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Oxidative Preconditioning Affords Protection Against Carbon Tetrachloride-induced Glycogen Depletion and Oxidative Stress in Rats

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Key words: ozone; oxidative preconditioning; carbon tetrachloride hepatotoxicity; oxidative stress; reactive oxygen species, metabolic disorders; glycogen depletion; anaerobic glycolysis.

The rectal insufflation of a judicious dose of ozone, selected from that used in clinical practice, is able to promote oxidative preconditioning or oxidative stress tolerance preventing the hepatocellular damage mediated by free radicals. In order to evaluate the effects of ozone oxidative preconditioning on carbon tetrachloride-mediated hepatotoxicity, the following experimental protocol was designed: group 1 (negative control, sunflower oil i.p.); group 2 (CCl_4 in sunflower oil, 1 ml kg^{-1} i.p.); group 3 (15 ozone–oxygen pretreatments at a dose of 1 mg kg^{-1} via rectal insufflation + CCl_4 as in group 2); group 4 (ozone control group, 15 ozone–oxygen pretreatments + sunflower oil i.p.). Ozone pretreatment prevented glycogen depletion (as demonstrated by biochemical and histopathological findings) and avoided lactate overproduction associated with the hepatotoxic effects of CCl_4. The administration of CCl_4 increased lipid peroxidation (as measured by thiobarbituric acid-reactive substances) and uric acid levels and inhibited superoxide dismutase activity. All these deleterious effects induced by CCl_4 were prevented by ozone pretreatment. The administration of ozone without CCl_4 (ozone control group) did not produce any changes in the evaluated parameters. Our results showed that ozone treatment, in our experimental conditions, was able to prevent anaerobic glycolysis and oxidative stress induced by CCl_4. **Copyright © 2001 John Wiley & Sons, Ltd.**

INTRODUCTION

Carbon tetrachloride (CCl_4) is a well-known environmental biohazard. It is particularly toxic to the liver, where it causes hepatocellular degeneration, centrilobular necrosis[1,2] and impairs different enzymatic systems.[3] The generation of free radicals appears to be pivotal in CCl_4 hepatotoxicity: CCl_4 is metabolized by cytochrome P-450 to produce the trichloromethyl radical, which initiates a cascade of free radical reactions resulting in an increase of lipid peroxidation and a reduction in some enzyme activities.[4] Many investigators have looked for protective agents against CCl_4 toxicity, and a variety of compounds with potential antioxidant activity have been tested.

Ozone (O_3) has been used as a therapeutic agent for the treatment of different, apparently non-related diseases and beneficial effects have been observed in cerebrovascular ischaemia,[5] chronic ulcers,[6] arteriosclerosis obliterans,[7] retinitis pigmentosa,[8] hepatic steatosis[9] and heart ischaemia.[10] In spite of these encouraging results obtained with O_3 therapy, its clinical use remains controversial due to the limited knowledge on the biochemical and pharmacodynamic mechanisms that underlie its therapeutic action and the efficacy in such heterogeneous pathologies.

Recently, our research group has demonstrated that O_3 oxidative preconditioning is able to afford protection against cellular damage mediated by free radicals.[11,12] The aim of the present study was to demonstrate the capability of O_3 oxidative preconditioning to preserve hepatic glycogen content, to reduce lactic acidosis, to prevent ATP breakdown and to control oxidative stress induced by CCl_4 administration to rats.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats (200–250 g) were used for this study ($n = 40$). Rats were housed in Plexiglass cages (five per cage) and maintained in an air-filtered

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and temperature-conditioned (20–22 °C) room with a relative humidity of 50–52%. Rats were fed with standard laboratory chow and water *ad libitum* and were kept under an artificial light/dark cycle of 12 h.

Treatment schedule

Ozone (O_3) was generated by an OZOMED equipment manufactured by the Ozone Research Centre (Cuba) and was administered by rectal insufflation. The O_3 obtained from medical-grade oxygen was used immediately and it represented only about 3% of the gas ($O_2 + O_3$) mixture. The O_3 concentration was measured using a UV spectrophotometer at 254 nm. The O_3 dose is the product of the O_3 concentration (expressed as mg l^{-1}) and the gas ($O_2 + O_3$) volume (l). By knowing the body weight of the rat, the O_3 dose is calculated as 1 mg kg^{-1}. Rats received 15 O_3 treatments, one per day, of 4.4–5.0 ml of O_3 (concentration 50 μg ml^{-1}) before challenge with CCl_4. After the last O_3 treatment, rats received CCl_4 (1 ml kg^{-1}) by intraperitoneal administration of a solution containing 10% CCl_4 in sunflower oil.

Animals were allocated randomly to the following treatment groups: group 1, control ($n = 10$), treated only with sunflower oil i.p.; group 2, positive control ($n = 10$), treated with 1 ml kg^{-1} of 10% CCl_4 solution in sunflower oil i.p.; group 3, ozone group ($n = 10$), receiving 15 O_3/O_2 pretreatments (1 mg kg^{-1}) and after 24 h of the last ozone treatment animals received CCl_4 as in group 2; group 4, ozone control group ($n = 10$), receiving 15 O_3/O_2 pretreatments + sunflower oil.

Sample preparation

The animals were euthanized by ether anesthesia 24 h after CCl_4 administration. Immediately after, liver was collected, weighed and some representative samples of different liver portions were taken for histological study, glycogen content determination and tissue homogenates.

Liver homogenates were obtained using a tissue homogenizer (Edmund Bühler, LBMA) at 4 °C. The homogenates were prepared using a 50 mM KCl–histidine buffer (pH 7.4) (1 : 10, w/v) and were spun down using a Sigma centrifuge (2K15) at 4 °C and 8500 x g for 20 min. The supernatants were taken for biochemical determinations.

Biochemical determinations

All biochemical parameters were determined by spectrophotometric methods using an Ultraspect Plus spectrophotometer from Pharmacia LKB.

Hepatic glycogen content was measured as follows: briefly, 1 g of tissue was digested using 60% KOH (w/v) and glycogen was precipitated (using absolute ethanol), centrifuged and dissolved in hot distilled water.[13] Afterwards, acidic glycogen hydrolysis was conducted in order to obtain free glucose. Glucose concentration was determined using a commercial kit from Boehringer Mannheim (Munich, Germany) and compared with those obtained from known hydrolysed glycogen amounts. Thus, the hepatic glycogen content can be determined indirectly. This assay was conducted according to the method described by Hawk *et al.*[14]

Lactate concentration was measured using a commercial kit from Boehringer Mannheim (Munich, Germany). In the presence of lactate dehydrogenase (LDH), lactate was oxidized by NAD to pyruvate. The amount of NADH formed during this reaction, equivalent to lactate concentration, was determined on the basis of its absorption at 365 nm.

Uric acid concentration was measured according to a commercial kit obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Total superoxide dismutase (Cu, Zn and MnSOD) activity in the supernatant was determined by measuring the inhibition of pyrogallol autooxidation,[15] where a unit of activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol autooxidation by 50%. To determine the SOD activity the change in absorbance per minute at 420 nm in the presence of the antioxidant was compared with that of the control.

The catalase activity was measured by following the decomposition of H_2O_2 at 240 nm at 10-s intervals for 1 min.[16]

Lipid peroxidation was assessed by measuring thiobarbituric acid-reactive substances (TBARS) according to Buege and Aust.[17]

Total protein concentration in liver homogenates was determined using the standard Coomassie Blue method.[18]

Histological study

Liver samples were taken and fixed in 10% neutral buffered formalin, processed and embedded in paraffin and then 5-μm sections were stained for glycogen using the PAS (Periodic acid–Schiff base) method.

Statistical analysis

Results are presented as means ± SEM. All data were analysed by one-way analysis of variance (ANOVA). If the F values were significant, the Student–Newman–Keuls test was used to compare groups. The level of significance was accepted at $P < 0.05$.

RESULTS

As shown in Fig. 1, CCl_4 administration to rats led to significant hepatic glycogen depletion. Glycogen content in the CCl_4-treated group (7.85 ± 1.14 mg g^{-1} tissue) decreased by 60% compared with the control group (19.65 ± 1.54 mg g^{-1} tissue; $P < 0.05$). Ozone treatment (1 mg kg^{-1}) maintained the glycogen content (21.28 ± 2.49 mg g^{-1} tissue) comparable to that of the control in spite of the presence of CCl_4. The administration of O_3 without CCl_4 (ozone control group) did not produce any change in glycogen content (18.94 ± 2.45 mg g^{-1} tissue) as compared with the control group.

The histological study of hepatic glycogen using the PAS method was in accordance with the quantitative findings (Fig. 2). In comparison with group 1 (negative control, Fig. 2a), the samples taken from group 2, treated with 1 ml kg^{-1} of 10% CCl_4 solution in sunflower oil, showed a moderate depletion of glycogen deposits that was more evident in zone 3 of the acini (Fig. 2b). In group 3, receiving 15 pretreatments with O_3, the permanence of glycogen deposits in hepatic cells was proved and only a minimal

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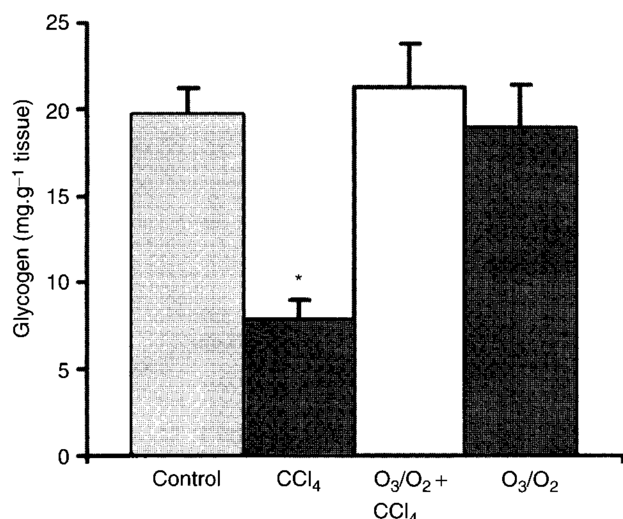

Figure 1. Hepatic glycogen content for all experimental groups. *Significant difference ($P < 0.05$) compared with control, $O_3/O_2 + CCl_4$ and ozone control group.

non-parenquimatous cell reaction co-existed around the central vein (Fig. 2c).

Table 1 shows a significant decrease ($P < 0.05$) in the liver weight (LW)/body weight (BW) ratio in those rats treated with CCl_4 ($3.25 \pm 0.07\%$) compared with the control group ($3.65 \pm 0.07\%$; $P < 0.05$). Ozone oxidative preconditioning was able to prevent the CCl_4-induced decrease in LW/BW ratio ($3.68 \pm 0.07\%$).

Lactate content in liver homogenate supernatants obtained from CCl_4-treated rats (7.10 ± 0.7 nmol g^{-1} tissue) increased by 55% and 44% compared with the control (4.57 ± 0.33 nmol g^{-1} tissue, $P < 0.05$) and O_3 treatment + CCl_4 (4.93 ± 0.33 nmol g^{-1} tissue, $P < 0.05$) groups, respectively, as shown in Table 2. On the other hand, uric acid concentration in the CCl_4 group (2.45 ± 0.46 mmol g^{-1} tissue) showed a significant increase (122%) in comparison with the control group (1.10 ± 0.36 mmol g^{-1} tissue; $P < 0.05$), whereas group 3 ($O_3 + CCl_4$) showed a uric acid concentration (0.79 ± 0.10 mmol g^{-1} tissue) similar to that of the control (Table 2). In the ozone control group, both lactate and uric acid levels were not different to the control group (Table 2).

Some oxidative stress parameters are presented in Table 3. Administration of CCl_4 decreased the SOD activity by 45% (12137.59 ± 2764.53 U mg^{-1} protein min^{-1}) and increased the catalase activity by 79% (829.82 ± 71.31 U mg^{-1} protein min^{-1}) compared with the control group (22051.18 ± 1965.61 and 462.16 ± 52.36 U mg^{-1} protein min^{-1}, respectively; $P < 0.05$). Although O_3 treatment attenuated the significant decrease in SOD activity (24719.45 ± 3352.61 U mg^{-1} protein min^{-1}), it was unable to prevent the CCl_4-induced increase in catalase activity (855.80 ± 66.02 U mg^{-1} protein min^{-1}). The

Table 1—Liver weight (LW)/body weight (BW) ratios

Experimental group	Liver weight (g)	Body weight (g)	LW/BW ratio (%)
Control	8.1 ± 0.6	223.3 ± 13.3	3.6 ± 0.07^a
CCl_4	7.7 ± 0.5	236.6 ± 10.7	3.2 ± 0.07^b
$O_3/O_2 + CCl_4$	8.2 ± 0.6	227.9 ± 12.8	3.7 ± 0.07^a
O_3/O_2	8.1 ± 0.6	228.2 ± 12.1	3.6 ± 0.06^a

Values are group means ± SEM ($n = 10$ per group).
[a] Significantly different ($P < 0.05$) compared with CCl_4 group.
[b] Significantly different ($P < 0.05$) compared with control, $O_3/O_2 + CCl_4$ and ozone control groups.

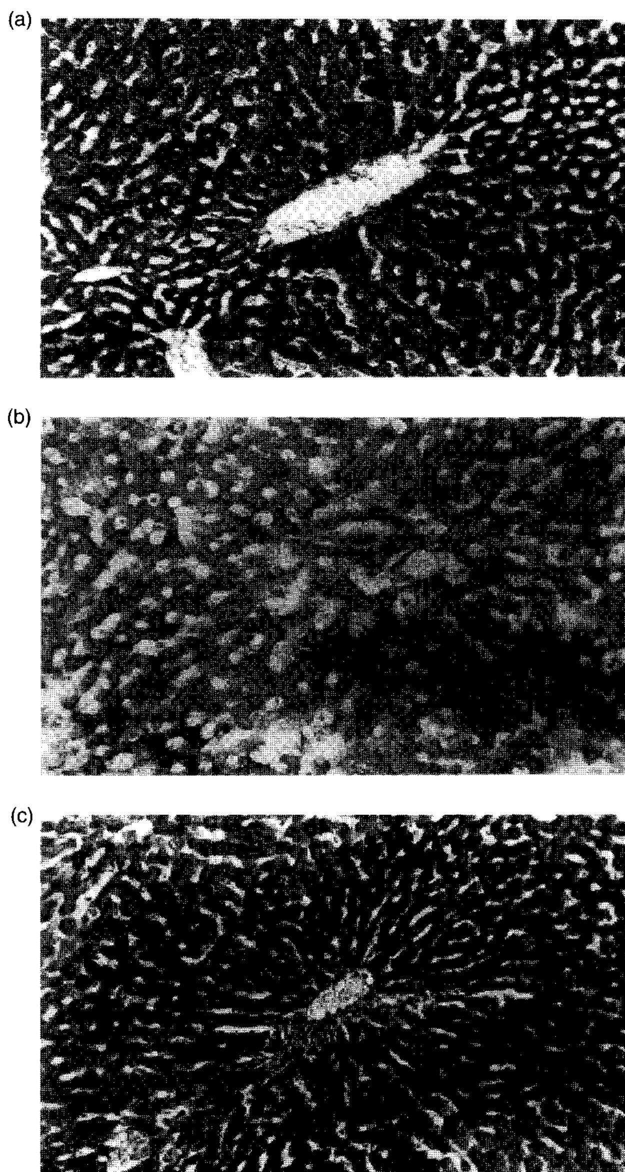

Figure 2. Histological study of hepatic glycogen.

Table 2—Metabolic parameters

Experimental group	Lactate (nmol g^{-1} tissue)	Uric acid (nmol g^{-1} tissue)
Control	4.6 ± 0.3^a	1.1 ± 0.3^a
CCl_4	7.1 ± 0.7^b	2.4 ± 0.4^b
$O_3/O_2 + CCl_4$	4.9 ± 0.3^a	0.8 ± 0.1^a
O_3/O_2	4.6 ± 0.3^a	1.1 ± 0.3^a

Values are group means ± SEM ($n = 10$ per group).
[a] Significantly different ($P < 0.05$) compared with CCl_4 group.
[b] Significantly different ($P < 0.05$) compared with control, $O_3/O_2 + CCl_4$ and ozone control group.

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Table 3—Parameters related to oxidative stress

	SOD ($U\ mg^{-1}$ protein min^{-1})	Catalase ($U\ mg^{-1}$ protein min^{-1})	TBARS (nmol g^{-1} tissue)
Control	22051.2 ± 1965.6^{a}	462.1 ± 52.3^{a}	1072.3 ± 94.5^{a}
CCl_4	12137.6 ± 2764.5^{b}	829.8 ± 71.3^{c}	1539.7 ± 77.3^{b}
$O_3/O_2 + CCl_4$	24719.4 ± 3352.6^{a}	855.8 ± 66.0^{c}	1060.9 ± 61.6^{a}
O_3/O_2	23048.1 ± 1904.5^{a}	466.2 ± 53.5^{a}	1088.4 ± 87.3^{a}

Values are group means ± SEM ($n = 10$ per group).
[a] Significantly different ($P < 0.05$) compared with CCl_4 group.
[b] Significantly different ($P < 0.05$) compared with control, $O_3/O_2 + CCl_4$ and ozone control group.
[c] Significantly different ($P < 0.05$) compared with control and ozone control groups.

ozone control group did not show any change in SOD or catalase activities compared with the control group, as shown in Table 3.

Thiobarbituric acid-reactive substances, as an index of lipid peroxidation, was kept under control in the O_3 oxidative-preconditioned rats (1060.86 ± 61.66 nmol g^{-1} tissue). Administration of CCl_4 increased the TBARS by 31% (1539.69 ± 77.33 nmol g^{-1} tissue) compared with the control group (1072.27 ± 94.55 nmol g^{-1} tissue; $P < 0.05$). Similar to previous findings, the pretreatment with a controlled dose of O_3 (ozone control group) was unable to increase the lipid peroxidation in comparison with the control group (1088.45 ± 87.33 nmol g^{-1} tissue).

DISCUSSION

Our experimental results have shown that CCl_4 induced a significant depletion in hepatic glycogen content (Fig. 1), promoting its conversion into lactate, the product of anaerobic glycolysis. Other studies have reported hepatic glycogen depletion after CCl_4 administration.[19,20] Ozone oxidative preconditioning was able to afford protection against glycogen depletion (Fig. 1) and prevented its breakdown to lactate (Table 2), thus reducing the intracellular acidosis associated with anaerobic glycolysis.

The decrease in LW/BW ratio observed in CCl_4-treated rats (Table 1) can be explained partially because of the reduced liver glycogen content shown in this experimental group (Fig. 1). Ozone treatment was able to maintain the LW/BW ratio comparable to that of the control, probably due to preservation of hepatic glycogen observed in this group (Fig. 1).

Anaerobic glycolysis could enhance CCl_4-mediated cellular damage due to intracellular acidosis and osmotic balance alterations. Moreover, ATP breakdown promoted by anaerobic glycolysis may increase reactive oxygen species (ROS) production via xanthine oxidase metabolism, with the corresponding generation of superoxide anion ($O_2^{\bullet}$) and other ROS capable of promoting deleterious effects. Ozone treatment (group 3) possibly controlled CCl_4-induced xanthine oxidase activation, as demonstrated by the uric acid levels (Table 2).

Both SOD and catalase are recognized scavengers of ROS.[21] The significant stimulation of endogenous SOD in the $O_3 + CCl_4$ group in comparison with the CCl_4 group suggests cellular protection most likely through the reduction in the availability of superoxide anion.

This result was somewhat expected on the basis of several findings[22–24] reporting increased activities of SOD, catalase and peroxidase after chronic O_3 exposure. It is noteworthy that plants also can express a protective response to O_3,[25,26] suggesting that living organisms chronically exposed to O_3 have the option of either programming their death or reacting and surviving by upregulating the antioxidant defence system, which is capable of readjusting the redox balance.

Moreover, in patients it was found[10,27,28] that calculated, transient oxidative stress such as that obtained during a cycle of ozonated autohaemotherapy also can induce a state of tolerance, characterized by a simultaneous overexpression of SOD and glucose-6-phosphate dehydrogenase and a reduction of the TBARS levels in plasma.

The rectal insufflation of O_3 (group 3) apparently is able to enhance the antioxidant system in a coordinate fashion because the increased activity of catalase on its own (group 2) is unable to quench CCl_4 toxicity.

Ozone treatment prevented the decrease in SOD activity induced by CCl_4 (Table 3), suggesting that oxidative preconditioning is a powerful mechanism able to reduce CCl_4-mediated oxidative stress as demonstrated by TBARS levels (Table 3). Marklund, in 1984, demonstrated that hydrogen peroxide overproduction inhibits SOD activity, whereas catalase is particularly active when excessive amounts of H_2O_2 are generated.[29]

Our results confirm again that prolonged administration of judicious doses of O_3 may promote the phenomenon of oxidative preconditioning or oxidative stress adaptation.[11,12]

Previous results demonstrated that O_2 administration (O_3 vehicle) was not only unable to confer protection but also increased the deleterious effects associated with CCl_4.[11] The greater cellular damage observed after oxygen administration + CCl_4 indicates the toxic effects of hyperoxygenation.[30]

As reported previously,[11] in our experimental conditions the administration of O_3 (15 pretreatments + sunflower oil) does not produce any effect without a CCl_4 oxidative challenge.

Oxidative preconditioning is a somewhat paradoxical cellular mechanism and it can be described as an induction of tolerance to O_3 and ROS generated by toxic agents. Oxidative preconditioning is analogous to other phenomena such as ischaemic preconditioning,[31] thermal preconditioning[32] and chemical preconditioning.[33] All these processes have in common that a repeated and nonlethal stress is able to confer protection against a prolonged and severe stress.

In summary, according to our results, ozone oxidative preconditioning has been proved to preserve glycogen content, to reduce lactate and uric acid formation and

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to control oxidative stress induced by CCl_4 administration to rats. The present study contributes to clarifying an important pharmacodynamic effect after prolonged ozone treatment to rats. Appropriate ozone therapy can upregulate the antioxidant system, representing a fundamental property of this complementary medical approach.

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