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—Original Article—

Modification of mitochondrial function, cytoplasmic lipid content and cryosensitivity of bovine embryos by resveratrol

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Abstract. Resveratrol is a potent activator of NAD-dependent deacetyltransferase sirtuin-1 (SIRT1) and affects lipid metabolism and ATP generation in somatic cells. In the present study, the effects of supplementing culture medium with resveratrol on lipid metabolism, ATP generation, and cryosensitivity of bovine *in vitro* produced embryos were investigated. Bovine early cleaved-stage embryos were cultured in medium containing 0 or 0.5 μM resveratrol for 1 or 5 days. Resveratrol treatment for both 1 day and 5 days increased the expression levels of SIRT1 and phosphorylated AMP-activated protein kinase (pAMPK) in the embryos. Furthermore, resveratrol treatment was effective to increase ATP generation and reduce lipid content of the embryos. The effects of resveratrol treatment were diminished by the SIRT1 inhibitor "EX527", and the reduced lipid content was reversed by treatment with etomoxir (a potent inhibitor of beta-oxidation). Blastocysts developed after resveratrol treatment showed low levels reactive oxygen species and increased cryotolerance. These results demonstrate that resveratrol improves *in vitro* development of bovine embryos, while reducing cytoplasmic lipid content through activation of beta-oxidation, thereby effective for production of bovine blastocysts with enhanced cryotolerance.
Key words: Cryopreservation, Embryos, Lipid, Mitochondria, Resveratrol

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Embryo quality is a crucial factor determining occurrence of successful conception in embryo transfer program in cattle. The quality of embryos produced *in vitro* is affected by the culture environment, and *in vitro*- and *in vivo*-produced embryos display differences in morphology [1], glucose levels [2], and amino acid metabolism [3]. One of the major factors in the *in vitro* culture milieu that causes these differential outcomes is the presence of fetal calf serum (FCS), which enhances embryo development but also leads to lipid accumulation in the embryo [4, 5]. It has been demonstrated that high lipid content in embryos reduces survival following cryopreservation [6]. It was also reported that culturing embryos with FCS increased the lipid content in blastocysts in a dose-dependent manner, and the lipid content in these embryos was much higher than that of *in vivo*-developed blastocysts [7]. In addition, maturing oocytes in medium containing high concentrations of non-esterified fatty acids reduced the post-vitrified-warming survival rate of the resultant blastocysts [8]. Several methods including mechanical [9, 10] and chemical procedures [11], have been used to reduce the lipid content of oocytes and embryos. For example, mechanical removal of cytoplasmic lipids from porcine oocyte improved their viability after cryopreservation [9, 10]. However, the mechanical treatments may imply a drawback effect on development of the oocytes/embryos. Men *et al.* [11] reported that forskolin treatment was effective to decrease lipid content of porcine embryos, thereby improving their cryotolerance.

The sirtuin family proteins are potent histone deacetylases that play versatile roles in early embryonic development. Additionally, the inhibition of sirtuins reduces the developmental ability of porcine embryos [12]. Lee *et al*. [13] reported that the supplementation of culture medium with resveratrol increased the blastocyst formation rate of porcine embryos; however, the molecular mechanisms underlying this positive effect were unclear. In addition, the activation of the SIRT1 protein via resveratrol increases ATP content in pig and cow oocytes [14, 15]. Furthermore, when oocytes collected from early antral follicles (0.5−0.7 mm in size) of gilts had been cultured in medium containing resveratrol, SIRT1 expression levels and ATP content in oocytes were upregulated. Interestingly, the authors showed that the lipid content in these oocytes was also reduced [16]. Based on these studies, it was hypothesized that SIRT1 activation in embryos induces ATP generation; it may also reduce lipid content and thereby improve cryotolerance of embryos.

In the present study, we conducted experiments where *in vitro* produced bovine embryos were cultured in the presence of resveratrol. We examined the effects of resveratrol treatment on ATP production, mitochondrial number, cytoplasmic lipid content, and cryotolerance of the embryos.

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Materials and Methods

Chemicals, medium and culture conditions

All chemicals were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. Medium 199 supplemented with 10% FCS (5703H; ICN Pharmaceuticals, Costa Mesa, CA, USA) and 5 mM taurine was used for *in vitro* maturation (IVM). *In vitro* fertilization (IVF) medium and *in vitro* culture medium were based on synthetic oviduct fluid (SOF) [17], with minor modifications, as reported previously [18]. The IVF medium consisted of SOF containing 4 mg/ml fatty acid-free bovine serum albumin (BSA) and 10 IU/ml heparin (Sigma-Aldrich, St. Louis, MO, USA). The *in vitro* culture medium used during the first 48 h after fertilization consisted of SOF containing amino acids (Sigma-Aldrich), 1.5 mM glucose, and 1% FCS (referred as IVC medium-1). Maturation, fertilization, and *in vitro* culture until 2 days post-insemination were performed at 38.5°C in an atmosphere of 5% CO_2 in air with saturated humidity. From 48 h to 7 days after fertilization, cleaved embryos (8–12 cell stage embryos with uniform blastomere size) were selected and cultured using *in vitro* culture medium in which the FCS concentration was increased 5% (referred to henceforth as "IVC medium-2"). Culturing was performed at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Resveratrol (Wako, Osaka, Japan) and 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX527; Sigma-Aldrich), an inhibitor of SIRT1, were each separately diluted in ethanol (20 mM), at 40,000 × and 20,000 × of final concentration, respectively. Etomoxir, a potent inhibitor of beta-oxidation, was diluted in dimethyl sulfoxide (100 mM) at 10,000 × of final concentration.

Oocyte collection and IVM

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at approximately 25°C. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (3−6 mm in diameter) using a 21-gauge needle connected to a 5-ml syringe. Only COCs surrounded by thick and compact cumulus cells were selected for subsequent culture. COCs were cultured in 100 µl of IVM medium (10 oocytes / drop) under paraffin oil (tissue culture grade; Nacalai Tesque) for 21 h.

In vitro fertilization, in vitro culture of embryos, and counts of blastocyst-stage embryos

Frozen-thawed semen from a Japanese black bull was washed with a 30−60% discontinuous Percoll gradient solution (Amersham, Uppsala, Sweden) by centrifugation for 10 min (800 × *g*). After centrifugation, sperm (2×10^6 cells/ml) were coincubated with COCs for 5 h in 100 µl of IVF medium (10−15 oocytes / drop). After insemination, these COCs were cultured for 48 h in 100 µl of IVC medium-1 (10−15 oocytes / drop). Forty-eight hours after insemination, surrounding cumulus cells were denuded to obtain cleaved embryos. Only cleaved embryos (8–12 cell stage embryos with uniform blastomere size) were selected; they were then randomly allocated to the experimental groups and cultured in 10 µl of IVC medium-2 (5 embryos / drop) for 5 days. At day 7 after insemination, development to the blastocyst stage and the total cell numbers per blastocyst were determined. For cell counting, blastocysts were fixed in 4% paraformaldehyde and mounted with antifade solution containing DAPI (4',6-diamidino-2-phenylindole) (Pro-long Gold Antifade Reagent with DAPI; Invitrogen, Carlsbad, CA, USA) on glass slides. They were then observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Immunostaining

Immunostaining of embryos was conducted as previously described [19]. In brief, embryos were fixed with 4% paraformaldehyde overnight and were permeabilized with 0.2% polyvinyl alcohol (PVA)-PBS containing 0.25% TritonX-100 for 1 h at room temperature. This was followed by incubation in blocking solution (PBS containing 5% BSA, 1% Tween 20, and 5% goat serum; Funakoshi, Tokyo, Japan) for 1 h at room temperature. Embryos were treated with primary rabbit polyclonal antibodies against either SIRT1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or phospho-AMP-activated protein kinase (pAMPK, 1:200; Cell Signaling Technology, Beverly, MA, USA), overnight, at 4°C. The embryos were then incubated with a secondary antibody (fluorescein-conjugated goat anti-rabbit IgG, 1:1000; Cell Signaling Technology Inc.) for 1 h at room temperature. The embryos were subsequently mounted with antifade solution containing DAPI on glass slides and were observed under a fluorescence digital microscope (BZ-8000; Keyence, Tokyo, Japan: for green fluorescence, sensitivity ISO 200, exposure 1/6 sec; for blue fluorescence, sensitivity ISO 200, exposure 1/2 sec), and fluorescence images of the embryos were captured. A negative control was generated using the above-mentioned procedure but omitting the primary antibody. Fluorescence intensities of whole embryos were measured using ImageJ (NIH, Bethesda, MD, USA).

ATP assay

The ATP content of embryos was measured based on luminescence generated in an ATP-dependent luciferin-luciferase reaction using an ATP Assay Kit (TOKYO B-Net; Tokyo Japan) as described previously [20]. Each embryo was transferred to water (50 µl) and stored at –20°C until needed. The ATP content for each individual embryo was then determined using a luminometer (Gene Light 55; Microtech, Chiba, Japan).

Reactive oxygen species detection

Reactive oxygen species (ROS) levels within embryos were measured using ROS Detection Reagents (Invitrogen), according to the manufacturer's instructions. In this procedure, dihydrocalcein-acetoxymethylester freely permeated cell membranes and was oxidized by ROS to emit green fluorescence. Embryo fluorescence was observed under a fluorescent digital microscope, and intensities of pixel fluorescence were quantified using ImageJ, as described above.

Mitochondrial DNA copy number

DNA in each embryo was extracted in 6 µl of lysis buffer (20 mM Tris, 0.4 mg/ml proteinase K, 0.9% Nonidet P-40, and 0.9% Tween 20) and incubated at 55°C for 30 min, followed by incubation at 95°C for 5 min. The mt-DNA copy number was then determined via real-time PCR using a Corbett Rotor Gene 6000 Real-Time Rotary Analyzer (Corbett Research, Sydney, Australia), as described previously [21]. Primers (5'-ATTTACAGCAATATGCGCCC-3' and 5'-AAAAGGCGTGGGTACAGATG-3') were designed us-

ing Primer-BLAST and a bovine mitochondrial ND5 sequence (LOCUS NC006853; 1.82-kb region extending over basepair positions 12,109–13,929). PCR was performed with initial denaturation at 95°C for 3 min followed by 40 cycles of 98°C for 5 sec and 59°C for 11 sec. SYBR green fluorescence was measured at the end of each extension step. A standard curve was generated for each run using 10-fold serial dilutions representing copies of the external standard. The external standard was the PCR product of the corresponding gene cloned into a vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen); the product was sequenced for confirmation prior to use. For data from two trials, all amplification efficiencies were greater than 1.95.

Cryopreservation protocol and evaluation of cryotolerance of embryos

Blastocyst-stage embryos at 7 days after insemination were used for cryopreservation experiment. The cryopreservation medium was Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 20% FCS, 1.8 M ethylene glycol (EG; Wako), and 0.2 M sucrose (freezing medium). Embryos were incubated in cryopreservation medium for 10 min at room temperature and then loaded into a 0.25-ml straw (Fujihira, Tokyo, Japan). The straws were then plunged into a –7.0°C ethanol bath stored in a programmable freezer (Fujihira), followed by ice-seeding. Straws were maintained at –7.0°C for 10 min and were then cooled to –30°C, at a rate of –0.3°C/min. After reaching –30°C, the straws were kept in liquid nitrogen (LN) vapor (2 cm above the surface of LN) for 3 min and were then plunged into LN. After being stored in LN for more than 1 day, the straws were thawed by keeping in air at room temperature for 7 sec, followed by immersion in water at 37°C. The embryos were then washed several times by IVC medium-2, and were cultured in 50 µl of IVC medium-2 (10 embryos/drop) under paraffin oil for 24 h, at 38.5°C, in an atmosphere of 5% CO_2 in air, with maximum humidity. After culture for 24 h, embryo survival was judged by their re-expansion or development beyond the hatching stage.

Cytoplasmic lipid content in embryos

Cytoplasmic lipid content in embryos was determined by staining with Nile red (Wako). Briefly, embryos were incubated in PBS containing Nile red (10 µg/ml) for 10 min. The embryos were then mounted with antifade solution containing DAPI on glass slides, and cross section of the equatorial region of each embryos was observed under the fluorescence digital microscope. Fluorescent intensities of whole embryo were measured using ImageJ (NIH).

Experimental design

Experiment 1: Effects of various concentrations of resveratrol on embryo development: Forty-eight hours after insemination, cleaved embryos were randomly selected and allocated to medium containing either 0 µM (vehicle only), 0.5 µM, or 5 µM resveratrol, and were then cultured for 5 days. The number of blastocysts in each group, and cell numbers per blastocyst were examined. Experiments were conducted four times.

Experiment 2: Effects of resveratrol on blastocyst characteristics: Forty-eight hours after insemination, cleaved embryos were randomly allocated to medium containing either 0 µM (vehicle only) or 0.5 µM resveratrol for 5 days, at which time embryos were analyzed for expression levels of SIRT and p-AMPK, ATP content, ROS level, cytoplasmic lipid content, mitochondrial DNA copy number, and cryotolerance.

Experiment 3: Effects of short resveratrol treatment on embryo characteristics: In experiment 2, resveratrol treatment enhanced SIRT1 and p-AMPK expression and affected ATP, lipid, and ROS content in the blastocysts. Here, we examined the effects of resveratrol treatment for shorter period on mitochondrial functions and lipid content of embryos. Forty-eight hours after insemination, cleaved embryos were randomly selected and allocated to medium containing either 0 µM (vehicle only) or 0.5 µM resveratrol for 1 day. The resveratrol-treated embryos were subjected to immunostaining against SIRT and p-AMPK, and their ATP content, ROS content, and lipid content were measured.

Experiment 4: Effects of SIRT1 inhibitor on ATP and lipid content of embryos: This experiment aimed at examining whether resveratrol induced high ATP and low lipid content in embryos was caused by upregulation of SIRT1. Embryos were allocated to the following four groups: 1) medium containing vehicle only (consisting of the same concentration of ethanol as used in the other experimental groups), 2) medium containing 0.5 µM resveratrol, 3) medium containing 0.5 µM resveratrol plus 1 µM EX527 (a potent inhibitor of SIRT1), and 4) medium containing 1 µM EX527 alone. Resveratrol treatments were performed as described for experiment 3.

Experiment 5: Effects of beta-oxidation inhibitor on lipid content of embryos: This experiment was carried out to the hypothesis that resveratrol treatment enhances lipid utilization via beta-oxidation. This hypothesis is supported by previous reports showing that the lipid content in embryos is closely linked to fatty acid oxidation [22]. Embryos were allocated to the following four groups: 1) medium containing vehicle only (consisting of the same concentrations of ethanol and DMSO as used in the other culture conditions), 2) medium containing 0.5 µM resveratrol, 3) medium containing 0.5 µM resveratrol plus 10 µM etomoxir (a potent inhibitor of beta-oxidation), and 4) medium containing 10 µM etomoxir alone. Resveratrol treatments were performed as described for experiment 3.

Statistical analysis

Comparisons of data among three or more groups were performed using analysis of variance (ANOVA), followed by Tukey's post-hoc test. Comparisons between two groups were conducted using the Student's *t*-test. Percentages were arcsine-transformed prior to analyses, and survival rates were compared using Chi-square tests. Values of $P < 0.05$ were considered significant.

Results

Resveratrol enhanced embryonic development

Medium containing 0.5 µM resveratrol significantly improved the rate of embryonic development to the blastocyst stage, while medium containing 5 µM resveratrol was detrimental to embryonic development (vehicle medium = 55.0 ± 2.9%; 0.5 µM resveratrol = 70.0 ± 4.1%; 5 µM resveratrol = 25.0 ± 2.9%; $P < 0.05$, Table 1). Additionally, total number of cells (TNC) per blastocyst was also decreased in the 5 µM resveratrol treatment group, whereas no

Table 1. Effect of resveratrol treatment on development of bovine IVF embryos

Resveratrol (μM)	No. of trials	No. of embryos	Development (Average ± SE)	
			Blastocysts (%)	Total numbers of cells
0	4	40	55.0 ± 2.9 [a]	65.5 ± 7.1 [a]
0.5	4	40	70.0 ± 4.1 [b]	65.4 ± 6.0 [a]
5	4	40	25.0 ± 2.9 [c]	38.3 ± 3.9 [b]

[a, b, c] Values with different superscripts differ significantly ($P < 0.05$).

differences were observed between the control and 0.5 μM resveratrol groups (vehicle medium: 65.5 ± 7.1, 0.5 μM resveratrol: 65.4 ± 6.0, 5 μM resveratrol: 38.3 ± 3.9, $P < 0.05$; Table 1).

Resveratrol enhanced expression levels of SIRT1 and pAMPK, improved mitochondrial functions, reduced lipid content, and improved cryotolerance of blastocysts

As shown in Figs. 1-A and B, resveratrol increased the expression levels of SIRT1 and p-AMPK in whole embryos (SIRT1: 1.52 (± 0.12)-fold increase per embryo, pAMPK: 1.26 (± 0.05)-fold increase per embryo, $P < 0.05$; representative images are presented in Supplementary Fig. 1: online only). To get better comparisons, expression levels per blastomere were evaluated. We found that resveratrol increased the embryonic expression levels of both SIRT1 and pAMPK compared with those levels in embryos treated with vehicle only (Supplementary Fig. 1).

We then examined the effects of resveratrol on mitochondrial quantity and function, and on lipid content, in blastocysts. Treating blastocysts with resveratrol increased their ATP content (resveratrol: 2.12 ± 0.11 pM, vehicle: 1.77 ± 0.12 pM, $P < 0.05$; Fig. 1-C), but decreased their ROS content (ROS: 0.67 (± 0.09)-fold decrease, $P < 0.05$; Fig. 1-D). However, mitochondrial DNA copy numbers, as determined by real-time PCR, were comparable between blastocysts developed with or without resveratrol (resveratrol: 345,696 ± 18,345.3, vehicle: 358,289 ± 16,250.7, Fig. 1-E). As shown in Fig. 1-F, the lipid content of blastocysts in the resveratrol-treated group was significantly lower than those in the control group (0.68 (± 0.04)-fold difference, $P < 0.05$; representative images are presented in Supplementary Fig. 2: online only). However, the total number of cells (TNC) per blastocyst did not differ between two groups (control: 75.0 ± 10.9 *vs*. resveratrol: 90.6 ± 7.1, $P > 0.05$), and the lipid content per cell was significantly lower in blastocysts developed with resveratrol compared to those developed without resveratrol (Supplementary Fig. 2). At 24 h after thawing, 68.3% (82/120) of the resveratrol treated

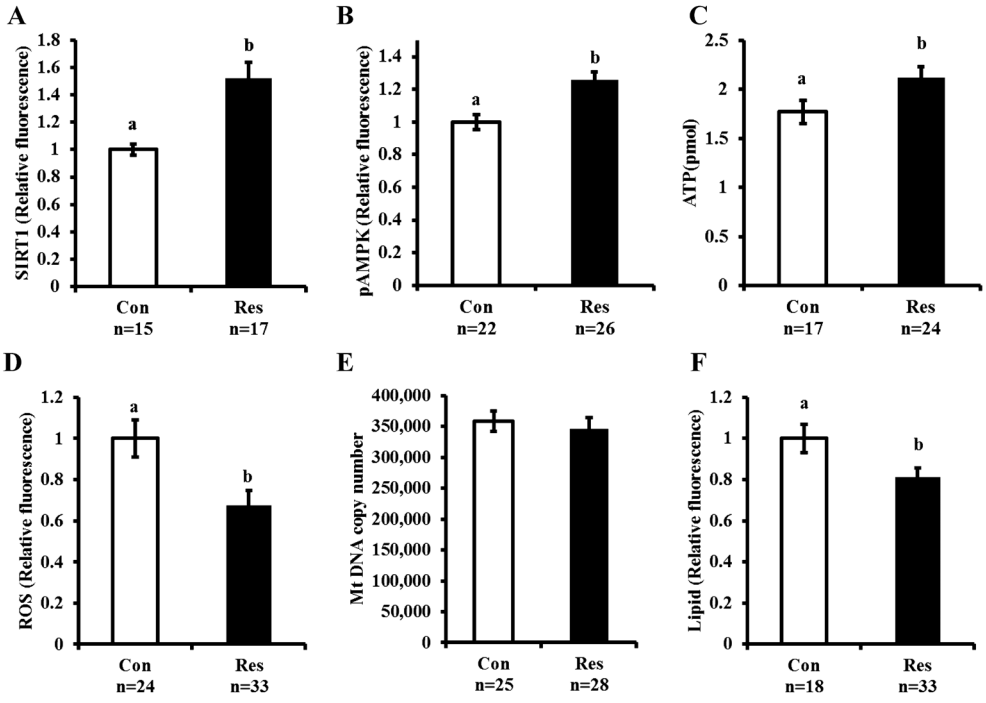

Fig. 1. Effects of resveratrol treatment on blastocyst characteristics. A, B: expression levels of SIRT1 (A) and pAMPK (B) proteins. C: ATP content. D: ROS content. E: mitochondrial DNA copy number. F: lipid content. At least 50 embryos were used for blastocyst productions in each group. In Figs. 1-A, B, D and F, the average fluorescence intensity of control blastocysts (treated with vehicle only) was defined as 1.0. Con.: vehicle group, Res.: resveratrol group.

blastocysts maintained viability (Table 2), which was significantly higher than the survival rate of the control (54.2%, 45/83, $P < 0.05$).

Treatment of embryos with resveratrol for 24h enhanced SIRT1 and p-AMPK expression levels, and affected mitochondrial function

As shown in Figs. 2-A and B, 1-day resveratrol treatment significantly enhanced the expression levels of SIRT1 and p-AMPK in embryos (SIRT1: 1.18 (± 0.02)-fold difference, pAMPK: 1.12 (± 0.03)-fold difference, $P < 0.05$; representative images are presented in Supplementary Fig. 3: online only). Additionally, resveratrol treatment of embryos significantly increased their ATP content (resveratrol: 2.76 ± 0.08 pM, vehicle: 2.47 ± 0.09 pM, $P < 0.05$; Fig. 2-C) and decreased both ROS content and lipid content (ROS: 0.83 (± 0.05)-fold difference, lipid content: 0.88 (± 0.03)-fold difference, $P < 0.05$; Figs. 2-D and E).

Resveratrol increased ATP content and decreased lipid content in embryos via activation of SIRT1

We examined whether SIRT1 upregulation is a potent factor capable of regulating ATP and lipid content in embryos. We found that the ATP content in the embryos was significantly increased by resveratrol treatment, and the increase in ATP content was reversed by the addition of EX527. Additionally, EX527 alone did not affect ATP content in the embryos (Fig. 3-A). Similarly, the effects of resveratrol on lipid content reduction were also reversed by EX527 treatment (Fig. 3-B).

Presence of beta-oxidation in embryos, and 1-day resveratrol treatment decreased lipid content via beta-oxidation

As shown in Fig. 4, 1-day resveratrol treatment significantly decreased the lipid content in embryos, and the addition of etomoxir to the culture medium increased their lipid content. Treatment of the embryos with etomoxir alone also significantly increased their lipid content.

Table 2. Effect of resveratrol on cryotolerance of bovine *in vitro* produced embryos

Resveratrol (μM)	No. of trials	No. of embryos	Embryos developed to blastocysts (%)	No. of blastocysts cryopreserved	No. of viable blastocysts (%)
0	20	200	88 (44.0%) [a]	83	45 (54.2%) [a]
0.5	20	200	122 (61.0%) [b]	120	82 (68.3%) [b]

[a, b] Values with different superscripts differ significantly ($P < 0.05$).

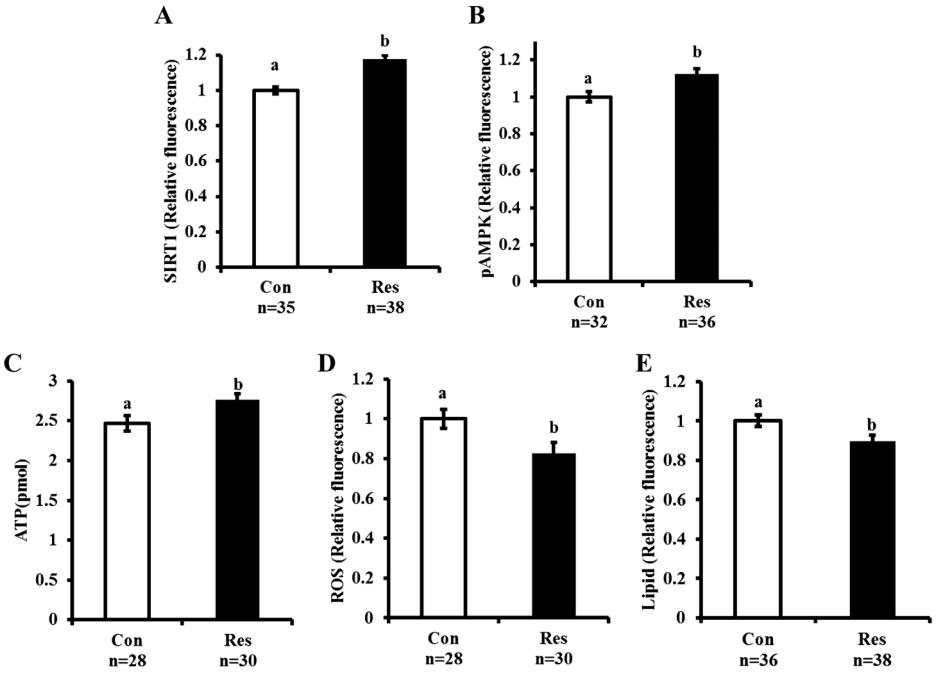

Fig. 2. Effects of resveratrol treatment of short duration (24 h) on expression levels of SIRT1 and pAMPK, and on mitochondrial function. Expression levels of SIRT1 (A) and pAMPK (B), ATP content (C), ROS content (D), and lipid content (E) in embryos cultured with or without resveratrol for 24 h; [ab] $P < 0.05$. In Figs. 2-A, B, D, and E, the average fluorescence intensity of control embryos was defined as 1.0. Experiments were repeated at least three times. Con.: vehicle group, Res.: resveratrol group.

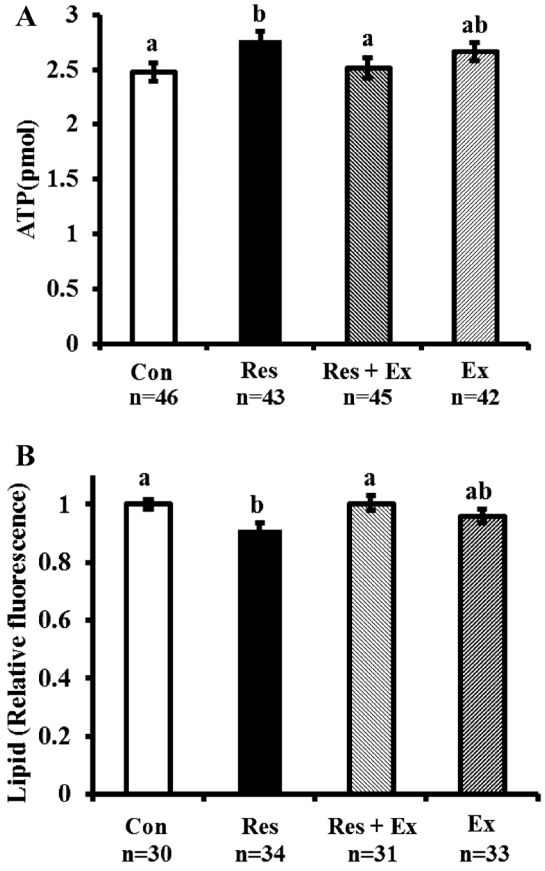

Fig. 3. ATP content (A) and lipid content (B) in embryos cultured under four conditions. Con: ethanol at the same concentration as used with resveratrol and EX527 treatment, Res: 0.5 μM resveratrol, Res + Ex: 0.5 μM resveratrol and 1 μM EX527 (specific inhibitor of SIRT1), Ex: 1 μM EX527 (^{ab} $P < 0.05$). In Fig. 3-B (lipid content), the average fluorescence intensity of control embryos was defined as 1.0. Ten 8-cell stage embryos were cultured for 1 day in each group. Experiments were repeated at least four times.

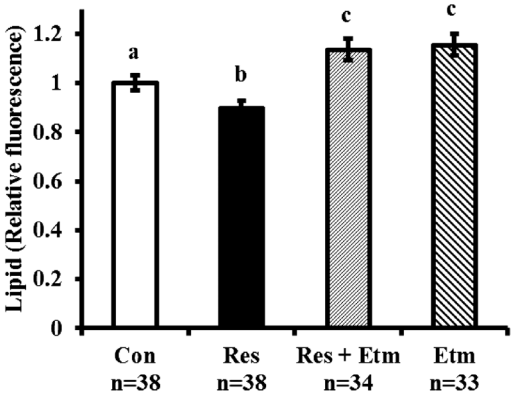

Fig. 4. Lipid content in embryos cultured in four types of media for 1 day. Con: vehicle media only, Res: 0.5 μM resveratrol, Res + Etm: 0.5 μM resveratrol and 10 μM etomoxir, Etm: 10 μM etomoxir (^{ab} $P < 0.05$). The average fluorescence intensity in control embryos was defined as 1.0. Experiments were repeated four times.

Discussion

The results of the present study demonstrated that supplementing culture medium with resveratrol enhances mitochondrial functions via SIRT1 expression, and reduces lipid content via beta-oxidation. Resveratrol treatment also improved the rate of embryonic development to the blastocyst stage, and improved blastocyst cryotolerance.

It has been reported that supplementing culture medium with 0.5 μM resveratrol improves porcine and bovine embryonic development [13, 23].

Resveratrol is a potent activator of SIRT1, and SIRT1 closely interacts with AMPK, cellular energy sensor [24, 25]. Based on immunostaining results, we determined for 5 days enhanced both SIRT1 and phospho-AMPK expression levels. Blastocysts that developed in the presence of resveratrol had high ATP content and low ROS content, though mtDNA copy number was unchanged. These results suggest that resveratrol treatment affects mitochondrial functions, but not mitochondrial quantity, in embryos. Reductions in ROS content due to resveratrol treatment have been reported in other cell types, such as ischemic-reperfused cardiomyocytes [26]. Resveratrol treatment also increases the expression levels of glutathione peroxidase 4 (GPX4), superoxide dismutase (SOD), and catalase in bovine granulosa cells [27]. Furthermore, SIRT1 activates ROS scavenger molecules via a mechanism involving manganese superoxide dismutase (Mn-SOD), a mitochondrial antioxidant [28]. Considering these results, we assert that one of the causal factors for the ROS reductions observed in blastocysts treated with resveratrol is the enhanced activity of antioxidant enzymes, via the activation of SIRT1. Additionally, because the upregulation of SIRT1 improves mitochondrial quality through mitochondrial biogenesis and degeneration in porcine oocytes [14], we speculate that another possible reason for the low ROS levels in resveratrol-treated blastocysts is that the treatment may improve overall mitochondrial quality.

It has been reported that the cytoplasmic lipid content of mammalian embryos relates to their cryosensitivity [8, 29–31]. Our results suggest that the reduction of cytoplasmic lipid content by chemical treatment may be an option to improve the efficiency of embryo cryopreservation.

We found that even 1-day resveratrol treatment enhanced SIRT1 and p-AMPK levels in embryos, and increased their ATP content. AMPK is a sensor of energy homeostasis in cells, and its activation induces ATP generation [25]. In previous reports, culturing bovine or porcine oocytes in medium containing resveratrol increased their ATP content [14, 15]. Furthermore, when bovine oocytes were cultured with a potent activator of AMPK, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), the ATP content increased [32]. In the present study, upregulation of ATP diminished by treatment with EX527, a SIRT1 inhibitor. This suggests that upregulation of ATP in resveratrol treated-embryos is derived from SIRT1 activation. Additionally, resveratrol treatment for 1 day reduced lipid content of embryos to similar extent observed after treatment for 5 days. This reduction was diminished by EX527 treatment, suggesting that the reduction of cytoplasmic lipid content was also derived from SIRT1 activation.

The reduction in embryo lipid content was reversed by treatment with etomoxir, an inhibitor of beta-oxidation. AMPK upregulation increases the usage of fatty acids in mitochondria [33], and AMPK activates beta-oxidation in oocytes [34]. Our results indicate that resveratrol activates SIRT1, which leads to AMPK activation and increases lipid usage in embryos via beta-oxidation, thereby reduces lipid content. Furthermore, treating embryos with etomoxir alone increased their lipid content, indicating that fatty acid oxidation is a major metabolic event in early embryonic development *in vitro*.

In conclusion, we demonstrated that supplementing embryo culture medium with resveratrol enhanced SIRT1 expression levels, increased beta-oxidation levels, and increased ATP generation in the bovine embryos. Additionally, treating embryos with resveratrol decreased their lipid content, improved their development, and improved their survival rates following cryopreservation.

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