# Apoptotic Effect of Green Tea Polyphenol (EGCG) on Cervical Carcinoma Cells

Farhan Asif Siddiqui, M.D.,[1*] Mohammed Naim, M.D.,[1] and Najmul Islam, Ph.D.[2]

*The objective of this study was to probe the apoptotic effect of green tea polyphenol epigallocatechin-3-gallate (EGCG) on cervical carcinoma cells. This study was conducted in Departments of Pathology and Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, India over a span of one and half years from January 2005 to August 2006. Caspase-3 assay was performed on monocytes isolated from cervical carcinoma patients and cultured with EGCG; cytosmears and sections from cervical carcinoma tissue cultured with EGCG were prepared for the morphological evidence of apoptosis. EGCG in a dose of 5 μg/ml and 10 μg/ml increased the caspase-3 levels in human cells. Cytosmears and sections from cervical carcinoma tissue cultured with EGCG showed better differentiation and increased number of apoptotic cells as compared to non EGCG controls. The number of such cells was increased more in 48 hours than in 24 hours. EGCG in a dose of 5 μg/ml and 10 μg/ml promoted apoptotic preparedness of human cells and induced apoptotic change in cervical carcinoma cells.* Diagn. Cytopathol. 2011;39:500–504. 

*Key Words:* green tea; cervical carcinoma; apoptosis; EGCG; caspase-3

In the recent years, green tea has emerged as a potential chemopreventive agent for many cancers, probably by inducing apoptosis in cancer cells. The most important polyphenol of green tea is epigallocatechin-3-gallate (EGCG).[1] In light of various studies being carried out globally, this work focuses investigation on inducing apoptosis by an economical natural antioxidant namely EGCG, a green tea polyphenol in cervical carcinoma cells.

[1]Department of Pathology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India
[2]Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India
*Correspondence to: Farhan Asif Siddiqui, M.D., Department of Pathology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India. E-mail: farhan414@rediffmail.com
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## Materials and Methods

This study was conducted in the Departments of Pathology and Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh over a period of one and half years from January 2005 to August 2006. Ninety-seven cytologically diagnosed patients of squamous cell carcinoma-cervix were initially selected for study. Cervical tissue and blood sample was taken under aseptic conditions from these patients. Cervical tissue was divided into two parts. One part in formalin was taken to Histopathological section of the Department of Pathology for confirmation of diagnosis, while other part of cervical tissue was washed with buffered normal saline to remove debris and transferred to a vial containing RPMI-1640 culture medium, which along with the blood sample were taken to the Department of Biochemistry for research study. Five histopathologically confirmed biopsies of non-neoplastic ectocervical lesion along with blood sample from 10 healthy comparable females were taken to serve as controls for serum values.

Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors and cervical cancer patients were isolated by density gradient sedimentation on Ficoll-Paque separation medium. By cytostaining, PBMC were comprised of 90% nonadherant lymphocytes and 10% adherent monocytes. Thereafter, PBMCs ($5 \times 10^6$ cells/well) were added in 12-well tissue culture plates (Costar Corp. Cambridge, MA) in complete RPMI-1640 medium, and were subsequently incubated at 37°C, 5% $CO_2$ for 1–2 hours for adherence, washed to remove nonadherant cells and rested for overnight in RPMI-1640 medium having 2% autologous serum. Then, the adherent monocytes were cocultured for 24 hours with varying doses of EGCG (1, 2, 5, and 10 μg/ml). Cultures devoid of EGCG served as control. Also, some cultures were pretreated with Z-VAD-FMK (R and D Systems, Europe) which is a known inhibitor of caspase-3. Following 24 hours, the above cells were lysed for 30 minutes at 4°C in 0.5 ml of protein lysis buffer, which was prepared using MLB

 

buffer (50 m*M* Mops, pH 7.0, 250 m*M* NaCl, 5.0 m*M* EDTA, 0.1% NP40, and 1.0 m*M* DTT) supplemented with protease inhibitors (1.0 m*M* PMSF, 5.0 μg/ml leupeptin, 10 m*M* NaF, 5.0 m*M* Na-pyrophosphate, 1.0 m*M* Na-orthovanadate, and 20 m*M* β-glycerophosphate). Thereafter, the suspension was centrifuged at 20,000 rpm for 15 minutes at 4°C and supernatants were stored at −20°C. Protein concentration was determined and the culture supernatants were stored at −20°C until use.

Caspase-3-related protease activity in cell lysates was determined with the help of Caspase-3 cellular activity assay kit plus (Biomol, USA). The effect of Caspase-3 inhibitor (Z-VAD-FMK) was also noted in the assay. The cleavage of the substrate was monitored spectrophotometrically at 405 nm and the activities were calculated according to the instruction of the manufacturer.

The cervical tissue in the department of Biochemistry was further divided into five parts. Tissues were kept in separate vials containing 1 ml RPMI-1640 medium, 2 m*M* HEPES buffer, and 2% autologous serum. Vials were treated with 1, 2, 5, and 10 μg/ml of EGCG, respectively, in different vials and taken as test samples while the one without EGCG was taken as control sample. They were then incubated for 24 hours at 37°C.

The vials were then taken to the department of Pathology. A 0.5 ml of the supernatant from the culture medium suspension after shaking nonvigorously for 10 minutes was taken and centrifuged at 500 revolutions per minute (rpm) for 5 minutes using Shandon Cytospin 4. The cytocentrifuged smears were stained using Hematoxylin and Eosin (H&E) and Papanicolaou stain. To the remaining tissue in test and control vials, 1 ml of RPMI-1640 culture medium were added with EGCG (test) and without EGCG (control), incubated for next 24 hours and processed for cytocentrifuged smears as were done earlier.

The remaining tissue (test and control) at 48 hours were fixed in buffered formol saline. After fixation, the tissues were processed in the histokinete and then they were transferred to a mould (Leuckhart's L pieces) filled with molten wax. Long ribbons of thin paraffin sections (3–5 μ in thickness) were cut by rotator microtome (Shandon Finesse 315) and stained with H&E.

Histopathological examination of the first part of the cervical tissue showed that out of 97 cases, 69 cases were cervical carcinoma of poorly differentiated squamous type without dyskeratosis and parakeratosis. These 69 cases were further selected for the main study while the rest 28 cases were excluded from the study.

## Results

The activity of caspase-3 in EGCG treated and untreated monocytes extracted from cervical carcinoma patients were measured in order to probe that whether EGCG induces apoptosis or not. Caspase-3 assay showed that the cells treated with EGCG have significantly higher activity of caspase-3 in comparison to cells not treated with EGCG (Fig. 1). As already known that caspase-3 is one of the key enzymes involved in apoptosis, these findings do indicate that raised caspase-3 levels in EGCG treated cells are due to the apoptotic effect of EGCG leading to increased levels of caspase-3.

The cytosmears prepared from non-neoplastic lesions showed that EGCG in a dose of 5 μg/ml as well as 10 μg/ml enhanced keratinization process in comparison with non-EGCG, control sample. No apoptotic cell was seen in cytosmears on cultured non-neoplastic ectocervical epithelium incubated with EGCG (Fig. 2).

The cytosmears prepared from neoplastic cervical biopsy treated with 1 μg/ml and 2 μg/ml showed no change as compared with control. The cytopathological findings in the test samples treated with 5 μg/ml as well as 10 μg/ml EGCG for 24 and 48 hours, in comparison with the control (Fig. 3) from the same patient (without EGCG) showed:

1. Better morphological appreciation of nuclear and cellular outlines; nuclear and cell contours were more rounded than the control smear cells. This change was seen in 25–40%.
2. Nuclear chromatin in the test samples appeared relatively compact and condensed in 15–20% in comparison to the control smears, and 10–15% of number of cells showing nucleoli were decreased.
3. 20–25% of cells showed increased eosinophilic cytoplasm from 24 to 48 hours in comparison to the control, with increased number of apoptotic as well as the keratotic cells (Fig. 4).

H&E stained sections rarely showed appreciable apoptotic bodies in the control sample while the test samples were positive for apoptotic changes showing isolated apoptotic cells (in most cases) and apoptotic cells in well defined groups (Figs. 5a and b).

## Discussion

Cervical cancer is the second most common cancer after breast cancer in females worldwide. In the year 2005, over 500,000 new cases of cervical cancer were diagnosed and about 260,000 women died of the disease.[2]

A number of agents are known to have apoptotic effects on the tumor cells and therefore deemed to be an aid in the treatment of many malignancies. These agents include *N*-(4-hydroxyphenyl) retinamide,[3] canthexanthin,[4] indole-3-carbinol,[5] genistein,[6] tamoxifen,[7] capsaicin,[8] sulindac,[9] indomethacin,[9] deguelin,[10] vitamin E succinate,[11] etc. In recent years, many studies have been conducted on green tea. Few have reported that green tea prevents the conversion of premalignant to malignant lesion.[12,13]

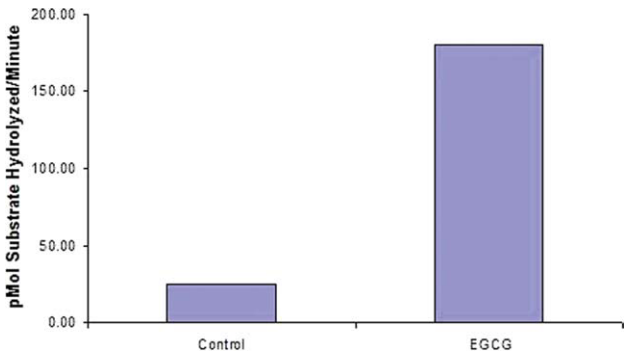


**Fig. 1.** Cell lysates showing increased caspase-3 levels in EGCG treated cells as compared with untreated cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

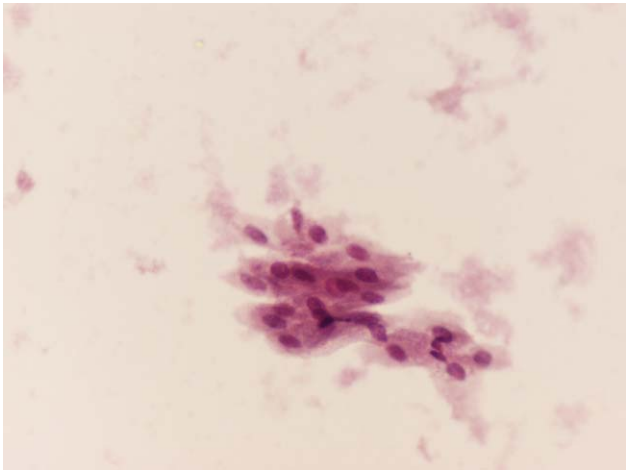

**Fig. 2.** Cytosmears from EGCG treated non-neoplastic ectocervical tissue culture showing well differentiated squamous cells (H&E, ×500). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

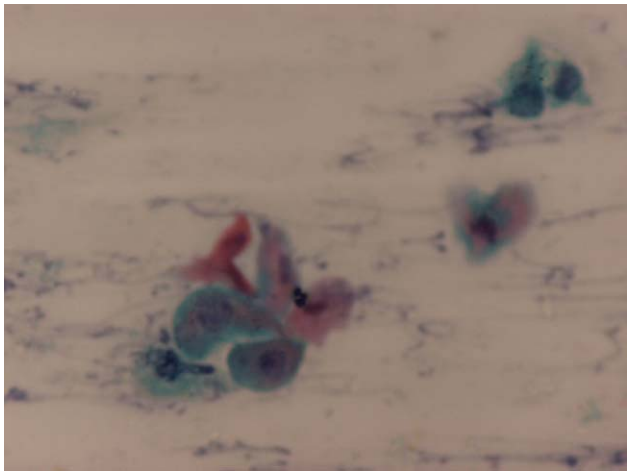

**Fig. 3.** Cytosmears from cervical carcinoma cells after 48 hours (control) showing pleomorphic malignant cells with irregular nuclei and basophilic cytoplasm (Papanicolaou, ×500). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

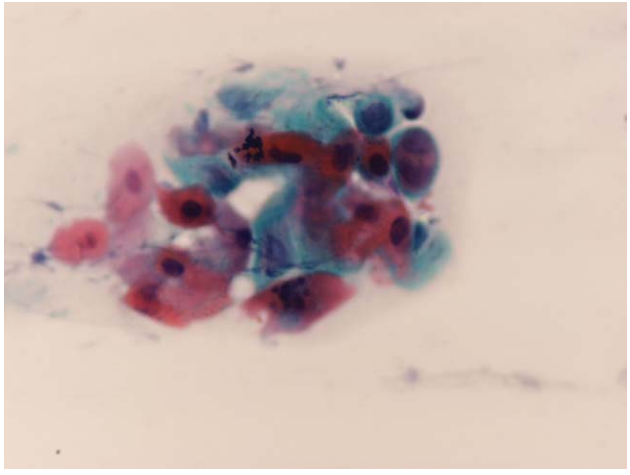

**Fig. 4.** Cytosmears from EGCG treated cervical carcinoma cells after 48 hours showing better nuclear and cellular outlines, increased eosinophila of cytoplasm and increased number of apoptotic cells (Papanicolaou, ×500). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Most of the green tea polyphenols are flavonols, commonly called as catechins. Some of the major green tea catechins are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), epicatechin, gallocatechin, and catechin. EGCG is the major component of the polyphenols in the green tea and is considered the most significant active compound.[1]

The chemopreventive effects of green tea are observed to target specific cancer types like prostate,[14] breast,[15] ovary,[16] urinary bladder,[17] lung,[18] stomach,[19] pancreas,[20] colorectum,[20] and esophagus.[21] In our study, we probed the chemopreventive apoptotic effects of EGCG on cervical carcinoma cells.

Caspases have been recognized as the key molecules in the apoptotic response. A cascade of molecular events has to be initiated in different ways to activate the caspases which further leads to apoptosis. The basis for changes in the apoptotic cell morphology is caspase-mediated cleavage of the target proteins. Out of the 14 different caspase identified, caspase-3 is activated during most apoptotic process and is believed to be the main effective caspase.[22]

The appearance of the active form of caspase-3 in the cell is a late event in biochemical cascade in the apoptotic pathway but precedes morphological alterations at the light microscopic level. Detection of caspase-3 enables detection of apoptosis even before the morphological changes of apoptosis appears and is therefore considered a specific marker of apoptosis.[23] Presently we measured the levels of caspase-3 in mononuclear cells extracted from blood of cervical carcinoma patients and cultured as test with EGCG and control without EGCG. In our study, we found that levels of cas-

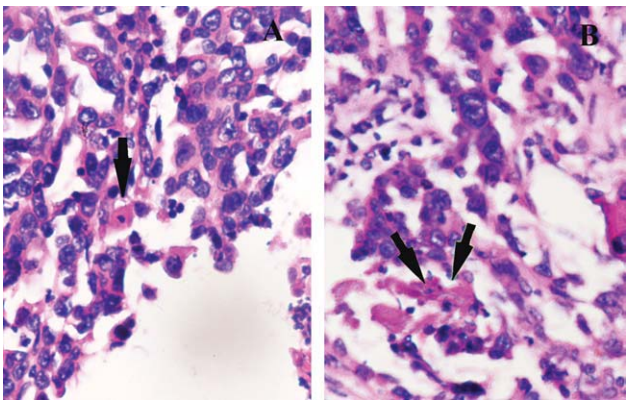


**Fig. 5.** (a,b): Section from EGCG treated cervical carcinoma cells after 48 hours showing apoptotic body (↑) (H&E, ×500). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pase-3 were significantly raised in cells treated with EGCG as compared to control, indicating that EGCG increased the apoptotic preparedness of the human cells.

Balasubramanian et al.,[24] in their study on human epidermal keratinocytes reported that EGCG neither promoted cleavage of procaspase-3 nor poly (ADP-ribose) polymerase (PARP), and concluded that EGCG treatment enhanced differentiation of cells and not promoted apoptosis in the normal keratinocytes. Our study was in accordance with findings of Balasubramanian as normal keratinocytes treated with EGCG showed better differentiation of cells and did not show apoptosis while cervical cancer cells treated with EGCG showed definite evidences of apoptosis.

Ahn et al.[25] studied the effects of EGCG in HPV-16 associated cervical cancer cell line. They observed that EGCG was growth inhibitory and observed significant number of apoptotic cells after 24 hours at dose 100 μm EGCG. Induction of apoptotic cells was insignificant at 35 μm EGCG. In further studies, Ahn et al.[26] studied EGCG in the form of ointment or capsule in HPV cervical lesions, untreated patients were taken as control. Seventy-five percent of the ointment-treated and 50% capsule treated-patients showed improvements.

Spinella et al.[27] studied effects of EGCG on ovarian carcinoma cell line and observed inhibition of factors governing cell proliferation, invasion, angiogenesis, endothelial growth and proteinase activation indicative of decreased cancer cell growth and progression.

Feugang et al.[28] studied apoptotic chemopreventive effects of EGCG in cultured immortalized cervical epithelial cells and cervical cancer cells. They found significant increase in apoptosis in EGCG and cactus mixed exposed cells at a dose of 50 μg/ml.

In our study, cytosmears from the non-neoplastic ectocervical biopsy culture with 5 μg/ml and 10 μg/ml EGCG showed only keratinization promoting effects of EGCG with no apoptotic change supporting the findings of Balasubramanian et al.[24] in normal squamous cells. Cytosmears prepared from cancer patients showed increased keratotic activity showing increased number of keratinized and anuclear squames in comparison to control. The number of such cells markedly increased at 48 hours in comparison with 24 hours in EGCG treated cultures. The tumor cells showed better cellular and nuclear outlines, better cell contours, condensation of chromatin and increased eosinophilia of cytoplasm in comparison to control at 24 as well as at 48 hours. Thus, the cytosmears from cancer biopsy culture with 5 μg/ml and 10 μg/ml EGCG showed well defined apoptotic changes. Apoptotic cells could be clearly distinguished by nuclear condensation, rounded contours with deep eosinophilia of the cell cytoplasm as against immature polymorphic nuclei with nucleoli and basophilic cytoplasm of neoplastic cells.

Histopathological sections from EGCG-treated test samples showed evidences of apoptosis, showing one apoptotic cell per 2–3 high power fields to more than one dispersed apoptotic cells in a single high power field. Occasionally, apoptotic cells were seen in groups implying foci of apoptotic changes. Dyskeratotic or parakeratotic changes as visible in cytocentrifuged smears were rarely visible in the tissue sections from the test samples. It could suggest that EGCG enhanced keratotic process in the cells at peripheral surface of biopsy causing desquamation. In view of the observation that EGCG promotes keratinization and not apoptosis in keratinocytes,[24] the present observations are exciting that EGCG induced apoptotic changes in the morphologically diagnosable cytocentrifuged smears from biopsy shake suspension and tissue section from culture vitalized biopsy directly from squamous cell carcinoma cervix. Why cervical squamous carcinoma cells (CSCC) desquamated from biopsy surface in cytosmears show keratanization, like in non neoplastic ectocervical biopsy while those deeper in the biopsy sections show apoptosis only, require further studies. The difference could be due to direct effect of EGCG on surface and indirect or diffusing effect in depth of biopsy.

Thus, this study shows the chemopreventive apoptotic effect of green tea polyphenol EGCG in cervical carcinoma cells. However, the exact mechanisms of the cancer preventive effects of green tea are yet to be clearly understood and demonstrated, requiring further researches.

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