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Deleterious effects induced by oxidative stress in liver nuclei from rats receiving an alcohol-containing liquid diet

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Highly purified rat-liver nuclei were previously shown to have nuclear ethanol (EtOH) metabolizing system able to bioactivate alcohol to acetaldehyde and 1-hydroxyethyl radicals. These reactive metabolites were able to covalently bind to nuclear proteins and lipids potentially being able to provoke oxidative stress of nuclear components. In this study, the above-mentioned possibility was explored. Sprague Dawley male rats (125–150 g) were fed a standard Lieber and De Carli liquid diet for 28 days. Controls were pair-fed with a diet, in which EtOH was isocalorically replaced with carbohydrate. The presence of a chlorzoxazone hydroxylase activity inducible by the repetitive EtOH drinking further suggested the presence of CYP2E1 in the highly purified nuclei. Nuclei from EtOH-drinking rats evidenced significantly increased susceptibility to a *t*-butyl hydroperoxide challenge as detected by chemiluminescence emission, increased formation of protein carbonyls, and decreased content of protein sulfhydryls. In contrast, no significant changes in the nuclear lipid hydroperoxides formation or even decreases in the 8-oxo-7,8-dihydro-2-deoxyguanosine were observed. No significant differences were observed in different parameters of the alkaline Comet assay. In immunohistochemical studies performed, no expression of p53 was observed in the livers of the animals under the experimental conditions tested. Since nuclear proteins and lipids are known to play a role in cell growth, differentiation, repair and signaling, their alterations by either oxidative stress, or by covalent binding might be of relevance to liver tumor promotion. *Toxicology and Industrial Health* 2008; **24**: 625–634.

Key words: *alcohol; CYP2E1; ethanol; nuclei and microsomes*

Introduction

In previous studies from our laboratory, the presence of an ethanol (EtOH) metabolizing–system (NEMS), which led to the production of acetaldehyde and 1-hydroxyethyl free radicals (1HEt), found in highly purified liver nuclei was reported (Castro, *et al.*, 1998). In further studies, these reactive EtOH metabolites proved to bind covalently to nuclear lipids and proteins (Díaz Gómez, *et al.*, 1999). The nuclear system was found to involve the potential participation of cytochrome P450 2E1 (CYP2E1) and being inducible when rats received the standard Lieber & De Carli diet (Lieber and De Carli, 1982, 1989) for 28 days (Díaz Gómez, *et al.*, 2002). Furthermore, not only the ethanol itself increased its metabolism upon repetitive alcohol drinking (Díaz Gómez, *et al.*, 2002) but also of

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N-nitrosodimethylamine (NDMA), the other relevant environmental carcinogen whose bioactivation process to the ultimate reactive moiety is CYP2E1 dependent (Guenguerich, 1995).

The EtOH promotion of free radicals at liver-nuclear level might spark an oxidative stress process in their lipids; proteins and nucleic acids, potentially having relevant toxicological consequences.

In the present studies, the possibility that, after repetitive drinking for 28 days, a standard Lieber & De Carli alcohol-containing liquid diet provoked alterations in the liver nuclear lipids, proteins, and DNA has been explored.

Materials and methods

Chemicals

EtOH (analytical grade) and methanol (high performance liquid chromatography [HPLC]-grade) were from Sintorgan (Villa Martelli, Argentina); Chlorzoxazone, 6-hydroxychlorzoxazone, 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodGuo), deferoxamine mesylate, tert-butylhydroperoxide (TBHP), and xylenol orange were from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of the best quality available.

P53 kit was from DakoCytomation, Inc. (Carpinteria, CA, USA) and p53 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Animals and treatments

Noninbred male Sprague Dawley rats were used. The procedures for breeding, housing, and handling animals were those established by the Food, Drug and Medical Technology National Administration (ANMAT; Buenos Aires).

Sprague Dawley male rats (125–150 g bodyweight) were fed with a nutritionally adequate liquid diet (Lieber and De Carli standard rat diet, purchased from Dyets, Inc., Bethlehem, PA, USA) (Lieber and De Carli, 1982, 1989). The rats were housed in individual cages and separated into two dietary groups, EtOH group and control group (Control). Both groups were pair-fed with the same diet except that in the control group, EtOH was isocalorically replaced with carbohydrate (dextrin-maltose). The liquid diet used provided 1 kcal/mL of which 35% of the total calories were derived from fat, 47% from carbohydrate, and 18% from protein. In the EtOH-treated animals, EtOH provided 36% of the calories replacing isocalorically carbohydrate. Feeding of the control and EtOH diet was continued for 28 days. The amount of ethanol was started with 30 g/L of the liquid diet for the first 2 days, 40 g/L for the subsequent 2 days followed by the final formula containing 50 g/L.

Isolation of highly purified nuclear preparations

Highly purified nuclei were obtained as previously described (Castro, *et al.*, 1998; Díaz Gómez, *et al.*, 1999, 2006). Briefly, the liver homogenate in 0.25 M sucrose in TKM buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, and 2.5 mM KCl), pH 7.5, was passed through a 100-mesh nylon cloth and centrifuged at 1,000 × g for 20 min. After washing the pellet twice by resuspending with 0.25 M sucrose-TKM and centrifugation at 1,000 × g for 5 min, the crude preparation was resuspended in 2.2 *M* sucrose-TKM, layered on 2.3 M sucrose-TKM and centrifuged for 20 min at 80,000 × g. The pellet was gently rinsed with 0.25 M sucrose-TKM and resuspended in 2.2 M sucrose-TKM and the ultracentrifugation step was repeated. Finally, the pellet was washed with 0.25 M sucrose-TKM. The purity of nuclei was assessed on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase), and by phase contrast microscopy in order to obtain an assessment of the purity of nuclear preparations.

Isolation of the microsomal fraction

Livers were homogenized using teflon-glass Potter-Elvehjem homogenizer with four volumes of 1.15% (w/v) KCl. The homogenates were centrifuged at 9,000 × g for 20 min. The resulting supernatants were centrifuged at 100,000 × g for 1 h and the microsomal pellets were recovered (Castro, *et al.*, 2002).

Chlorzoxazone hydroxylase activity in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Chlorzoxazone hydroxylase (CZ-ase) activity was determined essentially as described by Leclercq,

et al. (1998) with some modifications. Microsomes (~1 mg protein/mL) or nuclei (1–1.5 mg protein/mL) were incubated for 1 h at 37°C in 50 mM TKM buffer, pH 7.5, containing 0.18 mM chlorzoxazone, and NADPH-generating system (26 mM $NADP^+$, 66 mM D-glucose-6-phosphate, 40 U/mL glucose-6-phosphate dehydrogenase, 66 mM $MgCl_2$), with a final volume of 3 mL. Blanks were also run omitting the NADPH-generating system from the incubation mixture. The reaction was terminated by addition of 100 µL of 30% $ZnSO_4$ and centrifuged for 10 min at 4000 × g. The supernatant was used for the HPLC analysis.

Analysis of the metabolite 6-hydroxychlorzoxazone was performed by HPLC (Waters 1525, binary HPLC pump) on a C8 reversed-phase column (Hewlett Packard MOS Hypersil 5 µm, 200 × 2.1 mm). An ESA Coulochem III electrochemical detector was used (ESA, Chelmsford, MA), equipped with a 5011A analytical cell, set at 300 mV and ultraviolet absorbance was also monitored at 287 nm in a Waters 2996 photodiode array detector. The mobile phase, consisting of 75% water (0.01% phosphoric acid, pH 3) and 25% acetonitrile (Chen and Yang, 1996), was delivered at a constant flow-rate of 0.4 mL/min. Results were expressed as the amount of the metabolite 6-hydroxychlorzoxazone per mg of nuclear or microsomal protein.

Protein sulfhydryl determination in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Liver nuclei or microsomes were suspended in 0.15 M Tris-HCl, 1 mM KH_2PO_4, pH 7.4. A volume containing approximately 1 mg protein, was mixed with 5% trichloroacetic acid, 5 mM ethylenediaminetetraacetic acid (EDTA) and centrifuged at 13,000 × g. Pellets were used to determine protein sulfhydryls using the Ellman's reagent. Sulfhydryl content was calculated from the absorbance at 412 nm, using a molar absorption coefficient of 13,100 M^{-1} cm^{-1}(Galelli and Castro, 1998).

Protein carbonyl determination in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Liver nuclei or microsomes were suspended in 0.15 M Tris-HCl, 1 mM KH_2PO_4, pH 7.4. Protein carbonyl determination was carried out in a volume containing approximately 1 mg protein by the 2,4-dinitrophenylhydrazine technique (Levine, *et al.*, 1990). Carbonyl content was calculated from the spectrophotometric absorbance at 370 nm, using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} (Galelli, *et al.*, 1997).

Determination of TBHP-induced chemiluminiscence in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Chemiluminiscence was measured in a Wallac-1214 Rack Beta liquid scintillation counter at room temperature in an out of coincidence mode (Boveris, *et al.*, 1983). Liver nuclei (8.0–8.2 mg protein/mL) or microsomes (1.5–1.7 mg/mL) were suspended in 0.25 M sucrose, 50 µM deferoxamine mesylate in TKM buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM KCl), pH 7.5. Homogenates were incubated in flasks at 37°C for 10 min in a Dubnoff shaker. Chemiluminiscence measurement was started by the addition of 3 mM TBHP. Result were expressed as arbitrary units, obtained by quantification of the area under the emission curve as a function of time.

Measurement of lipid hydroperoxides by the xylenol orange method in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Liver microsomes and nuclei were homogenized in cold HPLC-grade methanol with an Ultra-Turrax (Jank & Kunkel, IKA-Werk, Stanfen, Germany), then centrifuged for 10 min at 1,000 × g and the supernatants were used for hydroperoxide determination. The ferrous oxidation-xylenol orange (FOX) reagent (100 µM xylenol orange; 0.25 mM ammonium ferrous sulfate hexahydrate; 25 mM H_2SO_4) was prepared just before use (Nourooz-Zadeh, *et al.*, 1994). For the determination of the hydroperoxides, samples aliquots (270 µL) were pipetted into screw cap tubes. A blank was run using methanol instead of the tissue extract. In order to discern color development due to authentic lipid hydroperoxide from that due to H_2O_2 or other interfering components, triphenylphosphine (TPP), a specific hydroperoxide-reducing agent that has no effect on H_2O_2, was added to a set of vials to reduce lipid

hydroperoxides (0.1 mM final concentration) and methanol was added to the remaining set of vials. All vials were then vortexed and incubated at room temperature for 30 min, prior to the addition of the FOX reagent (2.7 mL). After mixing, the samples were incubated again, in the darkness at room temperature, until the reaction was complete (270 min). Absorbance of the xylenol complex was measured at 560 nm. Levels of hydroperoxides were determined as the difference in vials with and without TPP. A standard curve was developed with different concentrations of TBHP in methanol. The levels of hydroperoxides were expressed as nanomole of TBHP equivalents/mg protein.

Determination of 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodGuo)

Approximately 500 mg of liver tissue was used to extract DNA using the chaotropic method and the DNA obtained was hydrolyzed with Nuclease P1 and alkaline phosphatase, essentially as described by Ravanat, *et al.* (2002).

The measurement of 8-oxodGuo was carried out in an HPLC (Hewlett-Packard 1090) coupled to a Coulochem III electrochemical detector (ESA, Chelmsford, MA). The isocratic mobile phase was 50 mM KH_2PO_4 (pH 4.5), 8% methanol. Separation of the nucleosides was performed using a C_{18} reversed-phase column (Spherisorb ODS-2 5 μm, 250 mm × 4.6 mm, Sigma-Aldrich). The 8-oxodGuo levels were expressed as the ratio of 8-oxodGuo per 10^6 dGuo (Pouget, *et al.*, 2000). All samples were analyzed by triplicate.

p53 Immunohistochemistry in liver from rats receiving an EtOH-containing liquid diet

Immunostaining for p53 was performed on formalin-fixed, paraffin-embedded tissues based on an avidin-biotin-peroxidase complex technique. Sectioned liver tissue (3 μm thick) was deparaffinized with xylene and rehydrated through descending strengths of alcohol.

The p53 technique used was based on the labeled streptavidin biotin (LSAB) method with DakoCytomation LSAB®, system-HRP (code K0679). The kit consisting of LSAB reagents was used for qualitative demonstration of antigens in paraffin-embedded liver tissue. An antigen-retrieval step was performed incubating 45 min at 95 °C in 0.01 M citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by first incubating the tissue slides for 5 minutes in 3% hydrogen peroxide. Slides were then incubated overnight at 40 °C in a humidified chamber with a primary antibody (p53 from rabbit, FL-393: sc-6243, Santa Cruz Biotechnology Inc.), followed by sequential incubations with biotinylated link antibody and peroxidase-labeled streptavidin. Staining was completed after incubation with substrate-chromogen solution (3,3-diaminobenzidine) and counterstained with hematoxylin. The slides were examined under an Eclipse 400 microscope (Nikon, Japan) at ×200 magnification. A positive evidence for p53 detection would be indicated by a brown color staining in nuclei. Control immunolabeling was performed with the same staining procedure but using the visualizing reagents in the absence of the primary antibody.

Comparative effect of an EtOH liquid diet on parameters of the Comet assay in hepatocytes

After sacrificing the animals (10 animals per group), a small piece of liver was place in 1 mL of cold HBSS containing 20 mM EDTA/10% dimethyl sulfoxide. Then it was minced into fine pieces, and after allowing it to settle for 10 minutes, the resulting suspension was filtered through four layers of gauze and centrifuged. The pellet was then suspended in phosphate buffer solution (PBS) and the alkaline (pH > 13) comet assay single cell gel electrophoresis (SCGE) was performed in duplicate for each animal on 5 μL of the cell suspension. Determination of cell yield and viability and the SCGE assay was carried out essentially according to Hartmann, *et al.* (2003). After staining with ethidium bromide, 50 cells per sample were examined with an Eclipse 400 microscope (Nikon) equipped with epifluorescence filters and at a magnification of × 20. Imaging was performed using a Nikon ACT-2U with a DS 5M digital camera for image acquisition and the software CASP downloaded from http://www.casp.of.pl, to determine tail length (TL), tail intensity (TI), and tail moment (TM), all parameters correlated with the degree of DNA damage in the single cell. Differences in these parameters between groups were analyzed by one-way analysis of variance test.

Protein concentrations

Protein concentrations were determined by the method of Lowry, *et al.*, 1951, using bovine serum albumin as standard.

Statistics

The significance of the difference between two mean values was assessed by unpaired *t*-test (Students *t* test) (Gad, 2001). Calculations were performed using GraphPad Software Differences were considered significant when $P < 0.05$.

Results

CZ-ase activity in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Results obtained for chlorzoxazone hydroxylation as determined by HPLC can be seen in Table 1. Nuclei from rats treated with EtOH liquid diet showed a significant increase in the formation of 6-hydroxychlorzoxazone as compared to the control group. The same was observed in microsomes, although in an even much greater magnitude.

Protein sulfhydryl and carbonyl content in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Liver nuclei and microsomes from rats receiving the EtOH-containing liquid diet exhibited a significant decrease in its protein sulfhydryl content (Table 2).

Liver nuclei from rats receiving the EtOH-containing liquid diet, but not microsomes, exhibited a significant increase in its protein carbonyl content (Table 3).

Table 1. Chlorzoxazone hydroxylase activity in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

Subcellular fraction[a]	Formation of 6-hydroxychlorzoxazone (nmol/mg protein)[b]	
	Control	EtOH
Nuclei	0.19 ± 0.02	1.08 ± 0.02
Microsomes	31.60 ± 0.16	72.53 ± 6.11

$P < 0.05$ (EtOH vs Control).
[a]Nuclei (control: 1, EtOH: 1.54 mg protein/mL) or microsomes (control: 0.98, EtOH:1.03 mg protein/mL) were incubated in 50 mM TKM buffer, pH 7.5, NADPH-generating system and 0.18 mM chlorzoxazone at 37 °C for 1 h. Then they were processed to determine enzymatic activity by HPLC with electrochemical detection (nuclei) or by UV at 287 nm (microsomes) as described in Methods.
[b]Results are the means of three separate determinations from three different livers.

Table 2. Protein sulfhydryl content in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

	Control (nmol/mg protein)	EtOH (nmol/mg protein)
Nuclei	39.84 ± 0.43	36.29 ± 1.08[a]
Microsomes	173.89 ± 8.92	123.79 ± 5.96[b]

Liver nuclei and microsomes from control and EtOH-treated rats were homogenized in 0.15 M Tris-HCl (pH 7.4), 1 mM KH_2PO_4, and used to sulfhydryl determination as described in Methods. Values are the means ± SD of four separate samples.
[a]$P < 0.05$ (Control vs Ethanol treated).
[b]$P < 0.005$ (Control vs Ethanol treated).

TBHP-induced chemiluminiscence in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

The TBHP-induced chemiluminiscence from EtOH-treated rats was significantly more intense than in Control. This result was obtained both in microsomes and in nuclei (Figure 1).

Formation of lipid hydroperoxides in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

The levels of hydroperoxides expressed as TBHP equivalents were measured in control as well as in rats treated with the EtOH liquid diet. After

Table 3. Protein carbonyl content in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

	Control (nmol/mg protein)	EtOH (nmol/mg protein)
Nuclei	0.88 ± 0.08	1.51 ± 0.30[a]
Microsomes	8.39 ± 0.29	7.89 ± 0.69[b]

Liver nuclei and microsomes from control and EtOH-treated rats were homogenized in 0.15 M Tris-HCl (pH 7.4), 1 mM KH_2PO_4, and used to carbonyl determination as described in Methods. Values are the means ± SD of four separate samples.
[a]$P < 0.005$ (Control vs Ethanol treated).
[b]$P > 0.05$ (Control vs Ethanol treated).

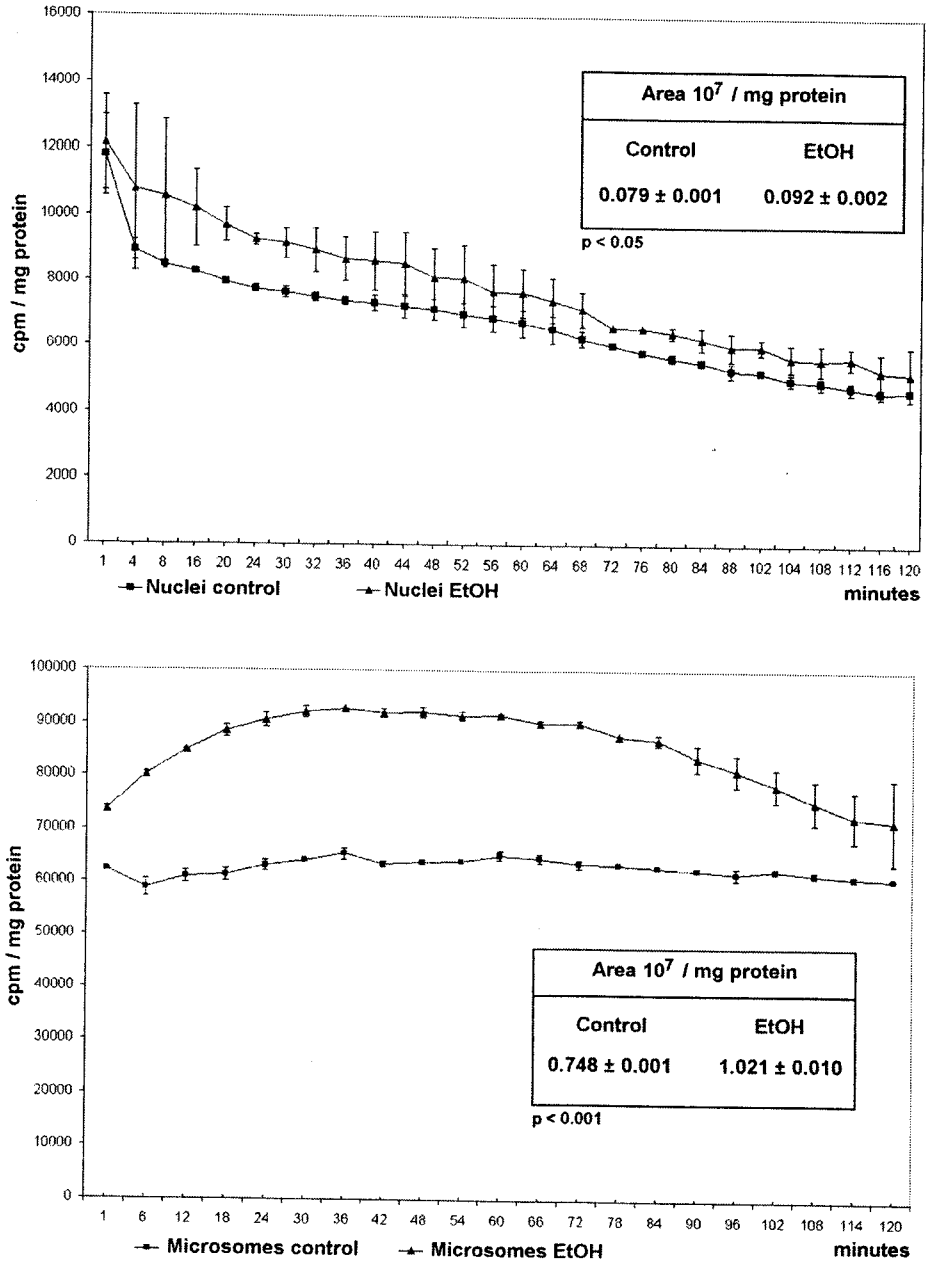

Figure 1 tert-Butylhydroperoxide (TBHP) induced-chemiluminiscence in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet. Liver nuclei (8.0–8.2 mg protein/mL) or microsomes (1.5–1.7 mg protein/mL) were homogenized in 0.25 M sucrose, 50 μM deferoxamine in TKM buffer, pH 7.5 were kept at 37 °C for 10 min in a Dubnoff shaker. Chemiluminiscence measurement was started by addition of 3 mM TBHP. Values are the means ± SD of four separate samples.

incubating with the reagent and subtracting the possible formation of hydroperoxides due to H_2O_2, a significant higher level of hydroperoxides in the EtOH-treated rats was observed in liver microsomes, as compared to control (Table 4). Nuclei suspensions didn't show any significant difference from control (Table 4).

Determination of 8-oxodGuo in liver from rats receiving an EtOH-containing liquid diet

8-oxodGuo was measured in DNA extracted from liver tissue from rats treated with the ethanol liquid diet for 28 days. 8-oxodGuo values in ethanol-treated rats were significantly lower than in control

Table 4. Measurement of lipid hydroperoxides by the xylenol orange method in liver nuclei and microsomes from rats receiving an EtOH-containing liquid diet

	Control (nmol TBHP/mg protein)	EtOH (nmol TBHP/mg protein)
Nuclei	0.30 ± 0.08	0.26 ± 0.09[a]
Microsomes	0.59 ± 0.38	2.24 ± 0.34[b]

Liver nuclei or microsomes from control and EtOH-treated rats were incubated with the xylenol orange reagent at room temperature as described in Methods. Values are the means ± SD of four separate samples.
[a] $P > 0.05$ (Control vs EtOH).
[b] $P < 0.001$ (Control vs EtOH).

rats. Results expressed as 8-oxodGuo × 10^6 dGuo were: Control: 5.82 ± 0.37, Treated: 2.79 ± 0.19, $P \leq 0.05$.

p53 immunohistochemistry in liver from rats receiving an EtOH-containing liquid diet

No significant difference in the expression of p53 were observed under the experimental conditions employed (Figure 2).

Comparative effect of an EtOH liquid diet on parameters of the comet assay in hepatocytes

The effect of an ethanol liquid diet on the induction of DNA single strand breaks in individual cells was measured using the alkaline comet assay. On the different parameters determined: TL, %DNA in tail (TI) and Olive TM no significant differences were observed, as seen in Table 5.

Discussion

In agreement with previous studies from others and from our laboratory, we observed that repetitive

Table 5. Comparative effect of EtOH liquid diet on parameters of the comet assay in hepatocytes

Parameters	Hepatocytes	
	Control	EtOH
Tail lenght (μm)	5.09 ± 1.30	5.20 ± 1.40[a]
Tail DNA%	0.46 ± 0.11	0.51 ± 0.13[a]
Tail moment (arbitrary units)	0.19 ± 0.05	0.23 ± 0.06[a]

[a] $P > 0.05$ when compared to control. Data are the mean ± SD.

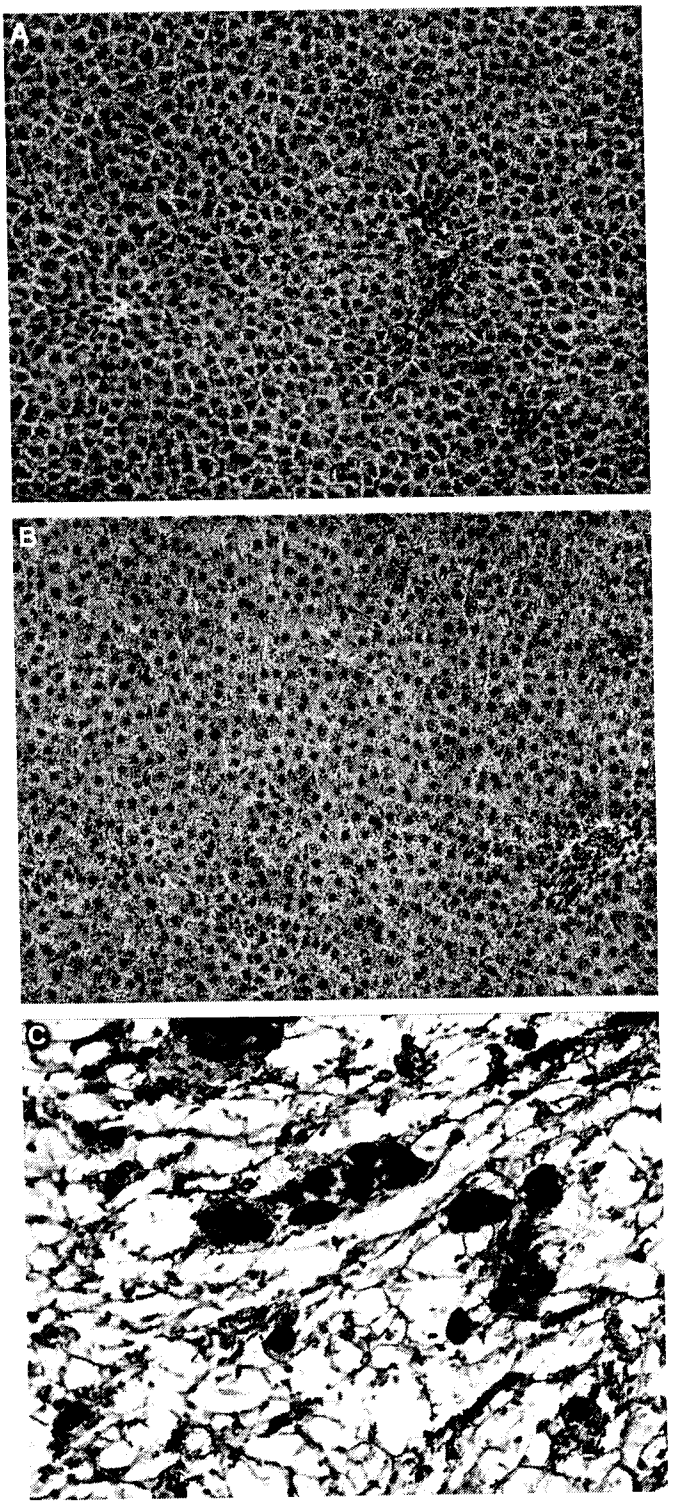

Figure 2 Immunostaining for p53 in liver from rats receiving an EtOH-containing liquid diet. (A) Slide from control animals treated with the Lieber De Carli diet (× 200). (B) Slide from EtOH-animals treated with the Lieber De Carli diet (× 200). (C) Slide from positive immunolabeling performed in mammary gland (× 200).

alcohol drinking is able to induce liver microsomal CZ-ase (Chen and Yang, 1996; Lucas, *et al.*, 1996; Leclercq, *et al.*, 1998; Tanaka, *et al.*, 2003). Now we report that highly purified liver nuclei also exhibit a small but detectable CZ-ase activity which is about 166 times smaller than the one in the microsomal fraction and inducible by repetitive alcohol drinking. CZ-ase determination has been widely used as a marker for the CYP2E1 activity (Chen and Yang, 1996; Lucas, *et al.*, 1996; Leclercq, *et al.*, 1998; Tanaka, *et al.*, 2003) and EtOH is known to be a well-known inducer of CYP2E1 (Lieber, 2004). The presence of CYP2E1 in the nuclei should not be unexpected in light of the fact that the outer nuclear membrane is continuous with the rough endoplasmic reticulum (RER), and the perinuclear space between both, the inner and the outer nuclear membrane is continuous with the lumen of the RER (Stryer, 1995).

In fact, other low molecular weight chemicals known to be metabolized by CYP2E1 (Bolt, *et al.*, 2003) such as EtOH itself; NDMA; CCl_4; $CHCl_3$; methanol; aniline, or toluidine can also be metabolized by highly purified liver nuclei (Castro, *et al.*, 1989, 1990, 1998, 2002; Díaz Gómez and Castro, 1980; Díaz Gómez, *et al.*, 1999, 2002, 2006). It has been previously reported that during EtOH metabolism by liver microsomal CYP2E1 not only acetaldehyde but also hydroxyl and 1-hydroxyethyl radicals are produced and iron is released from the cytochrome molecule (Lu and Cederbaum, 2008). During the liver nuclear metabolism of EtOH, our laboratory also reported the formation of acetaldehyde and the production of hydroxyl and 1-hydroxyethyl radicals (Castro, *et al.*, 1998).

Free radicals are known to lead to addition and H abstraction reactions (Castro and Castro, 1997). We previously reported that during the nuclear metabolism of EtOH, reactive metabolites bind covalently to macromolecules and lipids (Díaz Gómez, *et al.*, 1999). Now we have some indication that during EtOH drinking, changes in the liver nuclear components occur, which suggest that an oxidative stress process can also be observed in this organelle. In effect, on one hand liver nuclei showed increased production of reactive oxygen species (ROS) after repetitive EtOH drinking when challenged with TBHP and when their detection was made through the chemiluminiscence test previously described by others (Boveris, *et al.*, 1983; Török, 2004). The shape of the emission curve in the case of the nuclear samples (Control and EtOH) was similar, evidencing only changes in the intensity of the ROS production (see Figure 1). That suggests the occurrence of changes in the composition of the target molecules challenged by the TBHP, making them more susceptible to the oxidant attack. For the microsomal fraction, the situation was different. A more rapid and intense ROS production evidenced through chemiluminiscence emission was observable. That might be interpreted as a combined effect present in the microsomal fraction from EtOH-treated animals: decreased defenses against oxidative stress but also increased susceptibility at target molecules levels. Our present additional observations might shed some light on this hypothesis. However, not all the experiments made offer a full explanation to the above observations. For example, there were significant decreasing effects of oxidative stress on the nuclear protein sulfhydryl content (see Table 2). Further, the protein carbonyl content in the nuclear proteins of EtOH-treated animals was significantly increased as a consequence of the promoted oxidative stress (see Table 3). In contrast, no equivalent oxidative effect was observable in the microsomal fraction from EtOH-treated animals (Table 3).

The behavior of the lipid components from the liver nuclear and microsomal compartments after repetitive alcohol drinking was also different. No significant increase in the lipid hydroperoxides content was found in the nuclear fraction, whereas a highly significant one was observable in the microsomal compartment (Table 3).

The overall analysis of these observations related to ROS formation and the subsequent oxidative stress produced suggest that, apparently, there are significant differences about the nature of the major targets of the oxidation stress in the nuclei with respect to those in microsomes. Proteins appear to be uniquely targeted in the nuclei while lipids and proteins are preferentially involved in the case of the microsomal fraction.

Reasons for these important differences might be related to factors such as the different content of CYP2E1 at both cellular sites as well as to the composition of the targets and to the amount of the

cellular defenses against oxidative stress available at the neighborhood of the site of ROS production.

Under this line of reasoning, it should not be unexpected, our finding that as a result of the nuclear oxidative stress evidenced, there were no oxidative effects observable in DNA as detectable by either 8-oxodGuo formation or by the Comet Assay test, despite both being well-established biomarkers of effects of oxidative stress on DNA (Hussain, *et al.*, 2003; Marnett, 2000; Pouget, *et al.*, 2000; Ravanat, *et al.*, 2002). It is known that in resting liver cells a significant part of DNA is tightly bound to histones and other proteins (Castro, *et al.*, 1989) and in that way, they would protect it from oxidative stress attack. This hypothesis fits with the observable preferential attack on nuclear proteins observable in the liver nuclei from EtOH-treated animals. It also could give account for the failure to observe enhanced expression of p53 in the immunohistochemical studies made under the experimental conditions tested.

Whether our previously described alterations in liver nuclear components, derived from EtOH activation to metabolites covalently binding to its proteins and lipids or the here evidenced promotion of oxidative stress of critical proteins plays some role in the long-term effects of EtOH drinking remains as a purely speculative matter at present. Present views about the molecular mechanisms of alcohol-mediated carcinogenesis and cirrhosis were recently reviewed by Seitz and Stickel, 2007.

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