QUERCETIN AMELIORATES DIABETIC NEPHROPATHY IN RATS VIA MODULATION OF RENAL NA+, K+-ATPASE EXPRESSION AND OXIDATIVE STRESS

[1]Salah O. Bashir, [1,2]Mohamed Darwesh Morsy, [1]Hussein F. Sakr, [3,4]Hesham M. El Refaey, [5]Refaat A. Eid, [1]Mahmoud A. Alkhateeb and [1]Moataz A. Defallah

[1]Department of Physiology, College of Medicine, King Khalid University, Saudi Arabia
[2]Menoufyia University, Egypt
[3]Department of Pharmacology, Faculty of Pharmacy, King Khalid University, Saudi Arabia
[4]Al Azhar University, Egypt
[5]Department of Physiology, College of Medicine, King Khalid University, Saudi Arabia

ABSTRACT

Diabetic Nephropathy (DN) is one of most prevalent complications of Diabetes Mellitus (DM), therefore prevention of its development is a important field for researches. Quercetin is a plant flavenoid with hypoglycemic and antioxidant properties that is claimed to have a reno-protective effect in diabetes. This study was designed to investigate the reno-preventive role of Quercetin treatment in terms of biochemical and pathological changes in diabetic rats and to determine whether the effect is mediated through modulation of oxidative stress and Na+, K+ ATPase expression and activity. Sixty male Sprague-Dawley rats were distributed equally among 6 groups: (i) Control group (C), (ii) Quercetin treated Control group (CQ), (iii) Diabetic group (D), (iv) Diabetic Insulin treated group (DI), (v) Diabetic Quercetin treated group (DQ) and (vi) Diabetic Insulin and Quercetin treated group (DIQ). Systolic blood pressure was measured at the end of the experiments (8 weeks). Retro-orbital blood samples were used to determine the serum levels of glucose, HbA1c, urea, creatinine, Na+ and K+. Renal homogenate levels of Na+, K+ATPase activity, Malondialdehyde (MDA0, Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) were measured. Semiquantitative reverse transcriptase-PCR Na+, K+ATPase expression assays and kidney histopathological examination were conducted. Treatment with either insulin or Quercetin alone resulted in partial reversal of the biochemical and histopathological signs of nephropathy in diabetic rats. This was associated with partial but significant amelioration of indicators of oxidative stress and Na+, K+ATPase gene expression and activity. However only combined treatment by both insulin and Quercetin significantly improved all of the aforementioned parameters up to the control levels. These results suggested that combined therapy with insulin and Quercetin might be a useful preventive tool against development of DN.

Key words: Metabolic Diabetes Mellitus, Quercetin, Na+,K+Atpase Expression And Activity Semiquantitative Rt-Pcr For Na+, K+ Atpase Gene Expression

1. INTRODUCTION

Diabetes Mellitus (DM) has achieved a worldwide epidemic status associated with an increased risk of macro-vascular and micro-vascular complications (Tappy and Minehira, 2001). Microvascular complications of DM include retinopathy, neuropathy and nephropathy (Hakim and Pflueger, 2010). Diabetic nephropathy is one of the most serious and inevitable microvascular complications of DM. It occurs in 20-40%

Corresponding Author: Mohamed Darwesh Morsy, Department of Physiology, College of Medicine, King Khalid University, Saudi Arabia
Tel: +966544495223 Fax: +96672418194

of patients with type 1 diabetes mellitus leading to end stage renal failure in young age (ADA, 2005). To successfully cope with this challenging situations, there is an urgent need to search for treatment options that are readily available, safe and cost-effective.

Diabetic Nephropathy (DN) involves complex mechanisms and pathways. Chronic hyperglycaemia induced oxidative stress with consequence high lipid peroxidation in the vascular and cellular milieu is believed to be the most determinant factors responsible for the Development and progression of (DN) (Kedziora-Kornatowska *et al.*, 2003; Baig *et al.*, 2012). Free radicals and Reactive Oxygen Species (ROS) are formed disproportionately in diabetes and cause excessive production of advanced glycation end products, reduced antioxidant enzyme activities, impaired glutathione metabolism and formation of lipid peroxides (Soto *et al.*, 2003). Oxidative stress may also increase the formation of angiotensin converting enzyme, protein kinase C and Mitogen-Activated Protein Kinase (MAPK) all of which may contribute to the progression of nephropathy in diabetes (Hassan *et al.*, 2002).

Increased renal oxidative stress is associated with impairment of Na^+, K^+ ATPase activity and/or expression that is claimed to play an essential role in the pathogenesis and progression of DN (Tsimaratos *et al.*, 2001). Some studies have reported increased activity and/or expression in the units of this enzyme in diabetes (Wald and Popovtzer, 1988; Khadouri *et al.* (1987). Other studies have shown decreased activity and expression in various tissues including heart and kidney (Ng *et al.*, 1993).

Bioflavonoids are essential part of human diet and are present in plant extracts that are commonly used in oriental medicine. Quercetin is the most widely distributed bioflavonoid in foods like vegetables, fruits, tea, honey and many other dietary sources (Tong *et al.*, 2011). Quercetin is reported to normalize blood glucose level, augment liver glycogen content and significantly reduce serum cholesterol and LDL in diabetic rat models (Mahesh and Menon, 2004; Kim *et al.*, 2011). Exposure of isolated rat islets cells to Quercetin enhances insulin release by 44-70% (Hii and Howell, 1984). Quercetin is also a well known antioxidant directly scavenging ROS and free radicals (Tong-un *et al.*, 2013) and activating the function of antioxidant enzymes (Galati and Brien, 2004).

There has been only limited research on the role of Quercetin in ameliorating DN and the mechanism of its nephro-protective effect. To our knowledge there is no previous study of the possible role of Querecetin in regulating the activity and/or expression of renal Na+/K+ ATPase in diabetes. In the current study, we are hypothesizing that oral administration of Quercetin to StrepToZotocin (STZ) induced diabetic rats will improve

the renal function and ameliorate diabetic nephropathy by its hypoglycemic and antioxidant effects with subsequent amelioration of the renal Na+-K+ ATPase expression.

The aim of this study was to investigate the effect of Quercetin concomitantly with insulin as a protective agent against the development of nephropathy in diabetic rats and whether the effects are mediated by mechanisms involving anti-oxidant efficiency and the renal Na+-K+ ATPase expression and function.

2. MATERIALS AND METHODS

2.1. Animals and Experimental Design

This study followed a randomized controlled animal experiment design. The experimental procedures were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the ethical committee of the medical college at King Khalid University, Saudi Arabia. All procedures were also conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Fifty male Sprague-Dawley rats were obtained from the animal house of the physiology department, College of Medicine, King Khalid University, Saudi Arabia. The animals weighed between 150-200 gm and were fed with a standard chow diet, water, ad libitum. They were housed in the animal house of College of Medicine under laboratory conditions of: Temperature 22°C (±3°C), a 12:12-hour light/dark cycle. The rats were randomly divided into five groups (n = 10) as follow: Control group (C) where the rats received the vehicle (1 mL kg^{-1}) IntraPeritoneally (IP); Quercetin treated Control group (CQ), in which the rats received Quercetin (25 mg kg^{-1}) by oral gavage once daily. Diabetic model group (D) where diabetes was induced by a single IP injection of StrepToZotocin (STZ). Insulin treated Diabetic rats (DI), where rats were made diabetic as in group D and received mixtard insulin in a daily dose of one IU/kg. Quercetin treated Diabetic group (DQ), where rats were made diabetic as group D and received Quercetin in the same rout and dose as CQ group; Quercetin and Insulin treated Diabetic rats (DIQ) where rats were made diabetic as group D and received concomitantly insulin and Quercetin as groups DI and DQ respectively. All treatments continued daily for eight weeks. Hakam *et al.* (2006) have shown that diabetic nephropathy developes in streptozotocin treated rats after at least 6 weeks of diabetes induction.

2.2. Induction of Diabetes

Diabetes was introduced to rat groups D, DI, DQ and DIQ by I.P injection of a single dose of of 65 mg kg^{-1}

body weight STZ. The control group of rats were injected with the vehicle buffer alone. STZ was dissolved in 0.1 M cold sodium citrate buffer, pH 4.5 as a vehicle. The rats were then kept on 5% glucose solution bottles in their cages for the next 48 hrs to prevent hypoglycaemia. 48 hrs following the STZ injection, rats with non-fasting blood glucose level of ≥20 mmol L were considered diabetic and were included in this study. The failure rate for development of diabetes was 15% and the death rate was 5%. The failed and died rats were excluded from the experimental study groups from the start.

2.3. Measurement of Systolic Blood Pressure

At the end of the experimental protocol period (8 weeks), Systolic Blood Pressure (SBP) was measured using the rat-tail sphygmomanometer (Harvard Apparatus Ltd., England) in conscious rats prewarmed for 10 min in a thermostatically controlled restrainer (XBP1000; Kent Scientific) .The mean of at least three separate recordings on three occasions was taken (Wang *et al.*, 1995).

2.4. Blood Sampling for Biochemical Measurements

At the end of the experimental protocol and after overnight fasting, retro-orbital blood samples were obtained through non-heparinized capillary tubes and were divided into two parts. The first part of the blood was allowed to clot for 20 min in a 37°C water bath and centrifuged at 14,000 rpm for 10 min for serum separation and was used for different determination.

The other fraction of the blood was collected in tubes containing potassium oxalate and sodium fluoride and used for glycosylated Hemoglobin (HA1c) determination.

2.5. Renal Tissue Homogenates Preparation

Immediately after blood collection, animals were killed by lethal doses of thiopental sodium. The rats' abdomen was opened and right kidneys were quickly dissected and cut into small pieces. The kidney pieces were put in Phosphate Buffered Saline (PBS), pH 7.4, containing 0.16 mg Ml^{-1} of heparin to remove any red blood cells (erythrocytes) and clots. Some parts of the kidneys were homogenized with an Omni's homogenizer in cold phosphate buffer, pH 7.0 with Ethylene Diamine Tetraacetic Acid (EDTA).

2.6. Determination of Na^+, K^+-ATPase Assay in Renal Tissue Homogenate

A microsomal fraction was isolated from the renal cortex and medulla as previously described by Beltowski *et al.* (2004). Na^+, K^+-ATPase level was assayed by measuring the amount of inorganic phosphate (Pi) liberated from ATP in a buffer containing 100 mM NaCl, 20 mM KCl, 4 mM MgCl2, 1 mM EDTA, 40 mM Tris/HCl (pH 7.4) and 3 mM Na 2ATP and was calculated as the difference between the activities assayed in the absence and in the presence of 2 mM ouabain. Both samples contained 0.2 mM Sch 28080 to block ouabain-sensitive H+,K+-ATPase, which otherwise interferes with the Na^+,K^+-ATPase assay. Enzyme activity was expressed in micromolar of Pi liberated by 1 mg of microsomal protein during 1 h (micromolar per hour per milligram of protein). Pi and Effect of vanadium on renal Na+, K+-ATPase activity in diabetic.

2.7. Determination of Renal Tissue Levels of MDA, SOD and GPx

Lipid peroxidation was assessed by the measurement of secondary product MDA after precipitation of protein by addition of trichloroacetic acid then ThioBarbituric Acid (TBA) which reacted with MDA to form TBA reactive product, which was measured at 532 nm. A fresh solution of MDA was made by the hydrolysis of 1, 1, 3, 3-tetramethoxy propane and was used as a standard (Ohkawa *et al.*, 1979). SOD activity was assayed following the method of Kakkar *et al.* (1984). The sample containing 5 μg protein was mixed with sodium pyrophosphate buffer, Phenazine MeThosulphate (PMT) and Nitro Blue Tetrazolium (NBT). The reaction was started by the addition of NADH, incubated at 30°C and stopped by the addition of 1 mL of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity is defined as the enzyme concentration required for inhibition of the chromogen production by 50% in one minute per mg protein under the assay condition. The other parts of the kidney homogenate were homogenized in cold buffer consists of 50 mM tris-HCl, pH 7.5, 5 mM ED-TA, 1 nM DTT for Glutathione Peroxidase (GPx) activity analysis. All supernatants were kept in separate tubes and stored at -80°C. The left kidneys were cut into small pieces and preserved in a 10% formalin saline solution and sent for histopathological examination.

2.8. Determination of Plasma HbA1c%, Serum Glucose, Urea, Creatinine, Na+ and K+

Plasma glucose level was determined using glucose oxidase method. HbA1c% was determined according to the chemical separation and colorimetric method based on the phenol sulfuric acid reaction of carbohydrates (Nayak and Pattabiraman, 1981). Serum urea level was determined by enzymatic colorimetric method using a

specialized kit. The method was based on urease (urea amidohydrolase)/glutamate dehydrogenase coupled reactions and used a two-point fixed time kinetic scheme for monitoring the rate of consumption of NADH at 340 nm (Bretaudiere *et al*., 1976). Serum creatinine level was determined by the Jaffe reaction, where creatinine reacts with picrate ion in an alkaline medium to yield an orange red complex, which was measured at 490 nm (Spencer, 1986). Serum Na^+ and K^+ levels were estimated using a specific kit.

2.9. RNA Extraction and RT-PCR:

Published sequences of PCR primers used for the detection of ATPase α1 (forward: 5'GAAGCTCATCAT-CAGGCGACG3' reverse: 5'CCAGGGTAGAGTTCC-GAGCTC3', Atp1A1, rattus norvegicus; NCBI accession number NM-012504 product size 159 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward: 5'-AGAAGGCTGGGGCTCACT-3', reverse 5'-GGCATTGCTGACAATCTTGA-3' (GenBank accession no. NM-001034034), product size 134-bp (Weston *et al*., 2002, Herman *et al*., 2013). Total RNA was extracted from the frozen parts of kideny (30 mg) using the RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia) according to manufacturer's directions. The concentration of total RNA was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan). The purity was estimated by the 260/280 nm absorbance ratio. Single strand cDNA synthesis was performed as follows: 30 µL of reverse transcription mixture contained 1 µg of DNase I pre-treated total RNA, 0.75 µg of oligo d(T) primer, 6 µL of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor and 240 U of reverse transcriptase (Invitrogen). The RT reaction was carried out at 40°C for 70 min followed by heat inactivation at 95°C for 3 min. RT-PCR amplification was carried out in Biorad thermalcycler using 2 µL RT products from each sample in a 20 µL reaction containing *Taq* polymerase (0.01 U mL^{-1}), dNTPs (100 mM), MgCl2 (1.5 mM) and buffer (50 mM Tris-HCl). PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by 35 cycles of amplification, defined by denaturation at 96°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. A final extension cycle of 72°C for 15 min was included. A control reaction without reverse transcriptase was included for every sample to verify absence of contamination. PCR product (10 µL) were electrophoresed on 1.5% agarose gels containing 100 ng

mL^{-1} ethidium bromides and photographed with a Polaroid camera under ultraviolet illumination.

2.10. Histopathological Studies

Specimens sectioned from left kidneys from all experimental groups were fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethylalcohol (70-100%) and then prepared using standard procedures for hematoxylin and eosin staining.

2.11. Chemicals

Streptozotocin was purchased from Sigma (St Louis, Mo, USA). Assay kits for determination of Malondialdehyde levels (MDA, Cat No. 10009055), SuperOxide Dismutase (SOD, Cat NO.706002) and Glutathione Peroxidase (GPx, Cat NO.703102) activates were purchased from Cayman (Chemical, MI, USA). Assays kits for determination of serum glucose, HbA1c, Na^+, K^+, Urea and creatinine were purchased from Human diagnostic, Germany.

2.12. Statistical analysis

Statistical analyses were performed using Graphpad prism statistical software package (version 6). Data were presented as means with standard deviation (mean ± SD). Normality and homogeneity of the data were confirmed before ANOVA, differences among the experimental groups were assessed by one-way ANOVA followed by Tukey's t test.

3. RESULTS

Administration of Quercetin in control rats did not result any significant changes in all measured biochemical parameters execpt a significant increase (p<0.0001) in the activity of SOD compared with the Control group (C). Systolic blood pressure showed no signifcant changes in all experimental groups whether treated or not, compared with the control group.

3.1. Serum Glucose Levels

Eight weeks after induction of diabetes, the mean serum glucose level of Diabetic rats (D group) was significantly higher (p<0.0001) compared with the C group. Both Insulin and Quercetin treated diabetic groups showed significant decreases in their serum glucose levels compared with group D (p<0.0001). Concomitant administration of insulin and Quercetin (DIQ group) resulted in almost normal levels of blood glucose which were signifcant less ompared with the

diabetic insulin treaed (p<0.001), or diabetic Quercetin treated rats (p<0.0001) **Fig. 1**.

3.2. Plasma HBA$_{1c}$%

The Diabetic group (D) showed a significant increase in the percentage of HbA$_{1c}$ compared with the control group (p<0.0001). Diabetic insulin treated, diabetic querectin treated and concomittant diabetic insulin and quercetin groups showed significant decrease (p<0.0001) in the HbA$_{1c}$ levels compared with the diabetic group. However only concomittant treatment with insulin amd Quercetin normalized HbA$_{1c}$% levels. **Fig. 1**.

3.3. Serum Urea and Creatinine Levels

Eight weeks after induction of diabetes, there was significant increases (p<0.0001) in serum levels of both urea and creatinine in the D group compared with control group. Both insulin and Quercetin treatment (DI and DQ groups) resulted in significant reduction of the serum urea and creatinine levels compared with the diabetic group. Concomittant administration of both insulin and Quercetin resulted in significant reduction of both serum urea and creatinine levels (p<0.0001) down to the control level and significantly different compared with the diabetic or quercetin alone groups (DI and DQ) **Fig. 2**.

3.4. Serum Na$^+$ and K$^+$ Levels

There was significant reduction in serum Na$^+$ and significant elevation in serum K$^+$ (p<0.0001for both) in the Diabetic group of rats (D group) compared with the control group. Treatment with insulin or Querccetin (DI

and DQ groups respectively) resulted in significant elevation in serum Na$^+$ and significant reduction in serum K$^+$ compared with the diabetic group, however serum Na$^+$ was still significantly lower and serum K$^+$ was significantly higher (p<0.0001 for both) compared with the control group. Concomitant administration of insulin and Quercetin resulted in significant elevation in serum Na$^+$ significant reduction in serum K$^+$ groups (p<0.0001 for both) compared with the diabetic group but similar to the control group **Fig. 3**.

3.5. Renal Tissue homogenate Levels of MDA and SOD, GPx and Na+,K+-ATPase Activity Levels

Diabetic rats in group D showed a significant increase in kidney homogenate lipid peroxidation marker MDA level with a significant decrease in kidney homogenate anti-oxidants SOD, GPx and Na$^+$,K$^+$-ATPase activities compared with the control group (p<0.0001). Treatment with insulin (DI group) and Quercetin (DQ group) produced a significant decrease in kidney homogenate MDA levels and significant increases in kidney homogenate SOD, GPx (and Na$^+$, K$^+$-ATPase activity levels compared with the Diabetic group (D) (p<0.0001 for all). Compared with the control group, kidney homogenate MDA level remained significantly higher and SOD, GPx and Na$^+$, K$^+$-ATPase activity levels remained significantly lower in both DI and DQ groups. Levels of GPx and Na$^+$, K$^+$-ATPase activity in the DQ group increased almost to the levels in the control group.
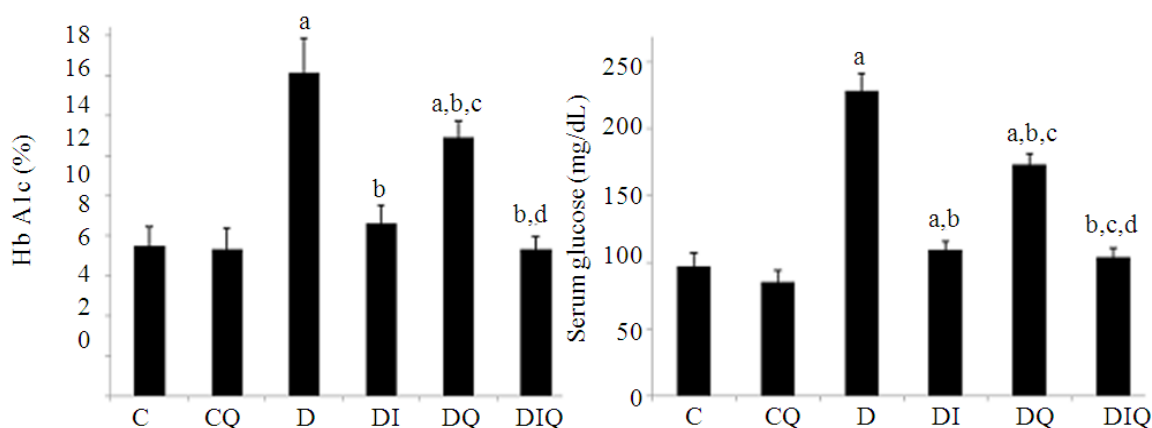

Fig. 1. Effect of Quercetin and insulin treatment for 8 weeks in a rat model of STZ induced diabetes on: Serum glucose and HbA1c. C: Control group; CQ: Control Quercetin treated group; D: Diabetic group; DI: Diabetic insulin treated group. DQ: Diabetic Quercetin treated group; DIQ: Diabetic insulin and Quercetin treated group. Results are expressed as means ± SD (n=10). Significance was considered when P value was <0.05. [a]Significantly different from C and CQ groups. [b]Significantly different from D group. [c]Significantly different from DI group. [d]Significantly different from DQ group
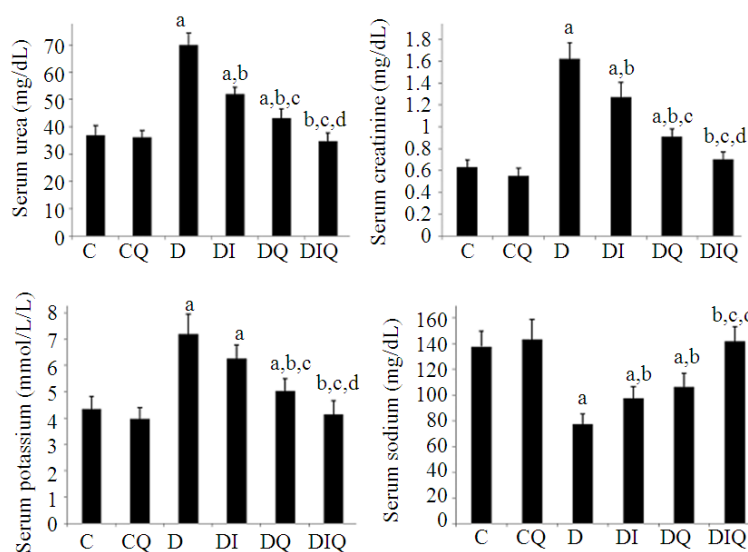
Fig. 2. Effect of Quercetin and insulin treatment for 8 weeks in a rat model of STZ induced diabetes on: Serum urea, creatinine. Sodium and potassium, C: Control group; CQ: Control Quercetin treated group; D: Diabetic group; DI: Diabetic Insulin treated group. DQ: Diabetic Quercetin treated group; DIQ: Diabetic Insulin and Quercetin treated group.Results are expressed as means ± SD (n = 10). Significance was considered when P value was <0.05. [a]Significantly different from C and CQ groups. [b]Significantly different from D group. [c]Significantly different from DI group. [d]Significantly different from DQ group
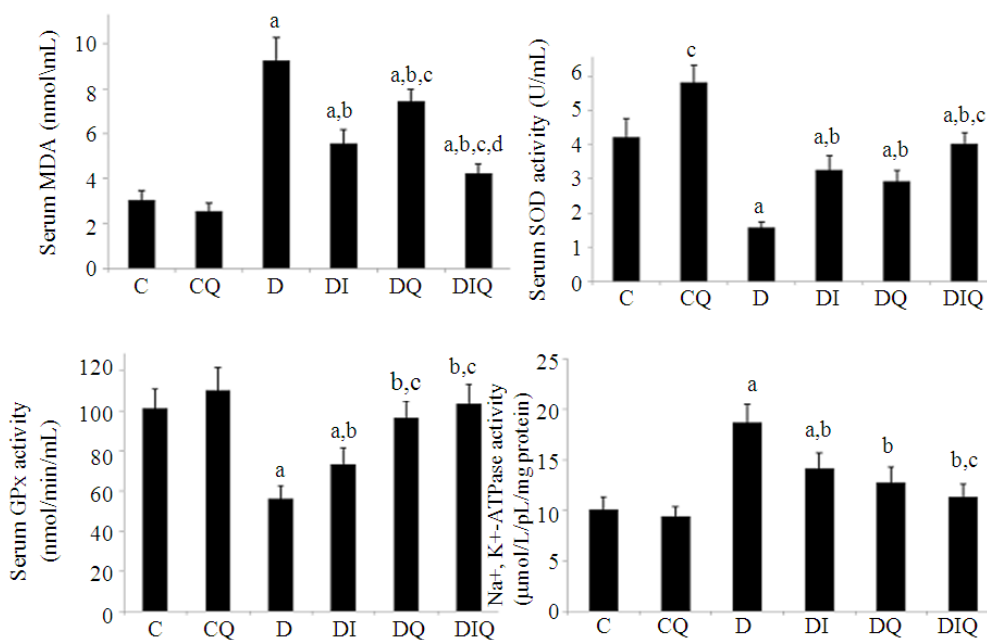

Fig. 3. Effect of Quercetin and insulin treatment for 8 weeks in a rat model of STZ induced diabetes on: Renal homogrnate level of MDA, SOD, GPx and Na+,K+-ATPase. C: Control group; CQ: Control Quercetin treated group; D: Diabetic group; DI: Diabetic Insulin treated group; DQ: Diabetic Quercetin treated group; DIQ: Diabetic Insulin and Quercetin treated group. Results are expressed as means ± SD (n = 10). Significance was considered when P value was <0.05. [a]Significantly different from C groups. [b]Significantly different from D group. [c]Significantly different from DI group. [d]Significantly different from DQ group. [e]Significantly different from C group
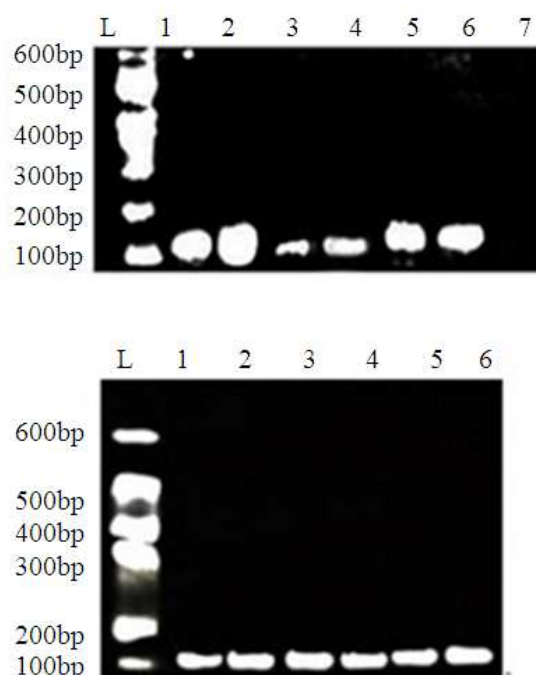
Fig. 4. Effect of Quercetin and insulin treatment for 8 weeks in a rat model of STZ induced diabetes on: RT-PCR amplificatin products of Na+,K+-ATPase α-1 (159 bp, Upper panel, A) mRNAs expression in the kidneys of all groups of rats. Na+,K+-ATPase α-1 mRNA expression were normalized to the internal reference gene, GAPDH (134 bp, Lower panel, B). All PCR product (10 µL) were electrophoresed on 1.5 % agarose gels containing 100 ng/ml ethidium bromides and photographed with a Polaroid camera under ultraviolet illumination. 1: Control group; 2: Control Quercetin treated group; 3: Diabetic group; 4: Diabetic insulin treated group. 5: Diabetic Quercetin treated group; 6: Diabetic insulin and Quercetin treated group. 7: Negative control (without reverse transcriptase)

Concomitant administration of both Insulin and Quercetin (DIQ group) resulted in a significant reduction in kidney homogenate MDA and significant elevation in SOD, GPx and Na$^+$,K$^+$-ATPase activity levels compared with both the Diabetic (D) and the Control (C,CQ) groups. The concomitant treatment normalized all the aforementioned parameters except for the kidney homogenate MDA level which remained significantly higher (p = 0.0430) compared with the control group **Fig. 3**.

3.6. mRNA Expression of Na$^+$, K$^+$ATPase α-1 Subunit by RT-PCR

Figure 5 shows the transcriptional changes in the levels of Na$^+$, K$^+$ ATPase α-1 subunit in the rats' kidneys of all groups of rats. GAPDH mRNA expression from same sample was used as internal control. All tested transcripts were detected and RT-PCR resulted in fragments similar in size to those expected (159 bp for Na$^+$, K$^+$ ATPase α-1 and 134 bp for GAPDH). GAPDH mRNA expression levels were similar in the kidney of all groups of rats (**Fig. 4**, Lower panel B). However, in the control group, thick prominent band for Na$^+$,K$^+$ATPase α-1 mRNA was seen (**Fig. 4**, **Band 1**). Quercetin administration to control rats resulted in a clear up-regulation of Na$^+$, K$^+$ ATPase α-1 mRNA as compared to control group and very thick band that is approximately double in size to that of the control was seen (**Fig. 4 and Band 2**). mRNA expression of Na$^+$/K$^+$ ATPase α-1 was inhibited in the kidney of diabetic rats and a very thin band was detected (**Fig. 4 and Band 3**). On the other hand, a small enhancement in Na$^+$, K$^+$ ATPase α-1 mRNA band that is narrower than that of the control was seen in diabetic rats treated with insulin alone (2 U kg^{-1}) (**Fig. 4 and Band 4**). Quercetin administration alone or incombination with insulin resulted in upregulation of Na$^+$, K$^+$ ATPase α-1 mRNA in the kidneys of diabetic rats (**Fig. 4, Bands 5 and 6**) The thickness of these bands were very close to that seen in the control group. No bands of amplification were seen in the negative control sample when reverse transcriptase was omitted (**Fig. 4 and Band 7**).

3.7. Histopathology of the Kidenys

Histological examination of renal tissue in Control or Control Quercetin (C,CQ) groups showed normal renal tissue structures (**Fig. 5A and B**). Eight weeks after induction of diabetes, the diabetic rats kidney sections showed thickening of the basement membrane along with changes in the density of mesenchyme, atrophy and complete degeneration of glomerular capillaries and tubular necrosis of both tubular and proximal tubules (**Fig. 5C**). Histological examination of kideny tissue in Insulin treated Diabetic group (DI) revealed partial damage of glomerular capillaries, intact epithelial cells with dilated Bowmann's capsule and some injury to both proximal and distal tubules (**Fig. 5D**). Treatement with qurecetin alone in DQ group resulted in hyperatrophy and partial damage of glomerular capillaries and little damage of proximal and distal tubules (**Fig. 5E**). Maximum improvement was seen in the histological examination of the kidney tissue of the concomitant group (DIQ) which showed almost the normal sturctures of the glomeruli, epithelial cell, glomerular capillaries, proximal and distal tubules (**Fig. 5F**).
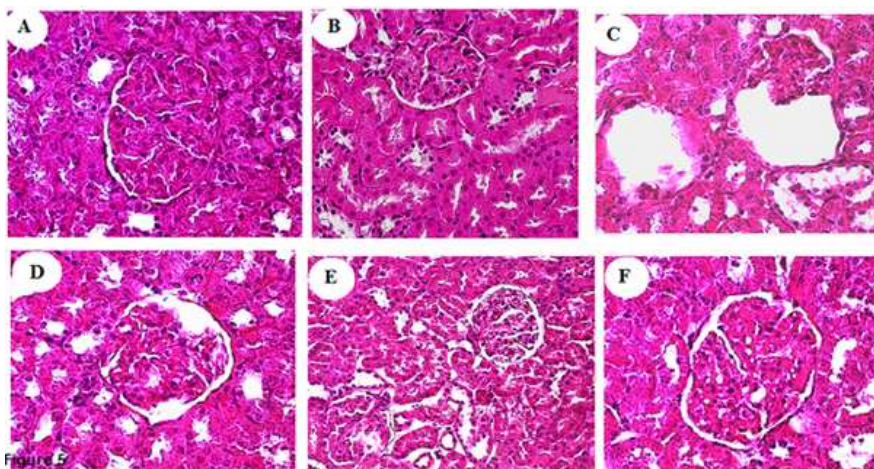
Fig. 5. Light microscopical photomicrograph of renal tissues from (A) control and (B) control Quercetin treated groups; both showed normal architecture of renal tissue with prominent Bowman's capsule, epithelial Cells and normal tubules. (C) Diabetic group showed thickening of the basement membrane along with mild change in the density of mesenchyme, complete degeneration of glomerular capillaries and tubular necrosis of both tubular and proximal tubules. (D) Insulin treated diabetic group showed normal partial damaging of glomerular capillaries, intact epithelial cells with the presence of injured dilated Bowmann's capsule. (E) Quercetin treated diabetic group showed hyperatrophied and partial damaging of glomerular capillaries and little damage of proximal and distal tubules. (F) Quercetin and insulin treated diabetic group showed normal kidney architecture with intact epithelial cell and tubules

4. DISCUSSION

The present study was carried out to evaluate the efficacy of oral administration of Quercetin (25 mg/kg/day) alone or in combination with insulin in combating the nephropathic effects of diabetes in (STZ) treated rats and whether any renal effect of Quercetin is mediated through modulation of hyperglycemia, oxidative stress and/or Na^+-K^+ ATPase gene expression.

After eight weeks of the induction of diabetes, there were definitive signs of nephropathy including both biochemical and histological changes. There was significant elevation of serum levels of urea, creatinine and K^+ with a significant reduction in serum Na^+ levels in diabetic rats compared with the control group (**Fig. 2**). The histological changes included thickening of the basement membrane along with pathological changes in the density of mesenchyme, degeneration of glomerular capillaries and tubular necrosis of both proximal and distal tubules (**Fig. 5**).

In the current study both insulin and Quercetin therapy ameliorated the DN but combination therapy had the most significant positive impact on renal function and histology.

We observed a positive effect for Quercetin alone or in combination with insulin, on the glycemic control in the diabetic rats as evident by the significant lowering of blood glucose and HbA1c (**Fig. 1**). Previous studies have reported that Quercetin has hypoglycemic effect on in a dose ranging from 10-50 mg kg^{-1} body weight/day in diabetic rats (Rivera *et al.*, 2008; Kim *et al.*, 2011). The exact mechanism of the hypoglycemic effect of Quercetin is not well known. Hii and Howell (1984) reported that exposure of isolated rat islets to Quercetin enhances insulin release by 44-70%. They suggested that, the effect is at least in part, via alteration in $Ca2^+$ fluxes and cyclic nucleotide metabolism. Quercetin supplementation was shown to induce regeneration of the pancreatic islets and increase insulin release in STZ-induced diabetic rats (Vessal *et al.*, 2003). Quercetin was also shown to exhibit an α-glucosidase inhibitory activity and adiponectin elevator effect in vitro that could contribute to its hypoglycemic effect (Jo *et al.*, 2009).

Persistent hyperglycemia induced oxidative stress due to overproduction of reactive oxygen is considered one of the main mechanism responsible for the development diabetic nephropathy (Hakim and Pflueger, 2010). Auto-oxidation of glucose, reduced antioxidant enzyme activities, impaired glutathione metabolism and formation of lipid peroxides and advanced glycation end products are the main factors responsible for such effect (Soto *et al.*, 2003). In the current study, the diabetic rats showed significantly decreased activities of GPx and

SOD and increased level of lipid peroxidation marker MDA in the renal homogenates, suggesting the development of oxidative stress in renal tissue (**Fig. 3**). Earlier studies reported a decrease in the activities of antioxidant enzymes SOD, CAT and GPx in the kidney and liver in diabetic animals (Morsy *et al*., 2010). In the same line, El-Khayat *et al.* (2011) reported decreased levels of endogenous anti-oxidatives in the kidneys of diabetic rats. There was significant reduction in the oxidative stress markers in rats treated with Quercetin or insulin with the maximum effect apparent in the group receiving combined therapy (**Fig. 3**). This was proved by the significant reduction in renal MDA levels with concomitant elevations in the renal antioxidant enzymes SOD and GPX activities. Rodriguez *et al*. (2009) reported that controlling hyperglycaemia in diabetic patients with insulin or other hypoglycaemic agent reduces oxidative stress-induced diabetic nephropathy complication in diabetes patients. Significant enhanced activity of SOD in kidneys of the control group treated with Quercetin alone may suggest another mechanism that is related to its potent antioxidant potential directly scavenging ROS and other free radicals (Mira *et al*., 2002; Tong-un *et al.,* 2013). It has been reported that Quercetin directly activates the function of antioxidant enzymes (Reddy *et al*., 2012). Also, in a recent study of Jeong *et al.* (2012), Quercetin at 0.04 and 0.08% of the diet increased the activities of hepatic SOD, CAT and GSH-Px in db/db obese mice.

It has been suggested that impairment in Na^+/K^+ ATPase expression and activity play an essential role in the pathology and progress of diabetic nephropathy (Sampathkumar *et al*., 2006). In experimental diabetes decreased Na^+, K^+ ATPase activity has been observed in various tissues including heart and kidney (Ng *et al*., 1993). The reduction usually occurs as part of the long term diabetic changes and could be due to altered enzyme kinetics and/or altered subunit production. The reduction in Na^+, K^+ ATPase expression in diabetic rats was only partially restored by insulin therapy (Vér *et al*., 1995). In the current study, there was significant decrease in both the activity of the Na^+, K^+ ATPase and the enzyme α-subunit mRNA expression in the renal tissue of diabetic rats after eight weeks of induction of diabetes. Insulin treatment partially ameliorated the changes in both activity and enzymes α-subunit expression. Quercetin treatment alone or in combination with insulin resulted in increased activity and up-regulation of the α-subunit of Na^+,K^+ATPase recovering to normal levels with the combined therapy. These results suggested a regulatory role for both insulin and Quercetin at the molecular level of Na^+, K^+ ATPase and

their synergistic effects. The magnitude of change in the enzyme activity and expression depends on the duration of diabetes and the organ involved (Scherzer and Popovtzer, 2002). Early diabetes (2-3 weeks) appear to induce increase in Na^+, K^+-ATPase activity due to increased protein expression (Ng *et al*., 1993). This increases in Na^+, K^+ ATPase activity was explained as an adaptive mechanism to the sodium load increase into distal tubules in the early stages of diabetic nephropathy (Khadouri *et al*., 1987). Long-term diabetes resulted in significant decrease the Na^+, K^+ ATPase activity, with a reduction in α1 and β1 isoforms abundance, particularly in the medullary thick ascending limb. In diabetes, in addition to alterations in activity of Na^+, K^+ATPase may result from parallel changes in the amounts of $α_1$ and $β_1$ subunits expression (Tsimaratos *et al*., 2001). Other possible mechanism of Quercetin activating Na^+, K^+ ATPase activity and expression effect have been suggested. Enzyme glycation is one of the mechanism that contribute to the decreased activity of Na^+, K^+ ATPase in diabetes (Ferretti *et al*., 2002), therefore Quercetin may have enhanced the enzyme activity through inhibition of glycation. There are also other mechanism which could contribute to or explain the effect of quercetin on Na^+,K^+ATPase activity. Quercetin could mediate its activation on Na^+, K^+ ATPase activity and expression through induction of C-peptide secretion in diabetes. The effect of Querectin could also be mediated through its suppressive activity on Mitogen-Activated Protein Kinase (MAPK) (Galuska *et al*., 2011). Another possible mechanism could be through Quercetin activation of ERK1/2 signaling pathway; that is significantly inhibited in diabetes (Upadhyay *et al*., 2003). The current study did not investigate theses alternative pathways and mechanisms due to technical and financial constraints.

An important observation in our study is that Quercetin significantly elevated Na+, K+-ATPase activity level as well as the expression of its α-subunit in control non-diabetic rats. This observation raises the possibility that Quercetin may induce some form of nephrotoxicity to normal kidneys which is potentially a serious problem with high doses and long term administration (Dunnick and Hailey, 1992).

The histological examination of the kidney tissues appears to confirm the protective role of both insulin and Quercetin. Insulin treated diabetic rats had only partial damage of glomerular capillaries, presereved glomerular epithelial cells with the presence of some dilatation of Bowmann's capsule. Where as Quercetin treated diabetic group showed some hyperatrophy and partial damaging

of glomerular capillaries but little damage of proximal and distal tubules. The maximal imrovement was observed in Quercetin and insulin treated group, which shows normal kidney architecture with intact glomerular epithelial cells and tubules.

5. CONCLUSION

The present study showed that treatment of diabetic rats with Quercetin in combination with insulin has a preventive role against the development of diabetic nephropathy. The renal protection seems to be mediated via hypoglycemic effect, antioxidants activity and enhancement of Na^+, K^+-ATPase expression and activity. In the current study we limited our investigations to the kidney and we did not study any possible changes in pancreatic histopathology, serum insulin level or pancreatic insulin gene expression. Also we did not investigate the other possible mechanisms that could be involved in the Quercetin nephron-protective effect. Further studies should be carried out on Quercetin nephron-preventive role in order to uncover the exact mechanism of action. Also, investigation of Quercetin with different doses and therapy regimens in other animal species is required to ensure its efficacy and safety.

6. ACKNOWLEGEMENT

7. CONFILICT INTEREST

The authors declare no conflict of interest.

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