

Protective effects of black tea extract against oxidative DNA damage in human lymphocytes

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Abstract. The aim of the present study was to examine the genoprotective and radioprotective effects of black tea extract (BTE) against the induction of single strand DNA breaks in human lymphocytes subjected to hydrogen peroxide (H₂O₂) or gamma-rays (2 Gy dose). Lymphocytes were incubated with or without different concentrations of BTE (0.005-500 µg/ml) for 30 min, followed by treatment with or without H₂O₂ (0.088 µmol/l) for 5 min. To examine the radioprotective effect of BTE, the lymphocytes were incubated with or without BTE for 30 and 60 min prior to and following *in vitro* irradiation. Oxidative damage to DNA was monitored using a comet assay. BTE at lower concentrations prevented H₂O₂-induced DNA damage. An increase in BTE concentrations resulted in increased formation of single strand DNA breaks. BTE also exerted significant protective effects against gamma radiation-induced total DNA damage in healthy lymphocytes during their 30 or 60 min incubation with BTE prior to or following irradiation. Therefore, the protective effect of BTE against irradiation was time-dependent. The results contribute to the research on potential beneficial effects of natural compounds, such as BTE, in cancer and its protective effects of normal tissue during radiation therapy.

Introduction

Radiation therapy is an important treatment for several diseases, including cancer, in humans (1). The primary obstacle in radiation therapy is the induction of normal tissue injury, resulting in cell death and acquired radioresistance in

cancer cells during radiotherapy (2). Hematological tissue, particularly lymphocytes, is one of the tissues, which is particularly sensitive to radiation (3). Ionizing radiation generates free radicals and reactive oxygen species (ROS). ROS can oxidatively damage biological macromolecules, including lipids, DNA and proteins and, as a consequence, cellular damage occurs resulting in genomic instability and leading to mutagenesis, carcinogenesis and cell death (4). The direct or indirect (free radical-induced DNA damage) interactions of gamma-rays with DNA can cause various lesions, including oxidized purines or pyrimidines, apurinic or apyrimidinic sites, as well as modifications of sugars (5-7). DNA can be attacked by endogenous and exogenous ROS, and by xenobiotics, resulting in DNA damage (8). Hydrogen peroxide (H₂O₂) is a reactive compound, which can generate hydroxyl radicals (·OH) at elevated concentrations via the Fenton reaction. ·OH has a high affinity to DNA causing the strand breaks, and this process can result in DNA instability, mutagenesis and ultimately carcinogenesis (9,10).

The use of natural products with antioxidant activity has gained momentum as possible radioprotectors in previous years due to their advantages, including lower toxicity and reduced cost (11-13). Black tea is rich in polyphenolic compounds, particularly theaflavins, and polyphenolic compounds considerably mitigate the effects of ionizing irradiation at the molecular, cellular and/or tissue level (14-16). However, there remains a lack of information regarding protective effects of black tea extract (BTE) against H₂O₂-induced DNA damage in healthy human lymphocytes.

The aim of the present study was to evaluate the total antioxidant capacity of BTE, and to examine its genoprotective and radioprotective effects against the oxidative DNA damage induced by H₂O₂ and ⁶⁰Co gamma-rays in healthy human lymphocytes. For this investigation, a comet assay was used to detect single strand DNA breaks, and a formamidopyrimidine glycosylase (Fpg)-assisted comet assay was used to detect total DNA damage (single strand DNA breaks and oxidized purines). Fpg creates strand breaks at oxidation-induced DNA lesions, increasing the specificity and sensitivity of the comet assay (17). The present study focused on determining possible protective effects of BTE against radiation injury during cancer therapy.

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Materials and methods

Chemicals and reagents. All solutions and chemicals used in the present study were purchased from Sigma-Aldrich (Irvine, UK).

BTE preparation. The BTE was provided by Dr Ján Keresteš (Molecule of Life, Ltd., Senec, Slovakia). The BTE consisted of a powder (0.1 g), which was dissolved in 10 ml distilled water (45–48°C) and mixed for 24 h using a magnetic stirrer (IKA, Staufen, Germany). The extract was then filtered through filter paper, and stock solution was prepared at a final concentration of 2 mmol/l, which was stored at 4°C. The BTE was diluted in the concentration range of 0.01–1 mmol/l appropriately with distilled water prior to treatment. The content of the active flavonoids was determined by pyrolysis of the product following determination of the individual compounds of the product by spectrophotometry and absorption chromatography (18). The content of active flavonoids in the BTE extract was at least 96%, containing catechins (60–75%), leucoanthocyanidins (25–40%), cyanidins and general flavone derivatives of diols. The complexes of the flavonoids were in mono and oligomeric forms.

Cell preparation. Blood samples were collected from healthy volunteers (n=9) from the antecubital vein. The volunteers included nine females (30–40 years old). Written informed consent was obtained from all volunteers. The study was approved by the ethics committee of the Faculty of Medicine, Comenius University and the University Hospital (Bratislava, Slovakia). Human lymphocytes were isolated from the fresh blood (10 ml) with the anticoagulant EDTA, by adding phosphate-buffered saline (PBS; 10 ml), and then underlaying it with Histopaque (10 ml) prior to centrifugation at 350 x g for 15 min at 4°C. The lymphocytes were separated as a layer at the top of the Histopaque, removed and resuspended in PBS (10 ml). Following centrifugation at 290 x g for 20 min at 4°C, the supernatant was removed. PBS was added to the sediment of lymphocytes and a suspension containing 2.5x10⁵ cells/gel was used for BTE treatment.

Treatment with H₂O₂. The lymphocytes (2.5x10⁵ cells/gel) were incubated with or without different concentrations of BTE (0.005, 0.05, 0.5, 5, 25, 50 and 500 µg/ml) for 30 min in the dark at room temperature. The samples were then centrifuged at 350 x g for 7 min at 4°C, washed with PBS and centrifuged again. The cells (2.5x10⁵ cells/gel) were treated with or without H₂O₂ (0.088 µmol/l for 5 min on ice) in the dark. Control samples were treated with PBS alone, without hydrogen peroxide.

Treatment with ⁶⁰Co gamma-rays. The lymphocytes were divided into two groups: Group A comprised BTE (0.005–500 µg/ml)-pretreated lymphocytes subjected to 2 Gy gamma-irradiation. Group B comprised 2 Gy gamma-irradiated lymphocytes, which were post-treated with BTE (0.005–500 µg/ml).

The lymphocytes with or without BTE pretreatment were isolated and embedded in a Petri dish, covered and irradiated for 2.36 min. Cobalt 60 (Co-60; Theratron Elite 100, Nordion Inc., Kanata, ON, Canada) radiation delivery was performed in

the Radiotherapy Department of St. Elisabeth Cancer Institute, (Bratislava, Slovakia). Radiation was administered at a dose of 2 Gy. The samples remained incubated with BTE at room temperature for 30 or 60 min immediately following or prior to irradiation.

Comet assay. DNA strand breaks and oxidative DNA damage were measured using an alkaline comet assay (19). The cell suspension was added to microscope slides precoated with 1% normal melting agarose. The slides were immersed in cold lysing solution (containing 2.5 mol/l NaCl, 100 mmol/l EDTA and 10 mmol/l of 1% Triton X-100, pH 10) for at least 1 h at 4°C. For the enzyme treatment, the slides were removed from the lysis buffer and incubated with enzyme reaction buffer (40 mmol/l Hepes, 0.1 mol/l KCl, 0.5 mmol/l EDTA and 200 mg/ml BSA, pH 8; Sigma-Aldrich) for 10 min at 4°C. Thereafter, 50 µl formamidopyrimidine glycosylase (Fpg; 1:3,000) was added to the slide and the slides were incubated at 37°C for 30 min. The enzyme treatment control slide was incubated with enzyme reaction buffer only. Following enzyme treatment, the cover slips were removed. The microscope slides were then placed in an electrophoresis tank, and the DNA was allowed to unwind for 40 min in freshly prepared alkaline electrophoresis buffer (containing 0.2 mol/l Na₂EDTA and 5 mol/l NaOH; pH 13). Electrophoresis was conducted at 4°C for 30 min at 25 V and 300 mA. The slides were washed twice at 4°C (10 min each) with neutralising buffer (0.4 mol/l Tris-HCl, pH 7.5). All chemicals were obtained from Sigma-Aldrich. Comets were visualized using fluorescence microscopy Olympus BX-41 (Olympus, Tokyo, Japan) following staining with 4',6-diamidine-2-phenylindole dihydrochloride (Merck, Darmstadt, Germany). The intensity of the comet tail relative to the head reflects the number of DNA breaks and is scored manually. A total of 100 cells from each of two replicate slides were analyzed for each BTE concentration and were classified into five classes, according to the relative intensity of fluorescence in the tail. These classes were scored between 0 and 4, with 0 indicating no damage and 4 exhibiting maximal damage. Therefore, the total score for the 100 comets had a potential range of between 0 (all undamaged) and 400 (all maximally damaged).

The total damage (TD) of the DNA was evaluated according to the following formula:

$$TD = \sum_{i=0}^4 i \cdot N_i$$

Where *i*, represents the class of damage and *N* represents the number of cells in the class. The percentage of DNA damage was calculated according to the following formula: % DNA damage = (TD / 400) x 100.

Total antioxidant capacity of BTE. The total antioxidant status of the BTE was determined using a Trolox Equivalent Antioxidant Capacity (TEAC) method (20). Briefly, ABTS solution (containing 14 mmol/l ABTS and 4.9 mmol/l K₂S₂O₈) (Sigma-Aldrich) was diluted with deionized water until an absorbancy of 0.7±0.02 at 734 nm was reached. To 1,980 µl of ABTS working solution 10 µl BTE sample was

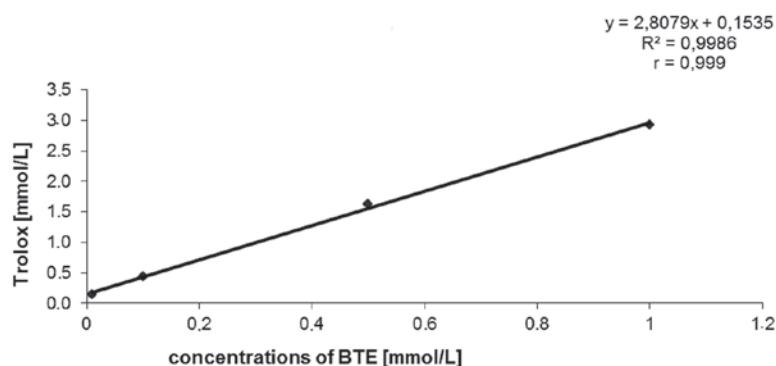


Figure 1. Total antioxidant status of BTE at concentrations of 1, 0.5, 0.1 and 0.01 mmol/l *in vitro*. BTE, black tea extract.

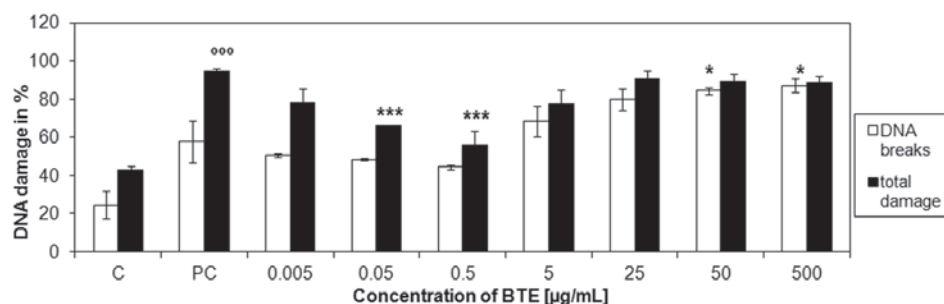


Figure 2. Effect of BTE on single strand DNA breaks and total DNA damage (oxidized purine bases and DNA strand breaks) in human lymphocytes. DNA damage was assessed as DNA strand breaks in cells treated with BTE (0.005-500 µg/ml) without Fpg enzyme, and total damage (total DNA damage) in cells treated with BTE (0.005-500 µg/ml) and Fpg enzyme. Human lymphocytes were preincubated with BTE (0.005-500 µg/ml) for 30 min, prior to exposure to H₂O₂ for 5 min. Results are presented as the mean ± standard error of the mean (n=7). *P<0.05 and ***P<0.001, compared with the PC group. ****P<0.0001, compared with the C group. C, control cells without exposure to H₂O₂ or BTE, PC, positive control cells exposed to H₂O₂ without BTE. BTE, black tea extract; Fpg, formamidopyrimidine glycosylase.

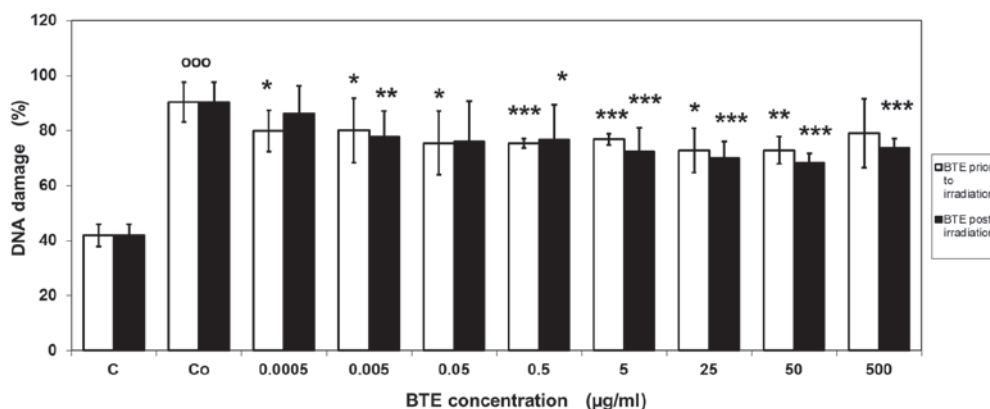


Figure 3. Single strand DNA breaks and total DNA damage (oxidized purine bases and DNA single strand breaks) induced by gamma irradiation in human lymphocytes during 30 min incubation with or without BTE prior to and following irradiation. White columns represent BTE incubation prior to lymphocyte irradiation; black columns represent BTE incubation post-irradiation. The cells were treated with BTE (0.0005-500 µg/ml) and incubated with formamidopyrimidine glycosylase enzyme. Results are presented as the mean ± standard deviation (n=9). *P<0.05, **P<0.01 and ***P<0.001, compared with the Co group. ****P<0.001, compared with the C group. C, control cells without irradiation or BTE treatment. Co, positive control-cells exposed to 60Co gamma-rays (2 Gy) without BTE; BTE, black tea extract.

added and the absorbance (734 nm) was recorded (UV-1700 PharmaSpec, Shimadzu Corporation, Tokyo, Japan) against deionized water at 0 and 10 min. The antioxidant ability of the BTE was determined from the analytical curve with trolox standard (Sigma-Aldrich), a synthetic, hydrophilic form of vitamin E. Total antioxidant capacity was expressed as mmol trolox/l.

Statistical analysis. Results are expressed as the arithmetic mean ± standard deviation of three separate experiments. Each experiment was performed with three parallels. Statistical evaluations were performed using a parametric unpaired t-test. P<0.05 was considered to indicate a statistically significant difference. StatsDirectR v.2.3.7 (StatsDirect Ltd., Sale, UK) was used for the statistical analysis.

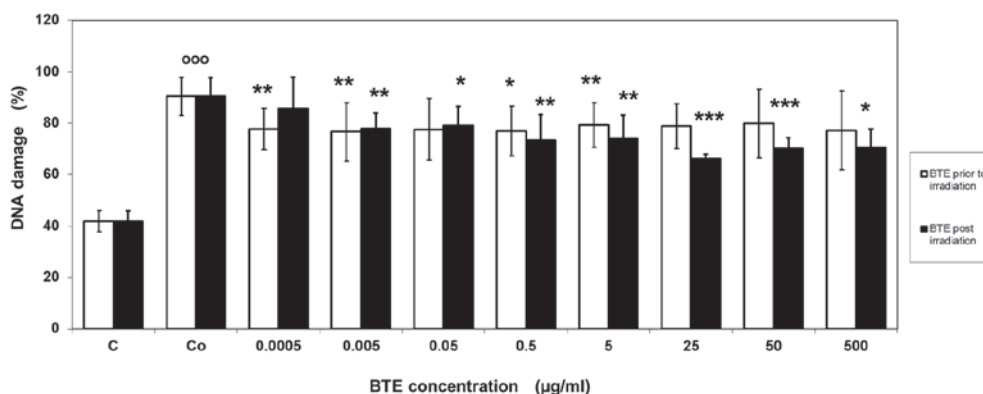


Figure 4. Total DNA damage (oxidized purine bases and DNA strand breaks) induced by gamma-irradiation in human lymphocytes during 60 min incubation with or without BTE prior to or following irradiation. White columns represent BTE incubation prior to lymphocyte irradiation; black columns represent BTE incubation post-irradiation. C, control cells without irradiation or BTE treatment, Co, positive control-cells exposed to ^{60}Co γ -rays (2 Gy) without BTE. The cells were treated with BTE (0.0005–500 $\mu\text{g/ml}$) and incubated with formamidopyrimidine glycosylase enzyme. Results are presented as the mean \pm standard deviation ($n=9$). * $P<0.05$ and ** $P<0.01$, compared with the Co group. ooo $P<0.001$, compared with the C group. BTE, black tea extract.

Results

Total antioxidant status of the BTE. The total antioxidant status of the BTE was determined using the TEAC method, and was found to be concentration-dependent within the concentration range of 0.01 and 1 mmol/l (Fig. 1). A BTE concentration of 1 mmol/l exhibited the same antioxidant ability as a 2.9 mmol/l concentration of trolox, indicating that the antioxidant effects of BTE were almost three times more effective than those of trolox.

Effect of BTE on H_2O_2 -induced DNA damage. The present study investigated the protective effect of BTE against H_2O_2 -induced DNA damage, which was achieved through monitoring single strand DNA breaks and the total DNA damage (oxidized purine bases and DNA strand breaks) using a comet assay and Fpg-assisted comet assay. Fpg is an enzyme, which cleaves DNA at sites with oxidized purine nitrogen bases (21). The total DNA damage (black bars in Fig. 2) in the lymphocytes incubated with H_2O_2 (PC-positive control; $94.5 \pm 1.7\%$) was significantly higher ($P<0.001$), compared with that in the lymphocytes without H_2O_2 (C-control; $43.1 \pm 2.0\%$; Fig. 2). Treatment with the BTE extract reduced the total DNA damage in the human lymphocytes, with the maximum effect observed at a concentration of 0.5 $\mu\text{g/ml}$ ($P<0.001$). BTE at low concentrations proved antioxidant properties by its ability to protect DNA against oxidative damage to purines. At concentrations between 5 and 500 $\mu\text{g/ml}$, the BTE had no significant protective effect against H_2O_2 -induced total DNA damage.

The H_2O_2 -induced single strand DNA breaks, which were determined in the absence of Fpg (white bars in Fig. 2) were not affected by BTE up to a concentration of 0.5 $\mu\text{g/ml}$. However, higher concentrations of BTE, between 5 and 500 $\mu\text{g/ml}$, induced the generation of single strand DNA breaks, thus contributing to the DNA damaging effect of H_2O_2 .

Effect of BTE on ^{60}Co gamma-ray induced DNA damage. The total DNA damage in the lymphocytes irradiated with 2 Gy ^{60}Co gamma-rays ($90.3\% \pm 7.3$) was significantly higher

($P<0.001$), compared with that in the control lymphocytes without irradiation or BTE treatment ($41.8\% \pm 4.2$; Fig. 3).

Incubation of the lymphocytes with BTE extract for 30 min prior to gamma-irradiation significantly reduced the total DNA damage ($P<0.05$) by 10.4% at the lowest concentration of BTE (0.0005 $\mu\text{g/ml}$; Fig. 3). The maximum reduction of DNA damage was observed at BTE concentrations of 0.5 and 5 $\mu\text{g/ml}$ ($P<0.001$). However, the highest concentration of BTE (500 $\mu\text{g/ml}$) had no protective effect on the lymphocytes against 2 Gy ^{60}Co gamma-ray-induced total DNA damage. When the BTE extract was incubated with the human lymphocytes 30 min following gamma-irradiation, the total DNA damage was reduced by 12.6% at a concentration of 0.005 $\mu\text{g/ml}$ BTE. The maximum effect against DNA damage was observed when the concentration of BTE was 25 $\mu\text{g/ml}$ (22%; $P<0.001$).

Lymphocytes from the healthy volunteers treated with BTE for 60 min prior to gamma radiation exhibited reduced levels of total DNA damage, which were 12.7% lower at the lowest BTE concentration (0.0005 $\mu\text{g/ml}$), compared with the control (Fig. 4). The levels of DNA damage did not change significantly within the BTE concentration range. The lymphocytes incubated with BTE for 60 min following gamma-irradiation exhibited the minimum level of DNA damage at a BTE concentration of 25 $\mu\text{g/ml}$ ($P<0.001$; Fig. 4).

Discussion

The present study demonstrated that the BTE had significant protective effects against gamma-irradiation-induced total DNA damage (oxidized purine bases and DNA strand breaks) and H_2O_2 -induced DNA damage in healthy lymphocytes. On examining gamma-irradiation-induced total DNA damage, BTE showed protective effects at all concentrations used in the present study, whereas in H_2O_2 -induced DNA damage, BTE protected DNA only at lower BTE concentrations.

The mechanisms by which tea polyphenols may act remain to be fully elucidated. The present study hypothesized that the inhibition of promutagen activation, inactivation of mutagens and carcinogens, inhibition and scavenging of reactive molecules, modulation of DNA replication or repair,

inhibition of promotion, and inhibition of the invasion and metastasis of tumor cells may be involved. As DNA is the major target of radiation-induced damage, the ability of BTE to prevent radiation-induced DNA damage was investigated in healthy lymphocytes in the present study. BTE exhibited an antioxidative capacity, which was three times higher than that of the trolox antioxidant, determined using a TEAC method *in vitro*. A previous study by Sun *et al* (22) found that black tea and its predominant polyphenols were effective in scavenging 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid and $\cdot\text{OH}$ free radicals *in vitro* in mice. DNA damage can be induced by ROS. H_2O_2 is a reactive compound that, at higher concentrations, can generate $\cdot\text{OH}$ free radicals via the Fenton reaction (23). $\cdot\text{OH}$ can attack DNA at the sugar residue of the DNA backbone, which leads to the generation of single strand breaks, double strand breaks, DNA protein cross-links and DNA instability, mutagenesis and ultimately carcinogenesis (24).

Several studies have demonstrated protective effects of epigallocatechin-3-gallate (EGCG), the predominant polyphenolic component of green tea, on the generation of DNA strand breaks in human lymphocytes (25-27). Therefore, the present study aimed to investigate the effects of BTE on DNA oxidative damage on human healthy lymphocytes, using a comet assay. The results obtained showed that pretreatment of human lymphocytes with BTE for 30 min prior to H_2O_2 treatment caused significant protection against total DNA damage at BTE concentrations of 0.05 $\mu\text{g}/\text{ml}$ and 0.5 $\mu\text{g}/\text{ml}$ ($P < 0.001$). By contrast, BTE concentrations $> 5 \mu\text{g}/\text{ml}$ increased the generation of DNA single strand breaks. Similar results with EGCG have been reported in previous studies (28,29). Johnson and Loo (28) treated human lymphocyte cells with EGCG, and found that treatment with a low concentration (10 $\mu\text{mol}/\text{l}$) decreased H_2O_2 -induced strand breaks, whereas higher concentrations (100 $\mu\text{mol}/\text{l}$) increased DNA damage. Concentration is a factor, which may determine whether tea polyphenols act as antioxidants or pro-oxidants *in vitro* (30). These effects have been suggested to be due to spontaneous H_2O_2 generation by polyphenols in solution present at higher concentrations (31).

Theaflavins present in BTE have also been reported to produce H_2O_2 and induce apoptosis in several cell lines (32-34). Feng *et al* (35) confirmed that theaflavins remain potent antioxidants, which can protect normal rat liver cells against oxidative stress induced by H_2O_2 and *tert*-butyl hydroperoxide, and prevent cellular oxidative DNA damage. Wei *et al* (36) showed that extracts of green tea and black tea enhanced the scavenging of H_2O_2 and quenching of 8-hydroxy deoxyguanosine, suggesting the importance of EGCG in the antioxidant activities of tea extracts.

In the present study, the radioprotective effect of BTE was also investigated, to determine whether BTE treatment for 30 or 60 min prior to or following gamma-irradiation was more effective. BTE exerted the maximum protective effects against gamma-radiation-induced DNA damage in lymphocytes when incubated following irradiation at a dose of 25 $\mu\text{g}/\text{ml}$ for 60 min, and at a dose of 50 $\mu\text{g}/\text{ml}$ for 30 min. Treating lymphocytes with BTE for 30 min (25 $\mu\text{g}/\text{ml}$) and 60 min (0.005 $\mu\text{g}/\text{ml}$) prior to exposure to 2 Gy gamma-radia-

tion resulted in a significant decrease in the protective effects, compared with incubation with BTE following irradiation. Higher concentrations of tea catechins present in the BTE may act synergistically with radiation due to their pro-oxidant activity (37).

Black tea accounts for 80% of the tea consumed worldwide, but its health advantages are less-well investigated compared with those of green tea. Black tea extract exhibited a three-times higher antioxidant capacity compared with trolox antioxidant, as determined by the TEAC method *in vitro*. The black tea extract obtained from *Camellia sinensis* exhibited genoprotective and radioprotective effects against oxidative DNA damage in human lymphocytes, possibly by decreasing oxidative stress due to its antioxidant nature. Protective effects of BTE are concentration dependent. Thus, BTE may be effective in reducing the risk of cancer development.

Ghosh *et al* (38) reported that efficient radioprotection is observed in normal lymphocytes at BTE concentrations as low as 5 $\mu\text{g}/\text{ml}$, which may be assessed in the future as a natural antioxidant supplement during radiotherapy. The ability of black tea to inhibit free radical generation, scavenge free radicals and chelate transition metal ions depends on its antioxidative properties. Black tea, as well as individual theaflavins, can affect the activation of transcription factors, including nuclear factor κB or activator protein-1 (39). AP-1 and NF- κB are known to be important in tumor promoter-induced transformation, tumor promotion, tumor progression and metastasis. Tea polyphenols can act as inhibitors of tumor necrosis factor, and control the development and progression of tumors. Chung *et al* (40) demonstrated that all polyphenols of green and black teas exhibited strong inhibition of cell growth and AP-1 activity. The association between the antioxidant activity of tea polyphenols and the cancer-preventive effects of tea consumption requires further investigation, and additional experiments are required to examine the effectiveness of tea as a genoprotective agent and its underlying mechanism of action.

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