Theriogenology 86 (2016) 1789–1798

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Relationship between the number of cells surrounding oocytes and energy states of oocytes

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ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 25 May 2016
Accepted 27 May 2016

Keywords:
Acetylation
ATP
Glucose
Lipid
Oocyte

ABSTRACT

Lipid content, ATP content, and histone acetylation are thought to reflect the energy state of cells. In addition, the energy state closely associates with growth and developmental ability of oocytes. Oocyte growth is accompanied by active proliferation of the surrounding granulosa cells (GCs), and GCs play a key role in the provision of energy substrates to the oocytes. In the present study, we first examined the relationship among the average number of GCs per follicle, the average number of cumulus cells (CCs) per oocyte, and the average lipid content in oocytes that developed *in vivo* within individual donor gilts. Second, we validated the relationship between the number of cells surrounding oocytes and the energy states of oocytes by using an IVC system of oocyte granulosa cell complexes (OGCs) derived from early antral follicles. We collected cumulus cells and oocyte complexes (COCs) from antral follicles (3–5 mm in diameter) and found that average lipid content in oocytes significantly correlated with the average number of both GCs/follicle and CCs/oocyte ($P < 0.05$). In the next series of experiments, we collected OGCs from early antral follicles (0.5–0.7 mm in diameter), and cultured them for 14 days, and then determined the cell number of OGCs, as well as the lipid content, ATP content, and acetylation level of H4K12 in oocytes grown *in vitro*. In addition, glucose consumption by OGCs was calculated from the sample media collected at Days 13 and 14. The lipid content of oocytes grown *in vitro*, significantly correlated with the number of cells surrounding the oocytes ($P < 0.01$) and with the level of glucose consumption by OGCs ($P < 0.01$). In addition, both ATP content and H4K12 acetylation levels of oocytes grown *in vitro* significantly correlated with the number of cells surrounding the oocytes ($P < 0.05$) and glucose consumption by OGCs ($P < 0.05$). In conclusion, the lipid content of oocytes depends on the number of cells surrounding the oocytes, and glucose uptake by OGCs is crucial for lipid content and ATP content, and H4K12 acetylation in oocytes.

1. Introduction

There are approximately 8000 granulosa cells in porcine early antral follicles (EAFs) [1]. This number increases during follicle development, so that as many as 1.0×10^6 granulosa cells are contained in the antral follicle (3–6 mm in diameter) [1]. Oocyte growth is supported by complex interactions between oocytes and the surrounding cells, through paracrine and autocrine factors and through small molecules that are transported via gap junctions [2]. Because oocytes have low glycolytic activity [3], glucose uptake by the surrounding cells and the provision of energy substrates from those cells to the oocytes are vital for oocyte homeostasis. The number of cumulus cells that surround the oocytes and the number of granulosa cells

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0093-691X/$ – see front matter © 2016 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2016.05.036

that are enclosed in the follicles vary to a great extent, and, in many laboratories, oocytes surrounded by thick layers of cumulus cells are selectively used for experiments. To the best of our knowledge, however, how the number of granulosa cells in the follicle, or the number of cells surrounding oocytes, affects the energy state of the oocyte has not been studied thus far.

Oocyte growth is accompanied by the active accumulation of mRNA, proteins, and lipids and by modifications of chromatin configuration and DNA methylation status, and these molecular reactions require enough energy. In addition, several markers of different energy states have been associated with oocyte growth and development. For example, Stojkovic et al. [4] categorized oocytes based on the number of cumulus cells and found that high ATP and high blastulation rates are found in oocytes with greater numbers of cumulus cells. In addition, ATP content in oocytes was closely associated with high embryogenesis and implantation in humans [5]. Lipids are important sources of energy during nuclear maturation and early embryonic development [6–9]; e.g., inhibition or activation of beta-oxidation inhibits or improves oocyte ability, respectively [6,7,10,11]. Furthermore, it has been well-documented that metabolic changes, from fatty acid synthesis to fatty acid oxidation, are crucial for meiotic maturation [12,13]. In addition, histone acetylation levels increase during oocyte development [14], such that porcine oocytes at the germinal vesicle (GV) stage exhibit a high level of H4K12 and H3K4 acetylation [15]. Although the significance of high levels of histone acetylation is unclear, acetylation in oocytes may be affected by energy sufficiency because, in somatic cells, protein acetylation is closely related to the availability of acetyl-CoA [16] and energy conditions [2,17]. Furthermore, a previous study [16] reported that glucose in the medium is used for lipid synthesis and H4K16 acetylation in cells. Based on these results, we hypothesized that the energy sufficiency of oocytes may reflect ATP content, lipid content, and histone acetylation of immature oocytes and that energy states may depend on the number of cells that surround the oocytes. To investigate the relationship among the energy states of oocytes, glucose consumption by OGCs, and the number of cells surrounding the oocytes, it is ideal to directly measure these factors using *in vivo*–developed follicles; however, it is difficult to examine these factors directly when using ovaries derived from slaughterhouses. Studies have shown that long-term preservation of organs under ischemic conditions significantly reduces ATP content [18]. Furthermore, we previously showed that the preservation of ovaries decreased glucose concentrations in the follicular fluid [19]. Taken together, these results suggest that testing oocytes that have been collected from slaughterhouse-derived ovaries does not provide accurate information about the relationship between the energy state of oocytes and granulosa cell numbers. In porcine, oocytes collected from EAFs grow to full size during 14 days of IVC [1,20–22] and can be used to examine the relationship among the energy state of oocytes, glucose consumption, and cell number.

In the present study, we collected OGCs from the antral follicle (AFs, 3–5 mm in diameter) of porcine ovaries and examined the relationship between the number of granulosa cells, the number of cumulus cells, and lipid content in oocytes. We also collected OGCs from EAFs, cultured them for 14 days, and then examined the relationship among the number of granulosa cells surrounding the oocyte, glucose consumption by OGCs, oocyte H4K12 acetylation, and oocyte energy states, including lipid and ATP content.

2. Materials and methods

2.1. Collection of ovaries

Ovaries were collected from individual prepubertal gilts that had been slaughtered at a local abattoir for public consumption and were transported to the laboratory (at approximately 37 °C in PBS, containing 100 μg/mL streptomycin and 10 IU/mL penicillin) within 1 hour. The ovaries were subsequently discarded without further use. This study was approved by the ethics committee for animal experimentation at the Tokyo University of Agriculture. Therefore, an ethics statement is not required in our article.

2.2. Chemicals and media

All reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. The medium used to culture OGCs *in vitro* (IVG medium) was α–minimum essential medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10-mM taurine, 1-μg/mL 17β-estradiol, 0.1-mAU/mL FSH (Kawasaki Mitaka, Tokyo, Japan), 2% polyvinylpyrrolidone 360K (Sigma–Aldrich), 2-mM hypoxanthine (Sigma–Aldrich), 1% insulin–transferrin–selenium (Gibco BRL, Grand Island, NY, USA), 3-mg/mL BSA (Fraction V), and antibiotics. The medium used for oocyte nuclear maturation (IVM medium) was prepared with North Carolina State University 23 (NCSU 23) [23] solution supplemented with porcine follicular fluid (10% v:v). Porcine follicular fluid samples were collected from AFs with diameters of 3 to 6 mm, centrifuged at 10,000 × *g* for 10 minutes, and stored at −20 °C. The medium used for embryo culture (IVC medium) was porcine zygote medium 3 (PZM3 solutions), as reported by Yoshioka et al. [24].

2.3. Collection of OGCs from EAFs

Ovarian cortical tissues were excised from the ovarian surface under a stereomicroscope, and OGCs were collected from EAFs (0.5–0.7 mm in diameter). Oocyte granulosa cell complexes containing oocytes with diameters ranging from 90 to 100 μm were then randomly selected under a digital microscope (BZ–8000; Keyence, Tokyo, Japan) and used for further experiments.

2.4. In vitro growth (IVG) of OGCs from EAFs

Oocyte granulosa cell complexes were cultured *in vitro* as reported previously, with slight modifications [1,21,22]. Using this culturing method, oocytes that were collected from EAFs successfully completed meiotic maturation and developed to the blastocyst stages. Oocyte granulosa cell