Original Research Article

Protective Effects of Dimedone Pyrone on Podocytes in Rats with Diabetic Nephropathy

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Abstract

Purpose: *To investigate the effect of dimedone pyrone (DP) on podocytes in rats with diabetic nephropathy (DN).*
Methods: *The rats were randomly assigned into 5 experimental groups (n = 10), viz, non-diabetic control with no treatment (ND/NT), diabetic with no treatment (DG/NT), diabetic treated with 5 mg/kg dimedone pyrone (DG/DP 5), diabetic treated with 10 mg/kg dimedone pyrone (DG/DP 10) and diabetic treated with 20 mg/kk dimedone pyrone (DG/DP 20) group. Clinical parameters, including 24 h urinary protein, blood urea nitrogen (BUN), serum creatinine (SCR), blood glucose (GLU), and kidney weight (KW)/body weight (BW) were determined after 12 weeks of treatment. Hematoxylin and eosin staining was used to examine renal pathological changes while transmission electron microscopy (TEM) was employed for evaluation of structural changes in the podocytes. The expression levels of nephrin and podocin were evaluated using immunofluorescence staining.*
Results: *Dimedone pyrone caused a significant decrease in SCR, BUN, GLU, KW/BW and 24 h urine protein in DG/DP 20 group compared to DG/NT group. Furthermore, incidences of glomerular disorders, chronic tubulo-interstitial damage and glomerular podocyte lesions decreased significantly following dimedone pyrone treatment. Glomeruli, tubules and podocytes exhibited pathomorphological improvements while nephrin and podocin protein expression levels were significantly higher in the nephridial tissue. Decrease in relative kidney weight (KW/BW) and 24 h urinary protein level were improved significantly on treatment with dimedone pyrone. Moreover, glomerular disorder, chronic tubulo-interstitial damage and glomerular podocyte lesions were also suppressed. The improvement was more significant in DG/DP 20 compared to DG/DP 5 and DG/DP 10 groups.*
Conclusion: *Dimedone pyrone exhibits a protective effect on the podocytes of rats and may be of therapeutic importance in the treatment of diabetic nephropathy.*

Keywords: *Dimedone pyrone, Podocin, Diabetic neuropathy, Nephrin, Glomerular disorders*

INTRODUCTION

Diabetic nephropathy (DN) is the main cause of end-stage renal disease (ESRD) and the disease is expected to increase in the coming years [1,2]. More than 30 % of all diabetic patients develop diabetic nephropathy (DN) [3], which is the most frequent cause of chronic kidney disease (CKD) and risk factor for stroke and heart attack [4]. The number of diabetic patients and those affected by DN is rapidly increasing [5]. Not all diabetic patients develop DN and the development and severity of DN vary greatly from one patient to another with familial clustering, suggesting that genetic factors play an important role [6].

The expensiveness of renal replacement therapy makes it unaffordable even for developed

countries [7]. Diabetic nephropathy also leads to glomerulosclerosis and ESRD worldwide in its advanced stage [8,9]. DN manifests as a clinical syndrome that is composed of albuminuria, progressively declining glomerular filtration rate (GFR) and increased risk for cardiovascular disease [10,11]. Persistent proteinuria is the hall-mark of diabetic nephropathy which is the leading cause of end-stage renal disease. Furthermore, a reduction in proteinuria is associated with a slowing of both the decline in the GFR and the progression to ESRD. As a result, a reduction in proteinuria has been widely used as a surrogate end point for reno-protection.

Pyrones are reported to exhibit a wide range of biological activities which include antimicrobial, antimalarial, anti-nematodal activities as well as calmodulin-dependent, estrogenic and anti-estrogenic, and acetylcholinesterase (AChE) inhibitory activities [12-14]. In the present study the effect of dimedone pyrone (Fig. 1) on podocytes in rats with diabetic nephropathy (DN) was investigated.
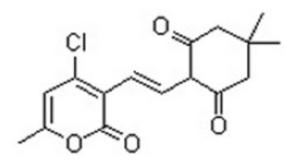

Fig 1: Structure of dimedone pyrone

EXPERIMENTAL

Animals

Fifteen week old male, adult Sprague Dawley rats (weighing around 200 g) from Chengdu Dashuo Biological Technology Co, Ltd, Chengdu, China were maintained in pathogen-free conditions and fed irradiated chow.

Reagents and instruments

Dimedone pyrone, TWP, and alloxan were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat urine albumin EIA assay kit, rabbit anti-rat nephrin antibodies, rabbit anti-rat podocin antibodies and goat anti-rabbit antibodies were obtained from Boster (Tarivid; Santen, Osaka, Japan). Accu-chek Blood glucose meter from Roche Diagnostics GmbH (Tarivid; Santen, Osaka, Japan) and 7150 automatic biochemical analyzer from Hitachi (BD Biosciences, Franklin

Lakes, NJ, USA). The JEOL-1230 transmission electron microscope was obtained from JEOL (BD Biosciences, Franklin Lakes, NJ, USA), Vanox multifunctional microscope was purchased from Olympus (BD Biosciences, Franklin Lakes, NJ, USA) and SDS-PAGE electrophoresis was obtained from Bio-Rad (Hercules, CA, USA).

Grouping and drug administration

After 15 days of acclimatization to laboratory environment, the rats were fasted 12 h prior to the experiment. Rats were randomly assigned into 5 experimental groups, with 10 animals each, as follows: Non-diabetic control (ND/NT) group consisting of normal rats without any treatment; Diabetic control (DG/NT) group consisting of diabetic rats without any treatment; Diabetic (DG/DP 5 mg) group consisting of diabetic rats treated with dimedone pyrone 5 mg/kg; Diabetic (DG/DP 10 mg) group consisting of diabetic rats treated with 10 mg dimedone /kg body weight and Diabetic (DG/DP 20 mg/kg) group consisting of diabetic rats treated with dimedone pyrone 20 mg/kg. The dimedone pyrone was administered intraperitoneally to the rats. During fasting alloxan was injected through tail veins at a dose of 42 mg/kg of body weight to four animal groups and 72 h latter blood samples were collected to measure blood glucose concentrations. The blood glucose in rats was maintained ≥16.7 mmol/L. The rats were weighed weekly to adjust the dose and dimedone pyrone was continuously administered for 12 weeks. During the experiment, the rats were fed a standard diet and were free to drink water. Use of laboratory animals was approved by the Local Committee on Animal Use and Protection and performed in accordance with the National Institutes of Health Guide for the Care (ref no: 2014-123).

Specimen collection

On the last day of experiment urine was collected for 24 h, centrifuged and stored at 80 ^{o}C. Through right common carotid artery blood samples were collected half of the samples were put in anticoagulant tubes and the rest was centrifuged. The plasma was stored at 20 ^{o}C. Weight of the animals was also recorded. The animals were anaesthetized and after sacrifice, kidney tissues were fixed in 4 % paraformaldehyde solution and embedded in paraffin. The paraffin-embedded kidney tissues were cut into 3 µm thick sections. For immunofluorescence examination the sections were treated with polylysine whereas for transmission electron microscopic studies 1 mm

sections were put in ethanol at 4 °C for 2 h before examination.

Analysis of biochemical parameters

Rat urine albumin EIA assay kit was used for 24 h urinary protein concentration determination as per the manufacturer's instructions. Clinical parameters like body weight, feed intake, water intake, and diuresis were recorded for every individual metabolic cage. The biochemical parameters including serum creatinine (SCR), blood urea nitrogen (BUN), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were detected in the serum using a 7150 automatic biochemical analyzer. Johnson & Johnson's standard enzymatic method was used for blood glucose determination.

Examination of renal tissue under light microscopy

Two mm sections of left kidney were fixed in 10 % formaldehyde and embedded in paraffin. The paraffin embedded sections were cut into 1 μm thick sections and after de-paraffinization were stained with hematoxylin and eosin (HE). The sections were subjected to light microscopy for pathological changes.

Ultrastructural changes

For examination of ultrastructural changes tissue specimens were cut into sections and washed in phosphate buffer followed by post fixation in glutaraldehyde. The tissues, after dehydration in graded acetone concentrations, were embedded in Araldite, cut into very thin sections, stained with uranyl acetate and aluminium citrate for examination under a JEOL 1230 transmission electron microscope.

Distribution of nephrin and podocin

The paraffin embedded tissues were cut into 1 mm thin sections, de-paraffinized and incubated overnight with rabbit anti rat nephrin (1:400) and rabbit anti rat podocin (1:400) antibodies at 4 °C. After washing in phosphate buffer the slices were treated with fluorescein isothiocyanate labeled goat, anti rabbit and antibodies observed under immunofluorescence microscopy.

Statistical analysis

All the results are expressed as mean ± standard deviation (SD). SPSS 11.0 software package (SPSS, Inc, Chicago, IL, USA) was used for statistical analysis of the data. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of dimedone pyrone on proteinuria in rats with DN

Examination of 24 h proteinuria in all the five groups of animals revealed a significantly higher level in DG/NT group compared to that in ND/NT and DG/DP 20 groups (Fig. 2). The 24 h proteinuria in DG/DP 10 group was lower than DG/VT 5 group which in turn showed the level comparable to that of DG/DP 20 (Fig. 2).
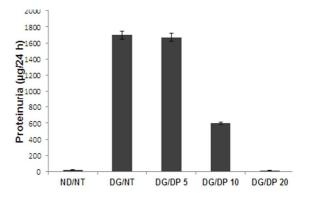

Fig. 2: The 24 h urine protein level comparison of non-diabetic, diabetic and dimedone pyrone treated diabetic rats

Effect of dimedone pyrone on kidney weight/body weight (KW/BW) in DN

After 12 weeks of treatment it was observed that compared to ND/NT group, the KW/BW ratios in DG/NT group was higher ($p < 0.01$) and that in DG/DP 20 group was significantly lower (Fig. 3). However the KW/BW ratio in DG/DP 5 group was slightly lower than that of DG/NT group.
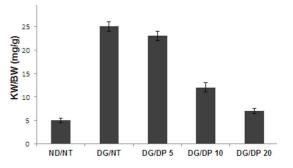

Fig. 3: Relative kidney weight (KW/BW ratios) comparison of non-diabetic, diabetic and dimedone pyrone treated diabetic rats

Effect of dimedone pyrone on serum biochemistry parameters of DN rats

In DG/NT group the SCR, BUN and fasting glucose levels were significantly higher ($p < 0.05$) compared to ND/NT group (Table 1).

Change of renal tissue pathology of DN

In DG/NT group the HE stained renal biopsy samples exhibited tubular deformation and glomerular hypertrophy. However dimedone pyrone treated animals showed a marked decrease in tubular deformation and glomerular hypertrophy on treatment with 20 mg/kg body weight of dimedone pyrone after 12 weeks (Fig 4).
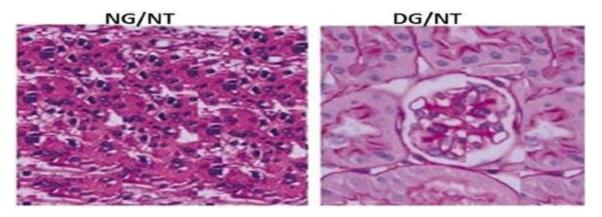
Effect of podocyte disease on DN rats

Compared to NG/NT group, the animals in DG/NT group showed abnormal morphological appearance of podocytes like fusion and decrease in foot processes. Treatment with dimedone pyrone caused a reversal of alloxan induced effect on podocytes. Although the reversal of effect started at a concentration of 5 mg/kg body weight but was significant at 20 mg/kg in DG/DP 20 group (Fig. 5).

Table 1: Dimedone pyrone improves SCR, BUN and fasting glucose levels

Group	Glucose (mmol/L)	BUN(mmol/L)	SCr(μmol/L)	AST (U/L)	ALT (U/L)
ND/NT	5.02 ± 0.56	5.98 ± 0.67	66.90 ± 4.88	58.23 ± 8.75	53.65 ± 6.59
DG/NT	25.14 ± 3.83	11.26 ± 1.86	95.57 ± 10.76	70.43 ± 8.46	78.79 ± 2.53
DG/DP 5	23.23 ± 2.78	10.67 ± 1.48	91.23 ± 10.43	68.65 ± 7.98	70.23 ± 5.11
DG/DP 10	18.02 ± 1.98	8.27 ± 1.74	74.12 ± 10.27	63.42 ± 8.12	61.67 ± 7.65
DG/DP 20	6.45 ± 1.06	6.91 ± 1.58	68.51 ± 10.96	59.84 ± 7.93	54.78 ± 6.43

The results are mean ± SD. **Key:** *Glu, glucose; BUN, blood urea nitrogen; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase; WBC, white blood cell*
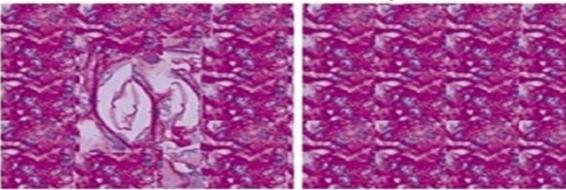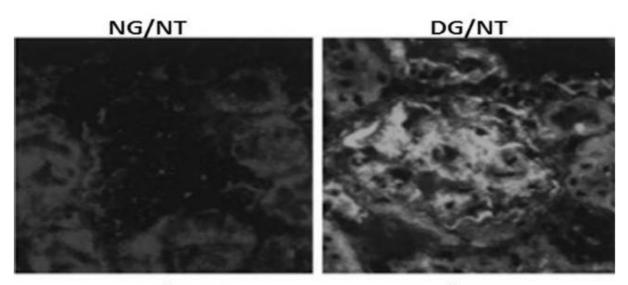

Fig 4: Renal pathological changes in control and dimedone pyrone-treated DN rats ND/NT, non-diabetic control no treatment; DG/NT, diabetic no treatment, DG/DP 5, diabetic group treated with 5 mg/kg dimedone pyrone treated and DG/DP 10, diabetic group treated with 10 mg/kg dimedone pyrone and DG/DP 20, diabetic group treated with 20 mg/kg dimedone purone
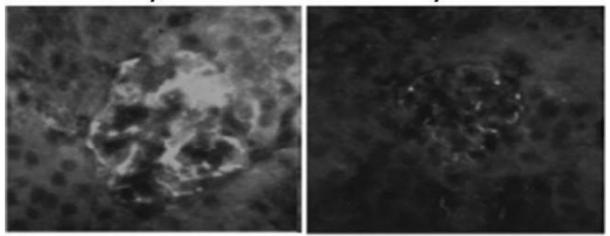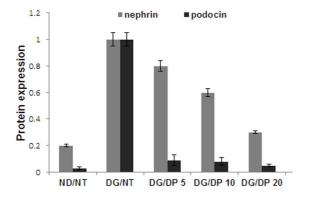
Fig 5: Podocyte lesions using electron microscopy in DN rats. ND/NT, non-diabetic control no treatment; DG/NT, diabetic no treatment, DG/DP 5, diabetic group treated with 5 mg/kg dimedone pyrone treated and DG/DP 10, diabetic group treated with 10 mg/kg dimedone pyrone and DG/DP 20, diabetic group treated with 20 mg/kg dimedone purone

Nephrin and podocin protein expression

Fig 6: Expression of nephrin and podocin proteins in podocytes of DN rats. ND/NT, non-diabetic control no treatment; DG/NT, diabetic no treatment, DG/DP 5, diabetic group treated with 5 mg/kg dimedone pyrone treated and DG/DP 10, diabetic group treated with 10 mg/kg dimedone pyrone and DG/DP 20, diabetic group treated with 20 mg/kg dimedone purone

In DG/NT group of rats, nephrin and podocin protein expression levels were significantly low compared to NG/NT group. Treatment with dimedone pyrone increased the level of nephrin and podocin protein expression. The increase in expression was significant and comparable to that of NG/NT group at a concentration of 20 mg/kg body weight in DG/DP 10 group (Fig 6).

DISCUSSION

It is reported that proteinuria on accumulation in mesangial cells enhances glomerular sclerosis [15,16]. Urinary proteins also regulate tubular cell

function, which changes the growth characteristics of cytokines and matrix proteins, as well as their phenotypic expression and induction of fibrosis [17]. The present study demonstrates that dimedone pyrone decreases the level of proteinuria in the DN rats.

Changed podocyte ultrastructure and associated molecules induce DN proteinuria [18,19]. Micro-albuminuria in DN decreases podocyte count which leads to compensatory hypertrophy to cover basement membrane, thereby increasing permeability. These changes produce abundant proteinuria and subsequently increase podocyte injury. Detachment of podocytes exposes the basement membrane and damage fenestrated membrane and allows large number of proteins to pass [14]. This ultimately leads to glomerular sclerosis and progressive loss of renal function [20]. The results from our study showed that dimedone pyrone at a concentration of 20 mg/kg body weight in rats reversed the effect of damage caused to podocytes in DN rats.

Slit diaphragm (SD) is the main barrier which filters protein macromolecules and is composed of neph 1, nephrin, podocin and FAT1 [21]. In animal experiments, researchers have found that DM worsens kidney damage in rats, reduces nephrin expression and increases albuminuria in urine [22]. The decreased expression of nephrin preceded glomerular tissue damage and is an early event in DN. In the DN model, changes in podocin were associated with protein and mRNA expression levels of nephrin [23].

We observed that dimedone pyrone treatment decreased glomerular hypertrophy and tubular deformation in DN rats after 12 weeks. There was also a decrease in fusion of the podocyte foot processes, disappearance of membrane slits and reduced number of slits in DG/DP group of rats. Dimedone pyrone also increased the expression level of nephrin and podocin in DG/DP 5 and DG/DP 10 groups. However, the increase was significant in DG/DP 20 group.

CONCLUSION

Dimedone pyrone exhibits a protective effect on the podocytes of rats and can be of therapeutic importance in the treatment of diabetic nephropathy.

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