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# Ethanol Suppressed the Expression of Steroidogenic Acute Regulatory Protein mRNA in the Prepubertal Rat Ovary

**Sang-Soo Kang, Gyeong-Jae Cho and Wan-Sung Choi†**

*Department of Anatomy, College of Medicine, Gyeongsang National University, Chinju 660-751, Korea*

# 미성숙 흰쥐난소에서의 에탄올에 의한 Steroidogenic Acute Regulatory Protein 유전자 발현 억제

강상수 · 조경제 · 최완성†

경상대학교 의과대학 해부학교실

**ABSTRACT:** The present study was undertaken to examine the effects of ethanol on the ovarian steroidogenic acute regulatory protein(StAR) gene expression during prepubertal and onset of puberty. From day 25, each rat began receiving either a control saline or ethanol. Animals were sacrificed on day 27 and 32, and their ovaries and blood were collected. In the present results, ethanol treatment significantly decreased serum luteinizing hormone contents at both time points. Uterine weights of ethanol-treated group were significantly lighter than control group at early time point, while there was no noticeable discrepancy at late time point. Vaginal openings, a marker of onset of puberty, also clearly delayed in ethanol-treated group. Using an in situ hybridization histochemistry, we determined the expression of mRNAs encoding StAR. Ovaries from ethanol-treated rats showed a suppresed expression of StAR mRNA. These results demonstrate that ethanol can disturb the prepubertal ovarian function and onset of puberty, at least in part, through the inhibition of ovarian StAR gene expression.

**Key words:** Ethanol, Rat, Ovary, StAR, Puberty.

**요 약**: 본 연구는 사춘기전과 사춘기가 시작될 무렵의 흰쥐난소에서 에탄올이 steroidogenic acute regulatory protein(StAR)의 유전자 발현에 어떠한 영향을 미치는지 조사하고자 수행되었다. 생후 25일 째부터 매일 흰쥐에 에탄올 또는 생리식염수를 복강주사하고 생후 27일, 32일 째에 실험동물을 희생시켜 혈액과 난소를 적출하였다. 실험결과 에탄올은 혈중의 황체호르몬 함량을 유의하게 감소시켰으며, 자궁의 무게는 27일 째에는 에탄올처리군에서 유의하게 적었으나, 32일 째에는 차이를 나타내지 않았다. 사춘기의 시작을 나타내는 지표의 하나인 viginal opening은 에탄올처리군에서 현저히 지연되었으며, 난소에서의 StAr mRNA 발현 또한 유의하게 감소됨을 알 수 있었다. 따라서 본 연구결과는 에탄올이 적어도 사춘기 난소의 기능 및 사춘기의 시작을 교란시킬 수 있으며, 이러한 영향의 일부는 난소내 StAR 유전자 발현을 억제함으로써 나타난다고 사료된다.

## INTRODUCTION

It has been well known that alcohol suppressed the reproductive activity at the various level of hypothalamus-pituitary-gonadal axis(Lee & Rivier, 1997). Dees & Skelley (1990) reported that ethanol administration during the peri-pubertal period caused a delay in the onset of puberty in the female rat, and provided an evidence that this delay was due to ethanol's action at the hypothalamic level. They also showed in vitro that ethanol was capable of interfering with the release of gonadotropin-releasing hormone(GnRH) from the median eminence of prepubertal female rats. In support of this result, we also have some unpublished data that ethanol treatment reduced the expression of hypothalamic GnRH mRNA levels in the adult male rat. In fact, there have been numerous reports that ethanol primarily acts at the level of brain. However, there were still little evidences that how ethanol induced lower activity of hypothalamic-pituitary reproductive axis affects the normal

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†교신저자: 경상남도 진주시 칠암동 92, 경상대학교 의과대학 해부학교실 (우) 660-751 (전) 055-751-8716 (팩) 055-759-0779 e-mail: choiws@nongae.gsnu.ac.kr

cycling and prepubertal ovarian function, especially the steroidogenesis. Actually, it has been well known that ethanol treatment inhibitory affected the hypothalamic GnRH secretion, pituitary gonadotropin release, and gonadal steroid secretion (Ogilvie & Rivier, 1997). There were also plenty amount of studies related to the effects of ethanol on the onset of puberty and ovarian function. However, the mechanism underlying the suppression of steroid secretion by ethanol is still controversial and not clear.

The first reaction in the biosynthesis of steroid hormones is catalyzed by the cholesterol side-chain cleavage enzyme, P450scc. This reaction is acutely stimulated by tropic hormones acting through the intermediacy of cAMP. This acute steroidogenic response involves the translocation of cholesterol from the outer to inner mitochondrial membranes, a process that is effected by steroidogenic acute regulatory protein(StAR). Congenital lipoid adrenal hyperplasia, an autosomal recessive disease in which synthesis of adrenal and gonadal steroid is severely impaired, is caused by mutation in the StAR gene(Lin et al., 1995). Expression of StAR is directly correlated with steroidogenic activity in adrenal and gonadal cells(Stocco & Sodeman, 1991). The abundance of StAR mRNA in gonadal cells is regulated by cAMP(Clark et al., 1995).

In the present study, we tried to evaluate the effect of ethanol at the level of ovary by estimating the level of StAR mRNA during pubertal period using in situ hybridization.

## MATERIALS AND METHODS

### 1. Animals and Tissue Preparation

Immature female Sprague-Dawley rats were maintained under conditions of temperature(25℃) and lighting (14 h light; 10 h dark, light on at 06:00 h) and allowed free access to food and water. The ethanol group received 15% ethanol(3 g/kg/day) instead of saline from day 25. Animals were sacrificed at day 27 and 32. For in situ hybridization, rats were deeply anesthetized with penobarbital(12 mg/100g, i.p.) and perfused transcardially with ice-cold 4% paraformaldehyde for 10 min at a flow rate of 50~60 ml/min. Ovaries were removed immediately, immersed in a cold 4% paraformaldehyde for 2 days, and then washed in a series of cold 20% sucrose solution. Frozen sections were cut in 10 $\mu$m thickness on cryostat. Sections were thaw-mounted on the probe-on plus charged slides(Fisher) at room temperature for 10 min, and then stored at -70℃. Trunk bloods were collected for radioimmunoassay of serum luteinizing hormone(LH). Every day during experimental periods we checked the vaginal opening. Uterine weights were immediately measured when animals were sacrificed using micro top-balance.

### 2. Radioimmunoassay of Serum LH

Blood LH concentrations were assayed using a double antibody radioimmunoassay reagent kindly provided by the National Pituitary Agency. The tracers NIADDK-rLH-I9 were iodinated by the chloramine-T method. The antiserum was NIADDK-rLH-S-10 and the reference preparation was NIADDK -rLH-RP-2. LH concentrations were expressed as NIADDK RP2 units. The intra- and inter-assay coefficients of variation were approximately 7.6 and 10.0%, respectively.

### 3. *In situ* Hybridization

Sections of each ovary(thickness; 10 $\mu$m) on gelatin-coated slides were made with a cryostat(Leicar, Germany) at -20℃. These sections were fixed and dehydrated as described(Gerfen & Engber, 1992). Briefly, sections were fixed, acetylated, dehydrated, and treated with chloroform for lipid droplet removing. After slight rehydration, sections were stored at -70℃ in tight plastic box until hybridization. *In situ* hybridization was performed as described(Shin et al., 1999). The digoxigenin-11-UTP labeled cRNA probe was *in vitro* transcribed(BM, Germany) using cloned partial cDNA as template. Hybridization was performed with antisense and sense probe(50 ng/ml) in humidified chamber at 42℃ for 18 h. To remove unhybridized probes, sections were washed and treated with RNase A(20 $\mu$g /ml) in NTE buffer(0.5 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). After washing in Buffer I(0.1 M maleic acid, and 0.15 M NaCl, pH 7.5) and immersion in buffer containing 1% blocking reagent(BM, Germany) for 1 h at room temperature, these sections were incubated with buffer containing alkaline phosphatase-conjugated anti-DIG-antibody (1.5 U/ml, BM, Germany) in a humidified chamber at 37℃ for 1 h. After incubation, sections were washed with Buffer II(0.1 M Tris-HCl, 0.1M NaCl, 50 mM $MgCl_2$, pH 9.5) and I. For color development, sections were incubated in enzyme substrate containing 0.4 mM NBT/ BCIP(BM, Germany) in dark space for

24 h. The color reaction was stop with distilled water when color development was adequate. *In situ* hybridization was undertaken independently at least three times for individual animal.

## RESULTS

In the present study, we try to confirm the inhibitory effect of ethanol on the onset of puberty, and to test the hypothesis that ethanol-induced suppression of steroid secretion and delayed ovarian cyclicity are related to the expression of StAR.

Ethanol treatment significantly lowered the serum LH levels at both time points(Fig. 1). The mean content of serum LH was 0.48 ± 0.11 ng/ml in control group and 0.31 ± 0.04 ng/ml in ethanol-treated group at day 27, 0.63 ± 0.13 ng/ml in control group and 0.32 ± 0.09 ng/ml in ethanol-treated group at day 32. We also measured serum PRL levels, but there was no significant changes between two tim points and only slightly increased tendency in ethanol-treated groups(data not shown).

Ethanol treatment reduced the uterine weight at first time points(day 27). But in the late time point(day 32), there was no significant discrepancy between ethanol-treated and control group(Fig. 2).

Since vaginal opening is well used to assess the timing of puberty, we monitored vaginal opening from day 25 with 10 animals in each group. In the control group, vaginal opening started at day 29, and 8 animals showed this at day 32. Vaginal opening time was significantly delayed in ethanol-treated group(Fig. 3).

Using in situ hybridization histochemistry, we studied the

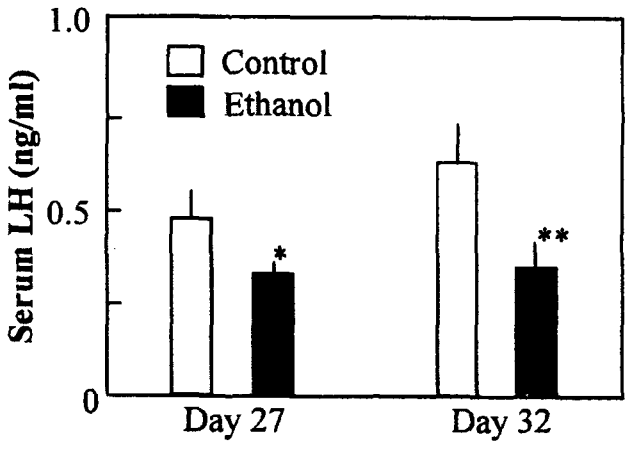


**Fig. 1. Effect of ethanol on serum LH levels.** From postnatal day 25, animals were received ethanol(15% in 0.9% saline, 3 g/kg/day) or saline at 10:00 h. Animals were sacrificed at day 27 and 32. Trunk bloods were collected for LH determination. Each bar represents the mean(± SEM, n=6) of serum LH levels. *P<0.05, **P<0.01.

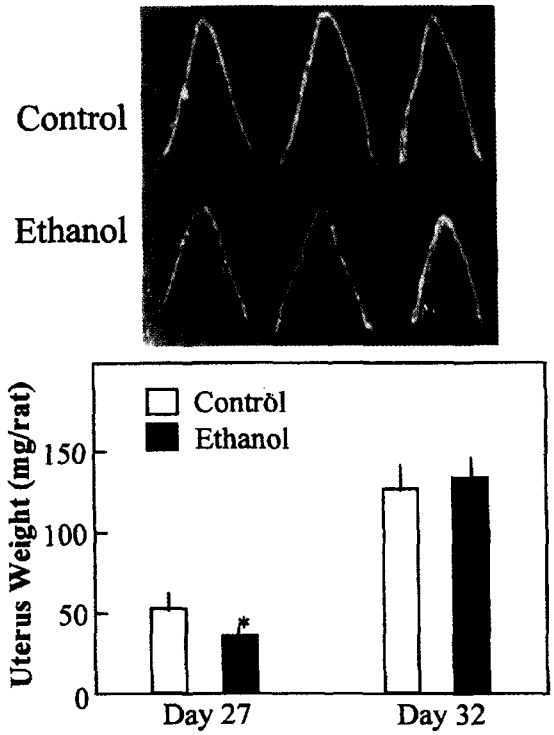


**Fig. 2. Effect of ethanol on the uterine weight.** Uterus was immediately excised, removed the fat, and weighed. Upper panel showed the photography of representative uterus from 27-day old animals. Lower panel represents the weight of uterus. Each bar represents the mean(± SEM, n=6) of uetrine weight. *P<0.05.

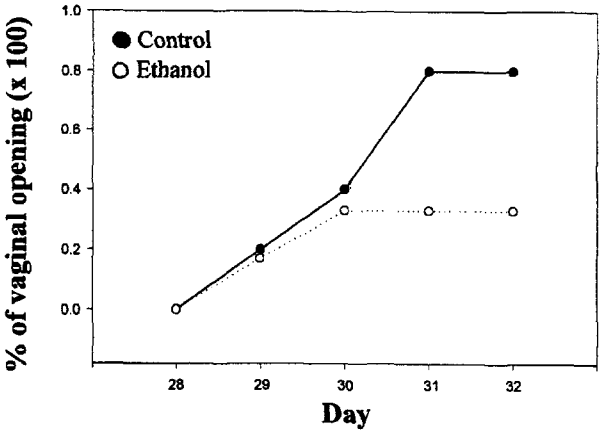


**Fig. 3. Effect of ethanol on the time of vaginal opening.** Ten animals in each group were monitored vaginal opening from day 25. Vaginal opening is used to assess the timing of puberty.

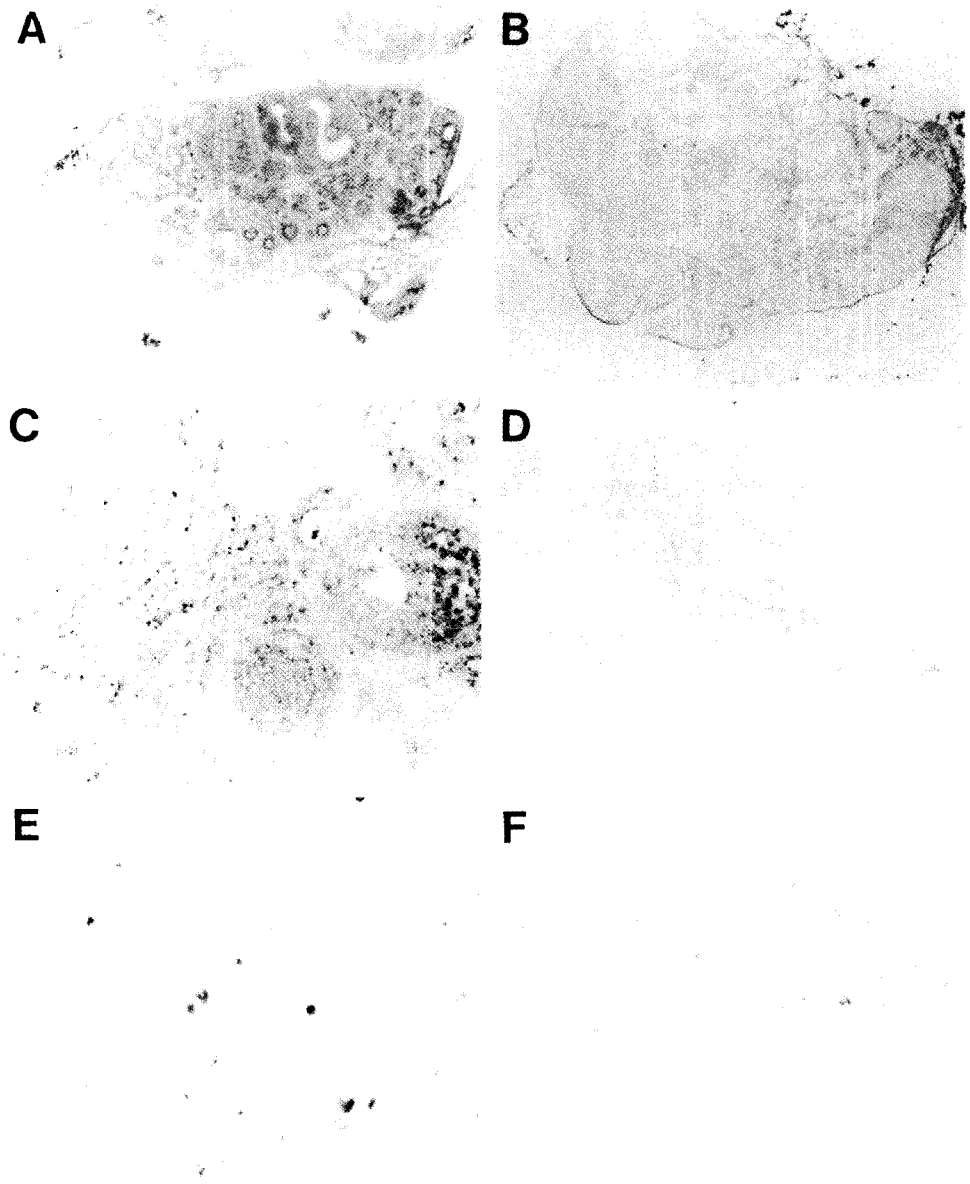


**Fig. 4. Effect of ethanol on the expression of StAR mRNA in the rat ovary.** In situ hybridization was performed with at least five individual ovaries at day 27. A, C, E: control group. B, D, F: ethanol-treated group. A, B: ×20 ; C, D:×100 ; E, F:×400.

effect of ethanol on the expression of ovarian StAR mRNA. As shown in Fig. 4. ethanol treatment clearly suppressed the expression of StAR mRNA. These data was further confirmed using Northern blot analysis(data not shown).

## DISCUSSION

The deleterious effects of ethanol on the hypothalamic-pituitary reproductive axis in adult male humans and animals have been well documented(Mello et al., 1989; Van Thiel & Gavaler, 1982). It is also well established that ethanol has toxic effects on testicular function in adult humans and animals (Gonzales-Reimers et al., 1994). Ethanol use and abuse by adolescents has been rising at an alarming rate. We used immature female rats to assess the effects of ethanol on the expression of StAR mRNA known to be involved in the rate limiting step for the steroidogenesis, critical for pubertal process.

Recently, Dees et al.(2000) augmented that ethanol affected

the development of a regular monthly pattern of menstruation through the detrimental effects on the activation of hormone secretion that accompanies puberty in female rhesus monkeys. They also suggest that the subsequent growth spurt and normal timing or progression of puberty may be at risk in human adolescents and teenagers consuming even relatively moderate amount of ethanol on a regular basis.

To assess more closely the physiological mechanism(s) by which ethanol delays the onset of female puberty, we have evaluated its inhibitory effects on uterine weight, the vaginal opening, and the serum concentrations of LH. These results are well agreement with others. From this result, ethanol might also affect the maturation and growth of uterus. However, the precise action mechanism of ethanol on the uterine growth and maturation needs further study. Dees & Skelley(1990) showed that ethanol produced varying detrimental effects on the onset of puberty and subsequent removal of ethanol from the diet resulted in rapid growth of the animals, followed by the onset of puberty.

In respect to the target site of action, the effects of ethanol on the reproductive system is multiple and variable along the hypothalamo-pituitary-gonadal reproductive axis (Canteros et al., 1995). For instance, treatment with alcohol-containing diet for 5 weeks to intact male rats resulted in decreases of serum LH, FSH, and testosterone levels, while expression of receptors for GnRH in the pituitary was not affected(Adams & Cicero, 1991; Little et al., 1992). In the female rats, treatment with alcohol resulted in a profound inhibition of the reproductive axis, including blockade of the preovulatory surges of GnRH and LH, suppression of ovulation, and perturbation of steroid hormone secretion(Ogilivie & Rivier, 1997). As shown in above studies, ethanol may act on the entire of hypothalamus-pituitary-gonad reproductive axis. Furthermore, Dees et al.(1990) demonstrated that ethanol-treated animals showed a significant increase in the hypothalamic content of GnRH with a significant decrease in the serum concentration of LH, but not follicle stimulating hormone. These results also demonstrate that chronic, prepubertal ethanol administration alters the concentrations of specific hypothalamic and pituitary hormones which are known to be involved in the female pubertal process. In the present study, ethanol treatment also reduced serum LH level implying the inhibitory action of ethanol on the hypothalamic GnRH neuronal activity. StAR is known to play a critical role in regulation of the rate-limiting step in steroid hormone synthesis, cholesterol side-chain cleavage(Sugawara et al., 1997). Furthermore, StAR gene expression is transcriptionally regulated in the gonads by gonadotropic hormones which was regulated by hypothalamic releasing hormone. It is presumable that delayed onset of puberty and ovarian acyclicity is due to the primary action of ethanol on the hypothalamic level, thereby reduced gonadotropin and ovarian expression of StAR. However, there was report that ethanol has direct inhibitory effect on steroidogenesis in human granulosa cells: specific inhibition of LH action. Saxena et al.(1990) demonstrated a direct effect of ethanol on cultured granulosa-lutein cells and suggest that ethanol may inhibit action of LH on the corpus luteum. A direct selective toxic effect of ethanol on the ovary may be responsible for some of the reproductive abnormalities observed in alcoholic women.

In conclusion, even though whether ethanol has direct or indirect effect on the ovarian StAR mRNA expression is still needs more studies, present results demonstrate that ethanol's detrimental effect on the female reproduction and onset of puberty, at least in part, mediated through inhibition of ovarian StAR gene expression.

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