TIH

Toxicology and Industrial Health
25(8) 525–538
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DOI: 10.1177/0748233709345937
tih.sagepub.com
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Metabolism of ethanol to acetaldehyde and increased susceptibility to oxidative stress could play a role in the ovarian tissue cell injury promoted by alcohol drinking

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Abstract

It is known that drinking alcohol can lead to reproductive problems in women. In this study, we analyzed the possibility that part of those effects were mediated through alterations of ovarian function related to ethanol oxidation to acetaldehyde occurring in situ. Biotransformation in the rat ovary cytosolic fraction was partially inhibited by allopurinol, suggesting the participation of xanthine oxidoreductase in the process. Microsomal pathway was of enzymatic nature, requiring nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), sensitive to oxygen and significantly inhibited by sodium diethyldithiocarbamate, 4-methylpyrazole and diphenyleneiodonium. Aldehyde dehydrogenase activity was detected by histochemistry in the ovarian tissue, in the strome surrounding the follicle while no alcohol dehydrogenase was detected. However, biochemical determination of alcohol dehydrogenase and aldehyde dehydrogenase activities in rat ovarian tissue revealed the presence of some activity of both enzymes but significantly lower than those found in the liver. By repetitive exposure of animals to ethanol, the microsomal metabolism to acetaldehyde was increased but not in the case of the cytosolic fraction. In these animals, *t*-butylhydroperoxyde-promoted chemiluminiscence was increased in comparison to control, revealing an increased susceptibility to oxidative stress due to alcohol drinking. Ultrastructure of ovarian tissue from rats exposed chronically to alcohol revealed alterations at the level of the granulosa; theca interna and pellucida zones. In the secondary follicle, alterations consisted of marked condensation of chromatin attached to the nuclear inner membrane. Intense dilatation of the outer perinuclear space could be observed. There was a marked dilatation of the rough endoplasmic reticulum accompanied of significant detachment of ribosomes from their membranes. Mitochondria appeared swollen. In the zona pellucida, most of the cell processes from oocyte and corona radiata cells were absent or broken totally or in part. Results suggest that in the rat ovary, metabolism of ethanol to acetaldehyde may play a role in alcohol effects on female reproductive function.

Keywords

Ovary, ethanol, alcohol, acetaldehyde, reproductive toxicity

Introduction

In primates, the ovary is responsible for the control of reproduction through its principal products, oocytes and steroid and protein hormones. These two processes are interdependent since follicular maturation and ovulation cannot occur without steroid hormone secretion. Unlike the male gonad, the female gonads

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have a finite number of germ cells at birth and are therefore uniquely sensitive to reproductive toxicants. Such exposure of the female can lead to decreased fecundity, increased pregnancy wastage, early menopause, infertility, depending of the component affected, the magnitude of the damage and the timing of the exposure. That is, ovarian functions are potentially susceptible to interruption by xenobiotic compounds. Several examples illustrating that possibility are available in literature (de Castro et al., 1989; Mattison, 1985; Mattison et al., 1990; Davis and Heindel, 1998; Keating et al., 2008; Smith et al., 1990).

It has been established that even moderate drinking in healthy women can lead to significant reproductive problems. Moreover, 60% of the heavy drinkers and 50% of moderate drinkers who consumed more than three drinks per day had significant problems, including delayed ovulation and failure to ovulate (i.e. anovulation). Shortening of the luteal phase was also observed. Menstrual problems did not appear to occur in the women who were occasional drinkers or who were moderate drinkers consuming fewer that two drinks per day. A close response relationship appears to exist between alcohol consumption and the frequency of menstrual problems. This notion is also supported by epidemiological surveys showing that the prevalence of menstrual disturbances grows with increasing alcohol consumption. Even moderate amounts of alcohol may cause infertility through suppressing ovulation and an increased risk for spontaneous abortion through interfering with the pregnancy-maintaining function of the corpus luteum. Furthermore, it has been observed that ethanol significantly decreases sexual responsiveness in women as well as in men. (Emanuele and Emanuele, 1997; Galvaõ-Teles et al., 1986; Garro et al., 1992; Gavaler, 1987; Mello et al., 1993; Mendelson and Mello, 1988;). Even more seriously, among alcoholic women, a teratogenic effect has been described, which has been named fetal alcohol syndrome (Garro et al., 1992).

The mechanisms of all these effects of alcohol drinking in women remains to be established. Some workers postulated that alcohol might lead to increased estrogen, inhibiting FSH and disrupting folliculogenesis and subsequently, the corpus luteum function. In addition, alcohol has been shown to suppress progesterone, the main secretion product of the corpus luteum (Garro et al., 1992).

In this study, we analyze the potential effects of alcohol in the ovaries related to ethanol bioactivation in situ to deleterious metabolites such as acetaldehyde.

Materials and methods

Chemicals

Ethanol (analytical grade) was from Sintorgan (Villa Martelli, Argentina). Acetaldehyde was from Fluka. Hypoxanthine, allopurinol, NAD^+, $NADP^+$ were from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were of the best quality available.

Animals and treatments

Non-inbred female Sprague–Dawley rats were used. 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). The starting breeding colony was from Charles River (Wilmington, MA, USA). For the studies on the metabolism in microsomal and cytosolic fractions, female Sprague–Dawley rats (250-280 g body weight) were used. Food was withdrawn 12-14 hours before sacrifice, but the animals had free access to water.

In the treatment with ethanol liquid diet, female Sprague–Dawley rats (125-150 g body weight, 5-6 weeks age) were fed with a nutritionally adequate liquid diet (Lieber & De Carli standard rat diet, purchased from Dyets Inc., Bethlehem, PA, USA). The rats were housed in individual cages and separated into two dietary groups: ethanol group (EtOH) and Control group (Control). Both groups were pair fed with the same diet except that in Control, ethanol was isocalorically replaced with carbohydrate (dextrin–maltose). The liquid diet used provided 1 kcal/mL where 35% of the total calories derived from fat, 47% from carbohydrate and 18% from protein. In the ethanol-treated animals, ethanol provided 36% of the calories replacing isocalorically carbohydrate. Feeding with the Control and EtOH diets 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 (Castro et al., 2006; Lieber and De Carli, 1982, 1989).

Isolation of ovarian tissue cytosolic, microsomal and mitochondrial fractions

Animals were killed by decapitation and their ovarian tissue was rapidly excised, separated from oviduct and processed to obtain cytosolic and microsomal fractions. Cytosolic and microsomal fractions were

obtained from whole ovarian tissue homogenates by cellular fractionation procedures via ultracentrifugation at 4°C (Castro et al., 2006). Mitochondrial fractions employed to determine aldehyde dehydrogenase (AldDh) activity were prepared according to Koivula and Koivusalo (1975).

Determination of alcohol dehydrogenase and aldehyde dehydrogenase activities in rat ovarian and liver tissue

Alcohol dehydrogenase (ADh) was measured in the cytosolic fraction of ovarian 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 ovarian tissue was measured by the method described by Koivula and Koivusalo (1975), with minor modifications. Cytosolic, microsomal or mitochondrial fractions were resuspended in pyrophosphate buffer pH 8, 1.67 mM pyrazole 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-sec intervals in quartz cuvettes termostatized at 37°C. Values obtained for ovarian tissue were compared to those in the liver of the same animals (five animals per group).

Histochemical procedure for alcohol dehydrogenase and aldehyde dehydrogenase activities detection in ovarian tissue

Animals were killed by decapitation or by cervical dislocation and 5 mm cubes of tissues were taken and immediately frozen on metal chucks maintained at –70°C in freezing mixture of solid carbon dioxide and hexane. Cryostat sections, 16-µm thick, were cut at –20°C and mounted on clean glass microscope slides. The sections were allowed to equilibrate to room temperature for 30 min and then placed in the incubation medium. Prior to staining, sections were dried at room temperature for 30 min, then incubated for 1 hour at 42°C in the dark in a staining solution containing 50 mM sodium phosphate, pH 7.6; 5 mM NAD^+, 11 mM pyruvic acid; 3.4 mM nitroblue tetrazolium and 500 mM ethanol as a substrate. After staining, the slides were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde neutral solutions for 10 min, dehydrated in ethanol/water, cleared in xylene and moistened in permount. In the case of the histochemistry for acetaldehyde dehydrogenase, same steps were performed, except that ethanol was replaced by 500 mM acetaldehyde. In both cases, liver tissue was processed simultaneously as a control (Allali-Hassani et al., 1997).

Histochemical procedure for xanthine oxidase activity detection in ovarian tissue

Portions of ovarian tissue from Control rats (five animals) were frozen at –70°C in hexane in a mixture of solid carbon dioxide and absolute alcohol. Tissue blocks were stored at –80°C until further use. Sections, 8-µm thick, were cut on a cryostat at –24°C. The sections were picked up onto clean glass slides and incubated immediately for xanthine oxidase activity using the cerium capture method in the presence of polyvinyl alcohol described by Frederiks et al. (1994). Briefly, the incubation mixture contained 100 mM Tris-maleate buffer, pH 8; 10 mM cerium chloride; 100 mM sodium azide; 0.5 mM hypoxanthine and 10% polyvinyl alcohol. Incubations lasted 60 min at 37°C. After that, sections were washed in hot distilled water (60°C). Visualization was performed by incubating sections for 30 min at room temperature in 50 mM acetate buffer, pH 5.3; 42 mM cobalt chloride; 100 mM sodium azide; 1.4 mM diaminobenzidine and 0.6 mM H_2O_2. After rinsing, the sections were embedded in glycerol jelly. Liver tissue was processed simultaneously as a control.

Ethanol metabolism to acetaldehyde in the microsomal fraction

Preparations containing microsomes (0.23-0.32 mg protein/mL), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) generating system (0.45 mM $NADP^+$, 4 mM d,l-isocitric acid trisodium salt and 0.25 units of isocitric dehydrogenase) and 0.14 M ethanol in 50 mM KH_2PO_4, pH 7.4, 3 mL final volume, were incubated for 1 hour at 37°C under air. Three samples per group were run, each consisting of microsomes from a separate lot of pooled ovarian tissue (three animals each). Incubations were performed in aluminum-sealed neoprene-septum-stoppered glass vials. The reaction was terminated by plunging in ice. After adding 1 mL of saturated NaCl solution, samples were kept at 37°C for 10 min and an aliquot (100 µL) of the headspace was analyzed by GC-FID.

Chromatographic conditions were column: GS-Q, 25 m × 0.53 mm i.d. (J&W Scientific, CA, USA); temperature: 110°C isothermal; injection port temperature: 150°C and FID: 200°C (Castro et al., 2003).

Ethanol metabolism to acetaldehyde in the cytosolic fraction

Incubation mixtures containing cytosol (1.58-1.65 mg protein/mL) in STKM buffer (0.25 M sucrose/ 50 mM Tris-HCl, pH 7.5/2.5 mM KCl /5 mM $MgCl_2$); 0.25 mM hypoxanthine; 0.3 mM NAD^+ and 0.14 M ethanol (3 mL final volume) were conducted for 1 hour at 37°C under air atmosphere. Three samples per group were run, each consisting of cytosol prepared from a separate lot of pooled ovarian tissue (three animals each). Incubations were performed in aluminum-sealed neoprene-septum stoppered glass vials (15 mL). Samples were processed as described above. Acetaldehyde was quantified in the head space by GC-FID in the same conditions as above (Castro et al., 2001).

Determination of t-butylhydroperoxide-induced chemiluminiscence in rat ovarian tissue homogenates

Chemiluminiscence was measured in a Wallac-Rack Beta 1214 liquid scintillation counter at room temperature in an out of coincidence mode (Boveris et al., 1983; Castro et al., 2006, 2008). Rat ovarian tissue was homogenized (7 mg protein/mL) 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 in flasks that were kept at 37°C for 10 min in a Dubnoff shaker. Chemiluminiscence measurement was started by addition of 3 mM *t*-butylhydroperoxide (*t*-BHP). Three samples per group were run, each consisting of a homogenate from a separate lot of pooled ovarian tissue (five animals each).

Transmission electron microscopy

Five female rats per group (Control and EtOH-treated animals) were anesthetized by diethyl ether. The estrus cycle was followed in each animal by observing the changes in types of cells in vaginal smears. The ovary gland was rapidly removed and immediately placed in chilled 2% formaldehyde: 2% glutaraldehyde in 100 mM cacodylate buffer containing 0.02% $CaCl_2$, pH 7.4 and promptly cut under the fixative. After adequate fixation, 10 cubes (1 mm^3) per each rat ovary gland, was washed with barbital buffer and post fixed with 1% osmiun tetroxide. Then, they were stained as a whole with uranyl acetate, dehydrated with graded ethanol and embedded in epoxy resin. Sections 1-μm thick were stained with toluidine blue and examined with a light microscope in order to select epithelial areas for thin sectioning. Thin sections were cut with a diamond knife and mounted on copper grids (300 mesh), stained with uranyl acetate and lead citrate and examined in a Philips EM300 transmission electron microscope (Castro et al., 2006; de Castro et al., 1989). The microscopic observation of the ovary sections was carried out in a blinded fashion by two observers who were unaware of the treatment group. The photomicrographs were representative average of observations.

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 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

Alcohol dehydrogenase and aldehyde dehydrogenase activities in rat liver and ovarian tissue

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

Alcohol and acetaldehyde dehydrogenase activities in rat ovarian tissue

Our histochemical studies were not able to detect an alcohol dehydrogenase activity in ovarian tissue. Instead, acetaldehyde dehydrogenase was revealed in the strome surrounding follicular structures (Figure 1).

Table I. Alcohol dehydrogenase and aldehyde dehydrogenase activities in rat ovary and liver tissue

	ADh activity nmol NADH/mg protein/min[a]	AldDh activity nmol NADH/mg protein/min[b]		
		Microsomes	Cytosol	Mitochondria
Ovary tissue	0.56 ± 0.14	4.38 ± 0.26	0.30 ± 0.02	4.45 ± 0.01
Liver	3.74 ± 0.42	24.63 ± 0.70	2.86 ± 0.53	27.78 ± 0.02

AldDh, aldehyde dehydrogenase; ADh, alcohol dehydrogenase; NADH, nicotinamide adenine dinucleotide phosphate-oxidase.
[a] The ADh activity was determined in the rat ovary 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.
[b] The AldDh activity was determined in the rat ovary tissue cytosolic, microsomal and mitochondrial fraction by measuring NADH formation at 340 nm. Each value is the mean from three separate samples.

Xanthine oxidase activity in rat ovarian tissue

Xanthine oxidase activity was not revealed by the histochemical studies. This could be due to a lack of sensitivity of the procedure.

Biotransformation of EtOH to acetaldehyde in the microsomal fraction

Results depicted in Table 2 show that the ovary microsomal fraction was able to biotransform ethanol into acetaldehyde in the presence (+NADPH) or absence of NADPH (–NADPH). The activity in the absence of NADPH was about 43% of that observed when NADPH was present. Both processes were found to be partially enzymatic in nature, in light that about 54% of the +NADPH and about 87% of the –NADPH activities were suppressed by heating microsomes for 5 min at 100°C. Oxygen from air was required for a part of the +NADPH-requiring process (49%) and for 56% –NADPH (Table 2).

Known inhibitors of P450 catalyzed oxidations, like SKF 525A, DDTC and 4 MP, were able to significantly deplete acetaldehyde production in microsomes, both in the presence or in the absence of NADPH. However, no effect was observed for chlormethiazole. Diphenyleneiodonium chloride (DPI), a potent inhibitor of flavoproteins, inhibited most of the ability of ovarian microsomes to oxidize ethanol into acetaldehyde. In the absence of NADPH, DPI blocked 57% the biotransformation process under air and 69% in its absence.

Partial inhibitory effects of thiobenzamide (TBA) were also observed for the +NADPH pathway (32% inhibition) and for the –NADPH metabolism of ethanol to acetaldehyde (39% inhibition; Table 2). Nordihydroguaiaretic acid (NDGA) and esculetin (two inhibitors of lipoxygenase activity) did not inhibit the +NADPH metabolism of ethanol to acetaldehyde.

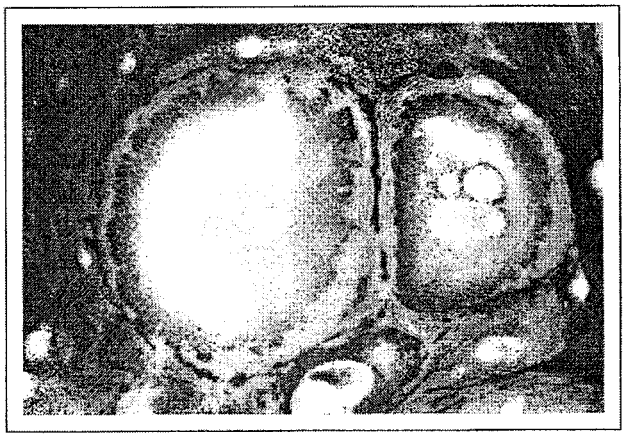

Figure 1. Rat ovary treated with phosphate-NAD-pyruvate-nitroblue tetrazolium. A Graaf follicle in the upper zone and a secondary follicle below can be observed. Acetaldehyde dehydrogenase activity is observed in the strome surrounding these structures (violet-blue staining). ×160.

Biotransformation of ethanol to acetaldehyde in the cytosolic fraction

Results on acetaldehyde levels in incubation mixtures containing the cytosolic fraction of rat ovary tissue are summarized in Table 3. The reaction occurring in the absence of NAD^+ was partially inhibited by pyrazole (45%), an inhibitor of alcohol dehydrogenase, and allopurinol (65%), an inhibitor of xanthine oxidoreductase. The generation of acetaldehyde was enhanced (60%) when disulphiram, an inhibitor of aldehyde dehydrogenase, was present in the incubation media.

The ethanol metabolism to acetaldehyde was significantly enhanced by hypoxanthine and this process was also inhibited by allopurinol, to the same level as above.

Table 2. Ethanol biotransformation to acetaldehyde in rat ovary microsomes

Experimental[a]	Acetaldehyde (nmol/mg protein)	
	−NADPH	+NADPH
Control	1.97 ± 0.12	4.62 ± 0.87
Nitrogen	1.00 ± 0.07	2.05 ± 0.57
Heated (100°C, 5 min)	0.90 ± 0.05	0.60 ± 0.06
3 mM SKF 525A	1.26 ± 0.05	3.15 ± 0.03
1 mM DDTC	1.16 ± 0.06	2.94 ± 0.08
1 mM 4MP	1.65 ± 0.10	3.18 ± 0.09
100 μM chlormethiazole	2.29 ± 0.39[b]	4.62 ± 0.55
10 μM DPI	0.84 ± 0.01	1.44 ± 0.04
1 mM TBA	1.35 ± 0.28	2.81 ± 0.91
100 μM NDGA	2.68 ± 0.07	5.06 ± 0.15[b]
50 μM esculetin	1.39 ± 0.01	4.79 ± 0.11

DPI, diphenyleneiodonium chloride; NADH, nicotinamide adenine dinucleotide phosphate-oxidase; NDGA, nordihydroguaiaretic acid TBA; thiobenzamide.

[a] Incubation mixtures containing microsomal preparations (2.26 ± 0.59 mg of microsomal protein/mL), NADPH generating system (0.45 mM $NADP^+$, 4 mM D,L-isocitric acid trisodium salt and 0.25 units of isocitric dehydrogenase) and 0.14 M ethanol were conducted for 1 hour at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 mL NaCl saturated solution. See Methods for details. Each result is the mean of three separate lots of pooled ovary tissue samples.

[b] $p < 0.01$, when compared to control.

Table 3. Ethanol biotransformation to acetaldehyde in rat ovary cytosolic fraction

Experimental[a]	Acetaldehyde (nmol/mg protein)	
	$-NAD^+$	$+NAD^+$
Control	0.95 ± 0.28	0.49 ± 0.05
Heated (100°C, 5 min)	0.18 ± 0.08	0.29 ± 0.11
5 mM pyrazole	0.54 ± 0.02	0.44 ± 0.02
0.2 mM disulphiram	1.60 ± 0.09	2.71 ± 0.01
Pyrazole + disulphiram	0.39 ± 0.03	0.31 ± 0.02
0.25 mM hypoxanthine	2.27 ± 0.02	0.16 ± 0.01
0.15 mM allopurinol	0.34 ± 0.06	0.39 ± 0.02
Hypoxanthine + allopurinol	0.31 ± 0.02	0.23 ± 0.01
Hypoxanthine + 10 μM oxypurinol	0.39 ± 0.04	0.14 ± 0.01
Hypoxanthine + 10 μM folic acid	0.34 ± 0.06	0.45 ± 0.04
Hypoxanthine + disulphiram	1.31 ± 0.01[b]	1.01 ± 0.01

[a] Incubation mixtures containing cytosol (3.78 ± 0.96 mg of cytosolic protein/mL) and 0.14 M ethanol, and when indicated, 0.3 mM NAD^+, were conducted for 1 hour at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 mL NaCl saturated solution. See Methods for details. Each result is the mean of three separate lots of pooled ovary tissue samples.

[b] $p < 0.05$, when compared to control.

Ethanol metabolism to acetaldehyde in the ovarian microsomal fraction from rats receiving an alcohol-containing liquid diet

Both, an NADPH-dependent and an NADPH-independent microsomal metabolism of ethanol to acetaldehyde was observed (Table 4). The former was significantly more intense than the latter. After chronic ethanol drinking, the activity of the NADPH-dependent pathway was significantly enhanced (505%). The metabolic pathway independent of NADPH presence was also enhanced significantly (43%).

Ethanol metabolism to acetaldehyde in the ovarian cytosolic fraction from rats receiving an alcohol-containing liquid diet

In contrast to the behavior observed with the microsomal pathway, chronic ethanol drinking, the activity of the cytosolic oxidation pathway from ethanol to

Table 4. Ethanol metabolism to acetaldehyde in the microsomal fraction of ovary tissue from rats receiving an alcohol containing liquid diet

	Acetaldehyde (nmol/mg protein)	
Experimental[a]	Control	EtOH treated
+NADPH	1.16 ± 0.11	3.21 ± 0.29[b]
−NADPH	0.81 ± 0.02[c]	0.53 ± 0.10[b,c]

EtOh, ethanol group; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase.
[a] Incubation mixtures containing microsomal fraction (1.56 ± 0.13 mg protein/mL), 0.14 M ethanol and, when indicated, NADPH generating system (0.45 mM $NADP^+$, 4 mM D,L-isocitric acid trisodium salt and 0.25 units of isocitric dehydrogenase) in KH_2PO_4 buffer, were conducted for 1 hour at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 mL NaCl saturated solution. Each result is the mean of three separate lots of pooled ovary tissue samples.
[b] $p < 0.05$ (EtOH treated vs Control).
[c] $p < 0.001$ (Control − NADPH vs Control +NADPH) (EtOH treated − NADPH vs EtOH treated + NADPH).

Table 5. Ethanol metabolism to acetaldehyde in the cytosolic fraction of ovary tissue form rats receiving an alcohol containing liquid diet

	Acetaldehyde (nmol/mg protein)	
Experimental[a]	Control	EtOH treated
−Hypoxanthine	3.17 ± 0.01	0.23 ± 0.01
+Hypoxanthine	3.23 ± 0.02	0.69 ± 0.14[b]
+Hypoxanthine + allopurinol	0.14 ± 0.01[b]	0.22 ± 0.04[b,c]

EtOh, ethanol group.
[a] Incubation mixtures containing cytosolic fraction (3.52 ± 0.20 mg protein/mL), 0.14 M ethanol and, when indicated, 0.25 mM hypoxanthine in STKM buffer, were conducted for 1 hour at 37°C. Acetaldehyde was measured in the head space of each sample after adding 1 mL NaCl saturated solution. See Methods for details. Each result is the mean of three separate lots of pooled ovary tissue samples.
[b] $p < 0.01$ (EtOH treated + hypoxanthine vs Control + hypoxanthine) (hypoxanthine vs hypoxanthine + allopurinol).
[c] $p < 0.05$ (EtOH treated + hypoxanthine + allopurinol vs Control + hypoxanthine + allopurinol).

acetaldehyde, significantly decreased. However, that activity was enhanced by the presence of hypoxanthine in the incubation mixture, and allopurinol was able to inhibit the metabolism either in Control or EtOH group (Table 5).

t-BHP-induced chemiluminiscence in homogenates of ovarian tissue from rats receiving an alcohol-containing liquid diet

In our studies on the total hydroperoxide-induced chemiluminiscence emitted by ovarian tissue homogenates, a significant difference in area and shape was observed between Control and those from rats receiving the ethanol-containing diet (Control: 2.011 ± 0.005 × 10^6; EtOH: 3.276 ± 0.038 × 10^6; $p < 0.01$; see Figure 2).

Ultrastructural alterations in the ovarian tissue of rats treated with ethanol liquid diet

Our structural studies in the ovary tissue from animals repetitively exposed to ethanol showed that alcohol drinking may also lead to deleterious effects in ovarian cells.

A section of part of a secondary follicle from a control rat ovary (see Figure 3) showed oval nuclei, abundant granular endoplasmic reticulum and free ribosomes (Rhodin, 1974). Mitochondria having different forms can be observed including many of elongated and parallel to the nuclear contour. In an equivalent section (Figure 4) from repetitive alcohol-treated rats, severe alterations can be observed. There was marked condensation of chromatin attached to the nuclear inner membrane. This was particularly evident around nuclear pores. Intense dilatation of the outer perinuclear space can be observed. There was a marked dilatation of the rough endoplasmic reticulum (RER) accompanied of significant detachment of ribosomes from their membranes. Mitochondria appeared swollen.

In the control rat ovaries, secondary vesicular follicles showed slender cell processes from the oocyte and the corona radiata cells penetrating the zona pellucida (Figure 5). In the case of ethanol-treated

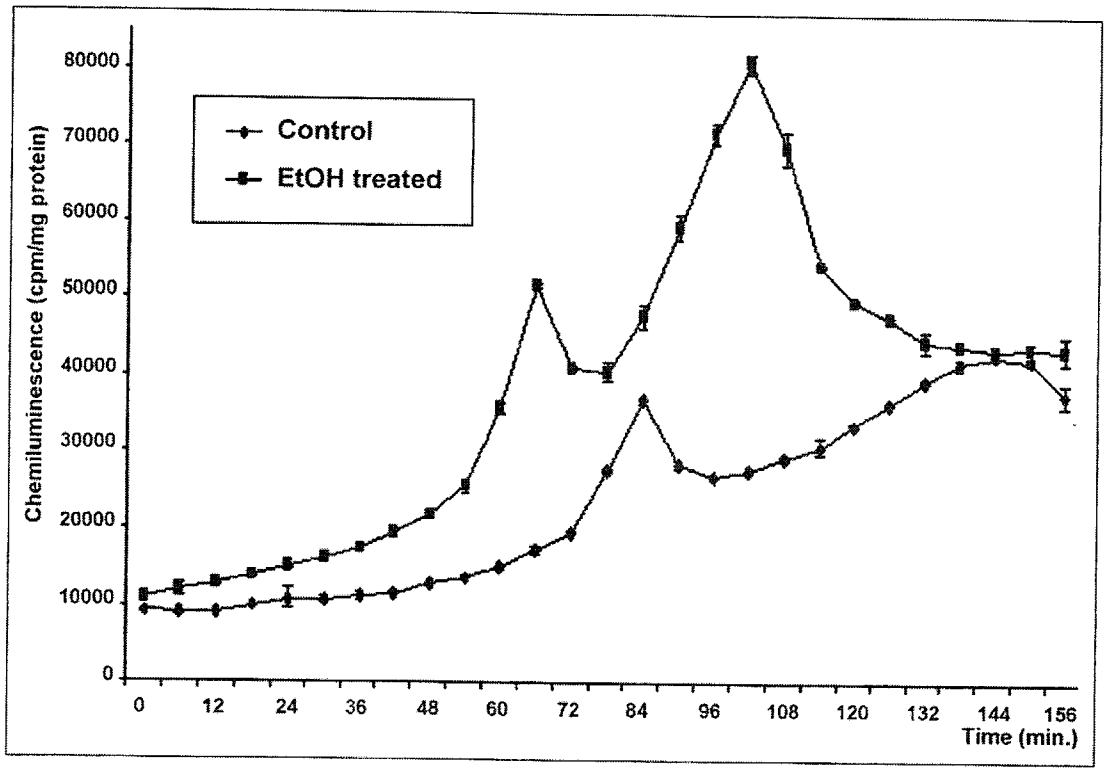

Figure 2. *t*-Butylhydroperoxide-induced chemiluminescence in ovary tissue homogenates from rats receiving an alcohol containing liquid diet. Rat ovary tissue homogenized in 0.25 M sucrose, 50 μM deferoxamine in TKM buffer, pH 7.5 (1.85 ± 0.19 mg protein/mL) were kept at 37°C for 10 min in a Dubnoff shaker. Chemiluminescence measurement was started by addition of 3 mM *t*-butylhydroperoxide. Values are means ± SD. Three samples per group were run, each consisting of a homogenate from a separate lot of pooled ovary tissue (five animals each).

animals, marked alterations in the zona pellucida can be observed (Figure 6). Most of cell processes from oocyte and corona radiata cells were absent or were broken totally or in part. The zona pellucida appeared less precisely defined.

Granulosa cells from control rats contained round or oval nuclei (Figure 7). Occasionally, some of them evidenced deep cytoplasmatic indentation. There were present vesicular RER containing abundant ribosomes attached to their membranes. Free ribosomes and scattered small tubular mitochondria were also observed. In the case of ethanol-treated rats (Figure 8a and b), granulosa cells frequently exhibited nuclei having different irregularly shaped forms. They might include elongated, atypical geometric forms; single or multiple bizarre forms. Most times they had highly dispersed chromatin. Vesicles of rough endoplasmic reticulum (RER) almost disappeared and many free ribosomes were present free in the cytoplasm. There were present large autophagic vacuoles occupying major parts of the cytoplasm.

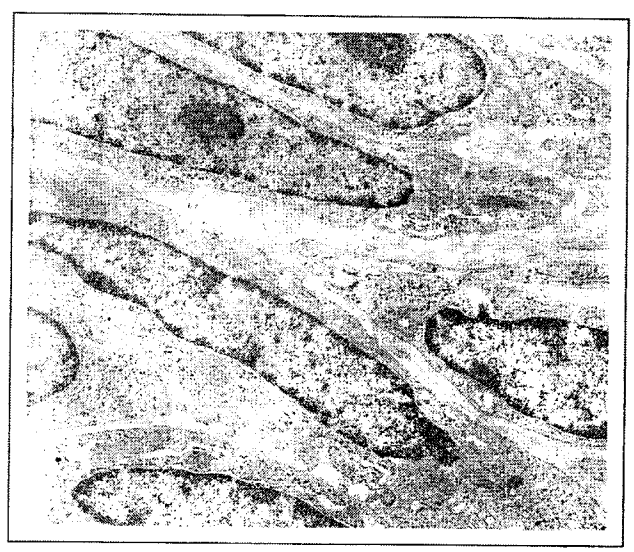

Figure 3. Electron micrograph of rat ovary (Control). Section of part of a secondary follicle from a control rat ovary showing oval nuclei, abundant granular endoplasmic reticulum and free ribosomes. Mitochondria having different forms can be observed including many of elongated and parallel to the nuclear contour. ×5984.

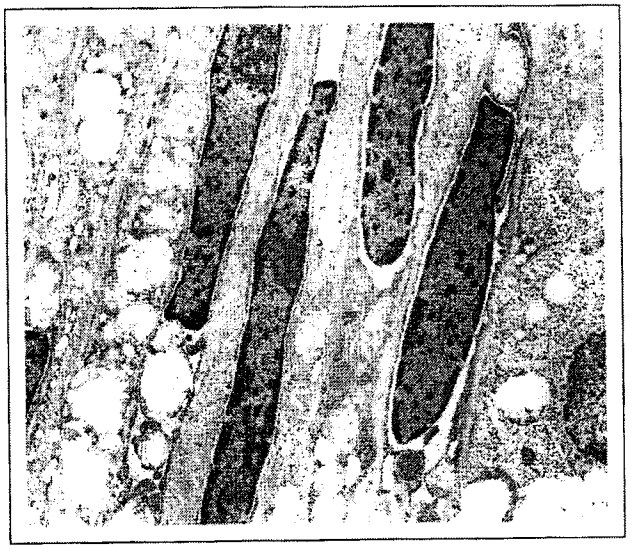

Figure 4. Electron micrograph of rat ovary (EtOH treated). Section of part of a secondary follicle showing marked condensation of chromatin attached to the nuclear inner membrane, particularly evident around nuclear pores. Intense dilatation of the outer perinuclear space can be observed. There is a marked dilatation of the rough endoplasmic reticulum (RER) accompanied of significant detachment of ribosomes from their membranes. Mitochondria appear swollen. ×5984.

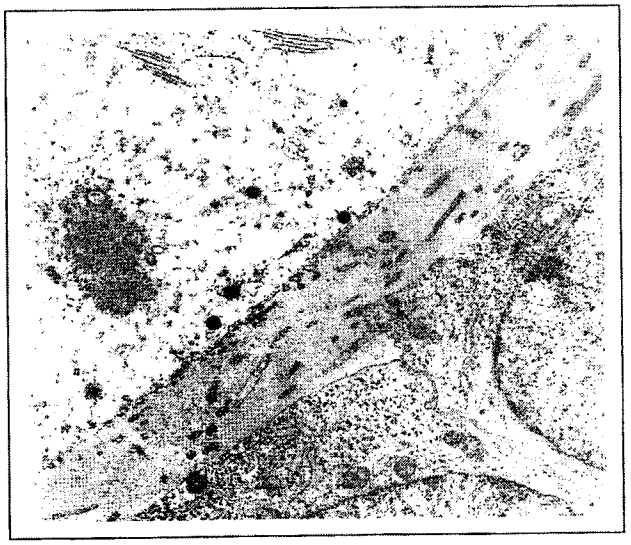

Figure 5. Electron micrograph of rat ovary (Control). Secondary vesicular follicle showing slender cell processes from the oocyte and the corona radiata cells penetrating the zona pellucida. ×8800.

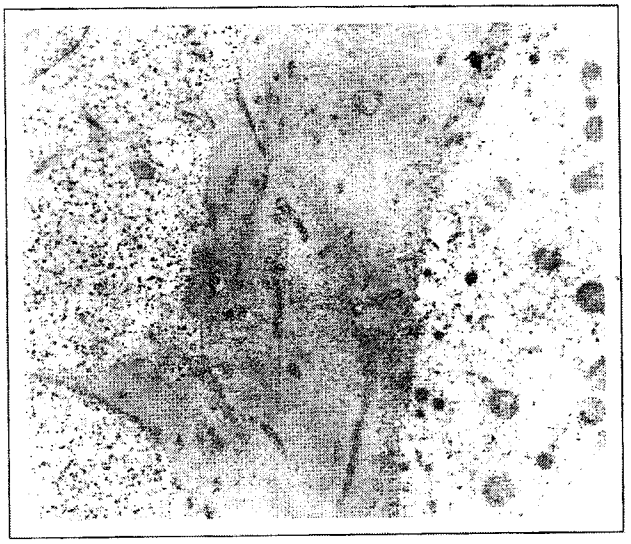

Figure 6. Electron micrograph of rat ovary (EtOH treated). Secondary vesicular follicle showing most of the cell processes from the oocyte and the corona radiata cells are absent or are broken totally or in part. The zona pellucida appears less precisely defined. ×8800.

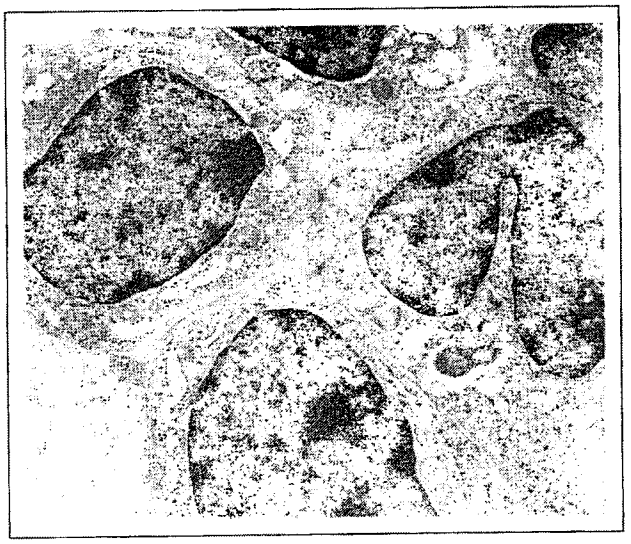

Figure 7. Electron micrograph of rat ovary (Control). Granulosa cells contain round or oval nuclei. Occasionally some of them evidence deep cytoplasmatic indentation. There are present vesicular rough endoplasmic reticulums (RER) containing abundant ribosomes attached to their membranes. Free ribosomes and scattered small tubular mitochondria are also observed. ×8800.

Discussion

It is well established that alcohol deleterious effects are linked to its metabolic activation to acetaldehyde and free radicals that upon interaction with different cellular components initiate the different toxic manifestations (Lieber, 2005). Several enzymes were described as involved in those alcohol metabolic activation pathways in different tissues, from either humans or rodents. They included alcohol

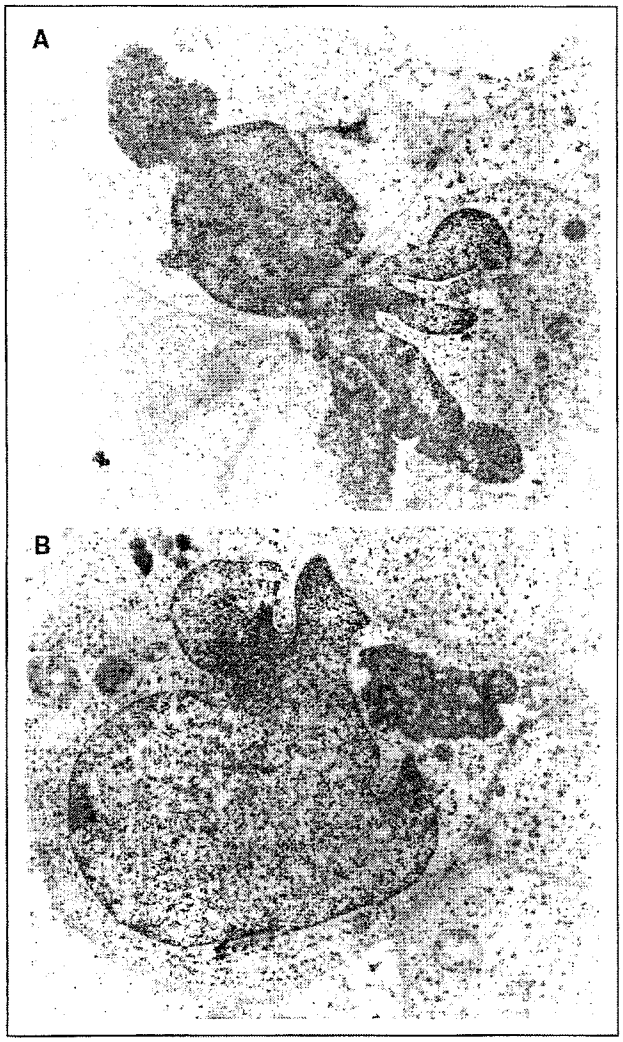

Figure 8. (a and b) Electron micrograph of rat ovary (EtOH treated). Granulosa cells frequently show nuclei having different irregularly shaped forms. They may include elongated and atypical geometric forms; single or multiple bizarre forms. Most of the times they have highly dispersed chromatin. Vesicles of rough endoplasmic reticulum (RER) almost disappear and many free ribosomes lie free in the cytoplasm. There are present large autophagic vacuoles occupying major parts of the cytoplasm. ×8800.

dehydrogenases; CYP2E1; catalase; NADPH oxidase; xanthine oxidoreductase; NADPH P450 reductase; peroxidase-like enzymes and a variety of tissues from the upper aerodigestive and digestive tract (Egerer et al., 2005) to pancreas, brain, testes, prostate, placenta and breast (Aleynik et al., 1999; Castro and Castro, 2005; Castro et al., 2006; Díaz Gómez et al., 2000; Kapoor et al., 2006; Quintans et al., 2005; Shibley et al., 1999).

In this study, we provide evidence that the microsomal fraction from the rat ovarian tissue has the ability to metabolize ethanol to acetaldehyde. A significant portion of that metabolism was of enzymatic nature as evidenced by heating microsomes at 100°C during 5 min. Several enzymatic pathways appear to be involved in the microsomal oxidation of ethanol to acetaldehyde. One fraction of the NADPH and oxygen-dependent metabolism could be attributed to the presence of P450 and P450 reductase in the microsomal fraction as suggested by the inhibitory effect of SKF 525A, a well-known inhibitor of P450-mediated biotransformations; and by the effects of DDTC and 4-methylpyrazole, two inhibitors of CYP2E1-mediated microsomal metabolism of ethanol to acetaldehyde in liver (Rendic and Di Carlo, 1997); and also to the highly significant inductive effects of repetitive alcohol drinking on the microsomal pathway here reported. The inhibitory effect of thiobenzamide might be attributed to the fact that this compound is also metabolized by CYP2E1 (Rendic and Di Carlo, 1997), and its competitive action on the oxidation of alcohol by this cytochrome.

The inductive effect of ethanol drinking on the microsomal CYP2E1 in liver has been repeatedly observed by others (Díaz Gómez et al., 2006, 2008; Lieber, 2005). The participation of NADPH P450 reductase was also suggested by the significant inhibitory effect of DPI on the oxygen-dependent pathway. This compound is well known as a potent inhibitor of flavin-dependent enzymes, including microsomal P450 reductase as a target (McGuire et al., 1998). This enzyme has both FMN and FAD in its active center and the semiquinone form of its flavine moiety not only activates DPI to give adducts with the enzyme (McGuire et al., 1998; Opsian and Coon, 1982) but was also shown by our laboratory to be able to activate ethanol to acetaldehyde (Díaz Gómez et al., 2000). This latter ability of P450 reductase might offer an explanation to the here-observed anaerobic transformation of ethanol to acetaldehyde and its susceptibility to DPI (Díaz Gómez et al., 2000).

The lack of response to NDGA and esculetin excludes the participation of a lipoxygenase in the microsomal metabolism of ethanol to acetaldehyde in ovarian tissue, since both compounds are potent inhibitors of these enzymes (Kulkarni, 2002). This behavior was completely different from the one reported by our laboratory for the case of other hormone-responsive tissue such as the mammary gland (Castro et al., 2003).

The presence of P450 or P450 reductase enzymes in the ovaries and also the ability of its microsomal

fraction to activate several CYP2E1 substrates was previously established by others and by our laboratory (Díaz Gómez et al., 1988; Keating et al., 2008; Villarruel et al., 1977).

The identity of the NADPH-independent microsomal enzymatic pathways of ethanol oxidation remains to be fully characterized. However, their susceptibility to DPI suggests that they may be flavoenzymes. The ability of those enzymes to operate in the absence or the presence of oxygen suggests that more than one metabolic pathway is present in the ovarian microsomal fraction.

It is interesting to point out that the ovarian microsomal fraction also has a small but detectable NAD^+-dependent AldDh activity. The microsomal fraction exhibited a NAD^+-dependent AldDh activity of the same order of magnitude (in terms of nmole NAD^+ consumed per mg protein) than the one of acetaldehyde formed in the NADPH-dependent microsomal pathway. That points to the relevance of the relative levels of both co-factors, NAD^+ and NADPH, under given experimental conditions. The ability to handle acetaldehyde in the microsomal fraction by AldDh is roughly equivalent to that of the mitochondrial fraction.

The response of the cytosolic enzymes able to generate acetaldehyde from ethanol in the presence of NAD^+ to inhibitors (Table 3) reflects the complexity involved in the interpretation of the obtained results. On one hand, when both pathways of acetaldehyde formation (in the presence or absence of NAD^+) were tested for the inhibitory effects of disulphiram, increased concentrations of acetaldehyde were detected. This effect of disulphiram might be attributed to its known inhibitory effect on AldDh. This is in agreement with the here reported presence of AldDh in the cytosolic fraction. The AldDh was also detected by histochemistry and showed to be present in the strome of the ovarian tissue. In addition, the ovarian cytosolic fraction evidenced to have a xanthine oxidoreductase-mediated pathway for the metabolism of ethanol to acetaldehyde. That was clearly shown using hypoxanthine rather than NAD^+ as cosubstrate, avoiding in that way the operation of AldDh that would process any acetaldehyde formed. As expected, acetaldehyde formation in the presence of hypoxanthine was strongly inhibited by allopurinol, oxypurinol and folic acid, which are potent inhibitors of XOR at low concentrations (Maciel et al., 2004). The repetitive alcohol drinking strongly decreased the XOR-mediated cytosolic metabolism of ethanol to acetaldehyde, using hypoxanthine as co-substrate. This behaviour of the ovarian cytosolic XOR enzyme when compared to that present in mammary tissue cytosolic fraction is interesting. In the latter case, the XOR-mediated oxidation of ethanol to acetaldehyde significantly increased its activity after repetitive alcohol consumption for 28 days (Castro et al., 2006).

The generation of acetaldehyde in situ may harm ovarian tissue. In effect, it is well known that acetaldehyde is a mutagenic, carcinogenic and toxic chemical able to react with DNA, proteins and lipids and with many other relevant molecules such as GSH (Dellarco, 1998; Garro et al., 1992). Those decreases in GSH content resulting from the acetaldehyde attack to this critical antioxidant molecule were blamed by other workers to be responsible for a significant part of the ethanol-induced oxidative stress in other organs (Lu and Cederbaum, 2008). A preliminary indication that an increased susceptibility to oxidative stressful condition could be present in ovaries after repetitive alcohol drinking was obtained in our experiments where ovarian tissue homogenates were challenged with *t*-BHP and the formation of reactive oxygen species (ROS) was followed by chemiluminiscence emission. It is considered that the intensity of the *t*-BHP-induced chemiluminiscence is an expression of the ROS formation in biological samples and of the defensive capacity of living systems against oxidative stress (Castro et al., 2006, 2008; Török, 2004).

In the present experiments on *t*-BHP-induced chemiluminiscence emission in ovarian homogenates derived from animals repetitively exposed to alcohol drinking for 28 days, we observed major changes in the chemiluminiscence emission curves. They included not only a significant enhancement in the intensity of the emitted chemiluminiscence but also important changes in the shape of the emission curve. The latter consisted of an anticipation of the initiation of the emission. Those two different responses to the *t*-BHP challenge might be interpreted as an indication of decreased presence of defences in the ovarian tissue as well as changes in the composition of the tissue itself attributable to the ethanol intoxication, which favoured ROS production when exposed to *t*-BHP.

The precise nature of those significant alterations remains to be established and is at present under careful study in our laboratory.

Results obtained showed that ovarian tissue is able to oxidize ethanol to acetaldehyde via different metabolic pathways and that as result of ethanol drinking

the ovary becomes more susceptible to oxidative stressful conditions. We also showed that the ovaries from the poisoned animals evidenced ultrastructural alterations as a consequence. The repetitive administration of the Lieber and De Carli diet for 28 days produced severe ultrastructural alterations in the different cell types of the ovaries. These alterations described, occurred irrespectively of the ovarian cycle stage of the animals at the time of their sacrifice. The deleterious effects included the secondary follicles, the oocyte, the corona radiata cells, the zona pellucida and the granulosa cells. The alterations observed (see Results for details) involved every cell compartment and organelles. The most intense deleterious effects were observed in the granulosa cells. Nuclear alterations included from marked condensations of chromatin attached to the nuclear membrane and intense dilatation of the outer perinuclear space to nuclei having very irregular forms or even bizarre forms.

RER alterations varied from marked dilatation and detachment of ribosomes to almost disappearance of their vesicles and total detachment of the ribosomal component. The cytoplasm of the granulosa cells was occupied by large autophagic vacuoles. Mitochondria appear swollen. The in situ formation of acetaldehyde and the increased susceptibility to oxidative stress exhibited by the rat ovarian tissue here described might play a role in these deleterious effects observed. In effect, acetaldehyde is a well-known mutagenic chemical that is known to attack DNA, proteins, lipids and other cellular components (Dellarco, 1998; Garro et al., 1992). Similarly, it was repetitively shown by many workers in the field that oxidative stress is a highly deleterious process provoking generalized derangement of almost every cellular component through its effects on DNA, proteins and other cell molecules (Lu and Cederbaum, 2008).

In light of these two simultaneous concurrent harmful effects of alcohol drinking on ovarian tissue, it appears likely that they play some role in the ultrastructural alterations described here. These severe alterations observed in tissue ultrastructute should significantly impair the function of the ovary, either reflected in a decreased number of healthy functional oocites but also in the altered hormone synthesis and secretion.

The significance of these early experiments rests on the fact that the harmful effects of alcohol drinking on rat ovaries here described could be reasonably linked to severe alterations in their known dual function of being responsible for both, the production and release of the cell ovum as well as the biosynthesis and secretion of the key steroid hormones, progesterone and oestrogen (Nordman and Litwick, 1987; Saxena et al., 1990).

There are other plausible alternatives or additional mechanisms potentially involved in the ovarian ethanol induced toxicity, beyond those linked to local activation to acetaldehyde and free radical-induced oxidative stress here described. One could be related to any acetaldehyde arriving via blood from liver to the ovaries and its potential accumulation there because of its limited ability to handle this deleterious molecule. However, previous work from our laboratory related to an equivalent situation described for the case of other tissue like the mammary one, evidenced that only limited concentrations of acetaldehyde remain in blood after even large doses of alcohol given to the rat (Castro et al., 2008). Further, other indirect mechanisms might also be involved in the alcohol-promoted effects in the ovaries and operating at the level of the central nervous system (Campillo, 2005; Davis and Heindel, 1998; Emanuele and Emanuele, 1997). The potential relevance of the different alternatives discussed above remain to be challenged with further experiments.

Acknowledgement

This work was supported by grants from ANPCyT (PICT-2004 25354) and CONICET (PIP 2005 5158), Argentina.

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