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DIRECT ACTION OF ETHANOL ON THE LUTEINIZING HORMONE-STIMULATED ANDROGEN SYNTHESIS BY THE ISOLATED INTERSTITIAL CELLS FROM MOUSE TESTIS[1]

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John Yuh-Lin Yu, Sheu-Yueh Roan, Zuey-Shin Hsu and Walter Chia-Mo Wan (1981) Direct action of Ethanol on the luteinizing hormone-stimulated androgen synthesis by the Isolated interstitial cells from mouse testis. *Bull. Inst. Zool., Academia Sinica* **20**(1), 67-74. Mice were used as a model for studying the effect of ethanol on reproductive function. The effect of ethanol on LH-stimulated androgen synthesis was investigated using the mechanically dissociated interstitial cells from the testes of 6-wk old mice. Various doses of ethanol, ranging from 0.02% to 20% of the incubation medium, were incubated with the interstitial cells in the tubes that contained 50 ng or 12.5 ng of rat LH for 3 hrs at 34°C, shaken at 100 cycles per min in Medium 199-xanthine-heparin incubation medium. The androgen produced was estimated by radioimmunoassay using testosterone antiserum. The results indicated that low doses of ethanol increased the LH-stimulated androgen formation whereas high doses decreased the hormone production. It is also demonstrated that high dose of ethanol (10% by vol) exhibited an instantaneous inhibition of androgen synthesis in the interstitial cells that have been stimulated with LH *in vitro* for various durations of time. It is concluded that the ethanol exhibits a biphasic actions on LH-stimulated androgen production under *in vitro* condition, and that the suppressive effect of high dose of ethanol is instantaneous.

It has been known that ethanol affects the liver and brain metabolisms as well as the reproductive functions in human[1,3~7,16~19,24~26]. Chronic exposure to ethanol markedly disrupts the hypothalamic-pituitary-gonadal axis, leading to hypogonadism, sterility, and impotency[23,24]. Studies have also shown that the plasma testosterone levels are lowered following chronic or acute exposure to ethanol[3~8]. Many of the ethanol-induced alterations in reproductive functions and the endocrine patterns in human have also been observed in several species of animals. As a consequence, rats and mice have been used as a model for studying the effects of ethanol on the reproductive system.

Studies indicated that the plasma levels of both luteininzing hormone (LH) and testosterone are decreased after acute treatment of ethanol[5~8]. While most of the studies showed that ethanol is inhibitory to testosterone synthesis, there are indications, from *in vivo* works, that low doses of ethanol stimulate the testicular steroid formation[6]. Very little information is available

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as to whether small doses of ethanol are stimulatory, inhibitory, or of no effects on the testicular synthesis of androgen under *in vitro* conditions. A study was therefore conducted to investigate the direct effects of ethanol on the LH-stimulated androgen formation by the dispersed Leydig cells isolated from the mouse testis, with special reference to the response by low doses of the agent. The present study indicates that low doses of ethanol stimulate the LH-induced androgen synthesis while higher doses exhibit an inhibitory action. The current investigation also show that the higher doses of ethanol exhibit an instantaneous suppression of androgen formation in the interstitial cells that have been stimulated with LH *in vitro* for various durations of time.

MATERIALS AND METHODS

Preparation of interstitial cells from mouse testis

Six-wk old mice, ICR, U. S. A., were purchased from the National Laboratory Animal Resources, National Taiwan University. The animals were raised in a temperature controlled room (22±2°C), fed *ad libitum* with Purina Chow; the lighting schedule was 12L:12D. The method in preparation of testicular interstitial cells was similar to that described previously[20] which is a modified procedure reported by Dufau *et al.*[10] and Van Dame *et al.*[22]. Briefly, the mice were sacrificed by cervical dislocation, the testes were removed and placed in a plastic Petri dish containing 3 ml of aerated preincubation medium (Medium 199 with Hank's salts, L-glutamine and 25 mM HEPES buffer, penicilin 10,000 units/100 ml, streptomycin 5 mg/100 ml, 0.2% bovine serum albumin, 10% sodium bicarbonate, 1.0 ml/100 ml, pH 7.40. The testes were cut with surgical blade into small pieces and 15 ml of the same medium was added. The tissues were gently dispersed for 15 min with a magnet stirrer surrounded by an ice-bath; the medium was repeatedly withdrawn into a fire-polished Pasteur pipet over several minutes until a homogeneous suspension was obtained. The cell suspension was then filtered through a fine nylon mesh, and preincubated for one hour at 34°C with gentle shaking (25 cycles/min). The cell suspension was cooled in ice-water, and centrifuged at 6°C, 250 g for 15 min. Sedimented cells were suspended in 2.5 ml/testis of incubation medium (preincubation medium + 0.125 mM methyl - isobutyl - xanthine + sodium heparin, 0.5 ml/100 ml). Incubation was performed in a tightly capped polyethylene tube (13 × 100 mm) at 34°C, shaken at 100 cycles/min in a Dubnoff metabolic incubator. The total volume in an incubation tube was 220 μl. Various doses of of ethanol and rat LH-RP-1 were incubated with the interstitial cells. After 3 hrs incubation, the tubes were placed in ice and 2.5 ml of 0.01 M PBS, pH 7.4, was added. Tubes were centrifuged for 30 min at 6°C, 1500 g. The suspernatant was collected and stored at −25°C until assay for androgen.

Assay of androgen

The radioimmunoassay procedure for androgen was a modification of Wingfield and Farner[27] and Anderson *et al.*[2]. The modified procedure quantitated total androgen, since a chromatographic separation of androgens was omitted. Briefly, 0.8 ml of the PBS diluted incubation medium was extracted once with 5 ml of diethyl ether (Merck) from a freshly opened can and allowed to freeze in the dry ice-ethanol medium. The ether layer was decanted into another tube and dried under ventilation hood at 38°C. The dried residue was dissolved in 1.0 ml of 0.01 M PBS (pH 7.4) containing 0.1% gelatin and incubated at room temperature for one hour. Tritiated testosterone (1, 2, 6, 7-^{3}H-testosterone, 88.5 Curies/m mole, Amershan) and testosterone antiserum were added and then incubated for 20 hrs at 4°C. Dextran-coated charcol was employed to separate the antibody-bound from the free steroid. Supernatant containing the bound labeled steroid was counted in a liquid scintillation spectrometer with counting effeciency of 55%. The assay was sensitive to 5 pg of testosterone per assay tube. The between-assay coefficient of variation was 14.2% and the within-assay coefficient of

variation was 4.5%. Standard and incubation samples produced parallel displacement of trtiated testosterone. The average recovery of added steroid was 91±4% (mean±SE) and individual recoveries were run with each sample.

The specificity of testosterone antiserum was described previously[2]; it cross-reacted with dihydrotestosterone, androstenedione, and androstenediol at 90-, 12-, and 11%, respectively, relative to testosterone (100%). The concentration of androgen in the sample was expressed as testosterone equivalent extrapolated from the standard curve. The data were statistically analyzed using the Least Significance Difference (LSD) to test the difference between controls and the ethanol treatments.

RESULTS

The numbers of total interstitial cells were adjusted to 0.3×10^6 per incubation tube for all incubations of the present study. The viability of the cells, as determined by trypan blue staining, was approximately 70%.

The responsiveness of the dispersed interstitial cells to various doses of rat LH is shown in Fig. 1. As indicated, a dose-response relationship was obtained from 2 to about 40 ng of LH under the experimental conditions. The rat LH from 50 to 250 ng tested in this study stimulated a maximal synthesis of androgen, approximately 7 ng per incubation tube. The effects of ethanol on androgen production by the dispersed interstitial cells were studied with stimulations by two doses of rat LH: 50 ng and 12.5 ng per tube.

As shown in Fig. 2, ethanol at final concentrations of 2.5% and 5% of the incubation medium partially inhibited the androgen production when stimulated by 50 ng of LH; while higher concentrations of ethanol (10% and 20%) exhibited complete inhibition of the androgen formation. Particular emphasis was placed to the effects of low doses of ethanol on the LH-stimulated androgen synthesis. Ethanol concentrations ranged from 0.02% through 0.3% exhibited a slightly stimulatory effect on

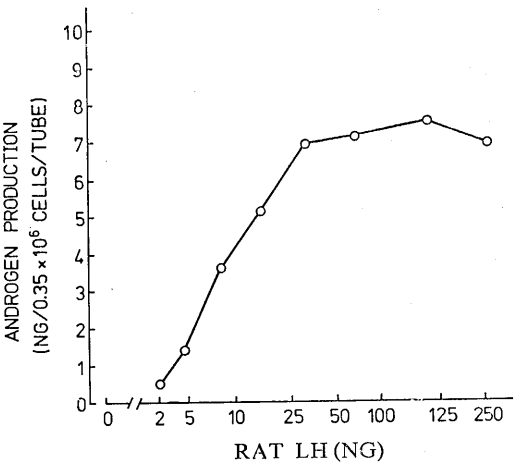

Fig. 1. Androgen response curve obtained by incubation of mouse testicular interstitial cells with increasing concentrations of rat LH from 2 to 250 ng. Each tube contained approximately 0.35×10^6 cells. The concentration of androgen was measured at the end of 3 hours incubation. See Materials and Methods for further details.

androgen production. Qualitatively similar patterns were observed when the corresponding doses of the ethanol were added to the interstitial cells incubated with 12.5 ng of rat LH. The relative changes of androgen production affected by various doses of ethanol, as compared to the controls (interstitial cells incubated with LH only), are shown in Fig. 3. Low doses of ethanol induced an average of 16% increase of androgen production from the interstitial cells incubated with 50 ng of rat LH over that of the controls, and an average of 42% increase of androgen over the controls when incubated with 12.5 ng of rat LH.

As described above, the higher concentrations of ethanol completely inhibited the LH-stimulated androgen synthesis when both LH and ethanol were simulataneously incubated with the cells. A preliminary experiment was also undertaken to demonstrate whether the inhibitory action by the high doses of ethanol on the androgen synthesis in the Leydig cells is instantaneous following addition to the Leydig

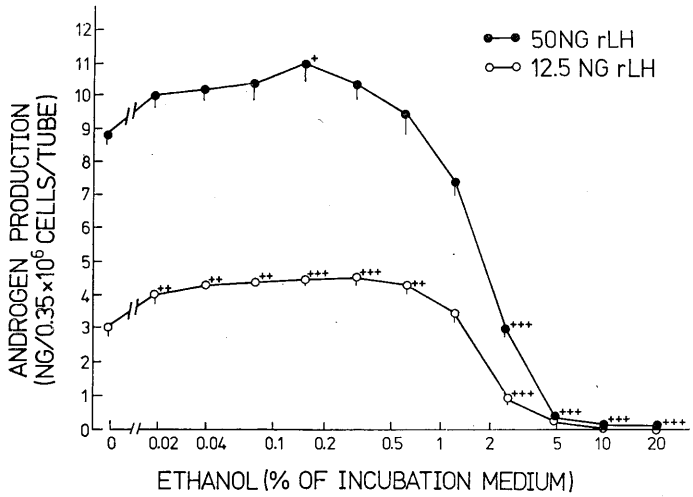

Fig. 2. Effects of ethanol on LH-stimulated androgen production by the dispersed interstitial cells from mouse testis incubated with 50 ng or 12.5 ng of rat LH. Ethanol was added at the begining of incubation. Each point represents the mean±SEM from 3 incubations carried out at different times. The symbols $^{+}$, $^{++}$, and $^{+++}$ denote the significance levels at $p \leq 0.1$, $p \leq 0.05$ and $p \leq 0.01$, respectively, as compared to the controls (cells incubated with LH only).

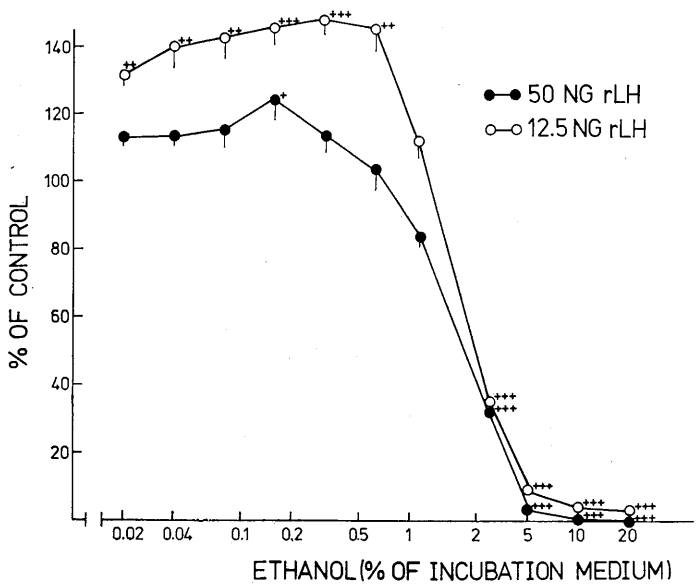

Fig. 3. The relative changes of the effects of ethanol on the LH-stimulated androgen production by the interstitial cells. All the values are relative to the controls. See Fig. 2 for further details. The denotions of the symbols, $^{+}$, $^{++}$, and $^{+++}$ are identical to those in Fig. 2.

cells that have been stimulated with LH *in vitro* for various durations of time. Ethanol of 10% of the incubation medium was thus added to incubation tubes in which the interstitial cells were previously incubated with 50 ng of rat LH for 0, 15, 30, 60, or 120 min, and were then continuously incubated for a total of 6 hrs. The androgen produced in the ethanol-treated tubes were compared to the androgen produced from the corresponding controls (cells incubated with 50 ng of LH only, and withdrawn from incubation at the time when ethanol was added to the parallel incubation tubes). As shown in Fig. 4, production of androgen was increased with incubation time in the controls (C_1-C_5); the synthesis of androgen was detectable as early as 15 min following incubation of the cells with LH. The androgen values in the ethanol-added tubes (E_1-E_5) were similar to those of the parallel control tubes. These results indicate that no further androgen was produced during the rest period of incubations following additions of ethanol.

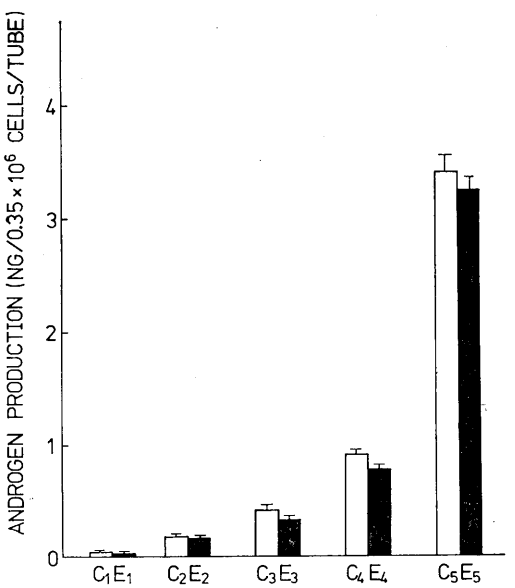

Fig. 4. The effects of ethanol on the androgen production by the dispersed mouse testicular interstitial cells that were previously incubated with 50 ng of rat LH for various durations of time. The androgen produced before ethanol added (the controls) was compared with androgen produced in the paralell ethanol-added tubes. C1, C2, C3, C4 and C5 denote the contol tubes (cells plus LH only) which were withdrawn from incubations at 0-, 15-, 30-, 60- and 120 min intervals, respectively; while E1, E2, E3, E4 and E5 denote the parallel tubes into which the ethanol was added at 0-, 15-, 30-, 60- and 120 min, respectively. The androgen produced in the control tube for 6 hrs was 9.50 ng. The data are expressed as mean±SEM for 3 incubations conducted at different times.

DISCUSSION

Studies have shown that the functions of both pituitary and gonad are affected by ethanol treatment[3~9]. It has been equivocal that what the primary locus or organ is in the reproductive system to which ethanol affects. Some investigations indicate that the primary site of ethanol suppression of testosterone production is at the testicular level[13,24]. Other studies support that the ethanol exerts its action primarily on the hypothalamic-pituitary axis, altering the LH release which, in turn, suppresses the LH-stimulated androgen synthesis in the testis[6~8,21]. Whether the hypothalamic-pituitary axis or the testis or both is the primary site of ethanol action affecting the reproductive function and capacity remains unclear.

The results from the present study clearly indicate that high doses of ethanol exhibit an inhibitory effect on the LH-stimulated androgen synthesis by the dissociated interstitial cells from the mouse testis. Such results obtained from the *in vitro* studies are in agreement with previous findings reported by other researchers[4,9,12] under similar experimental conditions. Ethanol has biphasic effects on many physiological processes such as neuronal firing patterns, sensory processing, and more complex behavioral function[28]. *In vivo* studies[6] also demonstrated that the ethanol has biphasic actions in the reproductive system: low doses of ethanol

increase LH and testosterone levels in the plasma while high doses decrease the release of both hormones. *In vitro* studies on decapsulated whole testis or dispersed testicular interstitial cells are useful for investigating whether the ethanol exerts its action directly on the gonad. Badr *et al.*[4] reported that small dose of ethanol appears to have a stimulatory effect on the LH-induced testosterone synthesis by the decapsulated mouse testes under *in vitro* conditions. In their study, however, the stimulatory effect was demonstrated by a single low dose of ethanol only. A systematic study was thus undertaken to demonstrate the direct effect of ethanol on the LH-stimulated androgen production by the dispersed mouse interstitial cells with special reference to the effect of low doses of ethanol. Our present study showed that a direct promotive effect on LH-stimulated androgen production was observed with small doses of ethanol. The biphasic actions of ethanol on the testicular androgen synthesis were demonstrated using the dispersed mouse interstitial cells.

The regulation of steroidogenesis in the gonad is complex, involving at least the factors such as receptor binding, cAMP formation, precursor availability, and enzyme activity. The mechanism under which the ethanol affects testicular production of androgen is little understood. *In vivo* studies showed that ethanol inhibits the binding of ^{125}I-HCG to rat Leydig cells[15]. However, such effect was not observed by the *in vitro* works where testicular homogenates were incubated with ethanol and ^{125}I-HCG[8]. Consequently, whether the ethanol influences the hormone receptor binding capacity remains inconclusive. In contrast, it is shown that ethanol can lower the intracellular level of pyridine nucleotide cofactors which are important in the biosynthesis of steroids[14]. Ethanol also inhibits the cAMP-induced androgen production, similar to the patterns obtained with LH-stimulated androgen formation, thus indicating the primary locus of ethanol action is intracellular rather than the plasma membrane receptors of the Leydig cells[11]. The results from the present study showed that the ethanol concentrations from 5 to 20% (by volume) resulted in nearly complete inhibition of androgen production when both LH and ethanol were simultaneously incubated with the interestitial cells. A preliminary experiment was made to determine whether the ethanol at such high concentration could exhibit an instantaneous inhibition on the androgen production by the interstitial cells which were previously incubated with LH *in vitro* for various intervals of time. Our data indicate that 10% of ethanol suppressed the androgen formation instantaneously following addition to the incubation tubes where the steroidogenesis were in progress in the Leydig cells as stimulated by LH (Fig. 4). However, such findings do not permit any conclusions as to the possible mechanisms by which high doses of ethanol inhibit the ability of the testes in response to LH stimulation *in vitro*. Clearly, this concentration of ethanol could never be reached in the blood or tissues in the living animals. Further studies using lower doses of ethanol are required to elucidate the mechanisms of ethanol interference with androgen synthesis in the Leydig cells.

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酒精對小白鼠睪丸雄性素生成之影響

余玉林　阮秀月　許瑞信　萬家茂

本研究以小白鼠爲模型實驗，研究酒精對生殖系統之影響。將 6 週齡之小白鼠睪丸精間細胞分離；以離體培養法觀察酒精對精間激素 (ICSH) 所促進之雄性素生成之效應。精間細胞與各劑量之酒精 (佔培養液之 0.02% 至 20%)，以及 50 或 12.5 Nanogram 之 ICSH 一齊培養於 Medium 199-Xanthine-Heparin 培養液；經 3 小時後，以放射免疫法定量雄性素之生成。

結果指出低劑量酒精稍能促進雄性素生成，而中等劑量則有部分抑制作用，高劑量完全抑制雄性素生成。本研究以離體方法，闡明酒精可直接影響睪丸雄性素之生成；其作用爲雙重性具促進與抑制作用，視其量而定。本實驗亦指出高劑量之酒精 (10%) 能卽刻抑制精間細胞雄性素之生成，但是否干擾細胞之一般活動功能或特異抑制雄性素生成，尙待進一步研究。