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Effect of supplementation of green tea polyphenols on the developmental competence of bovine oocytes *in vitro*

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Abstract

The objective of the present study was to examine the effect of green tea polyphenols (GTPs) supplementation during *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture on the developmental competence of bovine oocytes. Cumulus-oocyte complexes aspirated from the ovaries were matured *in vitro* (38.5ºC for 24 h) and fertilized (38.5ºC for 15-18 h) and embryos were cultured (38.5ºC for 192 h) in a defined conditioned medium with or without GTPs supplementation. The GTPs used in the present study contained 99% catechin derivatives, with the major components being 50% (-)-epigallocatechin gallate, 22% (-)-epicatechin gallate, 18% (-)-epigallocatechin, and 10% (-)-epicatechin. Four replicate trials were done for each type of experiment. GTPs supplementation (15 µM) of the maturation medium led to a significant increase in the rate of blastocyst formation (34.0 *vs* 21.4%, $P < 0.05$). However, the rate of blastocyst formation was not improved when higher GTPs concentrations (20 or 25 µM) were added to the *in vitro* maturation medium. During *in vitro* fertilization, supplementation with higher GTPs concentrations (20 or 25 µM) significantly reduced the rate of blastocyst formation ($P < 0.05$). Supplementation of the culture medium with 15 µM GTPs improved the rate of blastocyst formation, while higher GTPs concentrations (25 µM) significantly reduced embryo development ($P < 0.05$). In conclusion, these results demonstrate that supplementation with GTPs at low concentration (15 µM) during *in vitro* maturation and *in vitro* culture improved the developmental competence of bovine oocytes.

Key words
- Bovine oocytes
- *In vitro* culture
- Green tea polyphenols
- Embryo development

Introduction

The formation of reactive oxygen species (ROS) such as superoxide anions ($O_2^{-}\cdot$), hydroxyl radicals ($OH\cdot$) and hydrogen peroxide (H_2O_2) is a normal process that occurs in the cell when there is a deviation of electrons to oxygen (O_2) during electron transfer reactions (1). There is some evidence that ROS may be beneficial at some steps of reproduction to permit successful gamete interaction (2). However, an increasing number of *in vitro* studies have demonstrated the detrimental effects of ROS on reproduction. The main detrimental effects include reduced sperm motility and axonemal protein phosphorylation (3), *in vitro* two-cell block of embryos (4), and reduced embryo development (5,6).

In vitro oocyte or embryo culture results in higher O_2 concentrations than in *in vivo* environments, leading to increased ROS levels (7). ROS such as $O_2^{-\cdot}$ are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids. This can affect the early development of mouse, hamster, and bovine embryos (8-10). Living organisms possess natural protective equivalents known as ROS scavengers (antioxidants) that counteract the negative effects of ROS. These antioxidants include enzymes such as superoxide dismutase, which will eliminate $O_2^{-\cdot}$, catalase and selenium-dependent glutathione peroxidase, which will transform H_2O_2 into H_2O and O_2, as well as lipid- and water-soluble antioxidants such as vitamins C, E and uric acid (11). However, during *in vitro* oocyte and embryo culture the levels of antioxidants are lower than *in vivo* because the oocytes or embryos are divorced from the donor body and do not benefit from the maternal antioxidant protection. The addition of an antioxidant to the medium, therefore, may be important for *in vitro* oocyte maturation and *in vitro* embryo culture.

Tea (*Camellia sinensis*) is one of the most popular beverages consumed worldwide. Green tea polyphenols (GTPs) are the major water-soluble components of green tea infusions. The major GTPs are (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC), and (-)-epigallocatechin (EGC). These catechins have a strong antioxidant activity (12) and are potent scavengers of ROS superoxide, hydrogen peroxide, hydroxyl radicals, and nitric oxide produced by various chemicals (13).

There are no reports on the use of GTPs during *in vitro* maturation (IVM) of bovine oocytes and *in vitro* culture (IVC) of bovine embryos. Thus, the objective of the present study was to examine the effects of GTPs supplementation during IVM, *in vitro* fertilization (IVF) and IVC on the developmental competence of bovine oocytes.

Material and Methods

Reagents

All chemicals and media were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. The green tea polyphenols (from the Tea Department of Zhejiang University) used in the present study contained 99% catechin derivatives, with the major components being 50% EGCG, 22% ECG, 18% EGC, and 10% EC. The average molecular weight of GTPs was estimated on the basis of the percent presence of major components.

In vitro maturation

Cumulus-oocyte complexes (COCs) were aspirated from 2- to 6-mm follicles of bovine ovaries obtained from a slaughterhouse. Oocytes with intact cumulus cells and evenly granulated cytoplasm were selected and randomly assigned to each treatment. Ten COCs were washed and incubated with droplets of IVM medium, which consisted of modified synthetic oviduct fluid (m-SOF) supplemented with minimum essential medium non-essential amino acids (Gibco, Grand Island, NY, USA), minimum essential medium essential amino acids (Gibco), 1.5 mM glucose, and 1 mM glutamine. The SOF medium used in this study was based on the original formulation (14), with subsequent modifications (15). The IVM drops were covered with mineral oil and incubated at 38.5ºC in 5% CO_2 in air at saturated humidity for 24 h.

In vitro fertilization

The matured COCs were washed twice in warm HEPES-buffered Tyrode albumin lactate pyruvate solution (HEPES-TALP) (16) and once in IVF medium (a bicarbonate-

buffered modified TALP) (17) and then placed in 50-µL droplets (10-12 COCs per droplet) of IVF medium containing 10 µg/mL heparin. Frozen bull semen was thawed and prepared by the swim-up procedure (17). Sperm cells were added to the IVF drops at a final concentration of 2 x 10^6/mL. Incubation was carried out at 38.5ºC in 5% CO_2 in air at saturated humidity for 15-18 h.

In vitro culture

Between 15 and 18 h after insemination, surrounding cumulus cells of presumptive zygotes were denuded by repeated pipetting in phosphate-buffered saline (PBS) and subsequently washed three times in PBS before being transferred to the IVC drops (20-30 embryos/50-µL drops). In all experiments, embryos were cultured in m-SOF medium containing 0.8% bovine serum albumin at 38.5ºC in 5% CO_2 and 7% O_2 with high humidity.

Cleavage and development of embryos to the blastocyst stage were assessed at 48 and 192 h (i.e., on 8th day) post-insemination, respectively, under a stereomicroscope (60X). After removing the zona pellucida by immersing in acid Tyrode solution, pH 2.5, blastocysts were fixed in ethanol:acetic acid (3:1) and stained with 0.24% basic fuchsin. The number of cells in blastocysts was examined by phase contrast microscopy.

Experimental design

Three separate experiments were performed to evaluate the effects of supplementation with GTPs at different concentrations during different phases of the *in vitro* production of bovine embryos. In Experiment 1, the GTPs at different concentrations (0, 10, 15, 20, or 25 µM) were added during IVM in defined condition medium under 20% O_2 to reduce ROS production in the IVM medium. Oocytes cultured without GTPs supplementation were used as control.

In Experiment 2, bovine oocytes were matured in GTPs-free maturation medium. Oocytes were inseminated in the fertilization medium with different concentration of GTPs (0, 10, 15, 20, or 25 µM) under high (20%) O_2 concentration and then compared with the control group (GTPs-free fertilization medium).

In Experiment 3, oocytes were matured and inseminated in GTPs-free medium. Various concentrations of GTPs (0, 10, 15, 20, or 25 µM) were added to the culture medium under low O_2 (7%).

Statistical analysis

Data from four replicate trials were analyzed statistically for comparison of each treatment by ANOVA and the Fisher protected least significant difference test using the STATVIEW program (Abacus Concepts, Inc., Berkeley, CA, USA). All percent values were subjected to arc sine transformation before statistical analysis. Data are reported as means ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

Results

In the first experiment (Table 1), oocytes were cultured for 24 h in IVM medium

Table 1. Effect of green tea polyphenols (GTPs) concentration in the maturation medium on the development of bovine embryos *in vitro*.

GTPs concentration (µM)	No. of oocytes inseminated	No. of embryos		Number of cells in blastocysts
		Cleavage (%)[a]	Blastocyst (%)[b]	
0	109	79 (72.3 ± 1.3)	17 (21.4 ± 2.9)[d,e]	105 ± 7.2[c]
10	121	91 (75.2 ± 1.1)	27 (29.5 ± 1.2)[c,d]	123 ± 3.1[d,e]
15	115	85 (73.9 ± 2.7)	29 (34.0 ± 2.3)[c]	117 ± 4.9[d]
20	110	86 (78.3 ± 3.3)	22 (25.3 ± 3.1)[d]	129 ± 5.2[e]
25	107	78 (72.7 ± 0.9)	12 (15.2 ± 1.7)[e]	120 ± 4.3[d,e]

[a]Number of embryos is reported as median and percent of embryos cleaved as percent of the number of inseminated oocytes (mean ± SEM), or [b]percent of blastocysts as percent of the number of cleaved embryos (mean ± SEM). [c,d,e]Values with different superscripts within a column are significantly different from each other ($P < 0.05$, ANOVA followed by the Fisher protected least significant difference test).

supplemented with GTPs at various concentrations and then fertilized *in vitro* and transferred to IVC medium to assess the cleavage rates. There were no significant differences in cleavage rate among treatments. However, on the 8th day after transfer in 7% O_2, the presence of 15 µM GTPs during IVM significantly increased ($P < 0.05$) the rate of blastocyst formation and the cell number of blastocysts compared to control (34.0 *vs* 21.4% and 117 *vs* 105%). The cell numbers of blastocysts were significantly higher in all four treatment groups than in the control group ($P < 0.05$). However, treatment with GTPs at a concentration of 25 µM showed a significantly reduced number of blastocysts ($P < 0.05$).

In the second experiment (Table 2), oocytes were matured in GTPs-free maturation medium and fertilized in IVF medium supplemented with GTPs at various concentrations (0, 10, 15, 20, or 25 µM). No improvement in cleavage or blastocyst rate was found when 10 µM GTPs was added to the fertilization medium compared to control (78.3 *vs* 79.3% and 23.2 *vs* 25.1%). However, supplementation with GTPs at 15, 20, and 25 µM concentrations significantly decreased the cleavage rates compared to control ($P < 0.05$). Furthermore, higher concentrations of GTPs (20 and 25 µM) significantly reduced ($P < 0.05$) the rate of blastocyst formation. No improvement in the cell numbers of blastocysts was found when higher concentrations of GTPs (20 and 25 µM) were added to fertilization medium compared to control.

In the third experiment (Table 3), oocytes were matured *in vitro* and fertilized in GTPs-free medium. Subsequently, the zygotes were cultured in IVC medium supplemented with GTPs at various concentrations. There was no significant difference in cleavage rate between the treatment and control groups ($P > 0.05$). The addition of 15 µM GTPs during IVC increased the rate of blastocyst formation and blastocyst cell number compared to control. However, the blastocyst rate and the cell numbers in blastocysts were significantly reduced ($P < 0.05$) when 25 µM GTPs was added to the medium compared with 15 µM GTPs supplementation.

Table 2. Effect of green tea polyphenols (GTPs) concentration in the fertilization medium on the development of bovine embryos *in vitro*.

GTPs concentration (µM)	No. of oocytes inseminated	No. of embryos		Number of cells in blastocysts
		Cleavage (%)[a]	Blastocyst (%)[b]	
0	106	84 (79.3 ± 2.4)[c]	21 (25.1 ± 1.8)[c]	98 ± 5.2[c]
10	110	86 (78.3 ± 3.1)[c]	20 (23.2 ± 1.3)[cd]	113 ± 2.1[d]
15	117	74 (63.5 ± 2.5)[d]	14 (19.0 ± 2.0)[d]	117 ± 1.9[d]
20	121	76 (62.7 ± 3.3)[d]	12 (15.8 ± 2.1)[de]	106 ± 3.2[c]
25	108	65 (60.4 ± 2.7)[d]	5 (7.7 ± 1.9)[f]	103 ± 2.7[c]

[a]Number of embryos is reported as median and percent of embryos cleaved as percent of the number of inseminated oocytes (mean ± SEM), or [b]percent of blastocysts as percent of the number of cleaved embryos (mean ± SEM). [c,d,e]Values with different superscripts within a column are significantly different from each other ($P < 0.05$, ANOVA followed by the Fisher protected least significant difference test).

Table 3. Effect of green tea polyphenols (GTPs) concentration in the culture medium on the development of bovine embryos *in vitro*.

GTPs concentration (µM)	No. of oocytes inseminated	No. of embryos		Number of cells in blastocysts
		Cleavage (%)[a]	Blastocyst (%)[b]	
0	99	76 (76.7 ± 2.3)	16 (21.3 ± 1.1)[d]	109 ± 3.9[c]
10	111	88 (79.3 ± 1.9)	20 (22.8 ± 1.3)[d]	103 ± 2.2[c]
15	105	84 (80.1 ± 2.0)	26 (30.6 ± 1.8)[c]	113 ± 3.1[d,e]
20	110	86 (78.2 ± 2.7)	17 (19.7 ± 2.1)[d]	120 ± 3.2[d]
25	109	87 (79.8 ± 2.1)	11 (12.7 ± 0.9)[e]	105 ± 4.7[c]

[a]Number of embryos is reported as median and percent of embryos cleaved as percent of the number of inseminated oocytes (mean ± SEM), or [b]percent of blastocysts as percent of the number of cleaved embryos (mean ± SEM). [c,d,e]Values with different superscripts within a column are significantly different from each other ($P < 0.05$, ANOVA followed by the Fisher protected least significant difference test).

Discussion

The present study assessed the effects of GTPs supplementation during IVM, IVF and IVC on the developmental competence of bovine oocytes. The principal finding is that supplementation with 15 µM GTPs during IVM and IVC improves the rate of blasto-

cyst formation. However, the blastocyst rates were not improved by supplementation with 20 or 25 µM GTPs during IVM and IVC. Also, supplementation with GTPs at different concentrations (0, 10, 15, 20, or 25 µM) during IVF did not improve the proportion of embryos reaching the blastocyst stage.

To our knowledge, the present study is the first to demonstrate the beneficial effect of GTPs supplementation of IVM or IVC medium on early bovine embryo development. Tea polyphenols, especially GTPs, have been shown to be useful as antidiabetic, antitumor, antiarthritic, and antioxidant agents (18-21). The antioxidant effects of tea polyphenols are thought to be associated with their ability to stimulate the antioxidant defense metabolism through redox-regulated transcription factors and mitogen-activated protein kinase-dependent cell cycle regulation (22,23).

The present results indicate that the addition of 15 µM GTPs to the defined IVM medium (Experiment 1) significantly improved ($P < 0.05$) the rate of blastocyst formation. This enhancement could be attributed to the efficient GTPs-induced protection of oocytes against oxidative stress during IVM. It has been reported that other antioxidants such as ß-mercaptoethanol, cysteine and cystine added during IVM of bovine oocytes also improve the rate of embryo development to the blastocyst stage (24). These results suggest that supplementation with appropriate GTPs concentrations during IVM could lead to subsequent improvement of embryo development.

The results of Experiment 2 demonstrated that supplementation with low GTPs concentrations (10 µM) during IVF had no effect on the percentage of embryos produced, but higher concentrations of GTPs (15, 20, or 25 µM) significantly reduced the cleavage and blastocyst rates ($P < 0.05$). Ali et al. (24) and Luvoni et al. (7) reported that antioxidants such as superoxide dismutase and cysteine present during IVF could significantly reduce the percentage of embryos produced, suggesting that ROS might play a positive role during IVF. Furthermore, previous studies implied that ROS were involved in the control of capacitation (25), acrosomal reaction (26) and fertilization (22).

The results obtained here in Experiment 3 indicate that the proportion of oocytes developing to the blastocyst stage was significantly higher ($P < 0.05$) in the presence of a low GTPs concentration in a defined culture medium. This improvement in embryo development might be due to the antioxidant effect of GTPs, which scavenge ROS during *in vitro* embryo culture. Embryos are inevitably exposed to high oxygen *in vitro*, a fact that results in a higher production of ROS (7) than *in vivo* because of the necessary manipulations with transient exposure to atmospheric oxygen. ROS seems to be responsible for numerous types of embryo damage. ROS such as superoxide anions are able to diffuse and pass through cell membranes and alter most types of cellular molecules such as lipids, proteins and nucleic acids. The consequences are multiple, and include mitochondrial alterations, embryo cell-block, ATP depletion, and apoptosis (27). The detrimental effects of superoxide anion radicals on embryo development have been demonstrated in cattle (10,28). Fujitani et al. (29) reported that the development of bovine embryos to blastocysts was decreased in a dose-dependent manner when free radicals were generated *in vitro* by the addition of 2,2'-azobis (2-amindinopropane) dihydrochloride to the culture medium.

Interestingly, in our study, supplementation with higher GTPs concentrations (20 or 25 µM) during IVM (Experiment 1) or IVC (Experiment 2) significantly reduced the rates of blastocyst formation. As reported previously (30-32), GTPs have two different actions: an antioxidant action at lower concentrations, and a pro-oxidant action at higher concentrations. These results suggest that GTPs supplementation beyond the optimum

concentration ranges might have deleterious effects on the *in vitro* maturation events occurring in both the nucleus and the cytoplasm and on subsequent embryo development.

Our results demonstrate that 15 µM GTPs supplementation during IVM or IVC improves the developmental competence of bovine oocytes possibly by protecting the embryos from oxidative stress. However, the GTPs supplementation during IVF may not improve the rate of blastocyst formation.

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