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## Evolution of oxidative stress parameters and response to oral vitamins E and C in streptozotocin-induced diabetic rats

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### Abstract

Type I diabetes in humans and streptozotocin (STZ)-induced diabetes in rats has been associated with oxidative stress, but antioxidant therapy has given contradictory results, in part related to the absence of common conditions used to evaluate in-vivo antioxidant properties. This prompted the study of an experimental model of antioxidant therapy in STZ-treated rats. Adult female rats received STZ (50 mg kg<sup>-1</sup>) and were studied 7 or 14 days later. Adipose tissue weight progressively decreased with the time of treatment, whereas plasma triglycerides increased at 7 days, before returning to control values at 14 days after STZ treatment. STZ diabetic rats had increased plasma thiobarbituric acid reacting substances and  $\alpha$ -tocopherol levels, but the latter variable was decreased when corrected for total lipids. STZ diabetic rats showed a higher GSSG/GSH ratio at Day 14 and lower GSH + GSSG at Day 7 in liver. To evaluate the effect of short-term antioxidant therapy, rats received 5 doses of vitamins C and E over 3 days before being killed on Day 14. Treatment with antioxidants decreased plasma lactic acid and thiobarbituric acid reacting substances, as well as urine 8-isoprostane, and decreased plasma uric acid in controls. Vitamins increased the plasma  $\alpha$ -tocopherol/lipids ratio only in control rats, although the plasma and liver  $\alpha$ -tocopherol concentration increased in both groups. STZ diabetic rats showed moderate oxidative stress and treatment with antioxidant vitamins caused a significant change in a selected group of oxidative stress markers, which reflected an improvement in some of the complications associated with this disease. The present experimental conditions can be used as a sensitive experimental model to study the responsiveness of diabetes to other antioxidant interventions.

### Introduction

Type I diabetes is a physiopathological condition involving oxidative stress that can be used experimentally to evaluate the responsiveness to treatment with antioxidants intended to reduce the associated oxidative stress (Packer et al 2001). Streptozotocin (STZ)-induced diabetes in rats is an experimental model for type I diabetes (Ganda et al 1976; Ward et al 2001), in which increases in oxidative stress markers have been observed (Kakkar et al 1998; Feillet-Coudray et al 1999). This model has been compared with an animal model of oxidative stress and has recently been proposed as the most appropriate model of systemic oxidative stress for studying antioxidant therapies (Hermans et al 2007). However, although the evolution of several parameters has been studied in these animals, the most appropriate experimental design to evaluate antioxidant therapy has not been established. Vitamins C and E have been proposed as antioxidant therapy in numerous studies using a variety of animal models of diabetes. In general, vitamin C or E, either individually or in combination, decreased several parameters of oxidative stress such as isoprostanes and plasma malondialdehyde, and cellular markers of oxidative stress such as nuclear factor- $\kappa$ B in diabetic animals (Morrow 2000; Slatter et al 2000; Dias et al 2005). Vitamin E supplementation in STZ-induced diabetic rats protects against lipid peroxidation and contributes to preventing the elevation of plasma glucose levels; vitamin E deficiency leads to increased levels of hepatic thiobarbituric acid reacting substances (TBARS) in these diabetic rats (Kinalski et al 1999). Studies have shown that supplementation with either  $\alpha$ -tocopherol or  $\alpha$ -lipoic acid

reduces the peroxidation processes in diabetic patients (Fuller et al 1996). In addition, vitamin E supplementation in diabetic patients results in an improvement in both insulin effect and glycaemic control (Paolisso et al 1994; Jain et al 1996a; Sharma et al 2000), as well as a decrease in lipid peroxidation and low density lipoprotein oxidizability (Jain et al 1996b; Anderson et al 1999). However, contradictory results regarding antioxidant therapy for diabetes can be also found in the literature (Gallou et al 1993; Haffner 2000; Hartnett et al 2000; Vessby et al 2002). Moreover, endothelial function of type II diabetic patients that received oral vitamin E and C did not improve, whereas in type I diabetic patients, the same treatment was effective (Beckman et al 2003). Therefore, although vitamins E and C seem to be widely accepted as natural appropriate antioxidants, more investigation is required to establish the validity of this therapy.

The aim of this work was to establish conditions to detect changes in selected oxidative stress parameters associated with oral antioxidant treatment in STZ diabetic rats compared with their controls.

## Materials and Methods

### Chemicals

$\alpha$ -Tocopherol (*all-rac*) (vitamin E), ascorbic acid (vitamin C), STZ, triolein, Tween 80, *N*-ethylmaleimide, 2-thiobarbituric acid, trolox and standards of reduced glutathione (GSH), oxidized glutathione (GSSG), acetoacetic, 3-hydroxybutyric, lactic and uric acids, were obtained from Sigma (St Louis, MO, USA). Phenylododecane,  $H_3BO_3$ ,  $H_3PO_4$  85%,  $HClO_4$  70%, methanol and acetonitrile (high-performance liquid chromatography grade) were from Merck (Darmstadt, Germany). HCl 37%, NaCl, sodium hydroxide, trichloroacetic acid and EDTA- $Na_2$  were from Panreac (Montcada i Reixac, Spain). 2,2'-Azobis (2-amidine-propane)dihydrochloride (AAPH) was from Wako Pure Chemicals Industries (Richmond, VA, USA) and Na-fluorescein was from Geigy (Bale, Switzerland).

All the buffer solutions were prepared with water purified by Milli-Qplus185 (Millipore, Billerica, MA, USA), and saline solution contained 0.9 g of NaCl per 100 mL water. Tocol was kindly supplied by Roche (Basel, Switzerland), and 1,1,3,3-tetramethoxypropane was purchased from Aldrich (Steinheim, Germany).

### Animals and samples

Female Sprague–Dawley rats,  $12 \pm 2$  weeks old, from the animal quarters of University San Pablo-CEU were used. Throughout the experiments, the animals were kept in collective cages (<7 rats per cage) under controlled conditions ( $22 \pm 2^\circ C$  and  $55 \pm 10\%$  relative humidity). Animals had free access to tap water and food (Harlan Global Diet 2014; Harlan Interfauna Ibérica, Madrid, Spain).

To study the evolution of oxidative stress parameters, rats were distributed into three experimental groups: animals that received an intraperitoneal dose of STZ ( $50 \text{ mg kg}^{-1}$  dissolved in 50 mM citrate, pH=4.5) and showed blood glucose levels over  $200 \text{ mg dL}^{-1}$  4 days after treatment (diabetics, D) were

killed at either 7 (D7 group,  $n=22$  animals) or 14 days (D14 group,  $n=12$  animals) after STZ administration. Sex and age-matched rats that did not receive STZ were studied in parallel (controls, C,  $n=18$  animals).

To evaluate the antioxidant treatment effect, rats in the D14 and C groups received a mixture of vitamin C ( $1100 \text{ mg kg}^{-1}$ ) and E ( $125 \text{ mg kg}^{-1}$ ) dispersed in 1 mL vehicle (200 mg triolein and 20 mg Tween in 1 mL saline solution) by gavage at 72, 64, 48, 40 and 24 h before being killed. Therefore, four experimental groups were established: non-diabetic rats that received the vehicle (CV group,  $n=12$  animals) or the antioxidant mixture (CX group,  $n=12$  animals), and diabetic rats that received the vehicle (DV group,  $n=12$  animals) or the antioxidant mixture (DX group,  $n=12$  animals).

Animals were placed in individual metabolic cages during the last 24 h for urine collection. Blood was collected by cardiac puncture in EDTA- $Na_2$  while the animals were under anaesthesia with ketamine ( $75\text{--}100 \text{ mg kg}^{-1}$ )/azepromacine ( $2.5 \text{ mg mL}^{-1}$ ). The animals were then killed and the liver and lumbar adipose tissue were excised, and immediately frozen in liquid nitrogen, weighed and stored at  $-80^\circ C$ . Blood was rapidly centrifuged to separate plasma, which was stored at  $-20^\circ C$  until analysed. For glutathione determination, a blood sample was directly collected in diluted perchloric acid.

The experiments were approved by the Ethical Committee of the University San Pablo-CEU and they were in agreement with Amsterdam Treaty and Spanish legislation (RD 223/1988).

### Methods

Plasma glucose, triglycerides, cholesterol and urea were analysed with commercial kits from Spinreact (Gerona, Spain). 8-Isoprostane in 24-h urine was measured with a commercial kit from Cayman Chemical (Ann Arbor, MI, USA), using a Asys UVM340 plate reader (Asys Hitech GmbH, Eugendorf, Austria).

TBARS were measured by the specific fluorescence of the adduct malondialdehyde–thiobarbituric acid, using malondialdehyde from 1,1,3,3-tetramethoxypropane as standard according to Viana et al (1996). Total antioxidant capacity (TAC) was measured following the method described by Prior et al (2003), using a Cary Eclipse fluorescence detector (Varian, Courtaboeuf, France). Briefly, after adding AAPH to a cuvette with plasma and fluorescein, the area under the curve of fluorescence versus time was quantified and compared with the curve obtained with trolox.

Capillary electrophoresis equipments (P/ACE 5010 and P/ACE 5500) from Beckman-Coulter (Palo Alto, CA, USA) were used to determine glutathione and short-chain organic acids. Glutathione (both oxidized and reduced) in liver and blood was measured according to Maeso et al (2005), and uric, acetoacetic, 3-hydroxybutyric and lactic acids in plasma were determined as described by Baena et al (2004).

Plasma and liver  $\alpha$ -tocopherol was determined by previously reported methods (Rupérez et al 2004; García-Martínez et al 2007) with a high-performance liquid chromatography instrument that comprised a 125 Pump, a 507 autosampler, a 168 UV-DAD detector (all from Beckman-Coulter) and a Waters 470 fluorescence detector (Waters, Milford, MA, USA).

## Statistical analysis

To evaluate the influence of time following STZ administration, after one-way analysis of variance, the means of parameters were compared with the Student–Newman–Keul’s (SNK) multiple range test. When antioxidants were given, mean comparisons (SNK test) after one-way analysis of variance were performed, and also the results of two-way analysis of variance were evaluated. Statistical analysis was performed with StatGraphics Plus 5.1 (StatPoint Inc, Herndon, VA, USA).

## Results

### Effect of time after STZ administration

The results of the experiments are summarized in Table 1. At 7 or 14 days after STZ treatment, the bodyweight of the treated rats did not differ from the controls. The weight of lumbar adipose tissue was lower at Day 7 after STZ treatment (D7 group) and even lower at Day 14 (D14 group); the weight of the liver in the STZ diabetic rats at Day 14 was higher compared with both control (C group) and D7 rats (Table 1). As shown in Table 1, plasma glucose levels were very high in the two STZ-treated groups; plasma triglycerides were very high in rats studied 7 days after STZ but returned to control values at 14 days after STZ treatment. The plasma TBARS levels in STZ diabetic rats at both Day 7 and Day 14 were enhanced compared with their controls, and differences between D7 and D14 groups were not significant, mainly due to the high dispersion of the data in the D7 group (Table 1).  $\alpha$ -Tocopherol concentrations in plasma and liver in STZ diabetic rats were higher at 7 days than in the controls, but returned to control values by 14 days (Table 1). This pattern resembles that of plasma triglycerides, but values of  $\alpha$ -tocopherol corrected for plasma lipids (triglycerides plus cholesterol) were significantly lower in STZ diabetic rats at Day 7 compared with controls, whereas by Day 14 they had returned to values that were not different compared with controls.

Table 1 gives the results for GSH or total glutathione (GSH+GSSG) in liver, which were within the same range, because GSSG values do not represent more than 2% of the GSH. At Day 7 after STZ administration, the glutathione concentration was lower, but it had risen by Day 14 to values comparable with the controls. The GSSG/GSH ratio, on the other hand, was similar to controls at Day 7 after STZ treatment, but Day 14 values were significantly higher than those of either controls or Day 7 diabetic animals.

### Effect of antioxidant administration

The other set of control and diabetic (C and D14) rats were studied after receiving 5 oral doses of an antioxidant mixture (vitamins C and E) before being killed 14 days after STZ administration.

As shown in Table 2, administration of antioxidants did not modify plasma glucose levels in either STZ diabetic rats or in controls. Plasma levels of acetoacetic, 3-hydroxybutyric and lactic acids were higher in untreated STZ diabetic rats than in controls. When antioxidants were administered to diabetic rats (DX group), a significant decrease in lactic acid was

observed, whereas 3-hydroxybutyric and acetoacetic acids were slightly decreased (not significantly) compared with the non-treated diabetic group (DV), although values no longer differed compared with controls.

As shown in Table 2, both plasma TBARS and urine 8-isoprostane were significantly higher in the non-treated diabetic group than in controls, and, whereas antioxidant treatment reduced TBARS in diabetic rats, they did not reach control values. Antioxidant treatment in controls did not modify any of these parameters compared with untreated rats. The concentration of uric acid did not differ between untreated control and diabetic rats; however, whereas antioxidant treatment greatly decreased this variable in control rats (CX), it increased it in diabetic animals (DX) (Table 2). Plasma TAC values were lower in untreated diabetic rats than in controls; however, whereas it increased with antioxidant treatment in diabetic rats, it did not change in controls, negating the difference between them (Table 2). GSH+GSSG in blood but not in liver was found to be significantly lower in diabetic rats than in control rats not receiving antioxidant treatment (Table 3). In both blood and liver the GSSG/GSH ratio was significantly higher in STZ treated rats than in controls and antioxidant administration did not modify this variable in either group.

Values of  $\alpha$ -tocopherol should be analysed by taking into account that they correspond to 24 h after the last antioxidant treatment. As shown in Table 3,  $\alpha$ -tocopherol levels in plasma did not differ between diabetic and control rats, and antioxidant treatment similarly increased this variable in the two groups. However, when these values were corrected for plasma lipids, they were significantly lower in diabetic than in control rats, and the effect of increasing the  $\alpha$ -tocopherol/lipids ratio was only significant in control rats, with values in diabetic rats remaining significantly lower. In the liver, the  $\alpha$ -tocopherol concentration did not differ between the two untreated groups, but increased by 5 times after antioxidant treatment in controls; the effect in diabetic rats was milder.

## Discussion

### Effect of time after STZ administration

Although bodyweight in STZ diabetic rats has been reported to decrease (Feillet-Coudray et al 1999), in these experiments the decrease was not statistically significant because the investigation was carried out over a short time period. Nevertheless, 7 days after STZ administration was enough time to appreciate significant changes in the weight of lumbar adipose tissue. This finding was in agreement with the known effect of insulin reducing adipose tissue lipolytic activity, enhancing endothelial lipoprotein lipase (González Santos et al 1998) and raising low density lipoprotein receptor expression (Swami et al 1996), all facilitating fat deposit.

In contrast to fat depots, the liver size increased. This increase may be related to the previously described phenomena of hyperplasia following STZ administration, and subsequently less apoptosis, resulting in an increase in total cell numbers (Herrman et al 1999).

**Table 1** Physical, biochemical and oxidative stress parameters in control and diabetic rats after 7 and 14 days of diabetes

Group	Body weight (g)	Liver weight (g)	Lumbar adipose tissue weight (g)	Glucose (plasma, mgdL <sup>-1</sup> )	Triglycerides (plasma, mgdL <sup>-1</sup> )	TBARS (plasma, mM MDA)	α-Tocopherol (plasma, mgdL <sup>-1</sup> )	α-Tocopherol (liver, μg g <sup>-1</sup> )	α-Tocopherol/lipids (plasma, μmolmmol <sup>-1</sup> )	GSH (liver, μmol g <sup>-1</sup> )	GSSG (liver, μmol g <sup>-1</sup> )	GSSG/GSH (liver)	GSH+GSSG (liver, μmolGS g <sup>-1</sup> )
Control	241.0±4.6	8.2±0.2 <sup>a</sup>	1.6±0.1 <sup>c</sup>	177.8±9.8 <sup>a</sup>	62.9±5.4 <sup>a</sup>	1.3±0.1 <sup>a</sup>	1.09±0.05 <sup>a</sup>	28.9±2.3 <sup>a</sup>	5.4±0.3 <sup>b</sup>	7.8±0.2 <sup>b</sup>	0.18±0.02 <sup>a</sup>	0.023±0.002 <sup>a</sup>	8.2±0.3 <sup>b</sup>
D7	239.1±5.6	8.7±0.8 <sup>a</sup>	0.9±0.3 <sup>b</sup>	552.7±138.3 <sup>b</sup>	460.9±230.2 <sup>b</sup>	2.3±0.9 <sup>b</sup>	1.86±0.6 <sup>b</sup>	45.7±10.2 <sup>b</sup>	2.8±0.8 <sup>a</sup>	6.8±0.3 <sup>a</sup>	0.14±0.03 <sup>a</sup>	0.021±0.004 <sup>a</sup>	7.2±0.3 <sup>a</sup>
D14	234.0±7.5	9.9±0.3 <sup>b</sup>	0.3±0.0 <sup>a</sup>	701.6±39.2 <sup>c</sup>	115.7±18.4 <sup>a</sup>	2.8±0.2 <sup>b</sup>	1.18±0.06 <sup>a</sup>	27.6±5.3 <sup>a</sup>	4.8±0.5 <sup>b</sup>	7.8±0.7 <sup>b</sup>	0.27±0.02 <sup>b</sup>	0.042±0.011 <sup>b</sup>	8.4±0.7 <sup>b</sup>

Control, non-treated rats; D7, diabetic rats 7 days after streptozotocin injection; D14, diabetic rats 14 days after streptozotocin injection; TBARS, thiobarbituric acid reacting substances. After one-way analysis of variance, means were compared with the Student–Newman–Keul’s multiple range test. Different letters indicate significantly different mean values ( $P < 0.05$ ).

**Table 2** Plasma concentrations of glucose, lactic, acetoacetic, 3-hydroxybutyric and uric acids as well as total antioxidant capacity of plasma, total amount of 8-isoprostane excreted in urine, and plasmatic concentrations of uric acid and TBARS of control (C) and diabetic (D) rats that received either vehicle (V) or antioxidants (X)

Group	Glucose (plasma, mgdL <sup>-1</sup> )	Lactic (plasma, mM)	Acetoacetic (plasma, mM*10)	3-Hydroxybutyric (plasma, mM)	Uric acid (plasma, mM*10)	Total antioxidant capacity (plasma, mM trolox)	8-Isoprostane (urine, μg/day)	TBARS (plasma, mM MDA)
CV	174.0±15.9 <sup>a</sup>	2.4±0.4 <sup>a</sup>	0.6±0.1 <sup>a</sup>	0.4±0.1 <sup>a</sup>	0.4±0.1 <sup>bc</sup>	6.8±0.3 <sup>b</sup>	3.7±0.5 <sup>a</sup>	1.3±0.1 <sup>a</sup>
DV	701.6±39.2 <sup>b</sup>	5.4±1.0 <sup>b</sup>	1.2±0.2 <sup>b</sup>	0.3±0.1 <sup>b</sup>	1.2±0.2 <sup>b</sup>	5.5±0.3 <sup>a</sup>	20.1±2.3 <sup>c</sup>	2.9±0.2 <sup>c</sup>
CX	141.2±7.2 <sup>a</sup>	0.9±0.1 <sup>a</sup>	1.1±0.1 <sup>ab</sup>	0.1±0.0 <sup>a</sup>	0.3±0.0 <sup>a</sup>	6.3±0.3 <sup>b</sup>	3.4±1.3 <sup>a</sup>	1.0±0.1 <sup>a</sup>
DX	608.6±52.4 <sup>b</sup>	2.8±0.4 <sup>a</sup>	1.1±0.1 <sup>ab</sup>	0.6±0.1 <sup>ab</sup>	0.7±0.1 <sup>c</sup>	6.9±0.2 <sup>b</sup>	13.5±1.9 <sup>b</sup>	2.3±0.3 <sup>b</sup>

TBARS, thiobarbituric acid reacting substances. After one-way analysis of variance, means were compared with the Student–Newman–Keul’s multiple range test. Different letters indicate significantly different mean values ( $P < 0.05$ ).

**Table 3** GSH+GSSG and GSSG/GSH ratio in liver and blood, as well as α-tocopherol in plasma and liver of control (C) and diabetic (D) rats that received either vehicle (V) or antioxidants (X)

Group	GSH+GSSG (liver, μmolGSg <sup>-1</sup> )	GSSG/GSH (liver)	GSH+GSSG (blood, μmolGSL <sup>-1</sup> )	GSSG/GSH (blood)	α-Tocopherol (plasma, mgdL <sup>-1</sup> )	α-Tocopherol (liver, μg g <sup>-1</sup> )	α-Tocopherol/lipids (plasma, μmolmmol <sup>-1</sup> )
CV	8.4±0.3	0.027±0.002 <sup>ab</sup>	879.9±34.7 <sup>b</sup>	0.009±0.002 <sup>a</sup>	1.1±0.1 <sup>a</sup>	23.0±1.4 <sup>a</sup>	8.7±1.2 <sup>b</sup>
DV	8.4±0.7	0.042±0.011 <sup>b</sup>	671.4±41.3 <sup>a</sup>	0.034±0.007 <sup>b</sup>	1.2±0.1 <sup>a</sup>	27.6±5.3 <sup>a</sup>	4.8±0.5 <sup>a</sup>
CX	9.2±0.4	0.018±0.003 <sup>a</sup>	838.7±44.9 <sup>b</sup>	0.011±0.002 <sup>a</sup>	1.7±0.1 <sup>b</sup>	111.8±11.3 <sup>b</sup>	12.8±1.6 <sup>c</sup>
DX	9.0±0.3	0.046±0.004 <sup>b</sup>	681.9±57.3 <sup>a</sup>	0.029±0.007 <sup>b</sup>	1.5±0.1 <sup>b</sup>	77.6±17.3 <sup>b</sup>	6.7±1.0 <sup>ab</sup>

After one-way analysis of variance, means were compared with the Student–Newman–Keul’s multiple range test. Different letters indicate significantly different mean values ( $P < 0.05$ ).



It is remarkable that after the striking hypertriglyceridaemia seen at 7 days, the level of plasma triglycerides decreased at 14 days of diabetes to values that were not significantly different compared with those in control rats. Given the low weight of lumbar fat pad found in the D14 STZ rats, such a decline in hypertriglyceridaemia may result from a decreased availability of fat depots to be mobilized and subsequently to contribute to liver triglyceride production.

The TBARS concentration in plasma has been used as an oxidative stress biomarker associated with the presence and amount of final fat degradation products induced by free radicals, and it has been shown to be increased in diabetes and to decrease after treatment with antioxidants (Feillet-Coudray et al 1999; van Dam et al 2001; Baydas et al 2002). This parameter has been criticized because of its lack of specificity (Verhagen et al 2003), and although it should not be used on its own to draw conclusions, it may add information if used in combination with other oxidative stress biomarkers. In the present study, plasma TBARS were found to be increased at 7 days after the injection of STZ (D7), and the values were even higher 7 days later (D14). It is worth noting the high dispersion of the data for D7, suggesting that oxidative stress is not as uniform as at 14 days.

Vitamin E is nature's most effective lipid-soluble antioxidant (Ingold et al 1987; Serbinova & Packer 1994), and the principal membrane antioxidant in mammalian cells (Burton et al 1983). However, this is not the only function of this vitamin. There are studies demonstrating the effect of tocopherols or tocotrienols in cells and tissues that are not directly related with antioxidant activity (Traber & Packer 1995; Brigelius-Flohe & Traber 1999; Herrera 2000). Transport of vitamin E is highly dependent on lipid circulation changes in parallel with triglyceride levels (Thurnham et al 1986; Hacquebard & Carpentier 2005). Besides, the concentration of this vitamin in the liver is highly related to the concentration in plasma, the liver being the key organ for storage and regulation of circulating tocopherols (Mustacich et al 2007). In the present study, when diabetic animals were in the early stages of the disease (7 days), it was found that the levels of plasma lipids appeared with relatively low levels of tocopherol per lipid molecule. Thus, when diabetic animals develop hypertriglyceridaemia, the amount of circulating  $\alpha$ -tocopherol molecules per molecule of triglyceride is decreased and probably insufficient to deal with the increased amount of circulating free radicals and its consequent accelerated lipid peroxidation. In our experiment, 14 days after STZ administration the  $\alpha$ -tocopherol concentration (in both plasma and liver) did not differ from that of control rats, and this is in agreement with the plasma concentration of triglycerides and the size of the depots.

The glutathione antioxidant system has been proposed to be the most important intracellular defence of an organism against free radicals, and is known to be also affected by the generalized increase in oxidative stress associated with diabetes (Mak et al 1996). The glutathione concentration is widely used as an oxidative stress biomarker, but its determination is still controversial, mainly due to inadequate methods of sample treatments that artifactually increase GSSG levels (Maeso et al 2005). If properly measured, the GSSG concentration is less than 5% of the GSH (Maeso et al 2005). In the early stages of STZ treatment (7 days), the available glutathione in

the liver was found to be lower than in controls, although the ratio of oxidized/reduced glutathione was the same. However, as the disease progressed (D14), this ratio became greater, as did the total glutathione. Liver is the main contributor to plasma GSH (Ookhtens & Kaplowitz 1998), and there are many factors that may affect its synthesis in the liver and/or its release. It appears that in the first 7 days after STZ treatment the liver is not able to synthesize as much GSH as is needed. This may not be related to oxidative stress, because it has been shown that in diabetic rats the expression of the heavy subunit of the  $\gamma$ -glutamylcysteine synthetase (the key enzyme for GSH synthesis) is lower than in controls, and insulin restores it (Cai et al 1995). Nevertheless, 7 days later, the amount of GSH in liver recovered to the control values, suggesting enhanced synthesis of GSH. Oxidation of GSH at a higher rate at this time is suggested by the higher GSSG/GSH values, and this effect could be due to the higher production of free radicals during a more prolonged period of diabetes.

Again, it should be noted that most of the results determined on Day 7 after STZ administration were excessively dispersed. Individual differences may exist in the time necessary for the disappearance of all the  $\beta$ -cells, or even an enhanced activity of surviving  $\beta$ -cells may occur, and this dispersion means that 7 days after STZ is not a suitable time for further investigation of the effect of antioxidant therapies.

#### Effect of antioxidant administration

The results for bodyweight, liver and lumbar adipose tissue weights were in agreement with the results described above: with two-way analysis of variance it was found that there were no differences in bodyweight related to either diabetes or antioxidant administration ( $P > 0.1$ ), whereas diabetes was significant for liver weight (higher in diabetic rats,  $P < 0.001$ ) and for lumbar adipose tissue weight (lower in diabetic rats,  $P < 0.001$ ). Antioxidant administration did not induce any change in these parameters, or in plasma urea or triglycerides ( $P > 0.1$ ).

Vitamin E has been found to improve glycaemic control of insulin-treated diabetic patients (Fuller et al 1996), and other antioxidants such as resveratrol also have some insulin-like effects (Su et al 2006). From our results, antioxidant administration seemed to only slightly decrease plasma glucose in both control and diabetic rats, but this particular aspect needs further investigation. Differences with other studies could be related to the dose or time of treatment in our experiment not being enough to clearly appreciate the effects.

Acetoacetic and 3-hydroxybutyric acids are ketoacids that result from the incomplete oxidation of fatty acids in liver (Kaplan & Pesce 1996). As a consequence of metabolic disorders they tend to accumulate in blood and urine of individuals affected by starvation or uncontrolled diabetes mellitus (Herrera et al 1969; Foster & McGarry 1982; Webber et al 1994; Boden & Chen 1999). An alteration of carbohydrate and fat metabolism also leads to increases in plasma lactic acid levels (Jager & Tavares 2003). The similarities in plasma ketone bodies and lactic acid after the antioxidant treatment in STZ diabetic rats and their controls may be related to some insulin-like effect of antioxidants (as commented on for plasma glucose).

Treatment with antioxidants reduced the plasma TBARS levels, as is the case with other antioxidant therapies (Bonfont-Rousselot 2004). Nevertheless, it must be emphasized that antioxidant administration was performed only during 3 days, and that the last administration occurred 24 h before the rats were killed, whereas it took 10 weeks of antioxidant treatment to observe a reduction in TBARS in other studies (Baydas et al 2002; Demiyürek et al 2004). 8-Isoprostane is a product of free radical attack on arachidonic acid (Morrow 2000) and can be measured as a reflection of its final degradation. Our findings show an increase in total 8-isoprostane excreted in urine in 24 h in diabetic animals, which was less marked when antioxidants were administered. An increase in the amount of 8-isoprostane in the 24-h urine of STZ diabetic rats has been reported (Bachi et al 1997; Montero et al 2000), although there are contradictory results in the literature for plasma: higher in diabetic rats than in controls (Montero et al 2000) or similar between groups (Morrow 2000).

Uric acid is considered to be one of the most abundant water-soluble antioxidants, interacting with activated oxygen species and notably with the hydroxyl radical (Glantzounis et al 2005). Uric acid has been determined as a biomarker of oxidative stress, especially in relation to its main metabolic product, allantoin (Grootveld & Halliwell 1987; Tsahar et al 2006). We found that plasma uric acid levels are not modified by diabetes, but, whereas antioxidant treatment induces a decline in controls, it enhances its value in diabetic rats. Uric acid metabolism has been related with ascorbic acid in humans (Spitsin et al 2002), but in rats, where ascorbic acid is not a vitamin, this association has not been studied in depth and the administration of ascorbic acid could result in down-regulation of uric acid levels.

The TAC test consists of evaluating the capacity of whole blood or plasma to inhibit reactive oxygen species production in an in-vitro reactive oxygen species generating system (Ghiselli et al 2000). Reduced TAC in plasma of diabetic patients has been shown (Tsai et al 1994; Srinivasan et al 1997; Vessby et al 2002; Chuang et al 2006). In our diabetic rats, it was found that this happened too, but vitamins E and C were able to raise TAC values in DX rats to a value even higher (although not significant) than that of control rats. TAC values have been reported to be related to uric acid (Tsai et al 1994; Chuang et al 2006), but we found that with an exogenous contribution of antioxidants to control rats (CX), TAC was not different compared with non-treated rats, although the concentration of uric acid was the lowest among the four groups. The elevated concentration of  $\alpha$ -tocopherol (see below) may be also an important contributor for maintaining TAC values (Wayner et al 1987).

Antioxidant administration seems to be an effective way of increasing the  $\alpha$ -tocopherol concentration in plasma and liver, because such changes occurred not only in their total absolute concentration, but also in the plasma vitamin E/lipids ratio, which reached levels in the DX group that were not statistically different compared with CV rats.

It is known that the mean life of vitamin E is less than 24 h (Martinez et al 2002), therefore the plasma  $\alpha$ -tocopherol must come from stored vitamin E in the liver and not from the last antioxidant administration.

In our experiments, antioxidant administration did not improve the glutathione parameters in diabetic rats: that is, GSH did not increase and the GSSG/GSH ratio did not decrease either in liver or in blood after the treatment. The doses may not have been enough to appreciate changes in this variable, but it is worth noting that, depending on the chosen compartment (liver or blood), diabetes was or was not a significant factor when two-way analysis of variance of data was performed. Thus, in contrast with the results in liver, for GSH and total glutathione in blood, diabetes was found to be a significant factor, but antioxidant administration did not induce any change.

In summary, STZ diabetic rats have a severe oxidative stress condition, as shown by the high plasma levels of TBARS and excretion of 8-isoprostane in urine. They respond to a moderate dose of orally administered antioxidants (1.3 g ascorbic acid and 150 mg  $\alpha$ -tocopherol) over a short period of time (3 days) by decreasing the oxidative stress condition. This effect was clearly seen in certain parameters but not in others. Thus, a range of biomarkers for oxidative stress need to be analysed in order to assess the severity of oxidative stress in a given protocol under study. The antioxidant intervention reported was capable of decreasing the oxidative stress condition of the STZ diabetic animals but did not decrease the diabetic condition of the animals. The present experimental conditions can be used as a sensitive experimental model for studying the responsiveness to other antioxidant interventions. It has been shown that for consistent pathology more than 7 days after STZ administration may be necessary, but we found that 5 antioxidant doses in the last 3 days of a 14-day period were sufficient to detect changes in a selected set of parameters.

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