The Journal of Veterinary Medical Science

FULL PAPER

Theriogenology

Effect of ferulic acid supplementation on the developmental competence of porcine embryos during *in vitro* maturation

Fuminori TANIHARA[1)], Maki HIRATA[1)]*, Nguyen Thi NHIEN[1)], Takayuki HIRANO[1)], Toshiki KUNIHARA[1)] and Takeshige OTOI[1)]

[1)]Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima, Tokushima 770-8513, Japan

ABSTRACT. The value of laboratory and genetically-modified pigs is becoming increasingly clear; however, their *in vitro* development remains inefficient. Trans-ferulic acid (trans-FA) is an aromatic compound that is abundant in plant cell walls, and which exhibits antioxidant effects *in vitro*. Trans-FA is known to improve sperm viability and motility; however, its effects on porcine oocytes are unknown. Our aim was to investigate the effects of trans-FA supplementation during *in vitro* maturation on the meiotic and developmental competence of porcine oocytes. Oocytes were matured either without (control) or with trans-FA (10, 100 and 1,000 μM), fertilized, and cultured *in vitro* for 7 days. The maturation rate of oocytes cultured with 10 μM trans-FA (81.6%) was significantly higher than that of controls (65.0%; $P<0.05$). The fertilization rate of oocytes matured with 10 μM trans-FA (57.4%) was also significantly higher than that of controls (32.7%) and oocytes cultured with other concentrations (33.1% and 22.7% for 100 and 1,000 μM, respectively; $P<0.05$). Moreover, the blastocyst formation rate of oocytes matured with 10 μM trans-FA (6.9%) was significantly higher than that of controls (2.3%; $P<0.05$). Our results suggest that *in vitro* maturation with 10 μM trans-FA is beneficial for the *in vitro* production of porcine embryos and has the potential to improve production system.

KEY WORDS: antioxidant, developmental competence, embryo, oxidative stress, trans-ferulic acid

J. Vet. Med. Sci.
80(6): 1007–1011, 2018
doi: 10.1292/jvms.18-0131

Received: 10 March 2018
Accepted: 5 April 2018
Published online in J-STAGE: 16 May 2018

Recently, the value of pigs as laboratory animals has become widely recognized. Anatomically, and physiologically, pigs have close similarity to humans. Moreover, the genetic modification of these animals is expected to facilitate the generation of excellent human disease models, the production of suitable organs for xenotransplantation, and research on human regenerative medicine. Genetically modified pigs are generated through the molecular manipulation of embryos with subsequent implantation into surrogate pigs. In these animals, gene modification is normally performed using *in vitro* matured oocytes or subsequently fertilized zygotes. Therefore, *in vitro* matured oocytes are a critical resource for the generation of genetically modified pigs. To date, several researchers have tried to improve this *in vitro* production (IVP) system as well as the quality of *in vitro* matured oocytes, including their developmental competence after *in vitro* fertilization (IVF) and culture (IVC) [11, 17, 18]. However, the percentage of development to the blastocyst stage of *in vitro* produced embryos is still lower compared to that of *in vivo* produced ones [13, 26].

The high oxygen concentration and lack of antioxidant protection associated with *in vitro* conditions result in the increased generation of reactive oxygen species (ROS), and in turn, increased oxidative stress for the oocytes [2, 3]. Antioxidant supplementation during oocyte/embryo culture has been reported to reduce the effects of ROS, resulting in the protection of oocytes/embryos against damage caused by oxidative stress and improved IVP systems to generate high-quality porcine embryos [29]. In previous studies, the use of antioxidant such as flavonoids (quercetin and taxifolin) [16], melatonin [8, 10], selenium [30], vitamin E [30], resveratrol [21], chlorogenic acid (CGA) and caffeic acid [24] during *in vitro* maturation (IVM) has improved the production system for porcine embryos. Trans-ferulic acid (trans-FA) is an aromatic compound that is abundant in plant cell walls [7, 28]. Trans-FA removes ROS and exhibits an antioxidant effect *in vitro* [25, 28]. In humans, it was previously shown that supplementing the culture medium with trans-FA improves sperm viability and motility [35]. However, the effects of this compound on IVM, IVF, and IVC for porcine oocytes remains unknown. Therefore, in the present study, we investigated the effects of trans-FA supplementation during IVM on the meiotic and developmental competence of porcine oocytes.

*Correspondence to: Hirata, M.: mhirata@tokushima-u.ac.jp

©2018 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/)

MATERIALS AND METHODS

In vitro maturation and assessment of meiotic competence

Porcine ovaries were obtained from approximately 6-month-old gilts at a local slaughterhouse and were transported within 1 hr to the laboratory in physiological saline at 30°C. Ovaries were washed three times with modified phosphate-buffered saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU/m*l* penicillin G potassium (Meiji, Tokyo, Japan) and 0.1 mg/m*l* streptomycin sulfate (Meiji). The follicles of the ovarian surface were sliced using a surgical blade on a sterilized dish, and the cumulus-oocyte complexes (COCs) were collected under a stereomicroscope. Only COCs with a uniformly dark-pigmented ooplasm and intact cumulus cell masses were collected. Approximately 50 COCs were then cultured in 500 μl of maturation medium, consisting of tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, U.S.A.), supplemented with 10% (v/v) porcine follicular fluid, 50 μM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, U.S.A.), 2 mg/m*l* D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 10 IU/m*l* equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan), 10 IU/m*l* human chorionic gonadotropin (Kyoritu Seiyaku), and 50 μg/m*l* gentamicin (Sigma-Aldrich), covered with mineral oil (Sigma-Aldrich) for 22 hr in 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs were transferred into maturation medium without hormone supplementation and cultured for an additional 22 hr. The incubation of COCs was conducted at 39°C in a humidified incubator containing 5% CO_2 in air.

To assess the meiotic status of oocytes following IVM, some oocytes were denuded, fixed, and permeabilized in Dulbecco's PBS (DPBS; Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) triton X-100 (Sigma-Aldrich) at 25°C for 15 min. Permeabilized oocytes were then placed on glass slides and stained with 1.9 mM bisbenzimide (Hoechst 33342; Sigma-Aldrich), before being mounted with coverslips. After overnight incubation at 4°C, the oocytes were examined by fluorescence microscopy. Based on their chromatin configuration, they were classified as 'germinal vesicle,' 'condensed chromatin,' 'metaphase I,' or 'metaphase II'. Oocytes with the diffusely stained cytoplasmic characteristics of nonviable cells and those in which chromatin was unidentifiable or not visible were classified as 'degenerated'.

IVF and assessment of fertilization

IVF was performed according to methods described previously [24] with minor modifications. Frozen-thawed ejaculated spermatozoa were transferred into 5 m*l* of fertilization medium (PFM; Research Institute for the Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × *g* for 5 min. The pelleted spermatozoa were resuspended in fertilization medium and adjusted to 1×10^6 cells/m*l*. Next, approximately 50 COCs were transferred to 500 μl of sperm-containing fertilization medium covered with mineral oil in 4-well dishes and co-incubated for 5 hr at 39°C with 5% CO_2 and 5% O_2. After the co-incubation, the inseminated zygotes were denuded from the cumulus cells and the attached spermatozoa by mechanical pipetting.

To assess fertilization of the oocytes, some denuded zygotes were mounted on glass slides and fixed with acetic acid:ethanol (1:3 v/v) for 72 hr. The fixed zygotes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined by phase contrast microscopy. Oocytes containing both female and male pronuclei were considered fertilized and were categorized as monospermic or polyspermic according to the number of swollen sperm heads and pronuclei in the cytoplasm.

IVC and assessment of blastocyst quality

The remaining denuded zygotes were subsequently transferred to PZM-5 (Research Institute for the Functional Peptides Co.). Approximately 50 zygotes were cultured continuously in 500 μl of PZM-5 covered with mineral oil in 4-well dishes. After culturing the embryos for 3 days, they were subsequently incubated in 500 μl of porcine blastocyst medium (PBM; Research Institute for the Functional Peptides Co.) covered with mineral oil for 4 days in 4-well dishes. The incubation of zygotes and embryos was conducted at 39°C in a humidified incubator containing 5% CO_2, 5% O_2 and 90% N_2. To evaluate the development stage of fertilized oocytes, all embryos were fixed on day 7 (day 0=insemination) and were stained with Hoechst 33342 to assess the quality of embryos through cell counting.

Experimental design

To evaluate the effects of trans-FA supplementation during IVM culture on the *in vitro* maturation, fertilization, and development of porcine oocytes, COCs were cultured in maturation medium supplemented with 10, 100 and 1,000 μM trans-FA (Sigma-Aldrich). As a control, COCs were cultured in maturation medium without trans-FA. After maturation culture for 44 hr, COCs were fertilized *in vitro* and cultured continuously as described.

Statistical analysis

Experiments were repeated five times for oocytes matured with trans-FA. All percentage data were subjected to arcsine transformation before performing an analysis of variance (ANOVA). The transformed data were tested by ANOVA, followed by a Fisher's protected least significant difference (LSD) test, using StatView software (Abacus Concepts, Berkeley, CA, U.S.A.). Differences with a probability value (P) of 0.05 or less were considered statistically significant.

RESULTS

As shown in Table 1, the maturation rate of oocytes cultured with 10 μM trans-FA (81.6%) was significantly higher (P<0.05)

Table 1. Effects of trans-ferulic acid (trans-FA) supplementation during *in vitro* maturation culture on the maturation, fertilization and development of porcine oocytes*

Concentration of trans-FA (μM)	No. of examined oocytes	No. (%)** of matured oocytes	No. of examined oocytes	No. of oocytes		No. of examined embryos	No. of embryos		No. of cells in blastocyst
				Fertilized (%)	Monospermy (%)***		Cleaved (%)	Developed to blastocysts (%)	
0	72	49 (65.0 ± 3.6)[a)]	64	22 (32.7 ± 4.1)[a)]	18 (82.7 ± 8.4)	181	151 (83.1 ± 3.3)[a,b)]	4 (2.3 ± 0.6)[a)]	45.9 ± 10.6
10	72	60 (81.6 ± 5.6)[b)]	63	35 (57.4 ± 11.5)[b)]	26 (80.5 ± 10.7)	189	164 (86.3 ± 3.3)[a)]	14 (6.9 ± 2.1)[b)]	36.3 ± 4.1
100	70	46 (66.2 ± 8.1)[a,b)]	66	19 (33.1 ± 7.9)[a)]	13 (66.3 ± 11.6)	193	141 (72.4 ± 5.2)[b)]	9 (4.4 ± 1.5)[a,b)]	36.9 ± 4.1
1,000	69	48 (67.3 ± 5.9)[a,b)]	65	15 (22.7 ± 10.5)[a)]	13 (91.4 ± 5.1)	158	119 (75.2 ± 3.3)[b)]	5 (3.1 ± 0.9)[a,b)]	35.1 ± 3.3

*Five replicated trials were carried out. **Percentages are expressed as mean ± SEM. ***The monospermic fertilization rate was defined as a ratio of the number of monospermic oocytes and the total number of fertilized oocytes. a, b) Values with different superscripts in the same column differ significantly (P<0.05).

than that of controls (65.0%). Moreover, the fertilization rate of oocytes cultured with 10 μM trans-FA (57.4%) during IVM was significantly higher (P<0.05) than that of controls (32.7%) and oocytes cultured with 100 μM (33.1%) and 1,000 μM (22.7%) trans-FA. However, trans-FA treatment had no effect on monospermy rates among the groups. The cleavage rate of oocytes matured with 10 μM trans-FA (86.3%) was significantly increased compared to that with other concentrations of trans-FA (72.4 and 75.2% for 100 and 1,000 μM, respectively; P<0.05). Moreover, the blastocyst formation rate of oocytes matured with 10 μM trans-FA (6.9%) was significantly higher (P<0.05) than that of controls (2.3%).

DISCUSSION

In the present study, we confirmed the potential of trans-FA as an additive agent to maturation medium. We found that supplementation with 10 μM trans-FA significantly improved the rates of maturation, fertilization, and blastocyst formation in oocytes.

To date, many improvements have been made to *in vitro* culture systems to produce high-quality IVF embryos; however, the developmental competence of *in vitro* embryos remains insufficient compared to that with *in vivo* embryos [9, 18, 19]. One factor for the success of IVP has been suggested to be the oxidative conditions of the environment surrounding the oocytes and embryos [20]. The oxygen concentration in atmospheric air is approximately three-fold greater than that found under the lumen of the female reproductive tract [5, 23, 27]. Oxidative stress via ROS is a threat to oocytes and embryos *in vitro*, which have been removed from their natural habitat and place into a harsh environment that lacks maternal antioxidant factors. Oxidative stress during oocyte maturation, which gives rise to mitochondrial damage [31], DNA damage and apoptosis [29], can affect subsequent fertilization [4] and impair embryo development [20]. One study using human embryos demonstrated that the culture media itself can be a source of ROS [22].

Oocytes are protected from oxidative stress *in vivo* by external and internal protection systems [15]. External protection is mainly composed of non-enzymatic antioxidants such as hypotaurine, taurine, and ascorbic acid, which are contained in follicular and tubal fluids. Conversely, internal protection is mainly composed of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and gamma-glutamylcysteine synthetase. *In vivo*, an efficient reduction-oxidation reaction (redox) system can ensure an ideal environment for embryonic development by maintaining a balance between the production of ROS and antioxidant enzymes [1, 15]. However, *in vitro* conditions for oocyte culture lack external non-enzymatic antioxidants protection, which leads to increase ROS [15]. Therefore, antioxidant supplementation, as external protection against oxidative stress, could be an effective strategy for *in vitro* oocyte culture.

In our previous study, we suggested that CGA or caffeic acid supplementation during IVM are beneficial for oocyte maturation, fertilization, and the development to blastocysts [24]. CGA is a quinic acid conjugate of caffeic acid [14] and caffeic acid is the precursor to ferulic acid [6]. Trans-FA has a similar structure to CGA and caffeic acid, and therefore it is possible that trans-FA is also beneficial for the IVP of porcine embryos. In the present study, trans-FA supplementation significantly enhanced the nuclear maturation of oocytes and accelerated fertilization. Furthermore, embryonic development following IVF was significantly increased, compared to that in the control group. During porcine IVP, polyspermic penetration is a major obstacle for the production of normal and high-quality embryos [11, 12]. In this study, supplementation with 10 μM trans-FA significantly increased the fertilization rate compared to that in the control group; however, the monospermy rate was comparable to that in the control group, which indicated that trans-FA supplementation during IVM increased the number of normal fertilized embryos. These results suggested that this treatment enhances the nuclear maturation and normal fertilization of porcine oocytes, resulting in a higher blastocyst formation. However, the blastocyst formation rate was lower in all groups including control group compared to that of our previous study [14]. In this study, we did not add cysteine, well known as one of the effective additives to improve developmental competence of oocytes during IVM [32–34], to IVM medium to evaluate the effects of trans-FA supplementation clearly. We guess that the lack of supplementation of IVM medium with cysteine reduced embryonic development.

In conclusion, trans-FA is an effective additive that improves the maturation, fertilization, and developmental competence of porcine oocytes. It appears that trans-FA supplementation in maturation medium could be used in porcine IVP systems, and thus might facilitate further developments in biotechnology.

ACKNOWLEDGMENTS. The authors thank the Nippon Food Packer, K. K. Shikoku (Tokushima, Japan) for supplying the pig ovaries. This work was supported in part by Japan Science and Technology Agency/Japan International Cooperation Agency, Science and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS).

REFERENCES

1. Agarwal, A. and Majzoub, A. 2017. Role of Antioxidants in Assisted Reproductive Techniques. *World J. Mens Health* **35**: 77–93. [Medline] [CrossRef]
2. Agarwal, A., Saleh, R. A. and Bedaiwy, M. A. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil. Steril.* **79**: 829–843. [Medline] [CrossRef]
3. Agarwal, A., Gupta, S. and Sikka, S. 2006. The role of free radicals and antioxidants in reproduction. *Curr. Opin. Obstet. Gynecol.* **18**: 325–332. [Medline] [CrossRef]
4. Alvarez, G. M., Morado, S. A., Soto, M. P., Dalvit, G. C. and Cetica, P. D. 2015. The control of reactive oxygen species influences porcine oocyte in vitro maturation. *Reprod. Domest. Anim.* **50**: 200–205. [Medline] [CrossRef]
5. Amin, A., Gad, A., Salilew-Wondim, D., Prastowo, S., Held, E., Hoelker, M., Rings, F., Tholen, E., Neuhoff, C., Looft, C., Schellander, K. and Tesfaye, D. 2014. Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. *Mol. Reprod. Dev.* **81**: 497–513. [Medline] [CrossRef]
6. Boerjan, W., Ralph, J. and Baucher, M. 2003. Lignin biosynthesis. *Annu. Rev. Plant Biol.* **54**: 519–546. [Medline] [CrossRef]
7. Buanafina, M. M., Langdon, T., Hauck, B., Dalton, S., Timms-Taravella, E. and Morris, P. 2010. Targeting expression of a fungal ferulic acid esterase to the apoplast, endoplasmic reticulum or golgi can disrupt feruloylation of the growing cell wall and increase the biodegradability of tall fescue (Festuca arundinacea). *Plant Biotechnol. J.* **8**: 316–331. [Medline] [CrossRef]
8. Chen, Z., Zuo, X., Li, H., Hong, R., Ding, B., Liu, C., Gao, D., Shang, H., Cao, Z., Huang, W., Zhang, X. and Zhang, Y. 2017. Effects of melatonin on maturation, histone acetylation, autophagy of porcine oocytes and subsequent embryonic development. *Anim. Sci. J.* **88**: 1298–1310. [Medline] [CrossRef]
9. Dang-Nguyen, T. Q., Somfai, T., Haraguchi, S., Kikuchi, K., Tajima, A., Kanai, Y. and Nagai, T. 2011. In vitro production of porcine embryos: current status, future perspectives and alternative applications. *Anim. Sci. J.* **82**: 374–382. [Medline] [CrossRef]
10. Do, L. T., Shibata, Y., Taniguchi, M., Nii, M., Nguyen, T. V., Tanihara, F., Takagi, M. and Otoi, T. 2015. Melatonin Supplementation During In Vitro Maturation and Development Supports the Development of Porcine Embryos. *Reprod. Domest. Anim.* **50**: 1054–1058. [Medline] [CrossRef]
11. Funahashi, H. 2003. Polyspermic penetration in porcine IVM-IVF systems. *Reprod. Fertil. Dev.* **15**: 167–177. [Medline] [CrossRef]
12. Funahashi, H. and Day, B. N. 1997. Advances in in vitro production of pig embryos. *J. Reprod. Fertil. Suppl.* **52**: 271–283. [Medline]
13. Gajda, B. 2009. Factors and methods of pig oocyte and embryo quality improvement and their application in reproductive biotechnology. *Reprod. Biol.* **9**: 97–112. [Medline] [CrossRef]
14. Gonthier, M. P., Remesy, C., Scalbert, A., Cheynier, V., Souquet, J. M., Poutanen, K. and Aura, A. M. 2006. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed. Pharmacother.* **60**: 536–540. [Medline] [CrossRef]
15. Guérin, P., El Mouatassim, S. and Ménézo, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**: 175–189. [Medline] [CrossRef]
16. Kang, J. T., Moon, J. H., Choi, J. Y., Park, S. J., Kim, S. J., Saadeldin, I. M. and Lee, B. C. 2016. Effect of Antioxidant Flavonoids (Quercetin and Taxifolin) on In vitro Maturation of Porcine Oocytes. *Asian-Australas. J. Anim. Sci.* **29**: 352–358. [Medline] [CrossRef]
17. Kikuchi, K. 2004. Developmental competence of porcine blastocysts produced in vitro. *J. Reprod. Dev.* **50**: 21–28. [Medline] [CrossRef]
18. Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T. and Nagai, T. 2002. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.* **66**: 1033–1041. [Medline] [CrossRef]
19. Kikuchi, K., Kashiwazaki, N., Noguchi, J., Shimada, A., Takahashi, R., Hirabayashi, M., Shino, M., Ueda, M. and Kaneko, H. 1999. Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured in vitro. *Biol. Reprod.* **60**: 336–340. [Medline] [CrossRef]
20. Kitagawa, Y., Suzuki, K., Yoneda, A. and Watanabe, T. 2004. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* **62**: 1186–1197. [Medline] [CrossRef]
21. Kwak, S. S., Cheong, S. A., Jeon, Y., Lee, E., Choi, K. C., Jeung, E. B. and Hyun, S. H. 2012. The effects of resveratrol on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and in vitro fertilization. *Theriogenology* **78**: 86–101. [Medline] [CrossRef]
22. Martín-Romero, F. J., Miguel-Lasobras, E. M., Domínguez-Arroyo, J. A., González-Carrera, E. and Alvarez, I. S. 2008. Contribution of culture media to oxidative stress and its effect on human oocytes. *Reprod. Biomed. Online* **17**: 652–661. [Medline] [CrossRef]
23. Mastroianni, L. Jr. and Jones, R. 1965. Oxygen Tension within the Rabbit Fallopian Tube. *J. Reprod. Fertil.* **9**: 99–102. [Medline] [CrossRef]
24. Nguyen, T. V., Tanihara, F., Do, L., Sato, Y., Taniguchi, M., Takagi, M., Van Nguyen, T. and Otoi, T. 2017. Chlorogenic acid supplementation during in vitro maturation improves maturation, fertilization and developmental competence of porcine oocytes. *Reprod. Domest. Anim.* **52**: 969–975. [Medline] [CrossRef]
25. Pan, G. X., Spencer, L. and Leary, G. J. 1999. Reactivity of ferulic acid and its derivatives toward hydrogen peroxide and peracetic acid. *J. Agric. Food Chem.* **47**: 3325–3331. [Medline] [CrossRef]
26. Pomar, F. J., Teerds, K. J., Kidson, A., Colenbrander, B., Tharasanit, T., Aguilar, B. and Roelen, B. A. 2005. Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. *Theriogenology* **63**: 2254–2268. [Medline] [CrossRef]
27. Simon, M. C. and Keith, B. 2008. The role of oxygen availability in embryonic development and stem cell function. *Nat. Rev. Mol. Cell Biol.* **9**: 285–296. [Medline] [CrossRef]
28. Srinivasan, M., Sudheer, A. R. and Menon, V. P. 2007. Ferulic Acid: therapeutic potential through its antioxidant property. *J. Clin. Biochem. Nutr.* **40**: 92–100. [Medline] [CrossRef]
29. Takahashi, M. 2012. Oxidative stress and redox regulation on in vitro development of mammalian embryos. *J. Reprod. Dev.* **58**: 1–9. [Medline] [CrossRef]
30. Tareq, K. M., Akter, Q. S., Khandoker, M. A. and Tsujii, H. 2012. Selenium and vitamin E improve the in vitro maturation, fertilization and culture to blastocyst of porcine oocytes. *J. Reprod. Dev.* **58**: 621–628. [Medline] [CrossRef]

31. Velez-Pardo, C., Morales, A. T., Del Rio, M. J. and Olivera-Angel, M. 2007. Endogenously generated hydrogen peroxide induces apoptosis via mitochondrial damage independent of NF-kappaB and p53 activation in bovine embryos. *Theriogenology* **67**: 1285–1296. [Medline] [CrossRef]
32. Yoshida, M., Ishigaki, K. and Pursel, V. G. 1992. Effect of maturation media on male pronucleus formation in pig oocytes matured in vitro. *Mol. Reprod. Dev.* **31**: 68–71. [Medline] [CrossRef]
33. Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M. and Pursel, V. G. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. *Biol. Reprod.* **49**: 89–94. [Medline] [CrossRef]
34. Yoshida, M., Mizoguchi, Y., Ishigaki, K., Kojima, T. and Nagai, T. 1993. Birth of piglets derived from in vitro fertilization of pig oocytes matured in vitro. *Theriogenology* **39**: 1303–1311. [CrossRef]
35. Zheng, R. L. and Zhang, H. 1997. Effects of ferulic acid on fertile and asthenozoospermic infertile human sperm motility, viability, lipid peroxidation, and cyclic nucleotides. *Free Radic. Biol. Med.* **22**: 581–586. [Medline] [CrossRef]