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Journal of Nutritional Biochemistry 15 (2004) 688–693

THE JOURNAL OF Nutritional Biochemistry

Protective effect of Montilla-Moriles appellation red wine on oxidative stress induced by streptozotocin in the rat

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Abstract

This study evaluated the protective effect of Montilla-Moriles appellation red wine (Cordoba, Spain) on oxidative stress, course and intensity of symptoms in experimental diabetes induced by the injection of streptozotocin in male Wistar rats. The rats were injected with a single dose of streptozotocin (60 mg/kg i.p.) and given water and red wine separately. After 4 weeks of treatment, blood samples were obtained to determine sugar and fructosamine concentrations in blood plasma, serum insulin concentration, and percentage of glycosylated hemoglobin in blood. The kidney, liver, and pancreas were removed to determine lipid peroxidation levels, reduced glutathione content, and antioxidative enzyme activity. A significant increase of glucose concentration in urine was found in the rats after injecting the streptozotocin. The administration of red wine before streptozotocin elevated reduced glutathione content and antioxidative enzyme activity, while lowering the lipid peroxidation level. Moreover, the red wine induced decreased levels of glycemia, plasma fructosamine and percentage of glycosylated hemoglobin, while increasing levels of insulin. These data suggest that red wine has a protective effect against oxidative stress and diabetes induced by streptozotocin.

Keywords: Red wine; Diabetes; Oxidative stress

1. Introduction

Experimental evidence has supported the view that reactive oxygen species play a role in the numerous pathophysiological mechanisms that trigger diabetic complications. These include glucose autoxidation [1], nonenzymatic glycosylation of antioxidant enzymes and other proteins [2], polyol pathways [3], alteration of reduced glutathione and oxidized glutathione [4], intermediate products of cycloxygenase catalysis [5], and increased production of free radicals [6]. This fact suggests a clear link between oxidative stress and diabetes [7].

Streptozotocin is one of the most commonly used substances to induce diabetes in the rat. This molecule causes the death of β-cells by alkylation of DNA. Furthermore, 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 reactive oxygen species [8].

Dietary supplementation with natural antioxidants (α-tocopherol, β-carotene, vitamin C, uric acid, melatonin, etc.) attenuated the oxidative stress and diabetic state induced by streptozotocin [9–11]. Extensive experimentation has been performed with many known biological antioxidants, among them red wine polyphenols, using both in vivo and in vitro models applied to different tissues. These models were designed to test the effects of red wine on a variety of agents and/or situations leading to oxidative stress [12–14]. Accordingly, the polyphenolic compounds of wine, which are particularly abundant in red wine, could be implicated in enhancing the antioxidant system, since they behave as reactive oxygen species scavengers, metal chelators, and enzyme modulators [15]. In agreement with this view, it was demonstrated that polyphenols protect against the deleterious effect of oxidative stress and diabetes induced by alloxan in the Wistar rat [16]. Furthermore, some studies have shown the antidiabetic effect of flavonoids. These agents are naturally occurring phenolic compounds that are widely distributed in plants. As scavengers of reactive oxygen and nitrogen species, these agents inhibit peroxidation reactions and significantly reduce the oxidative

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0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2004.06.006

Table 1
Wine composition

	Red wine	
	Units	Values
Alcoholic degree	%	12.35
pH	20°C	3.41
Total acidity	g/L	4.64
Volatile acidity	g/L	0.41
Tartaric acid	g/L	2.01
Fix acidity	g/L	4.23
Reducing sugars	g/L	2.30
Density	g/mL	0.9923
Total SO_2	mg/L	85.00
Free SO_2	mg/L	31.00
Combined SO_2	mg/L	54.00
Total polyphenols index		56.00
Color density	ABS	5.00

ABS = Absorbance.

state [17,18]. Moreover, flavonoids have the capacity to inhibit enzymes such as cyclooxygenases and protein kinases involved in cell proliferation and apoptosis [19]. A recent study performed by Vessal et al. concluded that quercetin, a flavonoid present in red wine with antioxidant properties, leads to the regeneration of pancreatic islets and probably increases insulin release in rats with streptozotocin-induced diabetes, thus exerting beneficial antidiabetic effects [20].

The purpose of this study was to assess the effects of Montilla-Moriles appellation red wine (Cordoba, Spain) on the diabetic course and oxidative stress induced by the administration of streptozotocin in rats. The following parameters were used to evaluate diabetes and oxidative stress 4 weeks after the injection of streptozotocin: 1) for diabetes, we determined the changes produced in the levels of glycemia and fructosamine in plasma and the percentage of glycosylated hemoglobin; and 2) for oxidative stress, we analyzed the degree of lipid peroxidation, reduced glutathione content, and catalase activity in pancreas, liver, and kidney tissue.

2. Methods and materials

2.1. Wine samples

The red wine used in the present study came from the Montilla-Moriles wine-making region (Cordoba, Spain). Quantitative analyses were performed according to official methods [21]. The composition of the wine is shown in Table 1. All spectrophotometric determinations were made using a Shimadzu UV-1606 (Shimadzu Corporation Kyoto, Japan). Color density was calculated as the sum of absorbance at 520 and 420 nm.

2.2. Animals

The study was performed with male Wistar albino rats (250–300 g) purchased from Charles River (Crediffa S.A., Barcelona, Spain). The rats were maintained under a controlled light-dark cycle (12:12 h, lights on at 08:00) and constant temperature (20–23°C). Food (Purina® Rat Chow, Barcelona, Spain) and water were supplied ad libitum.

2.3. Experimental design

A total of 30 rats were used in the study. The rats were divided into five groups with six rats each and given the following: 1) water only (control), 2) red wine, 3) streptozotocin injection, 4) red wine given 2 weeks before injecting streptozotocin (red wine + streptozotocin), and 5) red wine and streptozotocin administered simultaneously (streptozotocin + red wine).

Streptozotocin was administered in a single dose (60 mg/kg i.p. in buffered citrate solution, pH 4.0) to produce diabetes. On day 3, urinary glucose was quantitatively determined in samples collected in metabolic cages. The animals were given free access to the red wine. The red wine or water were administered 2 weeks before injecting the streptozotocin and were present until the end of the experiment (4 weeks after injecting the streptozotocin). In some groups, red wine and streptozotocin were administered simultaneously at the beginning of the treatment and were present until the end of the experiment (4 weeks after injecting the streptozotocin).

At the end of the treatments, all the animals were sacrificed under light anesthesia by ether. Blood samples were collected from the vascular trunk of the neck to determine glucose and fructosamine levels and percentage of glycosylate hemoglobin. The pancreas, liver, and kidneys were removed immediately and homogenized.

2.4. Lipid peroxidation levels

Tissues were homogenized in ice-cold 20 mmol/L Tris-HCl, buffer, pH 7.4, to produce a homogenate. The homogenates were then centrifuged at 10,000 × *g* for 10 minutes at 4°C. The supernatant was collected and immediately tested for lipid peroxidation using the Bioxytech LPO-586 kit (OXIS International, Portland, OR, USA).

2.5. Reduced glutathione content

The tissue homogenates were diluted 1/20 (g/mL) in 5% metaphosphoric acid. The homogenates were then centrifuged at 2500 × *g* and 4°C, for 10 minutes. The supernatant was collected and immediately tested for reduced glutathione content (GSH) using the Bioxytech GSH-400 kit (OXIS International).

2.6. Antioxidant enzyme activity

Antioxidant enzyme catalase activity (E.C. 1.11.1.6) was measured in the tissues according to the method of Aebi [22]. Total superoxide dismutase (SOD; E.C.: 1.15.1.1) activity was assayed using the technique described by Sun et al. [23]. Glutathione peroxidase (GPx; E.C.: 1.11.1.9) activity was evaluated by the method of Flohé and Gunzler [24].

2.7. Protein estimation

Proteins were detected by the method of Bradford using bovine serum albumin as a standard.

2.8. Insulin levels

At the end of the study and under ether anesthesia, the animals were sacrificed and trunk blood was collected. Insulin levels in serum and renal tissue were measured by radioimmunoassay using rat-specific [^{125}I]-insulin as a tracer kit supplied by CIS Spain (Madrid, Spain).

2.9. Other parameters evaluated

Plasma glucose (glucose oxidase method, Boehringer-Mannheim, Mannheim, Germany) was measured. Fructosamine levels were estimated using a colorimetric assay; fructosamine, glycated serum protein reduces nitroblue tetrazolium (NTB) under alkaline conditions and forms a purple formazan with maximum absorption at 530 nm (Menarini S.A., Barcelona, Spain, Abbott Park, IL, USA). Percentage of glycosylated hemoglobin in total blood was determined by ionic capture (IMX System, Abbott, Abbott Park, IL, USA).

2.10. Materials

The reagents were purchased from Sigma Chemical Co. (St. Louis, MO) 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).

2.11. Statistical analysis

Statistical analysis of the data was accomplished by means of the SPSS statistical software package (SPSS Inc., Chicago, IL). 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 was performed followed by the Student *t* test using the Bonferroni correction for multiple comparisons. The level of statistical significance was set at $P < 0.05$. All results are expressed as mean ± SE.

Table 2
Effects of red wine on diabetic parameters (glucose, fructosamine, and percentage of hemoglobin) in plasma in the rat

	Glucose (mg/dL)	Fructosamine (μmol/L)	Glycosylated hemoglobin (%)
Control	132.14 ± 12.14	143.60 ± 5.45	9.28 ± 1.25
Red wine	148.86 ± 11.80	143.56 ± 7.56	9.54 ± 0.87
Streptozotocin	431.80 ± 55.67[a]	201.98 ± 55.06[a]	12.48 ± 1.12[a]
Red wine + streptozotocin	156.36 ± 37.72[b]	143.29 ± 9.97[b]	9.51 ± 1.05[c]
Streptozotocin + red wine	310.60 ± 78.68[d]	239.62 ± 40.05	12.72 ± 2.30

Values are means ± SEM of six rats and are expressed as milligram per deciliter, micromol per liter, and percentage, respectively.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.01$ vs streptozotocin group; d: $P < 0.05$ vs streptozotocin group.

3. Results

3.1. Diabetic state

The injection of streptozotocin induced a high degree of hyperglycemia ($P < 0.001$ vs control), as well as a significant reduction in insulin levels. Changes in glycemia and in serum insulin did not occur in normal rats after the administration of red wine. However, a significant decrease in glycemia concentration and a significant increase in insulin levels were found in diabetic rats, depending on the time when the red wine was administered (Tables 2 and 3). Thus, red wine administered before injecting streptozotocin prevented hyperglycemia, whereas the simultaneous administration of both red wine and streptozotocin led to a small increase in glucose induced by streptozotocin (Table 2).

Diabetic rats showed a highly significant increase in the percentage of glycosylated hemoglobin, which was significantly prevented when red wine was administered before injecting streptozotocin. Significant changes in the percentage of glycosylated hemoglobin were not observed in diabetic rats that were given red wine and streptozotocin simultaneously (Table 2).

Table 3
Effects of red wine on insulin levels

	Pancreatic tissue (ng/g tissue)	Plasma (ng/mL)
Control	51.62 ± 8.15	3.35 ± 0.77
Red wine	43.60 ± 7.87	2.83 ± 0.35
Streptozotocin	3.54 ± 1.12[a]	0.23 ± 0.02[a]
Red wine + streptozotocin	30.66 ± 1.05[b]	1.99 ± 0.03[b]
Streptozotocin + red wine	7.55 ± 1.30[b,c]	0.49 ± 0.03[b,c]

Values are means ± SEM of six rats.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.001$ vs red wine + streptozotocin group.

Table 4
Effects of red wine on lipid peroxides levels induced by streptozotocin.

	Lipid peroxides MDA + 4-HDA (nmol/L/mg protein)		
	Pancreas	Liver	Kidney
Control	9.24 ± 0.15	6.50 ± 0.28	9.92 ± 1.33
Red wine	10.90 ± 1.23	7.92 ± 1.06	10.53 ± 0.55
Streptozotocin	13.83 ± 1.29[a]	12.43 ± 1.00[a]	15.43 ± 1.58[a]
Red wine + streptozotocin	9.43 ± 0.86[b]	9.28 ± 0.70[b]	11.78 ± 1.23[c]
Streptozotocin + red wine	11.67 ± 0.82[c]	8.51 ± 2.00[b]	13.10 ± 1.15[c]

Values are means ± SEM of six rats and are expressed as nanomoles of MDA + 4-HDA per milligram of protein.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.05$ vs streptozotocin group.

Fructosamine levels increased markedly in diabetic rats. These values were prevented when red wine was administered before injecting the streptozotocin. However, no changes were observed when red wine and streptozotocin were simultaneously administered (Table 2).

3.2. Lipid peroxidation

Diabetes induced by streptozotocin led to a clear increase in lipid peroxide levels, although these changes were prevented when the red wine was administered before the streptozotocin. This effect was greater than when the wine and streptozotocin were administered at the same time (Table 4).

3.3. Reduced glutathione

Streptozotocin depleted the GSH content of pancreas, liver, and kidney tissues; an effect that was prevented by administering melatonin before injecting streptozotocin. This effect was greater than in the group simultaneously treated with red wine and streptozotocin (Table 5).

Table 5
Effects of red wine on reduced glutathione content in diabetes induced by streptozotocin

	Reduced glutathione (nmol/L/mg protein)		
	Pancreas	Liver	Kidney
Control	0.222 ± 0.04	0.668 ± 0.14	0.557 ± 0.07
Red wine	0.200 ± 0.02	0.680 ± 0.16	0.840 ± 0.14
Streptozotocin	0.122 ± 0.02[a]	0.276 ± 0.08[a]	0.395 ± 0.02[a]
Red wine + streptozotocin	0.226 ± 0.02[b]	0.564 ± 0.09[b]	0.693 ± 0.06[b]
Streptozotocin + red wine	0.172 ± 0.02[c]	0.484 ± 0.03[c]	0.653 ± 0.11[c]

Values are means ± SEM of six rats and are expressed as nanomoles of GSH per milligram of protein.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.05$ vs streptozotocin group.

Table 6
Effects of red wine on the activity of catalase in diabetes induced by streptozotocin

	Catalase activity AU/mg protein		
	Pancreas	Liver	Kidney
Control	0.035 ± 0.003	0.034 ± 0.004	0.013 ± 0.004
Red wine	0.023 ± 0.004	0.041 ± 0.004	0.023 ± 0.004
Streptozotocin	0.011 ± 0.003[a]	0.021 ± 0.004[a]	0.004 ± 0.001[a]
Red wine + streptozotocin	0.021 ± 0.003[b]	0.042 ± 0.007[b]	0.024 ± 0.004[b]
Streptozotocin + red wine	0.016 ± 0.003[c]	0.041 ± 0.003[b]	0.020 ± 0.006[b]

Values are means ± SEM of six rats and are expressed as activity units per milligram of protein.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.05$ vs streptozotocin group.

3.4. Antioxidant enzyme activity

Catalase activity, SOD, and GPx in pancreas, liver, and kidney tissues are shown in Tables 6–8. Enzyme activity dropped in all the tissues studied when streptozotocin was administered. After 4 weeks of treatment with red wine, this activity increased. This increase was greater when the treatment with red wine began before inducing diabetes.

4. Discussion

In the present study we report antidiabetic and free radical scavenging activity of Montilla-Moriles appellation red wine (Cordoba, Spain). It should be noted that the presence of oxidative stress induced by streptozotocin is inferred from the high degree of lipid peroxides in pancreas, liver,

Table 7
Effects of red wine on the activity of SOD in diabetes induced by streptozotocin

	SOD activity (AU/mg protein)		
	Pancreas	Liver	Kidney
Control	11.38 ± 0.89	19.93 ± 0.28	13.40 ± 0.10
Red wine	14.93 ± 0.98	20.89 ± 0.48	13.70 ± 0.75
Streptozotocin	7.24 ± 1.15[a]	17.21 ± 0.23[a]	10.00 ± 1.20[a]
Red wine + streptozotocin	15.83 ± 3.46[b]	22.20 ± 1.55[b]	14.40 ± 1.10[b]
Streptozotocin + red wine	15.63 ± 2.93[c]	20.18 ± 0.76[c]	14.00 ± 1.40[b]

Values are means ± SEM of six rats and are expressed as activity units per milligram of protein.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.05$ vs streptozotocin group.

Table 8
Effects of red wine on the GPx activity in diabetes induced by streptozotocin

	GPx activity (AU/mg protein)		
	Pancreas	Liver	Kidney
Control	3.98 ± 0.32	3.23 ± 0.51	2.76 ± 0.47
Red wine	4.07 ± 0.57	2.91 ± 0.05	1.79 ± 0.16
Streptozotocin	2.58 ± 0.14[a]	2.02 ± 0.28[a]	1.52 ± 0.21[a]
Red wine + streptozotocin	4.34 ± 0.39[b]	3.10 ± 0.21[b]	1.98 ± 0.33[c]
Streptozotocin + red wine	3.58 ± 0.41[c]	3.05 ± 0.26[b]	1.92 ± 0.33[c]

Values are means ± SEM of six rats and are expressed as activity units per milligram of protein.

Statistically significant differences are indicated by superscript letters: a: $P < 0.001$ vs control group; b: $P < 0.001$ vs streptozotocin group; c: $P < 0.05$ vs streptozotocin group.

and renal tissues, as well as from the depletion of GSH content and the reduction in the activity of glutathione peroxidase, superoxide dismutase, and catalase enzymes of these tissues. This oxidative stress coincides with biochemical signs typical of diabetes, such as high levels of glycemia and a high degree of protein glycosylation, as shown by significant increases in glucose and in percentages of glycosylated hemoglobin and plasma fructosamine. All of these changes once again support the clear link between hyperglycemia, protein glycosylation, and oxidative stress in experimental and clinical diabetes [7]. In addition to these data, we observed a significant decrease in pancreatic and serum insulin levels.

As mentioned above, hyperglycemia is currently considered to be the primary cause of autoxidative glycosylation as well as formation of hydroperoxides and free radicals [25]. It has been well documented that several molecular proteins, such as myoglobin, low-density lipoprotein (LDL), insulin, albumins, and certain antioxidant enzymes lose their functional properties when they are exposed to high concentrations of glucose, both in vivo and in vitro [26].

In this study, we observed a high degree of lipid peroxidation as well as a decrease in reduced GSH content and in the activity of the enzymes studied. However, it is difficult in this model to determine whether this is caused by hyperglycemia or by the toxic and specific effect of streptozotocin or by a combination of both factors. According to our previous studies, streptozotocin can cause a higher or lower increase in the formation of lipid peroxides depending on the dose administered [10]. Moreover, streptozotocin produces insulitis with macrophage activation and hydroxyl radicals, which are highly cytotoxic [6,27]. However, in light of the research, it should be noted that glucose autoxidation plays a pre-eminent role as a source of production of free radicals. This contributes significantly to oxidative stress as indicated by both the decrease of antioxidant reserves in diabetes and by the reduced efficiency of the defense systems against free oxygen radicals [27].

Having accepted the existence of a link between diabetes and oxidative stress in our model, we tested the effects of red wine on this process. According to our data, it is evident that depending on the time it is administered, this red wine acts as an antioxidant. Likewise, it reduces hyperglycemia, the intensity of lipid peroxidation, the depletion of GSH content, lowered catalase, glutathione peroxidase and superoxide dismutase activities, and protein glycosylation, and returns changes in pancreatic and serum insulin to normal levels. This leads us to conclude that red wine has a protective effect against streptozotocin, a highly cytotoxic and oxidative substance, and also against the diabetic state induced by streptozotocin, which in turn produces free radicals. In the current study, depending on the time at which it is administered, the protective effect of red wine is quite evident. This is demonstrated by the fact that hyperglycemia was reduced by 63.79% when the wine was administered 2 weeks before the injection of streptozotocin and by 27.94% when the wine and the streptozotocin were administered simultaneously.

It is surprising that the decreases in glycemic values observed in our study are similar to those obtained by Vessal et al. [20] in the streptozotocin-induced diabetic rat model treated with quercetin, a flavonoid that is widely present in red wines and plants. These and other authors indicate that quercetin and other chemicals with antioxidant properties and free radical scavengers can prevent autopoly (ADP-ribosyl)-ation of PARD, thereby stabilizing Reg gene transcriptional complex and inducing the regeneration of β-cells and the protection of pancreatic islets against streptozotocin or alloxan [8,20,28]. Vessal et al. also observed that quercetin reversed to normality the glucose tolerance test, hepatic glucokinase and hexokinase activities, lipid profile, and the number of pancreatic islets. Likewise, it is surprising that the decrease in degree of oxidative stress observed by this author is similar to that obtained by our group in the streptozotocin-induced diabetic rat model treated with melatonin [11].

Although this was not the aim of the current study, we also noted that red wine administered before and simultaneously with streptozotocin changed the pancreatic and serum insulin levels. However, the protective effect of the red wine was found to be more intense and significant when the wine was administered before the streptozotocin. These results indicate that: 1) the effect of red wine depends on the time it is administered; and 2) the wine has a protective effect on the anatomical and functional integrity of the pancreatic islets (possibly due to its phenolic compounds). Thus, the protective effect of red wine involves the inhibition of oxidative processes, prevention of insulitis with macrophage activation, and reduction of the cytotoxic effect. This hypothesis is supported by data from this same group that show the following: 1) the effect of streptozotocin is dose dependent [10]; 2) the changes induced by

streptozotocin are resistant to insulin administration [10]; and 3) in insulin-dependent diabetes (the type induced by the administration of streptozotocin), melatonin as well as vitamin E reduce the intensity of oxidative stress and severity of hyperglycemia, and consequently the degree of protein glycosylation, a process that is closely linked to glucose autoxidation and the formation of free radicals [11, 29]. Nonetheless, further research is needed to determine the possible mechanisms involved in these phenomena.

In short, red wine of the Montilla-Moriles appellation (Cordoba, Spain) reduces the intensity of oxidative stress and the severity of hyperglycemia in insulin-dependent diabetes, while enhancing stores of pancreatic insulin and increasing serum insulin levels. In accordance with the results and hypotheses of other investigators, and because of the flavonoid-rich composition of red wine, the findings of this study would seem to suggest that the protective effect of red wine is due to the presence of polyphenols.

Acknowledgments

We thank Omeyas warehouse S.A. (Montilla, Cordoba, Spain) for providing the red wine.

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