Resveratrol Improves the Mitochondrial Function and Fertilization Outcome of Bovine Oocytes

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Abstract. The aim of the present study was to address the effect of resveratrol-mediated upregulation of sirtuin 1 (SIRT1) during oocyte maturation on mitochondrial function, the developmental ability of oocytes and on mechanisms responsible for blockage of polyspermic fertilization. Oocytes collected from slaughterhouse-derived ovaries were cultured in TCM-199 medium supplemented with 10% FCS and 0 or 20 µM resveratrol (Res). We examined the effect of Res on SIRT1 expression in in vitro-matured oocytes (Exp 1); fertilization and developmental ability (Exp 2); mitochondrial DNA copy number (Mt number), ATP content and mitochondrial membrane potential in matured oocytes (Exp 3); and the time required for proteinase to dissolve the zona pellucida following in vitro fertilization (as a marker of zona pellucida hardening), as well as on the distribution of cortical granules before and after fertilization (Exp 4). In Exp 1, the 20 µM Res treatment upregulated protein expression of SIRT1 in oocytes. In Exp 2, Res treatment improved the ratio of normal fertilization and the total cell number of blastocysts. In Exp 3, Res treatment significantly increased the ATP content in matured oocytes. Additionally, Res increased the overall Mt number and mitochondrial membrane potential, but the effect was donor-dependent. In Exp 4, Res-induced zona hardening improved the distribution and exocytosis of cortical granules after in vitro fertilization. In conclusion, Res improved the quality of oocytes by improving mitochondrial quantity and quality. In addition, Res added to the maturation medium enhanced SIRT1 protein expression in oocytes and improved fertilization via reinforcement of the mechanisms responsible for blockage of polyspermic fertilization.

Key words: Fertilization, Mitochondria, Oocytes, Resveratrol

Successful fertilization is a crucial step in embryonic development. Normal fertilization depends on proper cortical granule distribution in matured oocytes and exocytosis following calcium oscillation triggered by sperm entry into the oocyte [1, 2]. Calcium release from the endoplasmic reticulum is achieved by well-orchestrated interactions between mitochondria and the endoplasmic reticulum [3–6]. Thus, mitochondrial quality profoundly affects calcium homeostasis [7]. In addition, mitochondrial quality and quantity are considered major markers of oocyte quality, and low mitochondrial DNA copy number and ATP result in low developmental competence of oocytes [8, 9].

Sirtuin 1 (SIRT1) belongs to the sirtuin family of class III nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases. SIRT1 is critical to cell survival owing to its interactions with a number of factors, including FOXO3A, p53 and PPARGC1 [10–12]. In addition, SIRT1 has a role in controlling mitochondrial function, biogenesis and degeneration via mitophagy [13–16]. Resveratrol is a specific activator of SIRT1. In our previous study, we showed that supplementation of maturation medium for bovine oocytes with resveratrol (Res) enhanced the protein expression of SIRT1 and improved in vitro fertilization (IVF) outcomes by increasing the rate of normal fertilization and decreasing the rate of abnormal fertilization. The inhibition of SIRT1 by EX527 increased the rate of abnormal fertilization [17].

In the present study, we address whether upregulation of SIRT1 during oocyte maturation by Res affects mitochondrial quality and quantity in oocytes and whether Res improves the mechanisms responsible for proper fertilization outcome, including cortical granule distribution and exocytosis.

Materials and Methods

Chemicals

All drugs used in this study were purchased from Nacalai Tesque (Kyoto, Japan) unless stated otherwise. Medium 199 supplemented with 10% FCS (FCS; 5703H; ICN Pharmaceuticals, Costa Mesa, CA, USA) and 5 mM taurine was used for maturation. Synthetic oviductal fluid (SOF) was used for IVF medium and in vitro culture (IVC) [18]. For IVF, SOF was supplemented with 5 mg/ml BSA (fatty acid free) and 10 IU/ml heparin (Sigma-Aldrich, St Louis, MO, USA). For IVC, SOF was supplemented with essential and nonessential amino acids (Sigma-Aldrich), 1% FCS, 5 mM taurine and 1.5 mM glucose. IVM, IVF and IVC cultures were maintained at 38.5°C with 5% CO2 in air at maximum humidity.
Ovary and oocyte collection

Japanese Black cows that were 26–30 months old were used as donors. Ovaries were collected and stored at 25 C in phosphate-buffered saline containing 10 mM glucose, 10 mM sucrose and antibiotics and were transported to the laboratory within 4 h. The preservation period of the ovaries used for all experiments was 3–4 h. Cumulus oocyte complexes (COCs) were collected from both ovaries of each cow by using a syringe with an 18-G needle.

In vitro maturation and fertilization

COCs were matured in IVM medium for 21 h (10 COCs/100 µl drop). After maturation, the complexes were washed with IVF medium and co-incubated with frozen-thawed semen. The semen was obtained from a Japanese Black bull and washed with a 45–60% Percoll solution (Amersham Biosciences, Uppsala, Sweden) to create a discontinuous gradient for centrifugation (800 × g for 10 min). The final sperm concentration in the IVF medium was 1 × 10⁶ cells/ml. After 6 h of co-incubation, the COCs were washed and transferred to IVC medium and then cultured for 12 h. To determine the fertilization rate, oocytes were denuded of cumulus cells by vortexing (6 min) and fixed in Carnoy’s fluid. Pronuclei (PNs) were observed under an inverted microscope (Olympus, Tokyo, Japan). The oocytes were divided into 3 groups based on the number of PNs. Oocytes with 2 PNs were considered to have undergone normal fertilization; oocytes with >2 PNs were considered to have undergone abnormal fertilization; oocytes with 0 PNs were defined as non-fertilized oocytes.

In vitro culture of embryos

After fertilization, COCs were washed three times and cultured in 100 µl of IVM medium (10 embryos/drop). Forty-eight hours post insemination, oocytes were denuded from surrounding cumulus cells by using a fine-pulled Pasteur pipette, and embryos with more than four cells were cultured in 50 µl of IVM medium (10 embryos/drop). The atmospheric culture conditions were 5% CO₂ in air for IVM, IVF and first IVC (until 2 days post insemination) and 5% CO₂, 5% O₂ and 90% N₂ for second IVC (from 2 days to 7 days post insemination). After 7 days of IVC, the ratios of development to the blastocyst stage 7 days post fertilization and total cell number (TCN) of the blastocysts was determined.

Assessment of mitochondrial DNA copy number

Mitochondrial DNA copy number (Mt number) in mature (after 21 h of culture) oocytes was determined by examining 10 oocytes from each donor. Each oocyte was lysed in 6 µl of lysis buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet-40 and 0.9% Tween 20) at 55 C for 30 min followed by 95 C for 5 min. Mt number was then determined by real-time PCR using a Rotor-Gene 6500 real-time rotary analyzer (Corbett Research, Sydney, Australia) as described previously [19]. The PCR primer set was 5'-ATTACAGCAATATGCGCCC-3' and 5'-AAAAGCGTGCTTACGATG-3'.

ATP assay

The ATP content of oocytes was measured as luminescence generated in an ATP-dependent luciferin–luciferase bioluminescence assay (ATP assay kit; TOYO B-Net, Tokyo, Japan) as described previously [20]. Individual oocytes were lysed, and luminescence was measured immediately using a luminometer (Gene Light 55; Microtech, Chiba, Japan).

Mitochondrial membrane potential

Oocytes were incubated in SOF containing 1 mg/ml BSA and 0.5 µM of MitoTracker Orange (Invitrogen, Eugene, OR, USA), a marker of mitochondrial membrane potential [21], for 10 min. After incubation, oocytes were washed and mounted on a slide for measurement of fluorescent intensity using a digital fluorescence microscope (BZ-8000; Keyence, Tokyo, Japan).

Assessment of cortical granule and zona pellucida hardening

After in vitro maturation and 18 h post fertilization, oocytes were fixed in 4% paraformaldehyde, and cortical granules were stained using a lectin from *Arachis hypogaea* (peanut) (Sigma-Aldrich). These oocytes were mounted with an antifade reagent containing DAPI (ProLong Gold Antifade Reagent with DAPI; Invitrogen, Eugene, OR, USA) on glass slides and observed under a fluorescent digital microscope (BZ-8000; Keyence, Tokyo, Japan). To assess zona pellucida (ZP) hardening, oocytes were incubated in SOF containing 1 mg/ml BSA and 0.1% proteinase 16h post IVF. The time required to dissolve the ZP was measured.

Oocytes used for experiments

In this series of experiments (except for experiment 1), the effects of Res were compared within the oocyte cohort collected from the same donor, and the experiment was repeated using 6–7 different donor cows because mitochondrial number and function were expected to differ among the donors. If we used oocytes randomly selected from pooled oocytes collected from numerous cows, any difference would mask the intrinsic difference. In addition, we have previously shown that the Mt number determined with 10 oocytes closely resembles that of a cohort of oocytes collected from the same donor cow. Oocytes were collected from all follicles (3–6 mm) on the surface of ovaries, divided into 2 groups and cultured in a medium containing 0 or 20 µM Res.

Experimental designs

Experiment 1: In a previous report, we showed that 2 µM Res enhanced the level of SIRT1 protein expression [17]. In a preliminary experiment, we examined the effect of supplementation of maturation medium with Res (0, 2 and 20 µM) on the expression level of SIRT1 in oocytes. We found that Res increased the level of SIRT in a concentration-dependent manner (Supplementary Fig. 1). Therefore, in subsequent experiments, we used 20 µM Res. Approximately 30 oocytes were randomly selected from an oocyte pool and cultured in IVM medium containing 0 or 20 µM Res for 21 h. Then, oocytes were immunostained for SIRT1. Experiments were repeated 3 times, and the levels of SIRT1 expression for total oocytes were compared between the 2 Res concentration groups. Detection of SIRT1 in oocytes was performed as described previously [17].

Experiment 2: The effect of Res on the ratio of nuclear maturation, fertilization, ratio of development to the blastocyst stage 7 days post fertilization and total cell number (TCN) of the blastocysts was examined. To examine the TCN of the blastocysts, embryos were
incubated in hypo-osmotic solution for 1 min, fixed and subjected to Giemsa staining. As mentioned above, oocytes collected from each donor cow were divided into 2 groups and cultured with or without 20 µM Res. Each experiment (nuclear maturation, fertilization and ratio of development to the blastocyst stage) was repeated 6 times using 6 different donor cows.

Experiment 3: The effect of Res on ATP content, Mt number and mitochondrial membrane potential (MMP) was examined. Oocytes collected from individual donor cows were divided into 2 groups and cultured with 0 or 20 µM Res. Seven, 8 or 7 donor cows were used for comparison of Mt number, MMP, or ATP content in oocytes, respectively.

Experiment 4: We examined the effect of Res in IVM medium on cortical granule (CG) distribution in oocytes both after maturation and fertilization, as well as on the solubility of the ZP following IVF. Six cows were used for each comparison.

**Statistical analysis**

To compare the effects observed with the 2 different Res concentrations, we used a 2-tailed Student’s t-test. The nuclear maturation, fertilization and developmental rates were arcsine transformed prior to analysis. A P value <0.05 was considered statistically significant.

**Results**

Addition of Res to the maturation medium enhanced the expression level of SIRT1 in *in vitro*-matured oocytes by 1.2-fold (Fig. 1A–C, P<0.05). In experiment 2, supplementation of maturation medium with Res did not affect the ratio of nuclear maturation (20 µM vs. 0 µM Res: 66 vs. 55 oocytes, ration of M2 oocytes of 81.9 ± 2.5 vs. 81.8 ± 2.1, respectively; data not shown), whereas addition of Res improved the ratio of fertilization, resulting in higher normal fertilization (63.5 vs. 46.7%, P < 0.01) and lower abnormal fertilization (18.4 vs. 44.6%, P < 0.01; Table 1). A similar ratio for successful development to the blastocyst stage was observed in the 2 groups (23.8 ± 3.1 and 24.5 ± 1.1, respectively), whereas Res treatment increased the TCN of blastocysts compared with that of oocytes cultured without Res (81.9 vs. 66.0, P < 0.01; Table 2).

Figure 2A shows the average ATP content of total oocytes. The ATP content in oocytes cultured with Res was 3.7 pM, which represented a significant increase compared with control oocytes (oocytes cultured without Res, 2.9 pM, P < 0.01). Figure 2B shows the average ATP content in oocytes of individual donor cows. Overall, the ATP content increased in most donor cows, and in 4 out of 7 donor cows, significant differences were observed in the presence and absence of Res.

Figure 3A shows the average Mt number of total oocytes. The Mt number of oocytes cultured with Res was 437,212, which did not differ significantly from that of control oocytes (385,332). Figure 3B shows the average Mt number in oocytes of individual donor cows. The Mt number differed substantially among the donor cows, ranging from 143,789 to 807,915. In 6 out of 7 cows, *in vitro*-matured oocytes treated with Res had greater Mt numbers than did control oocytes, and in 3 out of 7 cows, there were significant differences between the 2 groups (P < 0.05). However, Res treatment significantly decreased the Mt number in oocytes in one cow (P < 0.05).
Figure 4A shows the average fluorescence intensity of total oocytes stained with MitoTracker Orange. Oocytes matured with Res had a higher MMP than those matured without Res (P < 0.05). However, when we compared the MMP within each cohort of oocytes collected from individual donor cows, we found that in 3 out of 7 donors, the MMP increased significantly with the addition of Res, whereas the MMP of one donor cow decreased significantly (P < 0.05; Fig. 4-B). After *in vitro* maturation, the cortical granules were distributed either throughout the oocytes (Fig. 5A) or at the cortical region of oocytes (Fig. 5B). The ratio of oocytes with CGs distributed in the peripheral region of the oocyte was significantly higher for oocytes matured with Res than for those matured without Res (36.2% and 58.1%, P < 0.05; Table 3). After fertilization, we observed 2 types of oocytes: (1) oocytes with most of the CGs released (Fig. 5C) and (2) oocytes still containing some CGs in the peripheral region (Fig. 5D). The ratio of oocytes with most of the CGs released was significantly higher for oocytes matured with Res than for those matured without Res (79.0% and 55.5%, P < 0.05; Table 3).

Figure 6A shows the average time required to dissolve the ZP. Supplementation of IVM medium with Res significantly increased the time required to dissolve the ZP (327.8 sec vs. 384.2 sec, P < 0.001). Figure 6B shows the average time required to dissolve the ZP in each cohort of oocytes collected from individual cows in the presence and absence of Res. In all cows, the time was longer for oocytes matured with Res than for those matured without Res, and in 5 cows, the time was significantly longer (P<0.05).

### Discussion

The present study demonstrates that the addition of Res in the maturation medium upregulates SIRT1 expression and affects mitochondrial function in *in vitro*-matured oocytes. In addition, we found that Res improves the fertilization outcome via mechanisms responsible for prevention of polyspermic fertilization including cortical granule distribution and exocytosis.

Resveratrol is a potent activator of SIRT1 and has been reported to upregulate SIRT1 expression in cardiomyocytes [22]. In our previous study, we showed that 2 μM Res increased SIRT1 expression in oocytes. In addition, we observed that the addition of Res to IVM medium improved fertilization outcomes, while an inhibitor of SIRT1 (EX527) increased the ratio of abnormal fertilization [17].

In the present study, we confirmed similar effects of 20 μM of Res on fertilization outcome and discovered beneficial effects of Res.
supplementation of IVM medium on the quality of blastocysts.

Successful fertilization requires proper inhibition of polyspermic fertilization and oocyte activation. One of the major mechanisms underlying these events is calcium release from the endoplasmic reticulum. In addition, it has been reported that spatiotemporal calcium release and uptake depend on the interaction between mitochondria and the endoplasmic reticulum [6, 23]. Furthermore, calcium oscillation in the cytosol is accompanied by mitochondrial calcium oscillation and ATP production [4, 24]. Based on these observations, we hypothesized that Res affects mitochondrial function, which promotes physiological events responsible for successful fertilization.

In experiment 3, we examined the effect of Res on mitochondrial function. We found that the ATP content increased during IVM, which is consistent with previous reports [25]. In addition, the average ATP content of total oocytes was higher in Res-treated oocytes than in the untreated ones. Since the ATP content in oocytes is related to oocyte competence [26–28], our results suggest that supplementation of maturation medium with Res improved oocyte quality through improvement of mitochondrial function. To gain a better understanding of this mitochondrial function, we examined the effect of Res on Mt number and MMP. Because the Mt number in oocytes differed greatly among donor cows, there was no difference in the average Mt number for all oocytes between the 2 Res concentration groups. However, comparison within the same cohort of oocytes collected from each donor cow showed that the Mt number increased in response to Res in most donor cows, although treatment with Res decreased the Mt number in one cow. Based on these results, we concluded that the stimulatory effect of Res on mitochondrial biosynthesis depends on the individual conditions of donor cows and that there may be additional unknown factors in oocytes contributing to mitochondrial biosynthesis. In agreement with these results, when oocytes of early antral follicles were cultured
and the kinetics of Mt numbers in oocyte were examined, the ration of increase in Mt number in oocytes varied substantially among donor cows [29]. We observed a similar trend in our results for MMP. Res affected the level of MMP, and the kinetics depended on the individual cows. Although Simsek-Duran et al. [30] reported that high ATP content reflects high mitochondrial mass in oocytes,

**Table 3. Effect of resveratrol on CG distribution after IVM and IVF in bovine oocytes**

| Res 20 μM | No. of cows | No. of trials | No. of oocytes | After IVM | No. of trials | No. of oocytes | After IVF |
|----------|-------------|---------------|---------------|-----------|---------------|---------------|----------|
|          |             |               |               | Cortical area (%) |               |               | CG released (%) |
| +        | 13          | 6             | 50            | 58.1 ± 7.7a    | 7             | 57            | 79.0 ± 6.8a    |
| −        | 13          | 6             | 52            | 36.2 ± 2.4b    | 7             | 38            | 55.5 ± 4.3b    |

a–b, P<0.05.
CG distribution depends on microfilaments and actin in mice, and ATP has been shown to drive the function of the cytoskeleton in pigs [34, 35]. These results suggest that high ATP content in oocytes may contribute to the proper arrangement of CGs in oocytes. Furthermore, Res in maturation medium induced more ZP hardening following fertilization and improved CG exocytosis to the level that the amount of CGs remaining in oocytes after IVF was reduced by Res treatment. Cortical granule exocytosis is regulated by numerous pathways, including calcium-dependent proteins, the amount of calcium store, IP3 receptors and sensitivity to sperm entry [1, 2, 36]. Furthermore, there is growing evidence that mitochondrial distribution, function and interaction with the endoplasmic reticulum contribute to proper calcium homeostasis and cortical granule exocytosis [4, 5, 37, 38]. Thus, we concluded that proper mitochondrial function, due, in part, to the addition of Res, facilitates proper exocytosis in oocytes. Future experiments will be required to elucidate the molecular mechanism underlying Res-enhanced fertilization outcomes. In conclusion, Res affected oocyte mitochondrial function and improved fertilization outcome via reinforcement of the mechanisms responsible for the blockage of polyspermic fertilization.

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