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Variation of cholesterol contents in porcine cumulus-oocyte complexes is a key factor in regulation of fertilizing capacity

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Abstract

The aims were to explore substances affecting maturation of porcine oocytes and effects of cholesterol efflux by methyl-β-cyclodextrin (MBCD). Cumulus-oocyte complexes (COCs) were collected from ovaries with or without corpora lutea (CL). Ovarian cholesterol content was determined and histological sections were prepared for immunostaining of lanosterol 14α-demethylase (CYP51), a catalytic enzyme during cholesterogenesis. In addition, COCs collected from ovaries without a CL (prepuberal gilts) were subjected to *in vitro* maturation with MBCD for 22 h, followed by maturation without MBCD for 22 h. Fertilizability and developmental competence of matured oocytes were monitored. The cholesterol content in COCs from the ovaries with CL (2.73 μg/μg protein) was higher ($P < 0.05$) than that from the ovaries without CL (1.88 μg/μg protein). Immunoreactive CYP51 was localized mainly in cells within a CL and in proximity to the CL. In COCs from ovaries without a CL, the cholesterol content just before *in vitro* maturation was 1.29 μg/μg protein, but it was decreased ($P < 0.05$; 0.51 μg/μg protein) by culturing in MBCD-containing medium for 22 h, and subsequently increased (1.55 μg/μg protein) by culturing in MBCD-free medium for 22 h. When oocytes were matured with MBCD for 22 h and then matured without MBCD for the next 22 h, the fertilization rate improved ($P < 0.05$) to 76.9%, whereas the blastocyst rate (9.5%) decreased ($P < 0.05$; fertilization and blastocyst rates were 69.6 and 26.3%, respectively, in the control group). We concluded that ovarian cholesterogenesis depended on sexual maturity of the donor and that variation in cholesterol content in COCs during *in vitro* maturation of porcine oocytes affected their ability to be fertilized.

Keywords: Cholesterol; Embryo development; Fertilization; Oocyte maturation; Steroid hormone

1. Introduction

In *in vitro* embryo production, it has been reported that the developmental competence of a fertilized oocyte depends on the age (i.e., sexual maturity) of the oocyte donor [1,2]. Oocytes of post-pubertal animals have a higher developmental competence than those of pre-pubertal animals. Clarification of this difference would be very helpful for *in vitro* embryo production, particularly in pigs, because most pigs are slaughtered before the onset of puberty. Recently, our group reported that fertilizability and subsequent developmental competence of porcine oocytes were correlated with the number of corpora lutea (CL) in the ovary; therefore, we inferred that the progesterone (P_4) biosynthesis pathway was crucial to porcine embryogenesis [2].

Resumption of meiosis in porcine oocyte is induced by a certain amount of P_4 [3]. Steroid hormones required for oocyte maturation, e.g. P_4 and estradiol-17β (E_2), are provided by cumulus-oocyte complexes (COCs) during *in vitro* maturation (IVM) [4]. Steroid hormones including P_4 are converted from cholesterol (Cho) by cytochrome P450 enzymes and hydroxysteroid dehydrogenases [5]. There is enough Cho from circulating lipoprotein in the blood, cellular stocks in the cell membrane and cytoplasm, and/or from *de novo* synthesis in ovarian cells [6]. Cholesterogenesis is intermediated by some sterols, such as 4,4-dimethyl-5α-cholest-8,14,24-triene-3β-ol (FF-MAS) and 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol (T-MAS), which are commonly known as meiosis-activating sterols (MAS) [7,8]. Thus, cholesterogenesis at a nearby site of oocyte growth and maturation may also be involved in porcine embryogenesis.

The present study was conducted to examine the effect of the addition of P_4 into maturation medium on maturation of porcine oocytes. Furthermore, the effects of the

variation of Cho content on oocyte maturation and subsequent embryogenesis were also investigated.

2. Materials and methods

2.1. Collection of oocytes and follicular fluid

Porcine ovaries were collected at a local abbatoir and, within 3 h, were taken to the laboratory in sterile saline (0.9% NaCl) solution at 37 °C. Ovaries were classified with respect to the presence and number of CL, and were used for sample collection. After ovaries were washed twice in sterilized saline solution, COCs were aspirated from the ovaries using an 18-gauge needle attached to a 5 mL disposable syringe. Those COCs, having three or more layers of intact cumulus cells, and with uniform ooplasm, were rinsed three times in Hepes-buffered Tyrode's medium [9] containing 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA: TLH-PVA). In addition, some ovaries were used to collect COCs and follicular fluid for measuring Cho contents. Follicular fluid was centrifuged at 1200 x g for 15 min, and the supernatant used for the Cho assay.

2.2. Oocyte maturation

The selected COCs were washed three times in medium 199 (with Earle's salts, L-glutamine, and 2200 mg/L sodium bicarbonate; Sigma) supplemented with 0.05% (w/v) PVA, 3.05 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.91 mM Na-pyruvate (Wako), 100 μM cysteamine (Sigma), 10 ng/mL epidermal growth factor

(Sigma), and 75 mg/L kanamycin (Sigma). Ten to 15 COCs were cultured at 39 °C under 5% CO_2 in air in a 100 μL droplet of the same medium with 10 IU/mL eCG (ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/mL hCG (ASKA Pharmaceutical) under mineral oil (Sigma) for 22 h. Subsequently, COCs were transferred into the medium without hormonal supplements and cultured for another 22 h in a similar manner. During the first half of IVM (0 to 22 h), P_4 or methyl-β-cyclodextrin (MBCD) were added to the IVM medium according to the experiments (see Experimental Design).

2.3. Intracytoplasmic sperm injection (ICSI)

To circumvent polyspermy, ICSI was used as an insemination method, as described [10,11]. Briefly, pelleted frozen semen in the same batch from one boar was used throughout the experiments. The semen was thawed in prewarmed (39 °C) PBS containing 0.1% PVA (PBS-PVA) and was washed by centrifugation at 300 × g for 5 min in PBS-PVA. After IVM culture, oocytes were stripped of their cumulus cells by gentle pipetting in IVM medium. Manipulation for ICSI was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope. Then, drops (7 μL) of PBS-PVA containing sperm and TLH-PVA containing oocytes were placed on the lid of a 50 x 9 mm petri dish (Falcon 1006; Becton Dickinson Labware, Franklin Lakes, NJ, USA) and covered with mineral oil. A spermatozoon was aspirated into the injection pipette tail-first without an immobilizing treatment such as tail-scoring/cutting, and was transferred to the drop containing oocytes. After an oocyte was fixed in a position in which the first polar body was positioned at 6 or 12 o'clock, the aspirated single spermatozoon was injected into the oocyte cytoplasm and mixed with cytoplasmic components thoroughly by open tubing regulated by mouth.

2.4. Embryo culture

The injected oocytes were washed with Porcine Zygote Medium-4 (PZM-4) [12] supplemented with 2.77 mM myo-inositol (Sigma), 0.34 mM tri-sodium citrate (Merck, Darmstadt, Germany), and 50 μM β-mercaptoethanol (Sigma), and cultured in a droplet (10 to 12 oocytes/30 μL) of the same medium covered with mineral oil at 39 °C under 5% CO_2, 5% O_2, and 90% N_2 for up to 144 h.

2.5. Fixation of oocytes

At 12 h after ICSI, eggs were fixed in 25% (v/v) acetic acid (Wako) in ethanol (Wako) for 24 h and stained with 1% (w/v) orcein (Sigma) in 45% acetic acid solution. The eggs were examined under a phase-contrast microscope. Eggs having two pronuclei, two polar bodies, and a sperm tail were considered normally fertilized.

2.6. Cholesterol assay

Thirty COCs each before or during IVM was stored in 10 μL PBS-PVA at −30 °C until the assay. After being thawed at room temperature, the COCs were re-frozen in liquid nitrogen and thawed repeatedly five times to rupture the cells. Also, collected follicular fluid was stored at −30 °C and used for assay after being thawed at room temperature. The Cho content was quantified with an assay kit, the Cholesterol E-test Wako (Wako). Standard solutions were prepared at 0 to 50 mg/mL. Four microliters of each sample and of the standard solution were loaded onto a 96-well plate, followed by

the addition of 300 μL of reaction mixture in each well. The absorbance was recorded using a spectrophotometer (wavelength, 600 nm).

The Cho concentrations were corrected according to the protein contents of the samples [13]. Standard solutions were prepared using bovine serum albumin (0 to 10 mg/mL). Four microliters of each sample and of the standard solution were loaded onto a 96-well plate, and then 200 μL reaction liquid (Protein Assay CBB solution; Nacalai Tesque, Kyoto, Japan) was added to each well. Absorbance was recorded using a spectrophotometer (wavelength, 595 nm).

2.7. Immunohistochemistry

Porcine ovaries were fixed in 10% formaldehyde (Nacalai Tesque) and embedded in paraffin wax (Nacalai Tesque) to make histological sections. A catalytic enzyme, lanosterol 14α-demethylase (CYP51), involved in cholesterogenesis, was primarily combined with anti-CYP51 antibody (1:20; Abnova, Taipei, Taiwan). The antibodies were immunologically visualized using the STAT-Q HRP-DAB multivalent kit (Innovex Biosciences, Richmond, CA, USA) according to the manufacturer's instructions. After peroxidase activity was detected, slides were counterstained with Meyer Hematoxylin (Sakura Finetek Japan, Tokyo, Japan) for 10 min, and then washed in 1% hydrochloric acid (Nacalai Tesque) diluted with 70% ethanol.

2.8. Experimental design

First, the effect of P_4 (Sigma) on maturation of oocytes collected from ovaries without a CL was investigated. The COCs were matured in P_4-containing maturation

medium during the first half of the IVM culture. Because P_4 contents in follicular fluid collected from the ovaries with CL ranged up to approximately 1000 ng/mL according to a previous study [2], P_4 was dissolved in ethanol at 100, 500, and 1000 μg/mL, and final concentrations (100, 500, and 1000 ng/mL) were obtained by the addition of 0.1% stock solution into maturation medium. Medium containing 0.1% ethanol was used as a control for the solvent.

Secondly, Cho production in the ovaries was investigated under various conditions. Follicular fluid and COCs were collected from the ovaries without CL (CL0) and with 6 to 10 CLs (CL6-10), and their Cho contents were measured. In addition, Cho synthesis in ovarian cells was confirmed by immunohistochemical staining of CYP51. The sample was produced from the ovaries with 1 and 10 CLs (CL1 and CL10, respectively), as well as CL0, to document Cho production in ovaries with CL.

Finally, the effects of the variation of Cho contents of COCs on oocyte maturation and subsequent embryogenesis were investigated. For this purpose, COCs from the ovaries without CL were treated with MBCD at a concentration of 1 mg/mL for 1.5 h, because this will effectively remove Cho from the sperm plasma membrane [14-16]. In addition, the MBCD treatment was performed for 22 h. This treatment was experimentally reasonable, as the IVM medium was renewed at 22 h of IVM (see the section of Oocyte maturation, and Fig.1). Thus, COCs were cultured in IVM medium with MBCD for 1.5 or 22 h from the onset of IVM, and then cultured in IVM medium without MBCD for 42.5 or 22 h, respectively, according to the conventional IVM procedure. Because Cho could be synthesized *de novo* by cumulus cells [3,17,18], it was expected that Cho contents of COCs were compensated during the additional incubation in IVM medium without MBCD. The COCs cultured without MBCD were defined as the control. The Cho contents of COCs were measured at 0, 1.5, 22, and 44 h after the onset of IVM

culture. The fertilizability of oocytes after ICSI and developmental competence up to the blastocyst stage were evaluated.

2.9. Statistical analysis

All experiments were conducted at least four times. Statistical analyses were performed using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All percentage data were analyzed by logistic regression using the following model: $\ln(\alpha/1-\alpha) = \beta$ + main factor, where α = frequency of positive outcome and β = the intercept. The Cho levels were analyzed by general liner model. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Effect of P_4 on IVM of oocytes collected from the ovaries without CL

When P_4 was added to the maturation medium, oocyte maturation and subsequent fertilizability were adversely affected in a dose-dependent manner (Fig. 2). At the maximum dose (1000 ng/mL), the rates of maturation and fertilization were approximately half those of the control group. We speculated that the potency of oocytes might be regulated by the physiological event(s) during P_4 biosynthesis, but not by P_4 itself. Therefore, Cho, the precursor to P_4, was targeted in the following experiment.

3.2. Cho contents and localization in ovaries with and without CL

The Cho contents in COCs collected from the ovaries with CL (2.73 μg/μg protein) were higher ($P < 0.05$) than those of the ovaries without CL (1.88 μg/μg protein), whereas there was no significant difference in Cho content of follicular fluid (16.47 vs 19.08 mg/mL, respectively). In ovaries with CL, immunoreactive CYP51, an enzyme to convert lanosterol to cholesterol, was localized mainly at the CL and the surrounding cells (Fig. 3b). In ovaries with a CL (CL1), the immunoreactivity of CYP51 was low distal to the CL (Fig. 3d). Similarly, in ovaries with 10 CLs, immunostained CYP51 also faded away depending on the distance from CL (data not shown).

3.3. *In vitro* Cho synthesis of COCs collected from the ovaries without CL

When COCs were exposed to MBCD for 1.5 h (MBCD-1.5) and 22 h (MBCD-22) during IVM, meiotic maturation rates of oocytes (76.7 and 81.7%, respectively) were similar to that of the untreated control oocytes (76.0%). On the understanding that MBCD has no detrimental effect on the meiotic progression of oocytes, the Cho content of COCs was measured at various times during IVM (Fig. 4). In the control and MBCD-1.5 groups (Fig. 4a and b), there were no changes in the Cho contents of COCs during IVM, although they did increase slightly at 22 h after the onset of IVM. Conversely, the Cho contents of COCs in the MBCD-22 group (Fig. 4c) were decreased ($P < 0.05$; 0.51 μg/μg protein) at 22 h, but subsequently increased (1.55 μg/μg protein) at 44 h of IVM. The increment in Cho contents during 22 to 44 h of IVM was 1.04 μg/μg protein, comparable to basal Cho contents in COCs, (i.e., 1.29 μg/μg protein for 0 h of IVM).

When oocytes treated with MBCD during IVM were used for ICSI, oocytes in the MBCD-22 group (79.5%) had a higher fertilization rate compared to those in the control (65.0%) and MBCD-1.5 (51.3%) groups (Table 1). Nevertheless, rates of cleavage and

blastocyst formation were impaired ($P < 0.05$) in the MBCD-22 group (52.2 and 9.5%) compared to those in the control (69.6 and 26.3%) and MBCD-1.5 groups (66.5 and 23.0%).

4. Discussion

We previously reported that the presence of CL in the ovary was favorable for development of competent oocytes[2]. Our goal is to improve the quality of oocytes collected from the ovaries of pre-pubertal animals (without CL) by producing an *in vitro* environment similar to that in the ovaries with CL. Many studies have reported that MAS, the intermediate of Cho, are key molecules for oocyte growth [19-22] and its nuclear maturation [23-27]. Thus, Cho synthesis may also affect fertilizability and subsequent developmental competence of oocytes. In the present study, Cho synthesis in porcine ovaries/COCs was characterized using quantitative measurements and immunostaining, and variations in Cho contents of COCs were artificially induced during IVM (decreasing by culture with MBCD and then increasing by culture without MBCD), leading to higher fertilizability after ICSI.

Based on our previous results [2], P_4 was initially considered an effective factor in oocyte maturation. However, exogenous P_4 in the culture medium adversely affected nuclear maturation and fertilization (Fig. 2), even with physiological concentrations of P_4 in the follicular fluid of ovaries with CL [2]. Although P_4 is a key substance in the nuclear maturation of mammalian [3] and amphibian [28,29] oocytes, it seems unlikely that the addition of P_4 into maturation medium can directly improve cytoplasmic maturation, an indispensable prerequisite for fertilizability. This agrees with the conclusions of a previous study [4]. Therefore, the P_4 precursor, Cho, attracted attention

in subsequent experiments. Cho is supplied to ovarian cells from the blood, and from *de novo* synthesis [6]. Cho contents in follicular fluid have estrous cycle-dependent changes [30], suggesting that the Cho in follicular fluid may be transported into ovarian cells to produce steroid hormones. However, the presence of CL did not affect Cho content in follicular fluid. Therefore, we inferred that higher developmental competence in COCs collected from the ovaries with CL [2] did not result from Cho content in follicular fluid. Oocyte-derived growth factors, namely, growth differentiation factor 9 and bone morphogenetic protein 15, stimulated Cho production in cumulus cells [18]. Thus, perhaps Cho production in ovarian cells is involved in oocyte maturation. It is well known that CYP51 converts lanosterol to FF-MAS in the cholesterogenic pathway [31,32]. Ovarian CYP51 is increased by eCG administration [19,20], suggesting that Cho synthesis may be needed for competent oocytes. In the present study, the enzyme was utilized as a marker for Cho synthesis in the ovary, because the produced FF-MAS was metabolized rapidly to Cho [3] . Immunostaining for CYP51 revealed Cho synthesis in porcine ovaries with CL. Furthermore, CYP51 was localized mainly in CL and the surrounding cells (Fig. 3), suggesting that ovaries with multiple CLs could widely produce large amounts of Cho. Furthermore, Cho productivity in the ovary was consistent with Cho contents in COCs. Thus, we speculated that post-pubertal animals produce competent oocytes [2] as a result of exposure to Cho intermediates that originate from CL.

To mimic the environment in ovaries with CL, the variation of Cho in COCs was induced using MBCD during IVM. In the MBCD-22 group (Fig. 4c), a large amount of Cho was removed by MBCD during 22 h of IVM, and then the Cho content was recovered by additional culture in MBDC-free medium. The matured oocytes in this group had higher fertilizability compared to control and MBCD-1.5 groups (Table 1). This higher

fertilizability may have been induced by the change in the composition of the plasma membrane by Cho removal and/or by the variation of Cho contents in COCs during IVM. Based on the previous [2] and present results, we inferred that *de novo* synthesis of Cho during the later period of IVM (22-44 h) had a crucial role in fertilization. However, the detail on the mechanism underlying higher fertilizability in the present study remains elusive. Interestingly, the developmental competence of oocytes to the blastocyst stage in the MBCD-22 group declined severely (Table 1). Based on these results, we inferred that fertilizability was regulated by Cho synthesis at a nearby site of oocyte growth and maturation, and embryonic development was affected by the other factor(s). It is plausible that one of the other factors is steroidogenesis during IVM, because Cho removed by MBCD is the main component of steroid hormones. Regardless, MBCD treatment during IVM is a way to produce MII oocytes with higher fertilizability.

If Cho synthesis has a positive effect on oocyte maturation *in vivo*, it should be noted that this effect finishes before meiosis resumes (i.e., the luteal phase in the estrous cycle). Thus, the timing of MBCD treatment in the present study might be inadequate. It should be determined why the developmental competence of oocytes in the MBCD-22 group did not improve. Nevertheless, the cumulative findings of our present and previous [2] results demonstrated that the presence of CL affected ovarian cholesterogenesis and that the variation of Cho contents at a nearby site of oocyte growth and maturation was a key event to produce MII oocytes with higher fertilizability. This approach would provide a more natural environment for the improvement of *in vitro* oocyte maturation. Perhaps there is a rational system for oocyte maturation in which CL produced by ovulation have a positive impact on developing oocytes for the next estrous cycle.

Acknowledgments

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Figure legend

Fig. 1. Schematic representation of MBCD treatment during IVM.

Fig. 2. Oocyte maturation and fertilizability of pre-pubertal porcine oocytes matured with P_4-containing medium. Oocytes were collected from ovaries without CL. Matured oocytes were defined as those with the first polar body. Average numbers of examined oocytes for each group were 88.8 ± 2.8 and 53.0 ± 3.5 for meiotic competence and fertilizability, respectively.

$^{a\text{-}c;x,y}$Values without a common superscript differed ($P < 0.05$).

Fig. 3. Localization of immunoreactive CYP51 in pre- (a) and post-pubertal (b-d) porcine ovaries. The samples were produced from the ovaries of CL0 and CL1. In the CL1 group, these images were obtained from the same section: neighboring CL (b), opposite site of CL (d), and their intermediate site (c). a'-d') high-power field of each image. Bars = 100 μm.

Fig. 4. Transition of Cho contents in porcine COCs during IVM. Oocytes were collected from the ovaries without CL. As a control (a), COCs were cultured following a conventional IVM procedure. COCs were also cultured in MBCD-containing IVM medium for 1.5 h (b) and 22 h (c) from the onset of IVM. White bars indicate the values during MBCD treatment. The total number of examined oocytes was 90 for each group.

^{a,b}Values without a common superscript differed ($P < 0.05$).

Table 1. Fertilizability and developmental competence of porcine oocytes matured in MBCD-containing medium.

	No	Fertilization (%)	No.	Cleavage (%)	Blastocyst (%)
Control	80	52 (65.0 ± 1.3) [a]	144	101 (69.6 ± 4.8) [a]	34 (26.3 ± 4.7) [a]
MBCD 1.5*	80	41 (51.3 ± 9.6) [a]	102	68 (66.5 ± 4.2) [a]	24 (23.0 ± 3.8) [a]
22*	83	66 (76.9 ± 6.5) [b]	163	86 (52.2 ± 5.8) [b]	14 (9.5 ± 4.1) [b]

Data shown as mean ± SEM calculated from each replicate.

*COCs were cultured in MBCD-containing IVM medium for 1.5 and 22 h from the onset of IVM.

[a,b]Within a column, means without a common superscript differred ($P < 0.05$).

Fig. 1.

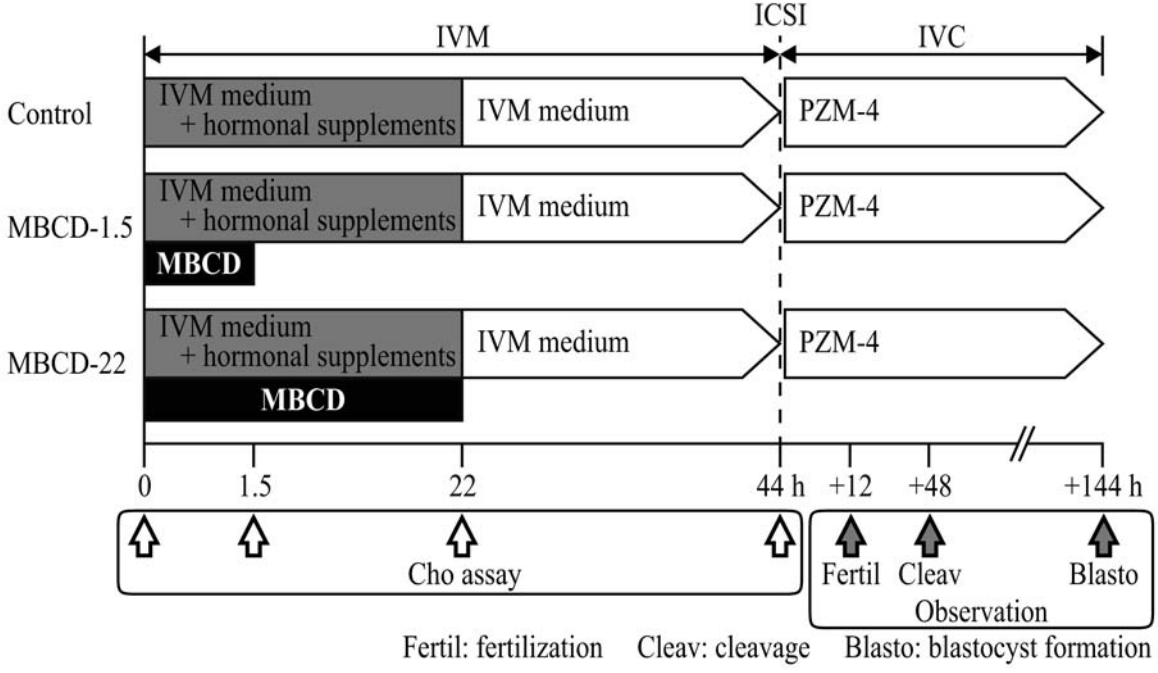

IVM
ICSI
IVC
Control
IVM medium + hormonal supplements
IVM medium
PZM-4
MBCD-1.5
MBCD
MBCD-22
0
1.5
22
44 h
+12
+48
+144 h
Cho assay
Fertil
Cleav
Blasto
Observation
Fertil: fertilization
Cleav: cleavage
Blasto: blastocyst formation

Fig. 2.

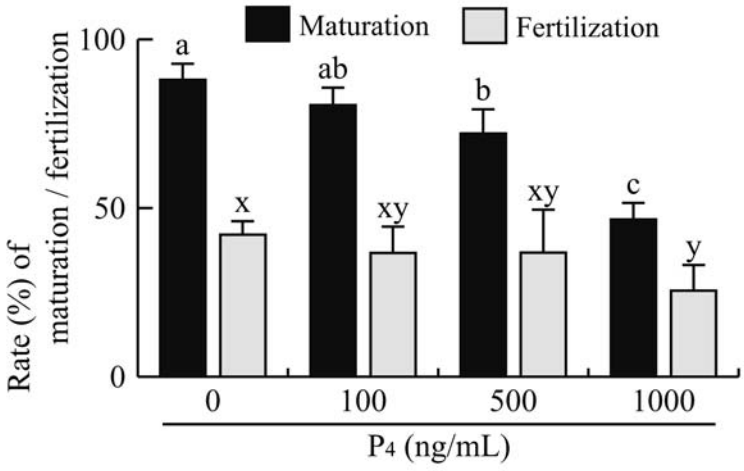

Maturation
Fertilization
Rate (%) of maturation / fertilization
100
50
0
a
ab
b
c
x
xy
xy
y
0
100
500
1000
P4 (ng/mL)

Fig. 3.
(Color figure in online)

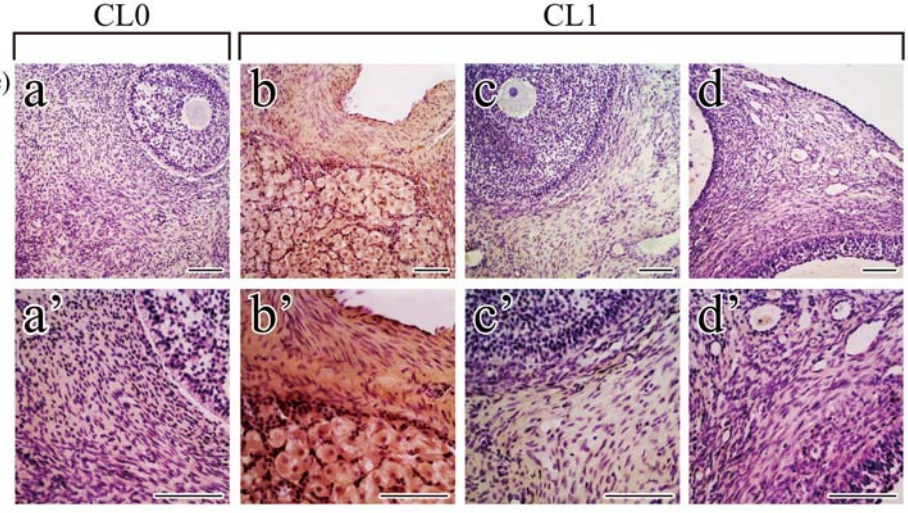
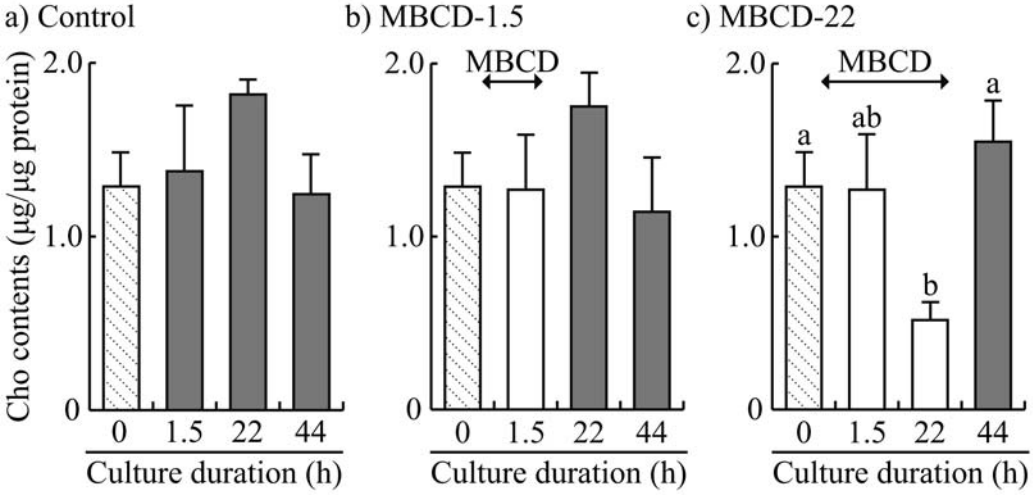


Fig. 4.


a) Control
b) MBCD-1.5
c) MBCD-22
Cho contents (μg/μg protein)
2.0
1.0
0
MBCD
a
ab
b
a
0
1.5
22
44
Culture duration (h)