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Oxidation of ethanol to acetaldehyde and free radicals by rat testicular microsomes

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Abstract A large number of epidemiological studies evidencing that excessive alcohol consumption is associated with impaired testosterone production and testicular atrophy are available in the literature. One hypothesis to explain the deleterious action of alcohol involves the in situ biotransformation to acetaldehyde, but it strongly suggests the need to learn more about the enzymatic processes governing alcohol metabolism to acetaldehyde in different cellular fractions since limited information is available in the literature. In this article we report studies on the metabolic conversion of alcohol to acetaldehyde and to 1-hydroxyethyl radicals in rat testicular microsomal fractions. The oxidation of ethanol to acetaldehyde in rat testes microsomal fraction was mostly of enzymatic nature and strongly dependent on the presence of NADPH and oxygen. Several compounds were able to significantly decrease the production of acetaldehyde: SKF 525A; diethyldithiocarbamate; esculetin; gossypol; curcumin; quercetin; dapsone; and diphenyleneiodonium. Microsomal preparations in the presence of NADPH were also able to produce both hydroxyl and 1-hydroxyethyl free radicals. Their generation was modulated by the presence of diphenyleneiodonium, gossypol, and deferoxamine. Results show that rat microsomal fractions are able to metabolize alcohol to deleterious chemicals, such as acetaldehyde and free radicals, that may be involved in ethanol toxic effects. Enzymes involved could include CYP2E1, P450 reductase, and other enzymes having lipoxygenase- /peroxidase-like behavior.

Keywords Alcohol · Ethanol · Acetaldehyde · 1-hydroxyethyl · Testes · Radicals · Microsomes

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Introduction

A large number of epidemiological studies evidencing that excessive alcohol consumption is associated with impaired testosterone production and testicular atrophy are available in the literature (Adler 1992; Emanuele and Emanuele 1998). Similar findings were observed in experimental studies in ethanol-treated rats (Akane et al. 1988; Van Thiel et al. 1987; 1989). Mechanistic in vitro studies on the testosterone production by isolated testes revealed that ethanol acts at least in part directly on the testes to affect this hormone production (Badr et al. 1977; Cobb et al. 1978).

One hypothesis about how alcohol may contribute to testosterone decreasing effects on those in vitro studies involved the metabolic transformation of ethanol to acetaldehyde. The rationale behind that hypothesis is that in some studies acetaldehyde was found to be more potent than alcohol in suppressing testosterone release (Badr et al. 1977; Cobb et al. 1978). Acetaldehyde might harm testicular function by directly inhibiting steroideogenic enzymes or indirectly by decreasing antioxidant defenses (e.g., GSH levels) and enhancing lipid and protein oxidation to cause damage as suggested by others (Rosenblum et al. 1989; Nordmann et al. 1990; Emanuele et al. 2001). Alternatively, the metabolism of ethanol to acetaldehyde by alcohol dehydrogenase (ADh) could also result directly in the formation of free radicals by changing the NADH level and the NADH/NAD^+ redox ratio, which in turn modulates the activity of the free-radical-generating enzyme xanthine oxidase (Mantle and Preedy 1999). Still, another possibility could be that the biotransformation of alcohol to acetaldehyde in the testes competed for cofactors with the process involved in testosterone production thereby preventing testosterone production (Ellingboe and Varanelli 1979; Gordon et al. 1980). In fact, Akane et al. (1988) reported that ethanol inhibited testicular steroidogenesis by suppressing at least two steps in the pregnenolone-to-testosterone pathway, the pregnenolone–

progesterone step and the 17α-hydroxyprogesterone to androstenedione step, both being NAD^+-requiring processes. Cicero and Bell (1980) also reported effects of both alcohol and acetaldehyde on the conversion of androstenedione to testosterone.

These hypotheses strongly suggest the need to learn more about the enzymatic processes governing alcohol metabolism to acetaldehyde in different cellular fractions since limited information is available in the literature (Chiao and Van Thiel 1986; Murono and Fisher-Simpson 1987).

In this article we report studies on the metabolic conversion of alcohol to acetaldehyde and to 1-hydroxyethyl radicals in rat testicular microsomal fractions.

Materials and methods

Chemicals

Absolute ethanol (analytical grade) was from Sintorgan (Buenos Aires, Argentina). N-*tert*-butyl-α-phenylnitrone (PBN), N,O-bis(trimethylsilyl)trifluoracetamide (BSTFA), and the drugs tested for their effects on the metabolism of ethanol were from Sigma (St. Louis, Mo.): α-phenyl-α-propylbenzeneacetic acid 2-[diethylamino]-ethyl ester hydrochloride (SKF 525A), 4-methylpyrazole (4MP), thiobenzamide (TBA), N,N-diethyldithiocarbamic acid sodium salt (DDTC), 2-mercapto-1-methylimidazole (MMI), NAD^+, $NADP^+$, and isocitric acid trisodium salt; isocitric dehydrogenase; acetylsalicylic acid (ASA), deferoxamine mesylate (DFA), gossypol, 3-amino-1,2,4-triazole (AT), diphenyleneiodonium chloride (DPI), curcumin, quercetin, dapsone, esculetin, nordihydroguayaretic acid (NDG); and 1-phenyl-3-pyrazolidone and indomethacin (IM). Esculetin was from Fluka (Switzerland). Nitrogen (ultra high purity) was from AGA (Buenos Aires, Argentina) and was further deoxygenated by bubbling through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1 N NaOH.

Animals and treatments

Random-bred Sprague-Dawley male rats (220–260 g, age range 8–9 weeks) were used. The starting breeding colony was from Charles River (USA). The animals were starved for 12 14 h before killing. Water was available ad libitum. 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).

Animals were killed by decapitation and their testes were rapidly excised and processed. Rat testicular microsomal fractions were obtained as previously described (Bernacchi et al. 1986). Briefly, testes were homogenized with 4 vol STKM buffer (0.25 M sucrose/50 mM Tris-HCl, pH 7.5/2.5 mM KCl/5 mM $MgCl_2$). The homogenate was centrifuged for 20 min at 9000 g and the supernatant fraction was further centrifuged for 1 h at 105,000 g to obtain microsomal pellet. Each pellet corresponded to the testes from two animals.

Ethanol oxidation to acetaldehyde in the microsomal fraction

Preparations containing microsomes (0.9 ± 0.2 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 in phosphate buffer (50 mM K_2HPO_4, pH 7.4), 3 ml final volume, were incubated for 1 h at 37°C under different atmospheres (air, nitrogen). Several drugs were tested for their effect on the ethanol microsomal metabolism to acetaldehyde: SKF 525A; 4MP and DDTC (known inhibitors of P450-mediated biotransformations); MMI and TBA (inhibitors of FMO-mediated oxidations); IM and ASA (inhibitors of prostaglandin endoperoxide synthase); AT (inhibitor of catalase); DPI (a selective inhibitor of flavoenzymes, e.g., P450 reductase); DFA (a potent iron chelating agent); dapsone (inhibitor of lactoperoxidase); NDG; 1-phenyl-3-pyrazolidone; gossypol; curcumin; quercetin; and esculetin (inhibitors of lipoxygenase activities). Incubations were performed in aluminum-sealed-neoprene-septum stoppered glass vials (15 ml). The reaction was stopped by placing the vials on ice. After adding 1 ml of saturated NaCl solution, samples were maintained at 37°C for 15 min and an aliquot (100 µl) of the headspace was analyzed by GC-FID. Chromatographic conditions were: column; Poraplot Q; 25 m×0.53 mm i.d. (Chrompack, Netherlands); temperature 140°C isothermal; injection port temperature: 150°C; FID: 200°C (Díaz Gómez et al. 2000).

Hydroxyethyl radical formation from ethanol by rat testicular microsomes

The spin adduct of the 1-hydroxyethyl (1HEt) radical was detected by the method described previously (Castro et al. 1997; Castro and Castro 2001). Microsomes (1.8 ± 0.5 mg protein per milliliter) were added to NADPH generating system, 0.15 M $MgCl_2$, 24 mM PBN, and 150 mM ethanol in phosphate buffer (50 mM K_2HPO_4 pH 7.4). After 1 h at 37°C, the volume (4 ml) was extracted with 500 µl toluene, centrifuged, and the organic layer was evaporated under nitrogen. The residue was silylated with BSTFA and analyzed by GC-MS. Chromatographic conditions were as follows: column; methylsilicone; 12 m×0.2 mm i.d., programmed from 100 to 150°C at a ramp of 10°C/min and then to 280°C at 4°C/min. Injection port was at 250°C and transfer line to MS, 300°C. Selected ion monitoring (SIM) of mass

spectra was employed to increase sensitivity. Selected masses were 250 (M - $CHCH_3OTMS$) and 194 (m/z 250 - C_4H_8). Dwell time was 50 ms for both masses.

Statistical analysis

The significance of the difference between two mean values was assessed by the Student's *t*-test (Gad and Weil 1982; Graph Pad Instat 1993).

Results

Ethanol oxidation to acetaldehyde in the testicular microsomal fraction

Results on acetaldehyde levels for incubations containing microsomes are summarized in Table 1. They show that microsomal fraction was able to metabolize ethanol to acetaldehyde in the presence (+NADPH) or in the absence of NADPH (−NADPH). The activity in the absence of NADPH was about 36% of that observed when NADPH was present. The NADPH dependent pathway was mostly enzymatic because about all the activity was destroyed by heating 5 min at 100°C. Oxygen from air was required for most of the +NADPH requiring process (80% of it) and for 65% of the −NADPH (Table 1). Including in the mixture 3 mM SKF 525A decreased the aerobic transformation of ethanol 20% (+NADPH). Other chemicals such as 4MP and DDTC, known for their specific inhibitory effects on P450 (CYP2E1) mediated reactions, were tested (Lieber 1996, 1999). Both compounds appeared to inhibit NADPH-dependent acetaldehyde production (48 and 30%, respectively). The DDTC was also able to decrease the microsomal oxidation process in the absence of NADPH (63%). Methylmercaptoimidazole inhibited the transformation to acetaldehyde in the absence of NADPH (45% inhibition) and slightly in its presence. In the case of thiobenzamide, the drug showed a minor inhibitory effect in the presence of NADPH and no effect in its absence. Inhibitors of prostaglandin endoperoxide synthase, such as ASA or IM (Degen et al. 2002), or of catalase, such as AT (Lieber 1996; Nicholls 1962) did not cause any significant decrease in the production of acetaldehyde. The potent inhibitor of flavoproteins DPI (McGuire et al. 1998) inhibited the NADPH-dependent ability of testicular microsomes to bioactivate ethanol into acetaldehyde (54%). In the absence of NADPH, DPI blocked 27% of the process.

The role of non-heme iron in the metabolism of ethanol to acetaldehyde was checked by the use of deferoxamine (DFA), although a slightly inhibition was observed. The inhibitor of lactoperoxidase dapsone (1 mM) was able to inhibit only the NADPH dependent process (39% inhibition).

Several inhibitors of lipoxygenase activities were tested for their effect on the microsomal oxidation of ethanol: NDG; 1-phenyl-3-pyrazolidone; and esculetin were able to decrease the NADPH dependent production of acetaldehyde (36, 41, and 52%, respectively). Esculetin was also able to diminish the response in the absence of NADPH (70%). Gossypol showed a strong ability to inhibit the formation of the aldehyde (+NADPH: 73% inhibition). In addition, two polyphenols were tested for their ability to inhibit the formation of acetaldehyde from ethanol. Quercetin (10 μM) decreased the NADPH-dependent response in about 47% and curcumin, at the same concentration, 67%.

Table 1 Ethanol oxidation to acetaldehyde by rat testicular microsomes

Experiment[a]	Acetaldehyde (×10[1]pmoles)/protein (mg)	
	+NADPH	−NADPH
Air	104.8 ± 20.0	37.3 ± 18.2
100°C, 5 min	24.3 ± 0.5	24.1 ± 1.6*
Nitrogen	21.4 ± 0.5	13.2 ± 3.6
3 mM SKF 525A	84.1 ± 0.7*	30.2 + 0.2*
1 mM 4-methylpyrazole	54.8 ± 1.6	24.8 ± 0.7
1 mMdiethyldithiocarbamate	73.0 ± 0.5	13.9 ± 0.2
1 mM methylmercaptoimidazole	91.1 ± 2.0*	20.7 ± 0.2
1 mM thiobenzamide	89.1 ± 0.7*	46.1 ± 0.7
30 μM indomethacin	174.3 ± 11.4	33.6 ± 3.4**
1 mM acetylsalicylic acid	102.5 ± 9.5**	44.1 ± 2.7**
5 mM 3-amino-1,2,4-triazole	95.5 ± 1.6**	38.2 ± 2.0**
10 μM diphenyleneoiodonium	48.2 ± 1.6	27.0 ± 4.1*
1 mM deferoxamine	85.5 ± 1.1*	23.9 + 0.2
1 mM dapsone	64.3 ± 4.3	35.0 ± 1.4**
100 μM nordihydroguaiaretic acid	67.3 ± 1.4	28.2 ± 1.6*
10 μM 1-phenyl-3-pyrazolidone	61.4 ± 0.9	52.5 ± 4.1**
50 μM esculetin	50.0 ± 3.6	11.1 ± 4.3
50 μM gossypol	28.2 ± 4.8	25.5 ± 5.0
10 μM quercetin	55.9 ± 0.2	30.0 ± 3.0**
10 μM curcumin	34.5 + 3.2	14.8 ± 3.6

*$p < 0.05$ when compared with control (air ± NADPH)
**$p > 0.05$ when compared with control (air ± NADPH)
[a]Incubation mixtures containing microsomal preparations (0.9 ± 0.5 mg of microsomal protein/ml), NADPH generating system, and 140 mM ethanol were conducted for 1 h at 37°C. Acetaldehyde was measured in the headspace of each sample after adding 1 ml NaCl saturated solution. See Materials and methods for details. Each result corresponds to the mean of three separate lots of pooled testes samples. GC-FID determination for each head space sample was made by triplicate

1-Hydroxyethyl radical formation by the testicular microsomal fraction

Figure 1 shows the capillary GC-MS analysis (SIM mode) of the reaction products formed when ethanol transformation by testicular microsomes was performed in the presence of the spin trap PBN. The spin adduct of the 1-hydroxyethyl radical (1HEt) was detected (Fig. 1a) when NADPH was present, while only traces of it were observed in its absence (Fig. 1c). Two additional peaks were detected (A and B in Fig. 1a and b). They correspond to adducts arising from the interaction between

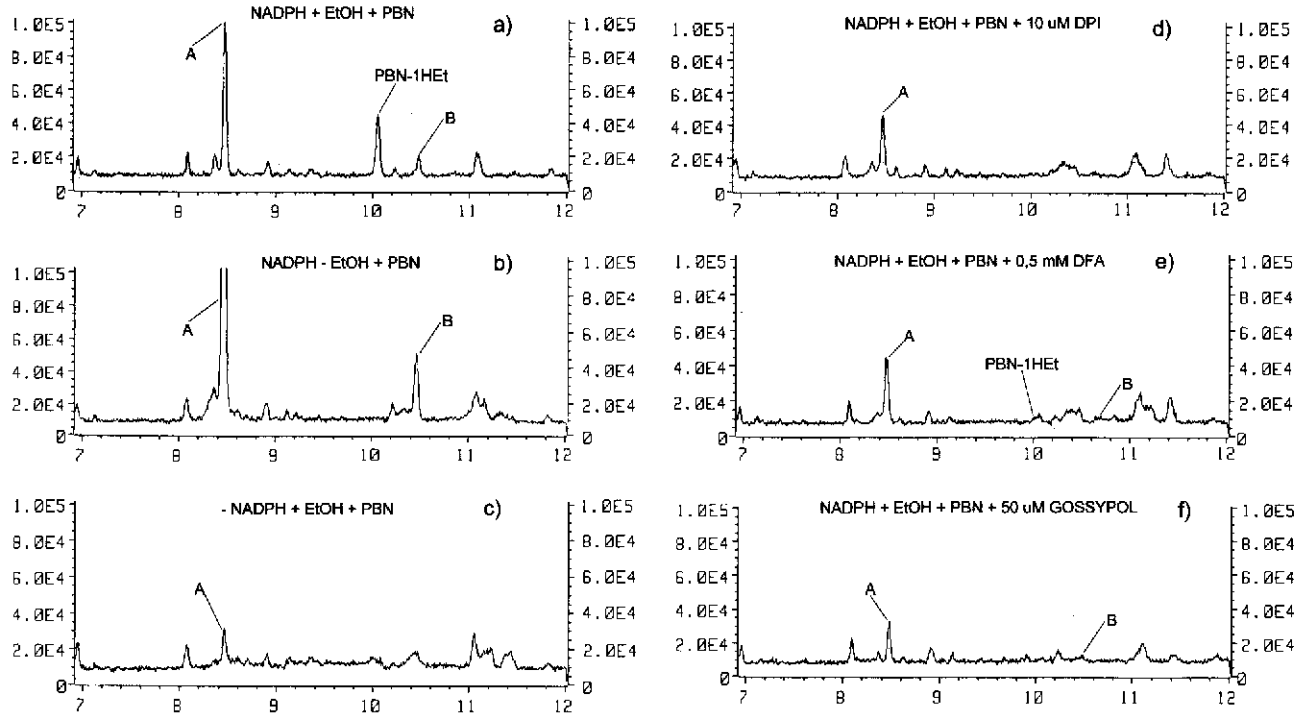

Fig. 1 Selected-ion current profile obtained from GC–MS SIM analysis of a sample of incubation containing microsomes and PBN. **a** NADPH and 150 mM ethanol. Peaks: A and B, hydroxyl-derived PBN adducts, PBN-1HEt, 1-hydroxyethyl-PBN adduct. **b** The same as in **a** but in the absence of ethanol. **c** The same as in **a** but in the absence of NADPH. **d** The same as in **a** with 10 μM DPI. **e** The same as in **a** with 0.5 mM DFA. **f** The same as in **a** with 50 μM gossypol

hydroxyl radicals and PBN (Castro and Castro 2001). They can be observed even in the absence of ethanol (Fig. 1b). Their formation was previously observed in different experiments and they were identified as aromatic-hydroxylation derivatives of PBN. Hydroxyl radicals are formed during NADPH/O_2-dependent microsomal metabolism (Castro and Castro 2001). The 1HEt formation was strongly decreased by the presence in the incubation media of 10 μM DPI (Fig. 1d), suggesting the involvement of a flavoprotein in the process. Non-heme iron would also have a role since DFA was able to pronouncedly decrease the formation of free radicals (Fig. 1e). As in the case of acetaldehyde studies analyzed above (Table 1), 50 μM gossypol also showed to be a potent inhibitor of the pathway leading to free radical formation (Fig. 1f).

Discussion

The results obtained evidenced the presence in the testicular microsomal fraction of an enzymatic NADPH and oxygen-dependent ability to transform ethanol to acetaldehyde. In fact, activity was destroyed by heating 5 min to 100°C and disappeared when the air atmosphere was replaced by oxygen-free nitrogen. Part of the activity would be cytochrome P450 (P450)-dependent and more specifically related to the known presence of the CYP2E1 isoenzyme in the Leydig cells (Jiang et al. 1998). This is suggested by the significant inhibitory effects of SKF525A, and specifically by 4-methylpyrazole and DDTC, two inhibitors of CYP2E1-mediated transformations (Lieber 1999). The participation of the P450 reductase in the process might be suggested by the marked inhibitory effect of DPI. In effect, DPI is a specific inhibitor of flavoenzymes and a known inhibitor of P450 reductase (McGuire et al. 1998). P450 reductase participation might be either indirect as a CYP2E1 reducing enzyme (Strobel et al. 1995) or direct via the participation of its reduced FAD semiquinone form to produce both acetaldehyde and 1-hydroxyethyl radicals, as previously reported by our laboratory (Díaz Gómez et al. 2000).

The inhibition by several of the chemicals tested also suggests the involvement of other enzymes or chemicals in the microsomal oxidation of ethanol to acetaldehyde. For example, the minor but significant inhibitory effects of MMI and TBA suggest the potential participation of the flavine monooxygenase system (FMO), since both have been proposed as relatively specific inhibitors of this enzyme (Hodgson et al. 1991; Poulsen and Ziegler 1995); however, other non P450-dependent oxidases and peroxidases are also inhibited by these two chemicals (O'Brien 2000; Lagorce et al. 1997; Petry and Eling 1987). Still other enzymes able to participate in the oxidation to acetaldehyde could be a lipoxygenase-like one. In effect, low concentrations of several compounds usually considered to be specific inhibitors of lipoxygenase were potent inhibitors of the alcohol metabolic transformation to acetaldehyde. They were NDG, gossypol, 1-phenyl-3-pyrazolidone, and esculetin (O'Brien

2000; Hover and Kulkarni 2000; Joseph et al. 1993; Skozypezak-Jankun et al. 2003). The participation of a peroxidase is also suggested by the inhibitory effect of dapsone. This compound has repeatedly been considered as an inhibitor of lactoperoxidase (Doerge 1986; Shin et al. 2000).

Part of the process of transformation of alcohol to acetaldehyde involves the participation of iron as suggested by the effect of deferoxamine. This compound has been described as a very potent inhibitor of iron-mediated reactions (Halliwell 1985).

Despite the difficulty to definitively establish the identity of all the enzymes involved in the microsomal transformation of ethanol to acetaldehyde, the present studies allow to postulate the partial involvement of CYP2E1 and P450 reductase, but also of other enzymes having lipoxygenase- /peroxidase-like behavior. The lack of response of AMT and of indomethacin or acetylsalicylic acid suggests that catalase or cyclooxygenases would not have any role in this activation process. In effect, AMT is a potent inhibitor of catalase (Lieber 1996; Nicholls 1962) and indomethacin or acetylsalicylic acid are general inhibitors of cyclooxygenases (Degen et al. 2002).

Rat testes microsomal preparations in the presence of NADPH were also able to produce both hydroxyl and 1-hydroxyethyl free radicals. Their generation was not modified by the presence of the general P450 inhibitor SKF 525A (3 mM), despite it slightly but significantly inhibits the oxidation to acetaldehyde. This would indicate that P450 may not be directly involved or that could have only a minor role in its production; however, the potent and specific inhibitor of flavoenzymes (e.g., P450 reductase) DPI fully suppressed free radical production at concentration as low as 10 μM. Our laboratory previously reported that P450 reductase is able to generate 1HEt and hydroxyl radicals when in presence of NADPH. That effect was attributed to the NADPH-mediated generation of a semiquinone at the FAD site of the enzyme (Díaz Gómez et al. 2000).

Gossypol at 10 μM concentration was also able to suppress the formation of free radicals and that could be interpreted as showing a participation in their formation of a lipoxygenase-like enzyme since gossypol is a powerful inhibitor of this enzyme (Joseph et al. 1993; Hover and Kulkarni 2000). Alternatively, gossypol may act as a free radical trapping agent. We were not able to discriminate between these two possibilities in our experiments.

A highly significant part of the free radical production requires the presence of iron. This has been evidenced not only because of the inhibitory effects of deferoxamine added to incubation mixtures, but also when microsomes were prepared using buffers containing DFA (Halliwell 1985).

Results obtained using DPI, DFA, or gossypol, when measuring either acetaldehyde or 1-hydroxyethyl formation, are not contradictory each other. In effect, the three of them decreased both processes. The quantitative differences observed in response may merely suggest that not all the microsomal pathways of acetaldehyde formation from ethanol have 1-hydroxyethyl as a necessary metabolic intermediate.

Irrespective of the detailed knowledge of the rat testicular microsomal enzymes involved in the bioactivation of ethanol to acetaldehyde and free radicals, the fact remains that both reactive moieties are able to interact with cellular components such as proteins, lipids, nucleic acids, and others. In effect, acetaldehyde is able to give adducts upon reaction with amino groups from amino acids (e.g., lysine); nucleic acid bases, or phosphatidylethanolamine and others (Brooks 1997; Lieber 1990, 1996; Díaz Gómez et al. 1999). It can also react with glutathione and decrease its content to diminish GSH-dependent cellular defenses against oxidative stress and allow a lipid peroxidation process (Mantle and Preedy 1999). Lipid peroxidation may also be initiated by the production of 1HEt and hydroxyl radicals evidenced here (Mantle and Preedy 1999). The occurrence of lipid peroxidation in testes has previously been shown in animals repetitively drinking alcohol (Nordmann et al. 1990; Rosenblum et al. 1989; Van Thiel et al. 1989). This might be of importance because spermatozoa are rich in unsaturated fatty acids their membranes being very susceptible to lipid peroxidation (Sikka 2001). Furthermore, the generation of reactive oxygen species was postulated to play a role in inhibition of sperm motility and in loss of fertility (Sikka 2001).

As a consequence of these interactions, some enzymatic reactions may result altered and cellular injury can occur. Ethanol feeding to rats has been shown to decrease the activity of at least three microsomal enzymes which are important in steroidogenesis: 3β-hydroxy steroid dehydrogenase / δ5–4 isomerase; desmolase; and 17α-oxidoreductase (Van Thiel et al. 1987). The present studies did not allow to establish whether the decreases in these enzymatic activities could be attributed to the microsomal ethanol oxidative pathways described in our studies; however, the finding of potent inhibitory non-toxic compounds able to block them, such as NDGA, curcumin, or quercetin, open the possibility in the future to test whether that might be the case.

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