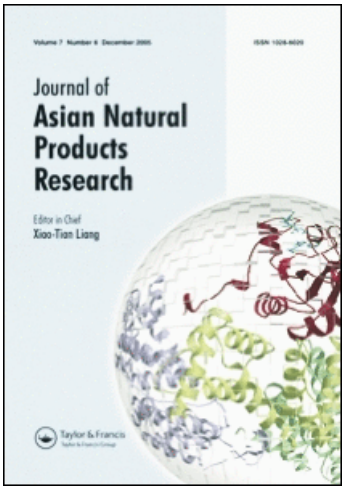

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Effect of polysaccharides from *Ganoderma lucidum* on streptozotocin-induced diabetic nephropathy in mice

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The effects of *Ganoderma lucidum* polysaccharides (GL-PS) on renal complication in streptozotocin-induced diabetic mice have been investigated in the present study. C57BL/6J mice were made diabetic by injection of streptozotocin and GL-PS (125 and 250 mg kg^{-1}) was administered for 8 weeks. Body weight was monitored every week. Serum glucose, creatinine (Cr), blood urea nitrogen (BUN), triglyceride (TG) and urinary albumin excretion (UAE) were measured after 8 weeks of treatment. Glomerular size and mesangial matrix index were assayed by morphometric analysis. Renal expression of transforming growth factor-β_1 (TGF-β_1) were determined by immunochemistry. Renal malondialdehyde (MDA) level and superoxide dismutase (SOD) activities were also evaluated. GL-PS was able to reduce the serum Cr and BUN levels and UAE compared with diabetic model mice in a dose-dependent manner. Increasing serum glucose and triglyceride levels in diabetic mice could also be lowered by GL-PS. Moreover, GL-PS had the capacity to improve the renal morphometric changes and oxidative stress state of diabetic mice. In summary, GL-PS can improve the metabolic abnormalities of diabetic mice and prevent or delay the progression of diabetic renal complications.

Keywords: *Ganoderma lucidum*; Polysaccharides; Diabetic nephropathy; Mouse

1. Introduction

Diabetic nephropathy is a major complication of diabetes and a leading cause of end-stage renal failure throughout much of the world [1]. Several mechanisms have been postulated for the development of diabetic nephropathy such as metabolic abnormality, oxidative stress, non-enzymatic glycosylation, and stimulation of protein kinase C [2], but the exact mechanism is not clear. *Ganoderma lucidum* polysaccharide (GL-PS) is one of the main active ingredients of *Ganoderma lucidum* (Leyss ex Fr) Karst, which has been documented to have hypoglycaemic, hypolipidaemic, antioxidant, immunomodulatory and anti-tumour activities [3–6]. The effect of GL-PS on diabetic nephropathy has not been reported. In the

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present study, we investigated the renal protective effect of GL-PS on streptozotocin-induced diabetic mice.

2. Results

2.1 General observations

Among diabetic mice, one mouse in the diabetic model group died spontaneously in the 4th week of the experiment. No mice in the other experimental groups died during the entire period of the experiment. The blood glucose levels were markedly raised in the diabetic groups as compared with non-diabetic control group mice ($P < 0.01$). Treatment of GL-PS for 8 weeks dose-dependently reduced the blood glucose levels; however, only at a dose of 250 mg kg^{-1} did it have statistical significance ($P < 0.05$). The serum triglyceride (TG) in the diabetic model group was significantly higher than that of control mice and GL-PS reduced it in a dose-dependent manner (table 1). After the 8-week period of treatment, the body weight of non-diabetic control group mice increased to 25.0 ± 1.5 g, which was much higher than that of the diabetic model group as well as groups treated with GL-PS (125 and 250 mg kg^{-1}) (18.6 ± 2.1 g, 19.3 ± 1.3 g, 19.6 ± 1.6 g, respectively). The body weight gain in diabetic groups was much lower than that of control group mice ($P < 0.01$), though it slightly increased by administration of GL-PS, but there was no statistical significance ($P > 0.05$).

2.2 Effect of GL-PS on renal function

The ratio of bilateral kidney weight to body weight was significantly higher in diabetic model group compared with the non-diabetic group ($P < 0.01$). Treatment of GL-PS partially prevented renal hypertrophy. The urine albumin excretion (UAE) level of the diabetic model group rose up to 10-fold compared with that of the non-diabetic group, but it was markedly ameliorated after treatment with GL-PS. The serum creatinine (Cr) and blood urea nitrogen (BUN) levels increased markedly in the diabetic model group mice and were reduced significantly by treatment of GL-PS (table 2).

2.3 Effect of GL-PS on renal morphological changes

Both glomerular area and mesangial matrix index were significantly increased in diabetic mice compared with those in non-diabetic group. However, a significant reduction of these

Table 1. Blood glucose, and TG levels in the non-diabetic, diabetic model, diabetic + GL-PS (125 and 250 mg kg^{-1}) groups after 8-week treatment in the STZ-induced diabetic mice.

Group	*Blood glucose mmolL^{-1}*	*TG mmol L^{-1}*
Non-diabetic	5.5 ± 1.4	1.1 ± 0.2
Diabetic model	26.5 ± 3.8**	1.8 ± 0.3**
GL-PS 125 mg kg^{-1}	24.0 ± 5.5	1.4 ± 0.4†
GL-PS 250 mg kg^{-1}	20.1 ± 6.1†	1.2 ± 0.4††

Data are means ± SD ($n = 10$–14). **$P < 0.01$ vs. non-diabetic group, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. diabetic model group.

Table 2. Renal hypertrophy, serum Cr, BUN and UAE levels in the non-diabetic, diabetic model, diabetic + GL-PS (125 and 250 mg kg^{-1}) groups after the 8-week treatment in the STZ-induced diabetic mice.

Group	*Kidney weight/Body weight mg g^{-1}*	*Cr μmol L^{-1}*	*BUN mmol L^{-1}*	*UAE μg per 24 h*
Non-diabetic	11.93 ± 0.56	52.0 ± 4.7	11.2 ± 1.2	9.1 ×/÷ 2.8
Diabetic model	15.23 ± 1.64**	79.5 ± 11.5**	15.6 ± 1.9**	250.7 ×/÷ 2.8**
GL-PS 125 mg kg^{-1}	14.61 ± 1.94	68.0 ± 6.6†	15.7 ± 4.5	175.9 ×/÷ 2.0
GL-PS 250 mg kg^{-1}	13.77 ± 1.04†	64.5 ± 8.3††	11.7 ± 1.3††	119.2 ×/÷ 1.7††

Data are means ± SD ($n = 10$–14).**$P < 0.01$ vs. non-diabetic group, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. diabetic model group.

two parameters was noted when treatment with GL-PS was compared with the diabetic model group (figure 1 and table 3).

2.4 Effect of GL-PS on expression of TGF-β$_1$ in the kidney

Integral intensity of TGF-β$_1$ in the renal cortex of diabetic mice was higher than that of non-diabetic control mice. Treatment with GL-PS dose-dependently reduced the TGF-β expression (figure 2 and table 4).

2.5 Effect of GL-PS on renal malondialdehyde levels and superoxide dismutase activity

The renal malondialdehyde (MDA) level and superoxide dismutase (SOD) activity are shown in table 5. The diabetic model mice had much higher MDA levels than non-diabetic mice. However, treatment of GL-PS at 250 mg kg^{-1} reduced the renal MDA level significantly. In addition, the renal SOD activity was notably lower in diabetic model mice than in non-diabetic mice, but was significantly increased by administration of GL-PS.

3. Discussion

Yotsumoto *et al.*[7] indicated that among various experimental models used to investigate diabetic nephropathy, streptozotocin (STZ)-induced hyperglycaemic mice were beneficial as they developed nephropathy earlier than rats, especially in evaluating the effects of various compounds on diabetic nephropathy. Therefore, mice were used in present study. Urinary albumin level is a marker of glomerular injury and development rates of albumin excretion are

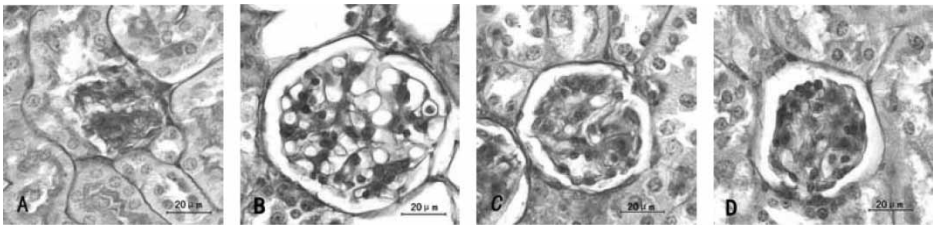

Figure 1. PAS staining of kidney section in non-diabetic mice (A), diabetic model mice (B) and diabetic mice treated with GL-PS (125 and 250 mg kg^{-1}, C and D, respectively). Glomerular size was measured first by tracing the tuft and mesangial area is defined as the PAS-positive area in the tuft area. The mesangial matrix index represents the ratio of mesangial matrix area divided by the tuft area. Original magnification ×400.

Table 3. Glumerular area and mesangial matrix index in the non-diabetic, diabetic model, diabetic + GL-PS (125 and 250 mg kg^{-1}) groups after the 8-week treatment in the STZ-induced diabetic mice.

Group	*Glomerular size μm^2*	*Mesangial matrix index %*
Non-diabetic	1607 ± 377	16.5 ± 4.1
Diabetic model	2551 ± 715**	21.7 ± 6.3**
GL-PS 125 mg kg^{-1}	1951 ± 551††	15.6 ± 3.9††
GL-PS 250 mg kg^{-1}	1941 ± 463††	14.9 ± 5.6††

Data are means ± SD (n = 100–140). **$P < 0.01$ vs. non-diabetic group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. diabetic model group.

a harbinger of progressive nephropathy. In this study, UAE of diabetic mice was 10-fold higher than that of non-diabetic mice; however, treatment with GL-PS could prevent the rise of UAE levels to a varying degree, as seen in diabetic controls. In addition, serum Cr and BUN, as a noticeable index for expressing the glomerular filtration rate, were significantly increased in the diabetic model and treatment with GL-PS likewise prevented the rising. This finding indicates that GL-PS may protect glomeruli from the injurious effects of diabetes, which is consistent with a recent report [8]. The result of the histopathological examination of the kidneys also showed that GL-PS could improve the glomerular damage in diabetic mice.

Recent studies strongly support the concept that the primary cause of diabetic nephropathy rests with the metabolic disorder. In particular, the importance of hyperglycaemia as a risk factor for diabetic nephropathy is supported by several observations and pieces of experimental evidence [9,10]. In this study, the increased blood glucose and triglyceride levels were significantly depressed by GL-PS in diabetic model mice, suggesting that the mechanism of renal protection by GL-PS is likely by modulating metabolic abnormality in the hyperglycaemic state.

In hyperglycaemic conditions glucose is prone to oxidation, and oxidative stress has been considered to be a common pathogenetic factor of diabetic nephropathy as in other complications [11]. A significant role is therefore ascribed to oxidative stress in the development of diabetic nephropathy. In our study, we measured the renal MDA levels and SOD activity to determine the effect of GL-PS on the oxidative stress in relation to the development of diabetic nephropathy. The MDA level was evidently increased and SOD activity was markedly decreased in diabetic model mice compared to non-diabetic mice, indicating an impaired homeostasis of oxidative stress state in the kidney. However, administration of GL-PS was able to effectively improve this imbalance.

The intrarenal TGF-β system plays a crucial role in the development of pathologic lesions of diabetic nephropathy. Two of the characteristic changes in diabetic nephropathy, namely glomerular basement membrane thickening and expansion of the mesangium with matrix, is

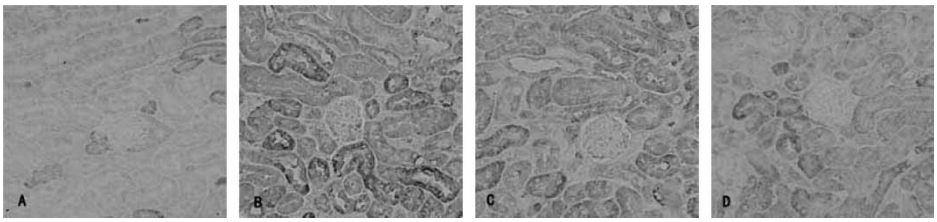

Figure 2. Expression of TGF-β_1 in renal cortex of non-diabetic mice (A), diabetic model mice (B) and diabetic mice treated with GL-PS (125 and 250 mg kg^{-1}, C and D, respectively) after the 8-week experimental period. Original magnification × 200.

Table 4. Integral intensity of TGF-β_1 positive area in the renal cortex of non-diabetic, diabetic model, diabetic + GL-PS (125 and 250 mg kg^{-1}) groups after the 8-week treatment in the STZ-induced diabetic mice.

Group	*TGF-β_1*
Non-diabetic	435 ± 93
Diabetic model	2535 ± 125**
GL-PS 125 mg kg^{-1}	1239 ± 461††
GL-PS 250 mg kg^{-1}	800 ± 203††

Data are means ± SD ($n = 100–140$). **$P < 0.01$ vs. non-diabetic group; $^{\dagger\dagger}P < 0.01$ vs. diabetic model group.

mediated in large part by the TGF-β system [12]. Treatment with the neutralizing anti-TGF-β antibody can prevent the development of diabetic nephropathy in STZ-induced diabetic mice [13]. It was found that TGF-β immunostaining was in greatest abundance in the renal cortex tubular area and less so in the glomerular area in the present study, just as reported by Gilbert *et al.* [14]; the cause may be related to the experimental period for primary glomerular pathology leading to tubular injury by many pathogenetic mechanisms, and progressive tubulointerstitial injury in diabetes may well develop as a consequence of advancing glomerulopathy [15,16].

In summary, our study demonstrated that GL-PS exerted a renal protective effect in mice with diabetic nephropathy through the amelioration of metabolic disorders, oxidative stress and renal dysfunction associated with renal lesions. As a plant drug, GL-PS has fewer undesirable side effects and our finding may provide a latent therapeutic agent to prevent or delay the progress of the diabetic nephropathy.

4. Experimental

4.1 Drugs

GL-PS, isolated from boiling water extract of the fruiting body of *Ganoderma lucidum*, is a polysaccharide peptide with a molecular weight of 584,900 with 17 amino acids. The ratio of polysaccharides to peptides is 93.51%:6.49%. The polysaccharide consists of rhamnose, xylose, fructose, galactose, mannose and glucose, with molar ratios of 4.7:5.7:17.6:1.0:2.3:47.5, and are linked by β-glycosidic linkages [17]. GL-PS was kindly provided by the Fuzhou Institute of Green Valley Bio-Pharm Technology.

4.2 Establishment of animal model and grouping

Experiments were conducted on inbred male 6–8 weeks old (body weight 20–22 g) C57bl/6J mice. All procedures were in accordance with the Institute's Ethical Committee for

Table 5. Renal MDA levels and SOD activity in n the non-diabetic, diabetic model, diabetic + GL-PS (125 and 250 mg kg^{-1}) groups after the 8-week treatment in the STZ-induced diabetic mice.

Group	*MDA nmol mg prot^{-1}*	*SOD U mg prot^{-1}*
Non-diabetic	1.42 ± 0.40	154.6 ± 18.2
Diabetic model	1.78 ± 0.41*	92.0 ± 14.8**
GL-PS 125 mg kg^{-1}	1.55 ± 0.34	106.1 ± 11.6†
GL-PS 250 mg kg^{-1}	1.09 ± 0.21†	111.3.1 ± 27.0††

Data are means ± SD ($n = 10–14$).*$P < 0.05$,**$P < 0.01$ vs. non-diabetic group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. diabetic model group.

Experimental Use of Animals. Experimental diabetes was induced by intraperitoneal injection of streptozotocin (STZ; Sigma Chemical, St. Louis, MO) at a dose of 100 mg kg^{-1} body weight per day for 2 days; non-diabetic control mice received the citrate buffer only. After 7 days, induction of diabetes was confirmed by measuring the blood glucose level using the oxidase method and hyperglycaemic mice with blood glucose levels >16.5 mmol L^{-1} were used. The diabetic mice were randomly divided into the following three groups (12–14 animals in each group): diabetic model group treated with vehicle and GL-PS groups treated with GL-PS (125 and 250 mg kg^{-1}, respectively). Ten control mice that had not been injected with STZ were treated with vehicle. Vehicle or GL-PS was given orally to mice by gastric gavage in the morning once a day.

4.3 Tissue preparation

After 8 weeks of study, 2 days prior to sacrifice, mice were individually housed in metabolic cages for 24 h for urine collection. Then blood samples were collected and the serum was separated for measurement of the biochemical parameters. Bilateral kidneys were rapidly removed and weighed. Half of the middle portion of the left kidney was immediately fixed in 10% formalin for H&E, periodic acid-Schiff (PAS) and immunochemistry staining. Remaining kidneys were stored at -70°C.

4.4 Biochemical study and renal function

Serum TG levels were studied by an enzymatic method using a spectrophotometer (Beckman DU640). The urinary volume was measured gravimetrically, and UAE concentrations were determined with an enzyme-linked immunosorbent assay using a mouse albumin quantitation kit (Bethyl Lab Inc., Montgomery, TX). Serum Cr and BUN were measured by an autoanalyser (Hitachi 7170).

4.5 Morphometric analysis of glomerulus

The method of Chen *et al.*, as previously described [18] was used for morphometric analysis of glomerulus. Briefly, renal tissues were fixed in 10% formalin and embedded in paraffin in routine fashion. Tissue sections were cut at 4-μm thickness, dewaxed, and stained with H&E and PAS. To evaluate glomerular size, 10 randomly selected glomeruli in the cortex per animal were examined under high magnification ($\times 400$), and the average was used for analysis. Glomerular tuft area was measured by manually tracing the glomerular tuft using an image analysis system (Leica Q550CW). Mesangial matrix index was defined as the PAS-positive area within the tuft area. The mesangial matrix index represented the ratio of mesangial matrix area divided by the tuft area.

4.6 Immunochemical staining for TGF-β_1 [14]

To evaluate the expression of TGF-β_1, sections were placed in xylene to remove the paraffin wax, hydrated in graded ethanol. After repairing the antigen, a rabbit anti-human polyclonal antibody to TGF-β_1 (Santa Cruz Biotechnology Inc.) was used as a primary antibody followed by a second reaction with horseradish peroxidase-labelled goat anti-rabbit IgG

(DakoCytomation); peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride as a chromogen. Ten fields of the renal cortex per animal were randomly selected under magnification ($\times 200$) using an image analysis system by two independent observers blinded to the disease status of the animal. Integral intensity of TGF-β_1 positive areas was evaluated by the QWIN software system.

4.7 Measurement of the renal MDA and SOD activity

The method of Kakkar *et al.* [19] was used as previously described for measuring the renal MDA and SOD activity. Tissues were homogenized in 10 volumes of 50 mmol L^{-1} phosphate buffer (pH 7.4) on ice for 20 s using an electronic homogeniser. The homogenate was then centrifuged at $4 \times 11{,}000 \times g$ using a refrigerated centrifuge. The resultant supernatant was used for measurement for MDA and SOD activity using commercial kits. The value of SOD was calculated in terms of units defined as the amount of SOD that inhibits the reduction of nitroblue tetrazolium (NBT) by 50%. Protein levels were evaluated by the Bradford method with bovine serum albumin as the standard.

4.8 Statistical analysis

Because of a positively skewed distribution, the 24-h urine albumin excretion (UAE) was logarithmically transformed before statistical analysis and expressed as the geometric mean $\times$ / $\div$ tolerance factor; other data are expressed as mean $\pm$ SD, subjected to analysis by one-way ANOVA employing the SPSS 10.0 statistical package. $P < 0.05$ was regarded as statistically significant.

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