FULL PAPER *Theriogenology*

Effect of Trans-ε-Viniferin on *In Vitro* Porcine Oocyte Maturation and Subsequent Developmental Competence in Preimplantation Embryos

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ABSTRACT. Trans-ε-viniferin is a naturally occurring polyphenol belonging to the stilbenoid family that has been isolated from *Vitis amurensis*, one of the most common wild grapes in Asia. We investigated the effects of trans-ε-viniferin on *in vitro* maturation (IVM) and developmental competence after *in vitro* fertilization (IVF) or parthenogenesis (PA). We observed that trans-ε-viniferin treatment during IVM did not improve nuclear maturation rates of oocytes in any group, but significantly increased (P<0.05) intracellular glutathione (GSH) levels and reduced reactive oxygen species (ROS) levels in the 0.5 μM treatment group. Trans-ε-viniferin treatment during IVM of recipient oocytes promoted higher (P<0.05) expression of DNA methyltransferase-1 (DNMT1) mRNA in the 0.5 μM treatment group as compared with the control group. However, the expression of essential transcriptional and apoptosis-related genes did not significantly differ from that of the control. In cumulus cells, pro-apoptosis gene expressions were changed as apoptosis decreased. Oocytes treated with trans-ε-viniferin during IVM did not have significantly different cleavage rates or blastocyst formation rates after PA, but total cell numbers were significantly higher (P<0.05) in the 0.5 and 5.0 μM treatment groups compared with those in the control group. IVF embryos showed similar results. In conclusion, these results indicate that trans-ε-viniferin treatment during porcine IVM increased the total cell number of blastocysts, possibly by increasing intracellular GSH synthesis, reducing ROS levels, increasing DNMT1 gene expression of oocytes and decreasing pro-apoptosis gene expressions of cumulus cells.

KEY WORDS: embryo, *in vitro* maturation, oocyte, oxidative stress, trans-ε-viniferin.

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In vitro production (IVP) of porcine embryos is a very valuable technology for agricultural and biomedical research. Pigs can be used as research disease models and disease-resistance animals and for creating genetically modified animals as potential donors of tissues and organs for xenotransplantation, because of their physiological similarities to humans. However, a large number of good quality, mature oocytes are required to perform studies in this area of research. Generally, immature porcine oocytes are collected from ovaries of slaughtered pigs, and *in vitro* maturation (IVM) is performed. Porcine IVM systems have improved quite a bit, but are still unsatisfactory due to low developmental rates and the low quality of oocytes as compared with *in vivo* oocytes.

Oocyte maturation includes nuclear as well as cytoplasmic maturation. These two processes must be considered interdependently [42]. However, although nuclear maturation appears to be completely established during IVM, cytoplasm maturation is still incomplete. Incomplete cytoplasmic maturation leads to polyspermy [44] and low developmental rates after *in vitro* fertilization (IVF) or somatic cell nuclear transfer (SCNT) of IVM oocytes. In general, cytoplasmic maturation involves the accumulation of mRNA, proteins, substrates and nutrients that are required to achieve oocyte developmental competence that fosters embryonic developmental competence [47]. Among them, glutathione (GSH) plays a role in sperm function, oocyte maturation, fertilization and embryonic development [6]. In particular, an important event that must occur during porcine oocyte maturation is the synthesis of intracellular GSH, which functions in DNA and protein synthesis and amino acid transport in mammalian cells [34] and has beneficial effects on subsequent embryonic development [1]. Reactive oxygen species (ROS) are also important factors that influence oocyte maturation and subsequent development of oocytes after IVF or SCNT [13, 45]. ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radicals (OH), are produced by aerobic organisms during metabolic processes. ROS are formed as natural by-products of normal oxygen metabolism and play important roles in cell signaling [52]. However, too much ROS can damage oocytes and embryos. Damage caused by increased ROS production includes cell membrane damage, mitochondrial dysfunction, RNA damage and cytoskeletal alterations [10, 48]. Finally, this damage can lead to early embryonic death [13, 14, 46]. GSH protects cells from ROS toxicity and regulates the intracellular redox balance. In pigs and cattle, GSH levels increase during oocyte maturation [8, 49].

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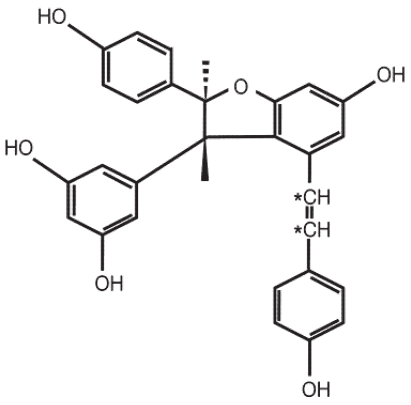

Fig. 1. Chemical structure of trans-ε-viniferin.

Therefore, many researchers have attempted to increase intracellular GSH concentrations or decrease ROS formation. Various antioxidants, such as β-mercaptoethanol, cysteine and cysteamine, have been used during IVM [1, 6, 7, 51, 52]. These antioxidants play an antioxidative role and enhance the viability of *in vitro* embryos. Trans-ε-viniferin (Fig. 1) is a component of *Vitis amurensis*, one of the most common wild grapes in Korea, Japan and China. Its fruits are used to make juice and wine, whereas the root and stem are used as a traditional medicine for treating pain, such as stomachache, neuralgic pain, abdominal pain and cancer [17]. The root and stem of *V. amurensis* have antioxidant and anti-inflammatory activities and neuroprotective effects in pheochromocytoma (PC 12 cells) [20, 21]. Additionally, trans-ε-viniferin extract has antioxidant and anti-inflammatory activities and neuroprotective effects in neuronal cells [25].

Many studies have reported the antioxidant effects of *V. amurensis* and its extract in somatic cells [20, 21, 24, 25]. However, there is limited information regarding the effects of *V. amurensis* and its extract on oocyte maturation and embryonic development. The aim of this study was to investigate the effects of trans-ε-viniferin treatment during IVM on oocyte maturation and subsequent developmental competence in preimplantation embryos.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, U.S.A.) unless stated otherwise.

Plant material, preparation and isolation of trans-ε-viniferin: The leaf and stem of *V. amurensis* were gathered on Keryong Mountain in Daejeon, Korea. Botanical identification and isolation of trans-ε-viniferin were performed by Professor Ki-Hwan Bae at the herbarium of the college of Pharmacy, Chungnam National University, Korea. Dried leaf and stem of *V. amurensis* (4.6 kg) were extracted using methanol (MeOH) (15 *l* × 24 hr × 3 times) at room temperature, filtered and concentrated to yield an MeOH extract (658 g). Trans-ε-viniferin (1,148 mg) was obtained from an MeOH extract after purification by silica gel column chromatography. Trans-ε-viniferin was provided to us for experiments.

Ovary collection, recover and in vitro oocyte maturation: Ovaries of prepubertal gilts were collected from a commercial abattoir and transported to the laboratory within 2 hr in 0.9% (w/v) NaCl solution supplemented with penicillin-G (100 IU/m*l*) and streptomycin sulfate (100 mg/*l*) at 30 to 35°C. The follicular fluid with oocytes was aspirated from 3- to 6-mm antral follicles with a 10-m*l* disposable syringe and 18-gauge needle and collected in a 15-m*l* centrifuge tube. Cumulus-oocyte complexes (COC) were recovered under a stereomicroscope; those with at least three layers of compact cumulus cells and homogenous cytoplasm were selected for IVM. The selected COCs were washed three times in a HEPES-buffered Tyrode's medium containing 0.05% (w/v) polyvinyl alcohol (TLH-PVA) and transferred into 500 μl of tissue culture medium 199 (Life Technologies, Rockville, MD, U.S.A.) supplemented with 26 mM sodium bicarbonate, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 10 *n*g/m*l* epidermal growth factor, 0.5 IU/m*l* porcine luteinizing hormone, 0.5 IU/m*l* porcine follicle stimulating hormone, 10% (v/v) porcine follicular fluid (pFF), 75 μg/m*l* penicillin-G and 50 μg/m*l* streptomycin. The pFF was aspirated from 3–7-mm follicles of prepubertal gilt ovaries. After centrifugation at 1,600 × *g* for 30 min, the supernatants were collected and filtered sequentially through 1.2- and 0.45-μm syringe filters (Gelman Sciences, Ann Arbor, MI, U.S.A.). The prepared pFF was then stored at −20°C until use. For maturation, the selected COCs were washed three times in oocyte maturation medium containing hormone supplements, and approximately 50–60 oocytes were transferred into each well of a 4-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 μl of culture medium and equilibrated at least 2 hr with 5% CO_2 at 39°C in a humidified atmosphere. After 22 hr of maturation with hormones, the oocytes were washed twice in a maturation medium without hormone supplements and then cultured for 22 hr without hormone supplements at 39°C under 5% CO_2 in air.

Assessment of nuclear maturation: After 44 hr of culture, oocytes were stained with 10 μg/m*l* Hoechst 33342 in absolute alcohol, visualized under epifluorescence microscopy (330–385 nm; at a magnification of 400×) and assessed for nuclear progression. Oocyte nuclear maturation status was classified as germinal vesicle (GV), metaphase I, anaphase-telophase I and metaphase II (MII) according to meiotic maturation stage.

Measurement of ROS and intracellular GSH levels: The IVM oocytes were sampled 44 hr after IVM to determine intracellular ROS and GSH levels. ROS and GSH levels were measured by methods previously described [37, 41]. Briefly, H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate; Invitrogen) and CellTracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen) were used to detect intracellular ROS as green fluorescence and GSH level as blue fluorescence, respectively. Ten oocytes from each treatment group were incubated (in the dark) for 30 min in TLH-PVA supplemented with 10 μM H2DCFDA and

10 μM CellTracker. After incubation, oocytes were washed with D-PBS (Invitrogen, Carlsbad, CA, U.S.A.) containing 0.1% (w/v) PVA and placed into 10 μl microdrops, and fluorescence was observed under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan) with UV filters (460 nm for ROS and 370 nm for GSH). Fluorescent images were saved as graphic files in tiff format. The fluorescence intensities of oocytes were analyzed with the Image J software (Version 1.41o; National Institutes of Health, Bethesda, MD, U.S.A.) and normalized to the control. We performed another GSH measurement method for more accurate determination of each oocyte's GSH value. After IVM (42–44 hr), the oocytes were stripped of surrounding cumulus cells by repeated pipetting, and matured oocytes (defined as oocytes in which the first PB was visualized under a stereomicroscope) were selected for GSH measurement. Intracellular GSH was measured as described by Baker *et al.* (1990) [3] with some modification. Briefly, MII oocytes from each group were washed three times in 0.2 M sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4 and 10 mM EDTA-2Na, pH 7.2), and groups of 50–60 oocytes (per sample) in 10 μl sodium phosphate buffer were transferred to 1.7-m*l* microfuge tubes; 10 μl of 1.25 mM phosphoric acid (final concentration of 0.625M H_3PO_4) in distilled water was added to each sample. Tubes containing the samples were frozen at −80°C until analysis. GSH concentrations in the oocytes were determined using a 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB)-GSH reductase (GSSG) recycling assay. Before the assay, the frozen samples were thawed at room temperature, vortexed, centrifuged and microscopically evaluated to ensure complete lysis of the oocytes. The supernatants were transferred to a 96-well microtiter plate, and for each sample, 700 μl of 0.33 mg/m*l* NADPH in 0.2 M assay buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μl of 6 mM DTNB in the stock buffer and 180 μl of distilled water and 1 U per sample of GSSG (Sigma G3664, 441 U/m*l*) were added in a conical tube, mixed and immediately added to the sample. The plate was immediately placed in a microtiter plate reader, and optical density was measured with a 405-nm filter (Emax, Molecular Devices, Sunnyvale, CA, U.S.A.). The formation of 5-thio-2-nitrobenzoic acid was monitored every 30 sec for 3 min. Standard curves were prepared for each assay, and GSH content per sample was determined using the standard curve. The GSH concentrations (pM/oocyte) were calculated by dividing the total concentration per sample by the total number of oocytes present in the sample.

Gene expression analysis by real-time polymerase chain reaction (RT-PCR): RT-PCR was performed with 120 matured COCs at a time. After IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase, and oocytes were washed three times in TLH-PVA. Isolated cumulus cells and cumulus-free (or denuded) matured oocytes were separately selected under a stereomicroscope for the gene expression study. At least 3 replicates were performed. Total RNA was extracted using the TRIzol Reagent (Invitrogen), according to the manufacturer's protocol, and the total RNA concentration was determined by measuring the absorbance at 260 nm. First-strand complementary DNA (cDNA) was prepared by subjecting 1 μg of total RNA to reverse transcription using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase (Invitrogen). To determine the conditions for logarithmic-phase PCR amplification of target mRNA, 1-μg aliquots were amplified using differing numbers of cycles. The housekeeping gene, cytochrome oxidase subunit 1 (1A), was PCR amplified to rule out the possibility of RNA degradation and to control for the variation in mRNA concentrations in the RT reaction. A linear relationship between the PCR product band visibility and the number of amplification cycles was observed for the target mRNAs. The 1A and target genes were quantified using 32 cycles. The cDNA was amplified in a 20-μl PCR reaction, which contained 1 U Taq polymerase (Intron Bio Technologies, Co., Ltd., Seongnam, Korea), 2 mM dNTP mix and 10 pM of each gene-specific primer. Quantitative real-time PCR was performed with 1 μl cDNA template added to 10 μl 2 X SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan) containing specific primers at a concentration of 10 pM each. The reactions were carried out for 32 cycles, and the cycling parameters were as follows: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. All oligonucleotide primer sequences are presented in Table 1. The fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was deemed the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of target gene was quantified relative to that of the internal control gene. The relative quantification was based on a comparison of Cts at constant fluorescence intensity. The amount of transcript present was inversely related to the observed Ct, and for every two-fold dilution in the amount of transcript, Ct was expected to increase by 1. The relative expression (R) was calculated using the equation R=2−[ΔCt sample−ΔCt control]. To determine a normalized arbitrary value for each gene, every data point was normalized to the control gene as well as to its respective control.

Parthenogenesis: For parthenogenesis (PA), oocytes that reached the MII stage at 44 hr of IVM were activated with two pulses of 120 V/mm DC for 60 μsec in 280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$. Following electrical activation, PA embryos were treated with 0.4 μg/m*l* demecolcine and 5 μg/m*l* cytochalasin B in *in vitro* culture (IVC) medium for 4 hr, respectively. The PA embryos were washed three times with embryo culture medium and cultured in 25 μl microdrops (10 gametes/microdrop) of porcine zygote medium 3 (PZM3) [50]. The embryos with cultured microdrops were covered with prewarmed mineral oil and incubated at 39°C for 168 hr under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. In all experiments, the culture media were renewed at 48 hr (day 2) and 96 hr (day 4) after PA.

In vitro fertilization: The IVF procedure performed was that reported by Kwak *et al.* [27]. For IVF, liquid semen was supplied weekly by the Veterinary Service Laboratory (Department of Livestock Research, Yong-in City, Gyeonggi-do,

Table 1. Sequences of the oligonucleotide primers and probe used in RT-PCR

Gene	Primer sequences	Product size (bp)	Gene Bank accession number
PCNA	F: 5'-CCTGTGCAAAAGATGGAGTG-3' R: 5'-GGAGAGAGTGGAGTGGCTTTT-3'	187	XM_003359883
BAK	F: 5'-GCGGAAAACGCCTATGAGTA-3' R: 5'-GCAGTGATGCAGCATGAAGT-3'	189	XM_001928147
BAX	F: 5'-TGCCTCAGGATGCATCTACC-3' R: 5'-AAGTAGAAAAGCGCGACCAC-3'	199	XM_003127290
DNMT1	F: 5'-CCTCTATGGACGGCTTGAGT-3' R: 5'-GGTGCTTGTCCAGGATGTTG-3'	185	NM_001032355
OCT4	F: 5'-GCGGACAAGTATCGAGAACC-3' R: 5'-CCTCAAAATCCTCTCGTTGC-3'	200	NM_001113060
Bcl2	F: 5'-AGGGCATTCAGTGACCTGAC-3' R: 5'-CGATCCGACTCACCAATACC-3'	193	NM_214285
Caspase-3	F: 5'-CGTGCTTCTAAGCCATGGTG-3' R: 5'-GTCCCACTGTCCGTCTCAAT-3'	186	NM_214131
1-A	F: 5'-CACCGTAGGAGGTCTAACG-3' R: 5'-GTATCGTCGAGGTATTCCG-3'	293	AP_003428

Republic of Korea) and kept at 17°C for 5 days before use. The semen sample was washed twice by centrifugation with Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.1% BSA at 2,000 × *g* for 2 min. After washing, the sperm pellet was resuspended in modified Tris-buffered medium (mTBM) that had been pre-equilibrated for 18 hr at 39°C under 5% CO_2. After 44 hr of IVM, the COCs were denuded by gently pipetting with 0.1% hyaluronidase and washed three times in TLH-PVA. Oocytes with a visible first polar body were used for all experiments. Groups of 15 oocytes were randomly placed into 40 μl microdrops of mTBM in a 35 × 10 mm Petri dish (Falcon; BD Labware, Franklin Lakes, NJ, U.S.A.) covered with pre-warmed mineral oil. After appropriate dilution, 5 μl of the sperm suspension was added to a 40 μl microdrop of fertilization medium (mTBM) to yield a final sperm concentration of 1×10^6 sperm/m*l*. Just before fertilization, sperm motility was assessed, and more than 80% motile sperms were used in every experiment. To use stored liquid semen, a modified two-step culture system was used. The oocytes were co-incubated with sperms for 20 min at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After 20 min co-incubation with sperm, the loosely attached sperms were removed from the zona pellucida (ZP) by gentle pipetting. The oocytes were then washed three times in mTBM and incubated in mTBM without sperm for 5–6 hr at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, gametes were washed three times with embryo culture medium and cultured in 25 μl microdrops (10 gametes/microdrop) of PZM3. The embryos with cultured microdrops were covered with pre-warmed mineral oil and incubated at 39°C for 168 hr under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. In all experiments, the culture media were renewed at 48 hr (day 2) and 96 hr (day 4) after IVF.

Experimental design: In Experiment 1, the effect of trans-ε-viniferin treatment during IVM on oocyte nuclear maturation was examined. Oocytes were randomly allocated and cultured in IVM media supplemented with different concentrations of trans-ε-viniferin (0, 0.1, 0.5, 1.0 and 5.0 μM) for the whole culture period (44 hr). After IVM, nuclear maturation was evaluated by Hoechst 33342 staining. In Experiment 2, the effect of trans-ε-viniferin treatment during IVM on the intracellular levels of GSH and ROS was examined. Oocytes were randomly allocated and cultured in IVM media supplemented with different concentrations of trans-ε-viniferin (0, 0.5 and 5.0 μM) for the whole culture period (44 hr). After IVM, the intracellular levels of GSH and ROS were evaluated. In Experiment 3, the effect of trans-ε-viniferin treatment during IVM on the expression of proliferating cell nuclear antigen (PCNA), octamer-binding transcription factor 4 (OCT4), DNMT1, Caspase-3, Bcl-2 homologous antagonist killer (BAK) and Bcl-2–associated X protein (BAX) mRNA in matured oocytes and BAK, BAX, Caspase-3 and B-cell lymphoma 2 (Bcl2) mRNA in cumulus cells were analyzed. The mRNA expression was compared in the control group and a treated (0.5 μM) group. In Experiment 4, the effect of trans-ε-viniferin treatment during IVM on subsequent developmental competence in PA and IVF embryos was examined. Trans-ε-viniferin was treated as in Experiment 2.

Statistical analysis: The statistical analysis was conducted using software from SPSS Inc. (PASW Statistics 17). A one-way analysis of variance with Duncan's multiple-range test was used to assess nuclear maturation rate, GSH and ROS levels, cleavage rate, developmental rate of blastocysts and total cell numbers. The *t*-test was used to assess mRNA expression. All data are presented as means ± SEM. Differences at $P<0.05$ were considered significant.

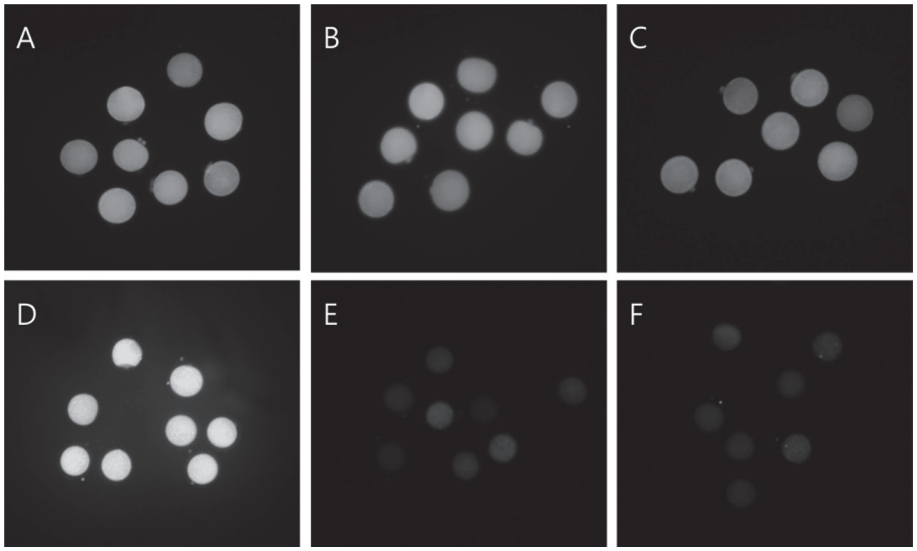

Fig. 2. Epifluorescent photomicrographic images of *in vitro* matured porcine oocytes. Oocytes were stained with CellTracker Blue (A–C) and H2DCFDA (D–F) to detect intracellular levels of glutathione and reactive oxygen species, respectively. Metaphase II (MII) oocytes derived from the maturation medium supplemented with 0.5 μM trans-ε-viniferin (B and E), 5.0 μM trans-ε-viniferin (C and F) or without trans-ε-viniferin (A and D).

Table 2. Effect of trans-ε-viniferin treatment during porcine IVM on nuclear maturation

Trans-ε-viniferin concentration (μM)	No. of oocytes cultured for maturation	% of oocytes at the stage of			
		Germinal vesicle	Metaphase l	Anaphase and Telophase l	Metaphase ll
0 (control)	120	5.0 ± 1.7	8.3 ± 1.0$^{a,b)}$	2.5 ± 1.6	84.2 ± 0.8$^{a,b)}$
0.1	120	4.1 ± 2.1	8.3 ± 1.0$^{a,b)}$	0.8 ± 0.8	86.6 ± 1.4$^{b)}$
0.5	114	5.1 ± 2.1	6.9 ± 1.4$^{a)}$	2.6 ± 0.9	85.5 ± 0.8$^{b)}$
1.0	120	5.8 ± 3.7	8.3 ± 2.2$^{a,b)}$	2.5 ± 1.6	83.3 ± 1.9$^{a,b)}$
5.0	120	4.1 ± 2.5	13.3 ± 2.4$^{b)}$	3.3 ± 1.4	79.2 ± 2.5$^{a)}$

The data are mean ± SEM of four replicates. a,b) Within a column, means without a common superscript differ (P<0.05).

RESULTS

Trans-ε-viniferin treatment during IVM did not improve the nuclear maturation of oocytes in the treated groups compared with the control group (Table 2). The control group and treated groups had similar proportions of matured oocytes (MII, anaphase and telophase I stage rates: 84.2, 86.6, 85.5, 83.3 and 79.2%; 2.5, 0.8, 2.6, 2.5 and 3.3%; and 0, 0.1, 0.5, 1.0 and 5.0 μM, respectively). However, significant differences among treatment groups were observed. There were significantly more immature oocytes in the 5.0 μM treatment group than in the 0.5 μM treatment group. The 5.0 μM treatment group (13.3%) had an increased (P<0.05) number of MI stage oocytes as compared with the 0.5 μM treatment group (6.9%). There were significantly fewer mature oocytes in the 5.0 μM treatment group than 0.1 than in the 0.5 μM treatment groups. The 5.0 μM treatment groups (79.2%) had a decreased (P<0.05) number of MII stage oocytes as compared with the 0.1 and 0.5 μM treatment groups (86.6 and 85.5%). The rates of other maturation stages were similar in all groups.

Trans-ε-viniferin treatment increased (P<0.05) intracellular GSH levels and decreased (P<0.05) ROS generation in MII oocytes after IVM (Figs. 2 and 3). The 0.5 and 5.0 μM trans-ε-viniferin treatment groups showed significantly higher GSH levels as compared with the control group (0.5 and 5.0 μM vs. control: 1.12 and 1.19 vs. 1.0 pixel/oocyte). Additionally, the trans-ε-viniferin treatment groups showed significantly reduced ROS levels as compared with the control group (0.5 and 5.0 μM vs. control: 0.14 and 0.13 vs. 1.0 pixel/oocyte). The GSH reductase recycling assay results revealed that the 5.0 μM treatment group (16.11 pM/oocyte) had similar GSH levels as compared with the control group (14.57 pM/oocyte) (Table 3). Only the 0.5 μM treatment group (16.77 pM/oocyte) showed significantly higher GSH levels as compared with the control group.

The effects of trans-ε-viniferin treatment during IVM on the expression of transcription factors and genes involved in apoptosis and cell proliferation in mature oocytes are shown in Fig. 4. Trans-ε-viniferin treatment during IVM promoted higher (P<0.05) DNMT1 mRNA expression in the 0.5 μM

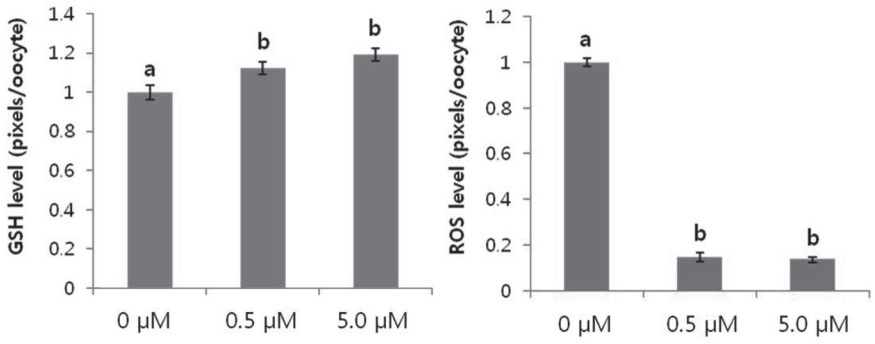

Fig. 3. Effect of trans-ε-viniferin in maturation medium on intracellular glutathione (GSH) and reactive oxygen species (ROS) levels in *in vitro* matured porcine oocytes. The experiment was replicated three times. a, b Values with different superscripts are significantly different (P<0.05).

Table 3. Effect of trans-ε-viniferin in maturation medium on intracellular glutathione (GSH) concentration in *in vitro*-matured porcine oocytes

	Trans-ε-viniferin concentration (μM)		
	0 (control)	0.5	5.0
GSH Concentration (pM/oocyte)	14.57 ± 0.25[a)]	16.77 ± 0.18[b)]	16.11 ± 0.58[a,b)]
(No. of oocytes examined)	(151)	(129)	(133)

The data are mean ± SEM of three replicates. a,b) Values with different superscripts are significantly different (P<0.05).

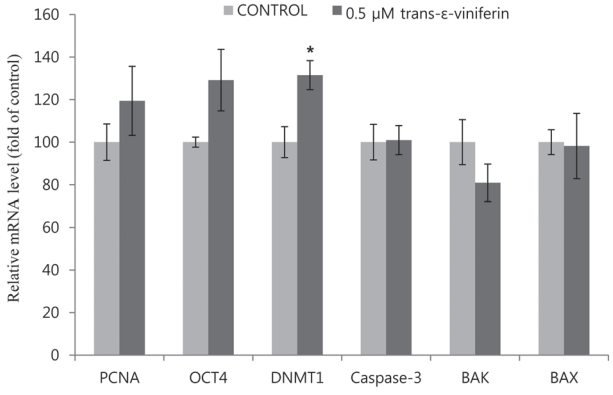

Fig. 4. Mean ± SEM expression of PCNA, OCT4, DNMT1, Caspase-3, BAK and BAX mRNA in matured oocytes treated with trans-ε-viniferin during *in vitro* maturation. The experiment was replicated three times. *P<0.05 vs. control.

treatment group as compared with the control. However, the expression of other genes (PCNA, OCT4, Caspase-3, BAK and BAX) did not significantly differ from the control.

The effects of trans-ε-viniferin treatment during IVM on the expression of apoptosis-related genes in cumulus cells are shown in Fig. 5. Trans-ε-viniferin treatment during IVM significantly reduced (P<0.05) BAX mRNA expression of cumulus cells in the 0.5 μM treatment group as compared with the control. BAK and Caspase-3 mRNA expressions were also reduced (P<0.07). Bcl2 mRNA expression was increased, but was not significant (P=0.67).

No significant differences in cleavage rates among the groups were observed for both PA and IVF embryos on day 2 (Table 4). About 75–80% of PA embryos and 60% of IVF embryos cleaved in all groups (Table 5). No significant differences in blastocyst formation were observed in PA and IVF embryos among the groups at day 7 (Tables 4 and 5). Approximately 50% of PA embryos and 20–25% of IVF embryos developed to the blastocyst stage in all groups. The numbers of cells in the blastocysts of PA and IVF embryos increased (P<0.05) in the trans-ε-viniferin treatment groups as compared with the control. Total cell numbers of PA embryos increased significantly in the 0.5 and 5.0 μM treatment groups (59.6 ± 4.2 and 60.8 ± 4.6) as compared with the control group (43.1 ± 2.1) (Table 4). IVF embryos showed similar results; total cell number increased significantly in the 0.5 and 5.0 μM treatment groups (53.6 ± 4.0 and 47.9 ± 3.1) as compared with the control group (36.4 ± 2.2) (Table 5).

DISCUSSION

In vitro swine embryo production systems are inefficient as compared with *in vivo* systems. In particular, current IVM-IVF systems have many problems. Incomplete cytoplasmic maturation is believed to result in abnormal fertilization, including polyspermy and asynchronous pronuclear formation [33], which are thought to be the major reasons for poor developmental competence of *in vitro* matured/fertilized embryos [18]. The addition of various antioxidants has been examined in an attempt to improve the quality of *in vitro* produced embryos due to their protective effects during culture

Table 4. Effect of trans-ε-viniferin treatment during IVM on embryonic development in porcine PA embryos

Trans-ε-viniferin concentration (μM)	No. of embryos cultured	Embryos developed to (%)		Total cell number in blastocyst
		≥2–cell	Blastocyst	
0 (control)	99	76.7 ± 3.7	50.0 ± 5.8	43.1 ± 2.1[a)]
0.5	106	75.8 ± 1.1	52.3 ± 1.7	59.6 ± 4.2[b)]
5.0	98	81.4 ± 2.9	50.3 ± 1.8	60.8 ± 4.6[b)]

The data are mean ± SEM of three replicates. a,b) Within a column, means without a common superscript differ (P<0.05).

Table 5. Effect of trans-ε-viniferin treatment during IVM on embryonic development in porcine IVF oocytes

Trans-ε-viniferin concentration (μM)	No. of embryos cultured	Embryos developed to (%)		Total cell number in blastocyst
		≥2–cell	Blastocyst	
0 (control)	140	58.1 ± 4.2	20.1 ± 1.6	36.4 ± 2.2[a)]
0.5	137	59.6 ± 3.1	26.3 ± 3.7	53.6 ± 4.0[b)]
5.0	131	57.3 ± 1.1	28.4 ± 6.4	47.9 ± 3.1[b)]

The data are mean ± SEM of three replicates. a,b) Within a column, means without a common superscript differ (P< 0.05).

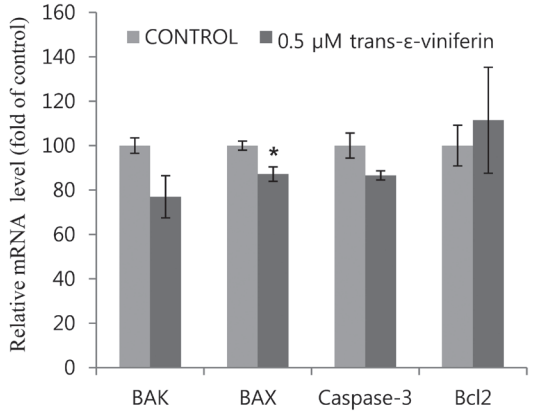

Fig. 5. Mean ± SEM expression of BAK, BAX, Caspase-3 and Bcl2 mRNA in cumulus cell treated with trans-ε-viniferin during *in vitro* maturation. The experiment was replicated four times. *P<0.05 vs. control.

[1, 6, 35, 51]. No previous reports are available regarding the influence of trans-ε-viniferin. We demonstrated that trans-ε-viniferin treatment during IVM had beneficial effects on oocyte maturation, increasing intracellular GSH synthesis, reducing ROS levels and increasing DNMT1 gene expression of oocytes; reduced BAX gene expression of cumulus cells; and increased the total cell number of blastocysts in subsequent embryonic development of PA and IVF embryos. However, nuclear maturation rate did not improve. Some differences were observed among the treatment groups, but there were no significant differences between the treatment groups and control group. The maturation rate did seem to be adversely affected by the concentration.

Trans-ε-viniferin is a resveratrol derivative. Resveratrol derivatives, including trans-resveratrol-4-O-β-D-glucoside, transresveratrol, (+)-ampelopsin A, trans-ε-viniferin, cis-ε-viniferin, γ-2-viniferin, gnetin H and suffruticosol A and B, have been isolated from more than 70 plant species including grapes, plums and peanuts [24, 39]. Among these plants, we extracted trans-ε-viniferin from *V. amurensis*. Trans-ε-viniferin may have antioxidant effects as a hydroxyl radical scavenger *in vivo* [25, 29]. Furthermore, trans-ε-viniferin may inhibit glutamate-induced increases in intracellular calcium ion concentrations, ROS generation, changes in apoptosis-related proteins and hypoxia-induced neuronal cell death in cultured neurons [21]. Jeong *et al.* [21] reported that trans-ε-viniferin protected against glutamate-induced neurotoxicity in cultured cortical neurons; pretreatment of mouse cortical neurons with 5 μM of trans-ε-viniferin reduced the neuronal death induced by 500 μM glutamate, and glutamate-induced neuronal death is usually associated with the elevation of intracellular calcium ion concentrations following NMDA receptor activation [21]. They also demonstrated that trans-ε-viniferin at concentrations of 5.0 μM showed significant inhibition of elevation of glutamate-induced intracellular calcium ion concentrations in cultured cerebral cortical neurons, the involvement of oxidative stress toxicity could be investigated by measurement of ROS accumulation and trans-ε-viniferin (0.5, 1.0 and 5.0 μM) showed concentration-dependent inhibition of the glutamate-induced ROS generation in cultured cerebral cortical neurons [21]. Similar antioxidative effects of trans-ε-viniferin on porcine oocytes were observed in the present study. The 0.5 and 5.0 μM trans-ε-viniferin treatment during IVM effectively reduced ROS levels and increased GSH levels, but did not show a concentration-dependent effect. It is estimated because of the difference between cell types and whether or not artificial induction of ROS.

This finding was most likely due to the antioxidative activity of intracellular GSH, which was increased by trans-

ε-viniferin treatment. We inferred that trans-ε-viniferin was involved in cytoplasmic maturation rather than nuclear maturation and increased intracellular GSH levels in IVM oocytes, which contributed to improve oocyte quality. However, no beneficial effect of trans-ε-viniferin treatment was found during blastocyst formation, even though trans-ε-viniferin effectively reduced ROS levels and increased GSH levels. Many antioxidant compounds have been used to avoid oxidative stress during *in vitro* culture. Transferrin, penicillamine, hypotaurine and taurine are often added to culture media, because positive effects of these compounds on embryo development have been observed [2, 4, 36, 38]. The positive effects of some plant extracts, such as anthocyanin and resveratrol, have also been reported [29, 51]. However, avoiding oxidative stress during oocyte and embryo culture is a complex problem. Simply adding the necessary ROS scavengers is insufficient, as the choice of antioxidant compounds to be used and their concentrations are difficult to ascertain. Some compounds, such as thiols and vitamins, must be used with care, as they too can have a negative impact on the embryo [32, 43]. Further, an excess of antioxidant compounds may have deleterious effects on the embryo. The effects of ROS on embryonic development are paradoxical. Most studies have shown that prolonged experimentally induced ROS production severely inhibits embryonic development [14]. ROS concentrations increase during the two–four-cell transition period in mice [37], indicating that an increase in ROS may be associated with the arrest of development at the two-cell stage. However, excessive reduction of ROS after treatment with a high level of antioxidants has toxic effects on bovine embryonic development and the viability of human cultured cells [12, 41]. Thus, ROS may play a pivotal role in the regulation of cell proliferation and embryonic development. Furthermore, an appropriate level of ROS may be necessary for embryonic development and cell proliferation. In this study, trans-ε-viniferin effectively reduced ROS, but the level of ROS was not appropriate ROS for embryonic development. The blastocyst formation rate did not change, and only total cell number increased, which is a meaningful result. Total cell number is a reflection of embryo quality. Increased total cell number in mouse embryos is associated with improved embryo quality and postimplantation developmental potential [28]. When Koo *et al.* [26] compared the total cell numbers of *in vivo* derived blastocysts to *in vitro* derived blastocysts and blastocysts derived from somatic cell nuclear transfer, total cell numbers varied from a mean of 122.5 for *in vivo* blastocysts (highest developmental potential) to 108.2 cells for *in vitro* derived blastocysts and 98.0 cells for nuclear transfer blastocysts (lowest developmental potential) in cattle [26]. Hence, the increased total cell numbers of blastocysts derived from COCs exposed to trans-ε-viniferin could reflect improved embryo quality.

Gene expression patterns can indicate oocyte status and can be influenced by the culture environment. Therefore, we analyzed developmentally important genes and gene expression related to antioxidant effects. PCNA is an essential component of the DNA replication and repair machinery [23], OCT4 is essential for early development of mouse and human embryos [5, 22], DNMT1 is involved in DNA methylation and cell proliferation [40] and Caspase-3, BAK, BAX and Bcl2 are associated with apoptosis [15, 21]. Cytochrome c released into cytosol forms the apoptosome to activate caspase-9 and caspase-3. Activated caspase-3 cleaves numerous proteins, triggering biochemical cascades that lead to cell death. PCNA and OCT4 mRNA expression did not differ significantly from the control. Trans-ε-viniferin (5.0 μM) significantly blocked the glutamate-induced decrease in Bcl-2 and increase in BAX expression in cultured cerebral cortical neurons [24]. In the current study, apoptosis-related gene (Caspase-3, BAK and BAX) expression in oocytes was not altered. In cumulus cells, BAX gene expression was significantly decreased in the treatment groups ($P<0.05$). During oocyte maturation, cumulus cells support oocytes via small molecule transport. Reducing apoptosis of cumulus cells could increase the subsequent capacity of the oocytes for development. DNMT1 expression was increased significantly in the trans-ε-viniferin-treated groups. DNMT1 accumulates in oocytes during the growth phase and is in the cytoplasm of mature MII oocytes, where it maintains DNA methylation and might also be involved in maintaining imprints. DNA methylation gradually increases during germ cell development before fertilization [9, 53]. Epigenetic markers, such as genomic methylation, regulate gene expression and development [19, 30]. Interfering with the proper establishment of methylation can result in tumorigenesis and death [11, 16, 31]. Therefore, maintaining appropriate methylation levels are important for embryonic development. Increased DNMT1 expression in MII oocytes maintains high DNA methylation before fertilization. In other words, trans-ε-viniferin treatment improved cytoplasm quality by increasing DNMT1 expression.

Supplementation with trans-ε-viniferin during IVM of porcine oocytes could help cytoplasmic maturation by increasing intracellular GSH concentrations and decreasing ROS levels, leading to appropriate gene expression. But, trans-ε-viniferin did not affect nuclear maturation, and it even showed adverse effects at a high concentration. Improvement of oocyte cytoplasmic quality might cause an increase of total cell number of blastocysts during subsequent *in vitro* development. However, trans-ε-viniferin treatment during IVM did not improve the subsequent blastocyst formation rate, and it was unclear how trans-ε-viniferin affected the oocyte cytoplasm. The mechanism underlying the effects of trans-ε-viniferin during IVM should be determined to identify ways to improve developmental competence during IVP.

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