Effects of Steroids on Nuclear Maturation of Porcine Oocytes

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ABSTRACT: The state of nuclear maturation was observed in porcine oocytes cultured with various steroids, in order to identify which steroids have a role of meiosis inducing substance (MIS) in porcine oocytes. In the oocytes cultured for 22 hrs, the percentage of oocytes with germinal vesicles (GVs) was significantly higher in those cultured with progesterone or 17α-hydroxyprogesterone (56.8 and 57.1 %) than in control oocytes (14.8 %), whereas the percentages of GV stage-oocytes cultured with cholesterol, pregnenolone, 17α-hydroxypregnenolone, 20α-hydroxyprogesterone or estradiol-17β (10.7 to 40.0 %) were similar to those of each control oocyte (14.8 to 67.9 %). At 44 hrs after maturation culture with various steroids, the percentages of oocytes with the MII stage nuclei were 42.9 to 91.7 % , showing no significant differences from those of each control oocyte (51.6 to 89.3 %). The developmental rates to 2-cell embryos of oocytes matured in the medium containing steroids also did not differ from those of each control oocyte, even in the use of either steroid examined. From the present findings, it was suggested that pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, estradiol-17β and cholesterol have no roles as MIS for porcine oocytes.

Key words: porcine oocyte, nuclear maturation, meiosis inducing substance, steroid

INTRODUCTION

It is known that steroids are involved in oocyte maturation[1-4]. Although not yet clarified in porcine oocytes, meiosis inducing substances (MISs) that induce the resumption of nuclear maturation have been determined to be estradiol-17β and progesterone in bovine[3] and human[4] oocytes, estradiol-17β in sheep oocytes[2] and progesterone in rhesus monkey oocytes[1].

On the other hand, it has been reported that the rates of porcine oocytes with the activities of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) with DHA as the substrate, 17β-HSD (using testosterone as the substrate) and 20β-HSD (20β-hydroxyprogesterone) did not change during maturation culture, whereas those with the activities of Δ^5-3β-HSD (pregnenolone and 17α-hydroxypregnenolone), 17β-HSD (estradiol-17β), 20α-HSD (20α-hydroxyprogesterone) and 20β-HSD (17α-hydroxyprogesterone) decreased as the time of culture was prolonged and reached 4, 0, 0, 2 and 0 % , respectively, after 44 hrs culture[5]. From these results, it is suggested that the nuclear maturation of porcine oocytes is related to the metabolism of some progestins and estrogen, and progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, 17α, 20β-dihydroxy-progesterone or estradiol-17β may possible serve as a MIS in porcine oocytes[5].

In the present study, the state of nuclear maturation was observed in porcine oocytes cultured with various steroids, in order to determine the substance which induces nuclear maturation in porcine oocytes.

MATERIALS AND METHODS

Collection of oocytes

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in

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0.9 % NaCl solution maintained at 39 ℃. The ovaries were washed in 0.9 % NaCl solution containing 200 i.u./ml potassium penicillin G. Immature oocytes covered with cumulus cells (COCs) were aspirated from medium-sized follicles (3-6mm in diameter) with a 21-gauge needle fixed to a 10-ml disposable syringe. Collected COCs were washed in phosphate buffered saline[6] (PBS, pH 7.4) and then in a culture medium[7] composed of TCM-199 (Gibco BRL, NY, USA) supplemented with 10 % (v/v) porcine follicular fluid, 10 % (v/v) fetal calf serum (FCS; Gibco BRL), 10 i.u./ml eCG (Serotropin; Teikoku Hormone Manufacturing Co. Ltd, Tokyo, Japan) and 10 i.u./ml hCG (Gonatropin; Teikoku Hormone Manufacturing Co. Ltd).

Observation of nuclear maturation

In order to investigate the effect of steroids on nuclear maturation, forty to 50 COCs collected from antral follicles were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) and cultured at 39 ℃ in an atmosphere of 5% CO_2 in air in a 400 μl of culture medium[7] containing pregnenolone (Sigma Chemical Co., St. Louis, MO, USA), progesterone (Sigma Chemical Co.), 17α-hydroxypregnenolone (Sigma Chemical Co.), 17 α-hydroxyprogesterone (Sigma Chemical Co.), 20 α-hydroxyprogesterone (Sigma Chemical Co.), estradiol-17 β (Sigma Chemical Co.) or cholesterol (Sigma Chemical Co.), which was previously dissolved in a solvent, acetone, ethanol or dimethylformamide, and then diluted with the culture medium up to 10 μM. Steroids examined and their solvents are shown in **Table 1**. The concentration of each solvent was adjusted to 0.1 %, and oocytes cultured in the medium containing a solvent at 0.1 % were used as controls. These culture media were previously covered with mineral oil (Sigma Chemical Co.) and equilibrated in a CO_2 incubator.

Table 1. Steroids and their solvents used for culture

Steroids (10 μM)	Solvents
Cholesterol	Acetone
Pregnenolone	Ethanol
Progesterone	Ethanol
Estradiol-17 β	Ethanol
20α-Hydroxyprogesterone	Ethanol
17α-Hydroxyprogesterone	Ethanol
17α-Hydroxypregnenolone	Dimethylformamide

At 22 and 44 hrs of culture, cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1 % hyaluronidase (Sigma Chemical Co.). Denuded oocytes were fixed in 25 % (v/v) acetic acid in ethanol for 48 hrs at room temperature. The fixed oocytes were stained with 1.0 % aceto-orcein and examined for evidence of nuclear maturation under a light microscope (OPTIPHOT-2, Nikon, Tokyo, Japan).

In vitro fertilization of oocytes

The ejaculated boar semen was treated by the method of WANG *et al.*[8], in order to induce capacitation of spermatozoa. The semen was washed three times in BO medium[9] containing 5 mM caffeine (Sigma Chemical Co.) and 0.3 % bovine serum albumin (BSA; Sigma Chemical Co.). Spermatozoa were resuspended in BO medium containing 5 mM caffeine and 0.3 % BSA to give a concentration of 5×10^5 live spermatozoa/ml, and a 400 μl of sperm suspension was covered with mineral oil in each well of a Nunc 4-well multidish.

At 44 hrs after maturation culture in the medium containing each steroid, COCs were washed twice in BO medium containing 5 mM caffeine and 0.3 % BSA. Forty to 50 COCs were introduced into the sperm suspension and cultured at 39℃ in a CO_2 incubator (5 % CO_2 in air).

In vitro development of inseminated oocytes

After 6 hrs culture with spermatozoa, COCs were washed three times in TCM-199 containing 10% FCS (culture medium). In order to observe the development of these oocytes to 2-cell embryos, forty to 50 COCs were introduced into the culture medium (400 μl) under mineral oil in each well of a Nunc 4-well multidish and cultured for 48 hrs at 39 ℃ in a CO_2 incubator (5 % CO_2 in air).

Statistical analysis

The rates concerning nuclear maturation of cultured oocytes and development to 2-cell embryos of inseminated oocytes were statistically analyzed using Chi-square test.

RESULTS

Nuclear maturation of steroid-treated oocytes

Nuclear maturation of porcine oocytes cultured in the medium containing each steroid is shown in **Tables 2** and **3.** Immediately after collection from antral follicles, 97.5 % of porcine oocytes were in the germinal vesicle (GV) stage (**Fig.1**) and the remaining oocytes were all in the diakinesis stage. In the oocytes cultured for 22 hrs, the percentage of oocytes with GVs was significantly higher in those cultured with progesterone or 17α-hydroxyprogesterone (56.8 and 57.1 %) than in control oocytes (14.8 %), whereas the percentages of GV stage-oocytes cultured with cholesterol, pregnenolone, 17α-hydroxypregnenolone, 20α-hydroxyprogesterone or estradiol-17β (10.7 to 40.0 %) were similar to those of each control oocyte (14.8 to 67.9 %). Nuclei of oocytes whose GVs had broken down were in the diakinesis stage to the metaphase stage of second maturation division (M II stage), mostly in the M I stage (**Fig.2**). At 44 hrs after maturation culture with various steroids, the percentages of oocytes with the M II stage nuclei (**Fig.3**) were 42.9 to 91.7 % , showing no significant

Table 2. Nuclear maturation of porcine oocytes cultured with steroids

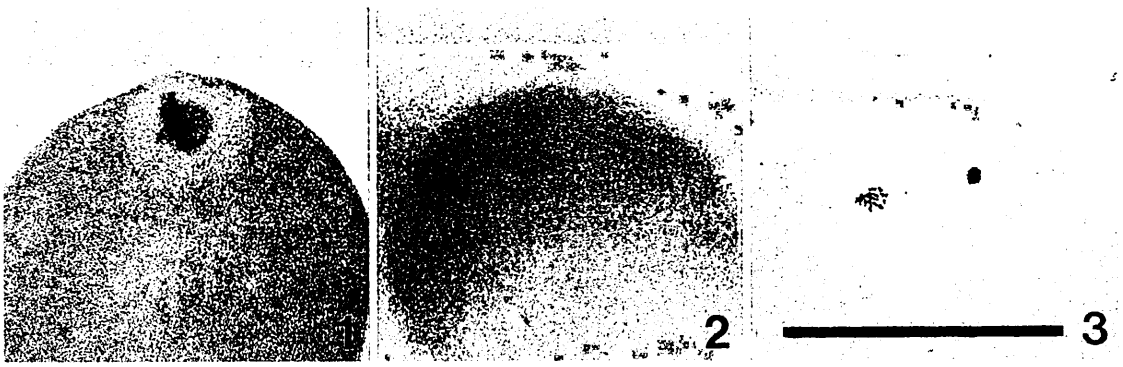
Treatments (Solvents)	No. of oocytes examined	Germinal vesicle	≦ Diakinesis	No. and (%) of oocytes at the stages of			
				Diakinesis	Metaphase I	Anaphase I and Telophase I	Metaphase II
None (Acetone)	50	18(36.0)[a]	32(64.0)[a]	4(12.5)	28(87.5)	0(0.0)	0(0.0)
Cholesterol	57	10(17.5)[a]	47(82.5)[a]	7(14.9)	39(83.0)	0(0.0)	1(2.1)
None (Ethanol)	27	4(14.8)[b]	23(85.2)[a]	2(8.7)	16(69.6)	5(21.7)	0(0.0)
Progesterone	37	21(56.8)[a]	16(43.2)[b]	0(0.0)	2(12.5)	14(87.5)	0(0.0)
Pregnenolone	32	11(34.4)[b]	21(65.6)[a]	0(0.0)	21(100.0)	0(0.0)	0(0.0)
Estradiol-17β	75	8(10.7)[b]	67(89.3)[a]	31(46.3)	28(41.8)	0(0.0)	8(11.9)
20α-Hydroxy-progesterone	42	10(23.8)[b]	32(76.2)[a]	2(6.3)	29(90.6)	0(0.0)	1(3.1)
17α-Hydroxy-progesterone	35	20(57.1)[a]	15(42.9)[b]	0(0.0)	14(93.3)	0(0.0)	1(6.7)
None (Dimethylformamide)	28	19(67.9)[a]	9(32.1)[a]	1(11.1)	8(88.9)	0(0.0)	0(0.0)
17α-Hydroxy-pregnenolone	27	11(40.0)[a]	16(59.3)[a]	1(6.3)	14(87.5)	0(0.0)	1(6.3)

The oocytes were observed after 22 hrs of culture.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

Table 3. Nuclear maturation of porcine oocytes cultured with steroids

Treatments (Solvents)	No. of oocytes examined	Metaphase II	≧Telophase I	No. and (%) of oocytes at the stages of			
				Germinal vesicle	Diakinesis	Metaphase I	Anaphase I and Telophase I
None (Acetone)	31	16(51.6)[a]	15(48.4)[a]	10(66.7)	0(0.0)	5(33.3)	0(0.0)
Cholesterol	21	9(42.9)[a]	12(57.1)[a]	3(25.0)	0(0.0)	9(75.0)	0(0.0)
None (Ethanol)	28	25(89.3)[a]	3(10.7)[a]	1(33.3)	0(0.0)	2(66.7)	0(0.0)
Progesterone	32	23(71.9)[a]	9(28.1)[a]	2(22.2)	1(11.1)	6(66.7)	0(0.0)
Pregnenolone	33	24(72.7)[a]	9(27.3)[a]	0(0.0)	0(0.0)	9(100.0)	0(0.0)
Estradiol-17 β	48	44(91.7)[a]	4(8.3)[a]	2(50.0)	0(0.0)	2(50.0)	0(0.0)
20 α-Hydroxy-progesterone	30	23(76.7)[a]	7(23.3)[a]	4(57.1)	0(0.0)	3(42.9)	0(0.0)
17 α-Hydroxy-progesterone	33	22(66.7)[a]	11(33.3)[a]	1(9.1)	1(9.1)	9(81.8)	0(0.0)
None (Dimethylformamide)	23	16(69.6)[a]	7(30.4)[a]	0(0.0)	1(14.3)	4(57.1)	2(28.6)
17 α-Hydroxy-pregnenolone	30	22(73.3)[a]	8(26.7)[a]	4(50.0)	0(0.0)	4(50.0)	0(0.0)

The oocytes were observed after 44 hrs of culture.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).



Explanation of Figures

All pictures are of porcine oocytes stained with aceto-orcein. Scale indicates 100 μm.

Fig.1. An oocyte just after collection. Germinal vesicle stage.

Fig.2. An oocyte cultured for 22 hrs in the medium containing 20 α-hydroxyprogesterone. Metaphase I stage.

Fig.3. An oocyte cultured for 44 hrs in the medium containing estradiol-17 β. Metaphase II stage.

differences from those of each control oocyte (51.6 to 89.3 %).

Development of inseminated oocytes to 2-cell embryos

When oocytes that had been matured in the medium containing each steroid were inseminated and then cultured for 48 hrs, 63.6 to 79.1 % of the inseminated oocytes developed to 2-cell embryos, showing no significant differences from those of each control oocyte (72.5 to 86.2 %) (**Table 4**). The developmental rates to 2-cell embryos of oocytes matured in the medium containing pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, estradiol-17β and cholesterol were not significantly different.

Table 4. Development to 2-cell embryos of porcine oocytes matured in the medium containing steroids

Treatments (Solvents)	No. of oocytes inseminated	No. and (%) of 2-cell embryos developed from inseminated oocytes
None (Acetone)	40	29 (72.5)[a]
Cholesterol	33	21 (63.6)[a]
None (Ethanol)	29	25 (86.2)[a]
Progesterone	30	21 (70.0)[a]
Pregnenolone	35	26 (74.3)[a]
Estradiol-17β	48	35 (72.9)[a]
20α-Hydroxyprogesterone	43	28 (65.1)[a]
17α-Hydroxyprogesterone	43	34 (79.1)[a]
None (Dimethylformamide)	37	27 (73.0)[a]
17α-Hydroxypregnenolone	43	34 (79.1)[a]

Development to 2-cell embryos was observed at 48 hrs after insemination.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

DISCUSSION

It has been clarified that estradiol-17β and/or progesterone play a role as a MIS in mammalian oocytes of several species[1-4], but not in porcine oocytes. On the other hand, we suggested that progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, 17α, 20β-dihydroxyprogesterone or estradiol-17β may be a candidate for MIS in porcine oocytes[5].

In the present investigation, the maturation rates of oocytes cultured for 44 hrs in the medium containing each steroid did not increase, compared to those of control oocytes. DODE and GRAVES[10] have also reported that estradiol-17β, progesterone and testosterone have no effects on the nuclear maturation of porcine oocytes. From the results of the present investigation and of DODE and GRAVES[10], it is considered that cholesterol, pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, estradiol-17β and testosterone have no roles as MIS of porcine oocytes.

Recently, it has been shown that C-29 sterols, intermediates in the cholesterol biosynthetic pathway, are involved in oocyte maturation[11]. Although not yet confirmed in porcine oocytes, meiosis-activating sterols (MASs) that induce the resumption of nuclear maturation have been determined as 4, 4-dimethyl-5α-cholesta-8, 14, 24-trien-3β-ol (FF-MAS) [12] and 4, 4-dimethyl-5α-cholesta-8, 24-dien-3β-ol (T-MAS)[13] in mammalian

oocytes of several species[11-17]. In the present study, some progestins and estrogen did not increase the maturation rates of porcine oocytes, suggesting a possibility that such MASs may play a role for maturation of porcine oocytes.

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ブタ卵母細胞の核成熟に及ぼす各種ステロイドの影響

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摘　　要

卵核胞期のブタ卵母細胞を各種ステロイドを含む培養液で培養し、核の成熟状態を観察した。

22時間培養したブタ卵母細胞において、卵核胞期のものの割合は、プロゲステロンあるいは17α-ヒドロキシプロゲステロンと共に培養したものでは56.8および57.1%であり、対照の卵母細胞の14.8%に比べて有意に高かったが、コレステロール、プレグネノロン、17α-ヒドロキシプレグネノロン、20α-ヒドロキシプロゲステロンあるいはエストラジオール-17βを含む培養液で培養したものでは10.7ないし40.0%であり、いずれもそれぞれの対照の卵母細胞の割合（14.8ないし67.9%）と相違なかった。また、各種ステロイドを含む培養液で44時間培養した卵母細胞において、第二成熟分裂中期に達したものの割合は、42.9ないし91.7%であり、いずれもそれぞれの対照の卵母細胞の割合(51.6ないし89.3%)と相違なかった。さらに、各種ステロイドを含む培養液で44時間培養した卵母細胞を媒精して48時間培養したところ、63.6ないし79.1%が2細胞胚に発生し、2細胞胚への発生率は、いずれもそれぞれの対照の卵母細胞の発生率（72.5ないし86.2%）と相違なかった。

以上の結果から、コレステロール、プレグネノロン、17α-ヒドロキシプレグネノロン、プロゲステロン、17α-ヒドロキシプロゲステロン、20α-ヒドロキシプロゲステロンおよびエストラジオール-17βは、いずれもブタ卵母細胞の成熟分裂を誘起・促進する作用を持っていないことが推察された。

キーワード：ブタ卵母細胞、核成熟、成熟分裂誘起物質、ステロイド

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