Original paper

Comparative efficacy of epigallocatechin-3-gallate against H_2O_2-induced ROS in cervical cancer biopsies and HeLa cell lines

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Aim of the study: Antioxidants play an important role in maintaining physiological homeostasis. Recent literature emphasises the potential therapeutic effects of natural antioxidants that play anti-inflammatory and antioxidant effects applicable in preventing oxidative stress-induced injury, which characterises their pathogenesis. The goal of this study was to evaluate the protective role of EGCG on the HeLa cell line and cancerous cells.

Material and methods: The HeLa cell line and cervical cancer biopsies (CCB) were treated with varying doses of antioxidants to determine their effects. Thereafter, hydrogen peroxide (0–10 nM) – an ROS-generating compound – was co-cultured with varying doses of epigallocatechin-3-gallate (EGCG). The effect of this compound on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity was assessed.

Result: The activity of SOD and GPx was protected significantly in the treatment of EGCG in cervical cancer biopsies and HeLa cell line.

Hypothesis: It is hypothesised that EGCG has free radical scavenging properties.

Conclusions: EGCG protected the activity SOD and GPx equally in cervical cancer biopsies (CCB) and HeLa cell line.

Key words: EGCG, SOD, GPx, HeLa, Cervical Cancer.

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Introduction

Globally, cervical cancer affects approximately 490,000 women each year, resulting in 270,000 mortalities [1]. Interestingly, in recent years the cervical cancer mortality rate has declined in developed countries due to health awareness, screening programs, and advances in therapy. Oxidative stress has been implicated in playing a crucial role in the aging and pathogeneses of a number of diseases, including cancer [2, 4]. Oxidative stress occurs due to an imbalance in pro-oxidant and antioxidant levels [3]. Reactive oxygen species (ROS) are highly reactive and may modify and inactivate proteins, lipids, DNA, and RNA and induce cellular dysfunctions. To prevent free radical-induced cellular damage, the organism has a defence mechanism: the antioxidative system. In this study, we emphasise superoxide dismutases (SOD) and glutathione peroxidise (GPx) activity. EGCG is a constituent of green tea, belonging to the genus *Camellia sinensis,* which is the most widely consumed beverage all over the world after water [6–8]. EGCG is a colourless, astringent, water-soluble, and readily oxidisable antioxidant [5]. Its catechol structure makes EGCG a strong chelator for metal ions [9]. EGCG can bind the transition metal ions, prevent formation of hydroxyl radicals, and thus inhibit exogenous ROS-potentiated tumour invasion [10]. EGCG could inhibit tumour cell invasion by scavenging oxygen radicals. It has high affinity for the lipid bilayers of the cell membrane and can easily enter the nuclei of cancer cells [11]. Because of the polyphenolic structure, EGCG has been shown to exhibit antioxidant properties, free radical scavenging, and chelation abilities [12–15].

As the active role-played by reactive oxygen species (ROS) in cervical cancer is well established, thus an attempt was made to probe a natural compound (EGCG) having antioxidant and anti-inflammatory properties in arresting ROS in cancer cells. Various researchers have extensively carried out such studies on cervical cancer HeLa cell line [16, 17], but no work has been done on cervical cancer biopsies. Thus, the protective role of EGCG was examined on SOD and GPx in cervical cancer biopsies and compared with HeLa cell line.

Material and Methods

All chemicals were obtained from Sigma-Aldrich (Milan, Italy) EGCG purchased from Sigma Chemical Company (St Louis, U.S.A.). Cell culture plates and Dulbecco's modified Eagle's media were from Himedia (India).

Cell culture

The human cervical cancer cell line (HeLa) was obtained from the National Centre for Cell Science, Pune, India. Cervical cancer biopsy was collected from

the Gynaecology Ward of Jawaharlal Nehru, Medical College, Aligarh Muslim University, Aligarh, India. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Hyclone), and antibiotic (Ampicillin) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

In the present study, we examined the activity of glutathione peroxidase and superoxide dismutase in normal biopsies (NB), cervical cancer biopsies (CCB), and HeLa cell line by treating them with EGCG. In another part of our study, we treated the cancer biopsies and HeLa cell line with hydrogen peroxide (H_2O_2), an ROS-producing compound, modulating with varying concentrations of EGCG.

Ethics Statement

This study was approved by the institutional Ethical Committee. All interviewed patients gave written, informed consent.

Preparation of a cell culture from biopsies

Cervical tissue samples were obtained from a total of 10 patients who had already had a positive pap smear test at the Department of Gynaecology, JN medical college, Aligarh. Mean patient age was 50 ±5 years. Tissue samples (about 5 g each) were collected from cervixes identified as normal and tumour specimens, by an expert. The nature of those areas was subsequently confirmed by histopathological evaluation. Tissues were placed in separated sterile 50-ml tubes with ice-cold culture medium and then transferred to a cell culture laboratory on ice.

Isolation protocol

1. In the laboratory culture cabinet, transfer the tissue to a 60-mm Petri dish. Using forceps and scissors, dissect off any fat, blood clots, and connective tissues from tumour tissues.
2. Cut the tissue sample into small pieces with scalpels.
3. Transfer the tissue fragments to a sterile 50-ml centrifuge tube, rinse them vigorously with ice-cold HBSS (contains EGTA that loosens cell junctions via calcium chelating action) and decant the supernatant. Repeat this last step until the solution is clear of blood.
4. Pour off the supernatant, transfer the fragments to a clean 60-mm Petri dish and finely mince the tissue into approximately 1-mm^3 pieces with a scalpel.
5. Re-suspend the small fragments in 25 mL of pre-warmed non-supplemented culture medium and combine it with the collagenase solution (1 mg/ml final concentration) in the incubation vessel. Incubate for 20 minutes at 37°C with gentle stirring.
6. Pass the digested tissue onto the first sieve (100 mm) into a 50-ml centrifuge tube. The same procedure is then applied to the following sieves (70 and 40 mm).
7. Wash the sieved cells by centrifugation (400 g, 5 min at 4°C), and re-suspend the pellet in Hank's buffered salt solution (HBSS). Repeat this process two more times, and then re-suspend the cell pellet in culture medium with supplements. Determine cell number and viability in a Neubauer haemocytometer using trypan blue solution.

Cell culture protocol

1. Seed the isolated cells on collagen-coated 75 cm^2 culture flasks at a density of 5 x 10^4 cells/cm^2 and incubate in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Dulbecco's modified Eagle's medium with nutrient mixture F-12 (DMEM/F-12) and GlutaMAX I™ supplemented with 10% heat-inactivated foetal bovine serum (FBS), penicillin/streptomycin (50 U/ml/50 mg/ml), amphotericin B (2.5 mg/ml), and human transferrin (5 mg/ml).
2. Change the medium 24 hr after initial seeding and at 48-hr intervals thereafter.
3. Allow the cells to grow to 80% confluence before they are sub-cultured or frozen. When cultured as described above, the cells reach confluence approximately 8–10 days after seeding.

Glutathione Peroxidase Assay

Control biopsies, cervical cancer biopsies, and Hela cell line were treated with EGCG (50 µg/ml) and H_2O_2 (10 nM) for 24 hr to estimate the activity of glutathione peroxidase (GPx), which was measured as described [18–20]. Cancerous cell lines were co-cultured for 24 hours with or without EGCG. Thereafter, cells were gently scraped with lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4; 1 mM EDTA; 500 mM PMSF). The cell suspension was homogenised and centrifuged at 10,000 g for 10 minutes at 4°C. Protein concentration in each supernatant was determined by the Bradford method with BSA as the standard, and subjected to GPx activity determination. The reaction mixture (1.0 ml) containing 50 mM potassium phosphate (pH 7.0), 1 mM sodium azide, 2 mM GSH, 1 unit/ml glutathione reductase, 1.5 mM cumene hydroperoxide, and 50 µl of samples was incubated at 25°C for 5 min. The reaction was initiated by the addition of (0.2 mM) NADPH. The kinetic change was recorded spectrophotometrically at 340 nm (37°C) for three minutes. GPx activity was calculated as µmol of NADPH oxidised/minute/mg protein (U/mg protein).

Superoxide dismutase Assay

SOD was measured using a superoxide dismutase assay kit provided by Oxis Research, USA SOD-525. The BIOXYTECH is based on the SOD-mediated increase in the rate of autoxidation of 5, 6, 6a, 11b-tetrahydro-3, 9, 10-trihydroxybenzo[c]fluorene R1 in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [21].

Statistical analysis

Results are expressed as mean ± SD of five individual experiments. Standard deviation (SD) was calculated using Microsoft Excel. A Dunnett multiple comparison test and paired *t*-test were used to compare different formulations, and $p < 0.05$ was considered to be significant.

Results

The effect of H_2O_2 and its protection by EGCG on SOD and GPx activity in control biopsies

Control biopsy cell cultures (CB) treated with varying doses of EGCG (0, 10, 20, 30, 40, and 50 µg/ml) for 24 hr

have shown similar activity for SOD and GPx, so a concentration of 50 µg/ml was selected for study, and we also used a reported dose of H_2O_2 (10 nM). Conversely, the control biopsies cells were treated with H_2O_2, and the activity of SOD and GPx was decreased by 30.22% and 39.85%, respectively. Thereafter, we treated the H_2O_2-induced control biopsies with EGCG (50 µg/ml) for 24 hr, showing that activity of SOD and GPx ameliorated by 22.99% and 29.97%, respectively (Table 1).

The effect of H_2O_2 and EGCG on the SOD and GPx activity in cervical cancer biopsies

The activity of SOD and GPx in cervical cancer biopsies treated with H_2O_2 was suppressed by 38.54% and 57.04%, respectively. Thereafter, we treated the H_2O_2-induced cancer biopsies with EGCG (50 µg/ml) showing that activity of SOD and GPx ameliorated significantly by 117% and 264.2%, respectively (Table 2).

The competitive effect of H_2O_2 and EGCG on the SOD and GPx activity in HeLa cell lines

The activity of SOD and GPx in HeLa cells treated with H_2O_2 was decreased by 52.57% and 58.19%, respectively. Thereafter, we treated the H_2O_2-induced HeLa cells with EGCG (50 µg/ml), which protected the activity of SOD and GPx ameliorates significantly, by 135.55% and 221.53%, respectively (Table 3).

Discussion

Many recent studies indicate that EGCG exerts an inhibitory effect on the activity of several enzymatic and metabolic pathways related to the development and progression of cancer [22]. EGCG is an important cancer chemopreventive agent by virtue of its ability to induce apoptosis in cancer cells and not in normal cells.

Thus, in the present study, EGCG, a natural antioxidant, was employed to explore the potential chemopreventive mechanism in cervical cancer. A variety of antioxidants and chemopreventive agents are cytotoxic to cancerous cells. Cellular growth inhibition by green tea has been established in many tumour cells where EGCG has been used as a prime candidate for mediating this effect. Recently, anti-proliferative and anti-cancer action of EGCG has been reported in cancer cell line [23–25]. A vast variety of naturally occurring substances are known to protect against experimental carcinogenesis. It is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, may have important cancer chemopreventive properties [26]. Some anti-inflammatory chemopreventive agents have been found to suppress growth and proliferation of transformed or malignant cells through induction of programmed cell death or apoptosis [27].

Various researchers have extensively carried out studies on cervical cancer HeLa cell line in multiple directions [16, 17], but no work has been done on cervical cancer biopsies. Thus, the present study is the first involving investigations on the effect of EGCG on cervical cancer biopsies from cervical cancer patients and cervical cancer HeLa cell line. The active role-played by reactive oxygen species (ROS) in cervical cancer is well established, thus an attempt was made in the present study to probe natural compounds having antioxidant properties in arresting ROS in cancer cells.

Superoxide dismutase and glutathione directly react with ROS, and GPx catalyses the removal of hydrogen peroxide [28]. Decreased activity of SOD and GPx indicate the impairment of hydrogen peroxide-neutralising mechanisms [29]. Co-culturing cancerous (cervical) cells with EGCG seems to protect apoptosis or act as an anti-inflammatory. Furthermore, apart from the above, a decrease in GPx activity was observed in cervical cancer biopsies and cervical HeLa cells that were untreated or treated with H_2O_2, thereby correlating with earlier reports that substantial amounts of ROS are generated in cancerous cells due to cellular activation [30]. Surprisingly, amelioration in SOD and GPx activity was observed when EGCG was co-cultured, indicating EGCG to be an effective natural antioxidant combating ROS, generated as a consequence of cellular activation in cancerous cells. When compared to normal biopsies, cervical cancer biopsies as well as HeLa

Table 1. Competitive effect of hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (50 µg/ml) on the SOD and GPx activities in control biopsy (CB) cell cultures after 24 hr

S. No.	SOD	GPx
CB	80.4 ±0.65	84.22 ±2.55
CB + H_2O_2	56.1 ±0.83 (–30.22%)	50.65 ±2.54 (–39.85 %)
CB + H_2O_2 +EGCG	69.47 ±0.91 (+22.99 %)	72.33 ±1.83 (+29.97 %)

Values are expressed as mean ± SEM of 5 experiments, the value in parentheses shows the percentage decrease and increase with respect to the control. The level of significance was p < 0.05

Table 2. Competitive effect of hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (50 µg/ml) on the SOD and GPx activities in cervical cancer biopsy (CCB) cell cultures after 24 hr

S. No.	SOD	GPx
CCB	39.12 ±1.56	37.78 ±1.36
CCB + H_2O_2	22.34 ±1.31 (–38.54 %)	16.23 ±0.73 (–57.04 %)
CCB + H_2O_2 +EGCG	48.51 ±1.15 (+ 117 %)	59.11 ±0.81 (+264.2 %)

Values are expressed as mean ± SEM of 5 experiments; the values in parentheses show the percentage decrease and increase with respect to the control. The level of significance was p < 0.05

Table 3. Competitive effect of hydrogen peroxide (H_2O_2) (10 nM) versus EGCG (50 µg/ml) on the SOD and GPx activities in HeLa cell line after 24 hr

S. No.	SOD	GPx
Hela	40.87 ±0.91	40.76 ±1.60
Hela + H_2O_2	19.35 ±1.139 (–52.57 %)	17.04 ±1.47 (–58.19 %)
Hela + H_2O_2 +EGCG	45.58 ±1.28 (+135.55 %)	54.79 ±1.37 (+221.53 %)

Values are expressed as Mean ± SEM of 5 experiments; the value in parentheses shows the percentage decrease and increase with respect to the control. The level of significance was p < 0.05

cell line exhibited an appreciable H_2O_2-mediated suppression in the SOD and GPx activity. Interestingly, EGCG was found to possess a higher potential ($p < 0.05$) to ameliorate the SOD and GPx activity significantly ($p < 0.05$) in cervical cancer biopsies than in HeLa cell line. The currently available modern medicines for treating cancers are very expensive, toxic, and less effective [31], so it is essential to investigate further, in detail, the agents derived from natural sources for the prevention and treatment of cancer. Voluminous clinical trials are needed to validate the usefulness of this agent either alone or in combination with existing therapy.

In conclusion, EGCG has free radical scavenging properties, which protect the proliferation of cancerous cells and boost the activity of SOD and GPx.

The author declares no conflict of interest.

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