

Effects of glutamine supplementation on oxidative stress-related gene expression and antioxidant properties in rats with streptozotocin-induced type 2 diabetes

Pei-Hsuan Tsai¹, Jun-Jen Liu², Chui-Li Yeh³, Wan-Chun Chiu¹ and Sung-Ling Yeh^{1*}

¹School of Nutrition and Health Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, ROC

²School of Medical Laboratory Science and Biotechnology, Taipei Medical University, Taipei, Taiwan, ROC

³Department of Food and Nutrition, Chinese Culture University, Taipei, Taiwan, ROC

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Abstract

There are close links among hyperglycaemia, oxidative stress and diabetic complications. Glutamine (GLN) is an amino acid with immunomodulatory properties. The present study investigated the effect of dietary GLN on oxidative stress-related gene expressions and tissue oxidative damage in diabetes. There were one normal control (NC) and two diabetic groups in the present study. Diabetes was induced by an intraperitoneal injection of nicotinamide followed by streptozotocin (STZ). Rats in the NC group were fed a regular chow diet. In the two diabetic groups, one group (diabetes mellitus, DM) was fed a common semi-purified diet while the other group received a diet in which part of the casein was replaced by GLN (DM-GLN). GLN provided 25% of total amino acid N. The experimental groups were fed the respective diets for 8 weeks, and then the rats were killed for further analysis. The results showed that blood thioredoxin-interacting protein (Txnip) mRNA expression in the diabetic groups was higher than that in the NC group. Compared with the DM group, the DM-GLN group had lower glutamine fructose-6-phosphate transaminase 1, a receptor of advanced glycation end products, and Txnip gene expressions in blood mononuclear cells. The total antioxidant capacity was lower and antioxidant enzyme activities were altered by the diabetic condition. GLN supplementation increased antioxidant capacity and normalised antioxidant enzyme activities. Also, the renal nitrotyrosine level and Txnip mRNA expression were lower when GLN was administered. These results suggest that dietary GLN supplementation decreases oxidative stress-related gene expression, increases the antioxidant potential and may consequently attenuate renal oxidative damage in rats with STZ-induced diabetes.

Key words: Glutamine: Diabetes: Thioredoxin-interacting protein: Glutamine fructose-6-phosphate transaminase 1: Nitrotyrosine

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia, disturbance of macronutrients, and the development of micro- and macrovascular complications. Increasing evidence from both experimental and clinical studies suggests that there are close links among hyperglycaemia, oxidative stress and diabetic complications^(1,2). There are four main hypotheses about how hyperglycaemia causes diabetic complications: (1) increased polyol pathway flux; (2) increased hexosamine pathway flux; (3) activation of protein kinase C (PKC) isoforms; (4) increased advanced glycation end-product (AGE) formation. These metabolic pathways are major contributors to reactive oxygen species (ROS) overproduction and high oxidative stress during hyperglycaemia^(2–4).

Glutamine (GLN) is the most abundant free amino acid in the circulation. Several studies have demonstrated that GLN has immunomodulating properties and suppresses inflammatory responses^(5,6). GLN is required during catabolic processes to manifest optimal tissue responses to catabolism, inflammation and infection. It is considered an essential amino acid during certain diseases^(7,8). A previous study has found that supplementing a high-fat diet with GLN attenuated hyperglycaemia in a diabetic rodent model⁽⁹⁾. A study by Greenfield *et al.*⁽¹⁰⁾ demonstrated that GLN improved glycaemic control in type 2 diabetic patients. A recent study performed by our laboratory has also shown that supplemental dietary GLN decreased leucocyte adhesion molecule expression and oxidative stress in organs of mice with type 1 diabetes⁽¹¹⁾.

Abbreviations: ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate); AGE, advanced glycation end product; AR, aldose reductase; BMC, blood mononuclear cell; DM, diabetes mellitus; GFAT, glutamine fructose-6-phosphate transaminase 1; GLN, glutamine; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; NC, normal control; PKC, protein kinase C; RAGE, receptor of AGE; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; Txnip, thioredoxin-interacting protein.

* **Corresponding author:** Dr S.-L. Yeh, email sangling@tmu.edu.tw

GLN is the precursor for the synthesis of glutathione (GSH). There is a significant correlation between GLN supply and intracellular GSH content⁽¹²⁾. There is increasing evidence that GLN supplementation enhances antioxidant capacity in various diseases^(13,14). Because interruption of the production of superoxide would normalise the polyol pathway flux, AGE formation, PKC and NF- κ B activation in diabetes⁽⁴⁾, we hypothesised that GLN supplementation reduces oxidative stress and thus decreases the associated pathway flux. Therefore, erythrocyte antioxidant enzyme activities and the total antioxidant capacity were measured. On the other hand, we analysed gene expressions of proteins in blood mononuclear cells (BMC) including aldose reductase (AR), glutamine fructose-6-phosphate transaminase 1 (GFAT) and PKC. AR is the first enzyme in the polyol pathway. It catalyses the NADPH-dependent reduction of various carbonyl compounds. GFAT is the rate-limiting enzyme in the hexosamine pathway that converts glucose to glucosamine^(3,4). Abnormal activation of PKC was implicated in decreased glomerular production of NO induced by diabetes⁽¹⁵⁾. Also, the receptor of AGE (RAGE) and thioredoxin-interacting protein (Txnip) gene expressions were measured in the present study. The RAGE is a multi-ligand receptor of cell surface molecules. Binding of AGE to the RAGE activates a number of pathways implicated in chronic inflammation and the development of diabetic complications⁽¹⁶⁾. Thioredoxin is a highly conserved thiol reductase that interacts with the endogenous inhibitor Txnip^(17,18). Hyperglycaemia inhibits thioredoxin's ROS-scavenging function by inducing Txnip⁽¹⁹⁾. A previous report has found that Txnip expression significantly increases in diabetic animals, and may have implications in the development of end-stage organ damage in diabetics⁽¹⁷⁾. The present study was designed to investigate the effects of dietary GLN on oxidative stress-related gene expressions and its antioxidant properties. Because the kidneys are the major site of AGE clearance⁽²⁰⁾, and diabetic nephropathy is a complication that usually occurs in patients with poorly controlled disease, we evaluated oxidative damage to the kidneys in this streptozotocin (STZ)-induced diabetic model.

Materials and methods

Animals

Male Wistar rats, 6-weeks old, weighing 180–200 g at the beginning of the experiment were used in the present study. All rats were housed in a temperature- and humidity-controlled room and were allowed free access to a standard chow diet for 1 week before the study. Care of the laboratory animals was in full compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and the protocols were approved by the Institutional Animal Care and Use Committee at Taipei Medical University.

Study protocols

Rats were assigned to one normal control (NC, n 8) group and two diabetic groups (n 10, each). Diabetes was induced

by an injection of 150 mg nicotinamide/kg (Sigma Chemical, St Louis, MO, USA) followed by STZ (Sigma) at a dose of 65 mg/kg after 15 min via an intraperitoneal route. The induction procedure was repeated 1 d later. Nicotinamide was dissolved in 0.9% saline, and STZ was dissolved immediately before use in sodium citrate (0.05 mol/l; pH 4.5). This is a well-established animal model to imitate type 2 diabetes⁽²¹⁾. Nicotinamide was used to protect pancreatic β -cells against STZ toxicity in order to build up a relative insulin-deficient state in rats that mimic type 2 diabetes⁽²²⁾. Animals were allowed to eat laboratory chow *ad libitum* for 1 week. Rats with 12 h fasting blood glucose levels exceeding 2000 mg/l were considered diabetic. Rats in the NC group were fed a regular chow diet. In the two diabetic groups, one group (DM) was fed a common semi-purified diet, while the other group (DM-GLN) was provided an identical diet except that part of the casein was replaced by GLN, which provided 25% of total amino acid N. This amount of GLN was found to have beneficial effects on DM rodents⁽¹¹⁾. The experimental diets were isonitrogenous and identical in energy and nutrient distributions (Table 1). After feeding the respective diets for 8 weeks, all rats were anaesthetised and killed by drawing arterial blood from the aorta of the abdomen. Blood samples were collected in tubes containing K₂-EDTA (Sigma) and immediately centrifuged at 1500 g for 10 min to separate the plasma. Packed erythrocytes were washed three times with isotonic saline. The kidneys were rapidly excised. All samples were stored at -80°C until further analysed.

Measurement of plasma biochemical parameters

Plasma glucose concentrations at the end of the study were determined by colorimetric methods after an enzymatic reaction with peroxidase (Randox, Antrim, Ireland). TAG, total cholesterol, HDL-cholesterol and LDL-cholesterol, creatinine and blood urea N were analysed by an autoanalyser (Hitachi 7170, Hitachi Limited, Tokyo, Japan). The procedures followed the manufacturers' instructions.

Table 1. Composition of the experimental diets (g/kg)

Components	DM	DM-GLN
Soyabean oil	100	100
Casein	200	150
GLN	0	41.7
Salt mixture*	35	35
Vitamin mixture†	10	10
Methyl cellulose	31	31
Choline bitartrate	2.5	2.5
Met	3	3
Maize starch	618.5	626.8

DM, diabetes without glutamine; DM-GLN, diabetes with glutamine; GLN, glutamine.

* The salt mixture contained the following (mg/g): calcium phosphate dibasic, 500; NaCl, 74; potassium sulphate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulphate, 0.55.

† The vitamin mixture contained the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL- α -tocopherol acetate, 20; cholecalciferol, 0.25; menaquinone, 0.005.

Measurement of the plasma total antioxidant capacity

The total antioxidant capacity was measured using an antioxidant assay kit (Cayman Chemical, Ann Arbor, MI, USA). The principle of the assay is the ability of aqueous and lipid antioxidants in the sample to inhibit the oxidation of 2,2'-azino-di-3-ethylbenzthiazoline sulphonate (ABTS) to ABTS⁺ by metmyoglobin. The capacity of the antioxidants present in the sample to prevent ABTS oxidation was compared with that of standard Trolox, a water-soluble tocopherol analogue. All samples were diluted 1:30 with the assay buffer before being assayed. Trolox standards and reagent were prepared according to the manufacturer's instructions. Then, 10 µl of Trolox standard and samples were loaded onto a ninety-six-well plate. Also, 10 µl of metmyoglobin and 150 µl of chromogen were added to the wells. The reaction was initiated by adding 40 µl H₂O₂ as quickly as possible. The plate was covered and incubated for 5 min at room temperature and was monitored at an absorbance of 750 nm in a spectrophotometer. Samples and Trolox standards were assayed in duplicate. Results are presented as mm Trolox equivalents.

Measurement of erythrocyte antioxidant enzyme activities, and reduced and oxidised glutathione contents

To 100 µl of packed erythrocytes, 300 µl of cold redistilled water were added to lyse the cells. Then, the lysed erythrocytes were centrifuged at 15 000 g for 10 min. The supernatants were used to analyse the antioxidant enzyme activities including those of catalase, glutathione peroxidase (GPx), glutathione reductase and superoxide dismutase (SOD; Randox). Total glutathione and oxidised glutathione (GSSG) were measured by a previously described enzymatic method⁽¹¹⁾. The content of GSH was calculated as the difference between total glutathione and GSSG. To assay GSSG, we added 4 µl of 10 mM-1-methyl-2-vinylpyridinium (Sigma) to 70 µl of packed erythrocytes (derivatised sample), mixed them and kept them at room temperature for 1 h. After that, buffer A (200 µM-NADPH, 2 units/ml glutathione reductase and 2 mM-EDTA in 50 mM-phosphate buffer) and buffer B (10 mM-5,5'-dithiobis-2-nitrobenzoic acid in 50 mM-phosphate buffer) were immediately added to 10 µl of the derivatised

sample. The change in the optical density at 5 min was measured with a spectrophotometer at 405 nm. To determine total glutathione, buffers A and B were added to erythrocytes, and absorption was measured at 405 nm. The glutathione content was calculated on the basis of a standard curve generated with a known concentration of glutathione. Enzyme activities, and GSH and GSSG contents are expressed per mg Hb. Hb was determined by an autoanalyser (Hitachi).

Total RNA extraction

Blood samples were applied to a Ficoll-Hypaque gradient (Amersham Biosciences, Piscataway, NJ, USA), and BMC were separated by density gradient centrifugation with a density of 1.077 g/ml for 30 min at 650 g and 4°C. BMC were washed twice with sterilised PBS (Sigma) and resuspended in 0.5 ml PBS. Total RNA from BMC was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Crawley, Sussex, UK) according to the manufacturer's protocol. Total RNA of the kidneys was obtained from 50 mg tissue by the guanidine isothiocyanate extraction method, using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Chloroform was added and allowed to stand at room temperature for 10 min. After centrifugation, the aqueous phase with RNA was transferred to a microtube. Cold isopropanol was added to the aqueous phase and precipitated by centrifugation. The RNA pellet was washed twice with 75% ethanol. The pellet was air-dried and dissolved in RNase-free water. The total RNA solution was stored at -80°C for a subsequent assay. The concentration of RNA was determined and quantified by measuring the absorbance at 260 and 280 nm on a spectrophotometer.

Real-time RT-PCR

The primers of the housekeeping gene (β -actin), AR, GFAT, PKC, RAGE and Txnip were purchased from Mission Biotech (Taipei, Taiwan). Primer sequences used for the quantitative RT-PCR assays are listed in Table 2. RNA was reverse-transcribed using an RT system (Fermentas, Vilnius, Lithuania). Briefly, 12 µl water containing 1 µg RNA were mixed with 1 µl oligo (dT) primer (0.5 µg/µl) and incubated for 5 min at 65°C. To the mixture, 4 µl of 5 × RT buffer,

Table 2. Primer sequences used for inflammatory mediator quantitative real-time PCR assays

Gene name	Accession no.	Primer sequence (5' → 3')	Product size (bp)
β -Actin	NM_031144.2	F: CACCAGTTCGCCATGGATGACGA R: CCATCACACCCCTGGTGCCTAGGGC	145
AR	X05884.1	F: GGCCAAGCCTGAGGACCCCT R: ACCGGATCAGCACCTGGGCT	95
GFAT	NM_001005879.1	F: CAGCGGTGTCGGCGGTTGA R: TGACGGGTTGCCATACCAAGCG	68
PKC- β	M19007.1	F: AGCCGGAGTGGATGGCTGGT R: CTCGCTTTCTCCGGCGGCA	85
RAGE	NM_053336.2	F: TGCCCAGCCTCCCCCTCAA R: GGGTGGCCACGCAGCTGTAG	128
Txnip	BC088411.1	F: TGAGCTTCTCAAGGGCCCT R: GTTGGCTGGCTGGGACGATCG	99

F, forward primer; R, reverse primer; AR, aldose reductase; GFAT, glutamine fructose-6-phosphate transaminase 1; PKC, protein kinase C; RAGE, receptor of advanced glycation end products; Txnip, thioredoxin-interacting protein.

2 μ l deoxyribonucleotide triphosphate mix (10 mmol/l), 1 μ l RNase inhibitor and 1 μ l MultiScribe-RT (200 U/ μ l) were added and incubated at 42°C for 60 min, and then the reaction was terminated by heating to 70°C for 5 min. Complementary DNA was used for the real-time PCR assay performed with an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to the supplied guidelines.

Analysis of nitrotyrosine in kidney homogenates

A 25% kidney homogenate was prepared in ice-cold lysis buffer (10% SDS and 10 mM-Tris base; pH 7.5) containing a protease inhibitor cocktail (Complete, Roche Diagnostics, Mannheim, Germany), using a homogeniser. The homogenates were centrifuged at 22 000 g for 20 min to remove cell debris. Supernatants were used for the analysis of nitrotyrosine. Nitrotyrosine concentrations were measured using a commercial ELISA kit (Millipore, Bedford, MA, USA). Nitro-bovine serum albumin (Sigma) was coated onto wells of microtitre strips, and nitrotyrosines were quantified using anti-nitrotyrosine antibodies. Competition was accomplished by adding 50 μ l organ supernatant samples and 50 μ l primary antibodies to the wells. Each competes with the coated nitrated proteins for antibody binding. The amount of antibody that binds to the coated nitro-bovine serum albumin is inversely proportional to the amount of nitrotyrosine present in the samples added to the well of the plate. The procedures followed the manufacturer's instructions.

Statistical analysis

Data are presented as mean values and standard deviations. All statistical analyses were performed using the SAS software package (SAS, Cary, NC, USA). Differences among different groups were determined by a one-way ANOVA followed by Duncan's *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Body weight and food intake

There were no differences in the initial body weights among the three groups. The food intake (DM group 27.9 (SD 2.4) g/d *v.* DM-GLN group 28.62 (SD 1.13) g/d; $P > 0.05$) or body weights (DM group 323.7 (SD 74.8) g *v.* DM-GLN group 332.5 (SD 90.1) g; $P > 0.05$) after feeding the diets for 8 weeks did not differ between the two diabetic groups.

Oxidative stress-related gene expressions by blood mononuclear cells

Txnip mRNA expression was higher in the NC group. Compared with the DM group, the DM-GLN group had lower GFAT, RAGE and Txnip gene expressions. There were no differences in AR or PKC- β mRNA expressions among the three groups (Fig. 1).

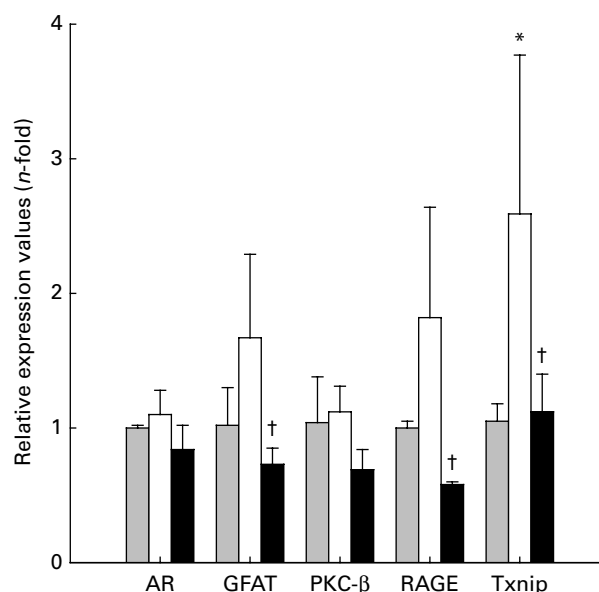


Fig. 1. Relative mRNA expressions of aldose reductase (AR), glutamine fructose-6-phosphate transaminase 1 (GFAT), protein kinase C (PKC)- β , receptor of advanced glycation end products (RAGE) and thioredoxin-interacting protein (Txnip) in blood mononuclear cells. Values are means of duplicate measurements, with standard deviations represented by vertical bars ($n = 4$). Differences among the groups were analysed by one-way ANOVA followed by Duncan's *post hoc* test. * Mean values were significantly different from those of the normal control group (NC, \square ; $P < 0.05$). † Mean values were significantly different from those of the diabetes mellitus group (DM, \square ; $P < 0.05$). DM-GLN (\blacksquare), diabetes with glutamine.

Plasma levels of biochemical parameters

Plasma glucose, total cholesterol, HDL-cholesterol, LDL-cholesterol, TAG and creatinine levels were significantly higher in the diabetic groups than in the NC group. There were no differences in glucose, total cholesterol, TAG, HDL-cholesterol, LDL-cholesterol, creatinine or blood urea N levels between the DM and DM-GLN groups (Table 3).

Plasma total antioxidant capacity

The total antioxidant capacity was significantly lower in the diabetic groups than in the NC group. The DM-GLN group had a higher total antioxidant capacity than the DM group (Table 4).

Erythrocyte antioxidant enzyme activities, and reduced and oxidised glutathione levels

GPx and SOD activities were significantly higher, while catalase activity was lower in the DM group than in the NC and DM-GLN groups. There were no differences in catalase, GPx or SOD activities between the NC and DM-GLN groups. The DM-GLN group had higher glutathione reductase activity than the NC and DM groups. There were no differences in GSH levels among the three groups; however, the GSSG level was significantly higher in the DM group than in the NC and DM-GLN groups (Table 4).

Table 3. Plasma concentrations of glucose, TAG, total cholesterol (TC), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C), creatinine and blood urea nitrogen (BUN) in the various groups at the end of the experiment
(Mean values and standard deviations, *n* 8 for the normal control group (NC) and *n* 10 for each diabetic group)

	NC		DM		DM-GLN	
	Mean	SD	Mean	SD	Mean	SD
Glucose (mg/l)	1843 ^a	137	4572 ^b	769	4431 ^b	629
TAG (mg/l)	409 ^a	71	806 ^b	484	756 ^b	388
TC (mg/l)	391 ^a	49	605 ^b	107	633 ^b	174
HDL-C (mg/l)	126 ^a	10	273 ^b	86	286 ^b	96
LDL-C (mg/l)	164 ^a	31	222 ^b	53	254 ^b	57
Creatinine (mg/l)	3.5 ^a	0.2	3.8 ^b	0.4	3.9 ^b	0.2
BUN (mg/l)	195	19	244	69	238	87

DM, diabetes without glutamine; DM-GLN, diabetes with glutamine.
^{a,b}Mean values within a row with unlike superscript letters were significantly different (*P*<0.05; one-way ANOVA with Duncan's *post hoc* test).

Txnip gene expression and nitrotyrosine levels in the kidneys

The DM group had higher Txnip mRNA expression than the NC and DM-GLN groups. The nitrotyrosine level was highest in the DM group. There were no differences in Txnip mRNA expressions or nitrotyrosine levels between the NC and DM-GLN groups (Table 5).

Discussion

Increased production of free radicals and/or an impaired antioxidant defence capability in diabetes indicate a central contribution of ROS to the pathological consequences of diabetes⁽²³⁾. Hyperglycaemia-induced overproduction of superoxide is the causal link between high glucose levels and diabetic complications. In the present study, we investigated the effect of GLN on key enzyme and protein expressions involved in major pathways responsible for hyperglycaemic damage.

The present results demonstrated that DM results in higher plasma glucose, lipid and creatinine levels, indicating that abnormal nutrient metabolism and renal function occurred. We also observed that the HDL-cholesterol level was higher in the DM groups than in the NC group. The rat is a species without endogenous cholesteryl ester or TAG transfer

capacity, because its lipid transfer protein activity is relatively low⁽²⁴⁾. Therefore, it is reasonable to have higher HDL-cholesterol when total plasma cholesterol is high in a diabetic condition. The findings of the present study did not show a decrease in glucose levels in diabetic rats after supplemental dietary GLN administration. The present results are inconsistent with previous reports which showed that supplementing GLN had positive effects on glucose metabolism by increasing insulin sensitivity which may lead to better glycaemic control^(9,10). However, the present study is consistent with a previous study performed by our laboratory which also showed that dietary GLN did not affect plasma glucose⁽¹¹⁾.

Hyperglycaemia results in increased enzymatic conversion of glucose to the polyalcohol sorbitol, with concomitant decreases in NADPH and GSH⁽⁴⁾. A previous study has found that AR inhibition prevented diabetic neuropathy in a 5-year animal study⁽²⁵⁾. AGE originally arise from non-enzymatic reactions between extracellular proteins and glucose. Intracellular hyperglycaemia is the primary initiating event in the formation of AGE. AGE bind to the RAGE on endothelial cells, mesangial cells and macrophages, inducing receptor-mediated production of ROS, and activate the transcription factor NF-κB and subsequent gene expressions⁽⁴⁾. Blockade of the RAGE was shown to inhibit the development of diabetic nephropathy^(26,27). Hyperglycaemia may also indirectly activate PKC isoforms

Table 4. Plasma total antioxidant capacity and erythrocyte antioxidant enzyme activities, reduced (GSH) and oxidised glutathione (GSSG) at the end of the experiment
(Mean values and standard deviations, *n* 8 for the normal control group (NC) and *n* 10 for each diabetic group)

	NC		DM		DM-GLN	
	Mean	SD	Mean	SD	Mean	SD
Trolox equivalents (mM)	1.84 ^a	0.33	0.98 ^b	0.36	1.36 ^c	0.16
Catalase (U/g Hb)	12.7 ^a	1.0	10.7 ^b	1.6	12.8 ^a	1.5
GPx (U/g Hb)	606.2 ^a	83.0	786.2 ^b	103.3	706.1 ^a	119.7
GRd (U/g Hb)	1.1 ^a	0.2	1.1 ^a	0.2	1.3 ^b	0.2
SOD (U/g Hb)	6123 ^a	1534	8669 ^b	1220	6922 ^a	1871
GSH (μmol/g Hb)	1.40	0.13	1.39	0.25	1.31	0.23
GSSG (μmol/g Hb)	0.10 ^a	0.02	2.97 ^b	0.70	1.19 ^c	0.27

DM, diabetes without glutamine; DM-GLN, diabetes with glutamine; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD, superoxide dismutase.
^{a,b,c}Mean values within a row with unlike superscript letters were significantly different (*P*<0.05; one-way ANOVA with Duncan's *post hoc* test).

Table 5. Kidney-relative thioredoxin-interacting protein (Txnip) mRNA expression and nitrotyrosine levels in kidney homogenates

 (Mean values and standard deviations, *n* 4 for Txnip, *n* 8 for the normal control group (NC) and *n* 10 for each diabetic group for nitrotyrosine, representative of duplicate measurements)

	NC		DM		DM-GLN	
	Mean	SD	Mean	SD	Mean	SD
Txnip (<i>n</i> -fold)	1.01 ^a	0.19	1.79 ^b	0.16	1.22 ^a	0.39
Nitrotyrosine (μg/ml)	17.33 ^a	3.04	22.81 ^b	6.35	17.80 ^a	3.00

DM, diabetes without glutamine; DM-GLN, diabetes with glutamine.

^{a,b}Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$; one-way ANOVA with Duncan's *post hoc* test).

through ligation of the RAGE and increased activity of the polyol pathway^(28,29). In early experimental diabetes, activation of PKC-β was shown to mediate retinal and renal blood flow abnormalities⁽³⁰⁾. Shunting of excess intracellular glucose into the hexosamine pathway plays an important role in hyperglycaemia-induced insulin resistance^(31,32). Also, it might cause several diabetic complications⁽³³⁾. Previous studies^(33,34) showed that inhibition of GFAT blocks hyperglycaemia-induced increases in the transcription of inflammatory mediators. In the present study, we demonstrated that the DM-GLN group had lower GFAT and RAGE gene expressions, which might indicate that oxidative stress was lowered when GLN was administered. The favourable effect of GLN supplementation may have resulted from its antioxidant capacity in a diabetic condition. GLN was found to be rate limiting for GSH synthesis, and the availability of GLN is critical for generating GSH stores⁽³⁵⁾. GSH represents the major source of cellular reducing equivalents, protecting cells against oxidative injury⁽³⁶⁾. Although erythrocyte GSH levels did not increase in the present study, we did observe a decrease in GSSG levels in the DM-GLN group, which may have increased the GSH:GSSG ratio and antioxidant potential. The present results also showed that the total antioxidant capacity increased, whereas BMC Txnip gene expression decreased in the DM-GLN group. Txnip is an endogenous inhibitor of the ROS-scavenging protein thioredoxin^(17,18). The thioredoxin X Txnip interaction plays an important role in the cellular redox balance⁽¹⁹⁾. Lower Txnip expression indicated that the ROS-scavenging activity of thioredoxin had increased.

The findings of the present study revealed that the activity of catalase decreased, whereas those of GPx and SOD increased in the diabetic condition. SOD converts superoxide anion radicals produced in the body to H₂O₂. GPx metabolises H₂O₂ to water using GSH as a hydrogen donor. Since DM is a disease with high oxidative stress, the antioxidant enzyme activities increased in response to high levels of free radicals, such as GPx and SOD, as observed in the present study. The effects of diabetes on antioxidant enzyme activities are erratic and depend on the species of animal, the duration of diabetes and the tissue studied⁽³⁷⁾. Also, there is not total agreement on the activities of these enzymes in a diabetic condition⁽³⁷⁾. The reason why catalase activity was suppressed in the diabetic group is not clear. However, the present results are consistent with previous reports^(38,39). The finding that GLN supplementation normalised changes in the antioxidant enzyme system indicates that the imbalance between ROS generation

and the antioxidant capacity was at least partly reversed by GLN administration.

Kidney nitrotyrosine levels were measured in the present study, because nitrotyrosine is considered a good marker of oxidative damage to proteins and of oxidative stress^(40,41). Nitration of the 3-position of tyrosine is the major product of a peroxy-nitrite attack on proteins. The findings of the present study showed that kidney nitrotyrosine levels were higher in the DM groups than in the NC group, indicating that the extent of NO oxidation increases with diabetes. The DM-GLN group had lower renal nitrotyrosine levels and Txnip gene expression, suggesting that oxidative damage to the kidneys was attenuated when GLN was administered in a diabetic condition.

In summary, the present study demonstrated that dietary GLN did not affect plasma glucose or lipid profiles. However, GFAT, RAGE, and Txnip mRNA expressions and renal nitrotyrosine levels were lower, whereas the total antioxidant capacity was higher and antioxidant enzyme activities were normalised in a diabetic condition when GLN was administered. These results suggest that dietary GLN supplementation decreases oxidative stress-related gene expressions, increases the antioxidant potential and may consequently attenuate renal oxidative damage in rats with STZ-induced type 2 diabetes.

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