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RESEARCH ARTICLES

(−)-Epigallocatechin-3-gallate induces apoptosis in human endometrial adenocarcinoma cells via ROS generation and p38 MAP kinase activation☆

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Abstract

(−)-Epigallocatechin-3-gallate (EGCG), the major polyphenol in green tea, has been shown to inhibit carcinogenesis of various tumor types. The aim of this study was to elucidate the antiproliferative potential of EGCG and its mechanism in human endometrial cancer cells (Ishikawa cells) and primary endometrial adenocarcinoma cells. The antiproliferative effect of EGCG was evaluated by cell viability assay. Apoptosis was measured by annexin/propidium iodide staining. Reactive oxygen species (ROS) generation was measured by using 2',7'-dichlorofluorescin diacetate dye. Expression of mitogen-activated protein kinases, proliferation and apoptotic markers were measured by immunoblot analysis. EGCG was found to inhibit proliferation in Ishikawa as well as in primary endometrial adenocarcinoma cells and effectively down-regulated the expression of proliferation markers, i.e., estrogen receptor α, progesterone receptor, proliferating cell nuclear antigen and cyclin D1. EGCG also decreased the activation of ERK and downstream transcription factors fos and jun. EGCG caused apoptotic cell death accompanied by up-regulation of proapoptotic Bax and down-regulation of antiapoptotic protein Bcl2. EGCG induced the cleavage of caspase-3 and poly(ADP-ribose) polymerase, the hallmark of apoptosis. EGCG significantly induced the ROS generation as well as p38 activation in Ishikawa cells, which appeared to be a critical mediator in EGCG-induced apoptosis. The apoptotic effect of EGCG and the p38 activation were blocked by pretreatment of cells with the ROS scavenger *N*-acetylcysteine. EGCG reduced the glutathione levels, which might be responsible for enhanced ROS generation causing oxidative stress in endometrial cancer cells. Taken together, these results suggest that EGCG inhibits cellular proliferation via inhibiting ERK activation and inducing apoptosis via ROS generation and p38 activation in endometrial carcinoma cells.

Keywords: (−)-Epigallocatechin-3-gallate; Endometrial cancer; Apoptosis; Reactive oxygen species

1. Introduction

Catechins are the key components of green tea that exert antineoplastic properties. Among them, (−)-epigallocatechin-3-gallate (EGCG) is found as the active component in terms of cancer chemopreventive potential [1]. Due to the lack of significant toxicity in normal cells, EGCG has gained much attention as a cancer preventive agent for humans. There are ample evidences that show potential chemotherapeutic efficacy of EGCG against cancers of the skin, lung, breast, colon, liver, stomach and prostate [2,3]. EGCG acts as a pleiotropic substance and influences various mechanisms that are involved in carcinogenesis such as mutation, cell proliferation, cell invasion and apoptotic cell death [4,5]. It addition to this, EGCG has been found to be effective for treatment of endometriosis [6]. Recently, it has been found that EGCG effectively inhibits proliferation and induces apoptosis in rat ELT3 uterine leiomyoma cells in vitro and in nude mice model. [7,8]. On this basis, we hypothesized that EGCG may have antiproliferative activity in endometrial adenocarcinoma cells and may also represent a promising therapeutic agent in the treatment of endometrial cancer.

Endometrial cancer is the most common gynecologic malignancy in the United States, with increasing incidents in Asian countries, too [9,10]. Due to absence of an effective adjuvant therapy, uterine cancer has a poor prognosis, and therefore, efforts are continuing in search of appropriate and effective therapy against this malignancy. Several plant products, i.e., isoflavonoids, have been identified for their preventive effects on estradiol-related endometrial carcinogenesis [11,12]. These isoflavonoids have been found to exert inhibitory effect on endometrial carcinogenesis through a cytokine- and estrogen-receptor-mediated pathway [12]. The only report by Dann et al. (2009) [13] showed that resveratrol and EGCG significantly reduced the vascular endothelial growth factor (VEGF) secretion in primary endometrial carcinoma cell culture. However, the detailed anticancer effects of EGCG on endometrial carcinoma have not been elucidated so far. In the present study, we have demonstrated the antiproliferative effect of EGCG on endometrial cancer cell line and primary endometrial adenocarcinoma cells. We tried to explore the mechanism of antiproliferative action of EGCG by studying apoptosis, reactive oxygen species (ROS) generation and mitogen-activated

☆ Conflicts of interest: none.

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protein kinase (MAPK) activation in endometrial cancer cells. Our study is the first to report the apoptosis-inducing activity of EGCG in endometrial adenocarcinoma cells.

2. Materials and methods

2.1. Chemicals

(−)-Epigallocatechin-3-gallate was purchased from Calbiochem, Merck; [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (MTT), fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) and *N*-acetylcysteine (NAC) were purchased from Sigma. All other reagents, media, buffered solutions and solvents were of the highest grade commercially available.

2.2. Cell culture

Human endometrial cancer cell line Ishikawa was purchased from European Collection of Cell Cultures, and normal cell line HEK-293 was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C in an atmosphere of 5% CO_2 in phenol red minimum essential medium (MEM)/Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) (Invitrogen , Carlsbad, CA, USA), 100 U/ml penicillin, 100 U/ml streptomycin and 100 U/ml neomycin, (Sigma Chemical Co., St. Louis, MO, USA). Prior to experiments, cells were cultured in phenol-red-free MEM/DMEM supplemented with 10% charcoal-stripped FBS.

2.3. Primary cell culture of human endometrial cancer

Human endometrial carcinoma samples were collected in the operating room of the Department of Obstetrics and Gynecology, Chhatrapati Shahuji Maharaj Medical University, Lucknow. A specific informed consent was obtained from each patient, and the study was approved by the local Ethics Committee. The cell isolation was done as described previously [14] with minor modifications. Briefly, endometrial tissue was collected immediately after surgery in DMEM, crushed in 1-mm pieces and incubated with 1 mg/ml collagenase and 2 mg/ml DNase for 2 h at 37°C with periodic mixing. Digested tissue was mechanically dissociated by slow pipetting and resuspended in 2 ml of DMEM. Digested tissue in DMEM containing 2% antibiotic–antimycotic solution was then centrifuged at least three times. Pellet was collected and poured on the top of 8 ml DMEM containing 2% antibiotic–antimycotic solution. Then digested tissue was allowed to sediment by gravity for 5–10 min. This process was repeated at least three times. Finally, cells were resuspended in DMEM containing 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and 2% of antibiotic–antimycotic solution (Sigma-Aldrich, USA) and then transferred into culture flasks (75 cm^2, Corning, USA). Cells were incubated at 37°C with 5% CO_2. Prior to experiments, cells were cultured in phenol-red-free DMEM supplemented with 10% charcoal-stripped FBS and 1% antibiotic–antimycotic solution.

2.4. Cell proliferation assay

Cell viability was determined by MTT assay. Cells were seeded in 96-well plate at a density of 3×10^3 cells/well and allowed 24 h for attachment. The Ishikawa cells were treated with 50-, 75-, 100-, 125- and 150-μM concentration of EGCG (Calbiochem, Merck) for 48 h and 72 h, whereas primary endometrial cancer cells were treated with 25-, 50-, 75-, 100- and 125-μM concentration of EGCG for 48 h. At the end of incubation, 100 μl of 0.5 mg/ml MTT was added to the cells and incubated for 2 h at 37°C. Following incubation, supernatants were removed, and 100 μl of dimethyl sulfoxide (DMSO) was added. The formazan crystals formed inside the viable cells were solubilized in DMSO, and the optical density was read with Microquant (Biotech, USA) at 540 nm. The IC_{50} values for EGCG was determined by Compusyn software. The experiments were performed three times with five replicates in each.

2.5. Annexin-V/propidium iodide (PI) labeling and flow cytometry assay for apoptosis

Annexin-V binding is indicative of early apoptosis. Human endometrial Ishikawa cells (2×10^5cells/ml) were cultured in six-well plates and treated with EGCG (100, 125 and 150 μM) in a dose-dependent manner for 48 h. In another experiment, the effect of NAC on EGCG-induced apoptosis was studied on both Ishikawa cells and primary endometrial cancer cells. For this, cells were treated with: (i) EGCG (125 μM for Ishikawa and 100 μM for primary culture cells) for 48 h, (ii) NAC for 2 h followed by EGCG for 48 h, and (iii) NAC alone for 2 h. At the end of incubation, cells were collected by trypsinization and washed with phosphate-buffered saline (PBS). Adherent and nonadherent cells were probed with florescein isothiocynate (FITC)-conjugated Annexin-V and PI (Sigma) for 15 min. The staining profiles were determined with FACScan and Cell-Quest software. The experiments were performed three times.

To elucidate whether the apoptotic effects were real, we also analyzed the apoptotic effects at earlier time points, i.e., 6, 12 and 24 h after the treatment of EGCG in Ishikawa cells.

2.6. ROS generation

Human endometrial Ishikawa cells were seeded (2×10^5 cells/well) into six-well plate and maintained overnight in phenol-red-free media containing 10% stripped FBS. Cells were treated with EGCG (100, 125 and 150 μM). Another group of cells was prepared where pretreatment of NAC (10 mM) was done for 2 h followed by EGCG (125 μM) for 48 h. A total of 400 μM H_2O_2 was used as a positive control. After 48 h, cells were collected by trypsinization and resuspended in PBS. DCFH-DA dye (1 μg/μl) was then added in each sample and incubated for 30 min at 37°C in the dark with continuous shaking. The oxidative burst (hydrogen peroxide) was detected using a FACScan flow cytometer (BD Biosciences, USA) with excitation and emission settings of 488 and 530 nm, respectively. The experiments were performed three times.

2.7. Glutathione (GSH) level detection by 5-chloromethylfluorescein diacetate (CMFDA) dye

For GSH measurement, human endometrial Ishikawa cells were seeded (10^6 cells/well) into six-well plate and maintained overnight in phenol-red-free media containing 10% stripped FBS. Cells were treated with EGCG (125 μM) for 48 h and NAC (10 mM) for 2 h. After 48 h, cells were collected by trypsinization and incubated for 30 min at 37°C in the dark with medium containing 10 μM GSH-reactive dye CMFDA (Invitrogen). Cells were then resuspended in fresh medium and maintained in the same conditions for 30 min. One hundred microliters of each group was then transferred into black 96-well plate, and fluorescence was detected by using fluorescence microplate reader (Biotron India) at excitation and emission settings of 485 and 535 nM, respectively [15]. The experiments were performed three times.

2.8. Western blot analysis

Endometrial cells were treated with EGCG at different concentrations. At the end of incubation, cells were lysed in lysis buffer (Tris–HCl 50 mM, NaCl 150 mM, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 μM sodium orthovanadate) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined by using Bradford assay [16]. Equal amounts of protein were separated by gel electrophoresis and then transferred to Immuno-Blot PVDF membrane (Millipore). The membrane was blocked with 5% skimmed milk and then incubated with primary antibodies specific for bax, bcl-2 (1:2000, Santa Cruz Biotechnology); proliferating cell nuclear antigen (PCNA), cyclin D1, estrogen receptor α (ERα), progesterone receptor (PR), (1: 5000, Santa Cruz Biotechnology); c-jun and c-fos

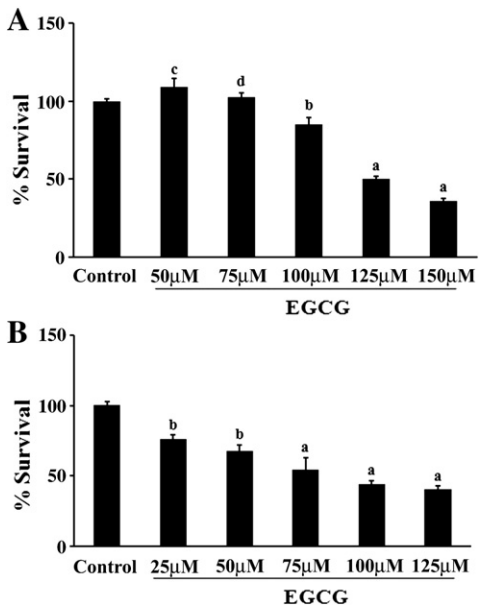

Fig. 1. Effect of EGCG on cell viability of human endometrial Ishikawa cells and primary human endometrial carcinoma cells. Ishikawa cells were treated with varying doses of EGCG, i.e., 50, 75, 100, 125 and 150 μM, for 48 h (A). Primary human endometrial carcinoma cells were treated with EGCG at 25-, 50-, 75-, 100- and 125-μM concentration for 48 h (B). Cell viability was determined by MTT assay. The percentage of viable cells was calculated as the ratio of treated cells to the control cells. Values are means±S.E., $n=5$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

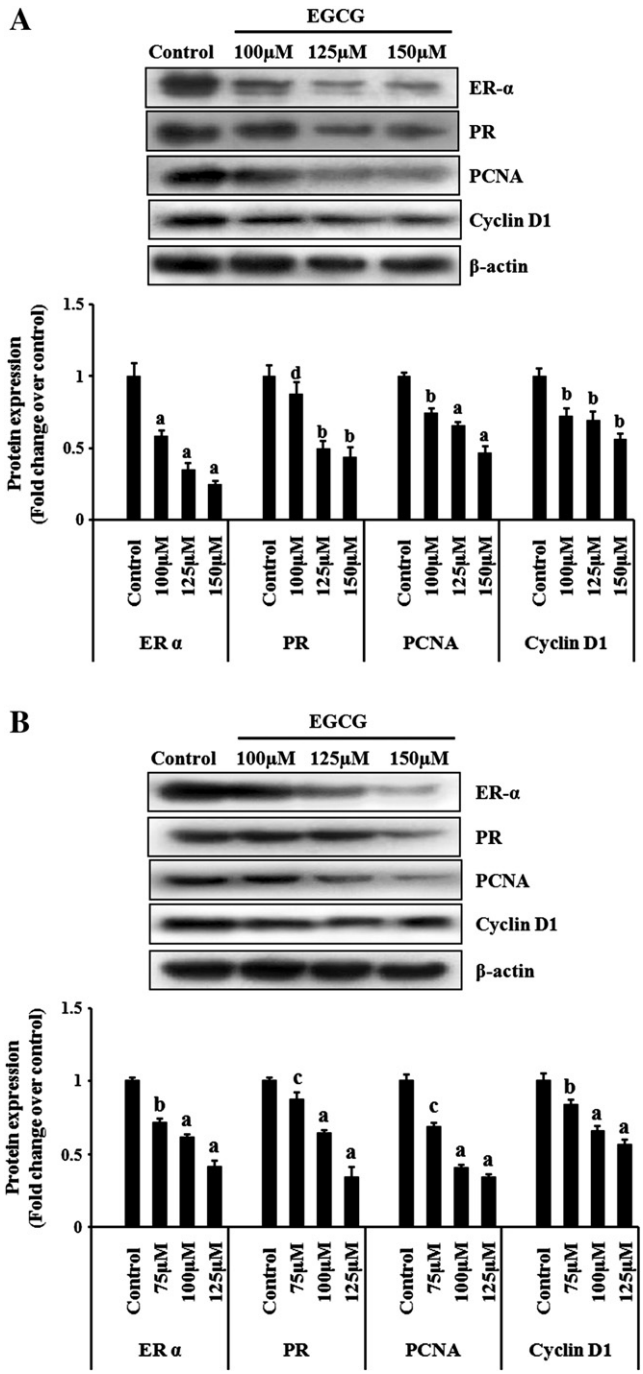

Fig. 2. Effect of EGCG on expression of ERα, PR, PCNA and cyclin D1 in human endometrial Ishikawa cells and primary human endometrial carcinoma cells by Western blotting. Ishikawa cells were treated with 100, 125 and 150 μM of EGCG (A); primary cancer cells were treated with 75, 100 and 125 μM of EGCG (B) for 48 h. Thirty micrograms of protein was loaded in each lane. β-Actin was used as internal control to correct loading error. Densitometric quantitation of protein expression levels is shown as fold changes. Results are expressed as mean±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

(1:1000, BD biosciences); cleaved caspase-3 (1:1000, Cell Signalling Technology); cleaved poly(ADP-ribose) polymerase (PARP) (1: 5000, Sigma); p-ERK, ERK, p-c-jun-NH2-kinase (JNK), JNK, p-P38 and P38 (1:2000, Santa Cruz Biotechnology) separately overnight at 4°C. The membrane was then washed and incubated with a secondary peroxidase-conjugated antibody for 1 h. Antibody binding was detected using enhanced chemiluminescence detection system (GE Healthcare). After developing, the membrane was stripped and reprobed using antibody against β-actin (1:10,000 Sigma-Aldrich) to confirm equal loading. Each experiment was repeated three times to assess for consistency of results. Quantitation of band intensity was performed by densitometry using Quantity One software (v.4.5.1) and a Gel Doc imaging system (Bio-Rad).

2.9. Statistical analysis

Each experiment was repeated three times, and results were expressed as mean±S.E.M. for the number of experiments indicated. Statistical analysis was performed using analysis of variance with Newman–Keuls test. $P<.05$ values were considered significant.

3. Results

3.1. Effect of EGCG on proliferation of human endometrial Ishikawa cells and human primary endometrial carcinoma cells

Antiproliferative potential of EGCG in human endometrial Ishikawa cells and primary endometrial carcinoma cells was evaluated by MTT assay. EGCG reduced the viability of endometrial cancer cells in a dose-dependent manner. In case of Ishikawa cells, EGCG significantly decreased cell viability at 100-μM and 75-μM concentration after 48 h ($P<.01$) (Fig. 1A), whereas in human primary endometrial cancer cells, EGCG significantly reduced cell viability even at 25-μM concentration ($P<.01$) (Fig. 1B). The IC_{50} value of EGCG in Ishikawa cells was found to be 132 μM (Fig. 1A) and 123 μM after 48 h and 72 h (Suppl. Fig. 1A), respectively, whereas in human primary endometrial cancer cells, it was calculated to be 91.3 μM at 48 h.

Cytotoxic profile of EGCG in normal HEK293 cells was also checked, and it was found that the IC_{50} value of EGCG in HEK293 cells was more than 200 μM, which suggested that EGCG was nontoxic for normal cells (Suppl. Fig. 1B).

3.2. Effect of EGCG on proliferation markers in human endometrial Ishikawa cells and primary endometrial carcinoma cells

For analysis of proliferation markers such as ERα, PR, PCNA and cyclin D1, Western blot analysis was performed (Fig. 2). The densitometric analysis showed that compound EGCG reduced the expression of these proliferation markers by ~76% in ER, ~57% in PR, ~54% in PCNA and ~44% in cyclin D1 expression at 150 μM concentration in Ishikawa cells ($P<.01$, $P<.001$). In primary cell culture, EGCG caused a reduction of ~59% in ER ,~66% in PR,~ 44% in PCNA and ~66% in cyclin D1 expression at 125-μM concentration ($P<.001$) (Fig. 2).

3.3. EGCG induces apoptosis in human endometrial cancer cells

We next assessed the effect of EGCG on the induction of apoptosis in Ishikawa cells by Annexin-V–FITC and PI staining.

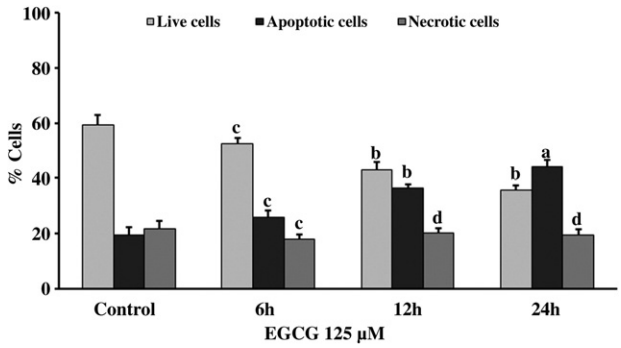

Fig. 3. EGCG induced apoptosis in Ishikawa cells in a time-dependent manner. Cells were treated with 125-μM concentrations of EGCG for 6, 12 and 24 h and analyzed by flow cytometry. AV^+/PI^- — intact cells; AV^-/PI^+ — nonviable/necrotic cells; AV^+/PI^- and AV^+/PI^+ — apoptotic cells. Values are means±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

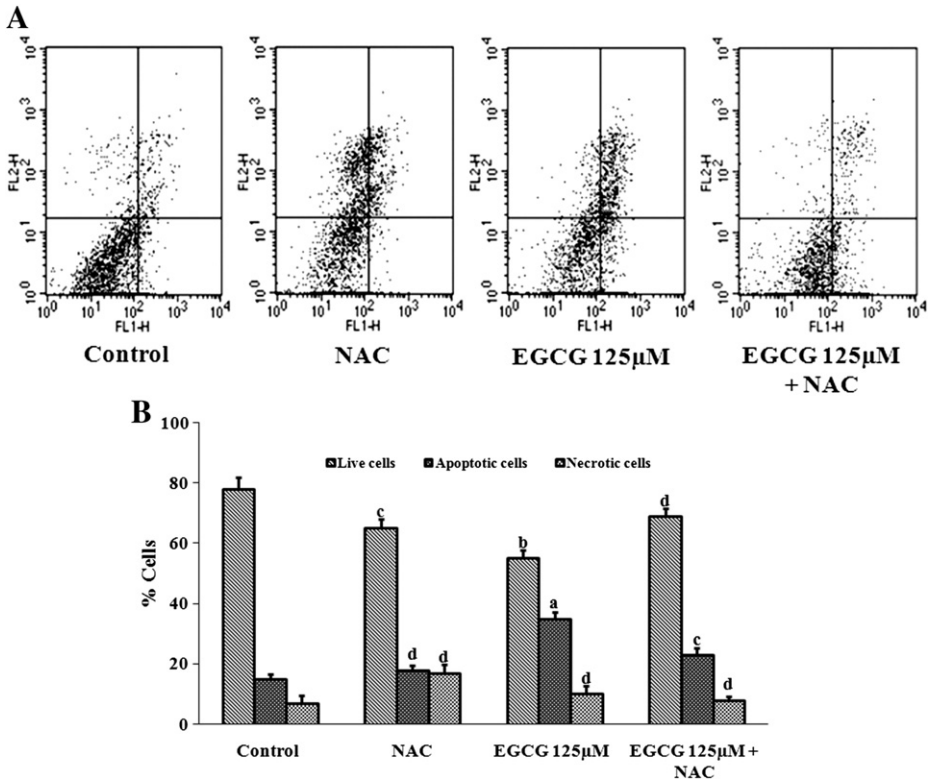

Fig. 4. Effect of EGCG on apoptosis in Ishikawa cells in the presence of ROS inhibitor NAC by flow cytometric analysis. Groups are control (no treatment), EGCG (125 μM), NAC 10 mM for 2 h and EGCG (125 μM)+NAC (10 mM). After 48 h, cells were stained with annexin V and PI dye. AV^+/PI^- — intact cells; AV^-/PI^+ — nonviable/necrotic cells; AV^+/PI^- and AV^+/PI^+ — apoptotic cells. Values are means±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

EGCG-treated Ishikawa cells were examined by FACS assay to determine the total DNA content of each cell. EGCG significantly increased the percentage of apoptotic cells [annexin V (+) ve] in dose-dependent manner ($P<.01$ to $P<.001$), and no significant change was observed in necrotic cells (PI stained) when compared with control (Suppl. Fig. 2).

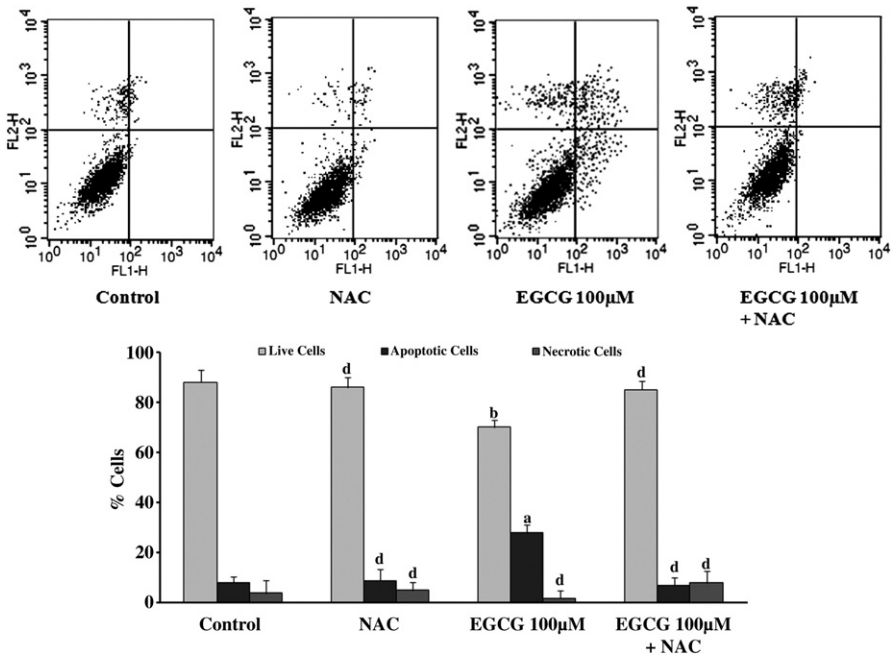

Fig. 5. Effect of EGCG on apoptosis in human primary endometrial cancer cells in the presence of ROS inhibitor NAC by flow cytometric analysis. Groups are control, EGCG (100 μM), NAC(10mM) for 2 h and pretreatment of NAC (10mM) for 2 h followed by EGCG (100 μM) treatment . After 48 h, cells were stained with annexin V and PI dye. AV^+/PI^- — intact cells; AV^-/PI^+ — nonviable/necrotic cells; AV^+/PI^- and AV^+/PI^+ — apoptotic cells. Values are means±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

To ascertain whether the EGCG-induced cell death was actual, we performed the experiment to analyze the effect of EGCG in time-dependent manner at 6 h, 12 h and 24 h. A significant induction in apoptosis was observed at all time intervals evaluated, i.e., 6, 12 and 24 h (Fig. 3).

3.4. NAC reduces EGCG-induced apoptosis in human endometrial cancer cells

Further, to see whether apoptosis is induced by ROS, we used NAC which is a ROS scavenger. For this, cells were divided into four different groups for various treatments — (a) control, (b) NAC for 2 h (c), EGCG and (d) pretreatment with NAC for 2 h followed by EGCG for 48 h — and percentage of apoptosis was determined. Results showed that the pretreatment of NAC significantly lowered the EGCG-induced apoptosis in Ishikawa cells (Fig. 4) as well as in primary endometrial cancer cells (Fig. 5). It was also observed that the NAC-treated group significantly induced necrosis as compared to control ($P<.05$) in Ishikawa cells. This may be due to induction of tumor necrosis factor α [17] which in turn caused necrosis [18].

3.5. EGCG induces ROS generation in human endometrial Ishikawa cells

As observed in the above experiment in which NAC reduced EGCG-induced apoptosis, we examined the production of ROS which triggers apoptosis. Ishikawa cells were treated with EGCG at 100-, 125- and 150-μM concentration for 48 h. At the end of incubation, ROS generation was observed using ROS-sensitive probe DCFH-DA. ROS levels were significantly induced in a dose-dependent manner ($P<.01$ to $P<.001$) (Suppl. Fig. 3). In a parallel experiment, pretreatment of 10 mM NAC along with EGCG (125 μM) significantly reduced ROS generation in Ishikawa cells as compared to the EGCG-treated group (Fig. 6A and B), whereas treatment with NAC alone did not alter ROS level in comparison to control.

3.6. EGCG reduces and NAC increases GSH level in human endometrial Ishikawa cells

NAC is a well-known precursor for GSH and also helpful for cell to decrease oxidative stress directly. Therefore, we analyzed the effect of EGCG on GSH levels. In the presence of NAC, GSH level was increased significantly ($P<.01$) as compared to cells of control group, whereas in EGCG-treated cells, GSH levels were reduced significantly ($P<.05$) as compared to those in control group (Fig. 6C).

3.7. Effect of EGCG on MAPKs

The activation of p38, JNK and ERK was detected 48 h after the treatment of EGCG in Ishikawa cells (Fig. 7A). It was observed that activation of p38 was increased significantly in a dose-dependent manner and reached maximum at 150 μM of EGCG ($P<.05$ to $P<.001$). EGCG was also found to decrease the activation of ERK in the same manner ($P<.01$ to $P<.001$), whereas activation of JNK was unaltered in EGCG-treated groups as compared to control.

3.8. Effects of ROS scavenger NAC on p38 activation

Increased ROS levels were observed to exert the activation of stress kinase p-38 [19]. To examine the function of EGCG-induced ROS in mediating the activation of p38 in Ishikawa cells, the expression of p38 protein in each of the treatment groups was analyzed. Activation of p38 in Ishikawa cells was observed after 150-μM EGCG treatment for 48 h (Fig. 7C). Pretreatment of cells with

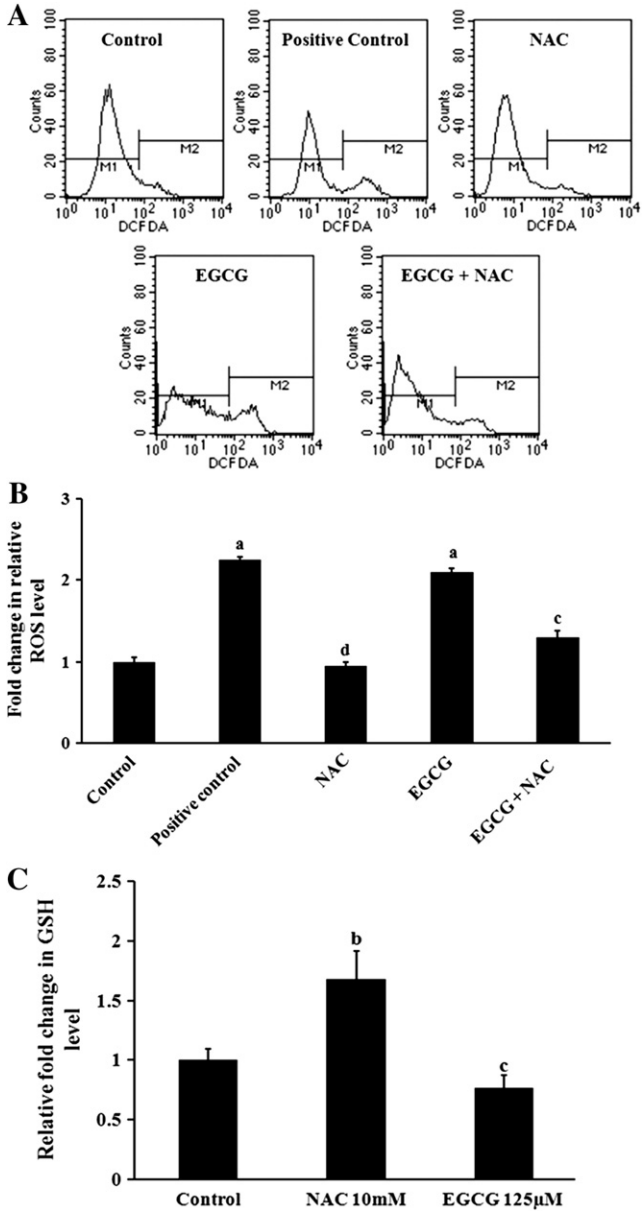

Fig. 6. (A & B) Effect of EGCG on ROS generation in Ishikawa cells in the presence of ROS inhibitor NAC. Groups are control (no treatment), positive control (H_2O_2 400 μM), EGCG 125 μM, NAC 10 mM for 2h, EGCG (125 μM)+NAC (10 mM). After 48 h, cells were stained with DCFH-DA dye (1 μg/μl) for 30 min at 37°C in the dark with continuous shaking. The oxidative burst (hydrogen peroxide) was detected using a FACScan flow cytometer (BD Biosciences,USA) with excitation and emission settings of 488 and 530 nm, respectively.Values are means±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control. (C) Effect of NAC on GSH level in Ishikawa cells. Ishikawa cells were treated with NAC (10 mM) for 2 h and EGCG (125 μM) for 48 h. Cells were stained with 10 μM of CMFDA dye for 30 min at 37°C. Fluorescence intensity was taken at excitation and emission settings of 485 and 535 nm, respectively. Results are expressed as mean±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

10 mM NAC for 2 h effectively abolished the EGCG-induced phosphorylation of p38, indicating the requirement of ROS for activation of p38.

3.9. Effect of EGCG on expression of transcription factors c-jun and c-fos

c-jun and c-fos are the downstream transcription factor of ERK signaling. Western blot analysis was performed to see the expression of c-jun and c-fos in EGCG-treated cells. A significant reduction in the

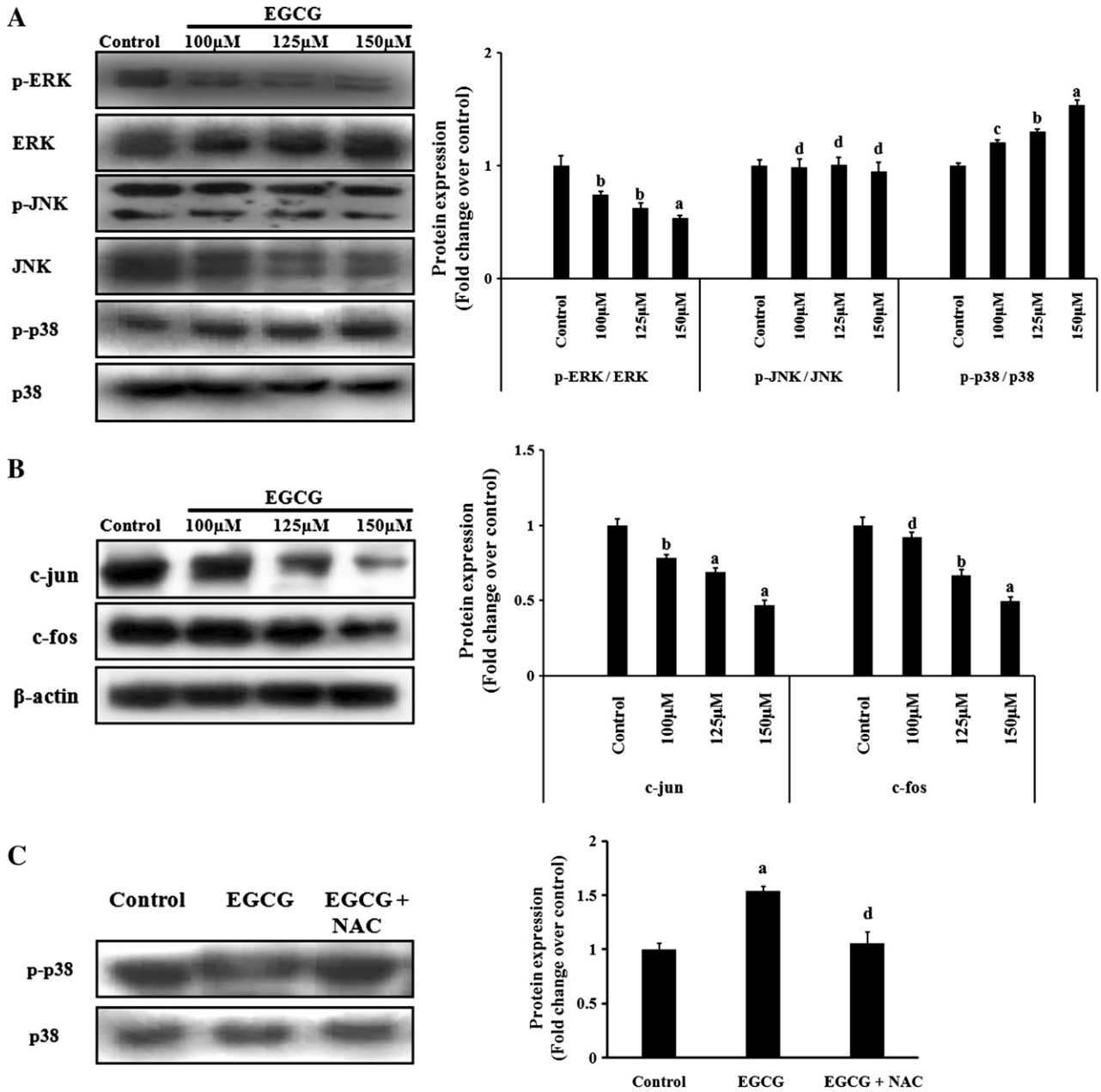

Fig. 7. Effect of EGCG on MAPK subfamilies. (A) Effect of EGCG on the activation of ERK, JNK and p-38 in human endometrial Ishikawa cells by Western blotting. Cells were treated with various concentrations of EGCG (100, 125 and 150 μM) for 48 h. (B) Effect of EGCG on expression of c-jun and c-fos transcription factors in human endometrial Ishikawa cells. (C) Effect of NAC on EGCG-induced activation of p-38. Cells were treated with 150 μM EGCG alone, and in another group, there was conjoint treatment of EGCG (150 μM) with NAC (10 mM). Densitometric quantitation of protein expression levels is shown as fold changes. Results are expressed as mean±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

expression of these transcription factor was observed (Fig. 7B). The densitometric analysis showed that EGCG reduced the expression of c-jun and c-fos by ~53% and ~45 % at 150-μM concentration ($P<.001$).

3.10. Effect of EGCG on cleaved caspase-3 and cleaved PARP in Ishikawa cells

For analysis of apoptotic markers such as cleaved caspase-3 and cleaved PARP, Western blot analysis was done. EGCG induced the expression of cleaved caspase-3 and cleaved PARP in a dose-dependent manner. Densitometric analysis showed that EGCG increased the expression of cleaved caspase-3 and cleaved PARP by about twofold at 150-μM concentration in Ishikawa cells ($P<.001$) (Fig. 8A).

3.11. EGCG increases Bax/Bcl-2 ratio in human endometrial cancer cells

To investigate whether EGCG induced apoptosis as a consequence of the altered expression of members of Bcl-2 family proteins, Western blot analysis for Bcl-2 and Bax was performed. The results revealed that EGCG caused significant induction of proapoptotic marker Bax and down-regulation of the antiapoptotic gene Bcl-2 in both primary adenocarcinoma cell culture and Ishikawa cells. The Bax/Bcl-2 ratio was found to increase by ~3.3-fold and ~2.8-fold in

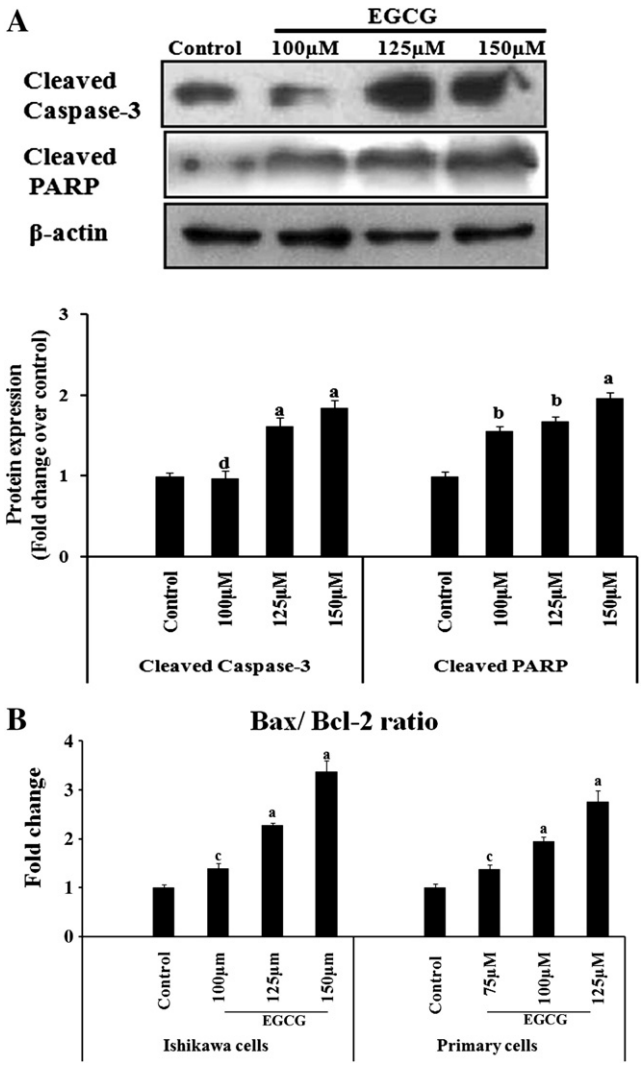

Fig. 8. (A) Effect of EGCG on expression of cleaved caspase-3 and cleaved PARP in human endometrial Ishikawa cells by Western blotting. (B) Effect of EGCG on ratio of bax and bcl-2 in human endometrial Ishikawa cells and primary human endometrial carcinoma cells as analyzed by Western blotting. Cells were treated with various concentrations of EGCG for 48 h. Thirty micrograms of protein was loaded in each lane. β-Actin was used as internal control to correct loading error. Densitometric quantitation of protein expression levels are shown as fold changes. Results are expressed as mean±S.E., $n=3$. P values are (a) $P<.001$, (b) $P<.01$, (c) $P<.05$ and (d) $P>.05$ vs. control.

Ishikawa cells and human primary endometrial cancer cells ($P<.001$and $P<.001$), respectively (Fig. 8B).

4. Discussion

Although tea leaves have several other polyphenolic compounds, EGCG has been found to be the more effective chemopreventive agent among them [1]. A sufficient number of studies have shown that EGCG could inhibit proliferation and induce apoptosis in various types of cancerous cells [20–23]. Various polyphenolic compounds such as resveratrol, genistein and daidzein have shown promising results for the treatment of endometrial carcinogenesis in *in vitro* model and *in vivo* mice model [11,12,24]. However, not many studies with EGCG on chemopreventive potential against endometrial cancer are available so far. Although it has been reported that EGCG inhibits VEGF secretion in endometrial tumor cells [13], the detailed mechanism of action of EGCG in endometrial cancer cells is not yet explored.

In the present investigation, we have examined the growth inhibitory effect of polyphenolic compound EGCG on endometrial adenocarcinoma cells. Results suggested that EGCG decreased cell viability in human endometrial Ishikawa cells and primary adenocarcinoma cells in both time- and dose-dependent manner. EGCG also reduced the expression of ER-α, PR, cyclin D1 and PCNA in Ishikawa and primary human endometrial carcinoma cell cultures, which are associated with cell cycle and tumor progression. EGCG induced apoptotic cell death which was accompanied by up-regulation of Bax and down-regulation of Bcl-2 protein in endometrial carcinoma cells. In our study, EGCG induced caspase-3 expression which subsequently increased the cleaved PARP and caused apoptosis.

ROS generation plays a key role in the apoptosis induced by various anticancer agents [25,26]. Although EGCG is reported to be antioxidant [27], it possesses significant prooxidant activity, too, as it has been observed that copper-mediated oxidation of EGCG leads to the formation of polymerized polyphenols [28]. We observed that EGCG caused the induction of ROS which may be considered as one of the mechanisms inducing apoptosis in endometrial carcinoma cells. High levels of ROS can also induce apoptosis by triggering mitochondrial permeability transition pore opening, release of proapoptotic factors and activation of caspase-9 [29,30]. It was interesting to note that pretreatment with NAC significantly reduced the EGCG-induced apoptosis in both Ishikawa cells as well as in primary human endometrial cancer cells. In the case of Ishikawa cells NAC-treated group, significant necrosis was observed possibly due to induction of tumor necrosis factor α [17] which in turn caused necrosis-induced cell death [18]. Further, to understand whether GSH is related to the EGCG-induced changes in ROS levels in endometrial cancer cells, changes in GSH levels were assessed. In the presence of NAC, a precursor of GSH that protects cells from ROS via reducing the oxidative stress [31], GSH levels were increased significantly, whereas in the EGCG-treated group, GSH levels were significantly reduced. There is a possibility that the EGCG-induced stimulation of ROS levels could result due to depletion in GSH levels, thus causing oxidative stress in endometrial cancer cells. Possibly, EGCG might regulate the degradation of GSH, in addition to inhibiting its biosynthesis. Similar observations are reported in 3T3-L1preadipocytes and adipocytes cells treated with NAC and EGCG [32]. Our findings suggest that EGCG might be responsible for causing oxidative stress due to excess of ROS generation by reducing the GSH levels in endometrial cancer cells, although the detailed mechanism of GSH-mediated action needs to be explored.

The MAPKs, a family of serine/threonine kinases, are also involved in apoptosis and cell survival [19,33]. In our study, to find out whether ROS generation induced by EGCG has a role in regulating the MAPKs, we analyzed the expression of MAPKs p-38, JNK and ERK in Ishikawa cells. Although the expression of both p-JNK and total JNK also appeared to be decreased, the ratio of p-JNK/JNK was not changed significantly in EGCG-treated cells. Further, ERK activation and the expression of downstream transcription factors c-fos and c-jun were found to be decreased, which in turn might be responsible for the reduced cellular growth and proliferation of the endometrial cancer cells. The increased ROS level is known to induce the activation of stress kinase p-38 [34]. The treatment of Ishikawa cells with ROS inhibitor NAC reduced the EGCG-induced activation of p-38, suggesting that p-38 activation was triggered by ROS generation. Thus, the activation of p-38 might be involved in the induction of apoptosis induced by EGCG in endometrial cancer cells [35].

ROS has been reported to activate Akt through a PI3-K-dependent mechanism [36,37], which in turn activates apoptosis signal-regulating kinase 1 (ASK1)/MAPK signaling pathway [38]. Eventually, ASK1 induces cell death by activating several proapoptotic signaling proteins, including JNK and p38 MAPK [39]. It may be postulated that ASK1 is involved in ROS-mediated activation of p38 in endometrial cancer cells

caused by EGCG, although experimental evidences would be required to prove this hypothesis in endometrial cancer.

Taken together, the results of our study have characterized the antiproliferative effects of naturally occurring phytocompound EGCG, the component of green tea, on human endometrial cancer cells. EGCG showed potential to inhibit ERK activation and subsequently reduced the expression of target genes involved in cell proliferation and cell survival. EGCG induced apoptosis via generation of ROS, which leads to activation of p-38, caspase-3 and the cleavage of PARP. In conclusion, the results suggest beneficial chemopreventive effects of EGCG on endometrial adenocarcinoma cells. Further studies are needed to confirm these findings in animal models in vivo to demonstrate the efficacy of EGCG in treating endometrial cancer.

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