Protective Effects of Adiponectin from Nephropathy and Atherosclerosis in Type 2 Diabetic Rats: Possible Mechanisms

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Abstract

Background: Diabetes mellitus and its associated complications have become a public health problem of considerable magnitude. Renal and vascular complications account for most of the excess morbidity and mortality in patients with diabetes mellitus. Clinical studies have demonstrated low plasma adiponectin level in diabetic cases closely linked to renal and atherosclerotic deterioration. The aim of this study is to investigate the effect of adiponectin on diabetic-induced nephropathy and atherosclerosis and the possible underlying protective mechanisms.

Methods: Six rat groups were assigned: Control, type 2-diabetic, non-diabetic-vehicle, diabetic-vehicle, non-diabetic-adiponectin and diabetic-adiponectin groups. The recombinant adiponectin gene was transfected into rats by a single intraperitoneal injection. After corresponding treatments for 8 weeks, body mass index, blood pressure, serum glucose, insulin, triglycerides, creatinine adiponectin and TNF-α, blood urea nitrogen (BUN) and urinary microalbumin were measured. Reactive oxygen species (ROS), endothelial nitric oxide synthetase expression (eNOS) in kidney and aorta, transforming growth factor-β1 (TGF-β1) and phosphorylated adenosine monophosphate kinase (pAMPK) in the kidney tissue and VCAM-1 expression in aorta were measured. The renal pathologic changes were observed by light microscopy.

Results: Diabetes significantly reduced serum adiponectin with concomitant significant increase in serum glucose, insulin, triglycerides, TNF-α and creatinine as well as in blood pressure, BUN and microalbuminuria with light microscopic evidence of nephropathy in all diabetic groups compared to the control group. Also ROS generation in aorta and kidney, renal TGF-β1 and aortic VCAM-1 expressions were significantly increased in diabetic groups compared to control group with significant decrease in renal pAMPK and aortic and renal eNOS expression (p<0.05). Compared with the diabetic group, the diabetic-adiponectin group showed a significant decrease in serum glucose, insulin, triglycerides, TNF-β and creatinine as well as in blood pressure, BUN and microalbuminuria with improvement of the renal microscopic pathology. Also adiponectin significantly decreased ROS generation in aorta and kidney, renal TGF-β1 and aortic VCAM-1 expressions in diabetic-adiponectin group compared to the diabetic group with significant increase in renal pAMPK and aortic and renal eNOS expression (p<0.05). These beneficial effects were absent in the diabetic-vehicle group indicating a direct role of adiponectin.

Conclusions: Adiponectin decreased TNF-α and ROS, relieved oxidative stress and up-regulated the expression of eNOS in renal and aortic tissues of diabetic rats. In addition adiponectin stimulated the renal AMPK signal pathway and down-regulated the expression of TGF-β1 in kidney and VCAM-1 in aorta, suggesting a protective effect of adiponectin from diabetic nephropathy and atherosclerosis.

Key Words: Diabetic nephropathy – Atherosclerosis – Adiponectin – VCAM-1 -TGF β1.

Introduction

DIABETIC nephropathy (DN) and atherosclerosis are common morbidity and mortality causes in patients with diabetes mellitus [1]. The pathogenesis of both complications is interrelated and has been linked with the components of the metabolic syndrome, oxidative stress, cytokines, reduced endothelial nitric oxide synthetase (eNOS) and changes in the extracellular matrix (ECM) [2,3].

Diabetic nephropathy (DN) is characterized by mesangial expansion, extracellular matrix deposition, basement membrane thickening of glomeruli and glomerulosclerosis associated with microalbuminuria and hypertension. Diabetic nephropathy has worse outcomes and is more difficult to be treated than nephropathy caused by other diseases, the reasons are unclear [4]. Furthermore, microalbuminuria has been reported as a risk marker for atherothrombosis and the associated decreased renal function and hypertension could accelerate atherosclerosis in patients with type 2 diabetes [5].

Transforming growth factor-β1 (TGF-β1) is an important cytokine secreted by different cell types, which directly or indirectly promote the components of ECM, and has been reported as the major
candidate to mediate the progression of diabetic nephropathy [6]. Treatment of diabetic mice with anti-TGF-β1 antibodies significantly attenuated the increase in TGF-β1 activity and extracellular matrix expression [7], and have been found to play a role in atherosclerosis and associated with an increased risk of development and progression of microalbuminuria [8].

Adiponectin, is a circulating plasma protein primarily secreted by adipocytes and its plasma levels have been observed to be reduced in patients with obesity, diabetes mellitus, and hypertension [9]. Clinical studies have demonstrated that circulating adiponectin levels were negatively correlated with proteinuria in type-2 diabetics in early stage and were increased in advanced diabetic nephropathy. Interestingly, in patients with type 1 diabetes, a single nucleotide protein in the adiponectin promoter showed linkage with diabetic nephropathy [10]. Adiponectin has been shown to have insulin-sensitizing metabolic effects suggesting its important role in diabetes mellitus, however, little is known about the exact protective mechanism of adiponectin from diabetic-induced atherosclerosis and nephropathy [11,12].

Therefore, the present study, investigated the effect of adiponectin gene delivery on diabetic-induced nephropathy and atherosclerosis in type 2 diabetic rats and the possible related protective metabolic, anti-oxidant and anti-inflammatory mechanisms.

Material and Methods

Animals and experimental design:

Animals were purchased from the animal Care Unit of Cairo Medical University, all procedures that involved animals were approved by this unit. 48 male white albino rats, 7-8 weeks old weighing 160-180g, were housed in wire mesh cages in a constant temperature (22-24°C) and light controlled room on an alternating 12:12h light-dark cycle and had free access to food and water.

Animals were divided randomly into the following groups (n=8/group):

- Control group: Rats receiving the standard diet.
- Diabetic group: Type 2 diabetic rats.
- Non-diabetic-vehicle group: Non-diabetic rats injected by lipofectamine vehicle.
- Diabetic-vehicle group: Type 2 diabetic rats injected with lipofectamine vehicle.
- Non-diabetic-adiponectin group: Non-diabetic rats injected with the adiponectin gene.
- Diabetic-adiponectin group: Type 2 diabetic rats injected with the adiponectin gene.

Induction of type 2 diabetes:

Beginning on day 0, animals were divided into two groups (24 rats/group): A group fed standard rodent diet (SD: 6.5% Kcal fat) and a group fed high fat diet (HFD: 58% Kcal fat) for a period of 2 weeks. On day 14, rats on the high fat diet (HFD) were injected intraperitoneally with a single low dose of streptozotocin (STZ, 45mg/kg i.p., in 0.01M citrate buffer pH4.3, Sigma, St Louis, MO, USA) to induce type 2 diabetes mellitus. Both the low dose of STZ and the high fat diet are essential elements to induce type 2 diabetes with insulin resistance [13]. Those fed on standard diet (SD) received only the buffer solution. Subsequently all rats had free access to food and water and were continued on their respective diets till the end of the study.

On day 21, type 2 diabetes was confirmed randomly in some HFD rats by measuring fasting serum glucose and insulin.

Preparation of adiponectin gene

Rat adiponectin gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) for 40 cycles and DNA was purified to prepare cloned DNA (cDNA)/lipid complex using a DNA purification kit. The purified DNA was then transfected through dilution of 0.2lg of DNA with 0.5-5 l of lipofectamine using the lipofectamine transfection kit (invitrogen, Carlsbad, CA, USA) then injected as 0.2ml in the rat tail [14].

Animal treatment:

On day 21, 8 rats from each of the two groups (diabetic and non-diabetic groups) were restrained manually and 0.2ml of the prepared adiponectin gene was administered intraperitoneally. This dose has previously been shown to result in persistent adiponectin gene expression for up to eight weeks [15]. Another 8 rats from each of the two groups (diabetic and non-diabetic groups) were injected only with 0.2ml of the lipofectamine vehicle.

Assessment of body mass index:

The body mass index (BMI) was calculated using the formula: Weight in g/body naso-anal length in cm² [16].

Systolic blood pressure (SBP) measurement:

Before measuring the systolic blood pressure, the body temperature of the rats had to be adjusted to 37°C using an incubator for 10min. The body
temperature was verified by colonic temperature measurement. SBP was measured by Harvard rat tail pressure monitor system, an electronic version of the traditional sphygmomanometer cuff method. The pressure was measured in conscious animals after being placed in the supplied restrainer [17].

**Blood and tissue samples:**

Eleven weeks after the beginning of the study (eight weeks after the administration of the adiponectin gene), retro-orbital blood samples (2ml each) were taken from the rats of all groups after overnight fasting (9p.m. to 8a.m.). Then, under anesthesia (100mg/kg ketamine and 2.5mg/kg acepromazine), both kidneys (one for histological examination and the other for biochemical analysis) and the aorta were dissected.

**Biochemical and hormonal assays:**

Fasting serum glucose was measured by the oxidase-peroxidase method using the spectrophotometer (Du 7400, Beckman Coulter Inc., CA, USA). Commercially available ELISA kits were used to measure serum insulin (Linco Research, St Charles, MO, USA), adiponectin (B-Bridge International Inc., Monroe, Washington, USA) and TNF-a (Quantakine High Sensitive, R&D Systems, Minneapolis, MN, USA) using ELISA reader (Stat Fax-2100 Awareness Technology Inc., Palm city, FL, USA). To estimate insulin resistance, the homeostasis model assessment for insulin resistance (HOMA-IR: insulin resistance index) was used, calculated as the product of fasting insulin (in µU/ml) and fasting glucose (in mmol/l) divided by 22.5, which has previously been used in rodents [18]. Serum triglycerides (TG) was measured (Biovision, Mountain View, CA, USA) and its absorbance was measured spectrophotometrically at 570nm.

Serum creatinine and blood urea nitrogen (BUN) were estimated by the QuantiChromTM creatinine and QuantiChromTM Urea (DIUR-500) assay kits respectively.

**Measurement of microalbuminuria:**

Microalbumin was detected in urine using ELISA kit supplied by Alpco immunoassays according to manufacture instruction [19].

**Determination of mitochondrial superoxide anion generation by dihydroethidium fluorescence:**

Superoxide anion generation from the kidney and aorta was determined using dihydroethidium (DHE) fluorescence. A portion of the kidney and from aorta was homogenized in 1:4 volumes of a buffer containing 20mM HEPES, 1mM EDTA and 0.1mM of phenylmethylsulfonyl fluoride followed by centrifugation at 3000g for 10 minutes at 4°C. The supernatant of kidney homogenate was incubated in a reaction mixture containing 10 µM DHE at 37°C for 30min. The cell permeable DHE stain is rapidly oxidized to fluorescent ethidium by superoxide anions, which is then intercalated into DNA. Fluorescent ethidium is therefore a presumptive marker of intracellular superoxide anion generation at that point in time. The fluorescent image was obtained using a fluorescence microscope with a 475-nm long-pass filter attached to an image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD).

Expression of the phosphorylated AMP-kinase (pAMPK) in the kidney was determined as previously described [20]. Kidney tissue (50mg) was homogenized using a polytron homogenizer in 1.5ml cold lysis buffer (50mmol/l Tris-HCL, pH 8.0, 150mM/l NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and 0.5mM/l phenylmethylsulfonylfluoride). The homogenate was centrifuged for 20min at 4°C and the supernatant was collected. Samples were stored at –80°C until use. After boiling at 95°C for 5min, samples (50µg/lane) were subjected to 7% SDS-PAGE gel and then transferred to polyvinylidene difluoride membrane (AMPK; Millipore, Bedford, MA, USA). The membranes were blocked in 7.5% non-fat dried milk in TBST (0.05% Tween-20 Tris-buffered saline) for 2h at room temperature and then incubated with primary antibodies overnight at 4°C with phospho-specific antibodies against AMPK Thr172 at 1:1000 (Cell Signaling Technology, Beverly, MA, USA). The membranes were washed and then incubated with a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:25 000, Bio-Rad, Hercules, CA, USA) for 1h at room temperature, followed by additional washing. Proteins were visualized by enhanced chemiluminescence (ECL plus; Amersham, Arlington Heights, IL, USA) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA, USA).

**Real-time polymerase chain reaction quantitative analyses for eNOS, TGF-ß1 and VCAM-1 gene expression:**

RNA-isolation and reverse transcription:

RNA-Isolation was conducted by means of the Qiagen Tissue Mini Kit (Qiagen, Valencia, CA,
USA). In the beginning the frozen kidney tissue was homogenized under liquid nitrogen using a mortar and pistle and than the cells were lysed and the RNA was released by centrifugation of the cell-homogenate through a biopolymer shredder (Qiashredder, Qiagen, Valencia, CA, USA). The quality and yield of the RNA was determined by spectrophotometry at 260nm and the integrity examined by agarose gel electrophoresis with ethidium-bromide staining. The Quantification of the total RNA was performed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDropTechnologies). cDNA was generated from 5µg of total RNA extracted with 1µl (20pmol) antisense primer and 0.8µl superscript AMV reverse transcriptase for 60min at 37°C.

Real-time polymerase chain reaction:

The relative abundance of mRNA species was assessed using the SYBR Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank. TGF-β1 forward primer was 5'-TCACTTTTTGTTGGATGC-3' and reverse primer was 5'-TTCTGTCTCTCAAGTCCCCC-3', eNOS forward primer was 5'-TCTAAGGAGTGCAGATATGGC-3' and reverse primer was 5'-TGGTGTGTCTGGTGATGATG-3', and VCAM-1 forward primer was 5'-GACTGGCAGCTGACCTATGTC-3' and reverse primer was 5'-AGTCTGATGAATCAACATCGTAGC-3'. All primer sets had a calculated annealing temperature of 60° C. Quantitative RT-PCR was performed in duplicate in a 25-µl reaction volume consisting of 2x SYBR Green PCR Master Mix (Applied Biosystems), 900nM of each primer and 2-3µl of cDNA. Amplification conditions were 2min at 50°, 10min at 95° and 40 cycles of denaturation for 15 s and annealing/extension at 60° for 10min. Data from real-time assays were calculated using the V17 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of each mRNA was calculated using the comparative Ct method. All values were normalized to the β-actin genes forward primer: 5'-ACTGCGCGAT CCTCTTCTTC-3' and reverse primer: 5'-ACTCTGCTCTGATCCACAT-3' and reported as fold change over background levels [21].

Histological examination:

Kidney tissues for paraffin sectioning were fixed in 10% formal saline, processed and finally imbedded in paraffin. Then, 3-µm-thick sections were cut, deparaffinized, hydrated and stained with hematoxylin and eosin reagents. Renal sections were examined in a blind fashion for glomerular congestion, tubular cell swelling, cellular vacuolization, pyknotic nuclei and necrosis.

Statistics:

The results were analyzed using SPSS computer software package, version 10.0 (Chicago, IL, USA). All data were parametric and presented as mean ± S.D except for the non-parametric microalbuminuria data which were expressed as median and interquartile range. Differences among groups were compared using one way ANOVA and post-hoc test for parametric data and kruskal-Wallis analysis for variances and Mann-Whitney test for the non-parametric data. The results were statistically significant at $p < 0.05$.

Results

Effect of diabetes and adiponectin gene transfection on metabolic parameters:

As shown in (Table 1) while there was no statistical significant difference in the body mass index between the different groups at the end of the study, diabetes induced a significant increase in the blood pressure in all the diabetic groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle group and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group ($p < 0.05$).

No statistical significant difference was observed in the blood pressure between diabetic-adiponectin group and untreated diabetic and diabetic-vehicle groups ($p > 0.05$).

Also diabetes induced a significant increase in serum glucose, insulin and triglycerides as well as in the HOMA-IR in untreated diabetic, diabetic-vehicle and diabetic-adiponectin groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle group and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group ($p < 0.05$).

Adiponectin gene delivery induced a significant decrease in serum glucose, insulin and triglycerides as well as in the HOMA-IR in diabetic-adiponectin group compared to the untreated diabetic and the diabetic-vehicle groups. Also adiponectin induced significant decrease in serum glucose in non-diabetic-adiponectin group compared to the control group ($p < 0.05$).
Table (1): Effect of Diabetes and adiponectin gene transfection on metabolic parameters.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Non-diabetic-vehicle</th>
<th>Non-diabetic-adiponectin</th>
<th>Untreated diabetic</th>
<th>Diabetic-vehicle</th>
<th>Diabetic-adiponectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (g/cm²)</td>
<td>0.72±0.05</td>
<td>0.69±0.04</td>
<td>0.71±0.04</td>
<td>0.71±0.05</td>
<td>0.74±0.06</td>
<td>0.76±0.06</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>116±4.1</td>
<td>126.88±7.9</td>
<td>120.63±6.2</td>
<td>201±6.6*</td>
<td>203.33±19.6*+</td>
<td>190±10.5*#</td>
</tr>
<tr>
<td>serum glucose (mmol/l)</td>
<td>5.22±0.75</td>
<td>4.8±0.8</td>
<td>4.85±0.72*</td>
<td>9.76±0.7*</td>
<td>9.89±0.5*+</td>
<td>7±0.5*##$</td>
</tr>
<tr>
<td>serum insulin (µU/l)</td>
<td>7±1.1</td>
<td>7.5±0.9</td>
<td>7.03±0.9</td>
<td>17.85±2.4*+</td>
<td>18.57±1.8*+</td>
<td>11.77±0.9*##$</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.65±0.4</td>
<td>1.63±0.3</td>
<td>1.52±0.3</td>
<td>7.7±0.8*</td>
<td>8.19±1.1*+</td>
<td>3.66±0.49##$</td>
</tr>
<tr>
<td>serum triglycerides (mg/dl)</td>
<td>62.7±6</td>
<td>62.5±4.9</td>
<td>63.22±6.5</td>
<td>110.2±9.6*</td>
<td>110.9±10.2*+</td>
<td>85.94±6##$</td>
</tr>
</tbody>
</table>

*: Significant compared to control group.
+: Significant compared to non-diabetic-vehicle group.
#: Significant compared to non-diabetic-adiponectin group.
@: Significant compared to untreated diabetic group.
$: Significant compared to diabetic-vehicle group.

Effect of diabetes and adiponectin gene transfection on serum adiponectin and TNF-α:

At the end of the study diabetes induced a significant decrease in serum adiponectin (Fig. 1, p<0.05) and a significant increase in serum TNF-α (Fig. 2, p<0.05) in the untreated diabetic and the diabetic-vehicle groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle group and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group (p<0.05).

Adiponectin gene delivery induced a significant increase in serum adiponectin (Fig. 1, p<0.05) in the non-diabetic-adiponectin group compared to the control and the non-diabetic-vehicle groups and a significant increase in serum adiponectin in the diabetic-adiponectin group compared to untreated diabetic and diabetic-vehicle groups (Fig. 1, p<0.05). Also adiponectin gene delivery induced a significant decrease in serum TNF-α (Fig. 2, p<0.05) in the diabetic-adiponectin group compared to the untreated diabetic and the diabetic-vehicle groups (p<0.05). Both serum adiponectin and TNF-α of the diabetic-adiponectin group were insignificantly different compared to the control group indicating a beneficial effect of the adrenomedullin gene delivery.
**Effect of diabetes and adiponectin gene transfection on renal functions:**

As observed in (Table 2), diabetes induced a significant increase in blood urea nitrogen (BUN), serum creatinine and urinary microalbumin (p<0.05) in all the diabetic groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group (p<0.05).

Adiponectin gene delivery induced a significant decrease in BUN, serum creatinine and microalbuminuria (p<0.05) in the diabetic-adiponectin group compared to the untreated diabetic and the diabetic-vehicle groups highlighting the effect of adiponectin on diabetic nephropathy.

**Table (2): Effect of diabetes and adiponectin gene transfection on renal functions.**

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Non-diabetic-vehicle</th>
<th>Non-diabetic-adiponectin</th>
<th>Untreated diabetic</th>
<th>Diabetic-vehicle</th>
<th>Diabetic-adiponectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dl)</td>
<td>42.4±9.5</td>
<td>46.46±6.5</td>
<td>40.81±9.2</td>
<td>81.44±13.1*</td>
<td>87.3±15.4++</td>
<td>64.4±3.6*##@$</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.12±0.02</td>
<td>0.12±0.03</td>
<td>0.13±0.05</td>
<td>0.8±0.3 *</td>
<td>0.87±0.21 **</td>
<td>0.3±0.08*##$</td>
</tr>
<tr>
<td>Microalbuminuria (mg/24h)</td>
<td>0.39 (0.06-1)</td>
<td>0.3 (0.06-1.02)</td>
<td>0.11 (0.04-1.04)</td>
<td>10.4 (6-20.5)*</td>
<td>11.2 (8.7-18.3)*+</td>
<td>1.1 (0.5-2.6)*##$</td>
</tr>
</tbody>
</table>

*: Significant compared to non-diabetic group.
+: Significant compared to non-diabetic-vehicle group.
#: Significant compared to non-diabetic-adiponectin group.
@: Significant compared to untreated diabetic group.
$: Significant compared to diabetic-vehicle group.

Results of BUN and serum creatinine are expressed as mean ± SD and results of microalbuminuria are expressed in median and interquartile range.

**Effect of diabetes and adiponectin gene transfection on renal and aortic tissue ROS generation and eNOS expression:**

Diabetes induced a significant increase in ROS generation (Fig. 3) and a significant decrease in eNOS expression (Fig. 4) in both renal and aortic tissues (p<0.05) in all the diabetic groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle group and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group.

Adiponectin gene delivery induced a significant decrease in ROS production (Fig. 3) and a significant increase in eNOS expression (Fig. 4) in both renal and aortic tissues (p<0.05) in the diabetic-adiponectin group compared to the untreated diabetic and the diabetic-vehicle groups indicating that the observed effect is attributed to adiponectin.

**Effect of diabetes and adiponectin gene transfection on renal TGF-β1 expression and pAMPK:**

Diabetes mellitus induced a significant increase in TGF-β1 expression (Fig. 5, p<0.05) associated with a significant decrease in pAMPK expression (Fig. 6, p<0.05) in renal tissue of all the diabetic groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group.

Adiponectin gene delivery induced a significant decrease in TGF-β1 expression (Fig. 5, p<0.05) associated with a significant increase in pAMPK expression (Fig. 6, p<0.05) in renal tissue of the diabetic-adiponectin group compared to the untreated diabetic and the diabetic-vehicle groups highlighting the mechanisms by which adiponectin improves diabetic nephropathy.

**Effect of diabetes and adiponectin gene transfection on aortic VCAM-1 expression:**

Diabetes mellitus induced a significant increase in VCAM-1 expression (Fig. 7) in aortic tissue of all the diabetic groups compared to the control group, in the diabetic-vehicle group compared to the non-diabetic-vehicle group and in the diabetic-adiponectin group compared to the non-diabetic-adiponectin group (p<0.05).

Adiponectin gene delivery induced a significant decrease in VCAM-1 expression (Fig. 7, p<0.05) in aortic tissue of the diabetic-adiponectin group...
compared to the untreated diabetic and the diabetic-vehicle groups highlighting a mechanism by which adiponectin improves diabetic atherosclerosis.

**Histological results of renal specimens:**

Histological examination of renal specimens of the diabetic group (Fig. 8d) revealed characteristics of nephropathy with many abnormal shrunken glomeruli, widening of bowman space, glomerular congestion, basement membrane thickening, vacuolated tubular epithelial cells and mononuclear lymphocytic infiltration compared with normal histological pattern in control group (Fig. 8a).

Normal glomeruli and tubules were observed in both non-diabetic-vehicle (Fig. 8b) and non-diabetic-adiponectin (Fig. 8c) groups without significant histological difference compared to the control group ($p>0.05$).

The diabetic group transfected with adiponectin gene (Fig. 8f) showed amelioration of these renal changes with many normal glomeruli, less congested glomeruli and vacuolated tubular (v) compared to the diabetic (Fig. 8d) and the diabetic-vehicle (Fig. 8e) groups although still significantly different from the control group ($p<0.05$).
### Protective Effects of Adiponectin from Nephropathy & Atherosclerosis

*: Significant compared to non-diabetic group.
+: Significant compared to non-diabetic-vehicle group.
#: Significant compared to non-diabetic-adiponectin group.
@: Significant compared to untreated diabetic group.
$: Significant compared to diabetic-vehicle group.

**Fig. (5):** Effect of diabetes and adiponectin gene transfection on renal transforming growth factor-\(\beta\) 1 (TGF-\(\beta\)1) expression.

**Fig. (6):** Effect of diabetes and adiponectin gene transfection on renal pAMPK expression. Western blot analysis (A) and means (B) of renal pAMPK compared to \(\beta\)-actin in the different studied groups.

**Fig. (7):** Effect of diabetes and adiponectin gene transfection on aortic VCAM-1 expression.
Fig. (8): Effect of diabetes and adiponectin gene transfection on renal tissue histological structure.

A- Control group showing normal glomeruli (G) and tubules (T) (x 400)
B- Non-diabetic-vehicle group showing normal glomeruli and tubules (x 100).
C- Non-diabetic-adiponectin group showing normal glomeruli (G) and tubules (x 100).
D- Diabetic group showing congested glomeruli with widening of bowman space (c) and thickening of basement membrane, necrotic tubular cells with pyknotic nuclei (arrows) and vacuolated tubules (v) and lymphocytic cells infiltration compared to the control group (x400).
E- Diabetic-vehicle group showing many abnormal shrunken glomeruli with widening of bowman space (*), glomerular congestion (C) and necrotic tubular cells with pyknotic nuclei compared to the control group (x 400).
F- Diabetic-adiponectin group showing many normal glomeruli (G) and less congested glomeruli (C) and vacuolated tubular (v) compared to the diabetic and diabetic-vehicle groups (x400).
Discussion

Diabetes is a leading cause of end stage renal disease and is also associated with accelerated atherosclerotic vascular diseases [22]. In this study adiponectin gene delivery to diabetic rats demonstrated a protective effect from both diabetic nephropathy and atherosclerosis by mechanisms related to its metabolic, anti-inflammatory and anti-oxidant actions.

At the end of the current study, diabetic rats developed criteria of diabetic nephropathy as evidenced by the significantly increased serum creatinine, BUN, microalbuminuria and blood pressure together with microscopic diagnosis of basement membrane thickening, tubular cells necrosis and congested glomeruli with lymphocytic cells infiltration compared to the control group. These results agree with previous studies demonstrating similar manifestations of diabetic nephropathy closely linked to vascular and glomerular dysfunctions [23].

Adiponectin gene transfection in the diabetic-adiponectin group at the onset of diabetes (preventive protocol) significantly increased serum adiponectin level and improved renal histology and functions compared to non-injected diabetic group. Such effects were absent in the diabetic-vehicle group indicating a direct action of the adiponectin gene itself. Similarly Yuan et al. [15] demonstrated renal glomerular hypertrophy, mesangial expansion, basal membrane thickening, tubular epithelial cells cavitations and exfoliation, and mononuclear lymphocyte infiltration in diabetic rats with significant amelioration of these changes in adiponectin transfected group.

The initiating steps in diabetic nephropathy were suggested to be intimately related to the metabolic and hormonal squalia of hyperglycemia, dyslipidemia, reactive oxygen species, inflammatory cytokines especially TNF-α and transforming growth factor β1 (TGF-β1) which is a pro-sclerotic cytokine widely associated with the development of fibrosis in diabetic nephropathy [24]. Indeed in the current study, diabetes mellitus induced significant insulin resistance with significant increase in serum glucose, insulin, triglycerides and TNF-α associated with a significant decrease in the serum adiponectin level in all diabetic groups compared to the control group. These results are supported by a previous study performed on Japanese individuals with obesity, type 2 diabetes, and cardiovascular disease, conditions commonly associated with insulin resistance, hyperinsulinemia and increased TNF-α [25].

In the present study, adiponectin was able to significantly improve serum glucose, insulin and triglycerides and subsequently insulin resistance in the diabetic-adiponectin group compared to the untreated diabetic group. The insulin-sensitizing effects of adiponectin have been linked to inhibition of hepatic glucose production [26], increasing fatty acids oxidation, reduction of lipid accumulation in muscle and reduction of cytokines like TNF-α which may cause insulin resistance [27].

It is worth to mention that adiponectin transfection significantly decreased serum TNF-α in the adiponectin-diabetic group compared to the diabetic group and even to a level comparable to that of the control group. These findings are supported by a previous study reporting that adiponectin treatment could suppress the hepatic production of TNF-α [28].

Similar to other inflammatory cytokines, the expression and synthesis of TNF-α is not limited to monocytes, macrophages, and T cells from where it is mainly secreted. Intrinsic renal cells, including hematopoietic mesangial, glomerular, endothelial, dendritic, and renal tubular cells are also able to produce this cytokine [29]. Reported actions of TNF-α on renal cells include the activation of nuclear factor kappa-B (NF-κB), synthesis of cytokines, growth factors, cell adhesion molecules and enzymes involved in the synthesis of other inflammatory mediators and acute phase proteins. This variety of biologic activities results in diverse effects with a significant role in the development of renal damage in diabetes [30]. Adiponectin was reported to reduce TNF-α production after lipopolysaccharide stimulation in macrophages through its ability to suppress NF-κB activity [31].

In the current study, renal specimens from all diabetic groups showed a significant increase in TGF-β1 expression compared to control group in agreement with a similar finding reported in rats with diabetic nephropathy [32]. TGF-β1 expression is increased through hyperglycemia-induced PKC activation, Amadori-albumin, advanced glycation end products (AGEs) and cytokine activation [33].

Transforming growth factor β1 (TGF-β1) exerts its effects via two transmembrane serine/threonine kinase receptors, type I and II, that are co-expressed on mesangial cells and can promote nephropathy by inducing glomerular and tubular changes resulting in progressive thickening of the glomerular basement membrane, expansion of the mesangial matrix, reduction of the glomerular filtration function and increase in proteins excretion [34]. In-
creased TGF-β1 expression in the diabetic kidney has been also recognized to attract monocytes/macrophages and T lymphocytes to inflammatory sites and to mediate the renal actions of high ambient glucose to promote cellular hypertrophy and stimulate extracellular matrix biosynthesis [35], providing additional explanation of the mechanisms by which diabetes can induce nephropathy.

In our findings, adiponectin independently from the vehicle was able to significantly reduce renal TGF-β1 expression in the kidney of the diabetic-adiponectin group compared to the untreated diabetic group although still elevated compared to the control group. These results together with those of Shintaro et al., [36] demonstrating that adiponectin decreased the progression of proteinuria by decreasing TGF-β1 expression in the renal cortex indicate an additional mechanism by which adiponectin can improve diabetic nephropathy.

In this study, renal pAMPK was significantly reduced in the diabetic group compared to control group and a significant increase in renal pAMPK was observed in the diabetic-adiponectin group compared to the untreated diabetic group related to the transfected adiponectin gene and independent from the vehicle. AMPK is a signaling kinase and a critical energy-sensing pathway with important functions to stimulate glucose uptake. In kidney, the AMPK plays a critical role in permeability of podocytes, the major cell type protecting the glomerulus from leaking albumin into the urinary space and its activity in podocytes is regulated by adiponectin [37]. Thus it is likely that the improved microalbuminuria observed in this work by adiponectin is likely caused by its activation of the AMPK signal pathway.

In support to these results, adiponectin administrated to cultured human podocytes, increased activity of AMPK via adiponectin receptor-1 and both adiponectin and AMPK reduced podocyte permeability to albumin and dysfunction, as evidenced by zona occludens-1 translocation to the membrane. One potential pathway by which adiponectin and AMPK activation may provide protection against albuminuria and podocyte permeability is via reduction of oxidant stress [38]. Adiponectin partly via AMPK activation reduced protein levels of the NADPH oxidase-4 (Nox4), the dominant NADPH oxidase in podocytes [39]. Furthermore, Ad / mice treated with adiponectin exhibited normalization of albuminuria, improvement of podocyte foot process effacement, via an AMPK dependent pathway, and reduced glomerular Nox-4 [37].

Indeed our results showed significantly increased ROS generation in the kidney tissue of the diabetic groups with significant decrease of eNOS expression compared to control group (p <0.05). Adiponectin transfection significantly reduced ROS formation and significantly increased renal eNOS expression in agreement with a previous study [36]. These findings in conjunction with the improved renal functions suggest that adiponectin is a key regulator of albuminuria, acting additionally by modulating renal oxidant stress.

Previous experimental and clinical evidence reported increased generation of reactive oxygen species (ROS) is in both types of diabetes [40]. NADPH is the major source of ROS overproduction in diabetes. NADPH-derived ROS mediate Akt/PKB and ERK1/2 activation, kidney hypertrophy, fibronectin expression [41] and is involved in vascular pathology caused by hypercholesterolemia or hypertension [42]. The precise mechanism by which oxidative stress may accelerate the development of renal and vascular complications in diabetes is a role of protein kinase C, AGEs and activation of transcription factors such as NFκB [43,44]. Furthermore, AGEs-modified proteins reduce eNOS expression by increasing mRNA degradation and inhibiting eNOS activity by suppressing eNOS phosphorylation. In contrast, AGEs increase iNOS and NADPH oxidase expression via a p38MAPK and NF-κB–mediated mechanism, thus triggering oxidative stress [45].

In the current study, diabetes induced significant increase of ROS generation and vascular cell adhesion molecule-1 (VCAM-1) expression in the aorta of the diabetic group compared to the control group. Similarly vascular ROS were reported to be increased by diabetes linked to the activation of NFκB, AGE/receptor leading to endothelial dysfunction and up regulation of VCAM-1 by endothelial cells [46,47]. VCAM-1 is a chemotactic and adhesion molecule which recruit monocytes to transmigrate into the subendothelial space, where the monocytes transform to macrophages and phagocytose trapped lipoproteins before developing into foam cells main mediators of atherosclerosis [48].

Liu et al. [49] suggested a role for adiponectin in early atherosclerosis by finding decreased serum C-reactive protein and suppression of VCAM-1 expression in aortic tissue following transfection of hyperglycemic mice with adiponectin adenovirus but the authors didn’t investigate the probable antioxidan effect of adiponectin. In the current study, adiponectin improved VCAM-1 expression in the
adiponectin transfected diabetic group compared to the diabetic group by significantly decreasing serum glucose and triglycerides as well as ROS generation in aortic samples of the diabetes-adiponectin group providing metabolic and antioxidant mechanisms of actions.

It was also reported in another studies that adiponectin reduced TNF-α-mediated expression of the adhesion molecules VCAM-1 in In vitro human endothelial cells [50] and in vivo in apolipoprotein E deficient mice [51]. The present study, provide an additional finding in diabetic rats by demonstrating concomitant reduction of serum TNF-α and aortic VCAM-1 expression by the transfected adiponectin. Thus adiponectin could protect endothelial cells and suppress the formation of foam cells the key step in the pathogenesis of atherosclerosis.

Furthermore in our work a significant decrease of aortic eNOS expression one of the atherogenic risk factor due to reduced availability of endothelial nitric oxide (eNO) was observed in all the diabetic groups compared to the control group. Adiponectin increased expression of eNOS the positive regulator of endothelial functions in the diabetic-adiponectin group compared to the untreated diabetic group in agreement with results of previous studies [52]. Adiponectin accumulate rapidly in the subendothelial space of the injured artery and enhances NO production by endothelial cells not only linked to AMP kinase activation, but is also dependent on signaling through the Akt kinase and its upstream mediator phosphatidylinositol 3-kinase [53]. The produced NO can inhibit leucocyte adhesion and rolling as well as cytokine-induced expression of VCAM-1, effects that are at least in part attributable to inhibition of the transcription factor NF-κB [54].

In conclusion adiponectin had protective effects from diabetic nephropathy and atherosclerosis related to inhibition of ROS generation and up-regulation of eNOS expression in renal and aortic tissues of diabetic rats. In addition, adiponectin stimulated the renal AMPK signal pathway, down-regulated the renal expression of TGF-β1 and decreased VCAM-1 expression in aorta, which may suggest future role for adiponectin in the prevention of these diabetic complications.

References
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