

Red Wine Prevents Brain Oxidative Stress and Nephropathy in Streptozotocin-induced Diabetic Rats

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We have studied the effects of red wine on brain oxidative stress and nephropathy in streptozotocin (STZ)-induced diabetic rats. Diabetes was induced in Wistar rats with a single intraperitoneally injection of STZ (50 mg/kg). Two weeks before and four weeks after injection, red wine was given orally in both normal and diabetic rats. Blood samples were taken from the neck vascular trunk in order to determine the glucose, triglycerides, total cholesterol, HDL-cholesterol (HDL-c), atherogenic index (AI), total protein, blood urea nitrogen (BUN), creatinine, insulin, lipid peroxidation products, reduced glutathione (GSH) and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities. As well, we estimated the lipid peroxidation, GSH and SOD, GSH-Px and catalase activities in brain and renal homogenates, and the excretion of albumin, proteins and glucose in urine over 24 h period. The administration of STZ caused significant increases in levels of glycosuria, proteinuria, albuminuria, glycemia, total cholesterol and AI, as well as in lipid peroxidation products in the brain, plasma and kidney, whereas it decreased the GSH content and SOD, GSH-Px and catalase activities. Treatment with red wine significantly prevented the changes induced by STZ. These data suggested that red wine has a protective effect against brain oxidative stress, diabetic nephropathy and diabetes induced by STZ, as well as it protects against hypercholesterolemia and atherogenic risk.

Keywords: Diabetes, Nephropathy, Oxidative stress, Red wine, Streptozotocin

Introduction

Diabetes mellitus is a common but serious metabolic disorder associated with many functional and structural complications (McCall, 1992; Gispen and Biessels, 2000). This disorder is associated with an increased production of reactive oxygen species (ROS) in both humans and animals. Experimental evidence has supported that ROS play a role in both pathogenesis and numerous pathophysiological mechanisms that trigger diabetic complications, primarily categorized into macroangiopathy and microangiopathy, the latter of which includes retinopathy, nephropathy, neuropathy, and microvascular damage to the cerebral artery (Hyslop *et al.*, 1988; Oberdley, 1988; Hunt *et al.*, 1990; Baynes, 1991; Wolf *et al.*, 1991; Ha and Kim, 1995; Abdel Wahab *et al.*, 1996; Guifliano *et al.*, 1996; Baynes and Thrope, 1999; Auslander *et al.*, 2002). Hyperglycemia-induced oxidative stress has been implicated in the development of diabetic neuropathy in the peripheral (PNS) and central nervous system (CNS) and nephropathy (Oberdley, 1988; Biessels *et al.*, 1994; 2002; Forbes *et al.*, 2003; Biessels *et al.*, 2004).

Streptozotocin (STZ) has broad spectrum antibiotic activity and is often used to induce diabetes mellitus in experimental animals through its toxic effect on pancreatic β -cells (Ruiz *et al.*, 1994). Besides its antibiotic and diabetogenic properties, STZ is genotoxic in a variety of assays (Ruiz *et al.*, 1994). STZ is a monofunctional nitrosourea derivative that was first isolated from *Streptomyces achromogenes* (Herr *et al.*, 1967). This toxin has been shown to be involved in the fragmentation of DNA, as well as other deleterious effects by means of the production of ROS (Szkudelski, 2001).

Additionally, free radical scavengers have been shown to protect neurons against a variety of experimental neurodegenerative conditions (Mosmann and Behl, 2002), as well as have been suggested for attenuation of the oxidative stress and diabetic state induced by STZ (Agustin *et al.*, 1993; Muñoz *et al.*,

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1993; Montilla *et al.*, 1998). As well, epidemiological and experimental studies have evidenced that moderate consumption of red wine is inversely correlated with the incidence of dementia, oxidative stress and diabetes (Constant, 1997; Tredici *et al.*, 1999; Virgili and Contestabile, 2000; Sabu *et al.*, 2002; Montilla *et al.*, 2004).

We hypothesized that the combination red wine (Montilla-Moriles, Cordoaba, Spain) would prevent diabetes-induced changes in cerebral oxidative stress. The present study was designed to look at the effect of red wine in streptozotocin-induced oxidative stress in the brain of rats.

Material and Methods

Drugs and reagents The STZ and reagents were purchased from Sigma Chemical Co. (St. Louis, USA) and were of the highest commercial grade available. The red wine used in the study was donated by the Omeyas Warehouse S. A. (Montilla, Cordoba, Spain).

Animals All animal cares and procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and the R.D. 223/1988, and were approved by Bioethics Committee of the University of Cordoba, Spain. Three month-old male Wistar rats weighing between 230- 250 g at the beginning of the study were purchased from Charles River, Barcelona, Spain. They were subjected to controlled conditions of temperature (20-23°C), illumination (12 h light/12 h dark cycle, lights on at 08 : 00 h) and were provided with food (Purina®, Barcelona, Spain) and water *ad libitum*.

Experimental protocols Twenty (20) three month-old Wistar rats were separated into four groups of five animals: Group 1 normal untreated control; Group 2 red wine treated; Group 3 STZ treated; and Group 4 red wine and STZ treated. The STZ treatment was a single i.p. injection of 50 mg/kg body weight. The red wine treatment was an oral dose (400 mL/70 kg body weight/day) administered daily for two weeks prior to the STZ injection and daily for four weeks after the STZ injection. Group 2 received red wine for six weeks.

Rats were fed with Purina® (Barcelona, Spain) rat chow (composition: crude protein 180 g/kg; fats 32 g/kg; crude fibre 36 g/kg; carbohydrate 598 g/kg and metabolisable energy 301 kcal/100g) throughout the experimental period. After the six week experimental period urine samples were collected one day prior to sacrifice, the animals were sacrificed with blood, brain, and kidney collected. The biochemical parameters determined included: Table 1- plasma: glucose, insulin, BUN, creatinine, total protein, lipid peroxidation, GSH, SOD, and GSH-Px; serum: total cholesterol, triglycerides, HDL-cholesterol and atherogenic index; urine: glucose, albumin and total protein. Table 2- brain lipid peroxidation, GSH, GSH-Px, SOD and catalase; Table 3- Kidney lipid peroxidation, GSH, GSH-Px, SOD, and catalase.

Evaluation of diabetic state Plasma and urinary glucose was determined using commercial kits (Roche Diagnostics Corporation, Basel, Switzerland) on a Roche-Hitachi 917 autoanalyzer.

Insulin levels in plasma was measured by radioimmunoassay using rat-specific [¹²⁵I]-insulin as a tracer kit supplied by CIS Spain (Madrid, Spain).

Table 1. Effects of red wine and STZ on diabetic parameters, lipid profile, urinary excretion and plasma oxidative stress in the rat

	Control	Red wine	Streptozotocin	Red wine + streptozotocin
Plasma				
Glucose (mg/dL)	128 ± 10	142 ± 12	443 ± 47 ^a	152 ± 18 ^c
Insulin (ng/mL)	3.2 ± 0.4	2.9 ± 0.5	0.32 ± 0.1 ^a	2.0 ± 0.2 ^c
BUN (mg/dL)	38 ± 6	40 ± 3	44 ± 4	44 ± 7
Creatinine (mg/dL)	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Total protein (g/dL)	6.4 ± 0.2	6.5 ± 0.2	5.9 ± 0.8	6.5 ± 0.1
Lipid peroxidation products (nmol/L)	6.3 ± 0.6	6.9 ± 0.7	9.2 ± 1.1 ^a	6.1 ± 0.5 ^c
GSH (nmol/L)	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1 ^a	0.3 ± 0.1 ^c
SOD (U/L)	5.9 ± 0.3	6.1 ± 0.6	3.3 ± 0.3 ^a	4.2 ± 0.2 ^c
GSH-Px (U/L)	4.6 ± 0.6	4.3 ± 0.6	2.9 ± 0.6 ^a	6.5 ± 0.5 ^c
Serum				
Total cholesterol (mg/dL)	52 ± 7	52 ± 5	69 ± 9 ^a	60 ± 5 ^b
Tryglicerides (mg/dL)	57 ± 12	55 ± 6	56 ± 17	64 ± 16
HDL-C (mg/dL)	31 ± 3	34 ± 2	31 ± 4	34 ± 5
AI	0.7 ± 0.2	0.5 ± 0.1	1.3 ± 0.4 ^a	0.7 ± 0.3 ^c
Urine				
Glucosa (mg/24 h)	1.9 ± 1.3	1.2 ± 2.9	179 ± 66 ^a	2.2 ± 1.1 ^c
Albumin (mg/24 h)	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.2 ^b	0.2 ± 0.1 ^d
Total protein (g/24 h)	6.6 ± 1.2	13 ± 3.7	26 ± 6.9 ^b	15 ± 2.5 ^d

Values are means ± SEM of five rats.

^a*p* < 0.001 vs control; ^b*p* < 0.05 vs control; ^c*p* < 0.001 vs streptozotocin; ^d*p* < 0.05 vs streptozotocin.

Table 2. Effect of red wine on changes in lipid peroxidation products levels, reduced glutathione (GSH) content; and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase activities brain tissue in rats treated or not with streptozotocin

	Control	Red wine	Streptozotocin	Red wine + streptozotocin
Lipid peroxidation products (nmol/mg protein)	8.7 ± 0.9	8.6 ± 1.1	15 ± 1.6 ^a	7.1 ± 0.8 ^c
GSH (nmol/mg protein)	0.3 ± 0.04	0.3 ± 0.04	0.2 ± 0.03 ^a	0.3 ± 0.04 ^c
Catalase × 10 (U/mg protein)	0.3 ± 0.01	0.3 ± 0.03	0.1 ± 0.01 ^a	0.2 ± 0.02 ^c
SOD (U/mg protein)	9.5 ± 1.0	9.2 ± 0.8	6.3 ± 0.5 ^a	8.3 ± 0.6 ^c
GSH-Px (U/mg protein)	3.0 ± 0.3	2.5 ± 4	1.4 ± 0.2 ^a	2.3 ± 0.3 ^c

Values are means ± SEM of five rats.

^a*p* < 0.001 vs control; ^c*p* < 0.001 vs streptozotocin.

Table 3. Effect of red wine on changes in lipid peroxidation products levels, reduced glutathione (GSH) content; and superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase activities renal tissue in rats treated or not with streptozotocin

	Control	Red wine	Streptozotocin	Red wine + streptozotocin
Lipid peroxidation products (nmol/mg protein)	9.9 ± 1.4	10 ± 1.6	16 ± 1.2 ^a	12 ± 1.1 ^c
GSH (nmol/mg protein)	0.6 ± 0.06	0.6 ± 0.09	0.3 ± 0.04 ^a	0.6 ± 0.05 ^c
Catalase × 10 (U/mg protein)	0.2 ± 0.04	0.2 ± 0.04	0.03 ± 0.01 ^a	0.2 ± 0.03 ^c
SOD (U/mg protein)	14 ± 0.9	14 ± 0.7	9.5 ± 1.0 ^a	15 ± 1.3 ^c
GSH-Px (U/mg protein)	2.4 ± 0.5	1.9 ± 0.3	1.3 ± 0.3 ^a	2.3 ± 0.3 ^c

Values are means ± SEM of five rats.

^a*p* < 0.001 vs control; ^c*p* < 0.001 vs streptozotocin.

Assessment of oxidative stress

Estimation of lipid peroxidation products

Brain and kidney 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. The supernatant was collected.

Supernatant and plasma were immediately tested for lipid peroxidation products using the Bioxytech LPO-586 kit (OXIS International, Portland, USA). The kit uses a chromogenic reagent which reacts with the lipid peroxidation products malondialdehyde and 4-hydroxyalkenals at 45 ± 1°C, yielding a stable chromophore with maximum absorbance 586 nm.

Reduced glutathione content

Reduced glutathione (gamma-glutamylcysteinylglycine or GSH) content was determined in both brain and renal homogenates and plasma. GSH content was carried out using Bioxytech GSH-400 kit (OXIS International, Portland, USA). The method is based on a chemical reaction which proceeds in two steps, obtaining with GSH a chromophoric thione which has a maximal absorbance wavelength at 400 nm.

Antioxidant enzyme activity

Catalase was measured in the cell cytosol fraction. Catalase was assayed following Aebi (1984), by the rate of decomposition for H₂O₂ at 240 nm. The brain and kidney were homogenized in ice-cold phosphate buffer (6.81 g KH₂PO₄ in water, made up to 1,000 ml). The homogenates were then centrifuged at 1,000 × *g* for 10 min at room temperature. H₂O₂ (10 mM) was used as reagent, with the rate of dismutation of H₂O₂ to water and molecular oxygen being proportional to the concentration of catalase and with

maximum absorbance at 240 nm.

Total superoxide dismutase (SOD; E.C.: 1.15.1.1) activity was assayed by the Sun *et al.* technique (1998). Brain and renal tissue was homogenized in ice cold isotonic saline. The homogenates were then centrifuged at 10,000 × *g* for 10 min at 4°C. SOD assay is based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) reduction by superoxide generator, with maximum absorbance at 560 nm.

Glutathione peroxidase (GSH-Px; E.C.: 1.11.1.9) activity was evaluated in plasma, and brain and kidney homogenates by the Flohé and Gunzler method (1984). The tissues were homogenized in ice-cold buffer (0.1 M KH₂PO₄/K₂HPO₄, pH 7.0, plus 29.2 mg ethylenediaminetetraacetic 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. The glutathione peroxidase assay is based on the oxidation of NADPH to NAD⁺, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm.

Protein estimation

Protein concentration was determined by the Bradford method (1976) using bovine serum albumin as a standard.

Other parameters evaluated Plasma BUN, creatinine, total protein and serum total cholesterol, triglycerides and high-density lipoprotein-cholesterol (HDL-c), as well as urinary levels of total protein and albumin were determined using commercial kits (Roche Diagnostics Corporation, Basel, Switzerland) on a Roche-Hitachi 917 autoanalyzer. The atherogenic index (AI) was calculated

as [TC/HDL-c] (Serrano-Martínez *et al.*, 2004).

Statistical analysis Statistical analysis of the data was accomplished by means of the SPSS® statistical software package. The Shapiro-Wilk test did not show a significant departure from normality in the distribution of variance values. To evaluate variations in data, a one-way analysis of variance (one-way ANOVA) was performed followed by Bonferroni correction for multiple comparisons. The level of statistical significance was set at $p < 0.05$. All results are expressed as mean \pm S.E.M. Student's *t*-test was used to compare differences between two groups.

Results

The biochemical parameters indicated that the STZ injection produce diabetic animals with low levels of insulin production, and high levels of plasma glucose, and glucose excretion (Table 1).

Oxidative stress was also induced in the STZ-treated animals with increased lipid peroxidation and decreased GSH, SOD, GSH-Px, and catalase in brain and kidney homogenates and in plasma (Tables 1-3). The determination of nephropathy was determined using BUN, creatinine, and total protein in the serum along with urine glucose, albumin, and protein levels (Table 1). STZ had little effect on the BUN, creatinine and serum protein levels although urine glucose, albumin, and protein were elevated (Table 1).

In all biochemical measurements, red wine alone had no significant effect, but red wine did mitigate all of the STZ-induced changes in the measured parameters (Tables 1-3).

Discussion

STZ-induced diabetes provides a relevant example of endogenous chronic oxidative stress and hyperglycemia. Thus, we evaluated the brain oxidative stress and nephropathy induced by STZ in Wistar rats and examined the potential protective effects of red wine against the changes STZ-induced.

Herein we report that: (i) STZ induced a diabetic state characterized by hyperglycemia, glycosuria and decrease in plasma insulin levels; (ii) STZ increased plasma total cholesterol levels and atherogenic index; (iii) STZ triggered a drop in GSH concentration, an increase in lipid peroxidation levels, and reductions in key antioxidative enzymes in the brain, kidney and plasma; (iv) STZ prompted proteinuria and microalbuminuria; (v) the administration of red wine prevented the changes induced by STZ. Likewise, the collected data indicate the important role of the oxidative stress in this experimental model and in the evolution and course of diabetes. And support the hypothesis that STZ induces brain oxidative stress and diabetic state, as well as that red wine has neuroprotective properties and prevents cerebral oxidative stress, diabetes and diabetic nephropathy. The results obtained

are in agreement with the reports from Montilla *et al.* (Muñoz *et al.*, 1993; Montilla *et al.*, 1998; 2004), Ruiz *et al.* (1994), Hunt *et al.* (1990; 1993), Baynes (1991), and other researchers (Kakkar *et al.*, 1995; Guigliano *et al.*, 1996; Rauscher *et al.*, 2000), who found that this toxin induced an oxidative stress. We also found a significant correlation among diabetic state, nephropathy and brain oxidative stress. Oxidative stress can both induce lipid peroxidation and oxidative damage leading to cell death and promote the formation of a variety of vasoactive mediators that can affect renal functions. The high degree of oxidative stress in brain and renal tissue coincides with biochemical signs typical of diabetes, such as high levels of glycemia and urinary glucose excretion, as well as dropped in plasma insulin levels. In addition to these data, we observed a significant decrease in plasma insulin levels.

In the current report, the biochemical phenomena triggered by STZ were prevented previously by red wine. These results are compatible with our previous study (Montilla *et al.*, 2004). In this work, we found that treatment with red wine induced decreased levels of glycemia, plasma fructosamine and percentage of glycosylated haemoglobin, while increasing levels of plasma and pancreas insulin (Montilla *et al.*, 2004). Moreover, other studies showed that quercetin, a flavonoid that is widely present in red wines and plants, administration to rats reversed to normality the glucose tolerance test, hepatic glucokinase and hexokinase activities, lipid profile, and the number of pancreatic islets, as well as it decreased the degree of oxidative stress (Vessal *et al.*, 2003). According to Anjaneyulu and Chopra quercetin treatment can play an antioxidative action showing a nephroprotective effect, attenuating diabetic renal damage, probably through its antioxidative action (Anjaneyulu and Chopra, 2004). Furthermore, Watkins' group found that quercetin reversed of compromised antioxidant status in different tissues such as liver, kidney, brain and heart (Sanders *et al.*, 2001; Coldiron *et al.*, 2002). All of these data findings support those found in our study and suggest the protective action of red wine.

Although it was not the main aim of the present study, our results seem to indicate that red wine may affect plasma total cholesterol and atherogenic index. In our study red wine, in combination with STZ dropped total cholesterol and atherogenic index. These data are in agreement with previous reports having shown protective effect of polyphenolic compounds on oxidative stress and hypercholesterolemia (Saini *et al.*, 1994; Magi-Caperyon 2002).

In short, the present results constitute the first in vivo evidence for a neuroprotective role of red wine of the Montilla-Moriles (Cordoba, Spain) in brain oxidative stress in streptozotocin-induced diabetic rats. Thus, red wine reduces the intensity of oxidative stress and the severity of hyperglycemia, while enhancing plasma insulin levels.

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