ACUTE EXPOSURE OF CULTURED NEURONES TO ETHANOL RESULTS IN REVERSIBLE DNA SINGLE-STRAND BREAKS; WHEREAS CHRONIC EXPOSURE CAUSES LOSS OF CELL VIABILITY

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Abstract – Aims: Ethanol can create progressive neuropathological and functional alterations of neurones. However, the influence of exposure duration is still debated. It is difficult to specify the level of alcohol consumption leading to alcohol-induced brain damage. Moreover, the mechanism of toxicity is assumed to combine direct and metabolically induced effects, although numerous uncertainties remain. Finally, the genotoxic power of ethanol has not fully been investigated in the brain. In the experiment reported herein, primary cultures of neurones were exposed either chronically or acutely to doses of ethanol within the range of blood alcohol levels in intoxicated humans. The impact on the integrity of neurones was assessed by cytotoxicity tests and DNA alterations by single-cell gel electrophoresis (Comet assay) and flow cytometry. Chronic ethanol exposure, even at a low dose, was more harmful to neurones than acute exposure. Both significant reductions in cell viability and DNA alterations were observed in this condition. On the other hand, DNA repair capacities seemed to be preserved as long as the viability measured by specific tests was not affected. Instead, neurones entered a death cell process compatible with apoptosis.

INTRODUCTION

The brain is a major target for ethanol and alcohol consumption has long been associated with brain damage. Numerous experimental studies have shown a wide range of structural and functional alterations in neurones as well as in astrocytes. For example, altered cell membrane fluidity and structural alterations of the membrane constituents have been observed (Barret et al., 1996; Diamond and Gordon, 1997). The particular impact on the ion channels can alter neurotransmission as well as cell homeostasis. Variations in gamma-aminobutyric acid-mediated inhibition, or in the concentration of glutamate, a major excitatory neurotransmitter, can increase the sensitivity of the cell to NMDA-stimulated cell-killing processes (Ledig et al., 1982; Lafon-Casal et al., 1993).

More recently, it has been shown that ethanol genotoxicity can contribute to cell death (Blasiak et al., 2000). Various studies have shown that products of ethanol metabolism interact with DNA, leading to a possibly lethal disruption of the genomic function. At the hepatic level, for example, nuclear and mitochondrial DNA strand-break formation was demonstrated (Mansouri et al., 1999; Navasumrit et al., 2000) as well as DNA adduct formation (Brooks, 1997; Navasumrit et al., 2001). Fewer studies have been conducted in the brain, although DNA strand breaks and DNA adduct formation have also been demonstrated (Singh et al., 1995; Vaudry et al., 2002). However, in practice, it is difficult to specify the levels of alcohol consumption that are likely to lead to ethanol-induced neurone death. In particular, the influence of exposure duration and dose continue to be debated (Nordmann et al., 1992).

For this reason, the present study was conducted using primary cultures of rat neurones in order to investigate the impact of ethanol on cell viability and DNA in different conditions of exposure to better evaluate the influence of exposure duration and ethanol dose on cell death.

MATERIALS AND METHODS

Materials

Poly-L-lysine, human transferrin, insulin, putrescine, progesterone, oestriadiol, Na selenite, acetaldehyde, MTT, red neutral, DMSO, comet products, aminotriazole, methylene blue and propidium iodide were obtained from Sigma Chemical (St Louis, MO, USA). Fetal calf serum, horse serum, PBS and antibiotic-antimycotic were obtained from Gibco (Grand Island, NY, USA). Dulbecco-modified Eagle’s medium (DMEM) and Ham’s F12 medium were obtained from ICN Biomedical Research (Costa Mesa, CA, USA). Tissue culture flasks were obtained from Costar (Cambridge, MA, USA) and tissue culture dishes were obtained from Becton Dickinson (Falcon, USA).

Neurone cell cultures

The neurone cell cultures were obtained from 18-day-old rat embryos using a modification of the method previously described by Duval et al. (1988). Pregnant Sprague-Dawley female rats (Iffa Credo, L’Arbresle, France) were anaesthetized and living embryos were excised by Caesarean section under sterile conditions. Cerebral hemispheres were dissected free of meninges and gently dispersed in a mixture of DMEM and Ham’s F12 medium (50:50) supplemented with 8% heat-inactivated fetal calf serum, 2.5% heat-inactivated horse serum, penicillin, streptomycin and amphotericin B. The cell suspension was then centrifuged at 700 g for 10 min. The pellet was redispersed in the same serum-supplemented medium and filtered through a fine nylon mesh (45 μm pore size). The final concentration of the cell suspension was adjusted to 2 × 10⁶ cells/ml of medium and aliquots of 1 ml of the cell. The suspension was transferred into 35 mm diameter Petri dishes (Falcon) pre-coated with poly-L-lysine (1 mg/100 ml),
dissolved in sterile water and pre-incubated at 37°C with 1 ml serum-supplemented medium before seeding. Cells were maintained at a constant temperature of 37°C in a humidified atmosphere of 5% CO₂/95% air. The following day, the culture medium was removed and replaced with a fresh hormonally defined serum-free medium consisting of DMEM/Ham’s F12 nutrient medium supplemented with human transferrin (1 mmol/l), insulin (1 mmol/l), putrescine (0.1 mmol/l), progesterone (10 mmol/l), estradiol (1 pmol/l) and Na selenite (30 nmol/l).

The growth, density and morphology of the cells were regularly observed using phase-contrast microscopy. The purity of cultures was estimated by immunocytochemical localization of neuron-specific enolase (NSE) for neurones and by immunocytochemical localization of glial fibrillary acidic protein (GFAP) for astrocytes (Hertz et al., 1982; 1985). Immunostaining identified at least 95% neurones in the preparation conditions detailed above.

**Ethanol exposure conditions**

All the exposed and non-exposed cells came from the same original pool of embryonic cells within each of the three experimental series. Three modes of ethanol exposure were tested in order to mimic different forms of ethanol intake. This was performed after a period of 6 days in vitro and free from ethanol exposure, in order to obtain matured neurones.

**Acute exposure.** When starting the exposure, the medium was aspirated from the cell cultures and replaced by fresh culture medium containing ethanol at three concentrations: 20, 50 or 100 mmol/l, corresponding to the range of concentrations of ethanol considered to affect behaviour. The culture dishes were then capped by Parafilm® to avoid evaporation and were replaced in an incubator at 37°C for 6 h. The medium containing ethanol and the control cell medium were then removed and replaced by fresh ethanol-free medium. The dishes were then replaced in the incubator for 24, 48, 72 or 96 h.

DNA strand breaks were evaluated immediately after the 6 h exposure and 24 h later, with cells maintained in fresh culture medium that did not contain ethanol.

**Chronic exposure.** After 6 days in vitro, cells were incubated in a culture medium containing 20 mmol/l ethanol for 3, 6 or 9 days. In order to avoid alcohol vaporisation during this incubation period, we used a previously described compensating system (Eysseric et al., 1997), consisting of saturating the atmosphere over the culture dishes by placing them inside an expanded polystyrene container with an open pan containing ethanol in aqueous solution. The container was closed but not sealed and did not prevent the passage of CO₂ and air through the polystyrene when placed in the incubator. This system almost completely stabilized the ethanol concentration in the culture medium for 3 days when compared to other incubation conditions. For example, more than 80% of the ethanol was lost in a 72 h period when the culture dishes were placed directly in an incubator at 37°C, and 20% was lost when the culture dishes were wrapped in Parafilm®, but with a slight impact on the cell viability in the latter (Eysseric et al., 1997).

During this exposure period, the medium was replaced every 3 days by fresh medium containing the appropriate concentration of ethanol. Controls were grown for the same period of incubation, and in the same conditions as the exposed cells, but in a culture medium that did not contain ethanol and that was replaced every 3 days.

**Chronic exposure followed by acute exposure.** After 9 days ethanol exposure in the above-mentioned conditions, some cells were exposed to another acute exposure. The cells were placed in the incubator for 6 h in fresh culture medium containing ethanol at the three concentrations already tested: 20, 50 and 100 mmol/l.

**Ethanol level determination.** Ethanol concentration was determined with a Carlo Erba Gas Chromatography 6000 Vega series 2 using isopropanol as the internal standard. One millilitre was treated with 1 μl trichloroacetic acid 10% containing 50 mmol/l isopropanol. After centrifugation, 1 μl supernatant was injected in split mode (1/40). The injector, oven and detector temperatures were 150, 90 and 250°C, respectively. The column used was a wideboreDbwax J.W. (length: 30 m, internal diameter: 0.32 mm, film: 3 μm) (Chromoptic, Auxerre, France). A standard curve was drawn from standard ethanol samples using the ratio of ethanol to isopropanol and was used to calculate the concentration of ethanol in the samples of the medium. Concentrations were calculated at the beginning of the exposure and after each replacement of the culture medium.

**Cytotoxicity assay**

Cell viability was determined by MTT assay (MTT) and by neutral red uptake assay (NR).

**MTT assay.** We assessed cell viability using a spectrophotometric method based on the reduction in the tetrazolium salt MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) into a blue formazan product, mainly by the mitochondrial enzyme succinate-dehydrogenase (Denizot and Lang, 1986). Therefore, the amount of formazan produced is proportional to the number of living cells. Neurones were incubated for 3 h at 37°C with MTT (500 μg/ml), washed with ice-cold phosphate-buffered saline (PBS), and lysed in dimethyl sulfoxide (DMSO). Optical density was measured at 519 nm (Uvikon 860; Kontron Instruments). Data were finally expressed as a percentage of controls for each duration of incubation.

**NR uptake assay.** The neutral red uptake assay (NR) (Borenfreund and Puerner, 1985) was also used. After intoxication, culture medium was replaced by DMEM/F12 medium containing NR at a final concentration of 50 μg/ml. Culture dishes were incubated for 3 h at 37°C. The dye medium was then removed and the cells were washed with 4% formaldehyde/1% CaCl₂ mixture, to remove unincorporated dye. Removing this mixture and adding a solution containing 1% acetic acid/50% ethanol then resulted in the extraction of the NR contained in the viable cells. Absorbency was measured with a spectrophotometer (Uvikon 860; Kontron) at a 540 nm wavelength, and the results are expressed as a percentage of controls for each incubation duration.

**Trypan blue exclusion assay.** Cells were counted 72 and 96 h after the end of acute ethanol exposure (100 mmol/l). The ethanol-free medium was removed from each dish and the cells were rinsed. The cells were detached from the substrate by trypsination (0.25% trypsin/0.1% EDTA: 1 min, 37°C). The trypsin was inactivated by adding medium containing...
10% FCS. The cells were gently dispersed and 0.5% trypan blue in PBS was added to stain dead cells. The total number of unstained (viable) and stained (non-viable) cells was estimated by light microscopy using a haemocytometer. Cells in each dish were counted at least three times. In each experiment \((n = 3)\), the cells of three dishes were counted. Cell viability was expressed as a percent ratio of unstained cells versus the total number of cells.

**DNA damage**

We used the alkaline Comet assay, considered a precious marker of DNA alteration (single-strand breaks detection), when cell viability was not affected, and cellular-DNA content measurement by flow cytometry when cell viability was affected.

**DNA strand breaks evaluation by Comet assay.** The presence of DNA fragmentation was examined by single-cell gel electrophoresis (Comet assay), with a slight modification of the method previously described by Singh \textit{et al.} (1988; 1991). Modifications were imposed by the particular sensitivity of neurones in culture. To prepare slides, a very thin agarose layer (150 \(\mu\)l 1% agarose) was quickly formed by the rapid application of the liquefied agarose to the slides, followed by the addition of microscope glass cover slips (Marienfeld, Germany). The agarose was allowed to solidify for a few minutes at room temperature, and then the glass cover slips were removed. The cells were detached from the substrate by trypsination. After the culture media were removed, the dishes were rinsed with PBS and a 0.25% trypsin/0.1% EDTA solution was added for 1 min at 37°C. Neuronal medium containing 10% FCS was added to inactivate trypsin activity. After trypsinization, centrifugations, rinses and cell counting, \(2 \times 10^6\) cells were mixed with 70 \(\mu\)l of 0.8% low-melting agarose, spotted on slides, flattened with a microscope glass cover slip and allowed to solidify for a few minutes on ice. After the cover slip was removed from the cell/agarose layer, the 'minigels' were immersed for 1 h at 4°C in ice-cold lysis solution (2.5 mol/l NaCl, 100 mmol/l Na\(_2\)EDTA, 10 mmol/l Tris-base, 1% N-Lauroylsarcosine sodium salt). We added 1% Triton X-100 and 10% DMSO to this lysing solution immediately before use. The slides were then gently transferred to a horizontal gel electrophoresis tank filled with freshly prepared electrophoresis alkaline solution (1 mmol/l Na\(_2\)EDTA, NaOH 300 mmol/l). DNA was denatured for 20 min in this high-pH buffer, and electrophoresis was then carried out at room temperature by adjusting the voltage to 25 V and the current to 300 mA, for 15 min. At the end of the run, the 'minigels' were neutralized in Tris–HCl 0.4 mmol/l, pH 7.4, stained with 50 \(\mu\)l ethidium bromide (3.3 \(\mu\)g/ml). The nuclei were visualized using a Zeiss \(\times 20\) objective with epifluorescent illumination (mercury vapour UV lamp HBO 50 W: emission 260–700 nm; filter Zeiss 15: excitation 546 nm, emission >590 nm) on a Zeiss Axioskop 20 microscope. Images of nuclei in random, non-overlapping fields were captured using a Pulmix TM 765® camera (Kinetic Imaging, Liverpool, UK) and a computer. Images were then analysed with Kinetic Imaging Komet 3.0 (Kinetic).

The DNA damage was quantified by determining the percentage of DNA in the tail, which is linearly related to DNA break frequency. Fifty cells were randomly examined in quadruplicate for each condition.

**Measurement of cellular DNA content by flow cytometry.** Flow cytometry measurements were taken with an Epics Profile 2 (Coulter) apparatus equipped with an argon-ion laser tuned to 488 nm. Histograms and cytograms were transferred to an IBM-compatible computer for data analysis and figures were prepared using software from Coulter.

DNA was analysed using propidium iodide (PI). After chronic ethanol exposure (3, 6 or 9 days), media and cells were recovered and cells were removed by trypsinization (0.25% trypsin/0.1% EDTA; 1 min, 37°C). After centrifugation, the supernatant was eliminated and the pellet was resuspended and fixed in 500 \(\mu\)l CAPS/ethanol 99% (vol./vol.) (CAPS buffer: 8.53 g saccharine \(+ 1.18\) g trisodium citrate \(+ 5\) ml DMSO \(+ 95\) ml H\(_2\)O, pH 7.6). Cells were centrifuged from the fixative, resuspended in 1 ml PBS containing 50 \(\mu\)g/ml PI and 100 \(\mu\)g/ml RNase, and incubated at 37°C for 15 min. Red fluorescence \((>620\) nm) was then recorded.

**Statistical analysis**

All the values were expressed as mean ± SEM from at least three independent experiments. The ANOVA test with Fisher’s PLSD test for selected groups were used for statistical analysis.

**RESULTS**

**Effect of an acute ethanol exposure**

Cell viability. According to MTT, a 6 h period of exposure did not significantly affect cell viability whatever the dose of ethanol (Fig. 1). We observed that cell viability tended to be reduced in the exposed cells in a more time-dependent manner (two-factors ANOVA; \(P < 0.001\)) than in a dose-dependent manner (two-factors ANOVA; NS).

The measures of viability obtained with NR had a different profile (Fig. 2a). Indeed, a significant increase in cell viability was observed as a function of the ethanol dose (two-factors ANOVA; \(P < 0.0001\)) and the duration of the exposure period (two-factor ANOVA; \(P < 0.0001\)) compared to controls. For example, the viability of cells exposed to 50 mmol/l ethanol

![Fig. 1.](https://example.com/fig1.png)
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Effect of a chronic ethanol exposure

Cell viability. Because it is difficult to maintain neurones in long-term culture, the duration of the chronic exposure was limited to 9 days, following an in-vitro 6 day period free from ethanol exposure. For the same reason, the values in the exposed groups of cells were strictly compared to those of controls having the same duration of culture growth. As determined previously (Eysseric et al., 1997), the ethanol concentration in the culture medium was stable when the compensatory system was used (data not shown).

In these conditions, cell viability was significantly reduced as a function of exposure duration, as assessed by MTT (Fig. 4). The number of viable cells in the exposed groups decreased from 80.2 ± 3.5 after 3 days of exposure to 24.8 ± 3.7 after 9 days. During the same period of time, the control values were also reduced but in a smaller proportion, from 100 ± 2.3 to 58.7 ± 1.9 (P < 0.0001; Fisher’s PLSD test). In the meantime, no significant difference in viability of control cells was observed between the start of intoxication and day 3 (data not shown).

The differences observed between the two populations (exposed and non-exposed groups of cells) were highly significant, whatever the duration of the exposure (P < 0.0001, two-factor ANOVA). Moreover, the interaction between the two factors (the population and the duration of exposure) was also highly significant (P < 0.0001, two-factor ANOVA). This confirmed that the effect on the cell viability was not due to the sole influence of the duration of the cell incubation but effectively related to ethanol exposure.

After 9 days intoxication, the reduction in viability was also evidenced by NR, but to a lesser extent (mean cell reduction in viability of 48.4 ± 3.6 for NR vs 29.1 ± 7.3 for MTT; P < 0.001, Fisher’s PLSD test). This effect was again strictly related to ethanol, as no difference was observed between the viability assessed for controls by either NR or MTT (Fig. 5).

Effect of a chronic ethanol exposure on DNA cellular content

Cellular DNA content was studied by flow cytometry using propidium iodide as DNA-binding dye after 3, 6 or 9 days ethanol exposure. Cell numbers were counted on about 20 000 cells for each condition. Cell cycle histograms (analysis of the cellular fluorescence intensity on 1024 channels) were then analysed and the results were compared to controls maintained in culture for the same duration.

The main modification consisted in an increased percentage of cells in the region below G0/G1, a region also called the sub-G0/G1 peak and considered to correspond to cells in DNA damage. After 6 h exposure to ethanol, the percentage of DNA in the tail of the exposed cells was significantly increased as a function of the dose of ethanol when compared to control cells (one-factor ANOVA; P < 0.0001) (Fig. 3a). For example, the percentage of tail DNA of the cells exposed to 100 mmol/l ethanol was more than three times as high as the control values (P < 0.0001, Fisher’s PLSD test). These results suggest significant DNA damage to the neurones despite the lack of impact on cell viability observed. Examples of comet photographs are shown in Fig. 3b.

This lack of impact on cell viability can be explained by the nearly full recovery observed 24 h later. Indeed, a slight persisting increased percentage of DNA in the tail was only observed for an ethanol dose of 100 mmol/l (Fig. 3a). After 48 h, the values were then strictly within the range of the control values (data not shown).

The trypan blue exclusion assay showed no significant changes in the number of total and viable cells during the recovery period, which could account for the differences observed between MTT and NR results (Fig. 2b). Indeed, the number of viable cells in the exposed groups (97.7 ± 4.4 at 72 h and 96.5 ± 4.0 at 96 h) was in the same range as the number obtained for controls (98.8 ± 3.1 at 72 h and 97.7 ± 4.3 at 96 h). The number of viable cells at the start of the experiment (day 6) was considered 100%.

DNA content. After 6 h exposure to ethanol, the percentage of DNA in the tail of the exposed cells was significantly increased as a function of the dose of ethanol when compared to control cells (one-factor ANOVA; P < 0.0001) (Fig. 3a).
apoptosis. This was followed by a subsequent decrease in the cell cycle fraction \(G_0/G_1\) in the exposed groups compared to controls. Moreover, these modifications were accentuated with the duration of exposure (Fig. 6).

For example, the percentage of increase in the cells present in the sub-\(G_0/G_1\) peak was 65% in the exposed group compared to controls after 3 days exposure (mean, 17.1% in controls versus 28.2% in the exposed group) and 105% after 6 days (mean, 27.3% in controls against 56% in the exposed group).

It should also be noted that this cellular fraction was increased by time in the control groups, especially after 9 days. However, this fraction remained predominant in the exposed groups despite the notable influence of incubation duration (mean, 57.9% in controls versus 84% in the exposed group after 9 days). This again stresses how important it is to strictly compare experimental groups to controls with the same duration of culture.

**Effect of acute ethanol exposure after a period of chronic exposure**

This new condition was tested to evaluate the impact of an additional acute exposure to different ethanol doses on cells.
Fig. 4. Evolution of neurones viability determined by MTT assay after chronic exposure (3- 6- 9-days) at a dose of 20 mM of ethanol. Results were compared with corresponding controls i.e. neurons free of ethanol exposure and having the same duration of incubation (***P < 0.0001 Scheffe post-test). The 100 baseline was defined as corresponding to the value calculated in controls after 3 days of incubation (n = 15 in three independent experiments).

Fig. 5. Comparison between MTT assay and neutral red assay in rat neurons after chronic exposure (9 days) of 20 mM of ethanol (**P < 0.001 Scheffe post-test between MTT and NR results). The 100 baseline was defined as corresponding to the value calculated in controls after 9 days of incubation (n = 15 in three independent experiments).

Fig. 6. DNA content frequency histograms of rat neurones after chronic exposure (3- 6- 9-days) at a dose of 20 mM of ethanol analysed by flow cytometry. The cells were stained immediately following fixation with propidium iodide (PI). (A) control cells 3 days; (B) exposed cells 3 days; (C) control cells 6 days; (D) exposed cells 6 days; (E) control cells 9 days; (F) exposed cells 9 days. Apoptotic cells appear in sub-G0/G1 region defined by cursor G. Normal cells appear in zone defined by cursor H. The results corresponded to three independent experiments.
sensitized by a previous long-term exposure to low-dose ethanol.

In such conditions, no significant additional impact was observed on cell viability whatever the test used. Interestingly, however, the tendencies already noted after a single acute exposure were confirmed. Indeed, when MTT showed a slightly increased reduction in cell viability (values varying from 29.1 ± 7.3 at the end of the chronic exposure to a maximum reduction of 28.4 ± 8.5 after this additional acute exposure), NR revealed a reduction in the loss of viability (values increasing from 47.6 ± 3.6 at the end of the chronic exposure to a range of 54.8 ± 1.8 to 56.9 ± 2.8 after this additional acute exposure) (Fig. 7).

All these results considered together support the hypothesis that the duration of exposure rather than by the dose to which the cells were exposed is a major toxic factor when this dose falls within the common range of ethanol absorbed by heavy drinkers.

**DISCUSSION**

Neurones are sensitive to excessive ethanol, which leads to progressive neuropathological and functional alterations. The influence of ethanol exposure duration continues to be debated. It is also difficult to specify the level of alcohol consumption leading to alcohol-induced brain damage. It is not certain that a heavy ethanol load is more harmful to neurones in terms of cell viability and outcome than a moderate but chronic one. Moreover, considering the potential genotoxicity of ethanol that was demonstrated on different cells (Blasiak et al., 2000; Navasumrit et al., 2001) or in neurones but in different exposure conditions (Singh et al., 1995; Vaudry et al., 2002), the present study also attempted to evaluate the influence of a chronic low dose of ethanol on the DNA integrity of neurones.

After acute ethanol exposure in concentrations considered being within the range of blood alcohol levels in intoxicated humans, no significant variation was observed in cell viability when MTT was used, in agreement with other studies. On a fibroblastic cell line derived from mouse connective tissue (L929 cells), Mikami et al. (1997) also observed no significant loss of viability following a 6 h ethanol exposure (12.5–200 mmol/l). The number of cells was only decreased after a 26 h exposure at 200 mmol/l ethanol. Similarly, 24 h cell incubation in progressively concentrated ethanol comparable with the present study (12.5–100 mmol/l) induced no loss of viability of cerebellar granule. A dose-dependent decrease in viability was only observed when ethanol concentrations ranged from 200 to 800 mmol/l (Vaudry et al., 2002). Mitchell et al. (1999) have also shown a concentration-dependent loss of rat cultured hippocampal neurones, but only after a long acute ethanol exposure (16 h) at relatively high doses (90–540 mmol/l).

On the other hand, the results obtained with NR showed a significant time-dependent increase in cell viability for the highest doses of ethanol compared to controls. This difference was not related to an increased number of cells, according to the results of the trypan blue exclusion assay. An explanation could be the inability of NR to distinguish between intact and damaged cells, but MTT also presents the same limitation (Yamashoji et al., 1992). It is then tempting to consider that these differences could reveal differences in the ethanol toxicity mechanism. Indeed, when the NR assay is mainly sensitive to any predominant membranous impact, MTT is sensitive to mitochondrial integrity. The capability of ethanol to alter the fluidity of the membrane, and in particular its phospholipid composition, is well known (Rottenberg et al., 1981; Boerescu et al., 1988). However, membrane damage is likely to be reversible and to occur in cells that are still alive; loss of respiration is not. Consequently, it is not surprising that NR gives a higher percentage of viable cells. In our view, more specific studies are thus necessary to precisely understand the underlying process explaining the observed differences between MTT and NR results.

The results observed after a chronic exposure to a low dose of ethanol maintained at a constant level by an appropriate system (Eysseric et al., 1997) were different. In these conditions, a significant loss of neurone viability was observed after periods of exposure lasting for 3, 6 or 9 days. The impact on viability depended on the duration of exposure, with a maximum reduction in cell viability for the longest period of exposure. Moreover, the changes in cell viability as a function of time were significantly different between the control and the exposed groups. The observed effect on cell viability in the exposed groups was thus independent of any effect related to the sole influence of the duration of the culture period.

Neurones therefore seem particularly vulnerable to chronic exposure, even with a low dose of ethanol. To evaluate whether this caused increased neurone vulnerability, 6 h of acute exposure was added to neurones already chronically exposed. In these conditions, the viability at the end of the chronic exposure was only slightly diminished by this additional exposure.

This combination of results strongly suggests that the main toxic factor of ethanol exposure is more the duration of exposure than the dose of ethanol, when this dose is in the range of the usual ethanol intake of chronic alcohol drinkers.
Different studies have also demonstrated that products of ethanol metabolism interact directly at the DNA level, leading to a disruption of the genomic function, potentially lethal. Thus, researchers have demonstrated DNA strand-break formation (Singh et al., 1995; Navasumrit et al., 2000); DNA adduct formation (Brooks, 1997; Navasumrit et al., 2001); and changes in the expression of transcription factors, immediate-early genes (IEG) promoting apoptosis, and mRNA (Moore et al., 1999; Cheema et al., 2000; Depaz et al., 2000).

The present experiment confirmed the genotoxicity of ethanol in the brain. DNA alteration assessed by Comet assay depended on the value of the acute ethanol load with the most altered parameters (percentage of the DNA in the tail) associated with the highest doses of ethanol. Normally, different cells possess elaborate DNA repair mechanisms to counteract DNA damage such as direct repair, base excision repair or nucleotide excision repair, which maintain the integrity and stability of the genome. When acute intoxication was short and moderate, the increase in DNA lesions did not exceed the capacities of the cellular repair systems, and the consequences for the cell remained minimal. In 1995, Singh et al. showed in-vivo DNA single-strand breaks in brain cells, 4 h after ethanol administration. This phenomenon was also shown to be reversible, as a few hours later, DNA strand-breaks levels were in the same range as control-strand breaks levels, as we have observed in different experimental conditions.

This genotoxic impact was also confirmed by DNA cellular content measurement using flow cytometry after chronic exposure, which resulted in an excessive action on the part of these DNA repair capacities, leading to cell death. In these conditions, DNA damage was too serious to be measured and quantified by the Comet assay.

Indeed, after a chronic exposure (3, 6 or 9 days) to a low dose of ethanol (20 mmol/l), the percentage of cells in the sub-G₀/G₁ fraction, corresponding to cells in apoptosis, was significantly increased as the duration of exposure increased. This percentage was increased from 28.2% after 3 days to 84% after 9 days. At the same time, the proportion of cells with a ‘normal’ DNA content was dramatically reduced.

The formation of a distinct and quantifiable cell cycle region below G₀/G₁ has been previously shown to represent cells undergoing apoptosis-associated DNA degradation (Telford et al., 1992), a phenomenon already described by other authors in different conditions of ethanol exposure (Cheema et al., 2000). However, a careful analysis of the profiles of the sub-region G₀/G₁ in the exposed groups suggests that besides apoptotic cells, other materials such as nuclear fragments, cell debris or damaged cells with different DNA content should have been taken into account, as suggested by other authors (Sun et al., 1992; Ormerod et al., 1993; Darzykiewicz et al., 1997). However, our in-vitro model contained a measured percentage of 95% neurones (i.e. post-mitotic cells) depending on culture conditions and media composition unsuitable for glial cells proliferation. The presence of damaged cells with different DNA content into the sub-region G₀/G₁ can thus be excluded. In our view, the difficulty of interpreting the sub-region G₀/G₁ profile stems from measurements that may be too distant in time and thus span different stages of the cellular impact of ethanol.

Numerous studies concerning ethanol effects on DNA and cell death also referred to an apoptotic process. For example, Vaudry et al. (2002) showed, following in vitro ethanol exposure, an increase in DNA laddering and caspase 3 activity associated with a decrease in mitochondrial activity. It is well known that these three observations are characteristic of an apoptotic mechanism. In an another recent in vitro study, Moulder et al. (2002) found that chronic ethanol treatment (6 days, ≤100 mmol/l) induced death of hippocampal neurones in a concentration-dependent manner. Moreover, the profile of dying neurones was characterized by condensed, fragmented nuclei, often associated with apoptotic cell death. On a model of explant cultures of developing cerebral cortex and of cerebellar granule progenitors (CGP), ethanol intoxication also led to a dose-dependent increase in apoptosis with no variation in the necrotic index (Cheema et al., 2000; Li et al., 2001). In 2000, Ikonomidou et al. observed a dose-dependent increase in the number of apoptotic neurones of ethanol-treated pups. According to these authors and in agreement with our findings, the apoptotic response induced by ethanol was not predicted by the dose but rather by the way the dose was delivered. The most toxic impact was linked to the amount of time during which the blood level of ethanol was over the toxic threshold values (above 45 mmol/l) (Ikonomidou et al., 2000).

In conclusion, the neurones were more sensitive to a low chronic ethanol exposure than to a heavy acute exposure, as both a significant reduction in cell viability and DNA alterations were observed in this condition. However, DNA repair capacities seemed to preserve neuronal integrity when genomic damage is transitory and relatively low. On the other hand, a permanent load of ethanol producing continuous cellular and nuclear damage led to a neuronal death process compatible with apoptosis and responsible for loss of viability.

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