Effects of epigallocatechin-3-gallate on the developmental competence of parthenogenetic embryos in the pig

Morteza Yavari,1,2 Hideaki Naot,1 Yukine Kaedei,1 Fuminori Tanihara,1 Zhao Namula,1 Vien Luu Viet,1 Takeshi Otoi1
1The United Graduate School of Veterinary Science, Yamaguchi University, Japan
2School of Veterinary Medicine, Bu-Ali Sina University, Iran

Abstract

This study was conducted to examine the effects of (-)-epigallocatechin gallate (EGCG) supplementation on the developmental competence and quality of parthenogenetic porcine embryos during culture. Parthenogenetic embryos derived from in vitro matured oocytes were cultured for eight days in a modified North Carolina State University (NCSU)-37 solution supplemented with EGCG at different concentrations (0, 1, 5, 10 and 50 µM). Supplementation of 1 and 5 µM EGCG during in vitro culture of embryos showed no significant influence on the rate of cleavage or that of blastocyst formation or on the total cell number and DNA fragmentation indices of blastocysts when compared to those of a control group. However, when 10 and 50 µM EGCG were supplemented into the culture medium, the cleavage and DNA fragmentation indices of blastocysts were significantly lower than those of a control group. No embryo developed to the blastocyst stage. Results suggest that treatment with low EGCG during in vitro culture has no influence on the developmental competence of porcine embryos but the presence of high concentrations of EGCG is apparently harmful for in vitro development of porcine parthenotes.

Introduction

The developmental competence of in vitro produced porcine embryos is much lower than that of in vivo-produced embryos (Beckmann and Day, 1993; Peters and Wells, 1993; Rath et al., 1995; Nagashima et al., 1996). Because in vitro culture conditions deviate from in vivo situations in many respects, many obstacles must be overcome to obtain a high frequency of in vitro development in porcine embryos following in vitro maturation (IVM) and in vitro fertilization (IVF) (Yoneda et al., 2004). High oxygen concentration during in vitro embryo culture is likely to be a problem that influences embryonic development. Endogenous antioxidants (or scavengers of free radicals) usually prevent or limit damage of oxygen radicals to living organisms. These antioxidants include enzymes such as superoxide dismutase, catalase and selenium-dependent glutathione peroxidase, as well as lipid-soluble and water-soluble antioxidants such as vitamins C and E, and uric acid (Knapen et al., 1999). However, the level of antioxidants during in vitro culture of oocytes and embryos is lower than that in vivo because the oocytes or embryos are divorced from the donor body and do not benefit from maternal antioxidant protection (Wang et al., 2007a). Consequently, the addition of an antioxidant might be an important means to prevent oxidative damage to the development of oocytes and embryos during in vitro culture.

Green tea, a popular beverage consumed worldwide, has attracted much attention because of its many scientifically proven health benefits, with functions against cancer and inflammation, and for improvement of cardiovascular function (Zaveri, 2006). The beneficial effects attributed to green tea consumption are believed to be related to its bioactive components, polyphenols (Sutherland et al., 2006). Green tea polyphenols (GTPs) are water-soluble components such as (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC) and (-)-epigallocatechin (EGC). These catechins have strong antioxidant activity (Rice-Evans, 1999; Dufresne and Farnworth, 2001; Higdon and Frei, 2003); they are potent scavengers of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radicals and nitric oxide produced by various chemicals (Guo et al., 1996; Vinson et al., 2002; Schroeder et al., 2003). It has been demonstrated that EGCG prevents spontaneous mutations (Mure and Rossman, 2001), LDL oxidation (Yamanaka et al., 1997) and chromosomal damage induced by ROS in somatic cells (Sugisawa and Umegaki, 2002; Roy et al., 2003). Sugisawa and Umegaki (2002) reported that a large amount of EGCG was present in cells after they were incubated with 0.3 µM EGCG. Wang et al. (2007a) demonstrated that supplementation with green tea polyphenols during in vitro maturation and in vitro culture improved the developmental competence of bovine oocytes. In contrast, the addition of EGCG to the maturation medium has been reported to have no beneficial effect on the ability of maturation and fertilisation of porcine oocytes (Spinaci et al., 2008). Moreover, it remains unclear whether EGCG supplementation during in vitro culture (IVC) affects the in vitro development of porcine embryos. Therefore, this study was undertaken to examine the possible effects of EGCG supplementation on the developmental competence and quality of parthenogenetic embryos during IVC.

Materials and methods

Recovery and in vitro maturation of oocytes

Ovaries from prepubertal-crossbred gilts, approximately six months old, were collected at an abattoir and transported to the laboratory in physiological saline (0.85% [w/v] NaCl) at 35°C. Cumulus-oocyte complexes (COCs) from follicles (3-6 mm diameter) were aspirated using an 18-gauge needle attached to a 5 mL syringe. After being washed twice with modified phosphate-buffered saline (mPBS) (Embryotec; Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan), only COCs with uniform ooplasm and compact cumulus cells were used. The selected COCs were transferred into...
a maturation medium, a modified North Carolina State University (NCSU)-37 solution (Peters and Wells, 1993) supplemented with 0.6 mM cysteine (Sigma, St. Louis, MO, USA), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan), 1 mM dibutyryl cyclic AMP (dbcAMP, Sigma), 10 U/mL equine chorionic gonadotropin (eCG; Teikoku-Zoki Co., Tokyo, Japan), 10 U/mL human chorionic gonadotropin (hCG; Teikoku-Zoki Co.), 50 μg/mL gentamicin (Sigma) and 10% (v/v) porcine follicular fluid (pFF). About 50 oocytes were cultured in each 500 μL of the maturation medium under a mineral oil for 22 h. Then they were transferred to the maturation medium without hormones and dbcAMP and cultured for an additional 22 h under the conditions described above. All cultures were performed in a 38.5°C humidified incubator containing 5% CO_{2} in air.

**Parthenogenetic activation of oocytes**

Electrical stimulation to induce oocyte activation was delivered (BTX 2001; BTX, San Diego, CA, USA); it was monitored using a graphic pulse analyser (Optimizor 500; BTX) in a chamber with two parallel platinum wire electrodes, spaced 1 mm apart, covered with Zimmerman medium (Wolfe and Kraemer, 1992). At the end of IVM culture, oocytes were denuded mechanically from cumulus cells in Dulbecco’s PBS (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 1 mg/mL hyaluronidase (Sigma). Only oocytes with the first polar body were used for the parthenogenetic activation. Then the denuded oocytes were transferred to Zimmerman medium and placed between the parallel electrodes of the chamber. A single DC pulse of 2 kV/cm for 50 μs was used for electrical stimulation. The oocytes were activated in NCSU-37 solution supplemented with 4 mg/mL bovine serum albumin (BSA; Sigma) and 10 μg/mL cycloheximide (Sigma) and incubated for 5 h.

**In vitro culture of activated oocytes**

Following cycloheximide treatment, the activated oocytes were cultured in NCSU-37 solution supplemented with 4 mg/mL BSA, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 50 μM β-mercaptoethanol and 50 μg/mL gentamicin. At 72 h after activation, all cleaved embryos were transferred into fresh culture medium; NCSU-37 supplemented with 4 mg/mL BSA, 50 μM β-mercaptoethanol, 5.55 mM D-glucose and 50 μg/mL gentamicin. The cleaved embryos were cultured for an additional 5 days to evaluate their ability to develop to the blastocyst stage. During IVC, (-)-epigallocatechin gallate (EGCG) (Sigma) was added at different concentrations (0, 1, 5, 10, and 50 μM) to each culture medium. Embryos were examined under an inverted microscope (TE2000-U; Nikon Instruments Co., Tokyo, Japan) every 48 h following activation. The number of embryos developed to the blastocyst stage was recorded, and the percentage of blastocyst formation was calculated by dividing the number of blastocysts by the total number of activated oocytes.

**Analysis of DNA fragmentation and cell number of blastocysts**

The status of fragmented chromatin in embryos was examined using a combined technique for simultaneous nuclear staining and the terminal deoxynucleotidyl transferase (TdT) nick-end labelling (TUNEL) and a modification of the procedures described previously by Otoi et al. (1999). Briefly, only blastocysts were fixed overnight at 4°C in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the blastocysts were permeabilised in PBS containing 0.1% (v/v) Triton-X100 for 40 min. They were incubated subsequently in PBS containing 10 mg/mL BSA (blocking solution) overnight at 4°C. Then they were incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics Corp., Tokyo, Japan) for 1 h at 38.5°C. The embryos were counterstained with 50 μg/mL propidium iodide after RNase treatment (50 μg/mL RNase for 60 min at room temperature) to label all nuclei. They were treated with an anti-bleaching solution (Slow-Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on a glass slide and sealed with clear nail polish. Labelled blastocysts were examined using a microscope (Diaphot; Nikon Instruments Co.) fitted with epifluorescent illumination. All cells and those cells with a DNA-fragmented nucleus were counted, and the DNA-fragmented nucleus index was calculated by dividing the number of cells with a DNA-fragmented nucleus by the total number of cells, which included DNA-fragmented nuclei.

**Statistical analysis**

Data are expressed as means±SEMs. The percentages of embryos cleaved and developed to the blastocyst stage from activated oocytes, the total cell numbers of blastocysts and the proportions of cells with a DNA-fragmented nucleus out of the total number of cells (DNA-fragmented nucleus index) were subjected to arcsine transformation before analysis of variance (ANOVA). Transformed data were tested by ANOVA followed by a post hoc Fisher’s protected least significant difference (PLSD) test using the Statview program (Abacus Concepts, Inc, Berkeley, CA, USA). Differences at a probability value (P) of 0.05 or less were considered to be significant.

**Results and discussion**

As Table 1 shows, supplementation of 1 and 5 μM EGCG during in vitro culture of embryos showed no significant influence on the rates of cleavage and blastocyst formation, or on the total cell numbers and DNA-fragmentation indices of blastocysts, when compared to those of the control group. However, when 10 μM

| Concentration of EGCG, μM | No. of oocytes examined | No. of embryos, % | Cleavage | Developed to blastocyst | Total cell n. | DNA fragmentation° |
|-------------------------|------------------------|------------------|---------|------------------------|-------------|-------------------|
| 0                       | 150                    | 130 (86.3±3.1)a  | 27 (19.1±2.9)b | 36.7±3.8              | 8.7±1.6     |
| 1                       | 150                    | 133 (88.4±4.3)a  | 23 (15.4±2.5)a | 35.5±5.3              | 7.0±1.1     |
| 5                       | 150                    | 124 (82.3±5.3)a  | 22 (13.9±4.9)b | 32.1±3.2              | 8.5±1.1     |
| 10                      | 150                    | 17 (13.8±4.8)b   | 0 (0.0±0.0)b   | NA                    | NA          |
| 50                      | 150                    | 0 (0.0±0.0)b     | 0 (0.0±0.0)b   | NA                    | NA          |

Table 1. Developmental competence of porcine embryos cultured in vitro with different concentrations of (-)-epigallocatechin gallate.*

EGCG, (-)-epigallocatechin gallate.* Data are expressed as the mean±SEM. Seven replicated trials were conducted; **data were calculated by dividing the number of cells with a DNA-fragmented nucleus by the total number of cells, including DNA-fragmented nuclei; °values with different superscript letters are significantly different (P<0.05).
and 50 µM EGCG were supplemented into the culture medium, the cleavage rates were significantly lower than those of the other groups. No embryo was able to develop to the blastocyst stage.

During IVM, the supplementation of antioxidant compounds such as β-mercaptoethanol, retinol and green tea polyphenols enhances the developmental competence of IVF embryos (Abeydeera et al., 1998; Livingston et al., 2004; Wang et al., 2007b). During in vitro culture, oocytes and embryos are invariably exposed to light and high oxygen concentration, which increase production of ROS (superoxide anion, hydrogen peroxide [H₂O₂] and highly reactive hydroxyl radicals); ROS damages proteins, lipids and nucleic acid components, causing mitochondrial alterations, embryo cell block and apoptosis (Guerin et al., 2001). The addition of antioxidants to IVM/IVF medium reportedly improves the developmental competence of embryos (Abeydeera et al., 1998; Livingston et al., 2004; Wang et al., 2007b).

EGCG is a potent natural antioxidant. It is the major and most abundant polyphenol component of green tea, with apparent low toxicity (Fiorini et al., 2005). Reportedly, antioxidant effects of tea polyphenols are associated with their ability to stimulate the antioxidant defence metabolism through redox regulated transcription factors and mitogen activated protein kinase-dependent cell cycle regulation (Jiao et al., 2003; Williams et al., 2004). In our study, however, the addition of EGCG to the culture medium had no positive effect on embryonic development and quality. This result apparently conflicts with those reported by Wang et al. (2007a), who reported an increase in the proportion of blastocyst formation after IVF when bovine embryos were cultured in the presence of 15 µM green tea polyphenols (GTPs). They suggested that beneficial effects of certain concentrations of GTPs (15 µM) in IVC medium on embryonic development are related to the antioxidant effects of GTPs. In contrast, Spinaci et al. (2008) showed that the addition of low EGCG concentration to the maturation medium had no positive effect on the ability of maturation and fertilisation of porcine oocytes. In their study, the maturation medium contained β-mercaptoethanol, which might act as an antioxidant during IVM of oocytes. In our study, NCSU-37 medium used for oocyte maturation and embryo culture also contained β-mercaptoethanol. We observed that the addition of EGCG to the culture medium did not improve the total cell number and DNA damage of blastocysts. Reactive oxygen species during IVC of embryos adversely affect their development and normal metabolic pathways, and compromise embryonic viability by inducing DNA fragmentation or apoptosis of embryonic cells (Goto et al., 1993; Johnson and Nasr-Esfahani, 1994). The level of apoptosis and cell number of blastocysts are important parameters for evaluating embryo development, viability and health (Brison and Schultz, 1997; de la Fuente and King, 1997; Brison and Schultz, 1998). Therefore, these antioxidants might be sufficient to protect oocytes and embryos from oxidative stress, decreasing differences between treated and untreated groups. However, supplementation with higher EGCG concentrations (10 and 50 µM) during IVC negatively influenced the developmental competence of parthenogenetic embryos. These results agree with those reported by Wang et al. (2007a), who reported that supplementation with high concentration of GTP during IVM or IVC significantly reduced the rates of blastocyst formation. Spinaci et al. (2008) also demonstrated that supplementation with high EGCG concentrations (25 µg/mL) during IVM decreased the developmental competence of porcine oocytes. Reportedly, GTPs have different actions with antioxidant action at lower concentrations and pro-oxidant action at higher concentrations (Yang et al., 2000; Sakagami et al., 2001). Therefore, addition of high EGCG concentrations to the medium beyond the optimum concentration ranges might have deleterious effects on the embryonic development.

Conclusions

In summary, this study assessed the effects of EGCG supplementation to the culture medium during IVC of parthenogenetic porcine embryos on their developmental competence and quality. Results showed that the treatment with a low concentration of EGCG (1 µM or 5 µM) during IVC had no influence on the abilities of cleavage and development to the blastocyst stage of parthenogenetic embryos and on the quality of blastocysts. Moreover, an additional increase in EGCG concentration from 10 µM to 50 µM might negatively affect the developmental competence of the embryos during IVC. It remains unclear whether treatment with a low EGCG concentration during IVC influences the developmental competence of porcine embryos, but the presence of high concentrations of polyphenol during IVC might be harmful for in vitro development of porcine parthenotes. Explanation of the negative effect on embryonic development resulting from high concentrations of EGCG necessitates further research.

References

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S., Day, B.N., 1998. Presence of beta-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology 50:747-756.

Beckmann, L.S., Day, B.N., 1993. Effects of medium NaCl concentration and osmolality on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium. Theriogenology 39:611-622.

Brison, D.R., Schultz, R.M., 1997. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biol. Reprod. 56:1088-1096.

Brison, D.R., Schultz, R.M., 1998. Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. Biol. Reprod. 59:136-144.

de la Fuente, R., King, W.A., 1997. Use of a chemically defined system for the direct comparison of inner cell mass and trophoderm distribution in murine, porcine and bovine embryos. Zygote 5:309-320.

Dufresne, C.J., Farnworth, E.R., 2001. A review of latest research findings on the health promotion properties of tea. J. Nutr. Biochem. 12:404-421.

Fiorini, R.N., Donovan, J.L., Rodwell, D., Evans, Z., Cheng, G., May, H.D., Milliken, C.E., Markowitz, J.S., Campbell, C., Haines, J.K., Schmidt, M.G., Chavin, K.D., 2005. Short-term administration of (-)-epigallocatechin gallate reduces hepatic steatosis and protects against warm hepatic ischemia/reperfusion injury in steatotic mice. Liver Transplant. 11:298-308.

Goto, Y., Noda, Y., Morí, T., Nakano, M., 1993. Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radical Bio. Med. 15:69-75.

Guerin, P., El Mouattassim, S., Menezo, Y., 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update 7:175-189.

Guo, Q., Zhao, B., Li, M., Shen, S., Xin, W., 1996. Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim. Biophys. Acta 1304:210-222.

Higdon, J.V., Frei, B., 2003. Tea catechins and polyphenols: health effects, metabolism,
and antioxidant functions. Crit. Rev. Food Sci. 43:89-143.
Jiao, H.L., Ye, P., Zhao, B.L., 2003. Protective effects of green tea polyphenols on human HepG2 cells against oxidative damage of fenofibrate. Free Radical Bio. Med. 35:1121-1128.
Johnson, M.H., Nasr-Esfahani, M.H., 1994. Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of pre-plantation mammalian embryos in vitro? Bioessays 16:31-38.
Knapen, M.F., Peters, W.H., Mulder, T.P., Merkus, H.M., Jansen, J.B., Steegers, E.A., 1999. Glutathione and glutathione-related enzymes in decidua and placenta of controls and women with pre-eclampsia. Placenta 20:541-546.
Livingston, T., Eberhardt, D., Edwards, J.L., Godkin, J., 2004. Retinol improves bovine embryonic development in vitro. Reprod. Biol. Endocrin. 2:83.
Mure, K., Rossman, T.G., 2001. Reduction of spontaneous mutagenesis in mismatch repair-deficient and proficient cells by dietary antioxidants. Mutat. Res. 480-481:85-95.
Nagashima, H., Grupen, C.G., Ashman, R.J., Nottle, M.B., 1996. Developmental competence of in vivo and in vitro matured porcine oocytes after subzonal sperm injection. Mol. Reprod. Dev. 45:359-363.
Otoi, T., Yamamoto, K., Horikita, N., Tachikawa, S., Suzuki, T., 1999. Relationship between dead cells and DNA fragmentation in bovine embryos produced in vitro and stored at 4 degrees C. Mol. Reprod. Dev. 54:342-347.
Peters, R., Wells, K., 1993. Culture of pig embryos. J. Rep. Fer. S. 48:61-73.
Rath, D., Niemann, H., Torres, C.R., 1995. In vitro development to blastocysts of early porcine embryos produced in vivo or in vitro. Theriogenology 43:913-926.
Rice-Evans, C., 1999. Implications of the mechanisms of action of tea polyphenols as antioxidants in vitro for chemoprevention in humans. P. Soc. Exp. Biol. Med. 220:262-266.
Roy, M., Chakrabarty, S., Sinha, D., Bhattacharya, R.K., Siddiqi, M., 2003. Anticlastogenic, antigenotoxic and apoptotic activity of epigallocatechin gallate: a green tea polyphenol. Mutat. Res. 523-524:33-41.
Sakagami, H., Arakawa, H., Maeda, M., Satoh, K., Kadofuku, T., Fukushima, K., Gomi, K., 2001. Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants. Anticancer Res. 21:2633-2641.
Schoeder, P., Klotz, I.O., Sies, H., 2003. Amphiliphilic properties of (-)-epicatechin and their significance for protection of cells against peroxynitrite. Biochem. Bioph. Res. Co. 307:69-73.
Spinaci, M., Volpe, S., De Ambrogi, M., Tamanini, C., Galeati, G., 2008. Effects of epigallocatechin-3-gallate on in vitro maturation and fertilization of porcine oocytes. Theriogenology 69:877-885.
Sugisawa, A., Umegaki, K., 2002. Physiological concentrations of (-)-epicatechin and (-)-epigallocatechin-3-O-gallate (EGCG) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. J. Nutr. 132:1836-1839.
Sutherland, B.A., Rahman, R.M., Appleton, I., 2006. Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. J. Nutr. Biochem. 17:291-306.
Vinson, J.A., Liang, X., Proch, J., Hontz, B.A., Dancel, J., Sandone, N., 2002. Polyphenol antioxidants in citrus juices: in vitro and in vivo studies relevant to heart disease. Adv. Exp. Med. Biol. 505:113-122.
Wang, Z.G., Yu, S.D., Xu, Z.R., 2007a. Effect of supplementation of green tea polyphenols on the developmental competence of bovine oocytes in vitro. Braz. J. Med. Biol. Res. 40:1079-1085.
Wang, Z.G., Yu, S.D., Xu, Z.R., 2007b. Improvement in bovine embryo production in vitro by treatment with green tea polyphenols during in vitro maturation of oocytes. Anim. Reprod. Sci. 100:22-31.
Williams, R.J., Spencer, J.P., Rice-Evans, C., 2004. Flavanoids: antioxidants or signalling molecules? Free Radical Bio. Med. 36:838-849.
Wolle, B.A., Kraemer, D.C., 1992. Methods in bovine nuclear transfer. Theriogenology 37:5-15.
Yamanaka, N., Oda, O., Nagao, S., 1997. Green tea catechins such as (-)-epicatechin and (-)-epigallocatechin accelerate Cu2+-induced low density lipoprotein oxidation in propagation phase. FEBS Lett. 401:230-234.
Yang, G.Y., Liao, J., Li, C., Chung, J., Yurkow, E.J., Ho, C.T., Yang, C.S., 2000. Effect of black and green tea polyphenols on c-jun phosphorylation and H(2)O(2) production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. Carcinogenesis 21:2035-2039.
Yoneda, A., Suzuki, K., Mori, T., Ueda, J., Watanabe, T., 2004. Effects of delipidation and oxygen concentration on in vitro development of porcine embryos. J. Reprod. Develop. 50:287-295.
Zaveri, N.T., 2006. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sci. 78:2073-2080.