Theriogenology 79 (2013) 680–686

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

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

Hiroyuki Watanabe [a,*,1,2], Saori Hirai [a,2], Hiroyuki Tateno [b], Yutaka Fukui [a]

[a] Laboratory of Animal Reproduction, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan
[b] Department of Biological Sciences, Asahikawa Medical University, Asahikawa, Japan

ARTICLE INFO

Article history:
Received 12 October 2011
Received in revised form 23 November 2012
Accepted 23 November 2012

Keywords:
Cholesterol
Embryo development
Fertilization
Oocyte maturation
Steroid hormone

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 histologic sections were prepared for immunostaining of lanosterol 14α-demethylase, a catalytic enzyme during cholesterogenesis. In addition, COCs collected from ovaries without a CL (prepubertal gilts) were subjected to *in vitro* maturation with MBCD for 22 hours, followed by maturation without MBCD for 22 hours. 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 lanosterol 14α-demethylase 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 hours, and subsequently increased (1.55 μg/μg protein) by culturing in MBCD-free medium for 22 hours. When oocytes were matured with MBCD for 22 hours and then matured without MBCD for the next 22 hours, the fertilization rate improved ($P < 0.05$) to 76.9%, and 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.

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 postpubertal animals have a higher developmental competence than those of prepubertal 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, 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

* Corresponding author. Tel.: +81 166 68 2731; fax: +81 166 68 2783.
E-mail address: h-wata@asahikawa-med.ac.jp (H. Watanabe).
[1] Present address: Department of Biological Sciences, Asahikawa Medical University, Asahikawa, Japan.
[2] These authors contributed equally to this work.

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.11.024

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, which are commonly known as meiosis-activating sterols (MAS) [7,8]. Thus, cholesterogenesis at a nearby site of oocyte growth and maturation might 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 abattoir and, within 3 hours, 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-ga 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% (wt/vol) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA). In addition, some ovaries were used to collect COCs and follicular fluid for measuring Cho contents. Follicular fluid was centrifuged at 1200 × *g* for 15 minutes, and the supernatant was 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% (wt/vol) PVA, 3.05 mmol/L glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.91 mmol/L Na-pyruvate (Wako), 100 μmol/L 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 hours. Subsequently, COCs were transferred into the medium without hormonal supplements and cultured for another 22 hours in a similar manner. During the first half of IVM (0 to 22 hours), P_4 or methyl-β-cyclodextrin (MBCD) was added to the IVM medium according to the experiments (see 2.8. Experimental design).

2.3. Intracytoplasmic sperm injection

To circumvent polyspermy, intracytoplasmic sperm injection (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 minutes 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 HEPES-buffered Tyrode's medium-PVA containing oocytes were placed on the lid of a 50 × 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 [12] supplemented with 2.77 mmol/L myo-inositol (Sigma), 0.34 mmol/L 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 hours.

2.5. Fixation of oocytes

At 12 hours after ICSI, eggs were fixed in 25% (vol/vol) acetic acid (Wako) in ethanol (Wako) for 24 hours and stained with 1% (wt/vol) 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 refrozen 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). 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 (0–10 mg/mL). Four microliters of each sample and of the standard