Effect of the Combination Between Metformin and Vitamin D on Experimentally-Induced Diabetic Nephropathy

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Abstract

Background: Diabetic nephropathy (DN) is one of the serious complications of diabetes caused by angiopathy of capillaries in the renal glomeruli. Metformin is an anti-diabetic agent that was shown to have renoprotective effects. Vitamin D was proven to have antioxidant and anti-inflammatory properties that may ameliorate DN. Aim: To study the effect of each of vitamin D and metformin alone and in combination on streptozotocin (STZ)-induced DN. Methods: Sixty male Wistar rats were divided into 6 equal groups: Control untreated group, STZ group, STZ + Metformin group, STZ + Vitamin D group, STZ+ Peanut oil group and STZ + Vitamin D + Metformin group. Kidney weight/body weight ratio, serum fasting glucose, C-reactive protein (CRP), glycosylated hemoglobin (Hb), blood urea, serum creatinine and creatinine clearance were determined. A part of the kidney was homogenized for determination of tissue catalase (CAT), glutathione peroxidase (GPx) and tumor necrosis factor alpha (TNF- α). The other part was subjected to histopathological and immunohistochemical examination. Results: Vitamin D and metformin alone and in combination induced significant increase in creatinine clearance, tissue CAT and GPx with significant decrease in serum fasting glucose, kidney weight/body weight ratio, glycosylated Hb, blood urea, serum creatinine, tissue TNF- α and serum C-reactive protein and alleviated the histopathological changes compared to STZ group. This effect was significant in vitamin D/metformin combination group compared to the use of either vitamin D or metformin alone. **Conclusion:** Vitamin D/metformin combination might represent a beneficial therapeutic modality for treatment of DN.

Keywords: Diabetic Nephropathy; Metformin; Vitamin D; Rats

Introduction

Diabetic nephropathy (DN) is an important complication of diabetes mellitus that usually occurs in a large proportion of diabetic patients. Its mechanisms are still not well known but may be attributed to elevation of the levels of glycosylated proteins and the hemodynamic changes that occur within the kidney tissue [1]. Chronic hyperglycemia leads to increased production of reactive oxygen species and attenuates the anti-oxidant mechanisms. Moreover, hyperglycemia leads to formation of advanced glycosylation end products (AGEs) and activation of protein kinase C, resulting in increased production of the inflammatory mediators such as tumor necrosis factor alpha (TNF- α) and interleukin 10 (IL-10) [2].

Metformin is one of the antidiabetic drugs that reduces intestinal absorption of glucose, increases its anaerobic metabolism, decreases glucagon release and improves the sensitivity of insulin receptors in the peripheral tissues [3]. Metformin was proven to decrease the hazardous effects of oxidative stress in various tissues of the body. Also, metformin has the ability to modulate the expression of the proinflammatory cytokines at both the biochemical and gene expression levels [4].

Vitamin D is an important regulator of calcium and phosphorus homeostasis [5]. Many studies have shown that the active form of vitamin D can improve the survival rate of patients with chronic kidney disease, and the mechanisms don't depend only on modulation of blood levels of calcium, phosphorus and parathyroid hormone. Furthermore, Tan et al [6], reported that 1,25-(OH)₂ vitamin D₃ can reduce glomerulosclerosis and interstitial fibrosis. However, the mechanisms of this protective effect in DN, have not yet been fully understood. The aim of this study was to investigate the effect of each of vitamin D and metformin alone and in combination on streptozotocin (STZ)-induced DN in rats.

Materials and Methods

Chemicals and drugs

Both streptozotocin (STZ) and metformin were obtained from Sigma Chemical Company (St. Louis, MO, USA). STZ was dissolved in 0.1 M citrate buffer (PH 4.5). Metformin was dissolved in normal saline. Vitamin D (0.25 μ g/tablet, Shanghai Roche Pharmaceuticals, Shanghai, China) was dissolved in peanut oil. Commercially available kits were purchased from Crescent diagnostics for estimation of blood glucose, blood urea and serum creatinine. Highly sensitive C-reactive protein kits were purchased from BioSystems.

Animals and groups

The present study was carried out on 60 male Wistar rats weighing about 150-200 grams. All the experiments were conducted according to the National Research Council's guidelines. The handling of the animals was followed according to Helsinki declaration of animal ethics. Rats were divided into six equal groups each of 10 rats as follows:

Group I: Control untreated group.

Group II: Received a single intraperitoneal injection of STZ in a dose of 65 mg/kg [2].

Group III: Received a single intraperitoneal injection of STZ (65 mg/kg) then received metformin orally by gastric tube in a dose of 500 mg/kg/day [2].

Group IV: Received a single intraperitoneal injection of STZ (65 mg/kg) then received 0.03 μ g/kg vitamin D in 0.05 mL peanut oil once daily orally by gastric tube [5].

Group V: Vehicle control group, received a single intraperitoneal injection of STZ (65 mg/kg) then received equivalent volume of peanut oil once daily orally by gastric tube.

Group VI: Received a single intraperitoneal injection of STZ (65 mg/kg) then received metformin orally by gastric tube in a dose of 500 mg/kg/day concomitantly with 0.03 μ g/kg vitamin D in 0.05 mL peanut oil once daily orally by gastric tube.

Determination of the biochemical parameters

Blood samples were collected from the retro-orbital sinus of rats one week after STZ injection and were used for estimation of serum fasting glucose level. Rats with fasting glucose level above 200 mg/dL were considered diabetic. Drugs were administered one week after STZ injection and continued for 12 weeks.

At the end of the work, rats were kept in special metabolic cages for 24 hours urine collection. After fasting overnight, samples of the blood were taken from the retro-orbital sinus of rats. Glycosylated hemoglobin (Hb) concentration was measured colorimetrically in 50 μ L of whole blood after being separated from the unbound hemoglobin using an affinity resin [7]. Blood samples were kept in glass tubes in a water bath for 30 minutes at 37°C until blood clotting occurred. Then, blood samples were centrifuged for 20 minutes for separation of serum to estimate serum fasting glucose level according to Trinder [8] and C-reactive protein (CRP) using CRP kits according to the instructions of the manufacturer. Blood urea was measured according to Patton and Crouch [9]. Serum and urinary creatinine was measured according to Henry [10]. Creatinine clearance was measured by using the following formula [11]:

Creatinine clearance (ml/min) = Urinary creatinine (mg/dl) X 24 hrs urine volume (ml)

Serum creatinine (mg/dl) X 1440

Rats were killed by decerebration and both kidneys were excised and weighed for estimation of kidney weight/body weight ratio. The kidneys were divided into two parts; one part was homogenized for determination of tissue catalase (CAT) according to Higgins et al [12], tissue glutathione peroxidase (GPx) using kits supplied by Crescent Diagnostics Co. according to the instructions of the manufacturer and tissue tumor necrosis factor-alpha (TNF- α) using rat TNF- α ELISA kits supplied by RayBiotech, Inc. according to the instructions of the manufacturer. The other part of the kidney was examined by a pathologist.

Histopathological and immunohistochemical examination

Sections from the kidney were fixed in 10% formalin, embedded in paraffin, stained with hematoxyline and eosin (H&E) and examined under light microscope and apoptotic indices were determined. The morphological criteria for apoptotic bodies were applied in this study according to Staunton and Gaffney [13]. A piece

of the renal tissue was homogenised and proteins were extracted and stored at -80 °C. The activity of caspase-3 was determined spectrophotometrically by the method described by Gurtu et al. [14].

Statistical analysis

For statistical analysis, the SPSS (version 16.0) package program was used. Results were expressed as mean ± standard error of mean (S.E.M.). To compare between the different studied groups, one-way analysis of variance (ANOVA) test followed by LSD test were used. The results were considered statistically significant when p-value less than 0.05.

Results

STZ administration induced significant decrease in tissue CAT, GPx and creatinine clearance with significant increase in serum fasting glucose, kidney weight/body weight ratio, blood urea, serum creatinine, glycosylated Hb, tissue TNF-α and serum CRP compared to the control untreated group.

Administration of peanut oil to STZ-treated rats resulted in non-significant effect on creatinine clearance, tissue CAT, tissue GPx, serum fasting glucose, kidney weight/body weight ratio, blood urea, serum creatinine, glycosylated Hb, tissue TNF-α and serum CRP compared to STZ group.

Administration of Vitamin D or metformin alone induced significant increase in tissue CAT, GPx and creatinine clearance with significant decrease in serum fasting glucose, kidney weight/body weight ratio, blood urea, serum creatinine, glycosylated Hb, tissue TNF-α and serum CRP compared to STZ group.

Vitamin D/metformin combination induced significant increase in tissue CAT, GPx and creatinine clearance with significant decrease in serum fasting glucose, kidney weight/body weight ratio, blood urea, serum creatinine, glycosylated Hb, tissue TNF-α and serum CRP compared to the use of vitamin D or metformin alone.

	Control	STZ	STZ+ Metformin	STZ + Vit D	STZ+ Peanut oil	STZ+ Metformin +Vit D
Serum fasting glucose (mg/dl)	78.26±1.48	215.63±6.52*	147.4±3.07#	176.35±3.06#	207.2±3.25^	110.82±2.6**\$
Kidney weight/body weight ratio	6.55±0.2	20.59±0.58*	13±0.54#	17.38±0.47#	19.36±0.45^	9.19±0.15**\$
Glycosylated Hb %	5.39±0.1	11.91±0.23*	8.25±0.19#	9.19±0.15#	11.08±0.26^	6.75±0.22**\$
Blood urea (mg/dl)	20.59±0.58	65.54±2.04*	40.17±0.67#	48.12±0.92#	67.51±2.16^	30.87±0.68**\$
Serum creatinine (mg/dl)	0.69±0.03	3.16±0.05*	1.77±0.04#	2.21±0.07#	3.21±0.2^	1.23±0.04**\$
Creatinine clearance (ml/min)	1.49±0.05	0.44±0.03*	0.83±0.04#	0.78±0.05#	0.49±0.02^	1.15±0.07**\$
Tissue CAT (U/mg tissue)	30.77±1.18	13.51±1.23*	20.59±0.58#	19.36±0.45#	13.00±0.54^	25.52±0.46**\$
Tissue GPx (U/g tissue)	1.44±0.02	0.66±0.02*	1.02±0.04#	0.82±0.03#	0.62±0.02^	1.2±0.02**\$
Tissue TNF-α (pg/g tissue)	210.01±6.75	920.5±22.5*	783.65±12.3#	731.1±14.64#	932.6±15.7^	604.7±11.2**\$
Serum CRP (mg/L)	440.4±10.4	3897.0±136*	743.55±9.2#	848.3±10.4#	3933.7±95^	642.4±12.3**\$

- * Significant compared to the con group
- # Significant compared to STZ group
- ^ Non significant compared to STZ group
- \$ Significant compared to STZ+Metformin group
- Significant compared to STZ+Vitamin D group

Table 1: Effect of metformin and/or vitamin D on the different parameters in the studied groups (Mean ± S.E.M.)

Histopathological results

STZ induced severe glomerulosclerosis and inflammatory cellular infiltration, tubular dilatation, casts, interstitial fibrosis and atrophy together with vascular arteriosclerosis (Fig. 1B) and significant increase in the apoptotic index and caspase-3 expression compared to the control group (Fig. 2). Peanut oil induced non-significant effect on the histopathological pic-

ture compared to STZ-treated group (Fig. 1C,2). Treatment with either metformin or vitamin D resulted in significant decrease in glomerulosclerosis, cellular infiltration and interstitial fibrosis (Fig. 1D,E) with significant decrease in the apoptotic index and caspase-3 expression compared to STZ group (Fig. 2). Metformin/Vitamin D combination resulted in significant improvement in the histopathological picture compared to the use of each of these drugs alone (Fig. 1F, 2).

Fig. 1: H&E stained sections from the kidney of A) control untreated group with normal appearance of the glomeruli, tubules and interstitium; B) STZ group showing glomerulosclerosis, interstitial fibrosis, tubular dilatation, casts and atrophy; C) STZ + Peanut oil group showing marked interstitial and glomerular fibrosis, casts and tubular atrophy; D) STZ+ Metformin group with

apparently normal glomeruli, mild interstitial fibrosis and minimal tubular necrosis; E) STZ + Vitamin D group with marked decrease in glomerulosclerosis, cellular infiltration and interstitial fibrosis with mild dilatation of the tubules; F) STZ+ metformin + vitamin D group with minimal glomerulosclerosis, minimal cellular infiltration and mild dilatation of the tubules (H&E X 200).

Fig. 2: Effect of different treatments on A) the apoptotic index and B) tissue caspase 3 expression (Mean±S.E.M.)

* Significant compared to the control group

Significant compared to STZ group

^ Non significant compared to STZ group

\$ Significant compared to STZ+Metformin group

• Significant compared to STZ+Vitamin D group

Discussion

In the present study, STZ administration resulted in development of DN manifested by significant decrease in creatinine clearance

with significant increase in kidney weight/body weight ratio, serum fasting glucose, glycosylated Hb, blood urea and serum creatinine and significant histopathological changes compared to the control group. These results are in the same line with Choi et al. [1] who attributed these changes to that STZ selectively damages insulin-producing beta cells of the pancreas and induces experimental hyperglycemia. This hyperglycemia leads to an increase in kidney weight, elevation of blood urea and serum creatinine due to interstitial atrophy and atrophic changes in the glomeruli and renal

tubules. With time, these changes may be an important leading cause of end stage renal failure ^[15].

It was reported that STZ-induced hyperglycemia causes DN by affection of several signal pathways such as nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase. This leads to increased expression of the genes that lead to overproduction of growth factors such as transforming growth factor beta (TGF- β) and advanced glycation end products. These factors lead to renal hypertrophy and deposition of extracellular matrix proteins that leads to glomerulosclerosis with subsequent albuminuria, high blood pressure, progressive decrease in the glomerular filtration rate and affection of various renal functions ^[16].

Oxidative stress is thought to play a crucial role in the pathogenesis of STZ-induced DN. Persistent hyperglycemia was thought to increase production of oxygen free radicals with inhibition of the activity of the antioxidant enzymes leading to oxidative stress which is associated with several health problems ^[2]. This was in agreement with the results of the present study where STZ administration resulted in significant decrease in renal CAT and GPx activity leading to severe cellular damage due to the deleterious effects of H₂O₂ ^[17].

Inflammation plays a crucial role in STZ-induced diabetic nephropathy. It is usually activated by the metabolic, biochemical and haemodynamic abnormalities that commonly exist in the diabetic kidney ^[15]. STZ has the ability to induce the expression of the proinflammatory cytokines by the leucocytes which in turn increase the production of the acute phase proteins such as CRP, serum amyloid A and fibrinogen which increase leukocyte infiltration and adherence in the glomeruli and tubules, along with marked increase in macrophage infiltration ^[18]. Moreover, there was a positive correlation between the glycemic status (Glycosylated Hb level), level of proteinuria and the levels of the markers of inflammation including CRP ^[19]. Roopakala et al^[20], suggested that estimation of serum CRP levels in diabetic patients can help in early intervention and prevention of the complications of diabetes including diabetic nephropathy. This was in the same line with the results of the present study where STZ resulted in increased serum CRP and tissue TNF- α levels compared to the control untreated group.

Caspase 3 is one of the caspase family that plays a central role in the execution of apoptosis. Recent studies reported that oxidative stress leads to activation of caspase 3 which was proven to play an important role in hyperglycemia induced proximal tubular apoptosis. Caspase 3 was thought to be responsible for the cleavage of poly ADP-Ribose polymerase (PARP) during cell death. Hyperglycemia was proven to induce apoptosis mainly in the proximal tubular cells through regulation of the pathway of Bcl2/caspase/ PARP ^[21]. This was in accordance with the results of the present study where administration of STZ resulted in significant increase in caspase-3 expression compared to the control untreated group leading to induction of apoptosis in the renal tubular cells.

Metformin is a biguanide drug that is recommended as the first-line therapy for type 2 diabetes, especially in overweight patients. Recently, a great attention had been made towards the possible

renoprotective effects of metformin. Recent studies have proven that metformin has potent antioxidant and anti-inflammatory properties. Reduction of apoptosis induced by oxidative stress in renal tissues and prevention of vascular dysfunction was found with metformin treatment^[22]. AMP-activated kinase (AMPK) enzyme which regulates the different aspects of the cellular metabolism is associated with the pleiotropic effects of metformin ^[23]. Recent data revealed that the beneficial effects of metformin are due to its mild inhibition of the mitochondrial respiratory chain. Moreover, metformin was able to decrease the production of reactive oxygen species and restore the normal levels of the enzymatic and non-enzymatic antioxidants in the renal tissues ^[24]. Kim et al^[25], reported that treatment of diabetic rats with metformin restored podocyte loss via its protective effects against oxidative injury. Furthermore, the improvement in the mitochondrial function and energy production induced by metformin may reduce the production of the inflammatory mediators such as CRP, TNF- α and IL-6 ^[2]. Moreover, metformin can affect the carbonyl stress which prevents AGEs formation and improves the free radical defense system. Also, Viollet et al. ^[26] suggested that metformin may reduce the expression of the growth factors such as TGF- β 1 which plays an important role in the pathogenesis of DN. Wang et al^[27], reported that metformin can suppress apoptosis of renal tubular epithelial cells in rats, possibly through inhibition of the expression of caspase-3 in the renal tissues. These studies were in agreement with the results of the present study where administration of metformin resulted in significant improvement in the renal function tests and the glycemic control, significant decrease in serum CRP, tissue TNF- α and kidney weight/body weight ratio with significant increase in tissue CAT and GPx with improvement of the histopathological and immunohistochemical picture compared to STZ-treated group.

The major limiting factor for the use of metformin in management of DN is the possibility of development of lactic acidosis which is a severe metabolic problem associated with high mortality and patients may need renal replacement therapy ^[28]. However, the risk of development of metformin-associated lactic acidosis could be decreased by avoiding its use in patients with high risk of sepsis, renal impairment, hypovolemia and old age patients. Also, supplementation with vitamins such as vitamin D and vitamin B complex can significantly reduce the possibility of development of lactic acidosis ^[29].

Vitamin D is one of the fat soluble vitamins. The kidney is the main target organ for vitamin D as vitamin D receptors are highly expressed in the renal tissues. Several studies suggested that vitamin D may have renoprotective effects especially in cases of complicated diabetes ^[30]. These studies were in the same line with the results of the present study where vitamin D resulted in significant improvement in the renal functions and the glycemic control, significant decrease in serum CRP, TNF- α and kidney weight/body weight ratio with significant increase in tissue CAT and GPx with improvement of the histopathological and immunohistochemical picture compared to STZ-treated group.

Nakai et al ^[31], reported that vitamin D attenuates the progression of DN by improving glucose metabolism, inhibition of rennin-angiotensin system activity, reduction of renal fibrosis and inhibition

of the pathways of oxidative stress. Also, vitamin D might delay the development of DN through suppressing the production of the inflammatory mediators such as CRP, TNF- α and IL-6 [32]. Moreover, Wang et al [33], found that signaling vitamin D receptors in the podocytes might protect against DN, possibly through affection of the apoptotic pathways including caspase-3 and bcl-2 expression. Also, Garsen et al [34], suggested that vitamin D attenuates proteinuria frequently encountered in DN by inhibition of the expression of heparanase enzyme in the podocytes.

In the present study, vitamin D/metformin combination induced significant improvement in the renal functions and the glycemic control with significant increase in tissue CAT and GPx with significant decrease in the levels of the inflammatory mediators together with improvement of the histopathological and immunohistochemical picture compared to the use of each of these drugs alone. This may be attributed to their combined potent antihyperglycemic, anti-inflammatory and antioxidant effects together with their ability to affect apoptosis. It was reported that the combination between vitamin D3 and metformin induced marked inhibitory effects on the cell proliferation and apoptosis induction. Also, Li et al [35], suggested that vitamin D3 supplementation potentiates the growth inhibitory effects of metformin by affecting AMP kinase/mTOR signalling pathway. Moreover, vitamin D may decrease the incidence of lactic acidosis which is the most common complication of metformin during treatment of diabetes mellitus [29].

Conclusion

The present study demonstrated the protective effects of each of vitamin D and metformin alone and in combination on STZ-induced DN in rats. This combination might represent a beneficial therapeutic modality for management of patients with DN.

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