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Vitamin E prevents ethanol-induced inflammatory, hormonal, and cytotoxic changes in reproductive tissues

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Abstract Ethanol causes decreased function of the hypothalamic–pituitary–gonadal (HPG) axis. Ethanol resulted in inflammatory changes in HPG manifested by increased concentrations of pro-inflammatory cytokines. Since, such cytokines have deleterious effects on functions of HPG, it seemed possible that ethanol's suppressive action could be due, at least in part, to this inflammation. Since oxidative stress can cause inflammation, we have used the antioxidant vitamin E to test, whether reducing inflammation might protect reproductive functions from ethanol. Rats were fed an ethanol diet or pair fed identically without ethanol for a 3-week period. For the last 10 days, animals were given 30 IU/kg or 90 IU/kg or vehicle. Ethanol significantly increased hypothalamic, pituitary and testicular TNF-α and IL-6, all changes prevented by the higher dose of vitamin E. Also, ethanol induced changes in LHRH, LH, testosterone, and testicular germ cell apoptosis were similarly prevented by vitamin E. These data strikingly show that vitamin E protects the HPG from deleterious effects of ethanol and suggests that the mechanism of this protection might be both anti-inflammatory and antioxidant.

Keywords Pituitary · Testes · Ethanol · Vitamin E · Apoptosis

Introduction

Several laboratories have shown that ethanol causes a decreased function of the hypothalamic pituitary gonadal (HPG) axis as reviewed in Emanuele [1] and detailed more completely in the Discussion. This disruptive effect probably has multiple mechanisms, including ethanol's ability to increase opiate tone [2–3], enhance sensitivity to nitric oxide [4–6], increase oxidative stress [7], and interfere with

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intracellular protein trafficking [8]. We have recently found that either acute or chronic ethanol administration results in inflammatory changes in HPG manifested by increased concentrations of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [9–10]. Since, such cytokines have been demonstrated by others to have deleterious effects on hormonal functions of HPG axis, it seemed possible that ethanol's suppressive action could be due, at least in part, to this inflammation. Since oxidative stress is well known to cause inflammation, we used the antioxidant vitamin E as a tool to determine, whether reducing inflammation might protect the HPG from the impact of ethanol.

Materials and methods

Animals

Adult 8-week-old male Sprague Dawley rats were obtained from Harlan Laboratories, Indianapolis, IN. All animas were singly housed and subjected to a 12-h light/12-h dark cycle at 22–24°C at Hines Veterans Administration Hospital's AALAC approved animal facility, which is operated and maintained by laboratory technicians and a veterinarian. All procedures are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, 1996).

Upon arrival animals were fed the Lieber-DeCarli liquid diet for 5 days to allow them to acclimate to the feeding paradigm. After 5 days, animals were weighed and separated into 2 groups, ethanol and pair fed control. The ethanol animals were given the Lieber-DeCarli diet containing 36% of the calories as ethanol, while the control animals received the same diet but with dextrimaltose in place of the ethanol. The ethanol groups were given increasing doses of ethanol for 5 days in order to become accustomed to the full diet. For the last 10 days of ethanol or pair feeding, the animals were further divided into vitamin E supplemented or nonsupplemented groups. Therefore, there were 6 experimental groups as follows: ethanol/vehicle, pair-fed control/vehicle, ethanol/30 IU vitamin E, pair-fed control/30 IU vitamin E, and ethanol/90 IU vitamin E, and pair-fed control/90IU vitamin E. The vitamin E ((+)-α-tocopherol acid succinate, Sigma, St Louis, MO, T3126) was diluted in oil, and made fresh daily. Rats were monitored daily for food intake, weight, and general grooming. After 10 days on the vitamin E and ethanol, the animals were sacrificed over a two day period starting at 8:30 AM. Trunk blood was collected and serum separated and stored at –20°C for subsequent radioimmunoassay (RIA). Similarly, anterior pituitaries, hypothalami, and testes were quickly removed and stored at –70°C for further study.

Tissue preparation and cytokine/LHRH determination

All tissues were homogenized in a ml of cold protease inhibitor phosphate buffer (Sigma P8340), centrifuged at 12,000 rpm, and the supernatant separated into aliquots and frozen for determination of TNF-α, IL-6, and luteinizing hormone releasing hormone (LHRH) by enzyme linked immuno sorbant assay (ELISA), as previously described [6]. The ELISAs for the cytokines were obtained from BD Biosciences (San Diego, CA) and the LHRH ELISA was from Peninsula Laboratories (San Carlos, CA). In all cases, the manufacturers' recommended protocols were followed. Protein was measured by a protein kit (Sigma 610A) following the procedure recommended by the manufacturer.

Serum LH, FSH, and testosterone determination

The determination of LH and FSH were accomplished using RIA kits purchased from Amersham Bioscience (Piscatway, NJ) and using the manufacturer's recommended protocol. The LH sensitivity was 80 pg/ml with an inter- and intra-assay coefficient of variation 5.1% and 4.0%, respectively. The FSH sensitivity was 160 pg/ml with an inter- and intra-assay coefficient of variation of 6.3% and 4.9%. Testosterone determination was conducted using a commercially available kit (DSL, Webster, TX) following the suggested protocol. The assay sensitivity was 100 pg/ml with an inter- and intra-assay CV of 4.8% and 3.2%.

TUNEL assay

This was performed essentially as described previously [11]. Briefly, testicular tissues were fixed in phosphate-buffered formaldehyde (Fisher Scientific) for 48 h and embedded in paraffin. Deparaffined and rehydrated sections were treated in 10 mM sodium citrate pH 6, using a microwave. Each section was incubated with 20 µl of terminal deoxynucleotidyl transferase (TDT) (Invitrogen) buffer for 10 min then 20 µl of TDT buffer plus 1 µM digoxigenin-11-dideoxyUTP, 49 µM didoxyATP and 1 Unit/µl of TDT for 60 min at 37°C in a humidified chamber. The sections were washed and blocked with 2% blocking agent (Boehringer Mannheim), incubated with a peroxidase-conjugated anti-digoxigenin antibody diluted to 0.5 Unit/ml for 45 min at room temperature. After washing,

the sections were immersed in a substrate solution containing 3′-amino-9-ethylcarbazole (AEC) and H_2O_2. After mounting with coverslips, sections were observed under a light microscope. TUNEL-positive cells and total number of seminiferous tubules on each cross-sectioned testis were counted. The data were expressed as: number of TUNEL positive cells per 1,000 seminiferous tubules.

Blood ethanol levels

Blood levels of ethanol were determined using a kit from Sigma (St. Louis, MO) following the protocol for serum samples.

Statistical analysis

Statistical analysis was by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. The 95% confidence limit was taken as significant ($P < 0.05$).

Results

Blood ethanol

The blood ethanol levels ranged from 115 to 125 mg/dl in all the ethanol treated groups, while the pair-fed groups had undetectable levels. The average amount of ethanol ingested over the 3-week period was 5.1 g/day (after the first week) to 6.0 g/day (at sacrifice).

Animal weights

All animals continued to gain weight during the feeding period. However, as we have seen in other studies, the rats in the ethanol groups had an attenuated rate of weight increase compared to pair-fed animals despite the fact that the pair-feeding paradigm provides equivalent calories. There was a statistically significant difference in body weight between treatment groups after two weeks of feeding ($P < 0.001$) which occurred in the ethanol/vehicle (273 ± 3.6 g) and the ethanol/90 IU vitamin E (269 ± 3.1 g) compared to their pair-fed mates (293 ± 2.7 g and 288 ± 3.1 g). At the time of sacrifice (3 weeks) all three ethanol groups (286 ± 5.8 g in ethanol/vehicle, 293 ± 4.0 g in ethanol/30 IU vitamin E, and 282 ± 4.1 g in ethanol/90 IU vitamin E) were significantly lower than their pair-fed mates (320 ± 3.4 g, 307 ± 4.3 g, and 313 ± 4.1 g, $P < 0.001$). All animals were well groomed and appeared active and healthy during the entire experiment.

Hypothalamus

Hypothalamic TNF-α concentrations were doubled by ethanol feeding ($P < 0.001$). Neither dose of vitamin E alone had any effect. However, both doses of vitamin E completely abrogated the ethanol induced cytokine increase (Fig. 1). In fact, the TNF-α levels in hypothalamus of ethanol-exposed, vitamin E treated animals were significantly lower than those who not given vitamin E ($P < 0.001$). Ethanol also significantly increased hypothalamic IL-6 concentrations by approximately 60% ($P < 0.01$). Again vitamin E, which alone had no effect on hypothalamic IL-6, blocked the ethanol-induced increase (Fig. 1B). IL-6 was significantly lower in ethanol-fed/vitamin E treated animals

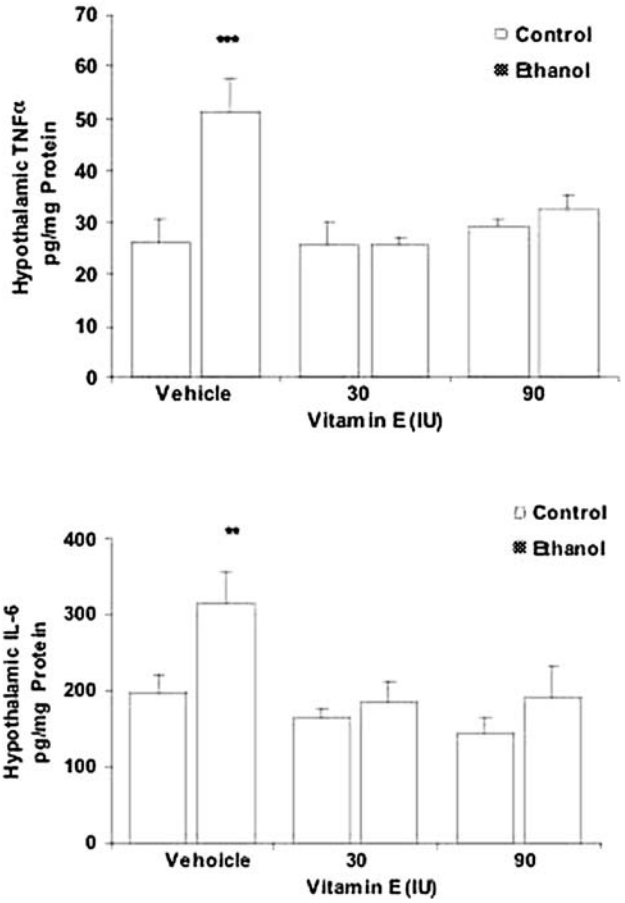

Fig. 1 (**A**) The effect of ethanol and vitamin E on hypothalamic TNFα. Both doses of vitamin E prevent the ethanol induced increase in hypothalamic TNFα. ***$P < 0.001$ from control/vehicle, ethanol/vitamin E 30 IU/kg and ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group. (**B**) The effect of ethanol and vitamin E on hypothalamic IL-6. Both doses of vitamin E prevent the ethanol induced increase in hypothalamic IL-6. ***$P < 0.01$ from control/vehicle, ethanol/vitamin E 30 IU/kg and ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group

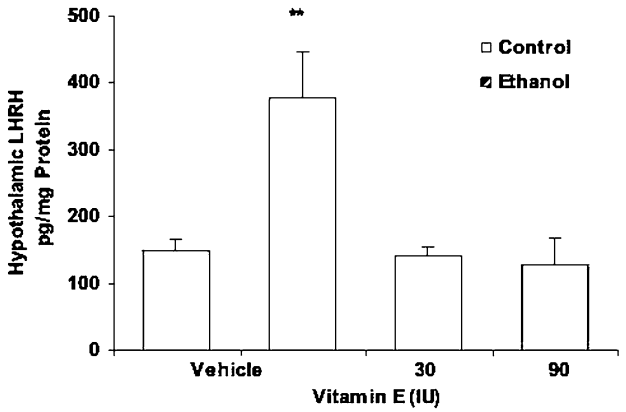

Fig. 2 The effect of ethanol and vitamin E on hypothalamic LHRH. Both doses of vitamin E prevent the ethanol induced increase in hypothalamic LHRH. ***$P < 0.01$ from control/vehicle, ethanol/vitamin E 30 IU/kg and ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–7 per group

compared those exposed to ethanol but not given vitamin E ($P < 0.01$). Finally, ethanol led to a robust 2.5 fold increase in LHRH content ($P < 0.01$), and this was completely prevented by both doses of vitamin E (Fig. 2). Unfortunately, not enough hypothalamic samples were left to determine LHRH levels in the rats given vitamin E without ethanol.

Pituitary

Pituitary TNF-α concentrations were increased 2.6 fold by ethanol feeding ($P < .001$). Although vitamin E alone had no effect on this pituitary cytokine, the TNF-α levels in pituitary of ethanol-exposed animals was significantly lower in those given 30 IU vitamin E ($P < 0.01$) or 90 IU vitamin E ($P < 0.001$) than the ethanol treated, non-vitamin E treated rats (Fig. 3). Ethanol also significantly increased pituitary IL-6 by 2.3 fold ($P < 0.001$). Vitamin E blocked the ethanol induced increase (Fig. 3), although it had no effect when given alone. IL-6 was significantly lower in ethanol-fed/vitamin E treated animals compared those exposed to ethanol but not given vitamin E. When compared to concentrations in ethanol-fed non-vitamin E treated rodents, pituitary IL-6 was significantly lower in rats given either 30 IU ($P < 0.01$) or 90 IU ($P < 0.05$) of vitamin E. Serum levels of the pituitary gonadotropin LH were reduced by almost 50% by ethanol ($P < 0.01$) as shown in Fig. 5A. Vitamin E had no effect on its own but the 90 IU dose prevented this reduction. However, the other gonadotropin, FSH, was not affected by ethanol or vitamin E (Fig. 5B).

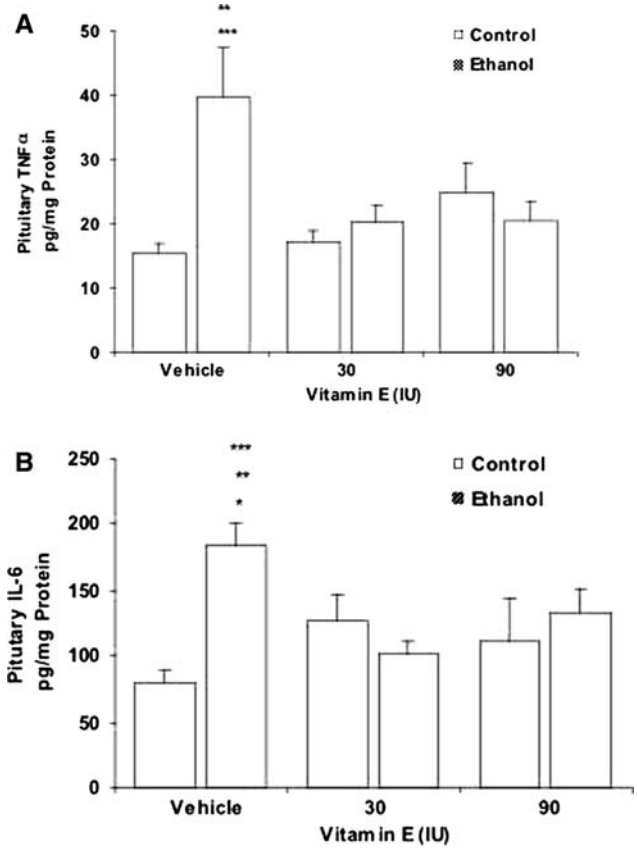

Fig. 3 (**A**) The effect of ethanol and vitamin E on pituitary TNFα. Both doses of vitamin E prevent the ethanol induced increase in pituitary TNFα. ***$P < 0.001$ from control/vehicle and ethanol/vitamin E 90 IU/kg and **$P < 0.01$ from ethanol/vitamin E 30 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group. (**B**) The effect of ethanol and vitamin E on pituitary IL-6. Both doses of vitamin E prevent the ethanol induced increase in pituitary IL-6. ***$P < 0.001$ from control/vehicle, **$P < 0.01$ from ethanol/vitamin E 30 IU/kg, and *$P < 0.05$ from ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group

Testes

Testicular TNF-α concentrations were almost tripled by ethanol administration ($P < 0.001$). While neither dose of vitamin E alone had any effect, both doses completely prevented the ethanol induced TNF-α increase (Fig. 4). The TNF-α levels in testes of ethanol-exposed animals were significantly lower in those given 30 IU vitamin E ($P < 0.02$) or 90 IU vitamin E ($P < 0.03$) than the ethanol exposed, non-vitamin E treated rats. Ethanol also significantly increased testicular IL-6 by 2.3 fold ($P < 0.001$). Vitamin E blocked the ethanol induced increase (Fig. 4). When compared to concentrations in ethanol-fed non-vitamin E treated rodents, testicular IL-6 was significantly lower in rats given either 30 IU ($P < 0.01$) or 90 IU ($P < 0.05$) of vitamin E. Uniquely, vitamin E alone seemed

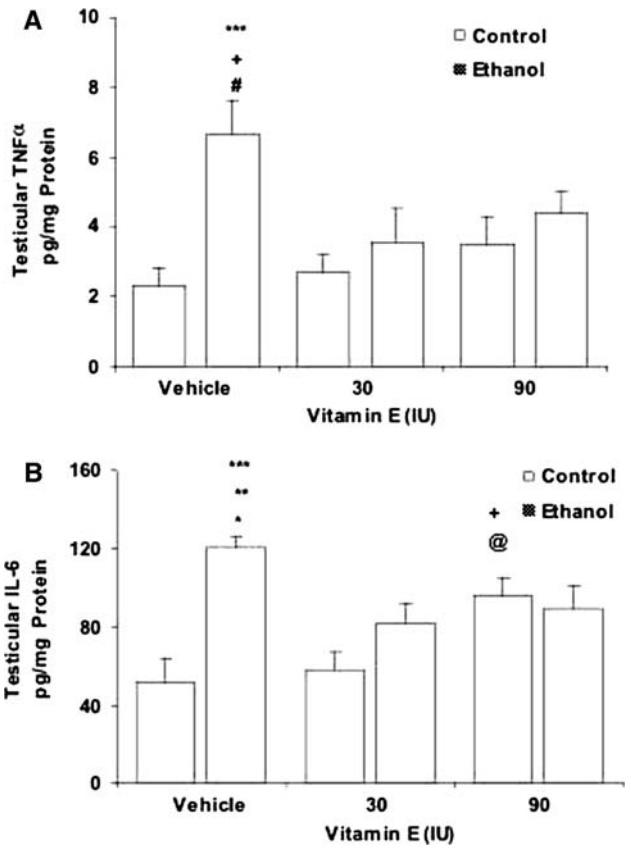

Fig. 4 (**A**) The effect of ethanol and vitamin E on testicular TNFα. Both doses of vitamin E prevent the ethanol induced increase in testicular TNFα. ***$P < 0.001$ from control/vehicle, +$P < 0.02$ from ethanol/vitamin E 30 IU/kg, and # $P < 0.03$ from ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. $n = 6$–10 per group. (**B**) The effect of ethanol and vitamin E on testicular IL-6. Both doses of vitamin E prevent the ethanol induced increase in testicular IL-6. ***$P < 0.001$ from control/vehicle, **$P < .01$ from ethanol/vitamin E 30 IU/kg, *$P < 0.05$ from ethanol/vitamin E 90 IU/kg, +$P < 0.02$ from vehicle control and @ $P < 0.05$ from vitamin E 30 IU control, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. $n = 6$–10 per group

to have an effect on testicular IL-6. The IL-6 levels in those rats that received 90 IU of vitamin E were significantly higher that those that received no vitamin E ($P < 0.02$) or 30 IU of vitamin E ($P < 0.05$).

Serum levels of the testosterone were reduced by almost 70% by ethanol ($P < 0.01$) as shown in Fig. 5. In and of itself, vitamin E did not affect serum testosterone. At the 30 IU dose, vitamin E did not alter ethanol's suppression of testosterone. However, 90 IU of vitamin E completely eliminated this reduction. Serum testosterone in animals given ethanol and 90 IU of vitamin E was no different than that in animals given 90 IU of vitamin E alone and was significantly higher than in rats given ethanol alone or ethanol plus 30 IU of vitamin E ($P < 0.01$).

Apoptosis in testes

Vitamin E supplementation reversed the apoptotic effects of ethanol (Figs. 6 and 7). In the present study, apoptotic cells within the testis were detected using the TUNEL method (Fig. 6). In the testis of rats fed control diet without vitamin E supplementation, apoptotic cells were detected among germ cells adjacent to the basement membrane. In the testis of rats fed ethanol diet with or without vitamin E, apoptotic cells were detected among cells adjacent to the basement membrane and among those that had migrated away from the basement membrane. However, it was obvious that rats fed ethanol and supplemented with vitamin E contained significantly less apoptotic cells. Cell counting experiments confirmed this observation. As shown in Fig. 7, the testis of rats fed ethanol-containing diet without vitamin E supplementation contained 124 ± 23 apoptotic cells per 1,000 seminiferous tubules, significantly higher than did animals fed isocaloric control diet without vitamin E (50 ± 6 cells per 1,000 seminiferous tubules $P < 0.01$). In contrast, there was no significant difference in the number of apoptotic cells in testes of ethanol fed rats supplemented with 90 IU of vitamin E. The level of apoptosis in rats given ethanol and 30 IU of vitamin E was higher than control fed animals given only 30 IU of vitamin E. This was primarily because this dose of vitamin E in itself reduced apoptosis. In fact, the apoptosis in rats fed ethanol and 30 IU vitamin E was significantly lower than those given ethanol alone ($P < 0.03$).

Discussion

We have reported here, for the first time, that vitamin E, a readily available and widely used anti-oxidant vitamin, can prevent the deleterious reproductive hormone changes that are known to be caused by ethanol in hypothalamus, pituitary, and testes [1]. We have also demonstrated that chronic ethanol feeding caused an inflammatory reaction in these reproductive organs, manifested by significant increases in two major pro-inflammatory cytokines, TNF-α and IL-6 and confirming previous findings from our laboratory [9, 10]. Furthermore, this was temporally correlated with biologically significant alterations in important reproductive hormones, LHRH in hypothalamus, serum LH from anterior pituitary, serum testosterone from testes and testicular germ cell apoptosis. Serum FSH from anterior pituitary was unchanged. The data, therefore, clearly show that vitamin E is beneficial in these circumstances and imply (but do not prove) that vitamin E's mechanism of action may be anti-inflammatory as well as anti-oxidant.

The three basic components of the male reproductive unit are the hypothalamus, the anterior pituitary and the testes.

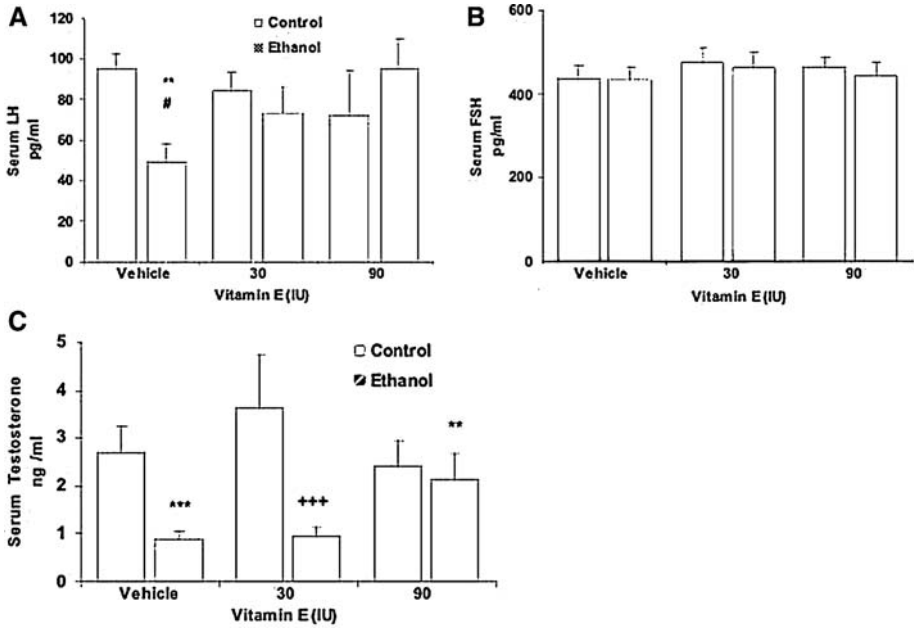

Fig. 5 (**A**) The effect of ethanol and vitamin E on serum LH. Both doses of vitamin E prevent the ethanol-induced decrease in serum LH. $^{**}P < 0.01$ from control/vehicle and # $P < 0.03$ from ethanol/vitamin E 90 IU/kg, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group. (**B**) The effect of ethanol and vitamin E on serum FSH. There were no significant differences between groups, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group. (**C**) The effect of ethanol and vitamin E on serum testosterone. The higher dose of vitamin E prevented the ethanol induced decrease in serum testosterone. $^{***}P < 0.001$ from control/vehicle, $^{+++}P < 0.001$ from control/vitamin E 30 IU, $^{**}P < 0.01$ from ethanol/vehicle and ethanol/vitamin E 30 IU, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group

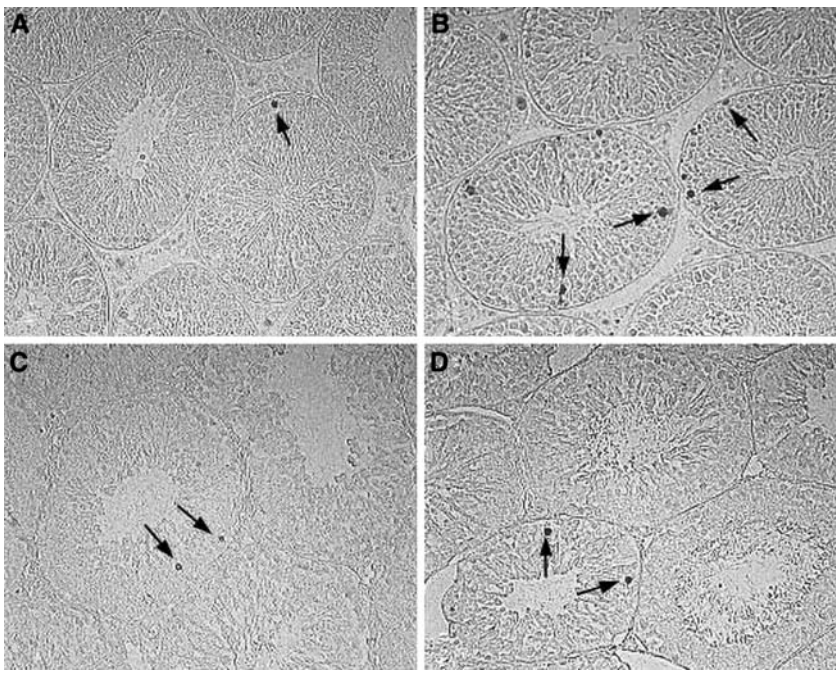

Fig. 6 Detection of apoptotic cells within the testis using the TUNEL method: (**A**) testes of rats fed the control diet for 21 days without vitamin E; (**B**) testes of rats fed ethanol-containing diet for 21 days without vitamin E; (**C**) testes of rats fed a diet containing ethanol for 7 days then a diet containing ethanol plus 30 IU vitamin E in the subsequent 14 days; (**D**) testes of rats fed a diet containing ethanol for 7 days then a diet containing ethanol plus and 90 IU vitamin E for the following 14 days. Arrows indicate apoptotic cells. More TUNEL-positive cells were present in the testes of rats fed ethanol than those fed the control diet. Vitamin supplementation reversed the apoptotic effects of ethanol, as demonstrated by fewer apoptotic cells in the two ethanol/vitamin E groups than those in ethanol no vitamin E group

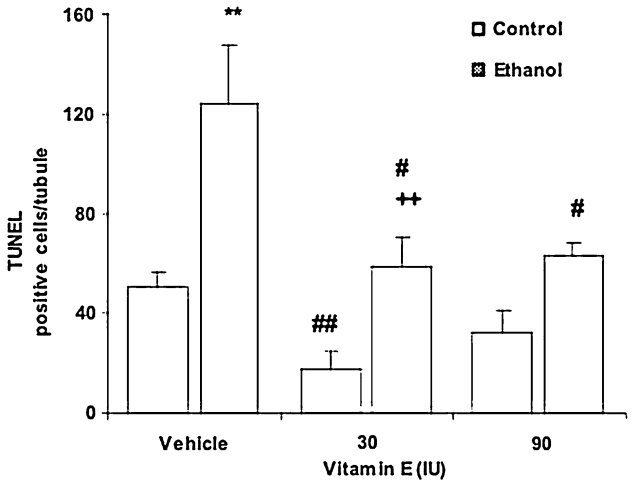

Fig. 7 The effect of ethanol and vitamin E on testicular germ cell apoptosis. The higher dose of vitamin E prevented the ethanol induced increase in apoptosis. $^{**}P < 0.01$ from control/vehicle, ## $P < 0.01$ from control/vehicle, # $P < 0.03$ from ethanol/vehicle, ++$P < 0.02$ from control/vitamin E 30IU, as determined by two-way ANOVA followed by Neuman–Keuls test. All values are expressed as mean ± SEM. n = 6–10 per group

The hypothalamus synthesizes and secretes LHRH into the hypothalamic–pituitary portal vessels, which deliver LHRH to the anterior pituitary. There LHRH attaches to specific cell surface receptors on the anterior pituitary and stimulates the synthesis and secretion of the two anterior pituitary gonadotropins, LH and FSH. In turn, LH and FSH stimulate the synthesis and secretion of testicular testosterone. Ethanol and pro-inflammatory cytokines each have effects at each level of the HPG.

Hypothalamus

Ethanol has been reported to decrease the LHRH secretion in several rodent studies [1, 12, 13]. In male rats, Ching et al. [12], observed that the LHRH concentration of hypothalamic–pituitary portal blood was significantly decreased by ethanol. In female animals, Ogilvie and Rivier [13] found that ethanol reduced LHRH in the hypothalamic interstitial space in living animals. This reproducible effect of ethanol was probably mediated either at an extra hypothalamic site or via conversion of ethanol to one or more of its metabolites [1]. In addition, repeated intraperitoneal injection of ethanol reduced LHRH gene expression assessed by both Northern blot analysis and in situ hybridization [13]. The increased levels of hypothalamic LHRH reported here in the ethanol animals not given vitamin E can be interpreted in several ways. One is that the increased tissue levels are due to decreased LHRH secretion; the second, increased synthesis; the third, decreased local LHRH degradation. These explanations, of course, are not mutually exclusive. Since ethanol reduces LHRH gene expression, it is unlikely that the ethanol-induced increase in hypothalamic LHRH content is due to increased synthesis [14]. Given the profound reduction in serum LH and testosterone and the available data on the effects of ethanol on LHRH, decreased secretion is the most likely explanation. This led to a decreased synthesis and release of LH with subsequent decreased serum testosterone. The restoration of hypothalamic LHRH by both vitamin E doses paired with ethanol feeding indicates that vitamin E overcame the ethanol-induced secretory blockade. The lack of concordance between LH and FSH has been seen before and McCann and colleagues have long suggested the possibility that there is a hypothalamic FSH releasing factor separate from LHRH [15]. The additional possibility that the inhibin/activin system is involved is also worth consideration.

Hypothalamus is one of the sites of TNF-α synthesis [16–19]. In rats, intravenous injection of the bacterial endotoxin lipopolysaccharide (LPS) suppressed pulsatile LH release, a reliable in vivo reflection of LHRH secretion [20]. This suppression was reversed by simultaneous intracerebroventricular injection of anti-TNF-α antibody, suggesting that LPS's effect involved TNF-α [20]. Interleukin 6 is made in the hypothalamus [21–23]. Although TNF-α seems to suppress LHRH release, reports show that IL-6 has either no effect [21] or a stimulatory effect [22, 24]. Since vitamin E reduced TNF-α and since TNF-α is inhibitory to LHRH, it is possible that vitamin E's action on LHRH is anti-inflammatory and anti-oxidant. It is of great interest that vitamin E has recently been shown to stimulate LHRH secretion from medial basal hypothalami of adult male rats in vitro [25].

Pituitary

There are many studies, from our laboratory and others, indicating that ethanol decreases secretion of the pituitary gonadotropins LH and FSH [26–31]. TNF-α is present and produced in the pituitary [16–18, 32–35]. Furthermore, functional surface TNF-α receptor protein, locally produced, is found in anterior pituitary as well [36–38]. Of relevance to this report is the finding that TNF-α suppressed LHRH stimulated LH release from dispersed pituitary cells in culture [21, 39]. Similarly, IL-6 decreases LHRH stimulated LH release from cultured male rat pituitaries, [21] an inhibitory effect also seen in human studies [40]. Since, vitamin E decreased pituitary levels of the two pro-inflammatory cytokines, it is quite conceivable that its protective effect on LH is anti-inflammatory as well as anti-oxidant. There is surprisingly little in the literature on the role of vitamin E in pituitary physiology. However, it is notable that pituitary

content and plasma levels of LH were significantly reduced in vitamin E deficient rats and that pituitary LH contents were increased in vitamin E supplemented rats [41]. Also, in vitro, vitamin E was able to prevent anterior pituitary cell apoptosis induced by cadmium [42].

Testes

Acute and chronic ethanol ingestion can cause an impaired testosterone production and testicular atrophy [43]. In many studies over the years, ethanol has consistently caused a significant fall in serum testosterone [29, 44–45]. Some of this effect is, no doubt, mediated via ethanol's effects at hypothalamus and pituitary. However, there is a direct ethanol effect as well. This is evidenced by demonstration of a direct testosterone suppressive effect of ethanol in isolated testes [46–47]. Ethanol reduced testicular concentrations of steroidogenic acute regulatory (StAR) protein mRNA, a central enzyme in testosterone biosyntheses providing some measure of mechanistic explanation for ethanol's effect [14].

There is a substantial body of work that supports an important role for TNFα in testicular pathophysiology. TNF-α is produced in germ cells [48–51] and resident macrophages of the testes [48, 52]. Testicular TNF-α receptors are located on both Leydig and Sertoli cells [48, 49, 51, 53–56]. In healthy men, TNF-α reduced serum testosterone without appropriate elevation of LH or FSH [56]. Abundant data, both in vivo and in vitro, in several animal species, have affirmed the ability of TNF-α to reduce testosterone and provided substantial insights into the mechanisms of this effect [48, 57–59]. IL-6 is produced in Leydig cells, Sertoli cells, and testicular macrophages [52, 60–62]. IL-6 injection reduces testosterone [40] and impairs spermatogenesis in humans [63–65]. Thus, like TNF-α, IL-6 inhibits testicular function. The relationship between vitamin E treatment, cytokines, and hormonal function is different in testes than in hypothalamus or pituitary. In both hypothalamus and pituitary, both doses of vitamin E prevented ethanol induced increases in cytokines and perturbation in hormonal function. In contrast, in the testes, there was discordance in that low dose vitamin E prevented ethanol associated cytokine increases but did not restore serum testosterone to control levels. Only the higher dose vitamin E did both. This suggests that in testes at least the protective effect of vitamin E may be both anti-inflammatory and anti-oxidant, though other mechanisms are not excluded. It is relevant that vitamin E deficiency has been reported to lead to decreased serum and testicular testosterone and decreased spermatogenesis in rats [66–67]. Furthermore, vitamin E supplementation significantly elevates testicular and blood testosterone in rats and circulating serum testosterone in humans [41].

The present study has demonstrated that vitamin E supplementation protected testicular germ cells from apoptosis. The results suggested that oxidative stress is primarily responsible for the increase in testicular apoptosis after ethanol exposure and that vitamin E can be used to prevent ethanol-induced tissue injury within the testis. Being a water and lipid soluble molecule, ethanol can easily penetrate the blood–testis barrier and reach germ cells. It is now well established that ethanol metabolism generates free radicals in various tissues, including the testis. Rosenblum et al. [68–69] have demonstrated that chronic ethanol exposure reduces testicular glutathione levels and increases testicular lipid peroxidation. Ikeda et al. [70] have reported that exogenously supplied oxygen free radicals induce apoptosis of isolated rat testicular germ cells in a dose-dependent manner. In vitro, vitamin E prevented oxidative stress and apoptosis of rat cardiomyocytes [71]. The protective effects of vitamin E observed in the present study were consistent with these earlier reports.

In this study, it was found that ethanol exposure increased testicular TNF alpha levels and vitamin E supplementation attenuated the effects of ethanol on testicular TNF alpha levels. In many tissues (such as the liver), TNF alpha is an apoptosis ligand. In the testis, TNF alpha is produced by round spermatids, pachytene spermatocytes, and testicular macrophages. The type 1 TNF receptor has been found on Sertoli and Leydig cells [72]. Pentikainen et al. [51] have shown that TNF alpha down-regulated Fas ligand levels and inhibited gem cell apoptosis when segments of seminiferous tubules were incubated with TNF alpha. Thus, the observed changes in TNF alpha levels do not seem to be directly related to germ-cell apoptosis. Instead, TNF alpha might be indirectly involved in the apoptotic effects of ethanol, via its inhibition of testosterone production [55, 59]. This is consistent with the facts that ethanol exposure reduces testosterone levels and vitamin E supplementation reversed the effects of ethanol on testosterone levels.

In summary, the widely available antioxidant vitamin E prevents the HPG hormonal changes caused by chronic ethanol ingestion. This is temporally associated with a reduction in hypothalamic and pituitary inflammatory cytokines supporting, but not proving the notion that inflammatory damage is a mechanism of ethanol's deleterious functional effects in theses organs. In testes, a low-dose vitamin E reduced the inflammation, but did not restore testosterone; only the higher dose of vitamin E was able to normalize both. This suggests the possibility that vitamin E's effect may be antioxidant or some other mechanism in addition to anti-inflammatory. These novel observations, therefore, pose new questions for future basic and clinical research.

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