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Resveratrol improves the quality of pig oocytes derived from early antral follicles through sirtuin 1 activation

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ABSTRACT

During oocyte growth, the number of mitochondria drastically increases and mitochondrial function profoundly affects the oocyte competence. Resveratrol is a well-known activator of sirtuin 1 (SIRT1), which has a role in cellular energy homeostasis and mitochondrial biogenesis. The main aim of the present study was to examine the effect of supplementation of culture media with resveratrol on oocyte development and mitochondrial number and functions. Lipid contents and developmental ability of the oocytes grown *in vitro* were also examined. Oocyte-granulosa cell complexes were collected from early antral follicles of gilt ovaries and were cultured in medium containing 0 or 2 μM resveratrol for 14 days. Immunostaining revealed that resveratrol enhanced SIRT1 expression in oocytes. Antrum formation during the culture period and survivability of the granulosa cells surrounding the developed oocytes did not differ between the two concentrations of resveratrol. In addition, the ability of oocytes to complete meiotic maturation did not differ between the two concentrations of resveratrol, whereas the ability of oocytes to develop to the blastocyst stage was improved significantly by resveratrol (7.4% vs. 1.6%; $P < 0.05$). Resveratrol upregulated the ATP content in oocytes grown *in vitro*, and the addition of 2 μM of the SIRT1 inhibitor 6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX527) diminished this effect although EX527 alone had no effect on ATP content. The mitochondrial DNA copy number in oocytes determined by quantitative real-time polymerase chain reaction increased during *in vitro* oocyte development, but resveratrol did not affect the kinetics of the mitochondrial DNA copy number. We found that resveratrol also increased the expression level of phospho-5′-adenosine monophosphate–activated protein kinase in oocytes but decreased the lipid content in oocytes grown *in vitro*. These results suggest that resveratrol increased the ATP content in oocytes *via* energy homeostasis and improved the developmental ability of oocytes grown *in vitro*.

1. Introduction

Almost all domestic embryos are produced from oocytes of large antral follicles by using *in vitro* fertilization or superovulation, and a certain number of blastocysts were successfully obtained. However, a huge number of small immature follicles are present in ovaries as a resource of embryos. Current improvements in culture technology have made it possible to develop oocytes of domestic animals from early antral follicles (EAFs) to fully grown follicles. However, the efficiency of the developmental process is still low, and further improvement of the culture conditions is required.

In addition, *in vitro* oocyte growth in optimal culture conditions may shed light on the molecular background underlying oocyte growth. In domestic animals, including pigs and cows, it takes about 2 weeks for oocytes derived from EAFs to develop into full-grown oocytes [1,2]. When

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oocyte-granulosa cell complexes (OGCs) are cultured *in vitro*, they form an antrum-like cavity, and this pseudofollicle structure is maintained during OGC development [3]. Therefore, cavity formation is a marker of oocytes development [4]. During long culture periods, OGCs morphologically start to degenerate and the follicle-like antrum structure disrupts, indicating that maintaining the antrum structure and the viability of the granulosa cells and oocytes is a crucial factor for *in vitro* follicle culture [1]. In addition, drastic changes, including increases in protein, transcripts, mitochondria, and lipid content, occur in developing oocytes [5–7].

Sirtuin 1 (SIRT1) is a member of the sirtuin (silent mating type information regulation 2 homolog) family of NAD-dependent histone deacetylases [8] and has an anti-aging effect on cellular homeostasis that involves cellular viability and energy metabolism [9,10]. In light of this, the upregulation of SIRT1 during stem cell culture with improved cellular viability has been demonstrated [11]. Another noteworthy role of SIRT1 is the upregulation of mitochondrial biogenesis [12]. Resveratrol, one of the polyphenols contained especially in grape peel, enhances SIRT1 activity by inhibiting phosphodiesterase [13]. Supplementation of *in vitro* maturation (IVM) media with resveratrol improved the fertilization outcomes and the developmental ability of bovine and porcine oocytes, most likely through the upregulation of mitochondrial biogenesis [14,15]. Furthermore, SIRT1 is closely related to adenosine monophosphate (AMP)-activated protein kinase (AMPK) activity, which is an energy sensor controlling cellular metabolism, including oxidative phosphorylation and fatty acid oxidization [16]. However, whether upregulation of SIRT1 expression by resveratrol improves the development of oocytes derived from EAFs, mitochondrial function, and mitochondrial number as well as energy metabolism in oocytes grown *in vitro*, has not been studied. The present study examined the effects of supplementation of culture media with resveratrol on the expression level of SIRT1 and phospho-AMPK (p-AMPK) in oocytes and the development of porcine OGCs derived from EAFs. The study also further examined the developmental ability, mitochondrial function, mitochondrial DNA copy number (Mt-number), and lipid content in the oocytes grown *in vitro*. These parameters were also compared with those of oocytes developed *in vivo*.

2. Materials and methods

2.1. Reagents and media

Unless otherwise stated, all drugs were purchased from Nacalai Tesque (Kyoto, Japan). The medium used for *in vitro* culture (IVC) of OGCs (growth medium) was α-modified minimal essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% wt/vol polyvinylpyrrolidone (Sigma-Aldrich), 10 mM taurine, 0.3% wt/vol BSA (Fraction-V), 1 μg/mL 17β-estradiole, 0.1 mAU/mL FSH (Kawasaki-Mitaka, Kawasaki, Japan), 2 mM hypoxanthine (Sigma-Aldrich), and insulin transferrin selenium (Gibco, Paisley, UK). The medium used for IVM of oocytes grown *in vitro* (IVM medium) was North Carolina State University 37 [17] supplemented with 10% v:v porcine follicle fluid. The porcine follicle fluid was collected from antrum follicles (3–6 mm in diameter), centrifuged (10,000 × *g* for 5 minutes), and stored at −30 °C until further use. The medium used for IVC of embryos (IVC medium) was porcine zygote medium 3 [18]. Resveratrol (Wako, Osaka, Japan) and 6-Chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX527), a selective inhibitor of the SIRT1 (Sigma-Aldrich), were diluted in ethanol (20 mM). Control media contained the same amount of ethanol.

2.2. Collection of ovaries and OGCs and IVC of the OGCs

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse (Kanagawa Meat Center, Kanagawa, Japan) and were transported to the laboratory in PBS at 37 °C within 1 hour. The OGCs were collected from EAFs (500–700 μm) by using an 18-ga needle connected to a sling. The OGCs were pooled and randomly selected for each experiment except for experiment 5 in which Mt-numbers in oocytes were examined. The OGCs were cultured in growth medium for 14 days. Every 4 days, half of the medium was replaced with fresh medium and OGCs were examined for antrum formation. At the end of the IVC period (14 days), OGCs with an antrum cavity were subjected to IVM. *In vitro* maturation of oocytes was conducted as described previously [15]. After IVM, oocytes were denuded from the surrounding granulosa cells and were parthenogenetically activated in IVC medium containing 10 μg/mL ionomycine for 5 minutes, followed by 6 hours of incubation in IVC medium containing 10 μg/mL cytochalasin B and 10 μg/mL cycloheximide for 6 hours. After activation, the embryos were cultured for 8 days and the ratio of blastulation was examined. *In vitro* growth of OGCs and IVM were performed at 38.5 °C in an atmosphere containing 5% CO_2 and 95% O_2. *In vitro* culture was performed at 38.5 °C in an atmosphere containing 5% O_2, 5% CO_2, and 90% N_2.

2.3. Assessment of antrum formation and survivability of the granulosa cells

Twenty OGCs were cultured individually in a medium containing 0 (vehicle, ethanol) or 2 μM resveratrol, and the number of OGCs forming an antrum was examined every 4 days. At the end of the culture period, OGCs that formed an antrum were selected and transferred to test tubes followed by vortexing for 5 minutes. The survivability of the granulosa cells was determined by using calcein-acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA) and propidium iodide (Calbiochem, La Jolla, CA, USA).

2.4. Immunostaining against SIRT1 and p-AMPK

The effects of resveratrol on the expression levels of SIRT1 and p-AMPK in oocytes were examined by immunostaining as described previously [15]. The primary and secondary antibodies used for immunostaining were rabbit polyclonal anti-SIRT1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-p-AMPKα (1:1000; Cell Signaling Technology Inc., Beverly, MA, USA), and fluorescein-conjugated goat anti-rabbit IgG (1:1000; Cell Signaling Technology Inc.). Oocytes were mounted on glass slides by using prolong Gold antifade reagent with