

Effect of Epigallocatechin Gallate on Oxidative DNA Damage in Human Lymphocytes

Sevtap AYDIN^{1*}, Dilek TOKAÇ¹, Nursen BAŞARAN¹, Arif Ahmet BAŞARAN²

¹Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, 06100 Ankara, TURKEY, ²Hacettepe University, Faculty of Pharmacy, Department of Pharmacognosy, 06100 Ankara, TURKEY

Green tea from *Camellia sinensis* is known to contain catechins, dietary polyphenolic compounds associated with a wide variety of beneficial health effects. The widely renowned biological actions of catechins are their antioxidant and free radical scavenging properties. However catechins are also reported to induce oxidative damage through the generation of reactive oxygen species. Among the bioactive chemicals in green tea leaves (-)-epigallocatechin gallate (EGCG) is the most abundant and active. In the present study, the antioxidant capacity of EGCG was determined by the trolox equivalent antioxidant capacity assay and the effect of EGCG on DNA damage induced by H₂O₂ in human lymphocytes were investigated by the standard comet assay and the formamidopyrimidine-DNA-glycosylase (Fpg) modified comet assay. Our results showed that above the concentrations of 0.01 μM EGCG showed significant antioxidant capacity. At all the concentrations studied above 5 μM of EGCG alone significant DNA damage was observed. However, within the concentrations of 0.01-10 μM EGCG significantly reduced oxidative DNA damage induced by H₂O₂. At the concentration of 5 μM EGCG induced Fpg sensitive sites indicating the increased oxidized purine base levels.

Key words: (-)-Epigallocatechin gallate (EGCG), Hydrogen peroxide, Trolox equivalent antioxidant capacity (TEAC) assay, Comet assay, Formamidopyrimidine-DNA-glycosylase (fpg)

İnsan Lenfositlerinde Epigallokateşin Gallat'ın Oksidatif DNA Hasarı Üzerine Etkisi

Camellia sinensis'den elde edilen yeşil çayın sağlık üzerine pek çok faydalı etkileri ile ilişkili diyetel fenolik bileşiklerden olan kateşinleri içerdiği bilinmektedir. Kateşinlerin en iyi bilinen biyolojik etkileri bunların antioksidan ve radikal süpürücü özellikleri olmasıdır. Ancak kateşinlerin reaktif oksijen radikallerin oluşumuna neden olarak oksidatif hasarı indüklediği de bildirilmektedir. Yeşil çayda bulunan biyoaktif kimyasallardan en fazla bulunan ve aktif olan (-)-epigallokateşin gallat (EGCG)'tır. Bu çalışmada EGCG'nin antioksidan kapasitesi troloks eşdeğer antioksidan kapasite deneyi ile ve insan lenfositlerinde H₂O₂ ile indüklenen DNA hasarı üzerine EGCG'nin etkisi standart comet yöntemi ve formamidoprimidin-DNA-glikosilaz (Fpg) ile adapte edilmiş comet yöntemi ile araştırılmıştır. Sonuçlarımız 0.01 μM konsantrasyon üzerinde EGCG'nin anlamlı olarak antioksidan kapasiteye sahip olduğunu göstermiştir. EGCG'nin 5 μM üzerinde çalışılan tüm konsantrasyonlarında anlamlı olarak DNA hasarı oluşturduğu saptanmıştır. Ancak 0.01-10 μM konsantrasyon aralığında EGCG H₂O₂ ile indüklenen oksidatif DNA hasarını anlamlı olarak azaltmıştır. 5 μM konsantrasyonda EGCG artmış okside pürin baz düzeylerinin göstergesi olan Fpg duyarlı bölgeleri indüklemiştir.

Anahtar kelimeler: (-)-Epigallokateşin gallat (EGCG), Hidrojen peroksit, Troloks eşdeğer antioksidan kapasite (TEAK) yöntemi, Komet yöntemi, Formamidoprimidin-DNA-glikosilaz (fpg)

*Correspondence: E-mail: sevtapay@hacettepe.edu.tr; Tel: +90 312 3052178

INTRODUCTION

The renewed interest in natural substances, rather than in synthetic agents has focused attention on plants used as food or spices which are a rich source of bionutrients or bioactive phytochemicals. Also questions concerning the safety of these compounds have encouraged more detailed studies of plant resources. Some aroma extracts and essential oils isolated from plants, formerly considered only as flavours and fragrances are now considered as natural remedies. Catechins are dietary polyphenolic compounds associated with a wide variety of beneficial health effects *in vitro*, *in vivo*, and clinically. The widely renowned biological actions of catechins are their antioxidant and free radical scavenging properties. However catechins are also reported to induce oxidative damage through the generation of reactive oxygen species (1). (-)-epigallocatechin gallate (EGCG) is the main constituent of the catechins polyphenols among the bioactive chemicals in green tea leaves. It is reported to inhibit the growth of acute myeloblastic leukemia cells or induce apoptosis in human cancer cells. It is suggested that EGCG can lower the risk of certain types of diseases and cancers. EGCG has been also reported to have a dual role for anti-oxidation and pro-oxidation (2,3).

Reactive oxygen species (ROS) react with cellular components, causing oxidative damage to critical cellular biomolecules as lipids, proteins, and DNA (4,5). Oxidative DNA damage may involve the breakage in single and double-strands, base modifications, fragmentation of deoxyribose, formation of DNA-protein cross-links as well as abasic sites (6,7). DNA strands breaks in eukaryotic cells can be detected by single cell gel electrophoresis (comet assay), which is commonly used for the assessment of protective effects of antioxidants on DNA damage in intervention studies with and without the addition of the repair enzymes endonuclease-III (Endo III), formamidopyrimidine N-glycosylase (Fpg), to characterize DNA lesions. Fpg initiates the repair of oxidized bases by excising them and cutting the sugar-phosphate backbone of the DNA molecule. Thus additional strand breaks

are induced at the location of oxidized base, causing DNA relaxation and migration. The detection of Fpg-sensitive DNA lesions revealed the presence of oxidized purine bases (8-11).

In the present study, the antioxidant capacity of EGCG was determined using the trolox equivalent antioxidant capacity (TEAC) assay and the modulating effects of EGCG against the oxidative DNA damage induced by H₂O₂ in human lymphocytes were investigated by the standard single cell gel electrophoresis (comet assay) and the Fpg-modified comet assay.

EXPERIMENTAL

Chemicals

The compound EGCG used in the experiments was from Teavigo™/Roche Vitamins Ltd.; the purity of the compound was 90%. The other chemicals were purchased from the following suppliers. Normal melting agarose (NMA) and low melting agarose (LMA) were from Boehringer Mannheim (Germany); sodium chloride (NaCl), potassium chloride (KCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hydrogen peroxide (H₂O₂), sodium hydroxide (NaOH), and hydrochloric acid (HCl) were from Merck Chemicals (Darmstadt, Germany); heparin, dimethylsulfoxide (DMSO), ethidium bromide (EtBr), Triton X-100, phosphate buffered saline (PBS) tablets, bovine serum albumin, formamidopyrimidine-DNA glycosylase (Fpg), potassium peroxodisulphate, and 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) were from Sigma (St. Louis, MO); ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA-Na₂), N-lauroyl sarcosinate, and Tris were from ICN Biochemicals (Aurora, OH, USA), HPLC grade ethanol was from Fluka Chemie AG., Histopaque-1077 was from Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) and 6-hydroxy-2,5,7,8-tetramethylcroman-2-carboxylic acid (trolox) was from Calbiochem/ Roche.

Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC method is one of the most often used methods for the determination of total antioxidant capacity (12-14). It is based on the neutralization of the radical cation formed by a single-electron oxidation of a synthetic ABTS chromophore to a strongly absorbing ABTS^{•+} radical. The radical reacts quickly with electron/hydrogen donors to form colorless ABTS. A decrease of the ABTS^{•+} concentration is linearly dependent on the antioxidant concentration including trolox as a calibrating standard. A mixture of 7 mM ABTS and 2,45 mM potassium persulfate (1:1, v/v) was left to stand for 12 h at laboratory temperature in the dark to form ABTS^{•+} radical. The working solution was diluted with ethanol to give an absorbance of around 0.8 at 734 nm. After preparing trolox and EGCG standards, they were mixed with equal volumes of ABTS solution and the decrease in absorbance was measured at 734 nm after 2 min. The trolox concentration is proportional to the change in absorbance of the ABTS solution. A standard calibration curve was constructed for trolox at 0.01-100 µM concentrations. 500 µL of EGCG solutions at concentrations of 0.01-100 µM in ethanol were mixed with 500 µL of ABTS. Samples were assayed in three replicates. The absorbance of the resulting oxidized solution was compared to that of the calibrated trolox standard.

Single cell gel electrophoresis (comet assay) and Fpg-modified comet assay

For each experiment, 5 mL heparinized (50 units/mol sodium heparin) whole blood was collected by venepuncture from a healthy 28-year-old non-smoker female donor not exposed to radiation or drugs. The replicate experiments were carried out with blood samples from the same donor collected at different time intervals.

Lymphocytes were isolated by Ficoll-Hypaque density gradient and washed with PBS (15). Cell concentrations were adjusted to approximately 2×10^5 /mL in the buffer. The cells were suspended in a total volume of 1 mL and each reaction contained 50 µL suspension ($\approx 10^4$ cells), varying micro liter

amounts of EGCG dissolved in PBS at the concentrations of 0.01-250 µM. The cells were incubated for 0.5 h at 37 °C in an incubator together with untreated control samples. Control incubations contained PBS. After incubation the lymphocytes were harvested by centrifugation at 800 x g for 3 min at 4 °C. Oxidative damage was introduced by replacing the medium with PBS containing at 0.05 mM concentration of H₂O₂ and the treatment was for 5 min on ice. Then the cells were harvested by centrifugation at 800 x g for 3 min at 4 °C. Again the cells were harvested by centrifugation at 800 x g for 3 min at 4 °C cells after washing with BPS. The cells were checked for viability by trypan blue exclusion.

The basic alkaline technique of Singh et al. (16), as further described by Collins et al. (10), was followed. The microscopic slides had been each covered with 1% NMA at about 45 °C in Ca²⁺-and Mg²⁺-free PBS before the experiment. This layer was used to promote the attachment of the second layer. For the second layer, around 10000 treated or control cells mixed with 75 µL of 0.65% LMA were rapidly pipetted onto this slide, spread using a cover slip, and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution, (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10), with 1% Triton-X 100 and 10% DMSO added just before use for a minimum of 1 h at 4°C but the cells treated with H₂O₂ and embedded on slides were immersed in other cold lysing solution. The alkaline comet assay using formamidopyrimidine-DNA glycosylase (Fpg), lesion-specific enzyme was used to detect oxidized pyrimidines as a result of oxidative stress-induced DNA damage as described with some modifications (8). The cell-agarose suspension slides were prepared as described above for the standard comet assay. In the fpg-modified comet assay, after lysing, the slides were washed with enzyme buffer (40 mM HEPES, 100 mM KCL, 0.5 mM EDTA and 0.2 mg/mL bovine serum albumin) at room temperature and then incubated with Fpg protein (1 mg/mL in enzyme buffer for 0.5 h at 37 °C in

an incubator and washed with the neutralizing solution. Then the slides were applied to electrophoresis.

The slides were removed from the lysing solution, drained, and placed side by side avoiding space and with the agarose ends facing each other nearest the anode in a horizontal gel electrophoresis tank. The tank was filled with fresh electrophoresis solution (1 mM Na₂EDTA and 300 mM NaOH, pH 13) to a level approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4 °C) for 20 min using 24 V and adjusting the current to 300 mA by raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were conducted under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were taken out of the tank and rinsed gently three times for 5 min with neutralization buffer (0.4 M Tris-HCL, pH 7.5) to remove excess alkali and detergents, then the slides were allowed to sit 5 min in 50%, 75 %, and 95% alcohol, successively.

The dried microscope slides were stained with Ethidium Bromide (EtBr 20 µg/mL in distilled water, 60 µL/slide), The slides were covered with a cover slip, placed in a humidified airtight container to prevent drying of the gel, and analyzed within 3-4 h.

The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd., Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, the slides were examined at 100x. DNA damage was expressed as tail intensity, tail migration, and

tail moment. 100 cells from each of two replicate slides were assayed. Analysis was performed by one slide reader, thus minimizing variability due to subjective scoring.

Statistical analysis

Statistical analysis was performed by the computer program SPSS for Windows 15.0. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences was performed by least significant difference (LSD) test. The Kruskal-Wallis *H* test was used in comparing parameters displaying abnormal distribution between groups. The results were given as the mean ± standard deviation and the *P* values of less than 0.05 were considered as statistically significant.

RESULTS

Cell viability assessed by trypan blue dye exclusion was more than 90 % for each treatment group.

EGCG demonstrated significant antioxidant capacity above 0.01 µM concentrations in a dose dependent with respect to ABTS radical solution diluted with ethanol giving an absorbance of around 0.8 at 734 nm. The antioxidant activity of EGCG within the range of 1-25 µM was also found to be significantly higher than the antioxidant activity of trolox (Figure 1).

The effects of EGCG on DNA strand breakage in human lymphocytes with or without H₂O₂ are shown in Figure 2. According to the data obtained from three separate experiments no additional DNA strand breakage in human lymphocytes was observed within the range of 0.01-5 µM EGCG alone in DNA tail moment and 0.01-1 µM EGCG alone in tail migration and tail intensity (Figure 2).

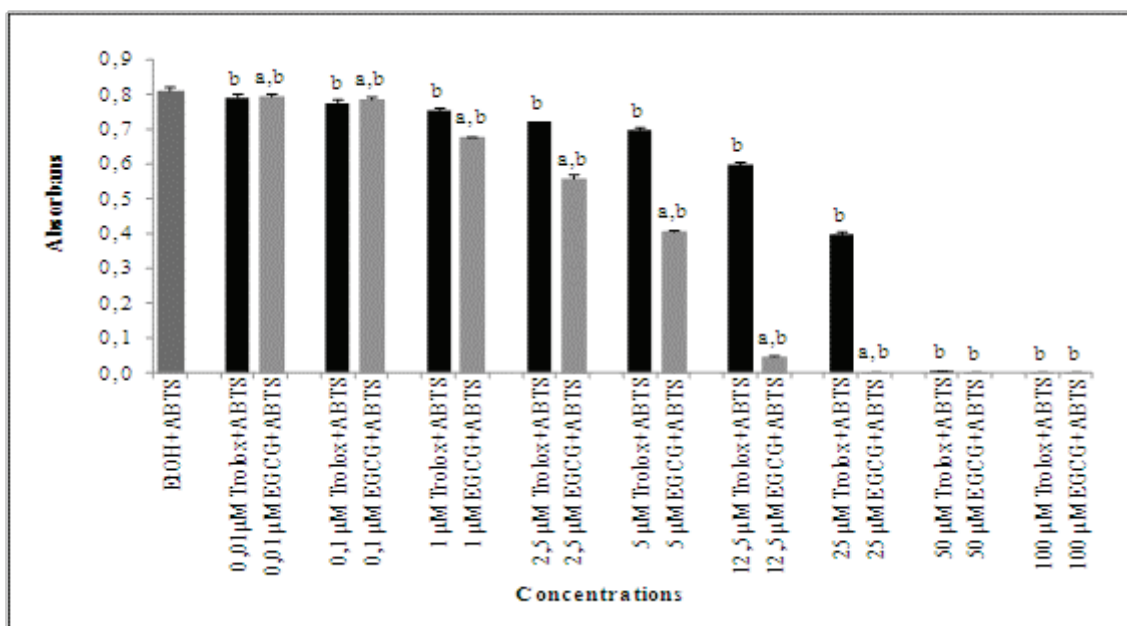


Figure 1. The antioxidant activity of EGCG and trolox on ABTS. Results were given as the mean \pm standard deviation. ^a $p < 0.05$, EGCG compared to trolox; ^b $p < 0.05$, EGCG or trolox compared to EtOH (ethanol) + ABTS.

EGCG, within the range of 0.01- 10 μM , significantly decreased oxidative DNA damage induced by 0.05 mM H_2O_2 in human lymphocytes. However 30 -250 μM of EGCG did not have a protective effect on oxidative DNA damage (Figure 2A, 2B, and 2C).

At the concentration of 50 μM EGCG, the level of Fpg sensitive sites seemed to be significantly increased for DNA tail moment. At the concentration of 5 μM and 50 μM of EGCG, the levels of Fpg sensitive sites seemed to be significantly increased for DNA tail migration and DNA tail intensity in the lymphocytes.

DISCUSSION

In recent years, many efforts have been focused on the safety and toxicity of natural dietary components. Green tea (*Camellia sinensis*) contains several bioactive compounds which protect the cell and prevent tumour development. Dietary intakes of green tea containing EGCG have been shown to be associated with the decreased risk of chronic diseases such as cancer and cardiovascular diseases in numerous studies (17). The inhibition of tumorigenesis by green or black

tea preparations has been demonstrated in animal models on different organ sites. However, epidemiological studies have not yielded clear conclusions concerning the protective effects of tea consumption against cancer formation in humans (18,19).

Phytochemicals in green tea extract (mostly flavonoids) scavenge free radicals, but also induce pro-oxidative reactions in the cell. But due to their diverse chemical structures, they are likely to possess different antioxidant capacities (20,21). Among tea catechins, EGCG is most effective in reacting with most reactive oxygen species. Under certain conditions, however, catechins may undergo autooxidation and behave like prooxidants. During the reactions of tea polyphenols with free radicals, several oxidation products are formed (22-27). In consistent with these studies, we found that EGCG above 0.01 μM concentrations had an antioxidant capacity against ABTS radical and the antioxidant activity of EGCG within the range of 1-25 μM was found to be significantly higher than the antioxidant activity of trolox using TEAC assay.

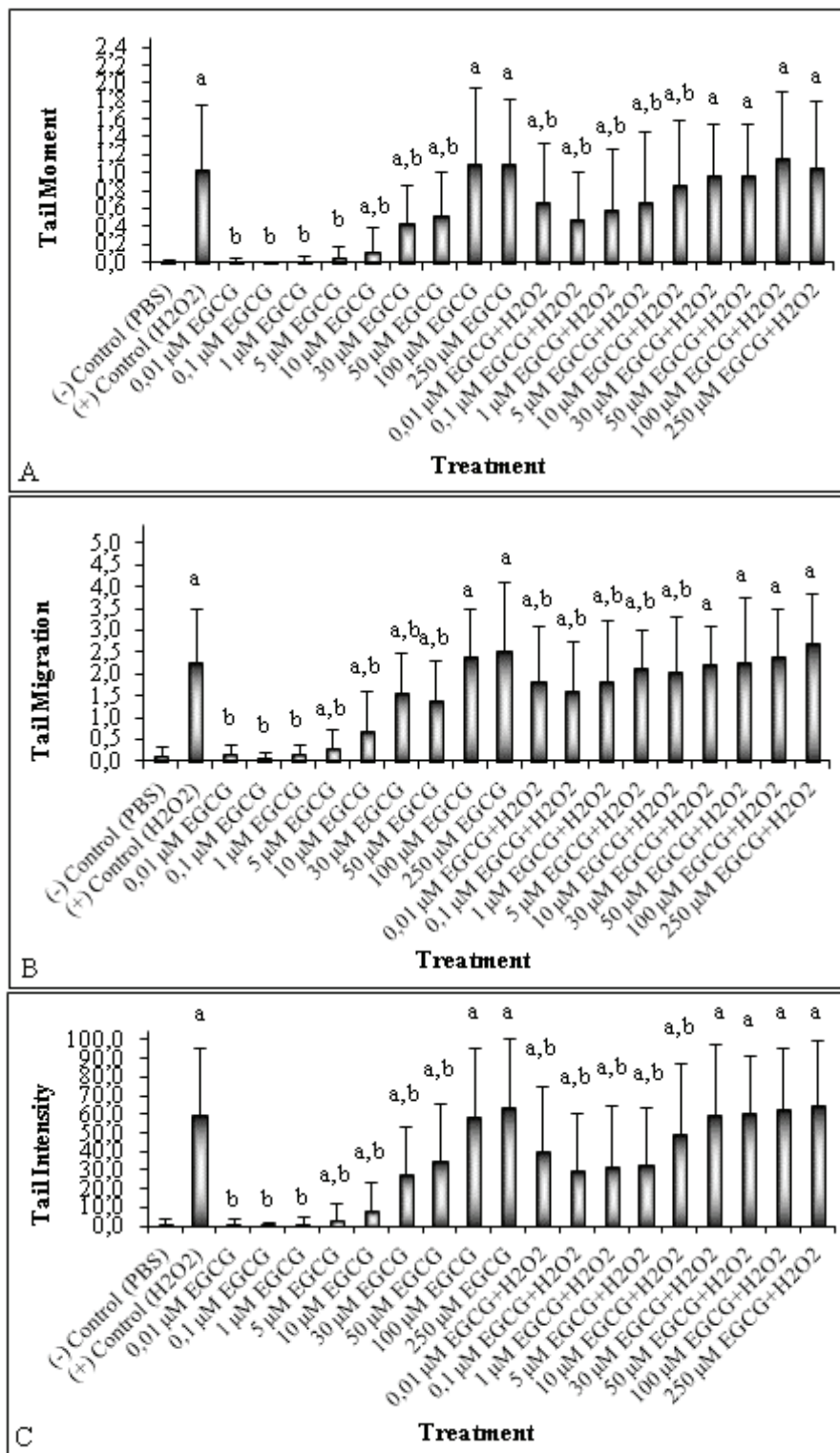


Figure 2. The effect of EGCG on H₂O₂-induced oxidative DNA damage in human lymphocytes. DNA damages were expressed as DNA tail moment (A), DNA tail migration (B), and DNA tail intensity (C) in the lymphocytes. Results were given as the mean ± standard deviation. ^ap < 0.05, compared with the negative control (PBS); ^bp < 0.05 compared with the positive control (0.05 mM H₂O₂).

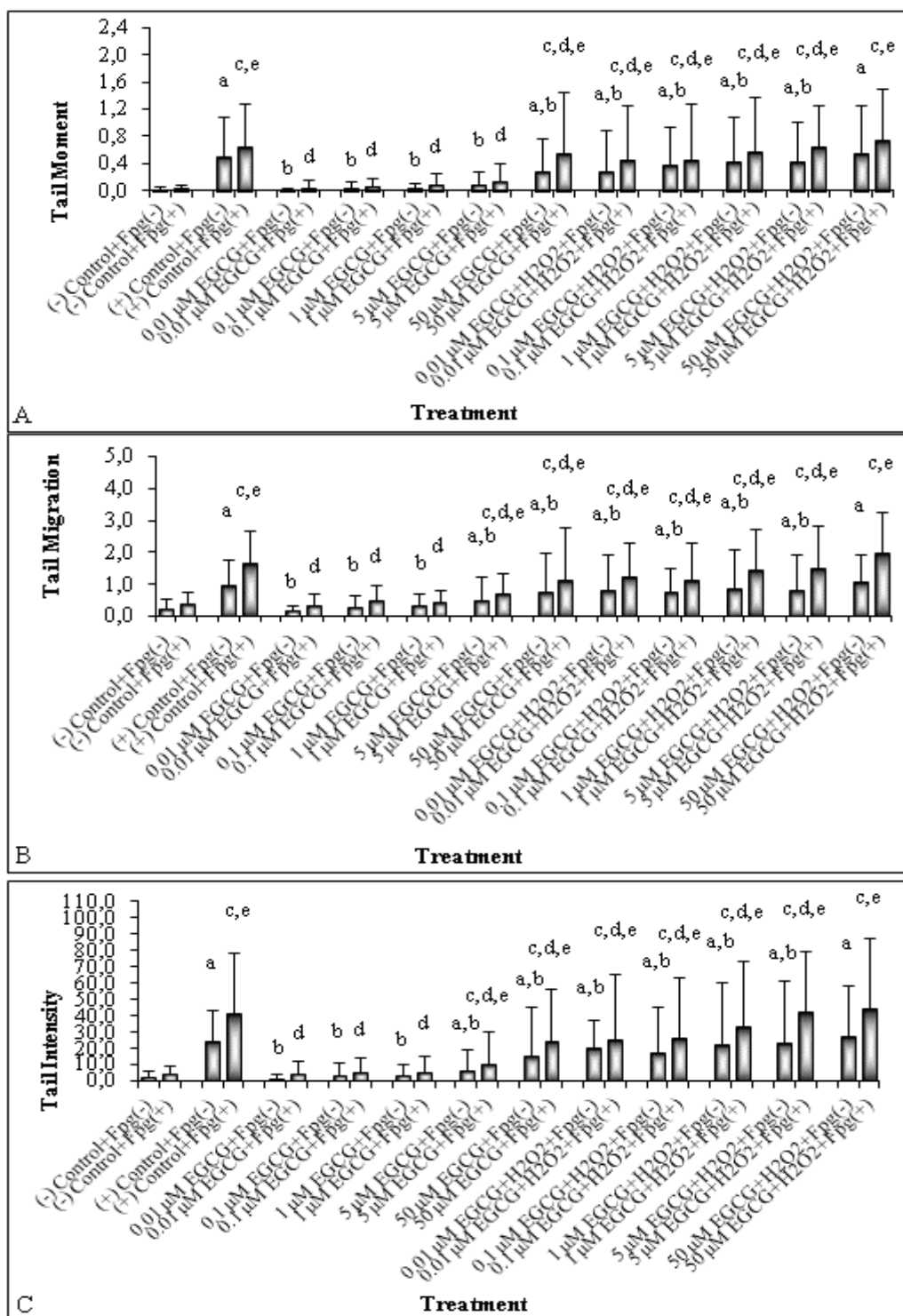


Figure 3.The effect EGCG on H₂O₂-induced oxidative DNA damage in human lymphocytes with/without Fpg protein. DNA damages were expressed as DNA tail moment (A), DNA tail migration (B), and DNA tail intensity (C) in the lymphocytes. Results were given as the mean ± standard deviation. ^ap<0.05, compared to negative control for the standard comet assay; ^bp<0.05, compared to positive control for the standard comet assay; ^cp<0.05, compared to negative control for the Fpg-modified comet assay; ^dp<0.05, compared to positive control for the Fpg-modified comet assay; ^ep<0.05, the standard comet assay was compared to the Fpg-modified comet assay.

In the study, we also evaluated by means of the standard and the fpg-modified comet assays whether EGCG induce or reduce oxidative DNA damage induced by hydrogen peroxide in human lymphocytes. EGCG alone was found to induce DNA damage above 5 μM concentrations in a dose dependent. At the concentrations of 0.01-10 μM , EGCG significantly decreased oxidative DNA damage induced by 0.05 mM H_2O_2 in human lymphocytes, however EGCG, within the range of 30 -250 μM , did not have a protective effect on the DNA damage.

The Fpg protein has been used widely for the detection of oxidative DNA base damage (28). However, besides its high sensitivity for detecting 8-OH-guanine and other oxidative damaged purines, Fpg enzyme also detects alkylation damage, such as abasic (AP) sites and ring-opened N-7 guanine adducts. Thus, it was concluded that enhanced DNA-damaging effects in the comet assay by Fpg after exposure to genotoxic agents with unknown mode of action should not be regarded as evidence for the presence of oxidative damage (29). Post-treatment with Fpg protein also revealed the increased DNA-damage for the positive control H_2O_2 (0.5mM). At 5 μM concentration of EGCG, the level of Fpg sensitive sites seemed to be significantly increased suggesting that the increase in DNA damage is the result of oxidative purine base damage.

It has been reported that catechins, primarily EGCG, prevented DNA damage at the low concentrations in few studies investigating the protective effect against oxidative DNA damage in different cells, however it acted as a prooxidant at high concentrations. It was found that 200 μM of EGCG increased oxidative DNA damage induced by H_2O_2 in the human lymphocyte DNA (1) and in Jurkat T-lymphocytes, above 100 μM concentrations of EGCG induced the oxidative DNA damage and 10 μM of EGCG inhibited DNA damage induced by H_2O_2 and 3-morpholinonydnonimine (SIN-1, a peroxydinitrite generator) (30). EGCG was also reported to induce DNA damage, cell death, and mutagenicity in human lung and skin normal cells (31). It has been suggested that low concentrations of EGCG scavenged free

radicals, thereby inhibiting oxidative damage to cellular DNA, but high concentrations of EGCG alone induced cellular DNA damage.

Kanadzu et al. (2) reported that EGCG prevented DNA strand breaks induced by mutagens in human lymphocytes at low doses, but induced the breakages at high doses. On the other hand, it has also reported that EGCG (1-25 μM) inhibited 12-O-tetradecanoyl phorbol-13-acetate (TPA)-mediated oxidative stress in HeLa cervical carcinoma cells and EGCG (50 μM) decreased the strand breaks in plasmid DNA (32,33).

CONCLUSION

EGCG have suggested ameliorating the diseases related to oxidative damage such as cancer, cardiovascular diseases, and neurodegenerative defects in various animal models and epidemiologic studies. On the other hand it should be considered that green tea might interact with some drugs (34-37). Our results compatible with most of the studies have suggested that EGCG should not consume at high doses. However EGCG might have protective effects on H_2O_2 -induced oxidative DNA strand breakage. There is much evidence pertaining to the wide beneficial health effects of catechins. It seems that our results highlight the potential benefit EGCG as a dietary supplement and a natural antioxidant. But our results concern only in vitro experiments with human lymphocytes and the full effects of the catechins are not be known; additional animal and human studies should be performed in order to clarify the potential effects of EGCG.

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