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Review

Alcohol consumption and reproductive toxicity: The role of *in situ* metabolism of ethanol in target organs

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ABSTRACT

Epidemiological studies conducted in different countries showed that alcohol abuse among the youth and the adolescent is a problem of growing concern and relevance. Consequently, it can be envisaged that the number of adult drinkers is going to increase in the years to come. The detrimental effect of alcohol consumption on the health of young people is particularly serious when one considers that, with respect to reproductive health, this is the age range more relevant in both sexes. In women, reproductive health is not a minor issue, considering the alarming increase in drinking at an age directly related to the fertility window. It is important to note that due to differences in the metabolism of ethanol, women, compared with men, are facing an increased risk of negative consequences associated with the consumption of large amounts of alcohol. Harmful consequences of alcohol abuse have been reported in women such as a significant risk of infertility and an increased risk for endometriosis. Other studies concluded that the probability of a successful pregnancy decreased with the increase in the consumption of alcohol. For men, a large number of epidemiological studies evidenced that excessive alcohol consumption is associated with impaired testosterone production and testicular atrophy. Reproductive disorders caused by drinking should certainly involve alterations in critical hormonal factors controlling reproductive functions but are also related to the direct toxic action of ethanol and its metabolites in the organs that constitute the reproductive system of both sexes.

KEYWORDS: ethanol, acetaldehyde, reproductive toxicity, alcohol, uterus, testes, prostate, ovary, oxidative stress

INTRODUCTION

The problem

Alcohol abuse can lead to serious consequences, such as social and psychological problems, several clinical pathologies, physical violence, risky sexual behavior and its related infectious diseases, increased suicides and homicides, and impaired growth among others [1].

At present, many countries recognize the serious problems in public health caused by the abuse of alcohol and have taken steps to adopt preventive policies, particularly oriented to reduce drunk-driving and the carnage that it causes. However, the complexity of the problem far exceeds this aspect.

Alcohol consumption starts at an increasingly young age. It has been shown that adolescents consume more alcohol in less time compared to adults, this being a worldwide phenomenon. Studies conducted over years in the United States

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revealed a direct association between drinking at an early age (before age 14) with the development of alcoholism in later stages and a higher percentage of episodes of violence and accidents [1-3]. The questions asked in epidemiological studies in adolescents focused on information related to family history and genetic vulnerability, socioeconomic characteristics, minority social groups, willingness to buy alcohol, temperament and other factors. These questionnaires lead to the identification of specific groups in the society that have a higher drinking problem. Other studies indicated that four out of five high schools boys consumed alcohol, two in five have had episodes of heavy drinking (five or more drinks in a row for men and four or more for women) in the past two weeks or 30 days depending on the questionnaire [2].

In 2005, worldwide consumption of alcoholic beverages was equivalent to 6.13 liters of pure alcohol consumed per person aged 15 years or older. Much of this consumption (28.6%, or 1.76 liter per person) was homemade and from illegal production (for example, unrecorded alcoholic beverages). Consumption of homemade alcohol may be associated with an increased risk of injury due to the presence of impurities or unknown contaminants. There is great variation between countries in per capita consumption of alcohol [1].

Strong and sporadic consumption of alcohol ("heavy episodic drinking") is another pattern that measures the risk of alcohol consumption, as it is associated with serious consequences of mortality and morbidity. The World Health Organization defines "heavy episodic drinking" as the act of drinking at least 60 grams of pure alcohol (corresponding approximately to a liter of beer or, a half liter of wine or 150 milliliters of whisky), at least once in the last seven days. Worldwide, about 11.5% of drinkers have weekly heavy episodic drinking occasions, with a clear predominance of men in comparison to women [1].

Although the use of alcohol increases gradually in both sexes, treatment programs tend to focus on men, disregarding the needs of women. Drinking was a habit traditionally associated with men and consumption control rested in the family. The situation today has changed, especially in the generation of young women who have significantly increased alcohol consumption. As a consequence, gender differences become increasingly smaller [1].

One of the factors to be taken into account to understand the susceptibility of women to alcoholic beverages is biology, at the level of toxicokinetics. In comparison to men, women have lower activity of gastric enzymes able to oxidize ethanol, a greater proportion of body fat (less volume of water for distribution of ethanol) and tolerance of the characteristic symptoms of the "hangover" is lower. Regarding cultural differences, in some countries acute and excessive consumption of alcohol is associated with the demonstration of masculinity, therefore, not allowing women to drink is a means of subjugation, preventing gender independence. However, with the feminist movement, women sought to occupy traditionally male roles and increased alcohol consumption, with adverse consequences [1].

Further, in recent years, the apparently favorable effects of moderate alcohol consumption for long-term health have received good publicity and widespread diffusion. In addition, it is argued that alcohol use is deeply integrated into the social and even religious traditions of many societies and that the manufacture of alcohol and related industries contribute significantly to employment and capital income through taxes. In particular, alcohol control policies are sometimes unpopular; no one believes in its efficacy or does not accept that the problems faced are so serious as to justify such interventions.

Scientific communications and the international and local press have repeatedly highlighted the growing problem of alcohol consumption, especially among youth and adolescents.

The detrimental effect of alcohol consumption on the health of young people is particularly serious when one considers that with respect to reproductive health, this is the age range more relevant in both sexes. Reproductive health of women is not a minor issue considering the alarming increase in the consumption of alcoholic beverages by this age group due to their association with the fertility window. The data shown in the report of the World Health Organization demonstrated that alcohol consumption in many countries requires a thorough analysis and decision-making in relation

to health policy. Among the American countries, Argentina revealed to be the first in terms of consumption and this has led to concern among public health authorities towards the need for an evaluation of the impact on the youth [4, 5].

Female reproductive system and the effects of alcohol

Epidemiological studies conducted in different countries including Argentina showed that alcohol abuse in women is a problem of growing concern. The harmful effect of drinking habits on health was specifically observed in young women [1]. Consequently, it can be anticipated that the number of adult drinkers would increase in the coming decades. In this regard, it is important to consider that due to differences in the metabolism of alcohol, women compared with men are facing a higher risk of negative consequences associated with the consumption of large amounts of alcohol.

Unlike the male gonad, female gonads have a finite number of germ cells at birth and are therefore uniquely sensitive to reproductive toxicants. Such exposure can lead to decreased fecundity, increased pregnancy wastage, early menopause, and infertility, depending on the component affected, the magnitude of the damage, and the timing of the exposure. In consequence, ovarian functions are potentially susceptible to interruption by xenobiotics. Several examples illustrating this possibility are available in literature [6-11].

Alcohol severely alters the normal menstrual cycle in both women and rats. It is known that alcoholic women present a variety of menstrual and reproductive disorders, from irregular cycles until the cessation of menstruation, lack of ovulation to infertility and early menopause [12, 13]. Menstrual problems did not seem to occur in women who were occasional drinkers or who were moderate drinkers consuming fewer than two drinks per day. However, 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 suppression of ovulation and an increased risk for spontaneous abortion through interference with the pregnancy-maintaining function of the corpus luteum. Further, it has been observed that ethanol significantly decreases sexual responsiveness in women as well as in men [12, 14-18]. Some researchers studied the cause of infertility in 3,800 women and reported that moderate alcohol intake was associated with a small but significant risk of infertility and an increased risk for endometriosis [19]. Moreover, other studies concluded that the probability of a successful pregnancy decreased with the increase in the consumption of alcohol [20-22]. A toxic effect on the functions of the ovary is one of the many reasons for infertility [23, 24]. Alcohol can also affect implantation in the early development of the blastocyst [25]. All these reproductive disorders caused by drinking certainly involve significant changes in critical hormonal factors [12, 13]. These alterations include an association with increased levels of plasma estrogens in pre- and postmenopausal women and with low levels of progesterone in premenopausal women [26]. A more serious consequence of heavy drinking is a teratogenic effect described among alcoholic women which has been named fetal alcohol syndrome [17].

Some researchers 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 [17]. However, not all the effects of alcohol on the reproductive organs and associated tissues can be explained only in terms of these endocrine alterations.

The possibility that both ethanol and its products of biotransformation are involved in these changes should be considered. This seems to be the case for the toxic effects of alcohol intake on other organs like the liver and the upper aero-digestive tract [27]. Research conducted in our laboratory showed that ethanol metabolism takes place *in situ* in mammary tissue, uterus, ovary, and the oviduct, and at the same time several manifestations of injury are expressed [24, 28-30].

Ovaries

In our studies performed with rat ovaries, we provided evidence that the microsomal fraction has the ability to metabolize ethanol to acetaldehyde,

a significant portion of this metabolism being enzymatic in nature [24]. A part of this metabolism showed a dependence on the presence of NADPH and could be attributed to the presence of P450 and P450 reductase in the microsomal fraction, as suggested by the inhibitory effect of SKF 525A, sodium diethylditiocarbamate and 4-methylpyrazole, suggesting the participation of CYP2E1 (in a similar way to the microsomal metabolism of ethanol to acetaldehyde in liver). Our research demonstrated also that microsomal oxidation of ethanol to acetaldehyde was significantly enhanced when animals were repeatedly exposed to alcohol. The participation of NADPH P450 reductase was also suggested by the significant inhibitory effect of diphenyleneiodonium (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 [31]. 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 [31, 32] but was also shown by our laboratory to be able to activate ethanol to acetaldehyde [33]. This latter ability of P450 reductase might offer an explanation for the evidence of an anaerobic transformation of ethanol to acetaldehyde and its susceptibility to DPI [33].

The presence of P450 or P450 reductase enzymes in the ovaries and also the ability of its microsomal fraction to activate several CYP2E1 substrates were previously established by others and by our laboratory [11, 34, 35].

The lack of response to nordihydroguaiaretic acid and esculetin excludes the participation of lipoxygenases in the microsomal metabolism of ethanol to acetaldehyde in the rat ovarian tissue, since both compounds are potent inhibitors of these enzymes [36]. This behavior was completely different from the one reported by our laboratory for the case of other hormone-responsive tissue like the mammary glands [37].

The NADPH-independent microsomal enzymatic pathways of ethanol oxidation remain to be fully characterized. However, their susceptibility to DPI suggests that they may be flavoenzymes. The ability of these 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 [24].

It is interesting to point out that the ovarian microsomal fraction also has a small but detectable aldehyde dehydrogenase (ALDH) activity. The microsomal fraction exhibited a NAD^+-dependent ALDH activity of the same order of magnitude (in terms of nmol NAD^+ consumed per mg protein) as that of the NADPH-dependent microsomal pathways producing acetaldehyde. This points to the relevance of the relative levels of both cofactors, NAD^+ and NADPH, under given experimental conditions. The ability to handle acetaldehyde in the microsomal fraction by ALDH is roughly equivalent to that of the mitochondrial fraction [24].

The response of the cytosolic enzymes, able to generate acetaldehyde from ethanol in the presence of NAD^+, to inhibitors reflects the complexity involved in the interpretation of the obtained results [24]. In fact, the effect of disulfiram revealed the formation of increased concentrations of acetaldehyde. This effect of disulfiram might be attributed to its known inhibitory effect on ALDH, which we revealed in the ovarian tissue 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. This was clearly shown using hypoxanthine rather than NAD^+ as cosubstrate, thereby avoiding the operation of ALDH which 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 xanthine oxidoreductase (XOR) at low concentrations [38]. Repetitive alcohol intake strongly decreased the XOR-mediated cytosolic metabolism of ethanol to acetaldehyde, using hypoxanthine as cosubstrate. It is interesting to compare this behavior of the ovarian cytosolic XOR enzyme to that present in mammary tissue cytosolic fraction. In the latter case the XOR-mediated oxidation of ethanol to acetaldehyde significantly increased its activity after repetitive alcohol intake for 28 days [28].

The generation of acetaldehyde *in situ* may harm ovarian tissue. It is well known that acetaldehyde is a mutagenic, carcinogenic and toxic chemical able to react with DNA, proteins, lipids and with many other relevant molecules such as glutathione

(GSH) [17, 39]. The decrease in GSH content resulting from the attack of acetaldehyde on this critical antioxidant molecule was blamed by other researchers to be responsible for a significant part of the ethanol-induced oxidative stress in other organs [40]. A preliminary indication that an increased susceptibility to oxidative stress could be present in ovaries after repetitive alcohol intake was obtained in our experiments, where ovarian tissue homogenates were challenged with *t*-butylhydroperoxide (tBHP) and the formation of reactive oxygen species (ROS) was followed by chemiluminiscence emission. It is considered that the intensity of the tBHP-induced chemiluminiscence is an expression of the ROS formation in biological samples and of the defensive capacity of living systems against oxidative stress [28, 41, 42].

Oxidative stress was evidenced in our experiments on tBHP-induced chemiluminiscence emission in ovarian homogenates derived from animals repeatedly exposed to alcohol for 28 days, we observed major changes in the chemiluminiscence emission curves. These included not only a significant enhancement in the intensity of the emitted chemiluminiscence but also important changes in the shape of the emission curve, characterized by an early initiation of the emission. These two different responses to the tBHP challenge might be interpreted as an indication of decreased presence of defenses in the ovarian tissue as well as changes in the composition of the tissue itself attributable to ethanol intoxication, which favored ROS production when exposed to tBHP [24].

An important consequence came up from the fact that ovarian tissue was found to oxidize ethanol to acetaldehyde via different metabolic pathways. As a result of ethanol consumption it becomes more susceptible to oxidative stressful conditions. Ovaries from poisoned animals evidenced ultrastructural alterations as a consequence of alcohol exposure. Repeated administration of the Lieber and De Carli diet for 28 days produced severe ultrastructural alterations in the different cell types of the ovaries [24]. These alterations occurred irrespective of the stage of ovarian cycle of the animals at the time of their sacrifice. The deleterious effects were oberved in the secondary follicles, the oocyte, the corona radiata cells, the zona pellucida and the granulosa cells. The alterations observed involved every cell compartment and organelle. The most intense deleterious effects were observed in the granulosa cells. Nuclear alterations varied from marked condensation of chromatin attached to the nuclear membrane, intense dilatation of the outer perinuclear space to nuclei having very irregular forms or even bizarre forms [24].

Alterations observed in the rough endoplasmic reticulum 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 appeared swollen. The *in situ* formation of acetaldehyde and the increased susceptibility to oxidative stress exhibited by the rat ovarian tissue described here might play a role in these observed deleterious effects.

It is likely that acetaldehyde and ROS produced by the exposure of ovaries to alcohol play some role in these ultrastructural alterations. These severe alterations observed in the tissue ultrastructure should significantly impair the function of the ovary, leading to a decreased number of healthy functional oocytes and also to altered hormone synthesis and secretion.

The significance of these early experiments rests on the fact that the harmful effects of alcohol consumption on rat ovaries described here could be reasonably linked to severe alterations in their known dual function of being responsible for both the production and release of the ovum as well as the biosynthesis and secretion of the key steroid hormones, progesterone and estrogen [43, 44].

There are other plausible alternatives or additional mechanisms potentially involved in the ovarian ethanol-induced toxicity beyond those linked to local activation by acetaldehyde and free radical-induced oxidative stress. One alternative mechanism could be related to any acetaldehyde arriving via blood from the 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 the rat mammary tissue, evidenced that only limited

concentrations of acetaldehyde remain in blood even after large doses of alcohol were given to the rat [42]. Further, other indirect mechanisms might also be involved in the alcohol-promoted effects in the ovaries, operating at the level of the central nervous system [10, 18, 45].

Uterus

Another target organ that attracted our interest was the rat uterus. In our experiments we provided evidence that the rat uterine horn has its own ability to oxidize alcohol to acetaldehyde, in the different purified subcellular fractions tested. These results are in agreement with previous findings of Messiha, who detected the presence of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase in the female rat genital system [46]. In addition, we found that the uterine horn cytosolic fraction has an XOR-mediated pathway for the metabolism of alcohol to acetaldehyde. This was clearly shown using hypoxanthine as cosubstrate and by inhibiting this pathway with allopurinol, ellagic acid or folic acid, that are potent inhibitors of XOR at low concentrations [38]. Our histochemical studies evidenced that this enzymatic activity was localized essentially in the epithelial cells [47]. In contrast, no ADH activity was detected histochemically in the uterine horn, despite the fact that a minor activity was biochemically measured in the cytosolic fraction. This activity was significantly lower than the one determined in liver.

We also showed that the microsomal fraction from the rat uterine horn has the ability to metabolize ethanol to acetaldehyde. A significant portion of this metabolism was of an enzymatic nature, requiring oxygen from air to proceed. NADPH was not required for the microsomal oxidation of alcohol to acetaldehyde and chlormethiazol did not inhibit this metabolism. This behavior completely excludes the participation of a CYP2E1 mediated process, in contrast to the liver microsomes [27]. Interestingly, the microsomal enzymatic process was significantly inhibited by DPI at low concentrations, as we observed in the case of the rat ovaries. The most frequent use of this inhibitor is to prove the participation of NADPH oxidase. In our experiments the microsomal enzymatic system did not require NADPH to proceed and that excludes NADPH oxidase as the enzyme involved. However, the potency of the DPI inhibitory effect still suggests that a flavoenzyme would be involved in the process. We were not able to define its nature [47].

Notwithstanding, the behavior of the microsomal metabolic process against given inhibitors might give a clue about the role this flavoenzyme could have in the transformation of ethanol to acetaldehyde in the uterine horn microsomal fraction. For example, we found that the presence of aminotriazole or sodium azide in concentrations known to inhibit catalase also significantly inhibited the production of acetaldehyde in microsomes [48]. The metabolic process by which catalase can produce acetaldehyde from ethanol has been thoroughly described and requires the participation of hydrogen peroxide [27]. Catalase proved to be present in uterine tissue [49].

Concerning the source of the needed hydrogen peroxide for the overall metabolic process to proceed, we envisaged a potential additional role for the above described flavoenzyme. According to this hypothesis the putative flavoenzyme should be a flavoprotein oxidase. Flavoprotein oxidases convert their substrates, (e.g. ethanol to acetaldehyde in our case) with the concomitant reduction of molecular oxygen to hydrogen peroxide [50]. This hydrogen peroxide would supply catalase with the necessary co-substrate to further metabolize ethanol to acetaldehyde.

As expected, levels of ethanol present in the uterine horn after an acute dose of alcohol tend to be closer to those occurring in plasma but higher than those found in its liver counterpart. This might reflect the fact that the liver has a greater ability to metabolize ethanol to acetaldehyde and to detoxify it to less harmful compounds than the uterus. Liver ALDH plays a critical role in this respect. In the case of the uterine horn we found that the ALDH activity present in the mitochondrial fraction is almost negligible and not detectable at all in the microsomal or cytosolic fractions. A minor ALDH activity proved to be present, in our histochemical studies, in the muscular and serous parts of the uterine horn [47].

The presence of metabolic pathways in the rat uterine horn able to oxidize ethanol to acetaldehyde in its microsomal and cytosolic fractions and its

low capacity to handle it through ALDH suggests a potential tendency for this tissue to accumulate very toxic levels of acetaldehyde during ethanol exposure, which proved to be the case in our experiments. This strongly suggests that any acetaldehyde produced *in situ* or arriving to the uterine horn via blood would remain in this organ long enough to have the opportunity to react with critical molecules to cause deleterious effects.

The mere macroscopic observation of the uterine horns from rats exposed to repetitive alcohol consumption revealed a very significant decrease in the diameter of the uterine horns, from animals at the same stage of the cell cycle. This effect can be quantified by the highly significant weight loss observed in the uterine horns from animals exposed to alcohol [30].

The harmful effects of repetitive alcohol exposure on the uterine horns were accompanied by severe alterations in the ultrastructure of their cellular components when compared to that in control animals, as revealed by our electron microscopy studies. Columnar epithelial cells from the uterine horn mucosa exhibited marked alterations of their organelles. This included marked vacuolization and dilatation of the nuclei, endoplasmic reticulum and Golgi apparatus membranes. There was a general disorganization of the cellular structure [30].

The wide derangement of the cellular structure observed in the rat uterine horn suggests the presence of a chemically-induced cell injury process beyond the unavoidable hormone-mediated effects promoted by alcohol drinking. This shows that not all the effects of alcohol in these target organs can be explained only in terms of endocrine disturbances [24, 28, 51].

In the uterine horn microsomal fraction from control animals and from those receiving ethanol via the Lieber and De Carli diet for 28 days, the presence of a NADPH-dependent pathway of oxidation of ethanol to acetaldehyde was observed. A little but significant increase in ethanol metabolism was found by repetitive alcohol exposure. In the course of our previous studies we reported the presence of metabolic pathways of oxidation of alcohol to acetaldehyde in the cytosolic fraction mediated by xanthine oxidoreductase and also activity of ADH [47]. The depletion exerted by NAD^+ or hypoxanthine on the cytosolic metabolism of alcohol to acetaldehyde at the uterine horn suggests that repetitive alcohol exposure induces an enzyme able to further oxidize acetaldehyde. This hypothesis rests on the fact that in these experiments we are measuring an intermediate metabolite, subject to a rapid degradation.

Acetaldehyde is a reactive molecule able to covalently bind to DNA, proteins, lipids and other molecules such as GSH. However, the production of reactive metabolites by ethanol is not limited to the generation of acetaldehyde. It is known that in the case of other tissues such as liver, prostate, testes, ovaries or mammary tissue, formation of free radicals occurs (eg., 1-hydroxyethyl, hydroxyl or acetyl) [33, 52-57]. This is of relevance because free radicals may lead to additional covalent binding processes and, further, to hydrogen abstraction reactions of oxidative nature in DNA, proteins and lipids (for example, lipid peroxidation) that might provoke oxidative stress if cellular antioxidant defenses are exceeded [58].

In our studies we found that some manifestations of oxidative stress occur in the uterine horn when animals were exposed chronically to alcohol. We detected the formation of hydroxyl free radicals in the microsomal fraction of uterine horn tissue when incubated in the presence of NADPH generating system. The generation process is less intense than the equivalent one occurring in the liver microsomal fraction. An additional source of hydroxyl radicals was found in the uterine horn cytosolic fraction in the presence of ethanol, when the incubation system included hypoxanthine as a cofactor.

The uterine horn cytosolic hydroxyl radical generation process is significantly less intense than the one occurring in the liver cytosolic fraction. These hydroxyl radical formation processes were mediated by xanthine oxidoreductase as evidenced by their complete inhibition by allopurinol, a specific inhibitor of this enzyme [30]. It is interesting to point out that the potential significance of these cytosolic free radical generating metabolic pathways might be favored under circumstances of alcohol consumption, since it is very well known that the formation of purine degradation products is enhanced during alcohol exposure and consequently, the availability of the necessary cofactors for these cytosolic metabolic pathways could be increased [59].

Decrease in cellular defenses might lead to the occurrence of oxidative stress at uterine horn level. This was suggested to occur in our experiments with uterine horn homogenates from rats chronically consuming alcohol for 28 days and measuring chemiluminiscence induced by tBHP. In effect, significantly higher levels of chemiluminescence were emitted by uterine horn homogenates challenged with t-butylhydroperoxide from alcohol treated rats than those from untreated control animals. This finding suggests that defenses against the oxidative challenge in alcohol treated animals could be significantly decreased [47].

At least a part of the diminished defenses can be attributed to a low decrease in GSH content and to a tendency to increase (but not significantly) the oxidized glutathione (GSSG) content and decreases in the GSH/GSSG ratio. The observed decrease in the activity of glutathione peroxidase (GSPx) was of relevance. This might be of particular interest to explain the intense response of the uterine horn tissue to the t-butylhydroperoxide observed in the chemiluminiscence test [30].

The significant roles of GSH, glutathione transferase (GST) and GSPx in the resistance of cells to oxidative damage are well known [60]. The behavior of the uterine horn in response to alcohol consumption for 28 days was different from that of the liver. The liver evidenced an adaptative response leading to increased GSH levels but not of glutathione reductase (GRed) activity. In addition, no significant changes in the level of GSSG, or in the ratio GSH:GSSG or in the GST and GSPx activities were observed. The generation of free radicals and a decrease in cellular defenses in the uterine horn tissue led to early indications of oxidative stress occurrence, such as decreases in the protein sulfhydryl content. However, no increases in protein carbonyl content were found after alcohol consumption for 28 days [30].

Acetaldehyde levels present in the uterine horn tissue, the production of reactive free radicals and the promotion of oxidative stress might be, at least, partially involved in the generation of the significant alterations occurring in the uterine horn tissue ultrastructure.

However, other relevant causes for these significant changes might arise from hormonal changes provoked by alcohol drinking on, for instance, the ovarian tissue, and from the increased levels of estrogen that it promotes [24, 61-63]. These questions remain to be answered in future experiments.

In relation to the carcinogenic risk, alcohol and its toxicity on the uterus is a relevant hypothesis for analysis and study. After all, we are talking about the presence of a mutagenic metabolite, acetaldehyde, formed in a tissue with a low capacity to remove it, along with a condition of oxidative stress that can act as a promoting factor. Several epidemiological studies have demonstrated clearly an increased risk for cervical, vulvar and vaginal cancer among alcoholics than in the general population [64]. However, these findings were not confirmed in population-based studies and adjusted for confounding factors. On the other hand, endometrial, uterine body and ovarian cancer did not seem to have a relationship with alcohol consumption [26].

Studies that have evaluated the role of alcohol consumption in relation to the risk for endometrial cancer have not provided clear evidence so far. However, it would be prudent not to discard a relationship and, in fact, there is a growing interest in the study of a potential epidemiological link between them [65].

Male reproductive system and the effects of alcohol

Testis

An important number of epidemiological studies evidencing that excessive alcohol consumption is associated with impaired testosterone production and testicular atrophy are available in the literature [66-68]. Studies using an isolated perfused rat testis preparation demonstrated that ethanol acts, at least in part, directly on the testis to harm its hormone production [69, 70]. Further, the hypothesis was advanced that the decreasing effects on testosterone production were related to the metabolic transformation of ethanol to acetaldehyde, since the latter was far more potent to inhibit testosterone release than the former under *in vitro* studies in isolated testicular preparations [69-71]. The hypothesis suggested the need to learn more about the enzymatic processes responsible for alcohol oxidation to acetaldehyde in different cellular

fractions, particularly in the microsomal one, since limited information is available in the literature [72, 73].

It has been reported that ethanol acutely reduces circulating testosterone levels in rodents and directly inhibits testosterone accumulation in isolated testes or interstitial cells [69, 70, 74, 75]. These effects were related by several authors in the field, to acetaldehyde formation in rat testes, particularly in their Leydig cells [73-75].

The ability of the testis to metabolize ethanol to acetaldehyde has been documented in several experimental models. Chiao and Van Thiel reported the presence of ADH, Dafeldecker and Vallee identified a testis specific alcohol dehydrogenase, and Juliá *et al*. characterized three isoenzymes of the ADH [72, 76, 77]. Other additional sources of acetaldehyde production from ethanol present in the testicular microsomal fraction were reported by our laboratory [57]. We reported that rat testicular microsomal preparations were able to metabolize ethanol to reactive metabolites such as acetaldehyde, or hydroxyl and 1-hydroxyethyl free radicals [57].

Acetaldehyde production was strongly dependent on the presence of NADPH and oxygen, and apparently involved several enzymes including CYP2E1, P450 reductase and other enzymes having a lipoxygenase or peroxidase-like behavior as suggested by their response to different inhibitors. Murono and Fisher-Simpson described, in purified rat Leydig cells, the presence of a microsomal NADPH-dependent enzyme metabolizing ethanol that was not CYP2E1-dependent [73]. However, it is noteworthy to point out that CYP2E1 was detected in immunochemical studies only in these cells and that this presence was considered linked to the specific testicular toxicity of some CYP2E1-bioactivated chemicals such as 1,3-butadiene [78, 79].

The formation of acetaldehyde and hydroxyl and 1-hydroxyethyl radicals reported in our studies might be of significance in the case of the CYP2E1-containing Leydig cells since acetaldehyde was shown to inhibit the synthesis and secretion of testosterone in these cells at concentrations as low as 5 μM [71]. Further, we reported that after a single high dose of ethanol, its levels present in testis are similar to those in the liver or in plasma. We also found that there is acetaldehyde accumulation in both liver and testes but not in plasma. The acetaldehyde present in testes remained significantly higher than in plasma for at least nine hours [80]. This sustained presence of acetaldehyde in testes might be related in part to a contribution of it via blood supply, but its *in situ* generation appears important considering the fact that plasma levels of acetaldehyde remained almost constant during the period of acetaldehyde accumulation. It is of interest to point out that acetaldehyde accumulation reported was of the same magnitude as those previously found to reduce steroideogenesis and secretion of testosterone by Leydig cells in culture [74, 75]. Emanuele and Emanuele clearly suggested the need for researchers to learn more about the cellular mechanisms underlying the toxic effects of ethanol in the male reproductive system in order to develop effective approaches to reverse or prevent these effects [67].

In our studies we focused our interest on the microsomal NADPH and oxygen-dependent testicular system leading to acetaldehyde production. This interest rests on the fact that during these studies the formation of hydroxyl and 1-hydroxyethyl free radicals was observed, and that in previous works by others oxidative stress manifestations were anticipated, like the occurrence of lipid peroxidation measured by formation of malondialdehyde (MDA) [81-83]. Grattagliano *et al.* produced additional evidence for occurrence of oxidative stress, showing that after repetitive alcohol exposure not only MDA promotion was observed, but also protein oxidation and a significant depletion of glutathione, alpha-tocopherol and ascorbic acid were also observed [84]. We confirmed these findings under two different experimental conditions, one being after a single dose of alcohol and the other after the exposure of the rats to a regular alcohol-containing Lieber and De Carli diet for 28 days. In both cases we found a significant increase in the formation of lipid hydroperoxides as determined by the xylenol orange method [80].

All these findings point to the potential significance of this microsomal NADPH and oxygen dependent system that generates deleterious moieties for the testicular functions: acetaldehyde and reactive

oxygen species [57, 80]. Their relevance rests on the well known fact that spermatozoa are rich in unsaturated fatty acids, their membranes being very susceptible to lipid peroxidation [85]. Furthermore, the generation of reactive oxygen species was postulated to play a role in inhibition of sperm motility and in loss of fertility [85]. In addition, both acetaldehyde and oxidative stress produced via the microsomal pathway might be involved not only in the decrease in testosterone generation, but also in the already observed enhancement of apoptosis of germ cells within the rat testes and in the increase in testicular levels of p53 mRNA, provoked by ethanol exposure [86].

The ability of plant polyphenols to inhibit metabolism of ethanol to acetaldehyde and free radicals in the microsomal fraction was clearly envisaged in our past work with gossypol, esculetin, quercetin and curcumin [57]. In the case of gossypol, even at 10 μM concentration, it was also able to suppress the formation of free radicals. This also was interpreted as indicating a participation of a lipoxygenase-like enzyme in these processes since gossypol may act as a free radical trapping agent. In addition, we further reported the effects of other plant polyphenols on the metabolism of ethanol to acetaldehyde in the testicular microsomal and cytosolic fractions. Representative flavonols, flavones, isoflavones, flavanols, flavanones, phenolic acids and derivatives, stilbenes, lignanes, anthocyanins and other polyphenols were studied [87].

Prostate

The prostate is also a target tissue for research in the field of alcohol toxicity. Prostate diseases such as prostate cancer and benign prostatic hyperplasia (BPH) are important health problems of the aging male. As age is the strongest risk factor for both diseases, their incidences are observed to significantly rise with the prolonged life expectancy of men [88-90]. Despite the obvious relevance of the problem, very little is known about what causes both diseases [89, 91].

Current existing evidence suggests that dietary and lifestyle factors might have significant roles in the incidence of prostate diseases [90, 92, 93]. The potential role of alcohol consumption in relation to the incidence of both diseases was considered in a large number of epidemiological studies [94-99]. One reason for this interest might be the well-established decreasing effect of ethanol consumption on the testosterone plasma levels and in the metabolism of testosterone by the prostate tissue [66, 67, 100-102]. Further, the prostates of ethanol-fed rats had significantly reduced concentrations of androgen receptor sets compared to control rats with a slightly reduced affinity for 5-alpha-dehydrotestosterone [103].

The overall evaluation of reviewed studies shows that alcohol consumption was inversely related to total BPH [96, 104] or that it did not predict BPH [99]. In the case of the reviewed studies concerning the risk of alcohol consumption in relation to an increase in prostate cancer risk, the situation might be different. These studies suggest that there is no association between the amount of alcohol consumed and the risk of prostate cancer [90, 93-95, 98]. In contrast, several large and detailed studies in the past and other recent studies differ and have reported a positive association between alcohol consumption and the risk of prostate cancer [97, 105-113]. Also, some authors suggested that the increased prostate cancer risk was occurring mainly in alcoholics [95].

These studies evidencing a positive association between alcohol consumption and risk of prostate cancer and the possibility that particular populations, for example, alcoholics had an increased susceptibility, led to the recommendation that further research on alcohol and prostate cancer should be continued.

Our studies performed using the model of rat ventral prostate gave evidence that after a single dose of ethanol some degree of acetaldehyde accumulation in prostate tissue can be observed. The increased acetaldehyde levels observed may derive from acetaldehyde produced *in situ* through our previously described cytosolic and non-CYP2E1 microsomal pathway [53, 55] as well as from the presence of a prostatic ADH activity evidenced in further work [114]. However, the contribution of acetaldehyde arriving via blood from other organs [for example, the liver] to prostate tissue might be relevant since prostatic ALDH activities are very low. The microsomal activity detected in control animals was about nine times

lower than the one in liver and far less responsive to induction after repetitive ethanol exposure than the liver. In effect, in the induced animals the liver to prostate ratio changed from 9 to 16 times. The p-nitrophenol hydroxylase activity has been considered a valuable marker of CYP2E1 activity [115]. As we already mentioned, CYP2E1 is known to be involved in the metabolism of ethanol to acetaldehyde and 1-hydroxyethyl radical [27]. This CYP2E1 microsomal mediated pathway of ethanol activation to reactive metabolites might be partially involved in our previously reported studies on the microsomal metabolism of ethanol in prostate tissue [55]. Both, acetaldehyde and 1-hydroxyethyl are able to covalently bind to macromolecules. Acetaldehyde is a mutagenic, carcinogenic and a toxic chemical able to react with DNA, proteins, lipids and other relevant molecular components. In addition, the 1-hydroxyethyl radicals have the possibility to form adducts and to also get involved in hydrogen abstraction reactions upon interaction with DNA, proteins, lipids and other cellular components and promote their further oxidation and get involved in chain reactions.

In rats exposed repetitively to alcohol, the increased susceptibility of prostate tissue to oxidizing conditions was shown by our experiments on the tBHP-induced chemiluminiscence in the rat ventral homogenates. In our experiments we observed that chronic alcohol ingestion increased the susceptibility of rat ventral prostate homogenates to oxidation when challenged with t-butylhydroperoxide. Further, when determination of lipid hydroperoxides by the xylenol orange method was performed in these prostate homogenates an increased production of lipid hydroperoxides was observed [114]. This might be of interest, since it has become apparent that oxidative stress may be an important etiological factor in the development and progression of prostate cancer [116] and it is a well known process involved in chemically induced cell injury and chemical carcinogenesis [117, 118].

A key reason for these increased risks includes DNA damage, protein and lipid modification encompassing the free radical generation overload as well as the changes in the transcriptional activation and/or repression of genes that are responsible for cellular homeostasis [117, 118].

In our observations of the rat ventral tissue ultrastructure from the chronically exposed rats to the Lieber and De Carli diet, we observed in the prostate epithelial cells a markedly dilated endoplasmic reticulum and a significant condensation of chromatin around the perinuclear membrane accompanied by very irregularly shaped nuclei with deep infoldings. Increased presence of apoptotic cells was also observed. These observations confirmed previous results from others (using brandy as the source of ethanol) as well as the work from our laboratory [51, 119].

Our past and present studies on acetaldehyde and free radical generation from ethanol metabolism at the cytosolic and microsomal level might be related to the ultrastructural alterations observed. In the particular case of the promoted apoptotic cell formation by ethanol consumption, the pathway of generation of both acetaldehyde and free radicals might have a special contribution. In effect, the *in situ* metabolism of ethanol to reactive moieties such as acetaldehyde and the very short lived 1-hydroxyethyl, has more chances to target components by either adduct formation or oxidative stress. An additional indirect contribution to the observed pro-apoptotic effects of repetitive alcohol exposure might arise from the well known toxic effects of chronic ethanol exposure on testes [57, 67, 80]. The balance between pro-apoptotic effects and tumor promoting actions of chronic alcohol consumption might partially explain the many conflictive results observed among the epidemiological studies available.

The proposed research study as necessary, according to Breslow and Weed, was what the authors named ‘targeted research’. Among them work on alcohol metabolism by prostate tissue and other studies of biological mechanisms were considered appropriate [94, 97]. The plausibility of alcohol as a risk factor was considered from the evidence that alcohol acts as a carcinogen or leads to formation of a carcinogen or modulates risk from known carcinogens. Another plausible criterion anticipated by Breslow and Weed was that of ‘analogy’ (for example, the numerous studies performed on alcohol and breast cancer showing a positive association with alcohol drinking) [94, 120]. Most prostate cancers are adenocarcinomas, which arise from the epithelial cells of the ducts or acini [90, 93].

All the considerations mentioned above on the relevance of the problem to health and the need to analyze critically the biological plausibility about an association between alcohol drinking and prostate cancer, led us to focus the present review on the effects of alcohol on prostate epithelial cells and on alcohol metabolism to carcinogenic or cancer-promoting metabolites.

CONCLUSIONS

Oxidation of ethanol to acetaldehyde and to other toxic metabolites is likely to occur by a variety of enzymatic pathways and therefore can be expected to occur in different target tissues, including those from the reproductive system. This metabolism does not respond to the well known enzyme profile of ethanol oxidation in the liver, but involves other transformations, which have been characterized only partially but they are responsible for an intrinsic capacity to generate toxic metabolites. The imbalance between the capacity to generate toxic metabolites and that to destroy them is also highly variable depending on the body site. In all the tissues studied, alcohol exposure provoked an oxidative stress process, consistent with the fact that pro-oxidant species are generated, and this affects critical cell components such as lipids and proteins. These tissues in general do not have efficient mechanisms for detoxification of acetaldehyde generated *in situ* or arriving through the blood, and this leads to a transient accumulation of the mutagenic metabolites exhausting cellular defenses, such as glutathione.

These changes may be the primary reasons for the alterations observed. Then, it is not difficult to imagine that each organ may have a different susceptibility to alcohol exposure, regardless of their local concentration (see a summarized analysis in Table 1).

Moreover, ethanol is a chemical with the ability to modify the metabolism of other toxins and endogenous substances like hormones. Organs like the ones constituting the reproductive system in both sexes can be affected by exposure to alcohol in an indirect way, when exposure to other toxic substances in the environment takes place, and when levels of hormones critical to the development and function of these organs are altered.

Other reasons might derive from epidemiological studies when the potential cooperative or synergistic effects of other simultaneous factors able to modulate the response to alcohol are evaluated. Diet could probably be one. For example, one of them might be related to high consumption of purine-rich food. High meat consumption is known to be by itself a relevant factor in prostate cancer promotion [93]. Another reason might be the high consumption of caffeine and/or methyl xanthine-rich containing drinks or beverages in conjunction with that of alcohol. In the case of uterus, a meta-analysis based on case-control studies suggests that consumption of meat (particularly red meat) increases the risk of endometrial cancer. Our laboratory previously evidenced that the XOR present in tissues like the rat uterine horn, ovary, mammary and prostate might use purines as co-substrates to further activate alcohol to acetaldehyde and free radicals [24, 28, 47, 53]. At present, the scientific literature does not provide evidence to establish an association with dairy products, and

Table 1. Hypothesis on the potential effects of alcohol consumption on reproductive organs.

Direct
• Generation of acetaldehyde and ROS *in situ*.
• Interaction with some diet components leading to production of free radicals (e.g. purines).
• Lesions in proteins, DNA and other biomolecules caused by acetaldehyde and free radicals (hydroxyl, 1-hydroxyethyl, acetyl).
Indirect
• Enhancement of pro-mutagen activating metabolisms.
• Changes in hormonal levels.
• Nutritional (eg. depletion of cell antioxidant defenses).
• Inhibition of repair in critical molecules such as DNA.

the available information is inconsistent for poultry, fish, and eggs. An inverse association with intake of dietary fiber was also suggested [121-125]. In all these cases, more prospective studies are needed.

In summary, alcohol toxicity in the reproductive systems of both sexes is subjected to multifactorial mechanisms that include genetic, dietary and environmental components. *In situ* metabolism, however, should be regarded as an important contributing factor because of a principle of similitude, that is, the same toxic metabolites can initiate the early steps of cellular alterations in any tissue, no matter where they are generated.

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CONFLICT OF INTEREST STATEMENT

Authors declare to not have conflicts of interest regarding the research mentioned in this article.

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