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# Metformin attenuates streptozotocin-induced diabetic nephropathy in rats through modulation of oxidative stress genes expression

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ABSTRACT

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or action. One of the most important complications of this metabolic disease is diabetic nephropathy. Hyperglycemia promotes oxidative stress and hence generation of reactive oxygen species (ROS), which is known to play a crucial role in the pathogenesis of diabetic nephropathy. Recent studies have established that metformin, an oral hypoglycemic drug, possesses antioxidant effects. However, whether metformin can protect against diabetic nephropathy has not been reported before. The overall objectives of the present study are to elucidate the potential nephroprotective effect of metformin in a rat diabetic nephropathy model and explore the exact underlying mechanism(s) involved. The effect of metformin on the biochemical changes associated with hyperglycemia induced by streptozotocin was investigated in rat kidney tissues. In addition, energy nucleotides (AMP and ATP), and Acetyl-CoA in the kidney homogenates and mitochondria, and the mRNA expression of oxidative stress and pro-inflammatory mediators were assessed. Our results showed that treatment of normoglycemic rats with metformin caused significant increase in ATP, Acetyl-CoA, and CoA-SH contents in kidney homogenates and mitochondria along with profound decrease in AMP level. On the other hand, treatment of diabetic nephropathy rats with metformin normalized all biochemical changes and the energy status in kidney tissues. At the transcriptional levels, metformin treatment caused significant restoration in diabetic nephropathy-induced oxidative stress mRNA levels, particularly GSTα, NQO1, and CAT genes, whereas inhibited TNF-α and IL-6 pro-inflammatory genes. Our data lend further credence for the contribution of metformin in the nephroprotective effect in addition to its well known hypoglycemic action.


## 1. Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or action. One of the most important complications of this metabolic disease is diabetic nephropathy (DN), which is now considered the principle cause of end-stage renal failure [1]. The functional disorders in nephropathy are manifested by early microalbuminurea, renal hyperfiltration, and increased permeability to macromolecules and protein urea [1]. Chronic hyperglycemia results in the development and accumulation of advanced glycated end products (AGEs) in the kidney of diabetic patients; in which glycation of proteins is regarded as one of the major contributors to the development and progression of DN [1,2].

Hyperglycemia promotes oxidative stress and hence generation of reactive oxygen species (ROS), which is known to play a crucial role in the pathogenesis of DN [3], in which increased ROS production and oxidative stress cause cell membrane damage, enzymes inactivation, apoptosis, and endogenous antioxidant altered gene expression [4]. In this context, it has been recently demonstrated that activation of intracellular antioxidants genes such as NAD(P)H quinone oxidoreductase (NQO1), glutathione *S*-transferase-α (GSTα), and heme oxygenase-1 (HO-1), suppressed hyperglycemia-induced ROS and metabolic dysfunction in human microvascular endothelial cells [5]. In addition, the level of glutathione (GSH), an endogenous antioxidant, superoxide dismutase, an enzyme that inactivate superoxide radicals, and catalase (CAT), an enzyme responsible for the removal of $H_2O_2$, are decreased significantly, whereas the serum level of malondialdehyde was increased, in uncontrolled diabetics in both humans [3] and animals [6] studies.

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The direct involvement of oxidative stress in the pathogenesis of DN is evidenced by the observations that administration of antioxidants such as vitamin C, lipoic acid, vitamin E, or glutathione causes a significant improvement of insulin sensitivity in insulin-resistant diabetics [7–9]. Therefore, one major defense mechanism against oxidative stress-induced DN is mediated through the induction of antioxidant genes and/or the inhibition of oxidative genes.

Drugs that inhibit angiotensin II play a significant role in the prevention of DN. Furthermore, insulin sensitizers such as troglitazone and rosiglitazone significantly reduce microalbuminurea independent glycemia [10–12]. Among those drugs, metformin, an aminoguanidine derivative hypoglycemic agent, is widely used in the management of type II DM. Several studies have shown that metformin has insulin sensitizing effect, decreases insulin levels, and controls hyperglycemia [13–15]. Furthermore, it has been reported that metformin causes a significant decrease in the formation of AGEs [16], a mild inhibition of respiratory complex 1 [17], and microalbuminurea in diabetic patients [18]. In addition, metformin possesses a direct scavenging effect against oxygenated free radicals and ROS generated in aortic endothelial cells through the reduction of both NAD(P)H oxidase, inhibition of protein C kinase activity, and/or the mitochondrial respiratory chain pathways [19,20].

In the view that DM is a chronic inflammatory state associated with induction of pro-inflammatory cytokines and chemokine genes [21], experimental studies have consistently reported that mRNA encoding tumor necrosis factor (TNF-α) and protein levels increased in glomerular and proximal tubule cells from diabetic rats [22,23]. Furthermore, it has been reported that that serum levels of interleukin (IL-6) were significantly higher in patients with type II DN than the levels observed in diabetic patients without nephropathy. These investigations demonstrated a significant role of TNF-α and IL-6 in the development of DN and renal dysfunction [23].

Unfortunately, the possibility that metformin attenuates DN have not studied yet. The overall objectives of the present study are to elucidate the potential nephroprotective effect and explore the exact underlying mechanism(s) involved in such effect. Accordingly, in the current study we have investigated the effect of metformin on the modulation of several oxidative stress and pro-inflammatory markers at the biochemical and gene expression levels. The results of the current study clearly showed that administration of metformin to STZ-induced DN rats reduces oxidative stress markers and improves renal antioxidant enzyme activity which highlights its potential nephroprotective effect against DN.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar Albino rats aging approximately 3 months ranging in weight from 200 to 230 g, were obtained from the Animal Care Center, College of Medicine, King Saud University, Riyadh, Saudi Arabia. The animals were housed in metabolic cages under controlled environmental conditions (25 °C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. The protocol of this study has been approved by Research Ethics Committee of College of Medicine, King Saud University, Riyadh, Saudi Arabia.

### 2.2. Materials

Metformin, streptozotocin (STZ), acetyl-CoA, CoA-SH, adenosine triphosphate and adenosine diphosphate, 2′,7′-dichloro-fluorescein-diacetate (DCF-DA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TRIzol reagent was purchased from Invitrogen® Co. (Grand Island, NY). The High-Capacity cDNA reverse transcription kits and SYBR® Green PCR Master Mix were purchased from Applied Biosystems® (Foster City, CA, USA). Metformin was dissolved in normal saline and administered orally at low dose (100 mg/kg) and high dose (500 mg/kg) according to previous published studies [24–26]. All other chemicals used were of the highest analytical grade.

### 2.3. Experimental design

A total of 60 adult male Wistar Albino rats were divided randomly into 6 groups of 10 animals each. Rats in the first group were injected with a single dose of normal saline (2.5 mg/kg, i.p.) and served as normoglycemic control (NG). The second and third groups received metformin daily for 8 weeks at low (100 mg/kg) and high dose (500 mg/kg), respectively. Animals in the fourth group received a single dose of STZ (65 mg/kg, i.p.) and served as a control DN group; whereas rats in groups 5 and 6 received a single dose of STZ (65 mg/kg) followed by meformin 100 and 500 mg/kg for 8 weeks, respectively.

At the end of the treatment protocol, animals were anesthetized with ether and blood samples were drawn from the orbital venous plexus after 4 and 8 weeks. Blood was collected to clot at room temperature and then serum was separated by centrifugation for 5 min at 300*g* and stored at −20 °C until analysis. All animals were then sacrificed by decapitation and their kidneys were rapidly excised, washed with saline, blotted with a piece of filter paper, and divided into four segments. One segment was homogenized, using a Branson sonifier (250 VWR Scientific, Danbury, Conn., USA), the second and third segments were utilized for isolation of mitochondria and gene expression, respectively, whereas the fourth segment was fixed in formalin for histopathology study.

### 2.4. Assessment of blood urea nitrogen, serum creatinine, and microalbuminurea

The concentrations of blood urea nitrogen (BUN) and serum creatinine (SCr) were measured spectrophotometerically [27,28], respectively. For microalbuminurea, metabolic cages were used to collect 24-h urine samples at the end of 4 and 8 weeks after treatment began. Urine samples were centrifuged at 1400 rpm for 5 min; after proper dilution, and the supernatant was collected to determine 24-h urinary protein levels using a colorimetric methods as described previously [29]. Microalbuminurea was assessed by measuring the Urinary Albumin Excretion Rate (UAER) using the following formula: UAER (mg/24 h) = 24 h total volume of urine (L) × urinary protein levels (mg/L).

### 2.5. Determination of adenosine triphosphate and adenosine monophosphate in kidney tissues

Adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were determined in kidney tissue homogenate using HPLC method [30]. In brief, kidney tissue was homogenized in ice-cold 6% perchloric acid, centrifuged at 300*g* for 15 min at 0.5 °C, and the supernatant fluid was injected into HPLC after neutralization to pH 6.7. Chromatographic separation was performed at a flow rate of 1.2 ml/min, using ODS-Hypersil, 150 × 4.6 mm I.D., 5 μm column (Supelco SA, Gland, Switzerland) and 75 mM ammonium dihydrogen phosphate as mobile phase. The peak elution was followed at 254 nm.