THYMOQUINONE AND PROANTHOCYANIDIN ATTENUATION OF DIABETIC NEPHROPATHY IN RATS

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of morbidity and mortality in diabetic patients. To prevent the development of this disease and to improve advanced kidney injury, effective therapies directed toward the key molecular target are required. Proanthocyanidin (PA) and thymoquinone (TQ) have been reported to be effective in treating DN, while little is known about their mechanism of action and their combination. Aim: This study was designed to investigate the possible beneficial effects of (TQ), (PA) and their combination to attenuate DN in rats. Materials and Methods: Rats were divided into five groups: group 1 (control), group 2 (diabetic untreated), group 3 (diabetic treated with PA), group 4 (diabetic treated with TQ) and group 5 (diabetic treated with PA+TQ). Diabetes was induced in groups 2-5 by a single dose of 65 mg/kg streptozotocin (STZ) in citrate buffer pH 4.5. Two days after STZ treatment, development of diabetes in the experimental groups was confirmed by measuring blood glucose. Rats in group 3 were given PA (250 mg/kg), rats in group 4 were given TQ (50 mg/kg) and rats in group 5 were given PA+TQ (250+50 mg/kg respectively) once a day orally for 12 weeks starting 2 days after STZ injection. Results: In this work, novel data correlate the relation between reactive oxygen species; advanced glycation end products; IL-6 and DN were obtained. Treatment of rats in groups 3-5 with PA, TQ and PA+TQ was significantly increased- the reduced body weight, the
reduced glutathione concentration and activity of superoxide dismutase. The elevated levels of urea, creatinine, nitric oxide, malondialdehyde and IL-6 in group 2 were significantly reduced as a result of the treatment. Conclusion: These findings suggest that PA and TQ treatment exerts a therapeutic protective effect in diabetes by decreasing oxidative stress and attenuating DN. Consequently, TQ and PA may be clinically useful for protecting diabetic kidney against oxidative stress.

INTRODUCTION

Diabetes is characterized by chronic hyperglycemia which is highly correlated to the initiation and progression of diabetic nephropathy and generation of oxidative stress (1, 2). Additionally, glucose can undergo autooxidation, resulting in the generation of intermediates, leading to production of reactive intermediates and may form adduct in proteins to form advanced glycation end products (AGEs) or Amadori products (2). Recently, a close association between oxidative stress and inflammation in the pathologies of diabetes was reported (3, 4).

Diabetic nephropathy is characterized by changes in both glomerular and tubular structure and function. Most studies have focused on alterations in the glomerulus, including abnormalities in glomerular permeability and capillary pressure, glomerular hyperplasia or hypertrophy and increase in mesangial volume (5).

Hyperglycemia induces oxidative insult in renal tubular epithelial cells and that injury initiates tubulointerstitial fibrosis, a characteristic feature of diabetic nephropathy, which then progressively results in renal failure (6, 7). Moreover, high glucose-exposed renal cells have been used to investigate the cellular and molecular mechanisms of diabetic nephropathy (8-10).

As one of the main causes of end-stage renal disease, the prevention and treatment of diabetic nephropathy in early stage, and the slowing down of diabetic nephropathy progression are of utmost importance and are topics of several ongoing research studies. Nutraceuticals endowed with antioxidant-anti-inflammatory properties may offer an opportunity of integrative treatment for this condition.
The ability of antioxidants to protect against the effects of hyperglycemia in vitro, along with the clinical benefits often reported following antioxidant therapy, supports a causative role of oxidative stress in mediating and/or worsening these abnormalities (11). Proanthocyanidin (PA) is a polyphenolic compound and has been reported to have anti-cancer (12) and cardioprotective effects (13), to protect against ulcers and gastric mucosal injury (14), to have a protective effect against diabetic retinopathy in streptozotocin-induced diabetic rats (15), to ameliorate diabetic macrovascular complications (16) and to protect against cisplatin-induced nephrotoxicity (17). However, the underlying protective mechanisms of PA against diabetic nephropathy have not been fully delineated.

Thymoquinone (TQ), the main constituent of the volatile oil from Nigella sativa seeds, is reported to possess strong anti-oxidant properties. Previous studies have demonstrated that TQ has a considerable protective effect against oxidative damage induced by a variety of free radical-generating agents, including doxorubicin induced cardiotoxicity (18). TQ has an analgesic and anti-inflammatory effect (19), protective effect against chemical induced carcinogenesis, and an inhibiting action on eicosanoids generation and membrane lipid peroxidation. Khan and Sultana proved that thymoquinone is a potent chemopreventive agent and suppresses Fe-NTA-induced oxidative stress, hyperproliferative response and renal carcinogenesis in Wistar rats (20).

To the best of my knowledge there is no previous work comparing the beneficial effects of TQ, PA and their combination in attenuating DN. In the present work, the possible beneficial role of PA, TQ and their combination to attenuate DN in rats was examined. The protective actions of PA and TQ against oxidative stress and inflammation using diabetic rat model was studied. Urea, creatinine, IL-6, lipid peroxidation (MDA), superoxide dismutase (SOD), nitric oxide (NO), and reduced glutathione (GSH) levels were measured in diabetic rats to clarify the mechanism by which the inhibitory effect of PA and TQ act on diabetic nephropathy related to oxidative stress and inflammation. Novel data correlate in vivo the role of MDA, IL-6,
AGEs in the progression of DN and the effect of PA, TQ, and their combination were obtained.

**MATERIALS & METHODS**

*Induction of experimental diabetes*

Nine week-old 200±20 g male albino rats (n=50) were housed in cages and received normal rat chow and tap water *ad libitum* in a constant environment (room temperature 28±2°C, room humidity 60±5%) with a 12-h light, 12-h dark cycle. The animals were kept under observation for one week prior to the start of the experiments. All procedures were done according to the Animal Ethics Committee. Ten rats were randomly selected as control group (group 1, n=10), which received a single tail vein injection of 0.1 mol/l citrate buffer only. The other 40 rats received a single dose of streptozotocin (STZ) (Sigma-Aldrich, Deisenhofen/Germany) through the indwelling catheters over 2 min, at a fixed dose of 65 mg/kg. Administration of STZ was performed after an 18-h overnight fast in conscious animals. STZ was weighed out in individual portions and dissolved in sodium citrate buffer pH 4.5, to a concentration of 65 mg/ml immediately before injection. Vomiting was seen in all animals during the first hours after administration of STZ. Only rats with blood glucose higher than 250 mg/dl after two days were considered as being diabetic in the fasting state. Blood glucose was measured by using Accu Check sensor Analyzer (Roche, Mannheim, Germany). Eight rats with blood glucose levels lower than 200 mg/dl were excluded from the study. All treatments were carried out two days after STZ had been injected.

**Experimental protocols**

All rats were divided into five groups. The groups were divided as follows: group 1, control rats (n=10); group 2, untreated diabetic rats (n=8); group 3, PA treated diabetic rats {PA, Alpha medicine, Cairo, Egypt, 250 mg/kg body weight/day [15], n=8}; group 4, TQ treated diabetic rats {TQ, Sigma/Aldrich (Deisenhofen, Germany) 50 mg/kg body weight/day [21], n=8}; and group 5, TQ+PA
Attenuation of Diabetic Nephropathy

treated diabetic rats [50+250 mg/kg/day respectively, (n=8)]. PA was
given in normal saline solution by intragastric administration for 12
weeks starting from day two after STZ administration (21). At the end
of the experiments, the final body weight of the various groups was
recorded. Then, animals were fasted overnight (18 h) and then
anesthetized intraperitoneally with 10% chloral hydrate 350 mg/kg
body weight (15). Blood was collected directly from the heart of each
animal. Serum was used for the determination of urea by
diacetylmonoxime (DAM) reagent using commercially available kit;
NO was measured by Griess reagent and creatinine was assayed by
alkaline-picric acid method using diagnostic kit of Bio-diagnostic
(Cairo, Egypt). IL-6 concentration was determined by using ELISA
kit from Promocell (Heidelberg, Germany), according to the
instructions of the manufacturer.

Kidney homogenate preparation.

To perform the biochemical analysis on the kidney
homogenate, kidneys from different groups were dissected and rinsed
thoroughly with ice-cold phosphate-buffered saline (PBS) to remove
blood components. Later, they were blotted dry and frozen
immediately in liquid nitrogen. Every kidney tissue was cut into small
pieces and washed by PBS. Moreover, it was ground in a
homogenization buffer {0.05 M Tris-HCl pH 7.9, 25% glycerol, 0.1
mM EDTA, and 0.32 M (NH₄)₂SO₄} containing a protease inhibitor
tablet (Roche, Mannheim, Germany). The lysates were homogenized
on ice using Polytron homogenizer (Brinkmann Instruments,
Cincinnati, OH, USA). The solution was sonicated (Hielscher
Ultasonics, Teltow, Germany) in an ice bath to prevent overheating
for 15 seconds followed by centrifugation at 12000 rpm, 4°C for 5
minutes. The supernatant was aliquotted and stored at −80°C and
assayed for protein concentration using BCA kit (Pierce, Rockford,
IL, USA) using albumin diluted in lysis buffer as standard (24).

Determination of reduced glutathione (GSH)

Reduced glutathione (GSH) plays a central role in protecting
mammalian cells against damage incurred by free radicals, oxidants,
and electrophiles. GSH was measured by colorimetric end point assay using dithionitrobenzoic acid method as described by Moron et al.\textsuperscript{(25)}. GSH concentration was expressed as µmol/g protein using GSH Kit from Roche (Mannheim, Germany) according to the instructions of the manufacturer.

**Determination of lipid peroxidation**

The concentration of TBARS was determined as MDA according to previous works of Sayed and Okhawa et al.,\textsuperscript{(24, 26)} The concentration of MDA was expressed in terms of nmol/g protein.

**Determination of IL-6**

IL-6 antigen was determined by enzyme-linked immunosorbent assay (ELISA). The ELISA for determination of IL-6 was performed using a commercially available kit from Promocell (Heidelberg, Germany) according to the instructions of the manufacturer.

**Determination of SOD activity**

SOD activity was determined as the volume of homogenate that is required to scavenge 50% of the superoxide anion generated from the photo illumination of riboflavin in the presence of EDTA (1 unit of SOD activity)\textsuperscript{(27)}. The activity was determined using the SOD available kit from Biodiagnostic (Biodiagnostic Co., Cairo, Egypt) according to the instructions of the manufacturer.

**Determination of Nitric Oxide (NO)**

NO as a free radical is relatively unstable in oxygenated solutions where it is rapidly and spontaneously reacts with molecular oxygen to yield a variety of nitrogen oxides. It was demonstrated that the only stable products formed by spontaneous decomposition of NO in oxygenated solutions are nitrites and nitrates, thus they were measured as indicators for NO production. Kidney nitrite content was measured after reduction of nitrates to nitrites with Aspergillus nitrate reductase (Sigma, Deisenhofen, Germany)\textsuperscript{(28)}. 
**Statistical analysis**
Statistical analysis was performed using SPSS software II (Chicago, IL, USA). The effect of each parameter was assessed using the one way analysis of variance. Individual differences between groups were examined using Dunnett’s test and those at $p < 0.05$ were considered statistically significant.

**RESULTS**
The weights of the rats at the beginning of the study were similar in all groups. At the end of the treatment, diabetic animals presented a significant weight loss. The initial and final body weights were not different in control rats, PA and/or TQ treated diabetic rats. As a result of STZ injection, diabetic untreated animals exhibited a significant hyperglycaemia ($p < 0.01$). Treatment with PA and TQ caused a sharp and significant decrease in the elevated serum glucose ($p < 0.05$) (Table 1) in groups 3-5. Also, PA and/or TQ treatment (groups 3-5) significantly ($p < 0.05$) decreased the elevated serum NO, urea and creatinine in diabetic untreated rats (group 2). Levels of serum IL-6 was significantly decreased ($p < 0.01$) in the PA and/or TQ treated groups as shown in Table I.

**Table I.** Body weight (g), serum glucose (mg/dl), serum urea (mg/dl), serum creatinine (mg/dl), Serum NO (µmol/l) and serum IL-6 (pmol/ml) levels of group 1 (control), group 2 (diabetic untreated), group 3 (diabetic treated with PA), group 4 (diabetic treated with TQ) and group 5 (diabetic treated with PA+TQ)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>group 1</th>
<th>group 2</th>
<th>group 3</th>
<th>group 4</th>
<th>groups 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight</td>
<td>200±4</td>
<td>208±2</td>
<td>210±3</td>
<td>210±4</td>
<td>212±5</td>
</tr>
<tr>
<td>Final body weight</td>
<td>205±3</td>
<td>148±4</td>
<td>211±5</td>
<td>212±3</td>
<td>216±5</td>
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<tr>
<td>Initial serum glucose</td>
<td>99±5</td>
<td>97±5</td>
<td>96±4</td>
<td>99±3</td>
<td>98±2</td>
</tr>
<tr>
<td>Final serum glucose</td>
<td>95±7</td>
<td>295±12</td>
<td>170±10</td>
<td>168±10</td>
<td>127±10</td>
</tr>
<tr>
<td>Blood Urea</td>
<td>29±1.33a</td>
<td>58±2.6b</td>
<td>37±1.4c</td>
<td>38±1.1c</td>
<td>30±1.2c</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>0.45±0.25a</td>
<td>0.95±0.07b</td>
<td>0.61±0.04</td>
<td>0.61±0.02c</td>
<td>0.50±0.01c</td>
</tr>
<tr>
<td>Serum NO</td>
<td>4.74±0.50a</td>
<td>9.3±1.5b</td>
<td>5.45±0.4c</td>
<td>5.4±0.5c</td>
<td>4.82±0.4c</td>
</tr>
<tr>
<td>Serum IL-6</td>
<td>65±2.6a</td>
<td>540±22b</td>
<td>80±7.5a</td>
<td>76±2.4c</td>
<td>69±5.4c</td>
</tr>
</tbody>
</table>

Statistical analysis used one-way ANOVA with student's $t$-test. Values are expressed as means ± SD, n = 10 for A and 9 for B, C groups.
Measurement of IL-6 level in kidney homogenate (Figure 1) demonstrated a significant increase in IL-6 level in group 2 (diabetic untreated group) ($p < 0.01$) which contributes to the renal injury (29). This high level of IL-6 in the kidney homogenate was significantly reduced in groups 3-5 (PA, TQ and PA+TQ treated groups) compared with group 2 ($p < 0.01$).

The degree of lipid peroxidation in the kidney homogenate was determined as MDA in Figure 2. MDA level was significantly increased in group 2 ($p < 0.01$). These high levels of MDA were significantly reduced as a result of PA and TQ treatment ($p < 0.05$).

In Figure 3, the level of GSH was determined. In group 2 GSH was significantly reduced as a result of STZ treatment ($p < 0.05$). In groups 3-5, GSH was further significantly increased as a result of PA and TQ treatment ($p < 0.05$). Similar data were obtained in Figure 4 for the SOD activity. As a result of STZ treatment, SOD activity in group 2 was significantly reduced. Treatment of animals with PA and TQ in groups 3-5 significantly increased the activity of SOD ($p < 0.05$).
Figure (2) Effect of 250 mg/kg PA, 50 mg/kg TQ and 250 mg/kg PA+50 mg/kg TQ on the level of MDA in the kidney homogenate. Vertical bars represent mean ± SEM from five replicate experiments.

* denotes P=0.01 vs control group, ** denotes p<0.05.
Ahmed Radwan & Abdulrahman Al-Malk

![Graph 1](image1)

**Figure (3)** Effect of 250 mg/kg PA, 50 mg/kg TQ and 250mg/kg PA+50 mg/kg TQ on the level of GSH in the kidney homogenate. Vertical bars represent mean ± SDM from five replicate experiments.

**P** means **P** = 0.01 vs control group, * denotes **p** = 0.05, *** denotes **p** = 0.01 vs diabetic untreated group.

![Graph 2](image2)

**Figure (4)** Effect of 250 mg/kg PA, 50 mg/kg TQ and 250mg/kg PA+50 mg/kg TQ on the activity of SOD in the kidney homogenate. Vertical bars represent mean ± SDM from five replicate experiments. * denotes **p** = 0.05 vs control group,

**P** means **P** = 0.05 vs diabetic untreated group.
DISCUSSION

Metabolic activity within the nephron produces a large amount of reactive oxygen species (ROS) that are counterbalanced by a large number of antioxidant enzymes and free radical scavenging systems. ROS mediate many negative biological effects, including peroxidation of cell membrane lipids, oxidation of proteins, renal vasoconstriction and damage to DNA. Unfortunately, hyperglycemia tips the balance towards production of ROS, most of which seem to be generated in the mitochondria \(^{24,27}\). Glucose metabolism through harmful alternate pathways, such as via protein kinase C activation and advanced glycation end-product formation is reported to contribute to the development of diabetic nephropathy \(^{30}\).

In the present study, PA, TQ and their combination were tested \textit{in vivo} to examine their effect to ameliorate the progression of diabetic nephropathy related to their anti-oxidative actions in diabetic rats. The obtained results indicated that diabetic untreated rats showed body weight reduction during the 12 weeks experimental period, suggesting that these animals were undergoing growth retardation due to the obstruction of glucose uptake caused by the lack of insulin following STZ injection, but treatment with PA, TQ, and their combination increased the body weight from the initial value. The obtained data were in line with previous work \(^{7,21,31}\).

The levels of serum glucose, serum urea, and serum creatinine observed in untreated diabetic rats were increased beyond those of untreated control rats which contributed to the STZ injection and the diabetic nephropathy in rats under investigation. However, TQ, PA and TQ+PA treated diabetic rats showed a significant reduction in these elevated levels compared with untreated diabetic rats. Therefore, we supposed that TQ and PA could have a reno-protective effect which correlates with the body weight gain. Also levels of serum NO were significantly reduced in the PA and TQ treated groups. All these data were in line with previous work \(^{15,32,33}\).

As a result of diabetes, ROS and oxidative stress increases. These ROS react with proteins forming AGEs. Moreover, AGEs interact with their receptors RAGE and activate the nuclear
transcription factor kaba B (NF-κB) and its controlled genes like IL-6 (7). To test this hypothesis, MDA and IL-6 levels were measured. The renal MDA level, an index of lipid peroxidation and oxidative stress, was significantly increased in the diabetic untreated rats compared with control group and these data were in line with a previous study (34).

Inflammatory cytokines also contribute to the development and progression of diabetic nephropathy. Concentrations of all these cytokines were increased in models of diabetic nephropathy and seemed to affect the disease via multiple mechanisms (35, 36). IL-6 has a strong association with the development of glomerular basement membrane thickening as well as possible relationships with increased endothelial permeability and mesangial cell proliferation. Treatment of diabetic rats with PA, TQ, and their combination was significantly reduced the elevated levels of IL-6 which could be attributed to the antioxidative and anti-inflammatory effects of both. This reduction in the levels of IL-6 could play a major role in the attenuation of the progression of diabetic nephropathy and subsequently the significant reduction of the serum urea and creatinine in PA, and TQ treated groups. The obtained data were in line with previous studies (21, 37, 38).

It was observed that PA and TQ have anti-oxidant properties as evidenced by the significant increase in GSH and SOD activity and reduction of lipid peroxidation and NO levels in the diabetic treated groups (groups from 3-5), so it might improve the function of β-cells in the diabetic rats, and influenced insulin effects by directly acting on specific components of the insulin-signaling transduction pathway.

In all trials, treatment with PA (group 3) and TQ (group 4) have similar effect on all biomarkers investigated. On the other hand, combination of both PA and TQ with their native concentration gave a better effect on the reduction of diabetic nephropathy symptoms. The effect of the combination was significantly different from each individual treatment.

**CONCLUSION**

In conclusion, the present work demonstrates that in diabetic rats, TQ, and PA showed anti-oxidative and anti-inflammatory properties to
reduce lipid peroxidation, total ROS generation, NO• and IL-6. In addition the activity of antioxidant enzymes like GSH, SOD was increased. We therefore postulate that the dual effect of ameliorating oxidative stress and inflammation might be the possible mechanism by which TQ and PA act on diabetic nephropathy in diabetic rats. Further detailed mechanistic studies are necessary to unveil the beneficial role of TQ and PA as a useful natural, anti-diabetic antioxidant.

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REFERENCES


Attenuation of Diabetic Nephropathy


