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Biological effects of trilostane in vitro on oocyte maturation and fertilization in the hamster

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Summary. The effects of the inhibition of steroidogenesis by trilostane on oocyte maturation were examined by studying spontaneous maturation and fertilization in vitro. 10^{-6} M trilostane had no influence on the meiotic process, whether the oocytes were naked or not. At a concentration of 10^{-6} M and 10^{-7} M trilostane, low normal pronuclear formation and high polyspermy were found during in vitro fertilization. However, no retarded male pronuclear development could be detected in the trilostane-treated group. Thus, steroid producing activity within ova is apparently necessary to prevent multiple sperm penetration, but it has no effect on meiosis or the action of the so-called male pronucleus growth factor (MPGF).
Key words. Hamster oocytes; oocytes, hamster; trilostane; oocyte fertilization in vitro; oocyte maturation in vitro; steroidogenesis; pronuclear formation; polyspermy.

Introduction

There has been no definite determination of the various roles of steroids in mammalian oocyte maturation and subsequent fertilization. In particular, the significance of steroids in regulating the resumption of meiosis and nuclear maturation of oocytes is still controversia[1,5,10,11,20] with regard to the cytoplasmic maturation of oocytes, some reports have appeared on the importance of steroids for the completion of maturation and the synthesis of male pronucleus growth factor (MPGF)[12,18]. Steroid-producing activity in hamster[7] and human follicular oocytes[13] has been studied by cytochemical and indirect immunofluorescence and found to be that of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD). Adenylate cyclase activity has also been detected in hamster[7] and human follicular oocytes[13] by ultrastructural-cytochemical analysis. Thus, on the basis of these findings, steroidogenesis and the adenylate cyclase-cyclic adenosine 3′:5′-monophosphate (cAMP) system seem to be related. In our previous paper, the dose-dependent inhibition of Δ^5-3β-HSD activity by 10^{-6} to 10^{-8} M trilostane (Mochida Pharmaceutical Company, Tokyo, Japan) in hamster ova was reported[14]. In this study, we investigated the effects on meiotic maturation and fertilization in vitro of blocking the key enzyme, Δ^5-3β-HSD, inside ova by trilostane, in order to assess full maturation in the hamster.

Materials and methods

Female golden hamsters 9–12 weeks old with a normal reproductive cycle were used for the in vitro maturation experiments, and 6–10-week-old females and 8–12-week-old males were used for in vitro fertilization.

In vitro maturation of oocytes from preovulatory follicles. The females were injected i.p. with 30 i.u. pregnant mare serum gonadotropin (PMSG, Teikoku Zoki, Tokyo, Japan) and the ovaries were removed 48 h later[4]. Cumulus-oocyte complexes were recovered by puncturing the antral follicles with a 26-gauge needle in a watch glass filled with equilibrated medium. The complexes were washed and incubated in 20 µl of medium under paraffin oil in a 35 × 10 mm tissue culture dish (Falcon Plastics, Oxnard, CA) at 37°C in an atmosphere with 5% CO_2 for 16 to 18 h. Ten to 15 complexes were present in each drop. To prepare denuded oocytes, the surrounding cumulus cells were removed by passing them through a fine flame mouth-operated micropipette. The denuded oocytes were washed and incubated in the same manner as the cumulus-oocyte

complexes. Spheroidal cumulus-oocyte complexes along with darkish strict compact layers of cumulus cells presumably harvested from atretic follicles, and extremely small or naturally denuded oocytes, were not used in the present experiment. The GH-2 medium of Haidri and Gwatkin[2] was used.

Cumulus-oocyte complexes and denuded oocytes of the experimental groups were incubated in microdrops containing 10^{-6} M trilostane at which concentration inhibition of Δ^5-3β-HSD activity seemed to occur in the denuded oocytes. Trilostane was dissolved in sterilized water adjusted to pH 8.5 by adding 0.1 N NaOH solution, because of its poor solubility in water[8]. The stock solution of 10^{-3} M trilostane was neutralized to pH 7.4 by 0.1 N HCl and diluted with the GH-2 medium. Following incubation, the oocytes were mounted on a glass slide in 1–2 µl of medium, compressed gently with a coverslip smeared with vaseline at the corners, and examined under a Nomarski differential interference contrast microscope. The oocytes were fixed with 2.5% glutaraldehyde in phosphate buffered saline for 1 min and then transferred to 10% neutral formalin overnight. After fixation, the slides were washed, dehydrated and stained with 0.25% lacmoid solution for chromosome observation[19]. The oocytes were classified according to the following stages: degenerated; immature stage with an intact germinal vesicle (GV); germinal vesicle breakdown without polar bodies (GVBD) and polar body extrusions (PB). The oocytes were assessed as degenerated when nonspherical, brown in color, fragmented or vacuolated.

In vitro fertilization

Modified TAPL (m-TAPL)[23] containing 5.5 mg hypotaurine (Sigma Chemical Co., St. Louis) and 0.9 mg L-epinephrine (Sigma)/100 ml was used for the experiment. A prominent tubule of the cauda epididymis was cut with scissors and the sperm mass squeezed out of it was placed in 500 µl of the medium covered with paraffin oil in a plastic dish (Falcon) and incubated at 37 °C in 5% CO_2 air for 4 h until insemination. The sperm concentration and the motility of each sperm suspension were examined by a hemocytometer just before insemination and a sperm suspension with $1–8 \times 10^6$ sperm/ml, 70–90% motile, was used for the insemination. Female hamsters were made to superovulate by an i.p. administration of 30 i.u. PMSG on the day of the postestrous discharge, followed by an injection of 30 i.u. human chorionic gonadotropin (hCG, Teikoku Zoki) 48–72 h later. The oviducts were removed after 16–18 h. The ovulated ova were recovered by tearing the ampullae with a 26-gauge needle in paraffin oil in a plastic dish, and treated for 5 min with 0.1% hyaluronidase (Sigma) to disperse the cumulus cells. The ova were washed and transferred to 50 µl of m-TAPL containing 0 (control), 10^{-8} M, 10^{-7} M, 10^{-6} M trilostane in paraffin oil in a plastic dish and incubated at 37 °C in 5% CO_2 air for 1 h before insemination. Each drop contained 8–12 normal ovulated ova with first polar bodies. At insemination, 10 µl of the sperm suspension was added by micropipette to the ova in the microdrop. In all the experiments, one sperm suspension was always used for both the control and trilostane-treated ova to avoid any influence from the sperm suspension. After incubation for 4–4.5 h, the ova were washed, mounted on a glass slide and examined under Nomarski optics. The ova were closely observed after being fixed and lacmoid stained. The criteria for confirmation of fertilization are as follows: the penetration of the ovum, shown by the presence of spermatozoon in the perivitelline space or vitellus; pronuclear development evidenced by the presence of male and female pronuclei and a sperm tail in the vitellus; degenerated ova showing necrosis, vacuolization, irregular surface, fragmentation or loss of spherical shape; and polyspermy. Experimental results for the control group not satisfying the following criteria were discarded; the occurrence of normal pronuclear formation by more than 60%, and polyspermic fertilization less than 15%. To assess the effects of trilostane on sperm, a sperm suspension was incubated for 4 h in m-TAPL containing 10^{-7} M trilostane; 10 µl of the sperm suspension were then added to 50 µl of the ova in a microdrop. After 4 h incubation, the fertilization process was examined.

For the statistical analysis, overall values were calculated as shown in the 2×2 statistical table.

Results

Effects of trilostane on in vitro maturation of oocytes

Denuded oocytes and cumulus-oocyte complexes were cultured for 16–18 h in the GH-2 medium (control) or in the presence of 10^{-6} M trilostane. The results are shown in table 1. The percentages of polar body extrusion and germinal vesicle breakdown in the control denuded oocytes were 67% and 33% respectively and did not differ appreciably from 68% and 32% in the trilostane treated group. When the oocytes were cultured in a cumulus-oocyte complex, the values obtained were 82% and 18% as against 74% and 26%, respectively. Observation of chromosomes after staining with a 0.25% lacmoid solution indicated that the oocytes, after an almost complete germinal vesicle breakdown, had progressed to prometaphase I or metaphase I, and those from which polar bodies extruded were in metaphase II. The cytotoxic effect of trilostane on oocytes could be assessed on the basis of viability; that is, the proportion of degenerated oocytes was not statistically different for the control and trilostane-treated oocytes, whether surrounding cumulus cells were present or not. No particular morphological change was detected in the trilostane group. The lower rates of polar body extru-

Table 1. Effect of trilostane on hamster oocyte maturation in vitro

Oocyte	Trilostane (M)	No. of oocytes (%) Total	GV*	GVBD*	PB*	Degenerated
Denuded	0	99	0 (0)	28 (33)	56 (67)	15 (15)
	10^{-6}	102	0 (0)	29 (32)	61 (68)	12 (12)
In the	0	49	0 (0)	9 (18)	40 (82)	0 (0)
complex	10^{-6}	78	0 (0)	20 (26)	57 (74)	1 (1)

* Of oocytes which did not degenerate. GV, germinal vesicle; GVBD, germinal vesicle breakdown; PB, polar body extrusion.

sion and higher degeneration of denuded oocytes, compared with those in the cumulus-oocyte complex, were due possibly to mechanical injury incurred during denudation. Consequently, the addition of 10^{-6} M trilostane, which inhibited the Δ^5-3β-HSD activity of denuded oocytes toward the GH-2 medium, had no influence on the meiotic maturation, whether the oocytes were naked or not.

Effects of trilostane on in vitro fertilization

The results for this are shown in table 2. Observation of ova 4–4.5 h after insemination indicated the motility of the sperm to be the same as that of the control at any trilostane concentration. The addition of 10^{-8} M trilostane to the medium 1 h before insemination had no effect on penetration, pronuclear formation or polyspermic fertilization, in contrast to the control. At 10^{-7} M trilostane, the percentage of normal pronuclear formation slightly decreased and that of polyspermy increased significantly to 53% and 35% ($p < 0.001$, differed from control), respectively. This tendency was enhanced at a concentration of 10^{-6} M and only 30% of the ova developed 2 normal pronuclei ($p < 0.001$, vs control). When the fraction of penetrated ova decreased to 66%, the rate of polyspermy did not change ($p < 0.005$, vs control). The cytotoxic effect of trilostane on the ova themselves was assessed by the rate of degeneration, as in the case of in vitro maturation of oocytes, and was negligible since the proportion of degenerated ova was not different from that of the control for any trilostane concentration. Furthermore, ova with incomplete male pronuclei appeared at the same frequency both in the presence and in the absence of trilostane in the medium. 4h after preincubation of the sperm in 10^{-7} M trilostane, insemination was carried out in a microdrop supplemented with 1.7×10^{-8} M trilostane, but the ova were not affected. As shown in table 3, trilostane had no effect on sperm motility and subsequent fertilization.

Discussion

To date, there has been no detailed report on the significance of the steroid-producing activity of oocytes in certain mammals. In the present study, the inhibition by trilostane of the activity of Δ^5-3β-HSD, the key enzyme of steroidogenesis, showed no significant effects on the progress of meiosis. However, certain abnormalities were found during in vitro fertilization in golden hamsters. Trilostane, 4α-5-epoxy-17β-hydroxy-3-oxo-5α-androstane-2α-carbonitril, M.W. 329.4, is a recently-discovered competitive inhibitor of Δ^5-3β-HSD, which has no direct or indirect steroid hormonal activity[9].

The in vitro inhibition of Δ^5-3β-HSD by trilostane has been shown in beef adrenals by the use of thin layer chromatogram scanning[8]. In a previous study the authors, using indirect immunofluorescence techniques, found the activity of Δ^5-3β-HSD in hamster ova to be dose-dependently inhibited by a preincubation period of 1 h in a 10^{-8} M to 10^{-6} M trilostane solution[14]. In that study, the concentration of 10^{-8} M trilostane was critical for the inhibition of the enzyme, since the fluorescence intensity of the 10^{-8} M group did not differ significantly from the control in which the substrate of Δ^5-3β-HSD was not added to the culture medium. However, it differed significantly from the experimental group in which the substrate was added without trilostane. Inhibition was most pronounced at 10^{-6} M, at which concentration there were no direct cytotoxic effects which could cause degeneration of the ova.

In the present study, trilostane was added to the medium not supplemented with any steroids 1 h before in vitro fertilization to block the Δ^5-3β-HSD of the ovulated ova. The addition of 10^{-6} M to 10^{-7} M trilostane brought about the formation of normal male and female pronuclei to a lesser degree, and a polyspermic fertilization higher than the control and 10^{-8} M trilostane groups. The normal pronuclear formation increased in inverse proportion to the concentration of trilostane. The mechanism for polyspermy blocking is the zona reaction and the egg plasma membrane block in mammals[22]. In the hamster, a primary block occurs at the zona pellucida[21]. Following activation of the egg by sperm penetration, the cortical granules are released exocytotically and change the sperm binding sites on the zona or the biochemical properties of the zona, causing, for instance, zona hardening[22]. The inhibition of steroidogenesis in the ova might induce an incomplete or abnormal zona block through certain effects on the cortical reaction and subsequent changes in the sperm binding sites and the zona. However, the precise mechanism for this is not completely understood. The results of ultrastructural observation on changes in the cortical granules and zona following trilostane treatment will be reported in a future paper.

Progesterone-induced polyspermy has been reported in the pig[3]. The polyspermic ova increased when progesterone was administered just before ovulation. Progesterone is considered possibly to exert certain indirect

Table 2. Effect of trilostane on hamster fertilization in vitro

Trilostane	No. of ova (%)				
	Total	Penetrated	Pronuclear	Polyspermic	Degenerated
0	87	73 (84)	61 (70)	10 (11)	4 (5)
10^{-8}	126	109 (87)	89 (71)	14 (11)	7 (6)
4	73	62 (85)	49 (67)	8 (11)	7 (10)
10^{-7}	75	68 (91)	40 (53)	26 (35)*	4 (5)
0	49	41 (84)	31 (63)	3 (6)	0 (0)
10^{-6}	71	47 (66)	21 (30)*	19 (27)**	1 (1)

* Significantly different from control, $p < 0.001$. ** Significantly different from control, $p < 0.005$.

Table 3. Effects of trilostane on sperm motility and subsequent fertilization in vitro

Trilostane (M) in sperm suspension	Sperm motility (%)	No. of ova inseminated in 1.7×10^{-8} M trilostane (%)				
		Total	Penetrated	Pronuclear	Polyspermic	Degenerated
0	70–90	41	34(83)	29(71)	4(10)	1(2)
10^{-7}	70–90	57	50(88)	40(70)	6(11)	5(9)

effects on ova by changing the conditions in the oviduct. But according to our data, progesterone produced in the ova of the hamster apparently plays an important role in preventing multiple sperm penetration.

Recently, Moor et al.[6] reported the importance of follicular steroids for full oocyte maturation in sheep. Oocytes were cultured in the follicle supplemented with gonadotropins, and the steroid synthesis inhibitors aminoglutethimide and SU 10603 (a blocker of 17α-hydroxylase enzymes). Insemination was carried out 24 h later in vivo to examine meiosis and fertilization. Groups with steroid inhibitors were found to show retarded meiosis and abnormal fertilization; low normal pronuclear development, high polyspermy and high monospermic penetration with abnormal sperm head decondensation. Our investigation of the effects on full maturation resulting from inhibiting steroid synthesis inside ova also showed abnormal fertilization.

It is well known that spontaneous matured oocytes in vitro lack the ability to synthesize MPGF and formation of male pronuclei is incomplete during in vivo or in vitro fertilization[15-17]. Our results indicate that trilostane may not alter the quality or mode of action of MPGF synthesized in ovulated ova.

In conclusion, the steroid producing activity of hamster ova may be required to prevent multiple sperm penetration during fertilization, but has no effect on meiosis in vitro or on the action of the so-called MPGF.

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