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PROTECTIVE EFFECT OF A POLY-PHYTOCOMPOUND ON EARLY STAGE NEPHROPATHY SECONDARY TO EXPERIMENTALLY-INDUCED DIABETES

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PROOF

Diabetic nephropathy (DN) is a severe and life-threatening complication of long-standing diabetes. As one of the main causes of end-stage renal disease, the prevention and treatment of DN in early stage, and the slowing down of DN 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. Male Wistar rats were randomly assigned to two groups. One group of rats (diabetic group) received a single tail-vein injection of STZ compound (50 mg/kg) under light anaesthesia. A protective dose of 0.5 ml of 5% dextrose was given intraperitoneally 30 min before the administration of STZ. One diabetic group was fed a normal pellet diet (group A) while group B was fed the diet added with DTS **(panax pseudoginseng, eucommia ulmoides), (Kyotsu Jigyo, Tokyo, Japan) in the proportion of 50/25 (% weight/weight),** at the dose of 50 mg/kg/day throughout the experimental period. **At the end of 8 weeks, 24-hour urine was collected for the measurement of the albumin concentration: blood samples were collected for serum biochemistry and the rats were sacrificed for kidney measurement of oxidative stress- and histo-morphological features. Nephrin and Macrophage Chemoattractant Protein-1 (MCP-1) gene expression were also assessed by fluorescence real-time quantitative PCR after RNA extraction and cDNA synthesis. STZ-treated animals showed significantly increased in lipid peroxidation in the kidney and in proteinuria. DTS supplementation did not affect plasma glucose but significantly decreased malonyldialdehyde (MDA) plasma level and the overall redox parameters together with a partial mitigation of proteinuria. Histological analysis showed also that DTS significantly reduced the glomerular volume together with glomerulosclerosis and interstitial fibrosis score (p<0.05), the latter two being correlated to proteinuria (p<0.05). DTS supplementation also enabled a reduction of diabetes-induced decrease of nephrin mRNA expression and a 67% reduction of MCP-1 mRNA up-regulation (p<0.01). Taken altogether, these data show that, besides the mandatory control of glycemia, intervention with a nutraceutical with antioxidant and anti-inflammatory properties may have beneficial effects when integrated in the mainstream of the therapeutic regimen.**

Diabetes mellitus (DM) is one of the world's fastest growing metabolic disorders that afflicts almost 7% of the world population (1). This number is expected to double by the year 2030 (2).

Key words: diabetic nephropathy, oxidative stress, DTS

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Hyperglycemia associated with diabetes impairs the natural antioxidative mechanisms through non-enzymatic glycosylation of the antioxidant enzymes (3) while the increased degradation of glycated proteins leads to enhanced production of reactive oxygen species (ROS) (4). ROS are known to play a major role in a variety of physiologic and disease-related processes and have been clearly implicated in the pathophysiology of diabetic nephropathy (5). A number of experimental and clinical studies suggest that increased oxidative stress may also contribute to the initiation and progression of diabetic nephropathy (6-7). Non-enzymatic glycation of long-lived structural proteins is one of the mechanisms involved in the pathogenesis of this complication (8-9) which is strongly associated with worsening of the whole clinical condition and premature death, mostly related to cardiovascular events. Therefore, the current challenge in the care of diabetes is targeted to design novel approaches aimed at minimizing the development of such complications. It develops in 5-10% of Caucasian patients with NIDDM, whereas among other racial groups the incidence of renal insufficiency due to NIDDM may be 50% or more since other genetic factors are likely to be involved. From the available scientific evidence, it would appear that the best predictive marker for vascular complications is represented by microalbuminuria (10) which is significantly linked with mortality and morbidity, including overcoming glomerulopathy (11). Indeed, once microalbuminuria appears, the risk of nephropathy remarkably and predictably increases (12). In this respect, nephrin, a specific podocyte protein, essential to maintain the integrity of the interpodocyte slit membrane structure and filtration barrier, seems also to be involved. It has been found that in diabetic nephropathy, the synthesis of the splice variant isoform of nephrin lacking a transmembrane domain causes a urinary loss of nephrin leading to a decrease of protein level.

Studies have shown that podocyte damage and decrease of nephrin expression are present even at the early stage of DN and play a key role in accelerating the development of DN especially when the levels of nephrin expression are downregulated and there is an increase in desmin expression (13).

We have recently shown that DTS, a novel functional food supplement used in clinics safely for general health-purposes, might exert a beneficial regulation of GSH/GSSG redox status (14-15) while conferring a protection against drug-induced DNA damage in the liver and kidney of either young or aged animals (16). This experimental study was designed in view of an integrated approach aimed at finding a possible natural protective counteraction against diabetes-induced early renal damage.

MATERIALS AND METHODS

Animals

Adult Wistar rats of either sex (150–180 g) were used in these experiments and were maintained under controlled standard conditions of light (12/24 h) and temperature (26±1°C). Food pellets (crude protein 180 g/kg; fats 32 g/kg; crude fibre 36 g/kg; carbohydrate 598 g/kg and metabolic viable energy 301 kcal/100g) and tap water were provided ad libitum. For experimental purposes animals were fasted overnight but were allowed free access to water. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

Drugs and reagents

The STZ and reagents were purchased from Sigma Chemical Co. (St. Louis, USA) and were of the highest commercial grade available.

Preparation of phytotherapeutic compound

DTS (panax pseudoginseng, eucommia ulmoides, 50/25 (%w/w), Kyotsu Jigyo, Tokyo, Japan) is produced under quality-controlled procedure from non-OGM-modified crops and ISO 9001 and 140001 regulation, registered as FOSHU (Food Of Specific Health Use) and it was kindly donated by the Institute of Health Care with Oriental Herbs and Medicine, Tokyo, Japan. This compound is composed of palatable tiny grains of medium consistency which can be easily mixed with food.

Study design

Male Wistar rats, 12 weeks old, weighing 220-250 g, were randomly assigned to two groups. One group of rats (diabetic group) received a single tail-vein injection of STZ (50 mg/kg) under light anaesthesia with diethyl ether after 18 h of fasting, as described by the National Institute of Diabetes and Digestive and Kidney Diseases Consortium for Animal Models of Diabetic Complications' protocol (available from http://www.amdcc.org). STZ was dissolved in a citrate buffered solution [0.15 M citric acid and 0.25 M sodium

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phosphate, (pH 4. 5). Approximately 80% of the STZ-treated rats developed stable hyperglycaemia. A protective dose of 0.5 ml of 5% dextrose was given intra-peritoneally 30 min before the administration of STZ. Only animals with blood glucose levels = 250 mg/100 ml were included in further experiments. All animals survived the study without any sign of illness or of apparent exhaustion during the course of the study.

One diabetic group was fed a common pellet diet (group A) while group B was fed the same diet supplemented with DTS at the dose of 50mg/kg/day throughout the experimental period. Another group of healthy animals received an equivalent volume of citrate buffer solution alone and were fed a normal diet representing a control group (C group), while a separate healthy control group of rats was fed a diet supplemented with DTS to check its effect in healthy animals.

Specimen collection

At the end of the 8th week, a 24-hour urine sample was collected through metabolic cages. Then the rats were anesthetized by sodium pentobarbital (50 mg/kg, ip), and blood samples were collected for serum biochemistry. Kidneys were completely perfused with normal saline before their removal. Part of the left kidney was immersed in 10% neutral formalin solution and 2.5% glutaraldehyde for patho-morphology analysis. The other part was divided into pieces and stored at –70 C for future quantitative real-time polymerase chain reaction (PCR).

Determination of plasma glucose and creatinine

Plasma glucose was measured by a colorimetric method using glucose oxidase (Boehringer Mannheim, Germany) in a Hitachi 917 autoanalyser. Serum creatinine level was measured by the enzymatic colorimetric method (17).

Determination of urinary albumin excretion

Urinary albumin concentration was measured by nephelometry using anti-mouse albumin antibody (ICN Pharmaceuticals, Aurora, USA). Results were normalized to the urinary creatinine levels (18).

Biochemical assessment in kidney tissue

Kidney tissue was homogenized in ice-cold buffer (0.1 M KH_2PO_4/K_2HPO_4, pH 7.0, plus 29.2 mg ethylen ediaminetetraacetic acid (EDTA) in 100 ml of distilled water and 10.0 mg digitonin in 100 ml of distilled water, final volume, 2,000 ml) to produce a homogenate. The homogenates were then centrifuged at 10,000 × g for 10 min at 4°C.

Glutathione peroxidase (GPx) activity was assayed at 340 nm by spectrophotometry and the amount of the enzyme converting 1 μmol GSH per min was taken as 1 activity unit (19). Catalase activity was measured in the cell cytosol fraction and was calculated at 240 nm by measuring the rate of H_2O_2 decomposition at 240 nm with the molar extinction coefficient for H_2O_2 being 43.6 M^{-1} cm^{-1}. The amount of the enzyme utilizing 1 μmol H_2O_2 per min was taken as 1 activity unit (20). For determination of malondialdehyde, the kidneys were homogenized in ice cold 20 mM Tris-HCl buffer (pH 7.4). The homogenates were then centrifuged at 10,000 × g for 10 min at 4°C. Concentration of malondialdehyde was measured by the thiobarbituric acid test. Briefly, 1 mM EDTA (Sigma, St. Louis, MO, USA) was added to a 0.5 ml haemolysate and was mixed with 1 ml cold 15% (w/v) trichloroacetic acid to precipitate proteins. The supernatant was treated with 1 ml 0.5% (w/v) thiobarbituric acid in a boiling water bath for 15 min. After cooling, the absorbance was read at 535 nm and the concentration of thiobarbituric acid reactive substance was calculated ~~by~~ using malondialdehyde as a standard. Results were expressed as nMol thiobarbituric acid reactive substances/mg of protein (21). Protein concentration was determined by the Bradford method using bovine serum albumin as a standard (22). To determine carbonyl level in proteins, 1 ml of haemolysate was placed in a glass tube and 3 ml of 8 mM 2, 4-dinitrophenylhydrazine in 3 M HCL was added. The tubes were incubated for 50 min at 37°C in the dark and vortexed every 10 min. Three ml of 25% (w/v) trichloroacetic acid was then added and the tube was left on ice for 8 min and then centrifuged for 3 min in a tabletop centrifuge to collect the protein precipitates. This pellet was washed using 3 ml 15% trichloroacetic acid followed by two washes with 4 ml of ethanol-ethyl acetate (1:1), (v/v). The final precipitate was dissolved in 2 ml of 5 M guanidine hydrochloride solution and was incubated for 15 min at 37°C with frequent mixing, and any insoluble materials were removed by repeated centrifugation.

Histological study

All formalin-fixed kidney sections (3 μm) were stained with hematoxylin (HE) and periodic acid-schiff (PAS). Digital images of glomeruli and interstitial areas were obtained by light microscopy (magnification, ×400). The glomerular cross-sectional area (AG) was measured in 50 glomerular profiles per rat by using computerized image analysis system (Olympus image-analyser, Japan). The glomerular volume (VG) was then calculated as: VG = β/K[AGI3/2, where β = 1.38 is the size distribution coefficient and K = 1.1 is the shape coefficient for glomeruli idealized as a sphere. The glomerulosclerosis index (GSI) in each glomerulus was scored semi-quantitatively as follows: 0, no sclerosis; 1, sclerosis in <25% of glomerulus; 2, sclerosis in 25%–

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50% of glomerulus; 3, sclerosis in >50% of glomerulus. To evaluate interstitial fibrosis, 20 fields for each section were assessed on PAS-stained sections (×200). Semi-quantitative analysis in each field was assessed as follows: 0, no fibrosis; 1, fibrosis in <10% area; 2, fibrosis in 10%–25% area; 3, fibrosis in 25%–50% area; 4, fibrosis in >50% area. Averages of glomerulosclerosis and interstitial fibrosis scores were calculated. All measurements and scoring were performed on blinded slides.

Assessment of Nephrin and Macrophage Chemoattractant Protein-1 (MCP-1) gene expression in the kidney

RNA extraction and cDNA synthesis. Frozen renal tissues samples were homogenized and total RNA was extracted with Trizol (Invitrogen, USA) one-step extraction protocol. The extracted RNA was quantitatively assessed by agarose gel electrophoresis and tested by spectrometry for quantity

At a wavelength of 280 nm.

The OD260/OD280 ratios were within the range of 1.8–2.0. Two □ of total RNA were reverse transcribed in the presence of random primers and M-MLV reverse transcriptase (Promega) according to the following sequence: 70□ for 5 min, followed by 37□ for 60 min, and 75□ for 15 min. Reverse transcription reaction system (BioGeneTech Co., Seoul) had a total volume of 20 µl (23).

Fluorescence real-time quantitative PCR

1000 ng mRNA sample was accurately added into the reverse transcription system with the resulting cDNA as standard. It was diluted 10-fold to establish the standard curve. The real-time PCR was carried out in ABI PRISM 7000 HT (Applied Invitrogen 11744-100, USA) in 25 µl reaction system: 12.5 µl SYBR Premix Ex TaqTM (2×Conc.), 0.5 µl ROX reference dye (50×), 0.5 µl PCR forward primer (10 µmol/L), 0.5 µl PCR reverse primer (BioGeneTech Co., Seoul). For nephrin mRNA quantification, primers (rNEF-Q1: 5'-TAATGTGTCTGCGGCCCAG-3' and rNEF-Q1R: 5'-TGTTGGTGTGGTCAGAGCCA-3') and TaqMan probe (rNEF-TAQ1: 5'-CCCTCTTCAAATGCACGGCCACC-3') were synthesized. For MCP-1 quantification: sense 5'-TGTGCCTGCTGCTCAC T-3', antisense 5'-GTTTGGGTTTGCTTGAA-3' (product size 368 bp,); β-actin sense 5’-AAGAGAGGCATCCTCACCGTG-3', antisense 5’-TACATGGCTGGGGTGTTGCTG-3' (product size 625 bp).The following two-step PCR procedure was applied: pre-denaturation at 95°C for 10 seconds and 45 cycles for 5 seconds at 95°C and 31 seconds at 60–63°C. Standard curves for target genes and internal reference gene were built under the same conditions, and the cycle threshold (Ct) values of the samples were used to calculate the corresponding gene copy number. The result was expressed as the ratio of target gene copy over housekeeping gene (GAPDH) copy.

Statistical analysis

Data was analyzed by ANOVA using Duncan’s post-hoc test for comparisons among means at p=0.05 when appropriate. If the data were not normally distributed, we used the Kruskal-Wallis test (non-parametric analysis of variance) followed by Dunn's multiple comparisons test. Statistically, $p < 0.05$ was considered significant.

RESULTS

Plasma and urine biochemistry

At the end of the 8th week, the biochemical parameters indicated that the STZ injection produced diabetic animals with low levels of insulin production, and high levels of plasma glucose and glucose excretion compared to healthy control rats ($p<0.01$, Table I). Oxidative stress was also induced in the STZ-treated animals with increased lipid peroxidation as compared to control ($p<0.001$). DTS supplementation (group B) did not affect plasma glucose but it significantly decreased MDA plasma level ($p<0.05$ *vs* untreated diabetic rats). STZ had no significant effect on creatinine while it caused a significant proteinuria ($p<0.01$, Table I). This abnormality was partially mitigated by co-administration of DTS ($p<0.05$ *vs* untreated diabetic rats).

Renal tissue biochemistry

Table II shows the effects of administration of DTS on the levels of kidney MDA, GSH and CAT in diabetic rats. STZ-induced diabetic rats showed a significant depletion of GSH and CAT with increased MDA ($p<0.01$; B *vs* C: $p<0.05$). The level of non-enzymatic and enzymatic antioxidants in both DTS-treated groups were found to be higher than the untreated rats with significantly lower MDA generation ($p<0.05$). In particular, DTS administration brought about a full restoration of all the above redox parameters ($p<0.001$).

Histological findings

The most consistent feature of glomerular lesion in diabetic rats was represented by thickening of the basement membrane, mesangial expansion and

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Table I. *Plasma and urinary measurements in diabetic rats: effect of DTS supplementation.*

	A	B	C
Glucose (mg/dl)	268 ± 8*	272 ± 11*	69 ± 3
Creatinine (mg/dL)	0.7± 0.1	0.7 ± 0.2	0.6 ± 0.1
U. albumin (mg/24h)	0.4 ± 0.1*	0.3 ± 0.2**	0.2 ± 0.1

*Effect of DTS phytocompound on plasma and urinary parameters in STZ-induced diabetes. *p<0.01 DTS treated group (group B) vs control animals (group C); ** p<0.05 vs untreated animals (group A)*

Table II. *Effect of DTS supplementation on oxidative stress parameters in the kidney of diabetic rats.*

	GPx U/mg	CAT U/mg	MDA nM/mg
A	2.8 ± 0.7*	0.1 ± 0.05*	0.37 ± 0.3*
B	3.9 ± 0.9**	0.2 ± 0.1**	0.15 ± 0.3**
C	4.4 ± 0.8	0.3 ± 0.1	0.13 ± 0.1

*Effect of DTS phytocompound on oxidative stress parameters in renal tissue in STZ-induced diabetes. *p<0.01 DTS treated group (group B) vs control animals (group C); ** p<0.05 vs untreated animals (group A).*

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interstitial sclerosis. The glomerulosclerosis score in diabetic rats was significantly higher than that of control rats (1.04±0.08 *vs* 0.15±0.03, p<0.01, Fig. 1). DTS significantly reduced glomerulosclerosis in diabetic rats (0.71±0.09, p<0.05 *vs* untreated DM. Concomitantly, DTS treatment significantly decreased (p<0.05) the over 50% increase of glomerular volume observed in diabetic rats (p<0.001 *vs* healthy control). Interstitial fibrosis was focal and mild but the interstitial fibrosis score was higher in untreated diabetic rats than in controls (0.44±0.02 *vs* 0.11±0.03, p<0.01; Fig. 1). Interstitial fibrosis was significantly reduced by DTS compared (0.29±0.04, p<0.05) with that of untreated diabetic rats. DTS did not produce any significant plasma or tissue biochemical changes in healthy control rats (data not shown). There was a significant correlation among 24-hour urinary protein on the one side and, glomerulosclerosis score (r=0.61, p<0.05), and interstitial fibrosis score (r=0.63, p<0.05) on the other one. DTS in healthy control rats did not produce any substantial histological change.

Assessment of nephrin and Macrophage Chemoattractant Protein-1 (MCP-1) gene expression in the kidney

Nephrin mRNA expression in diabetic rats was lower than that of control rats (66%, *p*<0.05). DTS significantly restored nephrin mRNA expression in diabetic rats (Fig. 2). MCP-1 mRNA expression in the renal cortex was significantly increased in diabetic rats as compared with that of control rats (288%, p<0.01). Over-expression of MCP-1 in diabetic rats was significantly reduced by treatment with DTS (67%, p<0.05). There was a significantly negative correlation between nephrin and MCP-1 mRNA (r: –0.75, p<0.01) and 24-hour proteinuria with either mRNA levels of nephrin (r=–0.63, p<0.01) or MCP-1 (r=0.59, p<0.01).

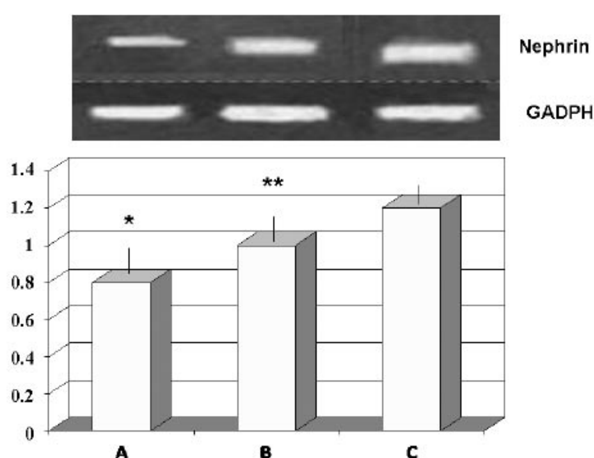

Fig. 1. *Effect of STZ-induced diabetes and phyto-compound treatment on glomerular parameters. Effect of DTS phytocompound on glomerular parameters in STZ-induced diabetes. GV: glomerular volume, GSI: glomerulosclerosis index; IF: interstitial fibrosis. *p<0.01 DTS treated group (group B) vs control animals (group C); ** p<0.05 vs untreated animals (group A)*

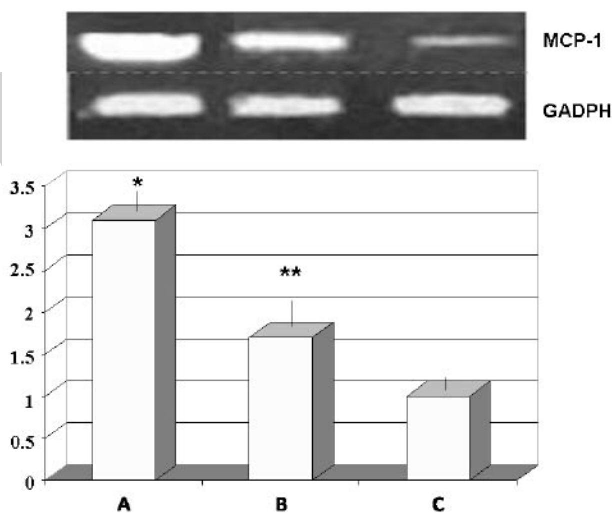

Fig. 2. *MCP-1 gene expression in renal tissue in STZ-induced diabetes: effect of nutraceutical treatment. Effect of DTS phytocompound on MCP-1 gene expression in renal tissue in STZ-induced diabetes. * p<0.01 DTS treated group (group B) vs control animals (group C); ** p<0.05 vs untreated animals (group A)*

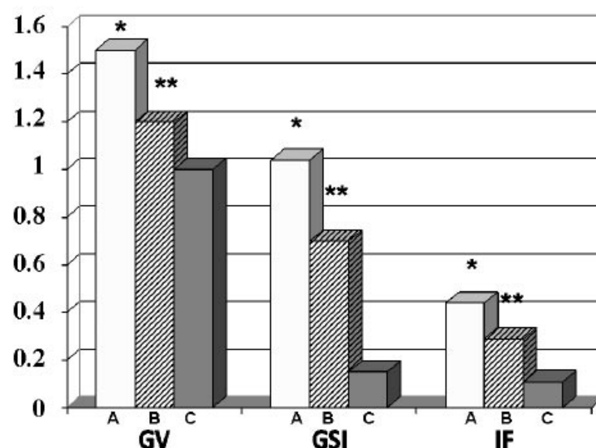

Fig. 3. *Modifications of nephrin gene expression in renal tissue in STZ-induced diabetes: effect of DTS nutraceutical. Effect of DTS phytocompound on nephrin gene expression in renal tissue in STZ-induced diabetes. *p<0.05 DTS treated group (group B) vs control animals (group C); ** p<0.01 vs untreated animals (group A)*

DISCUSSION

Diabetic nephropathy is a complication occurring in 30-50% of patients with type 2 diabetes after about 25 years of the disease and is the major cause leading to cardiovascular derangement and end-stage renal disease in the Western countries and in Asia, particularly in Japan. Early alterations in diabetic nephropathy consist in glomerular hyperfiltration hypertrophy also involving the tubular epithelium with consequent microalbuminuria. Although it is not fully clear how the structural changes bring about the microalbuminuria, the damage to the glomerular basement membrance (GBM) and mesangium is likely to result from the decreased density and sulfation of heparan sulfate proteoglycans (24). This in turn causes a loss of charge selectivity, and leakage of serum albumin across the barrier (25). Injury to the capillaries might result from multifactorial components, such as hemodynamic factors (26), glomerular hyperfiltration due to vasoactive control system abnormalities (27) and others. However, whatever the mechanisms are, the final GBM damage is the most consistent feature which develops during the glomerulopathy. These subtle changes are followed by thickening of glomerular basement membrane, accumulation of extracellular matrix, and overt proteinuria, eventually leading to glomerulosclerosis and end-stage renal disease (28). This sequel of events has been regarded as the

result of an immunological-inflammatory process. Indeed, the presence of monocyte chemoattractant peptide-1 (MCP-1), IL-1β, adhesion molecules, and macrophage infiltration in kidney has been reported in diabetic patients and animal diabetic models. MCP-1, a typical chemokine, recruits inflammatory cells to renal tissue and triggers inflammatory processes such as the stimulation of other cytokines and growth factors, which ultimately leads to kidney injury by induction of α1-type IV collagen synthesis. In this respect, STZ-induced diabetes provides a relevant model of endogenous chronic oxidative stress and hyperglycemia which, in turn promotes oxidative stress and up-regulates the expression of transforming growth factor-beta 1 (TGF-β1) in the glomeruli (29). Moreover, monocyte/macrophages glomerular infiltration, and glomerular hypertrophy bring about progressive damage of podocytes which are characterized by high differentiation but limited dividing ability and which are the major synthesis site of inflammatory cytokines, vascular endothelial growth factor, and chemokines receptors (30). In the present study DTS supplementation significantly mitigated oxidative stress in the kidney of STZ-treated rats. Furthermore, despite the overall diabetic features remaining unaffected (hyperglycemia, low insulin level), the supplemented nutraceutical significantly improved the proteinuria by a mechanism not yet fully understood which warrants further studies. In particular, proteinuria was directly correlated with GSI and IFI in untreated rats, while both scores significantly improved in DTS-treated rats.

DTS also mitigated the significant decrease of nephrin mRNA expression which was inversely correlated to proteinuria. It is generally accepted that podocyte damage is inversely correlated with down regulation of nephrin expression which suggests a switch-over to intermediate filament expression (31). Indeed, as a consequence of down regulation of nephrin expression, the structure of slit membrane in podocytes is destroyed and this represents one of the hallmarks of renal progressive dysfunction in an experimental setting (32) and in patients with diabetic nephropathy (33). It will be worthwhile in future studies to examine the possible effect of DTS on podocyte integrity. Although we did not conduct a detailed and specific morphological study on podocyte status, our study suggests that DTS could partially prevent the loss of podocytes and subsequently reduce urinary albumin excretion. The effect of DTS on podocyte integrity should be the focus of our new studies. Conversely, DTS significantly reduced glomerular expression of MCP-1 through a likely anti-oxidant/anti-inflammatory property exerted by the main components of the phytocompound (14-15), possibly resulting in nitric oxide-induced vasodilation as recently suggested for eucomnia ulmoides (34), although this hypothesis needs experimental confirmation.

While the primary prevention of nephropathy, and indeed of most diabetic complications, resides in the control of hyperglycemia and genetic predisposition to hypertension, micro-albuminuria and cardiovascular disease may be further complication factors in the course of this disease. The present study shows that DTS nutraceutical may offer a promising option to be further explored in clinical practice in a foreseeable integrative care of diabetic patients.

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