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Acetaldehyde accumulation in rat mammary tissue after an acute treatment with alcohol

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ABSTRACT: Previous studies reported the presence in rat mammary tissue of a cytosolic xanthine oxidoreductase pathway for the metabolism of alcohol to acetaldehyde and hydroxyl radicals and to the microsomal biotransformation of ethanol to acetaldehyde. It was also reported that after chronic ethanol drinking stressful oxidative conditions can be observed. The present work reports that even after single doses of ethanol, given at three different levels (6.3 g kg^{-1}; 3.8 g kg^{-1} or 0.6 g kg^{-1} p.o.), acetaldehyde accumulates for prolonged periods of time in the mammary tissue to reach concentrations higher than in blood (e.g. 5.1 ± 1.2 nmol g^{-1} versus 0.2 ± 0.1 nmol ml^{-1}, for 6.3 g kg^{-1} dose, 6 h after intoxication). The presence in rat mammary tissue of low activities of additional enzymes able to generate acetaldehyde was established (alcohol dehydrogenase: 0.97 ± 0.84 mU mg^{-1} protein; CYP2E1: 1.30 ± 0.12 × 10^{-2} pmol 4-nitrocatechol min^{-1} mg^{-1} protein) and a low activity of aldehyde dehydrogenase was observed in the cytosolic, mitochondrial and microsomal fractions (0.02 ± 0.04; 0.35 ± 0.09 and 0.72 ± 0.19 mU mg^{-1} protein, respectively). After a single high dose of ethanol, an increased susceptibility to oxidative stress was observed, as evidenced by changes in the shape of *t*-butylhydroperoxide induced emission of chemiluminescence in mammary tissue (6.3 g kg^{-1} dose; at 3 and 6 h). In summary, the results show that even after single doses of ethanol, acetaldehyde, either formed *in situ* or arriving via blood, tends to accumulate in mammary tissue and that this condition might decrease cell defenses against injury.

KEY WORDS: alcohol; breast; acetaldehyde; ethanol; mammary tissue

Introduction

It is at present well established that alcohol consumption is associated with a modest but significant increased risk of breast cancer (Stewart and Kleihues, 2003; Collaborative Group in Hormonal Factors in Breast Cancer, 2002; Singletary and Gapstur, 2001; Dumitrescu and Shields, 2005).

The mechanisms mediating this association, however, still remain unknown and speculative. Several studies support the hypothesis that ethanol (EtOH) use may increase breast cancer risk at least in part, through an effect in estrogen levels in women (reviewed by Ginsburg, 1999). However, most workers in the field consider that hormone-mediated mitogenic effects of EtOH on mammary epithelial cells play a promotional role in breast carcinogenesis, essentially by stimulating mitotic division of already initiated cells (Przlipiak *et al.*, 1996; Singletary *et al.*, 2001; Izevbigie *et al.*, 2002; Izevbigie, 2005; Etique *et al.*, 2004; Coutelle *et al.*, 2004; Dumitrescu and Shields, 2005). The nature of the mutational event responsible for the initiation step of the process is still less clear. Further, other factors considered to play a promotional role in the case of ethanol-induced cancer in target organs other than breast, e.g. oxidative stress in the case of liver (Garro and Lieber, 1990; Pöschl *et al.*, 2004) could also be involved in the case of mammary tissue. In effect, repetitive administration for 28 days also evidenced the ability of ethanol to promote oxidative stress in mammary tissue (Castro *et al.*, 2006).

Recent studies (Coutelle *et al.*, 2004; Freudenheim *et al.*, 1999; Zheng *et al.*, 2003) suggest that acetaldehyde (AC) produced elsewhere (e.g. in liver) and arriving at mammary tissue via blood or produced by metabolic transformation *in situ* (Triano *et al.*, 2003: Castro *et al.*, 2001, 2003, 2006) could be a key putative agent of the ethanol promoted breast cancer.

In the present study it is reported that even after a single dose of EtOH, acetaldehyde accumulates for prolonged periods of time in mammary tissue and that susceptibility to oxidative stress might be increased. The presence of some CYP2E1-mediated metabolic capacity and the low alcohol dehydrogenase and aldehyde dehydrogenase activities are also reported.

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Material and Methods

Chemicals

Ethanol (EtOH; analytical grade) and methanol (HPLC-grade) were from Sintorgan (Argentina). $NADP^+$, NAD^+, *p*-nitrophenol, 4-nitrocatechol, D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, deferoxamine mesylate and *t*-butylhydroperoxide were from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of the best quality available.

Animals and Treatments

Random-bred Sprague-Dawley female rats (280–300 g) were used in the experiments. The rats were postlactation young mothers (2 weeks after weaning of their pups). The starting breeding colony was from Charles River (Massachusetts, USA). The procedures used for breeding, housing and handling animals were those established by the Food, Drug and Medical Technology National Administration (ANMAT; Buenos Aires, Argentina). Food was withdrawn 12–14 h before ethanol was given, but the animals had free access to water.

Ethanol Single Dose Administration

Ethanol was administered p.o. in water (5 ml 200 g^{-1} b.w.) at three different doses: 6.3 g kg^{-1}; 3.8 g kg^{-1} or 0.6 g kg^{-1}. In the control groups, the calories provided by the alcohol were replaced by sucrose. After each time, the animals were killed for sample processing.

Isolation of Mammary Tissue Cytosolic, Microsomal and Mitochondrial Fractions

Animals were killed by decapitation and the mammary tissue was rapidly excised and processed. Cytosolic and microsomal fractions were obtained by cellular fractionation procedures via ultracentrifugation at 4 °C of the 9000 *g* supernatant of tissue homogenates as previously described (Castro *et al.*, 1989). Mitochondrial fractions employed to determine aldehyde dehydrogenase (AldDh) activity were prepared according to Koivula and Koivusalo (1975).

Determination of Acetaldehyde Levels in Rat Mammary Tissue After Single Dose Ethanol Administration

The animals were killed by decapitation and liver and mammary tissue were rapidly taken and frozen. Blood samples were obtained by bleeding and the plasma separated by centrifugation. In the case of liver and mammary tissues, frozen samples (1 g) were rapidly chipped and placed in aluminum-sealed-neoprene-septum stoppered glass vials. For blood samples, 1 ml plasma was used. After adding 1 ml of saturated $ZnSO_4$ solution, 1 ml saturated NaCl solution and 1 ml phosphate buffer, samples were kept at 37 °C for 30 min with shaking and then an aliquot (100 µl) of the headspace was analysed by GC-FID. Chromatographic conditions were: column, Supel-Q™ PLOT, 30 m × 0.53 mm i.d. (Supelco, CA); temperature 100 °C isothermal, injection port temperature: 150 °C, FID: 200 °C (Castro *et al.*, 2006).

Determination of *p*-Nitrophenol Hydroxylase Activity in Microsomes from Rat Mammary Tissue

p-Nitrophenol hydroxylase activity was determined essentially as described by Mishin *et al.* (1996) with minor modifications. Liver microsomes (~0.75 mg protein ml^{-1}) or mammary tissue microsomes (~1.0 mg protein ml^{-1}) were incubated for 30 min and 1 h, respectively, at 37 °C in 50 mM phosphate buffer, pH 7.4, containing 100 µM *p*-nitrophenol and NADPH generating system (26 mM $NADP^+$, 66 mM D-glucose-6-phosphate, 66 mM $MgCl_2$, 40 U ml^{-1} glucose-6-phosphate dehydrogenase), final volume 0.5 ml. Blanks were also run by omitting the NADPH generating system from the incubation mixture. The reaction was terminated by the addition of 100 µl trichloroacetic acid (TCA) and centrifuged for 10 min at 10 000 *g*. The supernatant was used for the HPLC determination. Analysis of the metabolite, 4-nitrocatechol, was performed by HPLC (Hewlett Packard 1090) on a reversed phase C18 column (HP ODS Hypersil 5 µm, 200 × 2.1 mm). An ESA Coulochem II electrochemical detector was used, equipped with a 5011A analytical cell, set at 700 mV and ultraviolet absorbance was also monitored at 345 nm. Elution was isocratic with a mobile phase consisting of 25% acetonitrile in 0.1% TCA. Results were the means of three separate determinations from three different livers and for mammary tissue from pools of ten organs each.

Determination of Alcohol Dehydrogenase and Aldehyde Dehydrogenase Activities in Rat Mammary and Liver Tissue

Alcohol dehydrogenase (ADh) was measured in the cytosolic fraction of mammary tissue by the detection of the NADH formed, at 340 nm. Under an excess of alcohol, the rate of NADH formation is proportional to enzyme concentration (Racker, 1950). Aldehyde dehydrogenase (AldDh) activity in mammary tissue was measured by the method described by Koivula and Koivusalo (1975) with

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minor modifications. Cytosolic, microsomal or mitochondrial fractions were resuspended in pyrophosphate buffer pH 8, 1.67 mM pyrazol was added and the mixture (3 ml) was incubated at 37 °C for 30 min. Immediately after adding 6 mM propanal and 0.67 mM NAD^+, absorbance at 340 nm was measured at 15 s intervals in quartz cuvettes thermostatized at 37 °C. Values obtained for mammary tissue were compared with those in the liver of the same animals (five animals per group).

Determination of *t*-Butylhydroperoxide-induced Chemiluminescence in Rat Mammary Tissue Homogenates

Chemiluminescence was measured in a Wallac-Rack Beta 1214 liquid scintillation counter at room temperature in an out of coincidence mode (Boveris *et al.*, 1983). Mammary tissue from rats receiving the alcohol was homogenized (7 mg protein ml^{-1}) in 0.25 M sucrose, 50 µM deferoxamine in TKM buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2.5 mM KCl), pH 7.5. The 600 *g* supernatant was kept at 37 °C for 10 min in a Dubnoff-shaker. Chemiluminescence measurement was started by the addition of 3 mM *t*-butylhydroperoxide. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (six animals each).

Protein Concentration Determination

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 mean values was assessed by unpaired *t*-test (Student's *t*-test) (Gad, 2001). Calculations were performed using GraphPad Software. Differences were considered significant when $P < 0.05$.

Results

Acetaldehyde Levels in Rat Mammary Tissue after Single Dose Ethanol Administration

The results obtained show that after a single dose of alcohol, acetaldehyde accumulated in mammary tissue to reach concentrations higher than in blood. Acetaldehyde concentrations in mammary tissue remained significantly higher than in plasma for several hours following EtOH administration (Fig. 1). This effect was observed for the three doses tested. In control animals acetaldehyde levels were very low, at any time, for the three doses of sucrose.

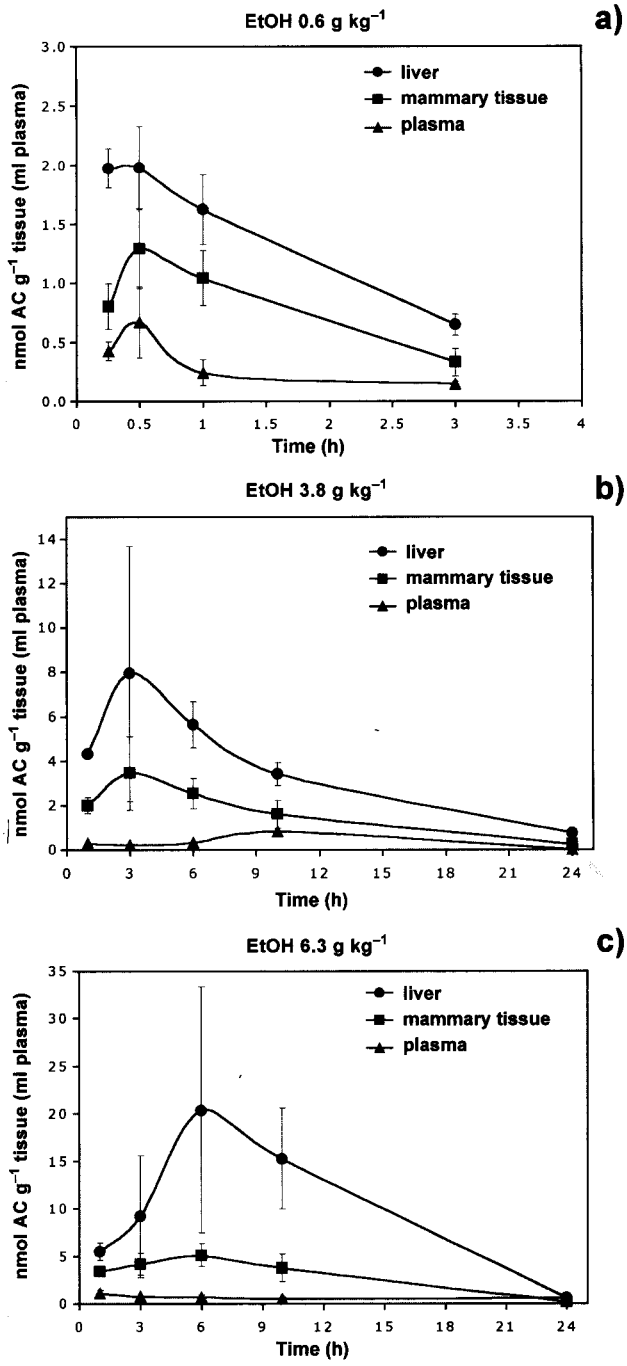

Figure 1. Acetaldehyde levels in mammary tissue, liver and plasma, after a single dose of alcohol. (a) low (0.6 g kg^{-1}); (b) medium (3.8 g kg^{-1}); and (c) high (6.3 g kg^{-1}). Animals (six per group) received alcohol p.o. as a solution in water (5 ml 200 g^{-1} b.w.). For the lower dose, acetaldehyde levels from 6 to 24 h were negligible (not displayed in graphic). In control groups, alcohol was isocalorically replaced with sucrose. Acetaldehyde levels in control groups were (expressed as a range): (a) Low dose: liver, 0.5–0.6 nmol g^{-1}; mammary tissue, 0.2–0.3 nmol g^{-1}; plasma, 0.1–0.2 nmol ml^{-1}. (b) Medium dose: liver, 0.3–0.7 nmol g^{-1}; mammary tissue, no detected; plasma, no detected. (c) High dose: liver, 0.1–0.8 nmol g^{-1}; mammary tissue, 0.1–0.3 nmol g^{-1}; plasma, 0–0.4 nmol ml^{-1}

Table 1. Alcohol dehydrogenase and aldehyde dehydrogenase activities in rat mammary and liver tissue

Organ	ADh activity (mU mg^{-1} protein[a])	AldDh activity (mU mg^{-1} protein[b])		
		Microsomes	Cytosol	Mitochondria
Mammary tissue	0.97 ± 0.84	0.72 ± 0.19	0.02 ± 0.04	0.35 ± 0.09
Liver	16.48 ± 2.96	7.52 ± 0.77	0.24 ± 0.15	4.92 ± 0.08

[a] The ADh activity was determined in the rat mammary tissue cytosolic fraction by measuring NADH formation at 340 nm. In the presence of an excess of ethanol the NAD reduction is proportional to the enzyme concentration. Each value is the mean from three separate samples. One enzymatic unit is defined by the change of 0.001 optical density per minute under these experimental conditions (Racker, 1950).

[b] The AldDh activity was determined in the rat mammary tissue cytosolic, microsomal and mitochondrial fraction by measuring NADH formation at 340 nm. Each value is the mean from three separate samples. One enzymatic unit is defined by the enzyme concentration necessary to catalyse the formation of 1 μmol NADH per minute under these experimental conditions.

Concentrations never exceeded 0.3 nmol g^{-1} in mammary tissue, 0.8 nmol g^{-1} in liver or 0.4 nmol ml^{-1} in plasma.

p-Nitrophenol Hydroxylase Activity in Liver and Mammary Tissue Microsomes

Mammary tissue microsomal fractions showed a *p*-nitrophenol hydroxylase activity of approximately nine times lower than that measured in the liver. Formation of 4-nitrocatechol in mammary tissue microsomes was $1.30 \pm 0.12 \times 10^{-2}$ pmol min^{-1} mg^{-1} protein versus $9.51 \pm 1.20 \times 10^{-2}$ pmol min^{-1} mg^{-1} protein in liver microsomes ($P < 0.05$).

Alcohol Dehydrogenase and Aldehyde Dehydrogenase Activities in Rat Liver and Mammary Tissue

Alcohol dehydrogenase and aldehyde dehydrogenase activities in rat mammary tissue were significantly lower than those found in the liver of the same animals (see Table 1).

t-Butylhydroperoxide induced Chemiluminescence in Mammary Tissue after Single Dose Ethanol Administration

No difference between the total hydroperoxide-induced chemiluminescence emitted by mammary tissue homogenates from EtOH-treated and control rats were observed at any one of the EtOH doses tested. For the higher dose, however, a significant difference in shape was observed in the graphics corresponding to 3 and 6 h after intoxication (Fig. 2).

Discussion

The obtained results evidence that acetaldehyde tends to accumulate in mammary tissue during long periods of time after single doses of orally given ethanol (Fig. 1). The intensity of this accumulative process and the period of time during which it can be observed depend on the dose of ethanol administered.

The levels of acetaldehyde in mammary tissue were higher than in plasma and lower than in liver for at least 15 h for the higher dose tested or 6 h for the medium dose or 2 h for the case of the lower one. The shape of the curve concentration of acetaldehyde versus time after p.o. ethanol administration in mammary tissue always mimicked that observed in liver, while levels of acetaldehyde in plasma were similar for the three ethanol doses given.

These results suggest that acetaldehyde present in the liver or mammary tissue reflect the balance between the ability of each of these tissues to generate acetaldehyde and the one to metabolize and excrete it. The liver is well known for its high capacity for processing ethanol to acetaldehyde via different metabolic pathways including ADh and the CYP2E1-dependent MEOS system (Lieber, 2004).

The liver is also able to get rid of the acetaldehyde formed with the participation of AldDh and GST (Lieber, 2004). In the case of mammary tissue the situation appears to be different. On one hand, ADh activity is about 16 times smaller than in the liver but perhaps more important, AldDh activity in the three subcellular fractions tested was in all of them at least ten times smaller than in the liver.

Interestingly, in previous studies by Guerri and Sanchis (1986) the authors did not detect any ADh activity in whole homogenates of rat mammary tissue. Further, Triano *et al.* (2003) reported that human mammary tissue contains a class I ADh having a limited potential to metabolize ethanol to acetaldehyde.

Previous studies from other laboratories reported the presence in human mammary tissue of CYP2E1 (Iscan *et al.*, 2001; Kapucuoglu *et al.*, 2003). CYP2E1 presence might also contribute to acetaldehyde formation in mammary tissue. CYP2E1 is also known to be induced by ethanol drinking and to be important in the production of 1HEt and reactive oxygen species (Lieber, 2004). In previous studies from our laboratory (Castro *et al.*, 2003),

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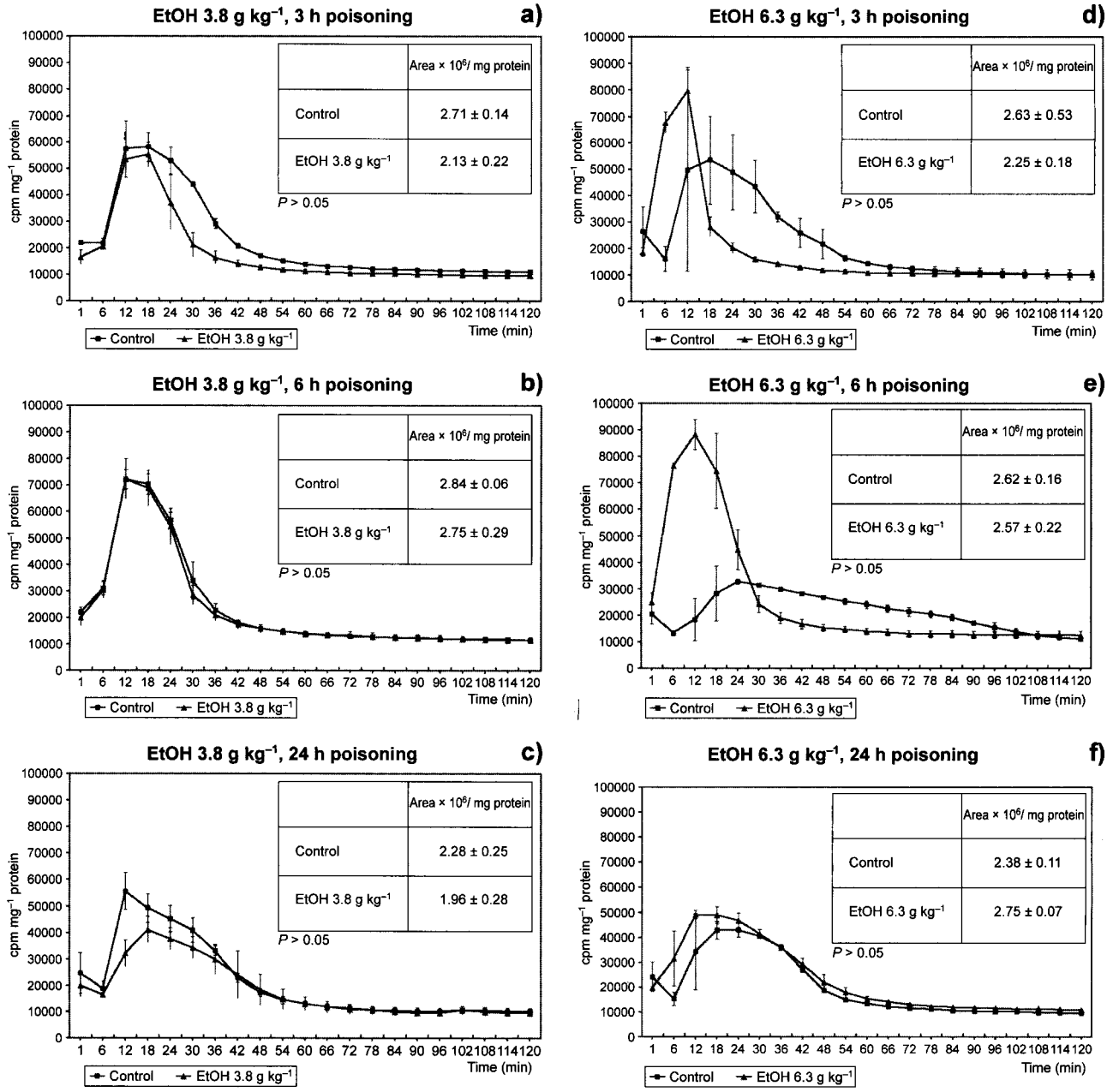

Figure 2. *t*-Butylhydroperoxide-induced chemiluminescence in rat mammary tissue homogenates from rats receiving a single alcohol dose. Rat mammary tissue homogenized in 0.25 M sucrose, 50 μM deferoxamine in TKM buffer, pH 7.5 (7 mg protein ml^{-1}) were kept at 37 °C for 10 min in a Dubnoff shaker. Chemiluminescence measurement was started by addition of 3 mM *t*-butylhydroperoxide. Values are the mean ± SD. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled mammary tissue (five animals each). Graphics (a) to (c) correspond to the 3.8 g kg^{-1} ethanol dose; 3, 6 and 24 h of poisoning respectively. Graphics (d) to (f) corresponds to the 6.3 g kg^{-1} ethanol dose; 3, 6 and 24 h of poisoning, respectively

however, the participation of P450 mediated pathways of metabolic transformation of ethanol to acetaldehyde in the microsomal fraction of rat mammary tissue was found to be not quantitatively significant when compared with that of the lipoxygenase pathway evidenced to be present in that cellular fraction (Castro *et al.*, 2003) and inducible by repetitive alcohol drinking (Castro *et al.*, 2006). However, its contribution should be considered since a non negligible CYP2E1-mediated activity was also detectable when it was determined by the very sensitive HPLC-coulometric detector method and using *p*-nitrophenol as substrate (Mishin *et al.*, 1996).

Its contribution must also be considered in the light of the well known ability of the CYP2E1-mediated pathway

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of EtOH bioactivation present in liver microsomes to promote a lipid peroxidation process (Lieber, 2004).

Another significant pathway contributing to acetaldehyde production from ethanol present in mammary tissue is that mediated by xanthine oxidoreductase (XOR) (Castro *et al.*, 2001; 2003). This metabolic pathway in the presence of adequate cosubstrates or cofactors is able not only to metabolize EtOH to acetaldehyde but also to generate hydroxyl radicals. In addition, it is also inducible in mammary tissue by repetitive alcohol drinking (Castro *et al.*, 2006). XOR is present in high amounts in control mammary tissue and is located in the epithelial cells as shown by previous histochemical studies (Castro *et al.*, 2006).

The accumulation of acetaldehyde in mammary tissue and the ability of two enzymatic systems, the XOR and the CYP2E1, present in it, to generate free radicals suggest the possibility that promotion of an oxidative stress condition were observable even after a single dose of EtOH. That possibility arose as feasible after our experiments on *t*-butylhydroperoxide-induced chemiluminescence emission by mammary tissue from EtOH treated animals when compared with that of control animals. In effect, while the obtained results showed that the total chemiluminescence emitted was not different in both groups for the three doses tested, there was a significant change in the shape of the chemiluminiscence emitted for the case of the highest dose tested, at 3 and 6 h after intoxication. Those findings might indicate that while the total content of the target molecule challenged by *t*-butylhydroperoxide producing the emission remained relatively constant, other components in the mammary tissue regularly delaying the response to the oxidative challenge of *t*-butylhydroperoxide, changed negatively.

In conclusion, the present studies would suggest that even a single but sufficiently high ingestion of ethanol may lead to a long lasting accumulation of acetaldehyde and to an oxidative stressful prone condition in mammary tissue.

If consideration is made to the fact that acetaldehyde proved to be a carcinogen and a highly mutagenic chemical, and able to interact with many cellular constituents including DNA, proteins, lipids, glutathione and others (Dellarco, 1988; Lieber, 2004; Pöschl *et al.*, 2004; Woutersen *et al.*, 1984, 1986), that acetaldehyde accumulation merits concern.

It remains as an interesting question to consider the possibility that repetitive high doses of ethanol, e.g. just in weekends such as those characteristic of the so called 'binge drinking' might lead, via cumulative alterations, to harmful effects on mammary tissue if the week days in between do not allow repair of any deleterious occurring actions.

Long term daily administration of ethanol to rats during 28 days produced in our hands similar deleterious effects in mammary tissue (Castro *et al.*, 2006).

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