Free Radical Research, Volume 38 Number 2 (February 2004), pp. 193–200

Taylor & Francis
healthsciences

Modulation of Tea and Tea Polyphenols on Benzo(a)pyrene-induced DNA Damage in Chang Liver Cells

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Accepted by Professor J. Yodoi

(Received 11 June 2003; In revised form 26 September 2003)

The protective effects of three tea extracts (green tea, GTE; oolong tea, OTE; and black tea, BTE) and five tea polyphenols (epicatechin, EC; epicatechin gallate, ECG; epigallocatechin, EGC; epigallocatechin gallate, EGCG; and theaflavins, THFs) on benzo[a]pyrene (B[*a*]P)-induced DNA damage in Chang liver cells were evaluated using the comet assay. B[*a*]P-induced DNA damage in Chang liver cells was significantly ($p < 0.05$) inhibited by GTE and OTE at a concentration of 10 μg/ml and by BTE at 25 μg/ml. At a concentration of 100 μg/ml, the % tail DNA was reduced from 33% (B[*a*]P treated only) to 10, 9, 13%, by GTE, OTE and BTE, respectively. EC and ECG did not cause DNA damage in cells according to the results of the comet assay; however, EGC, EGCG and theaflavins caused DNA damage in cells at a concentration of 100 μM. The results indicated that EC and ECG had protective effects against B[*a*]P-induced DNA damage in cells at a concentration of 10–100 μM. Although EGC, EGCG and the theaflavins caused DNA damage at a high concentration, but they had protective effects against B[*a*]P-induced DNA damage in cells at a low concentration of 10–50 μM. The results also showed that the DNA damage in cells induced by EGC, EGCG, and the theaflavins was due to the generation of superoxide during incubation with cells at a higher concentration. Therefore, tea catechins and THFs play an important role in enabling tea extracts to inhibit DNA damage in Chang liver cells.

Keywords: Tea; Tea polynenols; Comet assay; DNA damage; Chang liver cells

INTRODUCTION

Tea (*Camellia sinesis*) is the most widely consumed beverage by over two thirds of the world's population. Tea leaves are processed differently to give green tea, oolong tea or black tea. In Japan and China, green and oolong teas are mostly consumed. Tea has been reported to have antioxidant, antimutagenic and anticancer properties.[1–3] Animal experiments have also revealed that tea could inhibit tumor, cell matetic, and skin cancer.[3–6]. Lin *et al*.[7] reported that tea could inhibit cancer cell growth by interfering DNA genetic information, transcription and translation. All tea beverages are rich in polyphenols, particularly catechins, including epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and theaflavins (THFs).[8] Yoshizawa *et al*.[9] first reported that topical application of EGCG to inhibit teleocidin induced tumor promotion in the skin of mice previously initiated with 7, 12-dimethylbenz[*a*]anthracene. It was also found that oral administration of EGCG or tea fractions inhibited the carcinogen-induced tumors of various organs in animals, including the duodenum, stomach, lung, esophagus and colon. In addition, evidence has shown that catechins have antioxidant, antimutagenic and anticancer properties.[2,3,10]

The tea polyphenols in tea have a strong radical scavenging activity. They scavenge and detoxify radicals of various promoters of carcinogenesis and radicals produced in the process of exposure to radiation and light. They also inhibit the production of nitroso compounds with strong carcinogenic activity by reducing bisulfite in the digestive organs. Since tea polyphenols inactivate enzyme and virus

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760310001638001

activity, they may be effective against carcinogenesis caused by some virus.[11,12] Wei *et al.*[13] reported that tea polyphenols from GTE could inhibit ultraviolet light and peroxide-induced DNA damage. Rechner *et al.*[14] also reported that THFs from BTE tea have antioxidant and antimutagenic activities. Therefore, tea polyphenols are linked to the antioxidant activity of tea. However, other reports indicated that catechins show prooxidant properties in some test conditions.[15,16] Although catechins are the major compound of tea and are reported to have functional activities, the prooxidant properties and the moderating effects of those compounds on DNA damage in cells should be clarified. The purpose of this study was to investigate the moderating effects of tea extracts (GTE, OTE and BTE) and tea polyphenols (EC, ECG, EGC, EGCG and THFs) on B[*a*]P-induced DNA damage in Chang liver cells.

MATERIALS AND METHODS

Materials and Chemicals

Green tea, oolong tea and black tea were purchased from a local market in Taichung, Taiwan. EC, ECG, EGC, EGCG and THFs were purchase from Sigma Chemical Co. (St Louis, MO).

Preparation of Tea Extracts

Tea extracts were prepared according to the method described in our previous study.[1] Briefly, each tea (20 g) was extracted with boiled water (400 ml) for 5 min, and the filtrate was freeze-dried. The yields of crude tea extracts for GTE, OTE and BTE were 5.34, 5.12 and 4.34 g, respectively.

Total Antioxidant Activities Assay (Trolox Equivalent Antioxidant Capacity, TEAC Assay)

The total antioxidant activity of tea extracts and tea polyphenols was measured using the TEAC assay as described by Miller *et al.*,[17] with minor modifications. To measure antioxidant capacity, tea extracts were mixed with an equal volume of 2,2′-azino-bis[3-ethyln-benz -thiazoline-6-sulfonic acid] (ABTS), H_2O_2, peroxidase and deionized water. Absorbance was monitored at 734 nm for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. TEAC value is expressed as millimolar concentration of Trolox solution having the antioxidant equivalent to a 1000 ppm solution of the sample under investigation. The higher the TEAC value of the sample, the stronger is the antioxidant ability.

Cell Culture

Chang liver cells were obtained from the Culture Collection and Research Center (CCRC, Hsin Chu, Taiwan). Chang liver cells were cultured in complete minimal essential medium (containing 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, pH 7.4) at 37°C with 5% CO_2.

Cytotoxicity and Genotoxicity Analysis

To measure the acute cytotoxicity, 0.49 ml of each cell suspension was mixed with 10 μl of a 0.4% trypan blue solution and checked for viability 10 min later. The cells were analyzed through microscopic observation, and the percentage of viable cells was determined.

Genotoxicity of sample was determined according to the method of Aderson *et al.*[18] Sample (0.1 ml) in an eppendroff was mixed with 0.9 ml cells suspension at 37°C for 30 min. The cells were centrifuged and resuspended in preheated 1% low melting point agarose and then the DNA damage was determined using comet assay as described below.

Effect of Tea on B[*a*]P -induced DNA Damage

To evaluate the effect of tea extracts or tea polyphenols on modulating DNA damage in Chang liver cells induced by B[*a*]P, cells suspension was incubated with tea extracts (10–100 μg/ml) or tea polyphenols (10–100 μM) and B[*a*]P (480 μM) at 37°C for 30 min. After treatment with tea extracts or tea polyphenols, the cells were centrifuged and resuspended in preheated 1% low melting point agarose. The DNA damage was determined using comet assay as described below.

Alkaline Single-cell Gel Electrophoresis (Comet Assay)

The effect of tea extracts on DNA damage in Chang liver cells was investigated using the comet assay or single-cell gel electrophoresis under alkaline conditions according to the method of Yen *et al.*[19] After treatment with tea extracts or tea polyphenols, the cells were centrifuged and resuspended in preheated 1% low melting point agarose. The cell suspension was then added to Dakin fully frosted microscope slides (Menzel-Glaser, Germany), precoated with 1% normal melting agarose. After application of a third layer of 1% low melting point agarose, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosineaate, 1% Triton X-100, 10% DMSO, pH 10) for at least 1 h at 4°C. The microscope slides were then placed in an electrophoresis tank, and the DNA was allowed to

unwind for 20 min in freshly prepared alkaline electrophoresis buffer (1 mM EDTA, 0.3 N NaOH, pH 13). Electrophoresis was conducted at 4°C for 20 min at 25 V and 300 mA. The slides were then neutralized with Tris buffer (0.4 M, pH 7.5) and stained with ethidium bromide. All of the steps were performed under yellow light in order to prevent additional damage. The slides were observed using a fluorescent microscope attached to a CCD camera connected to a personal computer based image analysis system (Komet 3.0; Kinetic Imaging Ltd., UK). For each analysis, 50 individual cells were calculated, and in most cases, three separate experiments were conducted for each series. Single cells were analyzed under the fluorescent microscope as desired. The DNA damage was expressed as % Tail DNA, where % Tail DNA = Tail DNA/(Head DNA + Tail DNA) × 100. A higher % Tail DNA meant a higher level of DNA damage.

Determination of Glutathione S-transferase Activity

Glutathione S-transferase activity was determined using the method described by Habig *et al.*[20] Chang liver cells (150×10^4 cells/ml) were incubated with tea extracts at 37°C for 1 h and then the cells were lysed by sonication with the addition of 400 μl 0.5% triton X-100. Samples were then centrifuged at 12,000 ppm for 10 min at 4°C, and the aliquot of the supernatant was used for enzyme activity determined. In a cuvette, 100 μl cell lysates, 880 μl potassium phosphate buffer (100 μM, pH 6.5) containing 1 mM glutathione, and 20 μl 1-chloro-2,4-dinitrobenzene (CDNB) were combined. The change in absorbance at 340 nm was monitored for 5 min. The enzyme activity was calculated using the equation $E_{340} = 9.6\,mM^{-1}cm^{-1}$. The specific activity was expressed as nmole CDNB-GSH conjugated formed/min/mg protein.

Determination of Superoxide Generation

The generation of superoxide by EC, ECG, EGC and THFs was performed according to the method of Alanko *et al.*[16] In a cuvette, 100 μl of 2 mM tea compounds, 900 μl of 50 μM phosphate buffer (pH 7.4, containing 100 μM NBT and 100 μM EDTA) were combined. An increase in the absorbance at 560 nm was continuously recorded at 37°C. In a control group, superoxide dismutase (20 unit) was added 1 min after the test compounds. If the absorbance was decreased when compared with the absence of superoxide dismutase, indicating that the increase of absorbance was due to the generation of superoxide anion.

Statistical Analysis

Statistical analysis involved the use of the Statistical Analysis Systems software package. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple-rang tests.

RESULTS AND DISCUSSION

Total Antioxidant Activity of Tea Extracts and Tea Polyphenols

The total antioxidant activity of tea extracts was determined by TEAC (trolox equivalent antioxidant activity) assay. The TEAC values of GTE, OTE and BTE with a concentration of 1000 μg/ml were 3.6, 3.3 and 2.3 mM trolox, respectively. The scavenging activity on $ABTS^+$ of tea extracts was in the order of GTE > OTE > BTE. This is in a similar trend as the scavenging activity to DPPH radical.[1] The TEAC values of ECG, EGCG, THFs, EGC and EC with a concentration of 1 mM were 4.9, 4.0, 3.6, 3.1 and 2.5 mM trolox, respectively. In this study, the total catechins (EC, ECG, EGC and EGCG) concentration in tea extracts was also determined by HPLC. The total catechins in GTE, OTE and BTE were 258.4, 222.0 and 87.6 m/g, respectively. GTE had the highest content of EGCG while OTE and BTE had the EGC in the highest content. The total polyphenols content in GTE, OTE and BTE was 320, 281 and 228 mg/g, respectively. These results may explain the varied antioxidant activity of these three tea extracts.

Cytotoxicity and Genotoxicity of Tea Extracts and Tea Polyphenols on Chang Liver Cells

The cytotoxicity and genotoxicity of three tea extracts after reacting with Chang liver cells at 37°C for 30 min were investigated. The viability of Chang liver cells was higher than 90%, and at a concentration of 10–100 μg/ml, the tea extracts showed no toxicity toward these cells (data not shown). The % tail DNA of treated Chang liver cells was distributed between 0–10% at a tea extracts concentration of 10–100 μg/ml (data not shown). It was not significantly different ($p > 0.05$) from that of the control group.

The cytotoxicity of EC, ECG, EGC, EGCG and THFs was also evaluated. The cell viability was greater than 90% when these five tea polyphenols (10–100 μM) was incubated with cells at 37°C for 30 min (data not shown). This means that tea polyphenols showed no cytotoxicity to Chang liver cells at the test concentrations. The results also indicate that EC and ECG do not cause DNA damage at a concentration of 10–100 μM as compared with the control group. As the results

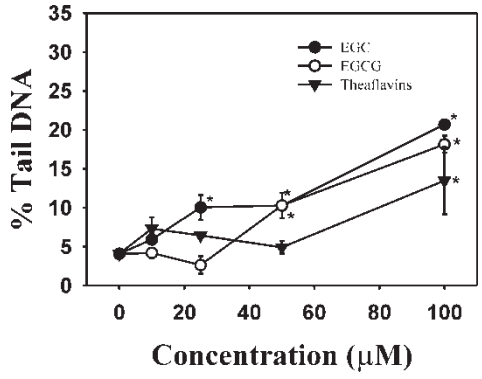

FIGURE 1 Genotoxicity of Chang liver cells treated with EGC, EGCG and THFs. DNA strand breaks were detected by the comet assay. Results are mean ± SD for $n = 3$. *Significantly different from untreated ($p < 0.05$).

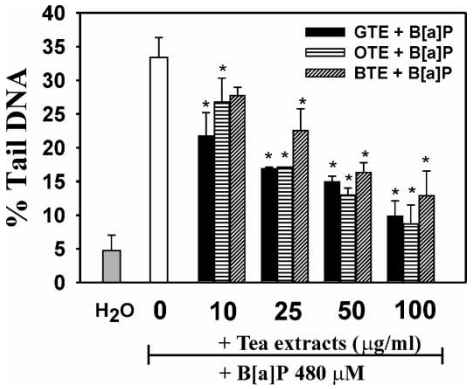

FIGURE 2 Effects of GTE, OTE and BTE on B[*a*]P-induced DNA damage of Chang liver cells. DNA breaks were detected by the comet assay. Results are mean ± SD for $n = 3$, independently. *Significiantly different form treated with B[*a*]P alone ($p < 0.05$).

shown in Fig. 1 reveal, EGC caused Chang liver cells DNA damage at a concentration of 25 μM ($p < 0.05$). The % tail DNA increased to 21% at an EGC concentration of 100 μM when compared with the control (4%). In addition, EGCG also induced Change liver cells DNA damage at the concentrations of 50–100 μM; the % tail DNA was distributed between 10–18%. The % tail DNA for THFs is 13% at a concentration of 100 μM. Therefore, EGC, EGCG and THFs caused Change liver cells DNA damage at a higher concentration (100 μM).

Effect of Tea Extracts on Cells DNA Damage Induced by B[*a*]P

In order to find the appropriate dose of B[*a*]P to induce the DNA damage in Chang liver cells, the cells were reacted with different concentrations of B[*a*]P. The % tail DNA of Chang liver cells treated with 160, 320, 480 and 800 μM B[*a*]P was 14, 18, 29 and 31%, respectively. Therefore, 480 μM B[*a*]P was used as positive control in the following study.

As shown in Fig. 2, GTE and OTE, at a concentration of 10 μg/ml, significantly ($p < 0.05$) inhibited the DNA damage induced by B[*a*]P; however, the BTE was a concentration of 25 μg/ml. At a concentration of 100 μg/ml, GTE, OTE and BTE decreased the % tail DNA from 33% (B[*a*]P treated only) to 10, 9 and 13%, respectively. These results clearly indicated that tea extracts significantly inhibited the B[*a*]P-induced DNA damage, especially the OTE.

Tea has been reported to inhibit the B[*a*]P-induced mutagenicity and tumorigenesis by inactivation of cytochrome P-450 (CYP-450) activity.[21–23] Gotze *et al.*[24] indicated that B[*a*]P would be readily self-oxidized to produce hydrogen peroxide. It would easily release the hydroxyl radical with the existence of Fe^{2+} and induce DNA damage. Bu-Abbas *et al.*[25] reported that green tea extracts would decrease the mutagenic response by inactivating cytochrome P-450 enzymes. Therefore, the inhibitory action of tea extracts on DNA damage should be due to the components in tea extracts that inhibit the CYP-450 activity in liver cells and scavenge the reactive oxygen species produced by B[*a*]P during metabolism.[26] On the other hand, the increase of glutathione S-transferase activity in cells may also be another way to inhibit the B[*a*]P damage.

Effect of Tea Extracts on Glutathione S-transferase in Cells

The chemopreventive mechanism by which tea extracts prevent B[*a*]P induced cancer is well understood, there is sufficient evidence to suggest that their effect may, at least in part, be due to their ability to increase GST activity.[27,28] As shown in Table I, the GST activity in Chang liver cells was increased after being treated with tea extracts, especially at a concentration greater than 50 μg/ml ($p < 0.05$). GSTs are a superfamily of multi-functional proteins which can catalyze the conjugation of GSH with a wide variety of electrophilic

TABLE I Effects of GTE, OTE and BTE on glutathione S-transferase activity in Chang liver cells

Concentration (μg/ml)	GST avtivity (nmol/min/mg protein)*		
	GTE	OTE	BTE
Control	152.6 ± 2.9	154.2 ± 12.4	165.4 ± 4.4
10	154.6 ± 5.4	163.2 ± 5.4	163.4 ± 5.7
25	153.4 ± 8.5	171.9 ± 4.4†	174.5 ± 2.3
50	167.9 ± 5.7†	171.3 ± 3.5†	174.7 ± 3.8†
100	176.1 ± 2.7†	176.3 ± 1.5†	177.7 ± 1.7†

*Values are means ± SD of triplicate determination of 1-chloro-2, 4-dinitrobezene conjugated form. Chang liver cells were preincubated with 0–100 μg/ml GTE, OTE and BTE, respectively, for 60 min.
†Significantly different from the control ($p < 0.05$).

xenobiotics, including the ultimate carcinogenic metabolite of B[*a*]P (benzo[*a*]pyrene-7,8-diol-9,10-epoxide, BPDE), generally leading to their detoxification.[29–31] Such a mechanism would decrease the level of reactive electrophiles available to bind to DNA, reducing the likelihood of the DNA damage and possible induction of the carcinogenesis process. Therefore, the tea extracts increased the GST activity in Chang liver cells and inhibited the DNA damage induced by B[*a*]P.

However, based on the results of this study, GST was induced by tea extracts at a concentration greater than 25 μg/ml, but inhibited B[*a*]P-induced DNA damage in all dosage (10–100 μg/ml). It revealed that the detoxification of B[*a*]P by enhanced GST activity could not the only candidate for tea's chemopreventive activity in the comet assay. Dipple *et al.*[32] indicated that there are many metabolism ways after B[*a*]P enters the bioorganism: the main metabolism way is through CYP-450 1A1 activation to produce the final carcinogens BPDE. Antimutagens such as GTE or BTE were related to their suppressing activation enzymes in a similar pathway.[33] Thus, inhibition of the conversion of B[*a*]P to BPDE may be another possible mechanism of the antigenotoxic effect of these tea extracts.[34] In addition, more than forming BPDE carcinogen through activation of CYP-450 for B[*a*]P, peroxides-B[*a*]P-7,8-dione (BPQ) also can be formed in liver cells through dihydrodiol dehydrogenase catalysis. The reaction process was proved to be with production of reactive oxygen species and semiquinone anion radicals and followed with ϕ X 174 DNA single-strand breaking.[35] Gotze *et al.*[24] indicated that B[*a*]P would be readily self-oxidized to produce H_2O_2. It would easily release the hydroxyl radical with existence of Fe^{2+} and induce 8-OH-dG formation in Sprague-Dawley rat liver cells. In our previous study, GTE had the characteristics of chelating Fe^{2+} and scavenging hydroxyl radical (1). Therefore, assuming that DNA damage induced by B[*a*]P in the comet assay is related to the free radicals produced in its activation process, antigenotoxicity of GTE may partly be from its antioxidant activity.

Effect of Tea Polyphenols on Cells DNA Damage Induced by B[*a*]P

Figure 3 shows the effects of EC and ECG on B[*a*]P induced DNA damage in Chang liver cells. These two compounds showed inhibitory effect in a dose dependent manner. At a concentration of 10 μM, the % tail DNA of EC and ECG was 25 and 28%, respectively, that was significantly different ($p < 0.05$) from the control group (35 and 33%). The inhibitory effect of EGC, EGCG and THFs on B[*a*]P induced DNA damage in Chang liver cells was also increased with the concentration range of 10–50 μM (Fig. 4). At a concentration of 50 μM, the % tail DNA of EGC, EGCG and THFs was 17, 12 and 19%, respectively, that was significantly different ($p < 0.05$) from the control group (29, 29 and 30%). However, at a concentration of 100 μM, the inhibitory effect of these three compounds was reduced, and EGC promoted the DNA damage.

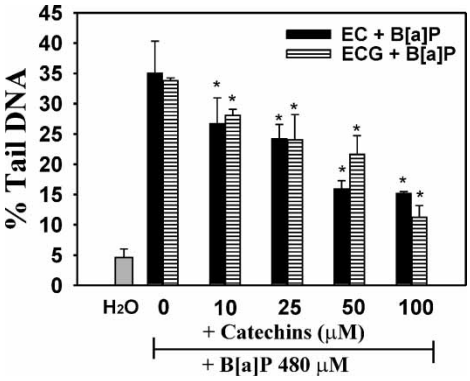

FIGURE 3 Effects of EC and ECG on B[*a*]P-induced DNA damage in Chang liver cells. DNA strand breaks were detected by the comet assay. Concentration of B[*a*]P was 480 μM. Results are mean ± SD for $n = 3$. *Significantly different from treated with B[*a*]P alone ($p < 0.05$).

B[*a*]P mainly through CYP-450 in liver cell to form epoxide as well as to produce free radical during the metabolism and then attack cell DNA. Wang *et al.*[36] indicated that catechins could inhibit the CYP-450 activity and scavenge the radicals formed during polyaromatic hydrocarbon (PAH) metabolism and in the order of EGCG > ECG > EGC > EC. As the results show in the present study, these five compounds showed significant inhibitory effect ($p < 0.05$) at a concentration of 10–50 μM; however, the inhibitory effect of EGC, EGCG and THFs was reduced at high concentration (100 μM) that might be

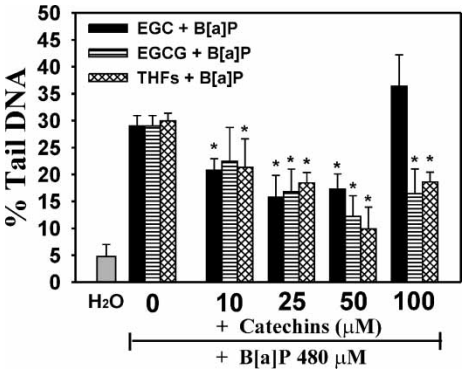

FIGURE 4 Effects of EGC, EGCG and THFs on B[*a*]P-induced DNA damage in Chang liver cells. DNA strand breaks were detected by the comet assay. Results are mean ± SD for $n = 3$. *Significantly different from treated with B[*a*]P alone ($p < 0.05$).

due to DNA damage effect of these three compounds themselves.

Pro-oxidant Effect of Tea Polyphenols: Superoxide Formation

As the above results show (Fig. 1), the % tail DNA of Chang liver cells treated with EGC, EGCG and THFs showed significant difference ($p < 0.05$) compared with the control group. However, EGC and EGCG significantly ($p < 0.05$) reduced the DNA damage induced by B[*a*]P at a lower concentration; however, the inhibitory effect was decreased at a higher concentration (Fig. 4). This means that the inhibitory effect of EGC and EGCG on Chang liver cells DNA damage induced by B[*a*]P was influenced by their damage to DNA at a higher concentration.

Alanko *et al.*[16] reported that the phenolic compounds with trihydroxybenzenes structure, such as pyrogall and 1,2,4-trihydroxybenzen, generated the superoxide and exhibited the prooxidant properties. The B ring structure of EGC and EGCG has

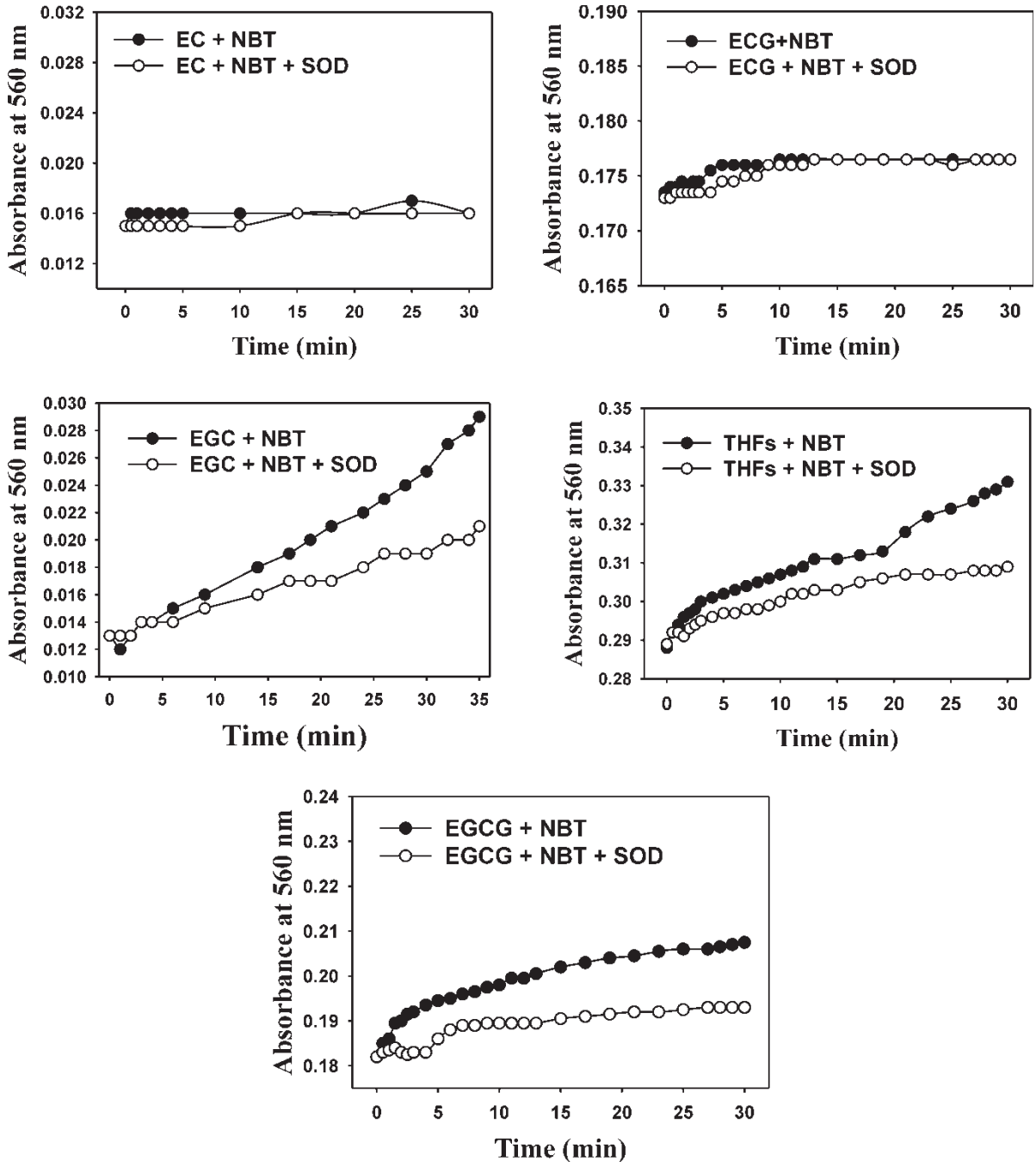

FIGURE 5 Superoxide-generating properties of EC, EGC, EGCG and THFs with superoxide dismutase (SOD) (○) and without SOD (●). Concentration of test compound is 200 μM. Different between closed and open circles is the amount of superoxide generated by the test compound. Results are mean for $n = 2$.

trihydroxybenzene structure as pyrogallol. Thus, the DNA damage induced by EGC, EGCG and THFs might be due to the generation of superoxide. Therefore, the generation of superoxide by EGC, EGCG and THFs was determined and compared with EC and ECG that did not cause DNA damage. As the results in Fig. 5 show, the absorbance at 560 nm in EGC, EGCG and THFs was increased in linearly with the increasing of time but EC and ECG did not. When the superoxide dismutase was added, the increase of absorbance became slow. This means that the absorbance increase in EGC, EGCG and THFs was due to the generation of superoxide.

Kondo *et al.*[37] indicated that EGC and EGCG could scavenge AAPH radical but also formed the superoxide during the inhibitory action. The superoxide can be further metabolized to form hydrogen peroxide.[38] Jiang and Millers[39] reported the generation of hydrogen peroxide during enzymatic oxidation of catechin. Thus, EGC and EGCG could generate superoxide and then lead to the formation of hydrogen peroxide. This hydrogen peroxide in cell can react with metal ions to form hydroxyl radical and cause DNA damage. In addition, superoxide also can attack protein, such as ferritin, to release Fe^{3+} and reduce to Fe^{2+}, which produced hydroxyl radical in the presence of reducing agents and hydrogen peroxide. Hydroxyl radical can act on and damage almost every molecule found in living cells, such as sugars, amino acids, lipids and DNA base. Cao *et al.*[40] indicated that the conjugated structure between A and B ring in flavonoids do not influence their antioxidant activity. However, the prooxidant activity of flavonoids was due to the reducing power of flavonoids, which reduce the transit metal and finally produce the hydrogen peroxide. Thus, flavonoids could generate hydrogen peroxide through redox reaction and further form hydroxyl radical to damage biomolecular. This may be the reason for EGC, EGCG and THFs to cause DNA damage. Although EGC, EGCG and THFs caused the DNA damage at a higher concentration, they showed inhibitory action on DNA damage induced by B[*a*]P at a lower concentration. The concentration of EGCG in human plasma is about 0.3–4 μM,[41,42] this means that the catechins level in human blood is not high but is showed the anti-tumor function. Therefore, the DNA damage caused by the high concentration of EGC, EGCG and THFs will not occur in human body.

Based on the results of this study, three tea extracts did not induce DNA damage in cells and showed inhibitory effect on DNA damage induced by B[*a*]P. Although tea catechins, EGC, EGCG and THFs, caused DNA damage in cells at a higher concentration by the formation of superoxide anion, the inhibitory effect of these three compounds at a lower concentration and the effect of EC and ECG should exhibit the overall inhibitory action of tea extracts. In addition, the human body also does not have high concentration of tea catechins. However, in a higher concentration, EGC, EGCG and THFs caused the DNA damage in cells as observed in comet assay. This result may provide some information on the pro-oxidant aspect of tea and the further research on tea functionality.

Acknowledgements

This work is part of a research project, NSC91-2313-B-005-055, supported by the National science Council, Republic of China.

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