Effect of acteoside on the re-localization and abnormal morphology of mitochondria in porcine oocytes during in vitro maturation

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Abstract

Purpose The aim of this study is to investigate the effect of acteoside, an antioxidant, on in vitro maturation (IVM) of oocytes to improve early parthenogenetic embryonic developmental competence.

Methods Porcine immature oocytes (total 770) were cultured in IVM medium with acteoside at various concentrations, 0 (control), 10, 30, and 50 μ M. Each group was assessed for maturation and subsequent development rates, reactive oxygen species (ROS) level (15 oocytes per group and four independent experiments performed), ultrastructure observation (15 oocytes per group), mitochondrial activity (30 oocytes per groups and three independent experiments performed), and expression patterns of apoptosis-related genes (100 expanded parthenogenetic embryos per group and three independent experiment performed). Main outcome measures

were the rates of IVM, blastocyst formation, ROS, mitochondria, and expression of apoptosis-related genes in oocytes treated with acteoside.

Result(s) Addition of acteoside during IVM did not change the maturation efficiency of oocytes but improved the rate of blastocyst formation with significantly decreased ROS level. Moreover, in acteoside-treated oocytes, cytoplasmic maturation was improved with morphologically uniform distribution of mitochondria and lipid droplets in cytoplasm. Acteoside supplementation also increased the mRNA expression levels of antiapoptotic genes and reduced those of pro-apoptotic genes. **Conclusion(s)** Acteoside supplementation in IVM medium improves the oocyte quality and subsequent development of pre-implantation embryos that would eventually contribute to produce embryos with high embryonic development competence.

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Keun Jung Kim and Ju Lan Chun contributed equally to this work.

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Keywords Acteoside · IVM · ROS · Mitochondria · Apoptosis-related genes

Introduction

Assisted reproductive techniques (ART) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been used as important tools for increased productivity in animal agriculture and human infertility treatment. In vitro maturation (IVM) is the initial step of ART that is highly correlated to further successful pre-implantation embryo development in vitro. IVM has also been recognized as an important step for achieving complete meiosis in embryos produced in vitro. In humans, IVM has been proposed as a strategy to optimize or improve the development of healthier oocytes [1–4]. Various methods have been suggested to improve the efficiency of IVM [5–10], which actually contributed to

increasing the rate of IVM. However, the developmental competence of oocytes matured in vitro is still known to be unstable; the cytoplasmic maturation was frequently found to be incomplete even after treatment with various chemical supplementations or substances such as hormones, vitamins, and cytokines [11–13].

Reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl radicals ($OH^\cdot$), and hydrogen peroxide (H_2O_2) are produced during regular metabolism of oxygen, and play important roles in cell signaling [14]. However, excessive ROS can induce lipid peroxidation and enzyme inactivation by hydroxyl radical formation, finally resulting in cell apoptosis [15, 16]. In the case of oocytes and embryos, endogenous ROS are produced by metabolic pathways and enzymes [17]. Overproduction of ROS in oocytes during IVM causes lipid peroxidation [18] and DNA fragmentation, which affect both RNA transcription and protein synthesis [19]. It has been reported that antioxidant supplementation of the IVM medium improves the oocyte quality and embryo developmental efficiency by enhancing nuclear and cytoplasmic maturation through regulation of ROS production and gene expression [20–23].

Acteoside (verbascoside) is a typical phenylethanoid glycoside, which is extracted from various plants including *Syringa vulgaris*. Acteoside has been known to have various biological functions including antioxidative, antiinflammatory, and antihypertensive activities and has been used pharmaceutically [24–27]. It has been well known as an especially powerful antioxidant that acts either by directly scavenging the ROS and nitrogen species or by acting as a chain-breaking peroxy radical scavenger [28]. However, to date, little is known about the effect of acteoside on oocytes during in vitro maturation and pre-implantation embryo development.

Mitochondria generate cellular energy to support metabolism in oocytes [29, 30] via the production of ATP by oxidative phosphorylation [31, 32]. They are involved in apoptosis [33], calcium signaling [34], ROS generation, and production of intermediary metabolites [35]. Mitochondria also play an important role in oocyte maturation and subsequent embryonic development. The intracellular distribution of mitochondria and their metabolic activity undergo substantial changes during oocyte maturation and embryonic development in many species [36–40]. For example, in a previous study, the enhancement of mitochondrial activity and lipid β -oxidation improved the oocyte developmental competence in mouse [41]. However, the relationship between mitochondria and ROS was not fully understood during oocyte maturation.

The present study aimed to investigate the effect of acteoside on oocyte maturation and embryo development, particularly in relation to mitochondrial distribution and

apoptosis. Porcine IVM medium was supplemented with acteoside, and then mitochondrial distribution and expression of apoptosis-related genes were observed.

Materials and methods

Chemicals and reagents

Acteoside was purchased from Chengdu Biopurify Phytochemicals Ltd. (China). Other chemicals and reagents used in this study were purchased from Sigma-Aldrich Co. (USA), unless otherwise stated.

The basic medium used for oocyte IVM was tissue culture medium-199 (TCM-199) supplemented with 2.5 mM fructose, 0.4 mM L-cysteine, 1 mM sodium pyruvate, 0.13 mM kanamycin, 10 % (v/v) porcine follicular fluid, 10.0 ng/mL epidermal growth factor (EGF), and 500 IU/mL gonadotropin hormone.

The medium used for in vitro culture was porcine zygote medium-3 (PZM-3) consisting of 108.0 mM NaCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 10.0 mM KCl, 0.35 mM KH_2PO_4 , 2 % basal medium Eagle essential amino acids, 1 % minimum essential medium non-essential amino acids, 5.0 mM hypotaurine, 25.0 mM $NaHCO_3$, 1.0 mM L-glutamine, 0.2 mM sodium-pyruvate, 2.0 mM $Ca(lactate)_2 \cdot 5H_2O$, 0.3 % bovine serum albumin (BSA), and 0.13 mM kanamycin (pH 7.3 and 285 Osm).

Oocyte collection and in vitro maturation

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in physiological saline containing 1 % penicillin/streptomycin (approximately 25 °C) within 3 h. The ovaries were washed three times with physiological saline. Antral follicles of 3–8 mm diameter were aspirated using an 18-gauge needle attached to a 10 mL disposable syringe. The aspirated follicular fluid was precipitated in a 37 °C water bath. The sediments were washed twice with physiological saline. Compact cumulus-oocyte complexes (COCs) surrounded by at least three layers of cumulus cells were selected. The selected COCs were washed with IVM medium. After washing, approximately 50 COCs per well were cultured in maturation medium supplemented with 0, 10, 30, and 50 μ M of acteoside for 22 h at 39 °C in 5 % CO_2 in air. They were then transferred into the IVM medium without hormones, and cultured for an additional 22 h under the same conditions. After IVM for 44 h, cumulus cells were removed by repeated gentle pipetting in 0.1 % hyaluronidase. For calculating the rate of maturation, oocytes that displayed the first polar body

were considered in the nuclear maturation stage metaphase II (MII).

Measurement of ROS level

After IVM, a total of 60 oocytes were collected to measure ROS level (repeated four times). The ROS level in each oocyte was measured by Image-iT™ LIVE Green ROS detection kit (Invitrogen, USA). Briefly, mature oocytes were washed in Dulbecco's physiological buffered saline (DPBS) supplemented with 0.10 g/L CaCl₂ and 0.10 g/L MgCl₂·6H₂O. After washing, oocytes were transferred into PZM-3 containing 100 μM *tert*-butyl hydroperoxide at 39 °C in 5 % CO₂ in air. After 60–90 min of incubation, oocytes were transferred into DPBS containing 25 μM 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) and were incubated for 30 min at 39 °C in 5 % CO₂ in air, protected from light. Oocytes were then washed in DPBS. The fluorescent emissions from the oocytes were recorded using the camera attached to a fluorescence microscope with filters at 495 nm for excitation and at 529 nm for emission. The recorded fluorescent images were analyzed for intensity using NIS-Elements BR software 3.00 (Nikon, Japan).

Parthenogenetic activation and in vitro culture

The electrical activation of the oocytes was executed according to the general method described in previous reports [7]. Briefly, denuded oocytes were allocated and equilibrated in electrical activation medium containing 0.3 M mannitol, 0.05 % BSA, 0.1 mM MgSO₄, and 0.1 mM CaCl₂. Equilibrated oocytes were placed between two electrodes overlaid with electrical activation medium; a direct-current (DC) pulse of 1.8 kV/cm for 30 μs was applied for single pulse, using a BTX electro-Cell Manipulator 2001. After activation by a single DC pulse, oocytes were washed thrice in PZM-3, and then cultured in PZM-3 containing 1.9 mM N-6-dimethylaminopurine (6-DMAP) for 3 h. The activated oocytes were again washed three times in PZM-3. Fifteen to 20 embryos were cultured in 20 μL of PZM-3 medium droplets overlaid with mineral oil in a 35 × 9 mm Petri dish for 7 days at 39 °C in 5 % CO₂ in air. The cleavage and development status were evaluated at 2 and 7 days after electrical induction of parthenogenesis.

Oocyte ultrastructure observation by transmission electron microscopy

The ultrastructure of oocytes in maturation process was observed by transmission electron microscopy (TEM). Briefly, immature (0 h), maturing (22 h), and matured (44 h) oocytes were collected (15 oocytes per each

group). For ultrastructural observation, oocytes were fixed with 2 % glutaraldehyde in PBS for 2 h at 4 °C. Fixed oocytes were washed five times with 0.1 M cacodylate buffer containing 0.1 % CaCl₂ at 4 °C. Oocytes were then post-fixed with 1 % OsO₄ in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 % CaCl₂ for 1 h at 4 °C. After rinsing with cold distilled water, oocytes were dehydrated slowly with ethanol immersion series and propylene oxide (50, 75, and 100 % for 5 min at 4 °C). The samples were embedded in EMbed-812 (EMS, USA). After polymerization of the resin at 60 °C for 36 h, serial sections (thickness ranging from 80 to 100 nm) were obtained from the mid part of oocytes with a diamond knife on an LEICA EM UC6 ultramicrotome (Leica, Austria) and mounted on formvar-coated slot grids. Sections were stained with 4 % uranyl acetate and lead citrate and examined under a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company, USA) and a JEM ARM 1300S high-voltage electron microscope (JEOL, Japan). The number of total and swollen mitochondria was determined from the TEM images of 5 sections of 15 oocytes from each group. Counting the number of mitochondria was performed at random areas of each section as representative samples.

Evaluation of mitochondrial distribution

For evaluation of mitochondrial distribution, 30 oocytes were stained with MitoTracker Red TM (Invitrogen, USA) according to the manufacturer's instructions. A stock solution of MitoTracker Red was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 1 mM and stored at −20 °C. After IVM, mature oocytes were incubated for 45 min in PZM-3 containing 100 nM of MitoTracker Red TM at 39 °C in 5 % CO₂ in air. Following staining, oocytes were washed twice with PBS for 10 min, and then observed under a laser scanning confocal microscope (Carl Zeiss, Germany). Images were captured using Zeiss LSM5 Live Release ver. 4.2. and analyzed using SP1 Image Browser software (Carl Zeiss, Germany).

RT-PCR

After culturing for 7 days following electrical parthenogenetic activation, expanded blastocysts from each group were collected and stored at −80 °C for further analysis. Total RNA was extracted from 100 blastocysts using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA samples were primed with oligo dT primer to generate complementary DNA (cDNA) using iScript reverse transcriptase (BIO-RAD Laboratories, Hercules, CA, USA).

Specific sequences of anti- and pro-apoptotic genes were prepared for reverse transcription polymerase chain reaction (RT-PCR). Primers are listed in Table 1. The total reaction mixture in 20 μ L volume consisted of 10 μ L premix Taq™ (Takara, Japan), 1 μ L each of forward and reverse primers (10 mol/L), 2 μ L of cDNA, and 6 μ L double-distilled water. RT-PCR consisted of 3 min denaturation at 94 °C, followed by 45 cycles of denaturation at 94 °C, annealing at 58 °C, and elongation at 72 °C for 30 s, respectively, and the final extension at 72 °C for 5 min. The PCR products were separated on 2 % agarose gels and visualized by ethidium bromide staining. The intensity of individual bands was semi-quantitatively analyzed using Image/J® software 1.43u (National Institutes of Health, USA), and the relative expression levels of each gene were obtained by normalization using the expression of GAPDH (Table 2).

Statistical analysis

All data were analyzed using one way-ANOVA software program (SPSS Statistics Inc., ver. 18.0.0). A P value <0.05 was considered statistically significant.

Results

Effect of acteoside on nuclear maturation during IVM

Acteoside is known to act in a concentration-dependent manner and displayed apparent activity at 30 μ M concentration [42]. To investigate the effect of acteoside on nuclear maturation of porcine parthenogenetic oocytes, acteoside was added to the IVM medium at concentrations of 10, 30, and 50 μ M. Supplementation of IVM medium with 10, 30, or 50 μ M acteoside did not improve the rate of recovery of MII stage oocytes in comparison with that of the oocytes without

acteoside treatment (0 μ M). There was also no statistically significant difference in nuclear maturation rate among the groups exposed to the three different concentrations of acteoside (P <0.05).

Acteoside supplementation during IVM affects the pre-implantation embryo development

After establishing that acteoside supplementation of IVM medium did not alter the rate of nuclear maturation of porcine oocytes, developmental ability of acteoside-treated oocytes subjected to electrical parthenogenetic activation was examined (Table 3). The cleavage rate of embryos did not differ significantly between the control group (89.89 %) and the groups supplemented with 10, 30, and 50 μ M acteoside (95.70, 96.04, and 88.38 %). However, acteoside supplementation at 10 μ M concentration significantly increased the rate of blastocyst formation compared to that in the control group (40.03 vs. 22.95 %) (P <0.05).

Effect of acteoside on ROS levels in mature oocytes

The ROS level in treated oocytes was examined to determine whether cytoplasmic competency was improved by acteoside supplementation during IVM. Varying levels of ROS were detected in most oocytes matured in vitro in the absence of acteoside (Fig. 1a). However, ROS was rarely detected in oocytes cultured in IVM medium supplemented with acteoside regardless of the concentration (Fig. 1b–d). The relative rate of ROS generation was also significantly lower in oocytes, which were matured in IVM medium supplemented with acteoside, compared to those matured without acteoside (P <0.05) (Fig. 1e).

Intracellular localization of mitochondria in oocytes after IVM in the presence of acteoside

To estimate cytoplasmic maturation level of mature oocytes after acteoside treatment, the intracellular localization of mitochondria was analyzed by labeling with a mildly thiol-reactive chloromethyl moiety. The total number of active mitochondria and the number of swollen mitochondria were counted in oocytes matured in the presence of 10 μ M acteoside at 0, 22, and 44 h during IVM (Fig. 2a). The total number of mitochondria was not affected by acteoside treatment, but decreased over time both with and without acteoside treatment (Fig. 2a). However, acteoside treatment delayed the decrease in the total number of mitochondria. The number of swollen mitochondria was significantly higher in mature oocytes (22 and 44 h) without acteoside compared to immature oocytes (0 h), but was not different in oocytes with acteoside (P <0.05) (Fig. 2a). In oocytes matured without acteoside treatment, active mitochondria were mainly localized in the

Table 1 Primer sequences used for reverse-transcription polymerase chain reaction

Gene	Primer sequences	Product size (bp)
<i>MCL-1</i>	F TTGCTGGAGTAGGAGCTGGT R TCCTCTTGCCACTTGCTTTT	197
<i>BCL-2</i>	F GCGATGACTTCTCTCGTCGCT R ACAGCCAGGAGAAATCAAATAG	350
<i>BCL-XL</i>	F ACAGCGTATCAGAGCTTTGAGCA R CGTCAGGAACCATCGGTTGAAG	296
<i>BAX</i>	F AAGCGCATTGGAGATGAAC R GGCCTTGAGCACCAGTTTAC	183
<i>BAK</i>	F CTGCCCTAGAACCTAGCAG R TTGATGCCACTCTCGAACAG	185
<i>GAPDH</i>	F TTCCACGGCACAGTCAAGGC R CATGGTCGTGAAGACACCAG	151

Table 2 Effects of acteoside supplementation of IVM medium on porcine oocyte maturation in vitro

Acteoside (μ M)	No. of GV oocytes	No. of MII oocytes	Maturation rate ($\% \pm$ SD) ^{NS}
0 (control)	193	137	71.13 $\pm$ 5.5
10	193	147	75.96 $\pm$ 3.1
30	192	140	72.95 $\pm$ 3.6
50	192	142	73.68 $\pm$ 4.7

Maturation rate: the number of MII/ the number of GV oocytes $\times$ 100. Experiments were repeated five times independently

NS not significant

peripheral regions of oocytes (Supplement Fig 1a). In oocytes matured in the presence of acteoside, mitochondria were distributed evenly within the oocytes (Supplement Fig 1b).

Acteoside supplementation induces rearrangement of organelles in mature oocytes

To check whether the ultrastructure of oocytes was affected by acteoside, transmission electron microscopy (TEM) was performed on immature (0 h of maturation; hm), maturing (22 hm), and mature oocytes (44 hm). Immature oocytes at 0 hm were characterized by peripherally located mitochondrial clusters (MC) along with cortical granules (CG) and conspicuously large cytoplasmic lipid droplets (LD) (Fig. 2b). In control maturing oocytes at 22 hm, MC, CG, and LD heterogeneous in their size were observed in the sub-olemmal cortex (Fig. 2c). However, mitochondria and CG were evenly distributed within the oocyte, and the size of LD was decreased in acteoside-treated oocytes at 22 hm (Fig. 2d). Finally, the organelle rearrangement in fully matured oocytes was observed at 44 hm. In oocytes fully matured for 44 h in the presence of acteoside, mitochondrial distribution in the ooplasm became more even, and a delimited region with well-developed smooth endoplasmic reticulum was observed with lipid droplets that were relatively homogenous and smaller (Fig. 2f) compared to those in the control oocytes (Fig. 2e). CG localized to the marginal region under the oolemma in both the control and acteoside-treated groups (Fig. 2e, f). In addition, hooded swollen mitochondria (SM) were arranged in large

groups and associated with lipid droplets at the periphery in control matured oocytes.

Apoptosis-related gene expression in blastocysts after IVM with acteoside

Blastocysts derived from parthenogenetically activated oocytes matured in the presence of acteoside were examined by RT-PCR, to investigate the expression patterns of apoptosis-related genes (Fig. 3a). The relative mRNA levels of *MCL-1*, *BCL-2*, and *BCL-XL* (antiapoptotic genes) were significantly higher in 10 μ M acteoside-treated blastocysts than non-treated blastocysts ($P < 0.05$). However, *BAX* and *BAK* (pro-apoptotic genes) were expressed at significantly higher levels in non-treated blastocysts than in 10 μ M acteoside-treated blastocysts ($P < 0.05$) (Fig. 3b).

Discussion

Oocytes from animals and humans have been used widely to study embryo development, and to develop methods for producing healthy embryos in vitro [43]. For in vitro embryo production, IVM of oocytes is the first crucial determinant step in the successful sequential embryonic development. In humans, IVM has been especially used for women who need urgent oocyte preservation prior to anticancer treatment or who are in need of the conventional IVF. However, there are many differences between in vivo and in vitro environments that differently affect oocyte maturation. For instance, the

Table 3 In vitro development of parthenogenetic embryos after IVM in the presence of acteoside

Acteoside (μ M)	No. of examined oocytes	No. of embryos ($\% \pm$ SD)	
		2-cell	Blastocyst
0 (control)	118	108 (89.89 $\pm$ 8.0)	24 (22.95 $\pm$ 12.8 ^a)
10	127	121 (95.70 $\pm$ 5.8)	48 (40.03 $\pm$ 11.5 ^b)
30	126	121 (96.04 $\pm$ 4.4)	44 (35.76 $\pm$ 12.5 ^{a, b})
50	129	115 (88.38 $\pm$ 8.4)	33 (24.72 $\pm$ 12.6 ^a)

Experiments were repeated six times independently

^{a, b} Values with different superscripts are significantly different ($P < 0.05$)

Fig. 1 Acteoside decreased the level of ROS in porcine oocytes matured in vitro with acteoside supplementation. The relative ROS production was detected in oocytes treated with **a** 0 μM (control), **b** 10 μM , **c** 30 μM , and **d** 50 μM acteoside by fluorescence microscopy after 44 h IVM (60 oocytes per group). **e** The relative intensity of fluorescence was measured using NIS-Elements BR software 3.00. Different superscripts above each bar represent significant difference ($P < 0.05$). The data shown represent the mean $\pm$ standard error of results from four independent experiments

quality of oocytes matured in vitro is significantly lower than that of oocytes matured in vivo [44]. Despite many efforts to delineate the mechanism of IVM, much of the IVM mechanism and the factors affecting IVM remain unknown.

In this study, acteoside, which has been known to have an effective antioxidative activity, was investigated in the experimental model of porcine oocytes to determine whether supplementation of the IVM medium with acteoside enhances the efficiency of oocyte maturation in vitro. Acteoside supplementation of IVM medium had no significant effect on IVM efficiency. However, the rate of blastocyst formation was increased, and it improved embryonic developmental competence in parthenogenetic embryos. The data indicated that acteoside supplementation of the oocyte maturation medium has a positive effect on in vitro development of parthenogenetic embryos. This finding implies that IVM is a critical initial event for the further successful development of parthenogenetic embryos.

ROS are produced in vivo under physiological conditions and stimulate cell growth and proliferation. However, excessive ROS induce oxidative stress and cellular injury (e.g.,

damage to DNA, lipid membranes, and proteins) [19]. Unlike the situation in vivo, ROS concentration has been reported to be higher in oocytes cultured in vitro compared to the in vivo ROS levels. ROS negatively affect oocyte quality, and limit the in vitro fertilization and embryonic developmental competence [45]. For example, excessive ROS cause peroxidation of the cell membrane lipids during oocyte maturation and embryonic development [18, 46]. Previous studies reported that the supplementation of IVM medium with antioxidants rescued oocytes and embryos from cellular injury by reducing ROS [47, 48]. Parthenogenetic embryos have widely used in scientific researches and medical sciences related to ART as somatic cell nuclear transfer to clone animals and to generate transgenic animals. Moreover, pluripotent stem cells have been tried to generate from parthenogenetic embryos to avoid ethical problem. In the present study, acteoside was used to treat immature porcine oocytes during IVM prior to parthenogenesis. Acteoside enhanced parthenogenetic embryo developmental competence by decreasing intracellular ROS levels. It is inferred that acteoside acted as a powerful antioxidant by

Fig. 2 Acteoside altered the distribution of cellular organelles of porcine oocytes during IVM. Oocytes were cultured for 0 (immature), 22 (maturing), and 44 h (mature) in IVM medium with or without 10 μ M acteoside (15 oocytes from each group were used for TEM). **a** The total number of mitochondria and the percentage of abnormally swollen mitochondria at 0, 22, and 44 h maturation, **b** immature oocyte (at 0 h and no acteoside), **c** maturing oocyte (at 22 h and without acteoside), **d**

maturing oocyte (at 22 h and with 10 μ M acteoside), **e** matured oocyte (at 44 h and without acteoside), and **f** matured oocyte (at 44 h and with 10 μ M acteoside). *CG* cortical granules, *LD* lipid droplets, *M* mitochondria, *MC* mitochondrial clusters, *SM* swollen mitochondria. *Bar* = 5 μ m. Different superscripts above each bar represent significant difference ($P < 0.05$). The data shown represent the mean $\pm$ standard error

scavenging ROS, which in turn improved the maturation of oocytes in vitro.

Mitochondria contain their own genomic DNA (mtDNA), which is maternally inherited [37]. These organelles play

Fig. 3 Expression of anti- and pro-apoptotic mRNAs was affected by acteoside in parthenogenetic blastocysts. **a** The result of RT-PCR was visualized by ethidium bromide staining after agarose gel electrophoresis. **b** The relative expression levels of genes were semi-quantitatively analyzed using Image/J® software 1.43u, and normalized to the expression of *GAPDH*. Control: parthenogenetic blastocysts

produced from oocytes cultured in IVM medium without acteoside supplementation. Acteoside: parthenogenetic blastocysts produced from oocytes cultured in IVM medium with 10 μ M acteoside. Different superscripts above each bar represent significant difference ($P < 0.05$). The data shown represent the mean $\pm$ standard error of three independent experiments

various essential roles in cellular functions by supplying energy, regulating apoptosis, and Ca^{2+} homeostasis [37, 49]. Mitochondria are an important determinant of the developmental competence of oocytes and embryos owing to their central role in cellular metabolism [50]. However, little is known about the mitochondrial morphology and activity in oocytes regarding ROS. During meiotic maturation, re-localization of mitochondria and lipid droplets occurs in oocytes. This redistribution, which is important for oocyte maturation and subsequent embryo development, might be in response to energy demands of oocytes during a given stage of development [49]. In this study, mitochondria in oocytes were observed and counted at random areas as representative samples. Mitochondria were found to be irregularly distributed and accumulated in the peripheral regions in oocytes during maturation. However, acteoside supplementation induced homogenous distribution of mitochondria with reduction of ROS level in oocytes matured in vitro. Moreover, the number of abnormal, swollen mitochondria decreased with the addition of acteoside to IVM medium. This implies that maintaining the number of normal mitochondria and re-localization of mitochondria from the periphery to a more uniform distribution positively affect the cytoplasmic maturation of oocytes and support subsequent embryonic development by more efficiently supplying energy essential for the formation of blastocysts. Similarly, it had been reported that the re-organization of mitochondria is correlated with increased ATP content in bovine oocytes during IVM [51]. In addition, it is possible that

clusters of swollen mitochondria, which were observed in oocytes matured in vitro without acteoside supplementation, are likely to cause cytoplasmic damage by oxidative stress [37, 52, 53].

Apoptosis is the process of programmed cell death triggered by extrinsic and intrinsic stimuli/responses, such as the perforin/granzyme pathway, that leads to cellular changes like cell blebbing, cell shrinking, DNA fragmentation, chromatin condensation, and consequent phagocytosis [54]. Apoptosis is strongly involved in both oocyte maturation and pre-implantation embryo development. The expression of apoptosis-related genes such as *p53*, *BAD*, *BCL-2*, and other *BCL* family genes, is known to regulate apoptosis during pre-implantation embryo development in murine and human systems [55–57]. In the present study, acteoside treatment during IVM decreased the expression of *BAK* and *BAX* in parthenogenetic blastocysts. On the other hand, the expression of antiapoptotic genes *MCL-1*, *BCL-2*, and *BCL-XL* was increased. This result implied that acteoside would be related to apoptosis in oocytes during early embryo development.

In conclusion, the acteoside supplement as an antioxidant in IVM medium supports pre-implantation developmental competence of parthenogenetic embryos by reducing ROS, improving oocyte cytoplasmic conditions, increasing expression of antiapoptotic genes, and decreasing expression of pro-apoptotic genes. Therefore, acteoside, an antioxidant is a supplement that has the potential to improve oocyte quality during IVM and subsequent embryo development competence

in vitro. This finding would contribute to the understanding of the involvement of mitochondria and ROS during the maturation of oocytes, which in turn will help improve the efficacy of IVM to achieve successful IVF.

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