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# Nuclear DNA strand breaks during ethanol-induced oxidative stress in rat brain

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**Abstract Free radical-mediated oxidative damage has been implicated in the pathophysiological mechanisms of apoptosis. In this study we report that statistically significant strand breaks were induced primarily in the hippocampus and cerebellum during chronic, and not acute, ethanol treatment. Damage to DNA observed in hippocampus and cerebellum was also correlated with significant modification in the activities of mitochondrial respiratory complexes I and IV and with a significant increase in lipid peroxidation products. This finding lends support to the fact that hippocampus and cerebellum are brain areas particularly vulnerable to redox changes induced by alcohol intoxication, suggesting lower threshold levels of ethanol tolerance.**

*Key words:* Ethanol; DNA strand break; Apoptosis; Redox balance

## 1 Introduction

Cell injury caused by effects of oxidative stress has led to a number of interesting working hypotheses which link cellular redox balance to induction of apoptosis [1]. Ulterior support for an association between cellular oxidative stress and apoptosis has been provided by observations in which chemical or physical agents frequently used to produce or inhibit apoptosis are often the same as those used to elicit or inhibit effects of oxidative stress, hence suggesting that the cellular redox might act as a signaling mechanism for the onset of metabolic events leading to apoptosis. The involvement of oxidative stress effects in temporal and programmed events in the cell has been a subject of much investigation, particularly with reference to the free radical theory as the basis of the aging process. Conceivably, the similarities for cellular aging [2] and apoptosis [3,4] might represent effects of the cellular redox state on various manifestations of cellular developmental programs. Hence, the final outcome of these programmed events would depend on oxidative signals, and the ability of the cell to respond to these programmed events would depend on its ability to maintain an oxidant–antioxidant balance.

While the actual mechanisms involved in the induction of apoptosis are not known, the morphological features of this event are very similar for the various cell types and conform to the idea that apoptosis is a distinct form of cell death, often referred to as programmed cell death, and distinguishable from general death-promoting physiological or pathological processes.

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An increasingly important area of antioxidant defense is based on sulfhydryl chemistry and on the role of -SH groups in the function of macromolecular structures as reported for enzymes and cellular membranes [5]. The susceptibility of these structures to inactivation by oxidation of their sulfhydryl groups has been well documented [6]. Furthermore, early increases in the production of reduced glutathione (GSH) during oxidative stress have been postulated as the main defense mechanism for maintaining essential -SH groups in the reduced state [7]. Thus, membrane damage caused by lipid peroxidation is an early event in cellular injury which is followed by oxidation of sulfhydryl enzymes. The onset of these mechanisms is presumably triggered by imbalances between production of reducing equivalents and cellular demands of the same.

These considerations led us to investigate the effectiveness of ethanol in promoting reactions of oxidative stress. In particular, we were interested in investigating the susceptibility of cerebral tissue to DNA strand breaks as a measure of effectiveness of the antioxidant defense system during ethanol intake. Ethanol is known to decrease the intracellular reduced glutathione pool as well as increase oxidation of bound thiol groups.

In previous studies [8], we have shown that ethanol can induce HPS 70 stress protein which can be related to a decrease in the intracellular free thiol content. In this study we report that statistically significant strand breaks were induced, primarily, in the hippocampus and cerebellum during chronic, and not acute, ethanol treatment. The interesting observation was that the damage to DNA observed in hippocampus and cerebellum was also correlated with significant modifications in the activities of mitochondrial respiratory complexes I and IV and lipid peroxidation products.

## 2. Materials and methods

### 2.1. Animals

Twelve male Wistar rats weighing approximately 220 ± 20 g were randomly divided into two groups of six per group. The rats were unfed overnight. The experimental animals received a single dose of ethanol equivalent to 5 g/kg b.wt. of rat. Ethanol was administered by stomach intubation as a 20% ethanol solution in saline (w/v). The control group received a single isocaloric dose of glucose in saline solution by stomach intubation. All animals were killed 6 h post-treatment by cervical dislocation. For chronic ethanol administration, 20 male Wistar rats weighing approximately 220 ± 20 g were randomly divided into two groups. The experimental group consisted of 12 rats whereas, the control group was made up of eight rats. The rats were individually housed in metabolic cages under a 12 h light/dark cycle, (lights on at 07:00 h). The experimental group was fed a vitamin/mineral-fortified diet containing 5% ethanol (w/v) for the entire experimental period, lasting 40 days. In the control group, isocaloric glucose was substituted for ethanol. The animals were allowed to eat approximately 50 g per day. Blood samples from the experimental


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group were routinely removed by cardiac puncture to monitor ethanol concentration. Animals were killed by cervical dislocation and exsanguinated by cardiac puncture. Plasma was prepared by centrifuging blood samples at 5000 rpm for 5 min. The brain was quickly removed, washed in cold 0.15 M NaCl and the various brain areas dissected on an ice-cold slab.

*2.2. Sample preparation and analysis*

The tissue was homogenized (1 : 1 w/v) for 2 min in a solution containing 10 mM Tris-HCl buffer (pH 7.4), 0.32 M sucrose and 1 mM EDTA. Homogenate was subjected to differential centrifugation, according to Renis et al. [9], to obtain the nuclei, the post-crude mitochondrial supernatant and the free mitochondria.

The nuclear and mitochondrial pellets were lysed by incubation in 300 μl of lysis buffer (20 mM Tris-HCl, pH 7.9, 25 mM EDTA, 0.5% SDS and 0.3 mg/ml proteinase K) for 15 h at 50°C and DNA extracted with phenol/chloroform/isoamylic alcohol (25 : 24 : 1). After precipitation with ethanol and digestion of RNA with 100 μg/ml, DNAse-free, RNAse for 1 h at 37°C, DNA was analyzed on 1% agarose gel electrophoresis. Moreover, DNA was analyzed by random oligonucleotide primers synthesis assay (ROPS) according to Basnakian and James [10].

Complex I, NADH reductase (EC 1.6.99.3) was measured as rotenone-sensitive rate of NADH oxidation according to the method of Ragan [11]. Complex IV (EC 1.9.3.1) was measured according to the method of Warton and Tzagoloff [12].

Ethanol concentration in blood was quantitated by head space gas chromatography as reported by Calabrese et al. [8]. Protein and non-protein sulfhydryl compounds in plasma were evaluated by the method of Sedlak and Lindsey [13]. Tests were performed, in vitro, with acetaldehyde and GSH standards, which revealed that acetaldehyde, in the range of 15–70 μM, did not interfere with -SH assay. Plasma levels of GSH were assayed by the NADPH-dependent GSSG reductase method as reported by Tietze [14] and modified by Adams et al. [15]. Lipid-soluble fluorescent products (LSFP) were assayed according to the method of Fletcher et al. [16]. Malondialdehyde (MDA) was measured by the thiobarbituric acid test as reported in Calabrese et al. [8]. Proteins were measured with the bicinchoninic acid assay as described by Smith [17].

## 3. Results

Acute administration of ethanol at a dose of 5 g/kg b.wt. of rat resulted in blood concentrations equivalent in humans to a greatly intoxicating dose estimated in the range of 200–400 mg/dl. Fig. 1 shows the blood ethanol concentration versus time for the chronic and acute ethanol-treated rats. The ethanol content in blood reached a constant level after the first week of ethanol administration and it remained approximately constant throughout the experimental period. In addition, the gain in body weight measured at the end of the experimental period of 40 days showed no significant differences between the ethanol-treated and isocaloric-fed rats; the mean weight ± SD for the ethanol-treated group was 420 ± 38 g and control group 446 ± 50 g. Chronic ethanol intake resulted in the build-up of steady-state levels of alcohol in blood equivalent to 40–62 mM or 0.18–0.28 g/dl through the experimental period up to sacrifice. These concentrations are similar to those reported in other chronic studies [18,19]. Chronic as well as acute ethanol treatment caused a marked reduction in plasma GSH concentration as well as free and/or protein-bound thiol content. A decrease in GSH and total thiol concentration post-ethanol treatment was an expected result. However, the interesting observation was that the blood values of GSH, during chronic ethanol treatment, were not suppressed but maintained a constant concentration lower than that of control animals.

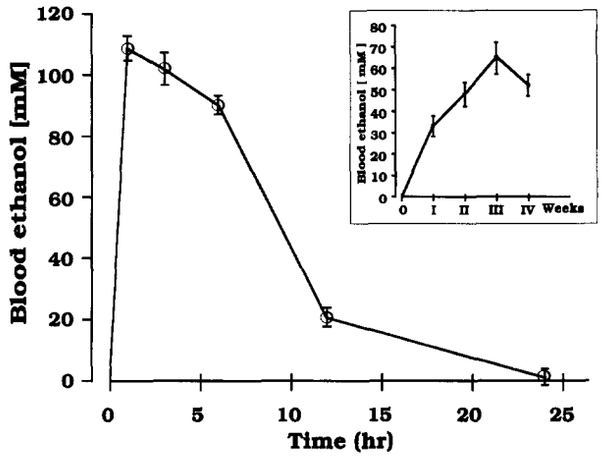


Fig. 1. Blood ethanol concentration versus time after oral administration of a single acute dose (5 g/kg) of ethanol (– ∘ –), or after chronic oral intake (inset). Ethanol in blood was determined by head space gas chromatography, as described in Section 2. Results represent the mean ± SD of at least six animals.

Free and protein-bound thiol concentration followed a similar pattern. The results are shown in Table 1.

The effect of ethanol administration on lipid peroxidation are shown in Tables 2 and 3. The data show that lipid peroxidation products were produced in all brain areas examined. However, the intensity of the response was dependent on the ethanol dose as observed for cortex, hippocampus and cerebellum. Table 4 shows the sensitivity of respiratory complex I and IV to the effects of ethanol, even at reduced dose, as shown in animals receiving a single acute dose of ethanol. ROPS analyses of phenol/chloroform-extracted nuclear or mitochondrial DNA are shown in Fig. 2a–d. The results show that statistically significant DNA strand breaks accumulate during chronic alcohol treatment, particularly in nDNA extracted from hippocampus and cerebellum. In contrast strand breaks in mtDNA were not observed post-treatment with acute or chronic doses of ethanol.

Table 1
Free and protein-bound thiols in plasma after acute or chronic ethanol treatment

| | Control | Acute ethanol | Chronic ethanol (weeks) | | | |
|---|---|---|---|---|---|---|
| | | | I | II | III | IV |
| Protein SH[a] | 428 ± 10 | 294 ± 12* | 354 ± 8* | 319 ± 16* | 328 ± 12* | 311 ± 7.9* |
| N-Prot. SH[a] | 49 ± 6.3 | 20.5 ± 6.1* | 26 ± 3.7* | 21.6 ± 4.6* | 30 ± 4.1* | 33 ± 3.2* |
| GSH[a] | 16.2 ± 0.9 | 6.7 ± 0.5* | 7.2 ± 0.5* | 6.7 ± 0.7* | 7.1 ± 1.1* | 7.6 ± 0.8* |
| Ethanol[b] | – | 90.5 ± 8.8 | 33 ± 9.6 | 48 ± 11 | 65 ± 15 | 52 ± 9.9 |

Values are expressed in nmol/ml plasma (a), or in μmol/ml blood (b). Results are the mean ± S.E.M. of six (acute) or 12 (chronic) experiments.
*Significantly different from control values.

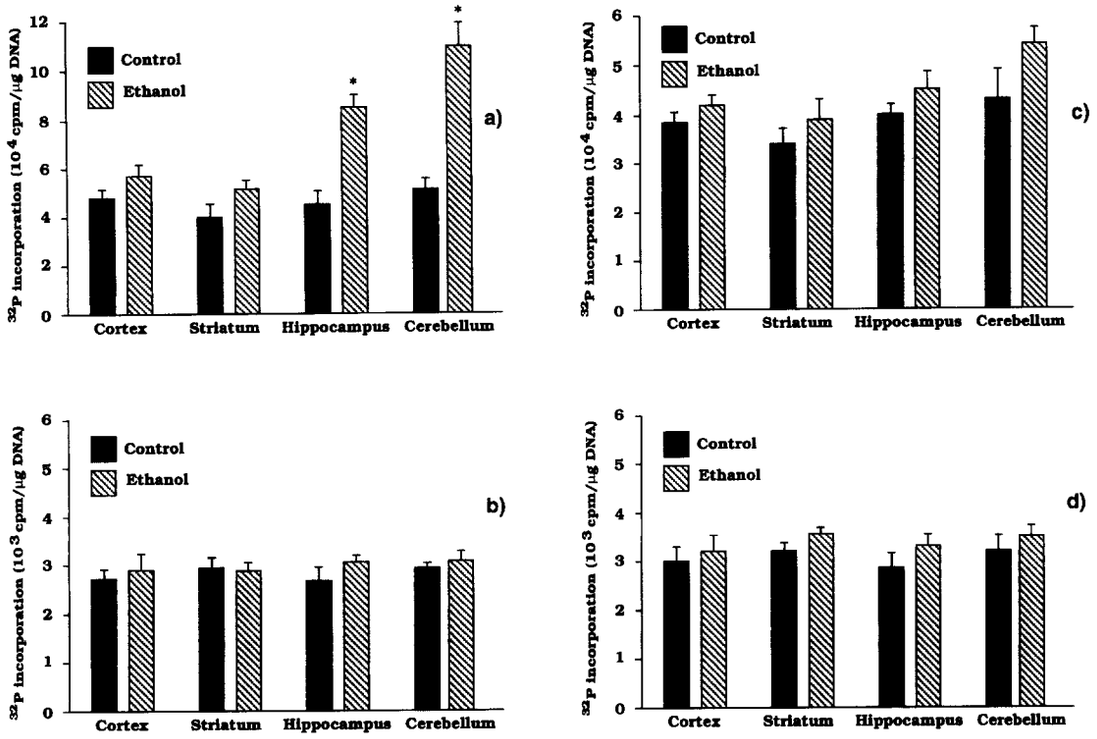


Fig. 2. DNA analysis by random oligonucleotide primed synthesis in different cerebral areas of isocaloric control and acute and/or chronic ethanol-treated rats. Nuclear DNA of acute (a) and chronic ethanol (c) rats. Mitochondrial DNA of acute (b) and chronic ethanol (d) rats. Results are expressed as mean ± SD of five animals; each experiment was performed in triplicate. *Significantly different from control ($P < 0.001$).

## 4. Discussion

This study clearly demonstrates that a number of ethanol-dependent biochemical alterations are consequence of ethanol administration. Predictably, some changes occur immediately after exposure to ethanol, as observed for the intracellular decreases in GSH concentration and the partial inactivation of respiratory chain complexes. Others are latent with low rate constants, in dependence on species, cell type, exposure regimen and many other factors. Potential targets such as nuclear DNA and mitochondrial DNA perhaps belong to the category of cytological or subcellular structures which require longer exposure times to the oxidative effects of ethanol. However, it is purely conjectural to ascribe, with any degree of significance, sequential responses of exposure to high ethanol concentrations in rats. Nevertheless, these considerations stem from the quantified changes observed after acute and chronic ethanol intake which show that hippocampus and cerebellum are more vulnerable to the effects of acute or chronic ethanol intake. In addition, these brain areas appear more severely damaged at the level of nuclear DNA, but not mitochondrial DNA, strand breaks and, however, only after a prolonged ethanol dosage regimen. Cortex and striatum did not show DNA chain breaks, suggesting higher threshold levels of ethanol tolerance.

While the cytotoxic effects of ethanol on brain tissue are not entirely understood, three possibilities should be considered. First, ethanol can exert its cytotoxic effects through its lipid-soluble properties and hence displays its biological effects by physical action as a denaturing or disaggregating agent in cellular macromolecular organization. Second, the cytotoxic effects of ethanol are linked to its metabolic fate and are most probably are mediated by oxygen-dependent generation of free radicals. These free radicals may directly react with vital cell constituents, or may be transformed into more reactive species. It is generally recognized that ethanol oxidation results in a burst of reduced pyridine nucleotides, NADH/NADPH. Increased reducing equivalents stimulate electron transport to oxygen which predispose the cell to increase the formation of oxygen free radicals. There is considerable evidence that free radicals may be formed during cell respiration [20], and when the rate of electron transport is increased there is an increase in the formation of these molecules. Third, the cytotoxic effects of ethanol result from a combined influence of its physical, chemical and metabolic properties. During heightened metabolic activity, the cellular membranes that are targets for the harmful effects of oxygen may shift into configurations that are more vulnerable to the oxidizing effects of free radicals. The latter possibility, although highly speculative, is not entirely unreasonable, since it has been

Table 2
Lipid-soluble fluorescent products (LSFP) tested in the cytoplasm of different cerebral areas of control and ethanol-treated rats

| | LSFP (UF/mg protein) | | |
|---|---|---|---|
| | Control | Acute ethanol | Chronic ethanol |
| Cortex | 2.12 ± 0.6 | 7.00 ± 1.1* | 8.40 ± 0.2* |
| Striatum | 2.24 ± 0.4 | 3.59 ± 0.78 | 5.70 ± 0.9*,[a] |
| Hippocampus | 1.41 ± 0.5 | 3.14 ± 0.8* | 5.11 ± 0.8*,[a] |
| Cerebellum | 1.56 ± 0.5 | 4.02 ± 0.4* | 5.70 ± 0.3*,[a] |

Results are the mean ± S.D. of six (acute) or 12 (chronic) experiments.
*Significantly different from control values ($P < 0.05$).
[a]Significantly different either from control or acute ethanol-treated ($P < 0.05$).

Table 3
Malondialdehyde (MDA) was assayed as TBA reactive material (as described in Section 2)

| | MDA (nmol/mg protein) | | |
|---|---|---|---|
| | Control | Acute ethanol | Chronic ethanol |
| Cortex | 0.020 ± 0.007 | 0.319 ± 0.04* | 0.086 ± 0.01* |
| Striatum | 0.014 ± 0.005 | 0.221 ± 0.06* | 0.082 ± 0.01* |
| Hippocampus | 0.018 ± 0.006 | 0.227 ± 0.07* | 0.180 ± 0.02* |
| Cerebellum | 0.015 ± 0.007 | 0.131 ± 0.02* | 0.098 ± 0.01* |

Results are the mean ± S.D. of six (acute) or 12 (chronic) experiments.
*Significantly different from control ($P < 0.05$).

Table 4
Enzymatic activities of respiratory chain complexes I and IV assayed in free mitochondria of different cerebral areas of control and ethanol-treated rats

| Treatment | Complex I (nmol/min per mg protein) | | | | Complex IV (k/min per mg protein) | | | |
|---|---|---|---|---|---|---|---|---|
| | CX | St | Hp | Cb | CX | St | Hp | Cb |
| Acute | | | | | | | | |
| Control | 60.30 ± 2.15 | 72.90 ± 3.30 | 85.40 ± 4.05 | 78.50 ± 4.60 | 23.00 ± 1.30 | 36.00 ± 2.05 | 31.05 ± 1.80 | 35.10 ± 1.55 |
| Ethanol | 59.50 ± 3.03 | 69.70 ± 4.10 | 61.40 ± 4.00* | 68.20 ± 3.35 | 21.70 ± 2.12 | 35.80 ± 3.10 | 26.90 ± 0.75* | 30.00 ± 0.90* |
| Chronic | | | | | | | | |
| Control | 51.20 ± 1.70 | 75.40 ± 2.35 | 80.30 ± 3.50 | 69.50 ± 2.13 | 24.20 ± 1.85 | 38.30 ± 2.25 | 32.20 ± 2.12 | 34.40 ± 1.75 |
| Ethanol | 43.05 ± 1.90* | 68.30 ± 3.30 | 56.40 ± 3.90* | 48.80 ± 3.25* | 17.30 ± 1.90* | 32.50 ± 2.45 | 22.40 ± 1.05* | 21.20 ± 2.15* |

CX = cortex; St = striatum; Hp = hippocampus; Cb = cerebellum. Each value is the mean ± S.E.M. of six (acute) or 12 (chronic) experiments.
*Significantly different from the control ($P < 0.05$).

shown, for example, that mitochondrial membranes undergo extensive alterations in structure when the respiration changes from resting state-4 to active state-3, [21]. Moreover, the disaggregating properties of the compound would further facilitate such structural modifications.

Although our study does not permit us to conclude that ethanol is an apoptosis-inducing agent, it does, however, demonstrate that ethanol can provide oxidative stress reactions in brain cells. At the present time such oxidative changes would appear non-specific signals due to the limitations in our experimental system. Nevertheless, in this study we have quantified the intensity of the oxidative stress produced by ethanol in terms of biochemical alterations in enzymatic activities, lipid peroxidation products, oxidation of free and protein-bound thiols, generation of free radical species and nucleic acid strand breaks. Hence, it appears that ethanol, through its lipophylic and free radical-generating properties, can provide a broader range of oxidative stress reactions. Conceivably, the specificity of the oxidative reactions induced by ethanol can be evaluated in immunologically competent cells by assaying the proliferative or apoptotic responses of these cells after exposure to ethanol.

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