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Peripubertal Paternal EtOH Exposure

Testicular Oxidative Injury, Fecundity, and Offspring

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Fetal alcohol syndrome usually implies effects on the offspring of *maternal* EtOH consumption during gestation, with fewer reports addressing the impact of *paternal* exposure on the progeny. One previous report has dealt with the impact of EtOH exposure on peripubertal male rats as a model of teenage drinking and the deleterious effects on the offspring. We report here findings examining the effect of 2 mo of EtOH feeding on male animals as they progressed through puberty on their ability to impregnate EtOH-naive female rats and characteristics of the subsequent litters. The EtOH-imbibing fathers weighed significantly less than pair-fed controls and animals ingesting a non-EtOH liquid diet ad libitum. Nevertheless, they were able to mate successfully, although fecundity was significantly reduced. The number of successful pregnancies, defined as carried to term, was diminished from 92% in controls to 75% in EtOH-fed animals ($p < 0.05$). There was increased paternal testicular oxidative injury demonstrated by enhanced lipid peroxidation, protein oxidation, and decreased ratio of reduced to oxidized glutathione. The litter size of the EtOH-exposed males was reduced by 46%. The average litter size was 12.4 ± 1.5 pups/litter in ad libitum animals, virtually identical to the 12.5 ± 0.6 pups/litter in the pair fed controls. This is in sharp contrast to the 6.7 ± 0.1 pups/litter from the paternal EtOH matings ($p < 0.001$). There was an increase in the average individual weight of pup offspring of paternally EtOH-exposed animals ($p < 0.01$ vs pair-fed controls and $p < 0.05$ vs ad libitum). Curiously, the male-to-female pup ratio was altered with a higher preponderance of male offspring from EtOH-fed fathers. There were no gross malformations noted among the pups. Insulin-like growth factor-1 levels in the pups at 10 d of age were unaltered between the groups. However, leptin was significantly elevated in the EtOH offspring. It appears that chronic EtOH exposure in the peripubertal fathers subsequently decreases fecundity and that this may be mediated by testicular oxidative injury, perhaps leading to accelerated germ cell apoptosis.

Key Words: Adolescent; paternal; EtOH; fecundity; testicular oxidative injury.

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Introduction

Maternal EtOH exposure during gestation is known to have deleterious consequences on the offspring and has been extensively studied *(1–3)*. Evaluations of the effects of EtOH paternal exposure are few but have similarly revealed abnormalities in fecundity *(4)*, litter size *(5)*, and malformations *(6)*. In these studies adult male rats were examined whereas only one previous report has investigated the impact of EtOH exposure in adolescent male rats *(7)*. A documented increase in EtOH abuse among adolescents has demonstrated that almost 35% of habitual drinkers are younger than 16 yr of age, and, thus, it is extremely important to study animal models relevant to this human age group *(8)*. To that end, we described the impact of 8 wk of EtOH exposure on the fertility and fecundity of young male rats as they progress through puberty. In addition to describing the deleterious effect of peripubertal EtOH exposure on fertility, we explored a potential mechanism for this impairment. This included the effects of EtOH on paternal gonadal oxidative injury. In the offspring, serum insulin-like growth factor-1 (IGF-1) and leptin levels were assessed, because these hormones are important triggers for puberty *(9–21)*.

Results

Paternal Weights

All animals steadily gained weight throughout the study (Fig. 1). However, the EtOH group consistently weighed

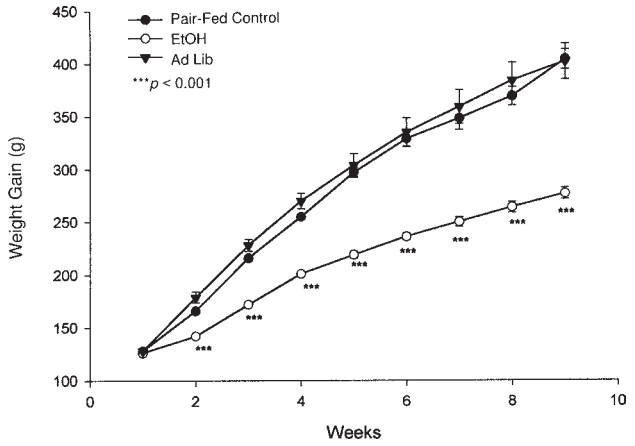

Fig. 1. Weight gain of prospective paternal rats during feeding. $n = 12$ for pair-fed and EtOH groups and 6 for ad libitum group.

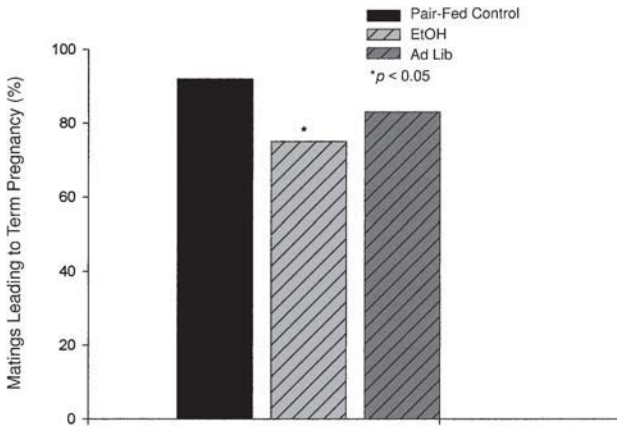

Fig. 2. Effect of EtOH on paternal fecundity. p value is for paternal EtOH animals compared with paternal pair-fed rats.

less than either the pair-fed or ad libitum groups. This difference was apparent by the end of the first week of feeding and continued for the duration of the feeding period ($p < 0.001$). The EtOH animals, however, appeared healthy with no evidence of malnutrition or illness based on their behavior. We suspect that this may have been owing to subtle malabsorbtion because the EtOH animals had an increased number of stool pellets. We have noted this in all our chronic-feeding studies in both male and female rats *(22–24)*. Ad libitum animals consumed an average of 151 ± 3.7 mL of liquid diet per day for a 2-mo period, whereas the rats in the EtOH group consumed an average of 127 ± 3.9 mL ($p < 0.001$ compared to ad libitum rats). We have commonly seen that the presence of EtOH in the diet reduces consumption. The average intake of the pair-fed animals was 127 ± 3.9 mL, by design, of course, equivalent to that of the EtOH group.

Mating Efficiency and Fecundity

Mating efficiency is defined as the ability to successfully complete copulation on the first exposure to a receptive (i.e., proestrous) female. This was not affected by EtOH. Mating efficiency was 100% in the 12 pair-fed controls, and 83% (5 of 6) in the ad libitum rats. The one rat that was unsuccessful at the initial attempt was able to copulate on the second attempt. Similarly, the mating efficiency in the EtOH-treated rats was 92% (11 of 12). The one rat that did not mate successfully on the first exposure was not able to copulate on three subsequent attempts with three different receptive females.

Although no quantitative sperm count was done, a semi-quantitative assessment was made by examining vaginal smears taken the morning after mating. Sperm were abundant in the vaginal smears from mates of the pair-fed control and ad libitum animals. By sharp contrast, the sperm from mates of the EtOH-fed rats were much less numerous. In non-EtOH-exposed animals, many sperm were seen in every microscopic field and far outnumbered vaginal epithelial cells. In EtOH-treated rats, sperm were frequently absent from entire microscopic fields and, even when seen, were less numerous than vaginal epithelial cells.

Although the EtOH-fed animals were able to impregnate their female partners, the number of successful pregnancies, defined as carried to term (fecundity), was diminished compared with both the pair-fed and ad libitum groups. In the EtOH group 75% of the matings resulted in successful pregnancies, in contrast to 92% success in the pair-fed control paternal impregnations (Fig. 2) ($p < 0.05$ compared with pair-fed controls). Fetal resorption was suspected to have occurred in this 25% of female partners that did not carry to term. This conclusion is based on the sperm positivity in the vaginal canal coupled with rapid weight gain by the female followed by weigh loss within a week. On sacrifice of the females, nonviable fetuses were detected in the uterine cavity in some, but not all.

Oxidative Injury

Testes were removed from animals treated identically in a separate series of experiments. Testes weight was 2.2 ± 0.1 g in the EtOH-fed group, 2.7 ± 0.2 g in the controls, and 2.8 ± 0.2 g in the ad libitum–fed rats ($p < 0.04$, comparing EtOH rats vs both other groups). EtOH feeding was associated with an almost twofold increase in testicular lipid peroxidation, as expressed by testicular malonaldehyde (MDA) levels ($p < 0.04$, EtOH vs control and ad libitum–fed rats) (Fig. 3A). EtOH-fed rats also had a significant increase in testicular total protein oxidation as measured by protein carbonyl content (PCC) ($p < 0.01$, EtOH vs control and ad libitum–fed rats) (Fig. 3B). EtOH intake decreased the total testicular glutathione (GSH) content by 50% (Fig. 3C). The testicular levels of GSSG, the oxidized form of GSH, were significantly increased by EtOH treatment (1.0 ± 0.1 μmol/mg of protein in EtOH vs 0.5 ± 0.1 μmol/mg of protein and ad libitum–fed 0.3 ± 0.05 μmol/mg of protein, $p < 0.02$). The GSH/GSSG ratio was found to be significantly decreased in the testis of the EtOH-fed rats (Fig. 3D).

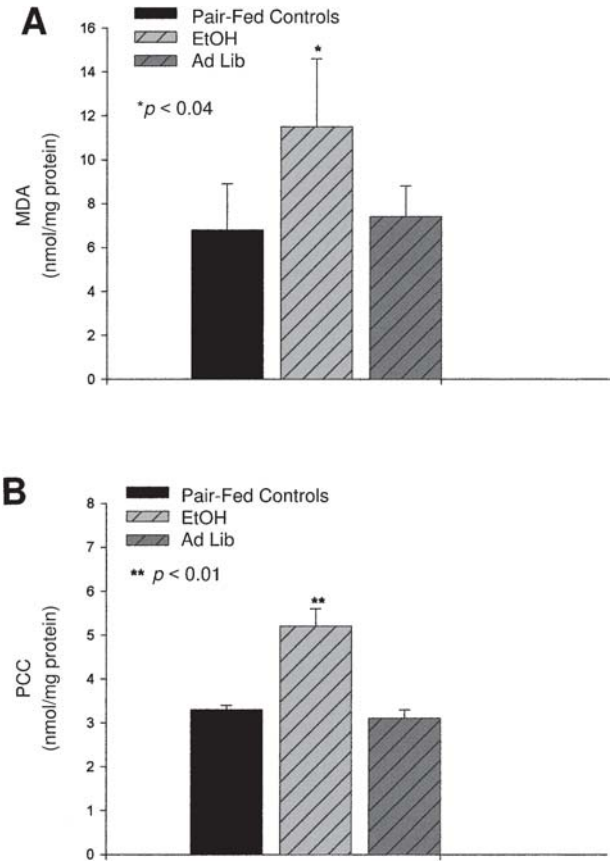

Fig. 3. (**A**) Effect of EtOH on paternal testicular lipid peroxidation, assessed by levels of MDA. *p* value is for EtOH-fed rats compared with both of the other groups. (**B**) Effect of EtOH on paternal testicular protein oxidation, assessed by levels of PCC. *p* value is for EtOH-fed rats compared with both of the other groups.

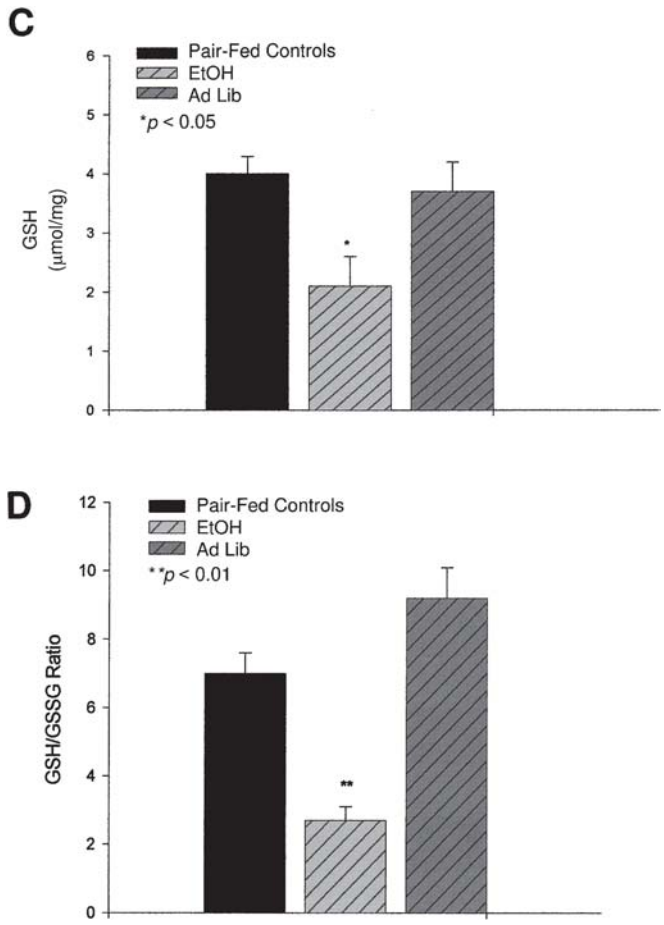

Fig. 3. (**C**) Effect of EtOH on paternal testicular glutathione (GSH). *p* value is for EtOH-fed rats compared with both of the other groups. (**D**) Effect of EtOH on paternal testicular ratio of GSH and the oxidized form of GSSG. *p* value is for EtOH-fed rats compared with both of the other groups.

Pups

There was a highly significant 46% decrease in the litter size from EtOH fathers compared with both pair-fed and ad libitum–fed controls. The average was 12.4 ± 1.5 pups/litter in ad libitum animals, virtually identical to the 12.5 ± 0.6 pups/litter in the pair-fed controls. This finding is in sharp contrast to the 6.7 ± 0.1 pups/litter from paternal EtOH matings ($p < 0.001$ compared with both ad libitum and pair-fed controls) (Fig. 4). This was associated with a slight but consistent and significant increase in the average individual weight of the pups. The pups of EtOH-exposed fathers weighed 6.67 ± 0.16 g, more than the offspring of the ad libitum (6.45 ± 0.08 g; $p < 0.05$) or the pair-fed controls (6.15 ± 0.08 g; $p < 0.01$) (Fig. 5). The litters of EtOH-fed fathers had a significantly greater percentage of male pups compared with the other two groups. The offspring of EtOH-fed fathers were 60% male compared with 47% in the ad libitum groups and 42% in the pair-fed controls ($p < 0.05$ for both groups compared to EtOH). There were no gross malformations noted in any of the pups from any of the three groups.

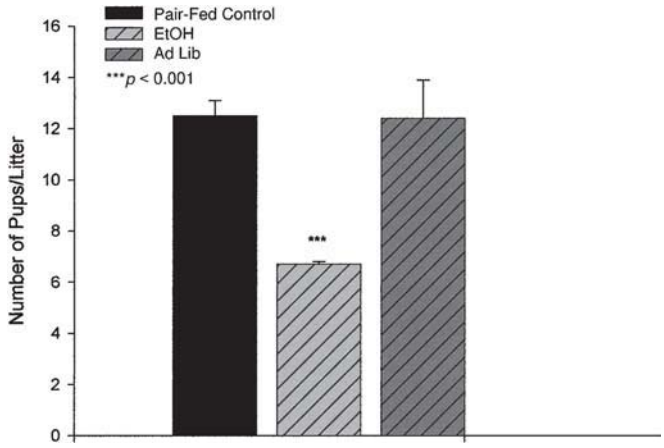

Fig. 4. Effect of EtOH on litter size. *p* value is for litters of EtOH animals compared with both of the other groups.

Serum IGF-1 and Leptin in Pups

There was no difference in serum IGF-1 levels among the pups at 10 d of age (33.0 ± 1.5 ng/mL in pair-fed controls, 33.5 ± 1.3 ng/mL in EtOH pups, and 36.6 ± 0.8 ng/mL for ad

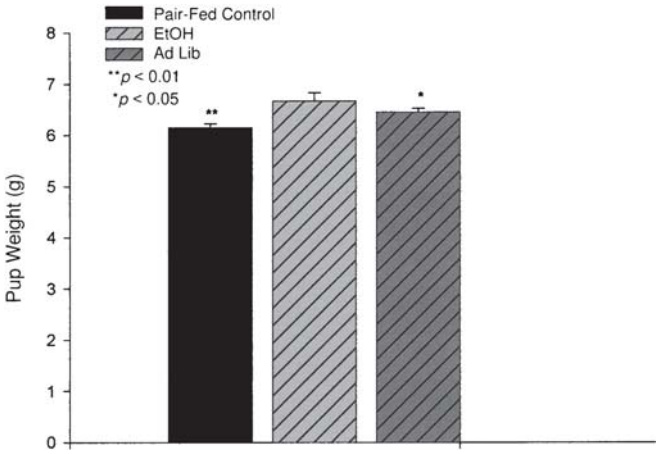

Fig. 5. Effect of EtOH on pup weight. *p* values are in comparison with those of pups from EtOH-fed fathers.

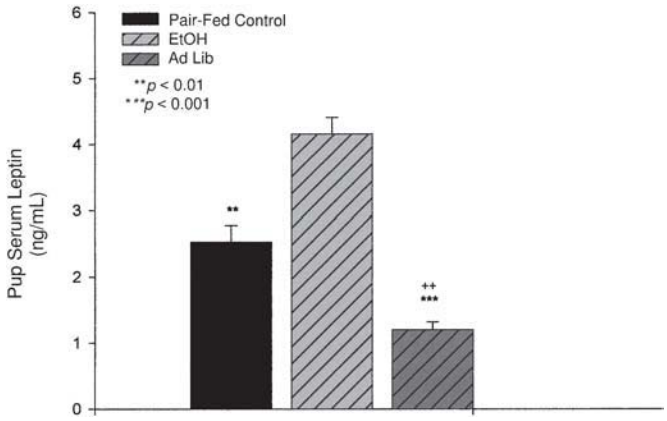

Fig. 6. Effect of paternal EtOH exposure on pup serum leptin levels. *Litters from EtOH vs pair-fed control rats; **Litters from EtOH vs ad libitum rats; ++Litters from pair-fed control vs ad libitum rats.

libitum pups). By contrast, serum leptin levels were markedly elevated in the pups sired by EtOH-exposed fathers compared with pups from pair-fed control fathers ($p < 0.05$) and to pups from the ad libitum males ($p < 0.01$) (Fig.6). Also, the pups from the pair-fed control fathers had significantly higher serum leptin than the pups from the ad libitum fathers ($p < 0.01$).

Discussion

The effects of maternal EtOH exposure during pregnancy on *in utero*–exposed fetuses have been well studied *(1,25–28)*. By contrast, less extensive evaluation has been done regarding paternal EtOH exposure. This is of interest because of the inherent possibility that EtOH, either acute or chronic, might be damaging to sperm and result in decreased fertility and deleterious consequences to offspring. In aggregate, research in this area has supported the idea that paternal EtOH exposure around the time of conception has a significant negative impact on offspring. However, specific findings have not always been consistent, probably because, at least in large part, there are substantial differences in protocol design. For example, some studies have been done on rats *(29)* whereas others have been done on mice *(30)*. Within species, there have been differences in animal strain *(29)*. The age of the animals studied (pubertal *[7]* vs adult *[31]*) has varied. There have also been major differences in feeding paradigms: mode of EtOH administration *(32,33)*, dose of EtOH *(31,34)*, and length of feeding *(29,35)*. Finally, the period of abstinence before mating and actual breeding procedures (i.e., one or two mating opportunities vs multiple opportunities until mating was accomplished) have varied considerably *(6–7)*. Given these differences, inconsistency in specific results is not surprising, but the fundamental concept that paternal, like maternal, EtOH exposure is deleterious to offspring clearly emerges.

In our study, we found that EtOH exposure of prospective fathers reduced fecundity (the percentage of pregnancies carried to term) and litter size. Although serum hormone levels were not accessed in this particular experiment, previous studies from our laboratory *(24)* as well as from Cicero et al. *(7)* are pertinent. In both of these previous studies, rats of the same age as examined here were fed EtOH for several months with consequent significant reductions in testosterone, inappropriate nonelevation of gonadotropins, and elevation in estradiol. Each of these alterations is rather quickly reversed. For example, in Cicero et al.'s *(7)* study, 3 d after withdrawal of EtOH, testosterone rebounded and was significantly elevated over the pair-fed controls ($p < 0.05$), to return to control levels within a few weeks. Thus, the fathers in our current study, 1 wk off EtOH at the time of mating, very likely had normal reproductive circulating hormones, and, therefore, any abnormalities in the litters were not owing to hormonal alterations at the time of mating. Clearly, reproductive problems could have arisen from hormonal changes during EtOH ingestion, and a detailed analysis of the mechanism of the paternal transmission of EtOH's deleterious effects should be the focus of much future study.

In our study, fecundity was decreased by EtOH. This is similar to the findings of some *(5,32,36)*, but not all *(35)* other groups. This decrease in fecundity suggests the possibility of some EtOH-induced damage to sperm, since the females in our study were EtOH-naive proven breeders. In the current studies, we noted, in a semiquantitative manner, that EtOH-fed rats had fewer sperm. Indeed, others have reported abnormalities in sperm of EtOH-imbibing rats as well. For example, Abel and Moore *(37)* reported morphologic abnormalities in sperm and Abel *(29)* reported and altered electrophoretic mobility of sperm DNA in animals that had consumed EtOH. Furthermore, Willis et al. *(38)*, in addition to observing sperm morphologic abnormalities in sperm reported decreased sperm motility and decreased

ability to fertilize mouse ova in vitro in EtOH-treated animals. Detailed analyses of sperm function may be the focus of future studies.

We have demonstrated that EtOH decreased litter size, similar to the findings of Abel *(29)* and Cicero et al. *(36)*. Interestingly, Bielawski and Abel *(6)* noticed an increased percentage of runts <5 g. This effect on litter size, however, has not been consistently seen *(6,30,33,35,39)*. Similarly, some researchers have found paternal EtOH associated with increased embryonic death *(5)* and increased fetal mortality *(36)* while we and others were not able to demonstrate increased pre- or postnatal mortality *(37)*. EtOH's effect on birth weight is also not consistent. We found that, although litter size was reduced by nearly 50%, there was a small but significant increase in the weight of the pups sired by EtOH-drinking fathers. We speculate that this was owing to increased nutrient availability secondary to decreased competition in the smaller litters. Others have reported either no effect on birth weight *(34,39,40)* or decreased birth weight *(5,32,34)*. We found, interestingly, that litters sired by EtOH fathers had an increased number of males, similar to the report of Abel *(4)*. However, others have found no EtOH effect on sex ratio *(30,33,35)*.

We found no gross malformations in the pups, similar to previous reports by Chauhan et al. *(33)* and Randall et al. *(30)*. However, others have found that pups from rats given higher doses of EtOH than we used did demonstrate malformations such as hydronephrosis and enlarged cerebral ventricles *(6)*. In addition, endocrine abnormalities have been reported in the pups from EtOH fathers. These include decreased testosterone *(7,29,34)* and reduced hypothalamic β-endorphin content *(7)*.

Furthermore, offspring fathered by EtOH males demonstrated a variety of behavioral abnormalities including lower levels of activity at lower EtOH doses *(29,39,40)* and less grooming behavior *(41)*. However, at higher levels of EtOH, offspring showed higher levels of activity *(31,42)*. Also, offspring of EtOH fathers preformed less well in a two-way shock avoidance paradigm *(40)* and had decreased facility in passive avoidance *(31,39)*.

Because EtOH can delay puberty *(9–21)*, we were interested in determining whether or not offspring of EtOH fathers demonstrated alterations in IGF-1 or leptin. These two hormones have been implicated in puberty and overall reproductive function *(9–21)*. EtOH has been shown to decrease IGF-1 *(43,44)*. Therefore, we determined whether or not this decline could be observed in EtOH-naive offspring of EtOH-imbibing fathers. There were no differences in IGF-1 levels among the pups. No other data are available on IGF-1 in offspring from paternally EtOH-exposed animals.

Significantly higher leptin levels were found in the pups, perhaps reflecting the increased weight of the pups from EtOH-exposed fathers. Other researchers have shown that EtOH increases leptin gene expression *(45)*. This is in accordance with our finding of elevated serum leptin in offspring of EtOH-fed fathers. Whether this is owing to EtOH-induced genetic damage to sperm or simply to the fact that the offspring of EtOH fathers weighed more cannot be determined from our study. Nonetheless, elevated leptin can inhibit the ability of gonads to respond to pituitary gonadotropins. It will be of great interest to study, in more detail, reproductive alterations in these offspring at more advanced ages.

Although it is well known that chronic EtOH abuse produces sexual dysfunction and impaired spermatogenesis, the mechanisms of EtOH-induced testicular alterations are not fully explained. Oxidative stress contributes significantly to the pathogenesis of organ and system injury, which results from EtOH abuse *(46)*. Lipid peroxidation and protein oxidation have been reported to occur at increased rates within the liver as a consequence of EtOH consumption *(47,48)*. Our findings confirm the observation reported by other investigators of increased lipid peroxidation, and protein oxidation and decreased glutathione levels in testis following chronic EtOH exposure *(49,50)*. These observations suggest that the enhanced oxidative injury in the testis that occurs following EtOH exposure may be an important factor in the pathogenesis of EtOH-associated gonadal injury. Apoptosis is presumed to represent the final common pathway of oxidative cell injury *(51)*. In other studies from our laboratories, Zhu et al. *(52)* demonstrated that young male rats fed EtOH did have increased testicular apoptosis, almost exclusively in germ cells. It is likely, therefore, that EtOH leads to a spectrum of oxidative injury to germ cells, culminating in apoptosis. It may be that some of those germ cells that are oxidatively damaged but not apoptotic impregnate females, yielding smaller litters with the abnormalities reviewed previously.

Understandably, EtOH exposure to pregnant females has been an area of great investigative activity. However, though not as thoroughly studied, paternal EtOH consumption may have profound and lasting implications as well. Indeed, decreased birth weights *(53)*, increased frequency in ventricular septal defects *(54)*, and hyperactivity *(55)* have been related to paternal EtOH consumption in *humans*. Our data and those of others suggest that these problems may be owing to oxidative damage to sperm DNA caused by EtOH *(29)*. They further suggest that the male rat may be a good animal model for this paternally mediated fetal alcohol syndrome, just as the female has been useful for the study of a maternally mediated fetal alcohol syndrome. It will be important to develop more consistent feeding paradigms to study the effects of various combinations of paternal and maternal EtOH exposure on fecundity and offspring. More consistent experimental design will allow better comparability of data. These studies should be conducted in concert with more extensive human epidemologic studies to better define what might be a significant public health problem.

Materials and Methods

Animals and Feeding

Thirty male Sprague-Dawley rats, age 30 d, were purchased from Harlan (Indianapolis, IN). Male animals were allowed to become acclimated to their environment for 5 d. They were housed in an AALAC-accredited facility and kept on a 12-h light/dark cycle at 22ºC ± 2ºC. The well-established Lieber DeCarli liquid without EtOH was administered 4 d before beginning the study to allow the animals to become accustomed to the new diet. The EtOH group (n = 12) received 36% of their calories as EtOH, as previously reported *(56)*. The pair-fed group (n = 12) received an equal number of calories as their EtOH-fed mate, with dextrimaltose substituted for EtOH. A third ad libitum group (n = 6) was given as much of the non-EtOH-containing liquid diet as the rats could consume, in order to observe any differences between the control pair-fed and ad libitum groups. Animals were weighed on a weekly basis and measurement of daily food consumptions was recorded. After 2 mo of feeding, all the male rats were switched to a standard rat chow and water ad libitum diet for 1 wk before mating them with a proven female breeder.

Thirty-five proven female breeders were also purchased from Harlan. On arrival, the females were injected with the leutinizing hormone–releasing hormone analog (D-Trp^6-Pro^9-Net-GnRH, generously donated by Dr. J. Rivier, Salk Institute, La Jolla, CA) to synchronize their estrous cycles *(57)*. Vaginal smears were taken daily to determine when each female would be at proestrus, and thus sexually receptive. Male rats, 1 wk off the liquid diets, were singly caged with a female, judged at proestrus by vaginal smear. The following morning the female was removed and a vaginal smear taken to determine the presence of sperm in the vaginal canal. Male animals that had not impregnated their female partner were mated with a different female within 2 d. When pregnancy was confirmed, the male animals were sacrificed by carbon dioxide inhalation. Females were allowed to come to term and their litter was counted, weighed, sexed, and checked for any major deformities by gross inspection. After 10 d, the rat pups were sacrificed by decapitation and the mothers by carbon dioxide inhalation. The pups were sacrificed in a different manner than their parents because hormone measurements were made on the pups and it was important to exclude stress and inhalation variables, which are known to alter hormone levels *(58–61)*. The trunk blood was collected from the pups and serum separated. Because of the small amount of serum collected, it was pooled by sex and litter and stored at –20ºC for subsequent radioimmunoassay (RIA). The brain was removed, weighed, and examined for possible irregularities.

In a separate series of experiments, peripubertal male rats treated exactly as just described were sacrificed by decapitation immediately after the 2-mo feeding period. Testes from these animals were utilized to assess oxidative injury.

All animal protocols were approved by the Joint Institutional Animal Care and Use Committee, Loyola University Stritch School of Medicine and Edward Hines Veterans Administration Hospital.

Radioimmunoassays

IGF-1 Radioimmunoassay

IGF-1 RIA was performed using an RIA assay kit from DSL (Diagnostic Systems, Webster, TX) following the suggested protocol. The assay sensitivity was 150 ng/mL, and the inter- and intraassay coefficient of variations (CVs) were 4.4 and 3.3%, respectively.

Leptin Radioimmunoassay

Leptin RIA was performed using a kit purchased from Linco Research (St. Louis, MO) following the suggested protocol. The assay sensitivity was 500 pg/mL, and the inter- and intraassay Cvs were 5.5 and 3.0%, respectively.

Oxidative Injury

Lipid Peroxidation

Testicular lipid peroxidation was assessed by determining the MDA level of testis homogenates using a colorimetric assay (Calbiochem, La Jolla, CA) according to the manufacturer's instructions. The results are expressed as micromoles/milligram of protein. The protein content for each sample was measured using the bicinchronic acid assay (Pierce, Rockford, IL).

Protein Oxidation

PCC was determined in testis homogenates as an index of protein oxidation by the reaction with 2,4-dinitrophenylhydrazine, according to Levine as previously described *(62)*. The results are expressed as nanomoles/milligram of protein.

Antioxidant Status

Reduced GSH is a major antioxidant in human tissues that provides reducing equivalents for the glutathione peroxidase–catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and alcohol. During this process GSH becomes GSSG. The GSSG is then recycled to GSH by reduction reactions catalyzed by glutathione reductase. When cells are exposed to oxidative stress, the ratio of GSH/GSSG will decrease as a consequence of GSSG accumulation. The measure of the GSSG level, or determining the GSH/GSSG ratio, is a useful measure of oxidative stress. GSH and GSSG content in the testis were determined by enzymatic assay according to the procedure described by Tietze *(63)* in tissue homogenates using a commercially available kit (Oxis International, Portland, OR). The results are expressed as micromoles/milligram of protein.

Statistical Analysis

Statistical analysis was by the Mann-Whitney U-tests and two-way analysis of variance followed by the Newman-Keuls post hoc with Tukey's follow-up and individual group com-

parisons by student's unpaired *t*-test. A p value of <0.05 was considered to be significant.

Acknowledgments

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