Effects of Steroids on Maturation of Denuded Porcine Oocytes

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Summary

The state of nuclear maturation and the distribution pattern of cortical granules (CGs) were observed in denuded porcine oocytes cultured with various steroids, in order to identify the effects of steroids on their nuclear and cytoplasmic maturation.

At 44 hrs after maturation culture with various steroids, the percentages of oocytes with the metaphase Ⅱ stage nuclei were significantly higher in those cultured with cholesterol, progesterone or 20 α-hydroxyprogesterone (72.7 to 89.1 %) than in each control oocyte group (49.3 and 58.3 %), whereas the percentages of such oocytes cultured with pregenolone, 17 α-hydroxyprogesterone, 17 α-hydroxypregnenolone and estradiol-17 β were similar to those of each control oocyte group. The distribution patterns of CGs in the oocytes cultured with steroids were similar to those in each control oocyte. The developmental rates to 2-cell embryos of inseminated oocytes matured in the medium containing steroids (50.0 to 73.5 %) also did not differ from those of each control oocyte group (60.0 to 69.0 %), even in the use of either steroid examined.

From these findings, it was clarified in denuded porcine oocytes that the nuclear maturation to the metaphase Ⅱ stage is accelerated by the treatment of some progestins and cholesterol, whereas the cytoplasmic maturation and the development after insemination are not affected by the treatment of steroids.

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It is known that steroids are involved in nuclear maturation of oocytes with cumulus cells[1-4]. In oocytes with cumulus cells, although not yet clarified in porcine, maturation inducing substances (MISs) that induce the resumption of nuclear maturation have been determined to be estradiol-17 β and progesterone in bovine[3] and human[4], estradiol-17 β in sheep[2] and progesterone in rhesus monkey[1]. Recently, DODE and GRAVES[5], and TAKANO and NIIMURA[6] have reported that cholesterol, pregnenolone, 17 α-hydroxypregnenolone, progesterone, 17 α-hydroxyprogesterone, 20 α-hydroxyprogesterone, estradiol-17 β and testosterone have no roles as MIS for porcine oocytes with cumulus cells. Since these studies have been done using oocytes with cumulus cells, the effects of steroids on maturation of oocyte itself are indistinct.

On the other hand, it has been reported that nuclei of oocytes without cumulus cells (denuded oocytes) in some mammals are able to mature to the metaphase Ⅱ stage *in vitro*, whereas their maturation rates are lower than those of oocytes with cumulus cells[7-13]. Furthermore, it has also been reported that the rates of fertilization and development after insemination are lower in denuded oocytes than those in oocytes with cumulus cells[10,11,14,15].

In the present study, the state of nuclear maturation and the distribution pattern of cortical granules (CGs) were observed in denuded porcine oocytes cultured with various steroids, in order to identify the effects of steroids on their nuclear and cytoplasmic maturation.

MATERIALS AND METHODS

Collection of oocytes

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 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 immersed in phosphate buffered saline[16] (PBS, pH 7.4) containing 0.1 % hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) to disperse cumulus cells from the oocytes. Denuded oocytes were washed in PBS and then in a culture medium[17] 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, denuded oocytes 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[17] containing pregnenolone (Sigma Chemical Co.), progesterone (Sigma

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Chemical Co.), 17α-hydroxypregnenolone (Sigma Chemical Co.), 17α-hydroxyprogesterone (Sigma Chemical Co.), 20α-hydroxyprogesterone (Sigma Chemical Co.), estradiol-17β (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 10μM. 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 (10μM)	Solvents
Cholesterol	Acetone
Pregnenolone	Ethanol
Progesterone	Ethanol
Estradiol-17β	Ethanol
20α-Hydroxyprogesterone	Ethanol
17α-Hydroxyprogesterone	Ethanol
17α-Hydroxypregnenolone	Dimethylformamide

At 22 and 44 hrs of culture, 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).

Observation of CG distribution

At 22 and 44 hrs after maturation culture, denuded oocytes were stained with PNA conjugated with FITC (E-Y Lab., CA, USA) according to the method of DUCIBELLA *et al.*[18] The denuded oocytes were immersed in PBS containing 0.2 % pronase (Sigma Chemical Co.) to dissolve the zonae pellucidae. The naked oocytes were washed in PBS and fixed in PBS containing 3.7 % paraformaldehyde for 30 min at room temperature. They were then washed 3 times in a blocking solution composed of 3 mg bovine serum albumin (BSA; Sigma Chemical Co.), 7.51 mg glycine (Wako Pure Chemical, Osaka, Japan) and 1 ml PBS, immersed in PBS containing 0.1 % Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 5 min at 20 ℃, and again placed in the blocking solution. These oocytes were finally immersed in a staining solution composed of 100μg PNA, 0.1μl Triton X-100, 3 mg BSA and 1 ml PBS for 30 min at 20 ℃. The stained oocytes were thoroughly washed in PBS containing 0.01 % Triton X-100 and 0.3 % BSA, and were placed in the center of four vaseline spots on a slide. A coverslip was then carefully placed on the vaseline spots and pressed gently to anchor the oocyte between the coverslip and the slide. Observation was carried out under an epifluorescence microscope (Nikon, Tokyo, Japan). Degenerated oocytes were eliminated from the observation.

In vitro fertilization of oocytes

The ejaculated boar semen was treated by the method of WANG *et al.*[19], in order to induce capacitation of spermatozoa. The semen was washed three times in BO medium[20] containing 5 mM caffeine (Sigma Chemical Co.) and 0.3 % BSA. 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 400μl 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, denuded oocytes were washed twice in BO medium containing 5 mM caffeine and 0.3 % BSA. These oocytes 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, inseminated oocytes 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, oocytes were introduced into the culture medium 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, and the number of oocytes with different distribution patterns of CGs were statistically analyzed using Chi-square test.

RESULTS

Nuclear maturation of steroid-treated oocytes

Nuclear maturation of denuded porcine oocytes cultured in the medium containing each steroid is shown in **Tables 2** and **3**. In the oocytes cultured for 22 hrs, the percentages of oocytes at the diakinesis to metaphase Ⅱ stages were significantly higher in those cultured with pregnenolone, progesterone, 17α-hydroxyprogesterone, 20α-hydroxyprogesterone or 17α-hydroxypregnenolone (70.0 to 86.8 %) than in each control oocyte group (65.0 and 35.0 %), whereas the percentages of such oocytes cultured with cholesterol and estradiol-17β (63.3 and 49.0 %) were similar to those of each control oocyte group (64.5 and 65.0 %). At 44 hrs after maturation culture with various steroids, the percentages of oocytes with the metaphase Ⅱ stage nuclei **(Fig.1)** were significantly higher in those cultured with cholesterol (72.7 %), progesterone (89.1 %) or 20α-hydroxyprogesterone (74.2 %) than in each control oocyte group (49.3 and 58.3 %), while the percentages of metaphase Ⅱ stage oocytes cultured with pregnenolone, estradiol-17β, 17α-hydroxyprogesterone and 17α-hydroxypregnenolone did not significantly differ from those in each control oocyte group.

Table 2. Nuclear maturation of denuded 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 and Telophase Ⅰ	Metaphase Ⅱ
None (Acetone)	62	22(35.5) [a]	40(64.5) [a]	4(6.7)	27(45.0)	4(6.7)	5(8.3)
Cholesterol	60	22(36.7) [a]	38(63.3) [a]	1(1.7)	28(46.7)	1(1.7)	8(13.3)
None (Ethanol)	40	14(35.0) [a]	26(65.0) [b]	2(5.0)	20(50.0)	2(5.0)	2(5.0)
Progesterone	51	9(17.6) [b]	42(82.4) [a]	5(9.8)	25(49.0)	3(5.9)	9(17.6)
Pregnenolone	53	7(13.2) [b]	46(86.8) [a]	1(1.9)	35(66.0)	7(13.2)	3(5.7)
Estradiol-17 β	47	24(51.0) [a]	23(49.0) [b]	5(10.6)	13(27.7)	2(4.3)	3(6.4)
20 α -Hydroxy-progesterone	50	15(30.0) [a]	35(70.0) [a]	1(2.0)	32(64.0)	2(4.0)	0(0.0)
17 α -Hydroxy-progesterone	56	12(21.4) [a]	44(78.6) [a]	5(8.9)	37(66.1)	2(3.5)	0(0.0)
None (Dimethylformamide)	40	26(65.0) [a]	14(35.0) [b]	2(5.0)	10(25.0)	0(0.0)	2(5.0)
17 α -Hydroxy-pregnenolone	54	12(22.2) [b]	42(77.8) [a]	2(3.7)	32(59.3)	5(9.3)	3(5.6)

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 denuded 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)	73	36(49.3) [b]	37(50.7) [a]	10(13.7)	5(6.8)	19(26.0)	3(4.1)
Cholesterol	55	40(72.7) [a]	15(27.3) [b]	2(3.6)	2(3.6)	10(18.8)	1(1.8)
None (Ethanol)	60	35(58.3) [b]	25(41.7) [a]	3(5.0)	2(3.3)	19(31.7)	1(1.7)
Progesterone	64	57(89.1) [a]	7(10.9) [b]	3(4.7)	0(0.0)	4(6.3)	0(0.0)
Pregnenolone	60	42(70.0) [b]	18(30.0) [a]	6(10.0)	0(0.0)	10(16.7)	2(3.3)
Estradiol-17 β	77	49(63.6) [b]	28(36.7) [a]	9(12.7)	3(3.9)	15(19.5)	1(1.3)
20 α -Hydroxy-progesterone	66	49(74.2) [a]	17(25.8) [b]	0(0.0)	3(4.5)	14(21.2)	0(0.0)
17 α -Hydroxy-progesterone	65	42(64.6) [b]	23(35.4) [a]	7(10.8)	2(3.1)	12(18.5)	2(3.1)
None (Dimethylformamide)	54	22(40.7) [a]	32(59.3) [a]	2(3.7)	5(9.3)	22(40.7)	3(5.6)
17 α -Hydroxy-pregnenolone	54	30(55.6) [a]	24(44.4) [a]	3(5.6)	3(5.6)	17(31.5)	1(1.9)

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

Changes in CG distribution of steroid-treated oocytes

When denuded porcine oocytes cultured with various steroids were stained with PNA, fluorescent granules appeared in their cytoplasm **(Fig.2a-c)**. Since these granules completely disappeared in 83 % (50/60) of inseminated oocytes **(Fig.2d)**, the PNA-reactive granules were determined to be CGs. Distribution patterns of CGs differed among oocytes and could classified into 3 types. In type Ⅰ, CGs were distributed over the cortical cytoplasm **(Fig.2a)**. In type Ⅱ, CGs were distributed in the cortical cytoplasm and also immediately beneath the plasma membrane **(Fig.2b)**. CGs in oocytes of type Ⅲ were all densely distributed just beneath the plasma membrane **(Fig.2c)**.

At 22 hrs after culture with steroids, types Ⅱ and Ⅲ oocytes appeared at 65 to 79 %, showing no significant differences from those of each control oocyte group (58 to

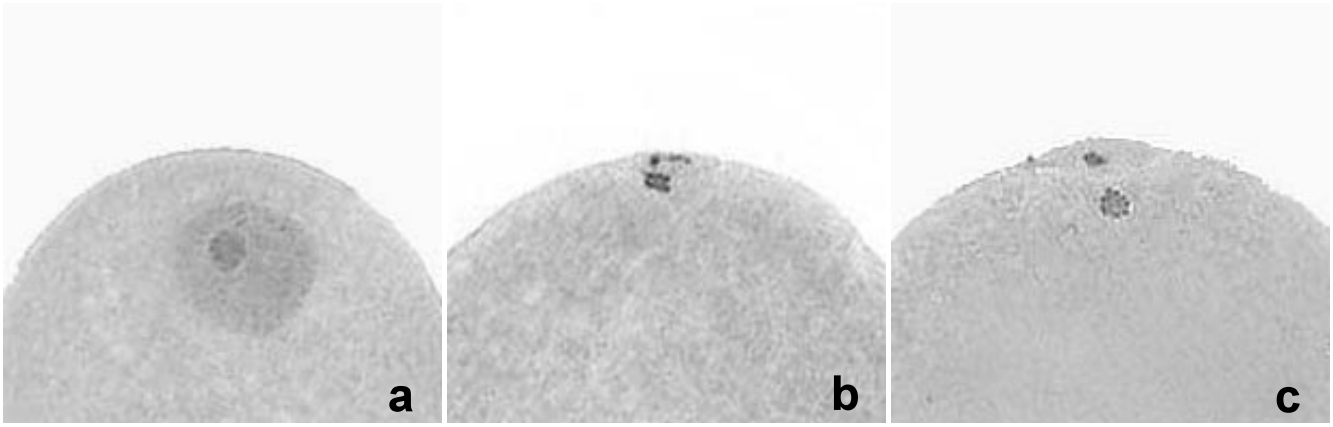

Fig.1. Whole mount preparations of porcine oocytes with a magnification of ×400 under a light microscope. All oocytes were photographed after staining with aceto-orcein.
a. An oocyte just after collection. Germinal vesicle stage.
b. An oocyte cultured for 22 hrs in the medium containing 20α-hydroxyprogesterone. Metaphase Ⅰ stage.
c. An oocyte cultured for 44 hrs in the medium containing progesterone. Metaphase Ⅱ stage.

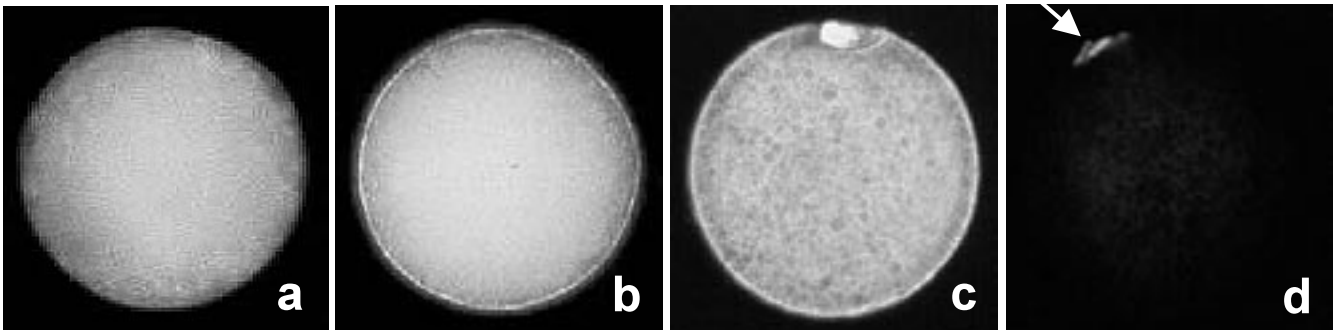

Fig.2. Whole mount preparations of porcine oocytes with a magnification of ×300 under an epifluorescence microscope. All oocytes were photographed after staining with FITC-conjugated PNA.
a. A type Ⅰ oocyte at the germinal vesicle stage just after collection. Cortical granules are distributed over the cortical cytoplasm.
b. A type Ⅱ oocyte cultured for 22 hrs in the medium containing cholesterol. Cortical granules are distributed in the cortical cytoplasm and also immediately beneath the plasma membrane.
c. A type Ⅲ oocyte cultured for 44 hrs in the medium containing progesterone. Cortical granules are densely distributed immediately beneath the plasma membrane.
d. A fertilized oocyte 6 hrs after insemination. No cortical granules are seen in the cytoplasm. First polar body (arrow) is seen.

68 %) (**Table 4**). Of oocytes cultured for 44 hrs with steroids, 46 to 75 % were found to be type Ⅲ, and there were no significant differences in the percentages of such oocytes between steroid-treated and control oocytes (**Table 5**).

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, 57.1 to 73.5 % of the inseminated oocytes developed to 2-cell embryos, showing no significant differences from those of each control oocyte group (60.0 to 69.0 %) (**Table 6**). The developmental rates to 2-cell embryos of oocytes matured in the medium containing pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyrogesterone, 20α-hydroxyprogesterone, estradiol-17β and cholesterol were not significantly different.

DISCUSSION

DODE and GRAVES[5] have reported that estradiol-17β, progesterone and testosterone have no effects on the nuclear maturation of porcine oocytes with cumulus cells. TAKANO and NIIMURA[6] have also reported that the maturation rates of porcine oocytes with cumulus cells cultured for 44 hrs in the medium containing various steroids do not increase, compared to those of control oocytes. From the results of these studies, it was suggested that cholesterol, pregnenolone, 17α-hydroxypregnenolone, progesterone,

Table 4. The changes in CG distribution of denuded porcine oocytes cultured with steroids

Treatments (Solvents)	No. of oocytes examined	No. and (%) of oocytes with different types* of CG distribution	
		I	II・III
None (Acetone)	35	14(40) [a]	21(60) [a]
Cholesterol	23	8(35) [a]	15(65) [a]
None (Ethanol)	31	10(32) [a]	21(68) [a]
Progesterone	33	7(21) [a]	26(79) [a]
Pregnenolone	32	7(22) [a]	25(78) [a]
Estradiol-17 β	31	8(26) [a]	23(74) [a]
20 α-Hydroxy-progesterone	29	9(31) [a]	20(69) [a]
17 α-Hydroxy-progesterone	28	7(25) [a]	21(75) [a]
None (Dimethylformamide)	36	15(42) [a]	21(58) [a]
17 α-Hydroxy-pregnenolone	35	11(31) [a]	24(69) [a]

The oocytes were observed after 22 hrs of culture.
*Type Ⅰ : CGs were distributed over the cortical cytoplasm.
Type Ⅱ : CGs were distributed over the cortical cytoplasm and also immediately beneath the plasma membrane.
Type Ⅲ : CGs were distributed immediately beneath the plasma membrane.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

Table 5. The changes in CG distribution of denuded porcine oocytes cultured with steroids

Treatments (Solvents)	No. of oocytes examined	No. and (%) of oocytes with different types* of CG distribution	
		I・II	III
None (Acetone)	24	12(50) [a]	12(50) [a]
Cholesterol	24	13(54) [a]	11(46) [a]
None (Ethanol)	30	13(43) [a]	17(57) [a]
Progesterone	36	12(33) [a]	24(67) [a]
Pregnenolone	28	7(25) [a]	21(75) [a]
Estradiol-17 β	28	14(50) [a]	14(50) [a]
20 α-Hydroxy-progesterone	31	13(42) [a]	18(58) [a]
17 α-Hydroxy-progesterone	31	8(26) [a]	23(74) [a]
None (Dimethylformamide)	33	22(67) [a]	11(33) [a]
17 α-Hydroxy-pregnenolone	20	8(40) [a]	12(60) [a]

The oocytes were observed after 44 hrs of culture.
*Type Ⅰ : CGs were distributed over the cortical cytoplasm.
Type Ⅱ : CGs were distributed over the cortical cytoplasm and also immediately beneath the plasma membrane.
Type Ⅲ : CGs were distributed immediately beneath the plasma membrane.
Values with different superscripts in the same column in each experimental lot are significantly different ($P<0.05$).

17 α-hydroxyprogesterone, 20 α-hydroxyprogesterone, estradiol-17 β and testosterone have no roles as MIS for porcine oocytes with cumulus cells. However, since these studies have been done using oocytes with cumulus cells, the effects of steroids on maturation of oocyte itself are indistinct.

In denuded porcine oocytes, although the maturation rate was lower than in oocytes with cumulus cells, 28 to 66 %[9] or 35 to 61 %[10] of those cultured in the routine medium for 44 hrs reached to the metaphase Ⅱ stage. In the present investigation, the percentages of denuded oocytes with the

Table 6. Development to 2-cell embryos of denuded 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)	29	20 (69.0) [a]
Cholesterol	34	23 (67.6) [a]
None (Ethanol)	40	24 (60.0) [a]
Progesterone	37	24 (64.9) [a]
Pregnenolone	34	25 (73.5) [a]
Estradiol-17 β	42	21 (50.0) [a]
20 α -Hydroxyprogesterone	35	20 (57.1) [a]
17 α -Hydroxyprogesterone	42	26 (61.9) [a]
None (Dimethylformamide)	42	28 (66.7) [a]
17 α -Hydroxypregnenolone	42	26 (61.9) [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 ($P<0.05$).

metaphase Ⅱ stage nuclei at 44 hrs after maturation culture with steroids were 55.6 to 89.1 %, and the percentages of such oocytes cultured with cholesterol, progesterone or 20 α -hydroxyprogesterone were significantly higher than those of control oocytes cultured with no steroids. From the findings of the present study, it was suggested in denuded porcine oocytes that some progestins and cholesterol accelerate the nuclear maturation to the metaphase Ⅱ stage, and that such steroids may play a role for maturation of oocytes.

Changes in CG distribution with maturation have been studied in porcine oocytes under electron microscopy[21,22] and also histochemically with lectin[23-25]. CGs are distributed over the cortical cytoplasm in the immature oocytes with germinal vesicles collected from antral follicles, and the CGs move to the cytoplasm immediately beneath the plasma membrane as nuclear maturation progresses[21-25]. In oocytes collected from antral follicles 20 hrs[21] or 24 to 36 hrs[22] after hCG injection and those cultured for 22 to 26 hrs[22-25], most CGs are observed immediately beneath the plasma membrane. Therefore, distribution pattern of CGs is considered to be a marker for cytoplasmic maturation. In the present investigation, changes in the distribution of CGs in denuded porcine oocytes cultured with various steroids were comparable to those in control oocytes.

In denuded porcine oocytes, the rate of development to 2-cell embryos after insemination was reported to be 20 to 43 %[10] or 28 %[15]. The developmental rates to 2-cell embryos of denuded oocytes cultured with and without steroids in the present study (57.1 to 73.5 %) were higher than those of previous studies[10,15]. Because the developmental rates to 2-cell embryos did not differ between steroid-treated oocytes and non-treated oocytes, the effect of steroids on the development was not obvious.

From the present findings, it was suggested that cytoplasmic maturation of denuded oocytes and the development after insemination are not affected by the treatment of steroids.

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ブタ裸化卵母細胞の成熟に及ぼす各種ステロイドの影響

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要　約

卵核胞期のブタ裸化卵母細胞を各種ステロイドを含む培養液で培養し、核の成熟状態と表層粒の分布状態を観察した。

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、コレステロール、プロゲステロンあるいは20α-ヒドロキシプロゲステロンと共に培養したものでは72.7ないし89.1％であり、いずれもそれぞれの対照の卵母細胞の割合（49.3および58.3％）に比べて有意に高かったが、プレグネノロン、エストラジオール-17β、17α-ヒドロキシプロゲステロンおよび17α-ヒドロキシプレグネノロンと共に培養したものでは、それぞれの対照の卵母細胞の割合と相違なかった。また、各種ステロイドを含む培養液で22および44時間培養した卵母細胞において、表層粒の分布状態は、対照のステロイド処置していない卵母細胞のものと相違なかった。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、50.0ないし73.5％が2細胞胚に発生したが、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（60.0ないし69.0％）と相違なかった。

以上の結果から、ブタの裸化卵母細胞では、第二成熟分裂中期への核の成熟はプロゲスチンのいくつかとコレステロールの処置によって促進されるが、表層粒の分布と媒精後の2細胞胚への発生は各種ステロイドの処置によって影響されないことが明らかにされた。

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キーワード：ブタ裸化卵母細胞、核成熟、細胞質成熟、ステロイド、表層粒

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