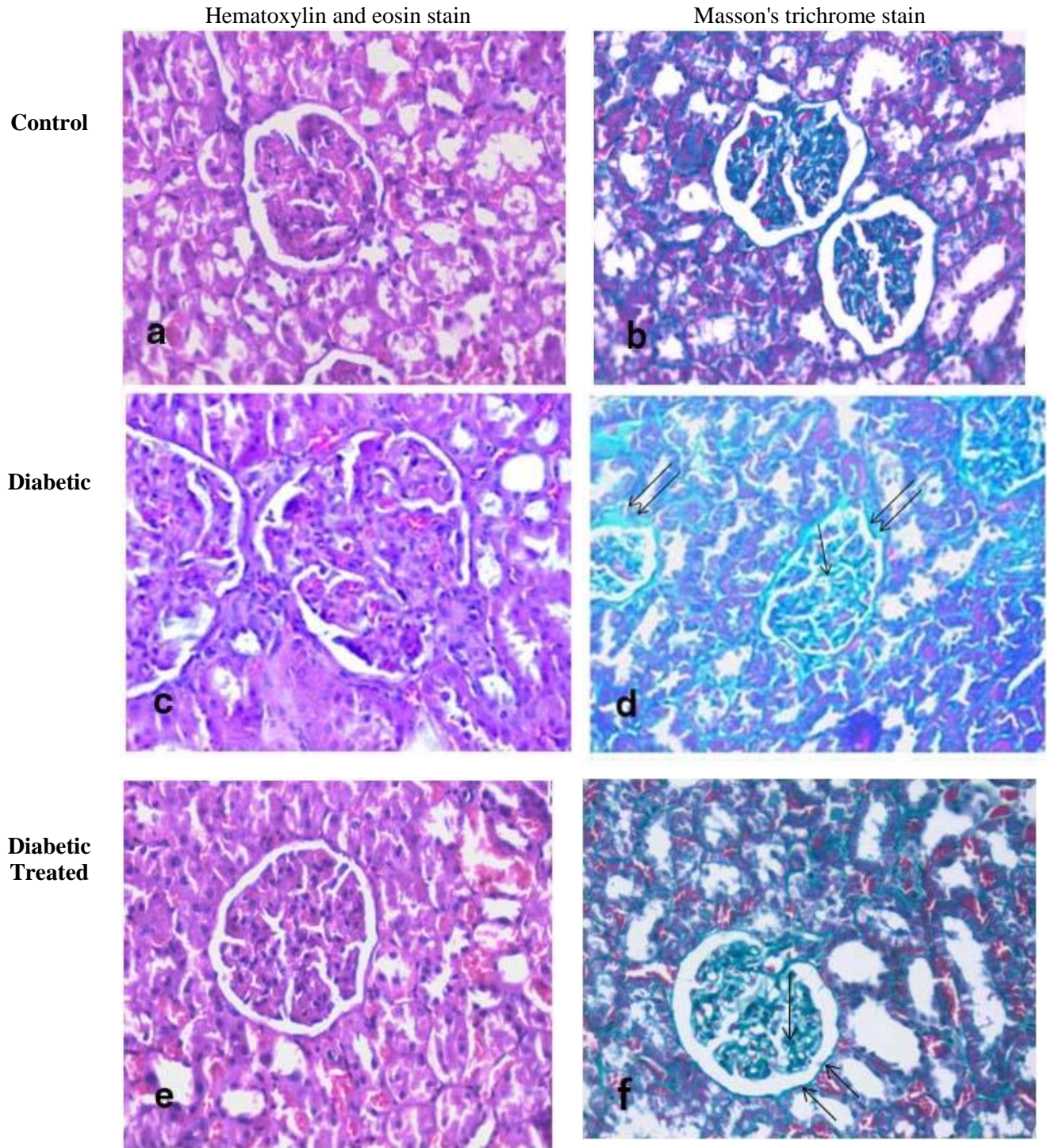


Fig. 1: Control animal (a,b). Diabetic animal: 2 glomeruli with marked expansion of the capillary tuft (c), increased mesangial matrix (arrow), and thickened glomerular basement membrane (double arrows) with highly increased collagen fibres (d). Treated animal: moderate expansion of capillary tuft (e), with increased mesangial matrix (arrow), and thickened glomerular basement membrane (double arrows) (f), (X400).

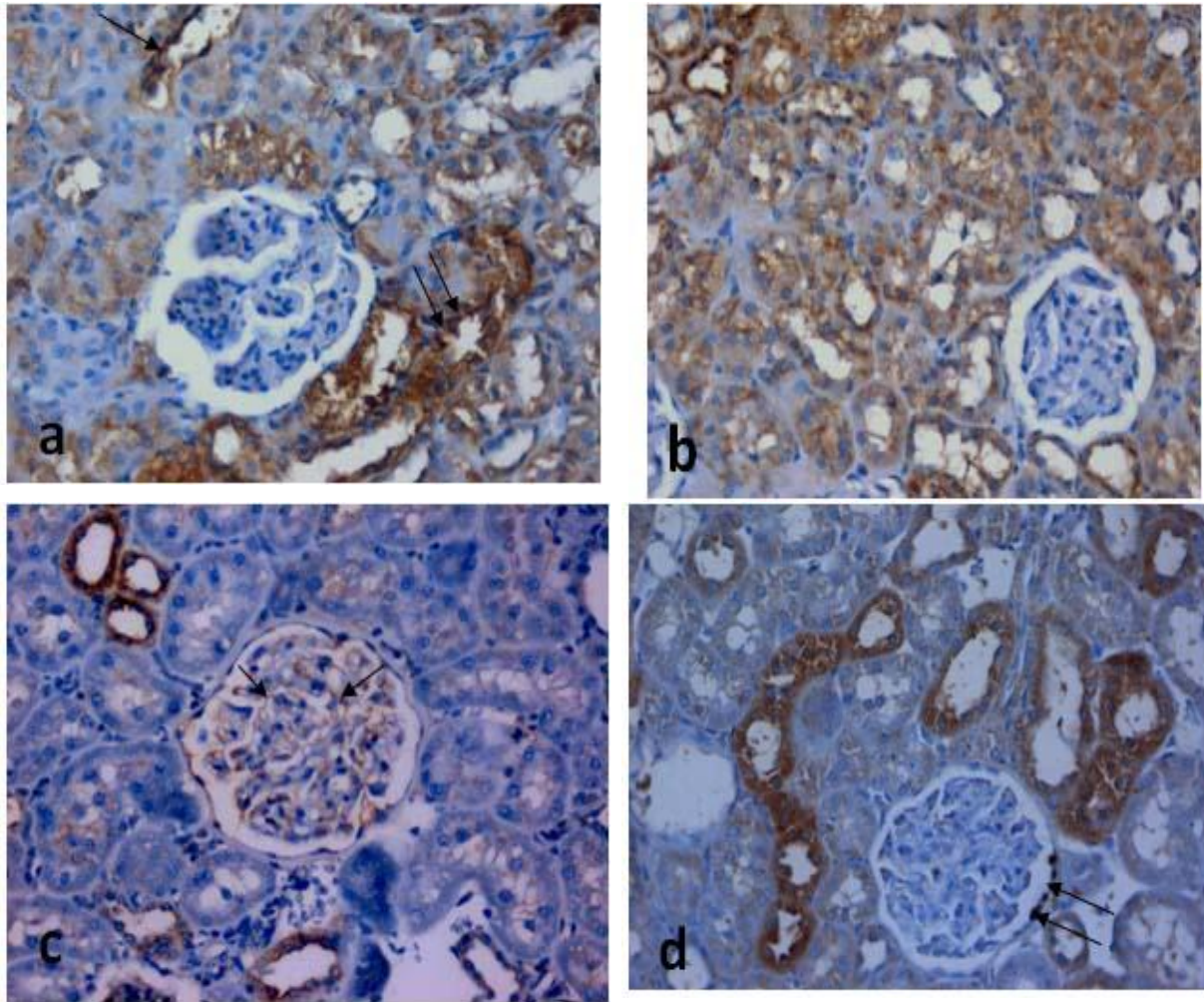


Fig. 2: Control animal: RAGE expression in the endothelial lining of blood vessels (arrow) and tubular epithelial cells (double arrows) (a). Diabetic animal: marked RAGE expression in the tubular epithelial lining (b), and along basement membrane of capillary tuft (arrow) (c). Treated animal: RAGE expression is detected in the epithelial lining of some tubules and focally in glomerular basement membrane (arrow) (Immunohistochemistry X400).