# Epicatechin Gallate Decreases the Viability and Subsequent Embryonic Development of Mouse Blastocysts

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**SUMMARY**

**Objective:** Catechins, a family of polyphenols found in tea, evoke various responses including cell death. We examined the cytotoxic effects of epicatechin gallate (ECG), a polyphenol extract from green tea, on the blastocyst stage of mouse embryos, subsequent embryonic attachment, and *in vitro* and *in vivo* outgrowth implantation after embryo transfer.

**Materials and Methods:** Mouse blastocysts were incubated in medium with or without ECG (12.5μM, 25μM or 50μM) for 24 hours. Cell proliferation and growth were investigated using dual differential staining, apoptosis was analyzed with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, and implantation and post-implantation development of embryos were measured by *in vitro* development analysis and *in vivo* embryo transfer, respectively.

**Results:** Blastocysts treated with 50μM ECG exhibited a significant increase in apoptosis and a corresponding decrease in total cell number. Importantly, the implantation success rate of blastocysts pretreated with 50μM ECG was lower than that of controls, and *in vitro* treatment with 50μM ECG was associated with increased resorption of post-implantation embryos and decreased fetal weight.

**Conclusion:** Our results collectively indicate that *in vitro* exposure to ECG induces apoptosis and retards early post-implantation development after transfer to host mice. The degree of teratogenic potential exerted by ECG in early human development is unknown at present and requires further investigation. [*Taiwan J Obstet Gynecol* 2010;49(2):174–180]

**Key Words:** apoptosis, blastocyst, embryonic development, epicatechin gallate

## Introduction

Catechins, a family of polyphenols found in tea, have antioxidative, antiallergic, antimutagenic and anticarcinogenic properties [1–3]. Recent reports demonstrate that catechins prevent cancer progression by inhibiting carcinogenesis, tumor growth, cancer cell invasion, and tumor angiogenesis [4,5]. At the cellular level, the major mechanisms underlying the antitumor effects of catechins involve apoptosis induction and cell cycle arrest [6–8]. Epicatechin gallate (ECG), one of the major polyphenols in green tea, reportedly induces apoptosis in human colorectal cancer. This compound may thus be an important contributor to the antitumorigenic activity of green tea. ECG significantly suppresses cyclin D1 expression in head and neck squamous cell carcinoma (HNSCC) cells [9]. Additionally, cell growth and apoptosis are triggered by the presence of ECG in HNSCC cells. These findings support the use of ECG as a

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potential chemopreventive agent in human cancers, such as HNSCC. However, to date, few studies have investigated the potential of ECG as a cytotoxic agent in embryonic development.

Apoptosis plays an important role in development and disease [10]. Although several studies have shown that apoptosis functions during normal embryonic development [11–13], mechanistically diverse teratogens can induce excessive apoptosis in early embryos, leading to developmental injury [14–18]. Recent investigations have shown that ECG induces or inhibits cell apoptosis [9,19–23]. In the present study, we investigated whether ECG has cytotoxic effects on mouse blastocysts.

## Materials and Methods

### *Collection of mouse morulas and blastocysts*

Imprinting control region mice were from the National Laboratory Animal Center, Taiwan. This research was also approved by the animal research ethics board of Chung Yuan Christian University, Taiwan. All animals received humane care, as outlined in the Guidelines for Care and Use of Experimental Animals [24]. All mice were maintained on breeder chow (Harlan Teklad chow; Harlan, Madison, Wisconsin, USA) with food and water available *ad libitum*. Housing was a standard 28 × 16 × 11 cm polypropylene cage with wire grid tops and kept under a 12-hour day/12-hour night regimen. Nulliparous females (6–8 weeks old) were superovulated by injection of 5 IU of pregnant mare serum gonadotropin (Sigma, St. Louis, MO, USA); 48 hours later, they were injected with 5 IU of human chorionic gonadotropin (NV Organon, Oss, The Netherlands), and then mated overnight with a single fertile male of the same strain. The day a vaginal plug was found was defined as day 0 of gestation. Plug-positive females were separated for experimentation. Morulas were obtained by flushing the uterine tubes on the afternoon of gestation day 3, and blastocysts were obtained by flushing the uterine horn on day 4; in both cases, the flushing solution consisted of CMRL 1066 culture medium (Gibco Life Technologies, Grand Island, NY, USA) containing 1mM glutamine and 1mM sodium pyruvate. The concentration of glucose in this medium was 5mM. Expanded blastocysts from different females were pooled and randomly selected for experiments.

### *ECG treatment and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay*

Blastocysts were incubated in medium containing the indicated concentrations of ECG for 24 hours. For apoptosis detection, embryos were washed in ECG-free medium, fixed, permeabilized and subjected to TUNEL labeling using an *in situ* cell death detection kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Photographic images were taken using a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

### *ECG treatment and cell proliferation*

Blastocysts were incubated with or without culture medium containing 12.5μM, 25μM or 50μM ECG (Sigma). After 24 hours, blastocysts were washed with ECG-free medium, and dual differential staining was used to facilitate counting of cell numbers in the inner cell mass (ICM) and trophectoderm (TE) [25]. Blastocysts were incubated in 0.4% pronase in $M_2$ medium containing 0.1% bovine serum albumin (BSA) (Sigma) for removal of the zona pellucida. The denuded blastocysts were exposed to 1mM trinitrobenzenesulfonic acid in BSA-free $M_2$ medium containing 0.1% polyvinylpyrrolidone at 4°C for 30 minutes, then washed with $M_2$ medium [26]. The blastocysts were further treated with anti-dinitrophenol-BSA complex antibody at 30 μg/mL in $M_2$-BSA at 37°C for 30 minutes, and then with $M_2$ medium supplemented with 10% whole guinea-pig serum as a source of complement, along with bisbenzimide at 20 μg/mL and propidium iodide (PI) at 10 μg/mL at 37°C for 30 minutes. The immunolysed blastocysts were gently transferred to slides and protected from light before observation. Under ultraviolet light excitation, the ICM cells, which took up bisbenzimide but excluded PI, appeared blue. The TE cells, which took up both fluorochromes, appeared orange-red. Since multinucleated cells are not common in preimplantation embryos [27], the number of nuclei was considered to represent an accurate measure of the cell number.

### *Annexin V staining*

Blastocysts were incubated in 12.5μM, 25μM or 50μM ECG for 24 hours, washed with ECG-free culture medium and then stained using an Annexin-V-FLUOS staining kit (Roche), according to the manufacturer's instructions. Briefly, the blastocysts were incubated in $M_2$-BSA for removal of the zona pellucida, washed with phosphate buffered saline containing 0.3% BSA, and then incubated for 60 minutes with a mixture of 100 μL of binding buffer, 1 μL of fluorescein isothiocyanate-conjugated Annexin V and 1 μL of PI. After incubation, the embryos were washed and photographed using a fluorescence microscope. Cells that were Annexin $V^+/PI^-$ were considered apoptotic, while those that were Annexin $V^+/PI^+$ were considered necrotic.

*Morphologic analysis of embryonic development*

Blastocysts were cultured according to a previously reported method with some modification [28]. Briefly, embryos were cultured in four-well dishes at 37°C. For group culture, four embryos were cultured per well. The basic medium consisted of CMRL 1066 supplemented with 1mM glutamine and 1mM sodium pyruvate plus penicillin at 50 IU/mL and streptomycin at 50 mg/mL (hereafter called culture medium). For treatments, the embryos were cultured with the indicated concentrations of ECG for 24 hours in serum-free medium. The embryos were then cultured for 3 days in culture medium supplemented with 20% fetal calf serum, and for 4 days in culture medium supplemented with 20% heated-inactivated human placental cord serum, for a total culture time of 8 days from the onset of treatment. Embryos were inspected daily under a phase-contrast dissecting microscope, and developmental stages were classified according to established methods [29,30]. Under these culture conditions, each hatched blastocyst attached to fibronectin and grew to form a cluster of ICM cells over the trophoblastic layer via a process called TE outgrowth. After a total incubation period of 96 hours, morphologic scores for outgrowth were estimated. Growing embryos were classified as either "attached" or "outgrowth", with the latter defined by the presence of a cluster of ICM cells over the trophoblastic layer. As described previously [31], ICM clusters were scored according to shape, ranging from compact and rounded ICM to a few scattered cells over the trophoblastic layer.

*Blastocyst development following embryo transfer*

To examine the ability of expanded blastocysts to implant and develop *in vivo*, the generated embryos were transferred to recipient mice. Imprinting control region females (white skin color) were mated with vasectomized males (C57BL/6J, black skin color; National Laboratory Animal Center, Taiwan) to produce pseudopregnant dams as recipients for embryo transfer. To ensure that all fetuses in the pseudopregnant mice came from embryo transfer (white color) and not from fertilization by C57BL/6J (black color), we examined the skin color of the fetuses at day 18 post coitus. To assess the impact of ECG on post-implantation growth *in vivo*, blastocysts were exposed to the indicated concentrations of ECG for 24 hours, and then eight embryos were transferred in parallel to the paired uterine horns of day 4 pseudopregnant mice. The surrogate mice were killed on day 18 post coitus, and the frequency of implantation was calculated as the number of implantation sites per number of embryos transferred. The incidence rates of resorbed and surviving fetuses were calculated as the number of resorptions or surviving fetuses, respectively, per number of implantations. The weights of the surviving fetuses and placentas were measured immediately after dissection.

*Statistics*

Data were analyzed using one-way analysis of variance and *t* tests, and are presented as the mean ± standard deviation, with significance taken at $p < 0.05$.

## Results

*Effects of ECG on mouse blastocysts*

To investigate the possibility of ECG-induced cytotoxicity, we treated mouse blastocysts with 12.5μM, 25μM or 50μM ECG at 37°C for 24 hours and monitored apoptosis using the TUNEL assay. Cell apoptosis was clearly evident in blastocysts treated with 50μM ECG (Figure 1A). Quantitative analysis revealed about tenfold more apoptotic cells in 50μM ECG-treated blastocysts compared with untreated control cells (Figure 1B). Our results clearly demonstrate that ECG induces apoptosis in mouse blastocysts.

*Effects of ECG on cell proliferation*

Differential staining followed by cell counting was used to assess cell proliferation in blastocysts treated with 12.5μM, 25μM or 50μM ECG for 24 hours, or those left untreated. We observed significantly lower cell numbers in 50μM ECG-treated blastocysts compared with control cells (Figure 2A). Annexin V staining revealed markedly higher numbers of Annexin $V^+/PI^-$ (apoptotic) cells in the ICM of treated blastocysts versus controls, but no such differences in the TE (Figure 2B). It seems that 50μM ECG significantly induces apoptosis in the ICM but not the TE of mouse blastocysts, further supporting the theory that ECG impairs the developmental potential of blastocysts.

*Effects of ECG on mouse embryonic developmental potential in vitro*

Untreated control morulas displayed about 86% development into blastocysts, whereas only 33% of the 50μM ECG-treated morulas developed into blastocysts under our experimental conditions (Figure 3A). To further determine the effects of ECG on post-implantation events *in vitro*, we treated blastocysts with or without 12.5μM, 25μM or 50μM ECG, and analyzed subsequent development for 8 days in culture. Importantly, the rate of embryo-only attachment to fibronectin-coated cultured dishes was markedly higher in blastocysts pretreated with 50μM ECG (Figure 3B). Additionally, these pretreated blastocysts displayed a lower incidence of post-implantation developmental milestones (Figure 3B).

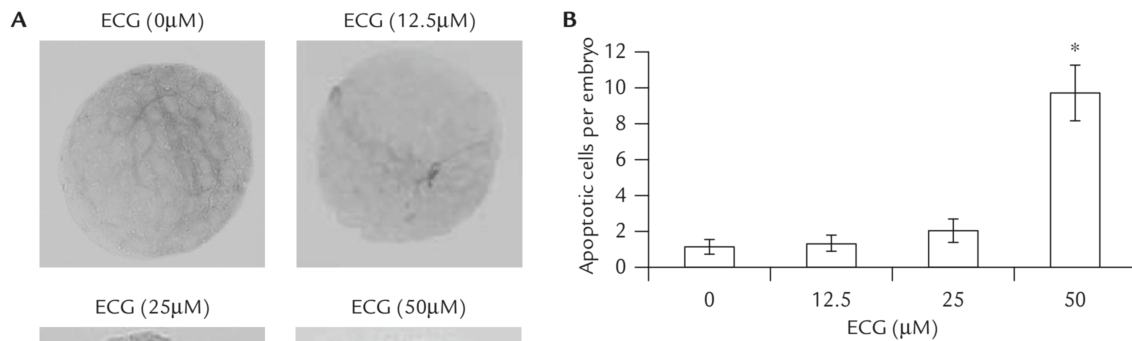


**Figure 1.** Epicatechin gallate (ECG) induces apoptosis in mouse blastocysts. (A) Mouse blastocysts were treated with ECG (12.5μM, 25μM or 50μM) for 24 hours or left untreated, and apoptosis was examined via transferase-mediated dUTP nick-end labeling (TUNEL) staining. Cells were visualized using light microscopy whereby TUNEL$^+$ cells are depicted in black. (B) The mean number of apoptotic (TUNEL$^+$) cells per blastocyst was calculated. Values are presented as mean ± standard deviation of ten determinations. *$p < 0.001$ versus the control group.

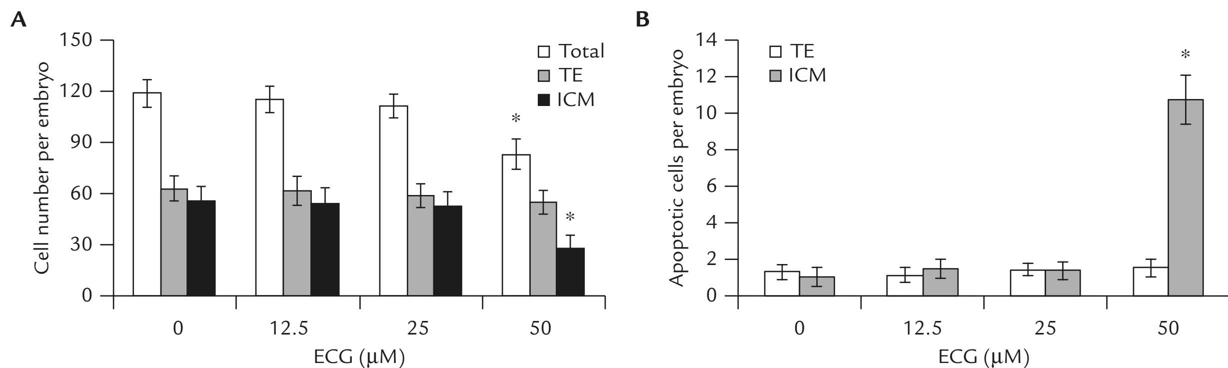


**Figure 2.** Effects of epicatechin gallate (ECG) on blastocyst viability. Mouse blastocysts were treated with or without ECG (12.5μM, 25μM or 50μM) for 24 hours. (A) The total number of cells per blastocyst and cell numbers in the inner cell mass (ICM) and trophectoderm (TE) were counted. Data are based on at least 180 blastocyst samples from each group. *$p < 0.001$ *vs*. the control group. (B) The percentages of Annexin V$^+$/propidium iodide$^-$ cells in the blastocysts of each group were examined. Data are based on at least 180 blastocyst samples from each group. *$p < 0.001$ *vs*. the control group.

These results indicate that ECG affects implantation as well as the *in vitro* potential of blastocysts to develop into post-implantation embryos.

### *Effects of ECG on the developmental potential of blastocysts in vivo*

To determine the effects of ECG on blastocyst development *in vivo*, we transferred control and ECG-pretreated mouse blastocysts and examined the uterine content at 13 days post transfer (day 18 post coitus). The implantation ratio in the 50μM ECG-pretreated group was significantly lower than that of the untreated control group (Figure 4A). Embryos that implanted but failed to develop were subsequently resorbed. However, the proportion of implanted embryos that failed to develop normally was markedly higher in the group treated with 50μM ECG (Figure 4A). Interestingly, there was no marked difference in placental weight between the 50μM ECG-treated and untreated groups (Figure 4B). However, fetal weight was lower in the 50μM ECG-treated group versus the control group (487 ± 58 mg versus 608 ± 73 mg, respectively). Previous studies, including a recent report by our group, have shown that 35–40% of fetuses weighed ≥ 600 mg, and the average weight of total surviving fetuses was about 600 ± 12 mg in the untreated control group at day 18 of pregnancy in a mouse embryo transfer assay [16,32–35]. Fetal weight is an important indicator of developmental status, and the average fetal weight of the untreated control group is used as a key indicator of the development of blastocysts treated with 50μM ECG. Interestingly, only 9% of the fetuses in the 50μM ECG-pretreated group weighed ≥ 600 mg, an important indicator of successful embryonic and fetal development, whereas 40% of control fetuses exceeded this threshold (Figure 4C). Together, these observations indicate that ECG exposure at the

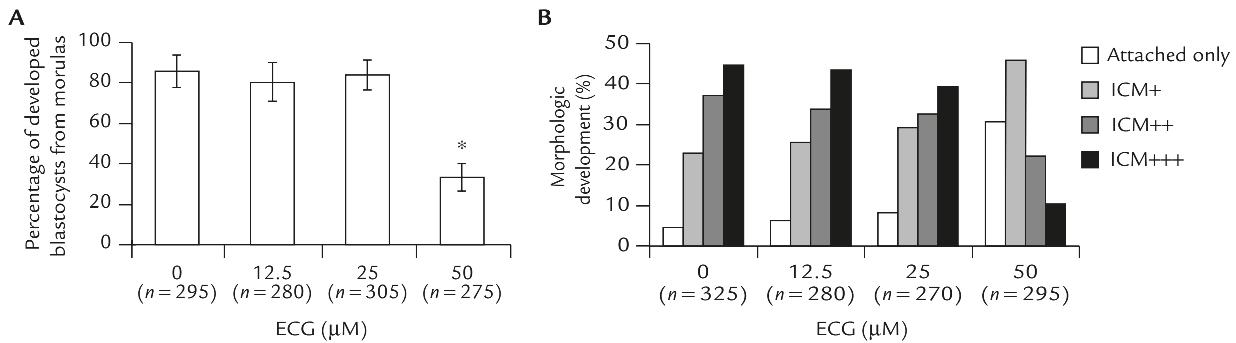


**Figure 3.** *In vitro* development of mouse embryos exposed to epicatechin gallate (ECG) at the blastocyst stage. (A) Mouse morulas were treated with ECG (12.5 μM, 25 μM or 50 μM) for 24 hours or left untreated and cultured for an additional 24 hours at 37°C. Blastocysts were counted and percentages calculated. Values are presented as means ± standard deviation of eight determinations. *$p < 0.001$ versus the control group. (B) Mouse blastocysts were treated with ECG (12.5 μM, 25 μM or 50 μM) for 24 hours or left untreated and cultured for 7 days post treatment. Blastocysts were identified as hatched, inner cell mass [ICM] (+), ICM (++) and ICM (+++) via morphologic assessment, where ICM ranged from compact and rounded (+++) to a few scattered cells (+) over the trophoblastic layer. Values are presented as means ± standard deviation of eight determinations.

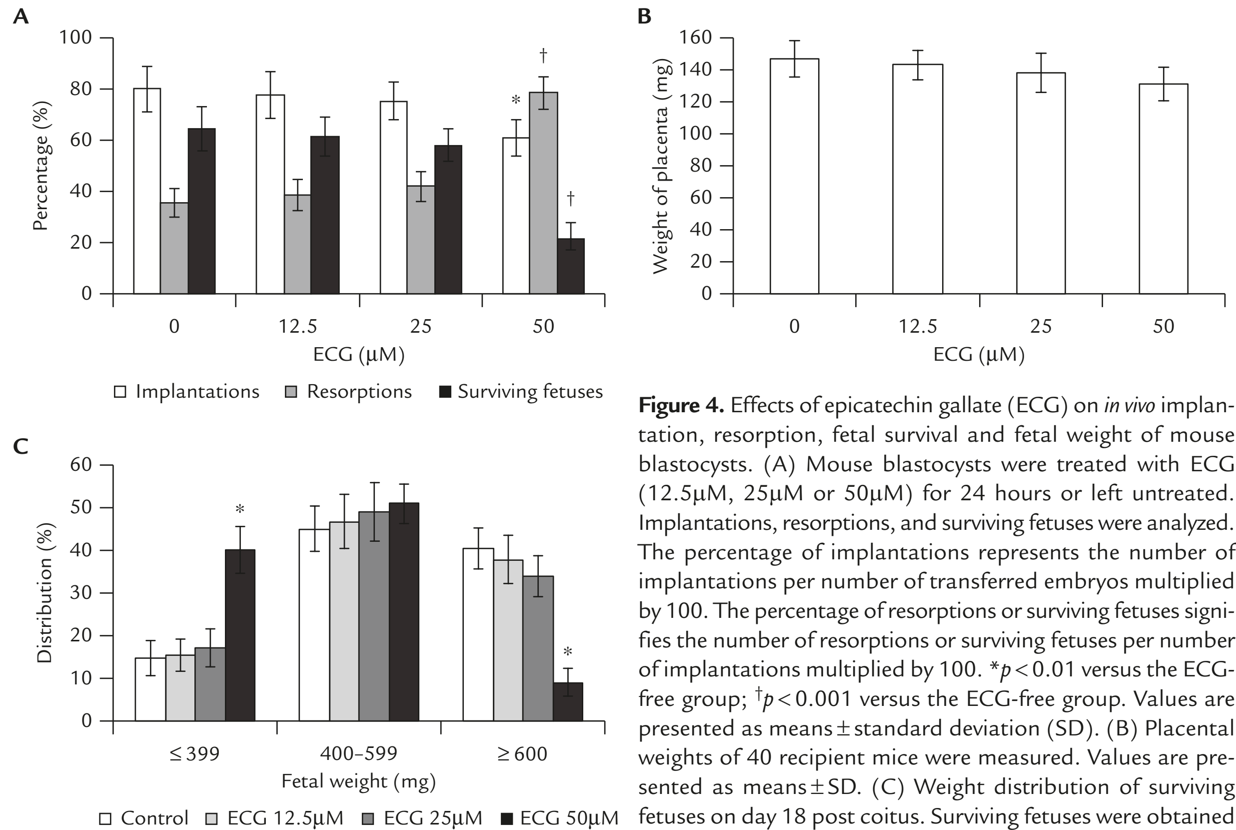


**Figure 4.** Effects of epicatechin gallate (ECG) on *in vivo* implantation, resorption, fetal survival and fetal weight of mouse blastocysts. (A) Mouse blastocysts were treated with ECG (12.5μM, 25μM or 50μM) for 24 hours or left untreated. Implantations, resorptions, and surviving fetuses were analyzed. The percentage of implantations represents the number of implantations per number of transferred embryos multiplied by 100. The percentage of resorptions or surviving fetuses signifies the number of resorptions or surviving fetuses per number of implantations multiplied by 100. *$p < 0.01$ versus the ECG-free group; †$p < 0.001$ versus the ECG-free group. Values are presented as means ± standard deviation (SD). (B) Placental weights of 40 recipient mice were measured. Values are presented as means ± SD. (C) Weight distribution of surviving fetuses on day 18 post coitus. Surviving fetuses were obtained by embryo transfer of control and ECG-pretreated blastocysts (320 total blastocysts across 40 recipients). *$p < 0.001$ versus the ECG-free group. Values are presented as means ± SD.

blastocyst stage reduced embryo implantation and the potential for post-implantation development.

## Discussion

During the complex and precisely orchestrated embryonic developmental process, chemical or physical injury can affect normal development and lead to malformation or miscarriage of the embryo. Thus, it is important to establish the possible teratogenic effects of various agents, including natural chemical compounds contained in food, such as ECG. Previous studies have shown that ECG induces apoptosis in various cancers, including human bladder cancer cells and human prostate cancer DU145 cells [19,36], and inhibits growth

and triggers apoptosis in HNSCC and human colon carcinoma LoVo cells [9,37]. These findings indicate that ECG has chemopreventive and/or chemotherapeutic effects on human cancers by decreasing cell viability and/or increasing cell apoptosis. In the present study, we investigated whether ECG adversely affects the blastocyst stage of mouse embryos and subsequent early post-implantation embryonic development. Preliminary data showed that ECG treatment for 24 hours induced apoptosis in mouse blastocysts (Figure 1). Based on this finding, we analyzed the effects of ECG on embryonic development by incubating blastocysts in medium containing 12.5μM, 25μM or 50μM ECG for 24 hours. Our results indicated that ECG treatment decreased cell viability in mouse blastocysts via apoptosis (Figures 1 and 2). Treatment of mouse blastocysts with 50μM ECG induced apoptosis (ninefold greater than the control level), as shown by the TUNEL assay data (Figure 1). Dual differential staining results showed that ECG-induced cell loss and apoptosis occurred primarily in the ICM (Figure 2).

The TE arises from the trophoblast at the blastocyst stage, and develops into a sphere of epithelial cells surrounding the ICM and blastocoel. These cells contribute to the placenta and are required for development of the mammalian conceptus [38], indicating that reduction in the TE cell lineage may suppress implantation and embryonic viability [25,39]. However, in our experiments, ECG induced cell apoptosis specifically in the ICM and not the TE, which may be indicative of deleterious effects on embryonic attachment and outgrowth *in vitro* or rate of implantation *in vivo* (Figures 2–4). Previous studies have shown that a reduction of at least 30% in the number of cells in the ICM is associated with a high risk of fetal loss or developmental injury, even in cases where the implantation rate and TE cell numbers are normal [40]. Additionally, the ICM cell number is essential for proper implantation, and reduction in the ICM cell lineage may decrease embryonic viability [25,39]. Although apoptosis is responsible for eliminating unwanted cells during normal embryonic development, this process does not normally occur at the blastocyst stage [41,42]. Excessive apoptosis before or during the blastocyst stage is likely to delete important cell lineages, affecting embryonic development and potentially leading to miscarriage or embryonic malformation [43]. Our observation that ECG treatment reduced the cell number and promoted apoptosis in the ICM of mouse blastocysts but had no effect on the TE led us to investigate the possibility that ECG induces mortality and/or developmental delay in post-implantation mouse embryos *in vitro* and *in vivo* (Figure 2). ECG-treated blastocysts displayed decreased embryonic development and increased embryonic death *in vitro* and implantation *in vivo* (Figures 3 and 4). Further studies are required to assess the effects of ECG on embryo development in animals during pregnancy.

A previous study [44] has shown that 7.4 g of tea polyphenol can be extracted from 100 g of dry tea leaves. If five cups of tea are drunk per day (2 g of dry tea leaves per cup), the intake of tea polyphenol will be about 740 mg per day [44]. However, the present study used higher concentrations of ECG (50μM) than those observed in human blood to induce apoptosis and impair embryonic development; because mouse embryos develop much quicker than those of humans, it might not be practical to assess the long-term effect of ECG in mice, especially at the concentrations detected in human blood. To evaluate the possible hazardous effects of ECG on embryonic development, the present study used higher concentrations of ECG than those observed in human blood in order to induce a cellular response in a relatively short period of time. In summary, we have shown for the first time that ECG induces cell apoptosis in the ICM of mouse blastocysts, leading to decreased embryonic development and viability. These findings indicate that ECG is an injury risk factor for normal mouse embryonic development. Although future studies will be required to elucidate the possible teratogenic actions of ECG on human embryogenesis, our present findings provide important new insights into potential ECG-induced safety risks for embryonic development.

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