A synergistic effect of GABA tea and copper(II) on DNA breakage in human peripheral lymphocytes

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ABSTRACT

GABA tea is a tea product that contains a high level of γ-aminobutyric acid (GABA). The oxidant and antioxidant roles of GABA tea in DNA damage were investigated in this study. DNA cleavage was observed by GABA-tea extract in the presence of copper ions. Comet assay revealed that combination of GABA-tea extract, but not pure GABA, and Cu2+ is capable of oxidatively degrading cellular DNA in human peripheral lymphocytes. Using various reactive oxygen scavengers, we found that catalase and sodium azide effectively inhibited GABA-tea extract/Cu(II)-induced DNA degradation, suggesting the essential role of singlet oxygen and H2O2 in the reaction. In addition, neocuproine inhibited the DNA degradation, confirming that Cu(I) is an intermediate in the DNA cleavage reaction. Therefore, we speculate that GABA-tea extract/Cu(II)-induced DNA damage is probably mediated through the formation of H2O2 and the reduction of copper. Furthermore, our data showed that GABA-tea extract was more genotoxic and pro-oxidant than its major catechin constituent, (−)-epigallocatechin-3-gallate (EGCG), leading to DNA cleavage in the presence of Cu2+. These findings will provide implications for the potential of GABA-tea extract in anticancer property, which may involve copper ions and the consequent pro-oxidant action.

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1. Introduction

Tea made from the leaves of the plant Camellia sinensis is one of the most commonly consumed beverage worldwide. GABA tea contains a high level of γ-aminobutyric acid (GABA). The basic steps of manufacturing GABA tea are similar to green tea, except in anaerobic condition (Jeng et al., 2007; Wang et al., 2006). The different production methods alter the chemical composition of the dried tea leaves. GABA has antioxidant activity, such as hydroxyl radical quenching activity (Chan et al., 1994). Its antioxidant activity has been proven to decrease oxidative stress through increasing intracellular glutathione (Ito et al., 2007; Lamigeon et al., 2001), superoxide dismutase and catalase (Sasaki et al., 1996). The cancer preventive activity of tea constituents have been also demonstrated in many animal models (Yang et al., 2007, 2009). Although little is known about the anticancer potential of GABA tea, a possible mechanism is suggested by its polyphenolic constituents, which are antioxidants (Yang et al., 2009). For instance, catechins are known as the characteristic polyphenolic compounds in tea. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant and active catechin in green tea (Jankun et al., 1997; Yang et al., 2007). In contrast to the anticancer activity demonstrated in animal models, the results from epidemiological studies assessing associations between tea consumption and human cancer risk have not yielded a clear conclusion (Yang et al., 2009). So far it has not been clearly elucidated whether EGCG plays a critical role in the process of anticancer. On the other hand, a serious concern

Abbreviations: EGCG, (−)-epigallocatechin-3-gallate; FOX, ferrous ion oxidation-xylenolyhdyrazyl radical, hydroxyl radical and superoxide anion (Jeng et al., 2007; Wang et al., unpublished data). These findings indicate that GABA might be involved in the biological activity of GABA tea. Intriguingly, a number of studies have shown the inhibitory effects of GABA on the cell growth and migration in gastric cancer (Tatsuta et al., 1992), colon carcinoma (Joseph et al., 2002), hepatocarcinoma (Sun et al., 2003) and cholangiocarcinoma (Fava et al., 2005). The cancer preventive activity of tea constituents have been also demonstrated in many animal models (Yang et al., 2007, 2009). Although little is known about the anticancer potential of GABA tea, a possible mechanism is suggested by its polyphenolic constituents, which are antioxidants (Yang et al., 2009). For instance, catechins are known as the characteristic polyphenolic compounds in tea. (−)-Epigallocatechin-3-gallate (EGCG) is the most abundant and active catechin in green tea (Jankun et al., 1997; Yang et al., 2007). In contrast to the anticancer activity demonstrated in animal models, the results from epidemiological studies assessing associations between tea consumption and human cancer risk have not yielded a clear conclusion (Yang et al., 2009). So far it has not been clearly elucidated whether EGCG plays a critical role in the process of anticancer. On the other hand, a serious concern
exists about possible toxicities in individuals with high EGCG supplement intakes. EGCG-induced liver damage has been reported in studies from France, Spain, and Canada (Gloro et al., 2005; Molinari et al., 2006). Many studies have observed both pro- and antioxidant effects of EGCG (Galati and O’Brien, 2004). Furukawa et al. (2003) have found that EGCG induced H2O2 generation and caused subsequent oxidative damage to isolated and cellular DNA in the presence of transition metal ions. Nakagawa et al. (2004) reported that EGCG-mediated generation of H2O2 primarily triggers Fe(II)-dependent apoptosis in Jurkat cells. EGCG also significantly induces DNA oxidation in HL-60 cells. DNA oxidation induced by EGCG is further increased in glutathione-depleted cells; however, this does not occur in H2O2-resistant HP100 cells (Ebbing et al., 2005). These findings suggested that the H2O2 induced by EGCG might participate in DNA oxidation and could explain why EGCG did not inhibit, but increased 1,2-dimethylhydrazine or 2,2′-dihydroxy-di-n-propyl-nitroamine-induced colon carcinogenesis in male F344 rats (Hirose et al., 2001, 2002).

Copper is an important structural metal ion present in chromatin (Lewis and Laemmli, 1982) and is necessary for human health. Among metal ions in +2 state, Cu(II) (Cu2+) ion is the strongest oxidant in the solution state (Ouyang et al., 2008). The Cu2+ ion alone does not cleave DNA, but it indeed significantly induces single- and double breaks in the presence of reducing agent and/or H2O2. It can propagate reactive oxygen species (ROS) chains. However, in vivo, copper ions are seques-tered in forms unable to catalyze free radical reaction (Halliwell and Gutteridge, 1990). Very low levels of free copper ions may be released by tissue injury (Smith et al., 1992) or possible hepatic Cu2+ overload diseases. Most importantly, several studies have also reported a great increase of copper ions in various malignant tumor, including breast cancer, colorectal cancer, bladder carcinoma, hepatic cancer, kidney cancer, and lung cancer (Dobrowolski et al., 2002; Ebara et al., 2000; Hadi et al., 2010). For this reason, there is increased interest to investigate the ability of Cu2+ ion in DNA-damaging reactions in vivo and in vitro. Many studies have focused on the interactions of catechins with metal ions. They have found that catechins could cause redox reactions or chelate with metal ions under different conditions (Azam et al., 2004; Furukawa et al., 2003; Hayakawa et al., 2004; Kumatomo et al., 2001; Okaiawa et al., 2003). Interaction of catechins with metal ions also changed their bioactivities and metabolisms, which might be an important way that catechins could prevent or cure cancer (Yu et al., 2007).

Therefore, in this study, using a cellular system of human peripheral lymphocytes and alkaline single cell gel electrophoresis (comet assay), we determined whether GABA-tea extract is capable of oxidatively damaging cellular DNA in the presence of copper ions. Furthermore, we elucidated the mechanism of GABA-tea extract (Cu(II)) system-induced DNA damage. Our findings have implications for the potential of GABA-tea extract as anticancer agents.

2. Materials and methods

2.1. Chemicals

Ammonium ferrous sulfate, cupric chloride, EGCG, glutathione, N-sodium lauryl sarcosinate, neocuproine, sodium azide, Triton X-100 and xylene orange were purchased from Sigma Chemical Co. (St. Louis, MO). Lymphocyte separation medium (Ficoll–Paque PLUS) was acquired from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Superoxofer 4X174 phage DNA was purchased from Promega Corporation (Madison, WI). Hydrogen peroxide (H2O2) was purchased from Wako Pure Chemical Industries, Ltd. Taiwan. GABA-tea extract was dissolved in deionized water before using as a stock of 100 mg/mL solution.

2.2. Preparation of GABA-tea extract

The experimental procedures were based on previously described method with slight modifications (Wang et al., 2004). GABA-tea leaves were purchased from a local tea market (I-Min Corporation, Ltd.) in Nantou, Taiwan. Six grams of GABA-tea leaves were steeped twice in 120 mL of boiling water for 5 min. After quickly cool-ing to room temperature, the infusion was filtered twice through a filter paper (Whatman No.1) and freeze-dried under vacuum condition (Kingmech Corporation, Ltd. Taiwan). GABA-tea extract was dissolved in deionized water using as a stock of 100 mg/mL solution.

2.3. Lymphocyte isolation

Human peripheral blood was obtained from healthy subjects in a 10 mL tube containing heparin. Lymphocytes were isolated by a slightly modified procedure according to the manufacturer’s instructions. Briefly, 3 mL of whole blood was diluted 1:1 with RPMI 1640 and carefully layered on the top of lymphocyte separation medium in a centrifugation tube in a ratio of 1:1. After centrifugation for 15 min at 400g, the white layer of lymphocytes at the interface between blood plasma and the medium was carefully transferred into a tube containing 5 mL of culture medium, RPMI 1640. The lymphocytes were then washed twice with RPMI 1640 and centri-fuged at 250g for 10 min. The cell pellet was resuspended in 6 mL of RPMI 1640 medium containing 10% fetal bovine serum and 2 mM glutamine. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO2.

2.4. Cell culture and chemical treatment

One milliliter of lymphocytes in RPMI 1640 medium was incubated with vari-ous concentration of GABA-tea extract in the absence or presence of Cu2+ for 1 h. The reaction mixture was centrifuged at 2000g. The supernatant was collected for the detection of H2O2 formation. The cell pellet was washed in phosphate-buffered saline (PBS) (Ca2+ and Mg2+ free) and used for the comet assay. Cell viability was determined before the start and end of the reaction.

2.5. Comet assay (single cell gel electrophoresis)

Comet assay was adapted from the method of Chuang and Hu (2004). One mil-liliter of lymphocyte pellet was mixed with 500 μL of 1% low-melting-point agarose (LMPA) in PBS at 37 °C, and 140 μL of this mixture was applied onto a frosted glass microscope slide pre-coated with a layer of 1% normal-melting-point agarose (150 μL). After application of a third layer of 1% normal-melting-point agarose (150 μL), the slides were immersed in cold-lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, 1% N-sodium lauryl sarcosinate, 1% Triton X-100, 100 DMSO) for 1 h at 4 °C. The slides were then placed in an electrophoresis tank, allow-ing the DNA to unwind for 15 min in the alkaline solution (300 mM NaOH and 1 mM Na2EDTA). Electrophoresis was then performed at 300 mA for 20 min in the same alkaline solution at room temperature. The slides were then neutralized with 0.4 M Tris–HCl buffer (pH 7.4) and stained with ethidium bromide. The image was then read with an Olympus BX51 fluorescence microscope equipped with inte-grated CC camera (Moticam 2000). To assess lymphocyte DNA breakage, Comets were scored at 400× magnification. Images from 60 cells (20 from each triple slide) were analyzed by computer using the Image Pro Plus 6.0 software (Media Cybernetics, USA). The percent of DNA fragmentation is used as DNA damage parameter for this study and defined as the percent of DNA in the Comet tail versus total DNA from both Comet tail (DNA fragments) and Comet head (cell nucleus).

2.6. DNA cleavage assay

The experimental procedures were based on previously described method with slight modifications (Wang et al., 2004). Briefly, supercoiled φX174 phage DNA was incubated with various concentration of GABA-tea extract in the absence or pres-ence of Cu2+ at 37 °C for 1 h. The total volume was 10 μL in 1 × PBS (Ca2+ and Mg2+ free). After incubation, each sample was mixed with 5 μL of the staining dye solution (bromophenol blue and xylene cyanole in 50% glycerol) and then the mixture was loaded onto a pre-prepared 1% agarose gel. The gel was run at 100 V for 30 min. Due to single-strand DNA cleavage, the supercoiled Form-I DNA was converted into a relaxed open circular Form-II DNA. The two DNA forms were separ-ated by agarose gel electrophoresis and quantified to obtain the percent of DNA cleavage (Wang et al., 2004). To understand DNA cleavage mechanism, scavengers used for different purposes were: catalase to remove H2O2, sodium azide to quench single oxygen, neocuproine to chelate Cu2+.

2.7. Detection of Cu(II) reduction by GABA tea

The experimental procedures were described previously (Bhat et al., 2006). The selective sequestering agent neocuproine was employed to detect the reduction of Cu2+ to Cu+ by recording the formation of neocuproine–Cu(I) complex which ab-sorbs maximally at 450 nm. The reaction mixture (3 mL) contained 3 mM Tris-
2.8. Detection of H$_2$O$_2$ in the incubation medium

The ferrous ion oxidation-xylene orange (FOX) method was adapted to detect and quantify the generation of H$_2$O$_2$ in the incubation medium RPMI 1640, as described by Nourooz-Zadeh et al. (1996). 100 μL of the incubation medium was mixed with 900 μL of the FOX reagent and incubated for 30 min. Solutions were then centrifuged at 15,000 g for 10 min at room temperature. The absorbance at 560 nm was measured. The results were calibrated against solutions of H$_2$O$_2$ of known concentrations.

2.9. Statistics

The statistical analysis was performed as described by Tice et al. (2000) and is expressed as median ± SD of three independent experiments. A student’s t-test was used to examine statistically significant differences. Analysis of variance was performed by using ANOVA. P-values <0.05 were considered statistically significant.

3. Results

3.1. GABA-tea extracts/Cu(II) mediated DNA breakage in human peripheral lymphocyte

In order to investigate the role of Cu$^{2+}$ on GABA-tea extract-mediated DNA breakage production, the Comet assay was performed under alkaline condition. Photographs of Comets seen on treatment of lymphocytes with GABA-tea extract either alone or in the presence of Cu$^{2+}$ are shown in Fig. 1. Cells are intact without a tail after exposure to 100 μM Cu$^{2+}$ (panel a, Fig. 1A), indicating Cu$^{2+}$ alone does not render any effect on DNA breakage in this condition. When cells treated with 0.5 mg/mL GABA-tea extract, a Comet with slight tail was induced (panel b, Fig. 1A). Interestingly, a Comet with a significant tail indicative of DNA breakage can be seen when lymphocytes were co-treated with GABA-tea extract (0.1–0.5 mg/mL) and Cu$^{2+}$ (100 μM) (panel c, Figs. 1A and B). Compared with GABA-tea extract alone, a 19-fold increase of DNA breakage was obtained in 100 μM Cu$^{2+}$ and 0.1 mg/mL GABA-tea extract co-treatment, while a 4.5-fold enhancement of DNA breakage occurred in 100 μM Cu$^{2+}$ and 0.5 mg/mL GABA-tea extract (Fig. 1B). To further investigate the effect of GABA-tea extract and Cu$^{2+}$ in DNA breakage, cells were treated with increasing concentrations of copper ions at 0.5 mg/mL GABA-tea extract. A significant increase of DNA breakage as showed by increased tail% of Comets is observed at 100 μM Cu$^{2+}$ (Fig. 1C). 200 μM Cu$^{2+}$ did not further enhance the tail%. These results indicate that GABA-tea extract/Cu$^{2+}$ co-exposure is capable of inducing DNA breakage in lymphocytes, although Cu$^{2+}$ alone did not induce significant DNA breakage. Furthermore, Cu$^{2+}$ could significantly enhance DNA breakages at low dosages of GABA-tea extract co-exposure.

3.2. Cleavage of supercoiled ΦX174 phage DNA by GABA-tea extract/ Cu(II)

To understand the chemical basis of DNA breakage by GABA-tea extract/Cu(II) system, we tested the ability of GABA-tea extract to cause cleavage of supercoiled ΦX174 phage DNA in the presence of copper ions. The results given in Fig. 2A showed that 10 μM Cu$^{2+}$ (lane 2) or 10 μg/mL GABA-tea extract (lane 6) alone did not cause DNA cleavage. In contrast, gradually increased open circular DNA (form-II) was accompanied with increasing concentrations of GABA-tea extract (1–10 μg/mL) in the presence of 10 μM Cu$^{2+}$ (Fig. 2A, lane 3–lane 5). Similar results were obtained when the concentrations of GABA-tea extract were up to 50, 100, and 500 μg/mL, respectively (data not shown). Moreover, nearly all DNA are converted to Form-II at 10 μg/mL GABA-tea extract in the presence of 10–500 μM Cu$^{2+}$ (Fig. 2B). These data suggest that induction of DNA cleavage by lower dosages of GABA-tea extract is dependent on the concentration of copper ions. Taken together, GABA-tea extract/Cu$^{2+}$ co-exposure indeed caused DNA breakage in a cell-free system and in vitro.

EGCG is one of the major tea catechins in GABA-tea extract (Wang et al., 2006). It is conceivable that the EGCG might contribute the major effect on GABA-tea extract-induced DNA cleavage. To address this idea, ΦX174 phage DNA was exposed to different concentrations of EGCG in the same condition as mentioned in Fig. 2A and the DNA cleavage was assessed. As shown in Fig. 2C, EGCG caused DNA cleavage in a dose-dependent manner (1–50 μM) in the presence of 10 μM Cu$^{2+}$, whereas 50 μM EGCG alone did not

![Fig. 1](image-url)
lead to DNA cleavage (Fig. 2C, lane 7). However, GABA, the other major constituent in GABA-tea extract (Wang et al., 2006), did not induce DNA cleavage in the presence of 10 μM Cu²⁺ (Fig. 2D), even though the GABA concentration was high as 500 μM. It is likely that EGCG might play an important role in GABA-tea extract/Cu(II)-mediated DNA cleavage.

3.3. ROS was involved in GABA-tea extract/Cu(II)-induced DNA damage

Several lines of evidence suggest that EGCG is able to interfere with the dynamic equilibrium of oxidant/antioxidant systems. To explore whether ROS participate in the molecular event of DNA cleavage induced by GABA-tea extract/Cu(II), various active oxygen scavengers were used in GABA-tea extract/Cu(II) co-exposure and the levels of DNA cleavage were determined by supercoiled ΦX174 phage DNA cleavage assay. As shown in Fig. 3, DNA cleavage was substantially inhibited by the addition of free radical scavengers. The GABA-tea extract/Cu(II)-induced DNA cleavage was significantly inhibited in the presence of glutathione in a dose-dependent manner (Fig. 3A). The similar inhibitory effect was also observed in the treatment of neocuproine (Fig. 3B). Furthermore, 58.6% and 51.1% decrease of GABA-tea extract/Cu(II)-induced DNA cleavage were obtained at 7.5 mM sodium azide, a agent to quench single oxygen, and at 50 μg/mL catalase, respectively (Fig. 3C and D). Based on the biological effects of sodium azide

![Fig. 2. Effect of GABA-tea extract (GT), epigallocatechin-3-gallate (EGCG), or GABA in the absence or presence of Cu²⁺ on DNA cleavage. ΦX174 phage DNA was incubated with GABA tea in the absence or presence of 10 μM Cu²⁺ at 37 °C for 1 h. Points significantly different from control untreated shown by asterisks (p < 0.05, by Student’s t test).](image)

![Fig. 3. Effects of active oxygen scavengers on GABA-tea extract (10 μg/mL)-induced DNA cleavage in the presence of Cu²⁺ (10 μM). Points significantly different from control GABA-tea extract (10 μg/mL) and Cu²⁺ (10 μM) treated shown by asterisks (p < 0.05, by Student’s t test). (A) Glutathione, (B) Neocuproine, (C) Sodium azide, (D) Catalase.](image)
and catalase, it is reasonable to postulate the essential role of singlet oxygen and \( \text{H}_2\text{O}_2 \) in the GABA-tea extract/Cu(II)-induced DNA cleavage. Moreover, neocuproine, a specific Cu\(^+\) sequestering agent, completely repressed DNA cleavage (Fig. 3B), suggesting that Cu\(^+\) is an essential intermediate in the DNA cleavage reaction induced by GABA-tea extract/Cu(II).

3.4. Formation of \( \text{H}_2\text{O}_2 \) by GABA-tea extracts/Cu(II) in the incubation medium RPMI 1640

Many studies have found that tea-related phenolic compound, EGCG, induced the generation of \( \text{H}_2\text{O}_2 \) to trigger DNA damage (Furukawa et al., 2003; Nakagawa et al., 2004; Oikawa et al., 2003). Therefore, such production of \( \text{H}_2\text{O}_2 \) could account for lymphocyte DNA breakage. To assess the contribution of \( \text{H}_2\text{O}_2 \) induced by EGCG in GABA-tea extract/Cu(II) system induced-DNA breakage, lymphocytes were exposed to a series of different concentrations of catalase (a scavenger of \( \text{H}_2\text{O}_2 \)) in the presence of GABA-tea extract/Cu(II) co-treatment. 50 \( \mu \text{g/mL} \) catalase completely abolished GABA-tea extract/Cu(II) system induced-DNA breakage in lymphocytes determined by Comet assay (Fig. 4A). Furthermore, using FOX assay to determine the formation of \( \text{H}_2\text{O}_2 \) in culture media, the results presented in Fig. 4B showed that GABA-tea extract/Cu(II) co-treatment remarkably enhanced the \( \text{H}_2\text{O}_2 \) production as compared to Cu\(^{2+}\) alone. In conjunction with previous studies, the increased fractions of \( \text{H}_2\text{O}_2 \) production were dependent on Cu\(^{2+}\) concentrations. Therefore, it is conceivable that cellular DNA breakage by GABA-tea extract/Cu(II) system might be through extracellular \( \text{H}_2\text{O}_2 \). In sum, \( \text{H}_2\text{O}_2 \) is an essential component in the molecular mechanism in which GABA-tea extract/Cu(II) system lead to DNA cleavage.

3.5. Reduction of Cu(II) to Cu(I) by GABA-tea extracts/Cu(II)

Neocuproine could significantly suppress DNA cleavage induced by GABA-tea extract/Cu(II) in our assay condition. Therefore, it is of interest to determine whether Cu\(^{2+}\) could be reduced to Cu\(^+\) in GABA-tea extract/Cu(II) co-treatment. To address this idea, neocuproine was used to sequester Cu\(^+\) ion to form the Cu\(^+\)–neocuproine complex that has an absorption maximum at 450 nm. In the presence of 100 \( \mu \text{M} \) Cu\(^{2+}\) with various concentrations of GABA-tea extract, the production of Cu\(^+\) was enhanced by GABA-tea extract in a concentration-dependent manner (Fig. 5A). Meanwhile, the increased Cu\(^+\) ion production in the presence of 100 \( \mu \text{g/mL} \) GABA-tea extract was augmented by Cu\(^{2+}\) in a concentration-dependent profile (Fig. 5B). These data clearly demonstrate that GABA-tea extract could reduce Cu\(^{2+}\) to generate Cu\(^+\) ion. Furthermore, Cu\(^+\) and \( \text{H}_2\text{O}_2 \) are the mediators to cause DNA breakage by GABA-tea extract in cells.

4. Discussion

In this study, we have elucidated that GABA tea exhibited pro-oxidant properties, leading to oxidative strand breakage in DNA in the presence of low dose of Cu\(^{2+}\) ion. Thus, our findings imply that GABA tea consumption could be associated with ROS-mediated genotoxicity. Similar to the finding of Ouyang et al. (2008), our data also show that the redox cycle between Cu(I) and Cu(II), singlet oxygen and hydrogen peroxide might play essential roles in GABA-tea extract/Cu(II)-induced DNA cleavage. Moreover, GABA tea might not act as radical scavenger, but may behave as a pro-oxidant and/or as a reducing agent that promotes free radical to damage DNA during a Cu\(^{2+}\)-Cu\(^+\) reducing reaction.

Our data show that neocuproine prevents DNA damage in the reaction mixture (Fig. 3B), demonstrating that Cu\(^+\) is essential for GABA tea-induced DNA cleavage. Significant inhibition of DNA damage was also observed by adding glutathione, implying that the redox between Cu\(^{2+}\) and Cu\(^+\) might have an important role in GABA-tea extract/Cu(II)-induced DNA cleavage. In a previous study, green tea extract showed a high rate of Cu\(^{2+}\) reduction and consequent hydroxyl radical formation (Malik et al., 2003). The reduction of Cu\(^{2+}\) was possibly through epoxidation requiring the involvement of a hydroxyl radical in the presence of molecular oxygen (Singh et al., 2001). Azam et al. (2004) reported that through the generation of superoxide anion and hydroxyl radical, epicatechin and EGCG caused oxidative DNA strand breakage in the presence of copper ions. However, in our experimental conditions, superoxide dismutase (superoxide anion scavenger) and hydroxyl radical scavengers (mannitol, potassium iodide, sodium benzoate) were ineffective in the prevention of DNA breakage (data not shown). This indicated that superoxide anion and hydroxyl radical may be not the main ROS in the reaction, since GABA itself has the superoxide anion and hydroxyl radical scavenging activity (Chan et al., 1994; Jeng et al., 2007; Wang et al., unpublished data). On the other hand, sodium azide (singlet oxygen scavenger) and catalase effectively decreased DNA damage (Fig. 3C and D), sug-
suggesting that singlet oxygen and hydrogen peroxide might participate in GABA-tea extract/Cu(II)-induced DNA damage. Our experimental results are in consistent with the finding of Frelon et al. (2003), that reaction of copper ion and H₂O₂ produces singlet oxygen as the predominant ROS which causes DNA degradation. Based on our findings, we propose that in the course of the reduction of Cu²⁺ to Cu⁺ through GABA-tea extract, the singlet oxygen radical will be indirectly produced while Cu⁺ reacts with oxygen or H₂O₂ oxidize other molecules, for example, hypochlorous acid or lipids. Oxidation of polyphenolic compounds will produce several kinds of semiquinone radicals, which will be further oxidized to generate O-quinone form and H₂O₂ (Azam et al., 2004; Furukawa et al., 2003; Oikawa et al., 2003). Thus, the more GABA-tea extracts are oxidized, the more Cu⁺ and H₂O₂ are produced, causing the probability of singlet oxygen production will be remarkably augmented (Fig. 6). Our proposal is supported by the report from Ryter and Tyrrell (1998). The authors have reviewed that singlet oxygen may originate from biological sources as well as photochemical reactions.

Several previous studies have found that plant polyphenols have pro-oxidant effects in vitro, particularly in the presence of transition metal ions such as iron and copper (Hadi et al., 2007; Nakagawa et al., 2004; Said Ahmad et al., 1992). Malik et al. (2003) also found that water extract of green tea efficiently cleaves DNA in the presence of copper ions. GABA tea contains many polyphenolic compounds, similar to those in green tea (Wang et al., 2006). Although the contents of total catechin, epicatechin and EGCG are significantly lower in GABA tea than those in green tea, the antioxidant activity of GABA tea is close to green tea (Wang et al., unpublished data). However, GABA tea (10 µg/ml) contains less EGCG (0.71 µM) and more GABA (175.5 µM) than green tea. Our data showed that GABA alone (500 µM) does not induce DNA cleavage in the presence of copper ions. In accordance with Azam et al. (2004) studies, EGCG induced DNA cleavage in our experiments. It is notable that GABA-tea extract is significantly more genotoxic and pro-oxidant than pure EGCG. It has been reported that the actions of pure compound alone do not explain the observed health benefits of diets rich in crude form (complex mixture), because, taken alone, the individual antioxidants studied in clinical trials do not appear to have consistent preventive effects (Liu, 2004). Due to loss of bioactivity or behavior not as the same way as the compound in whole foods, the isolated pure compound may generate synergistic, antagonistic, or additive effects. Our data show the more DNA damage promotion effect in GABA tea crude extract, but not pure EGCC alone. In this regard, we suggest that (1) other compounds than EGCG may also play an important role in pro-oxidant activity of GABA tea; (2) a synergistic oxidative effect may occur in the complex constituents of crude extract of GABA tea.

Most plant polyphenols possess both antioxidant as well as pro-oxidant properties. It is believed that the chemopreventive properties of polyphenols generally reflect their ability to scavenge endogeneous free radicals. However, the pro-oxidant action of plant-derived phenolics rather than their antioxidant action may be an important mechanism for their anticancer properties. Previous studies have suggested that the cell killing activity of plant polyphenols may be related to their pro-oxidant activity (Galati and O’Brien, 2004; Hadi et al., 2007; Nakagawa et al., 2004; Nazeem et al., 2009). Several studies have demonstrated that many polyphenolic compounds may mobilize endogenous copper and consequently degrade cellular DNA to exhibit apoptosis inducing properties (Azmi et al., 2005, 2006; Hadi et al., 2000, 2010; Hanif et al., 2008). Copper is a major metal ion present in the nucleus, serum and tissue (Hanif et al., 2008). A number of studies have found that both serum and tumor copper levels were significantly elevated in cancer patients compared to healthy subjects (Hadi et al., 2010; Mazdak et al., 2010). Therefore, Hadi et al. (2007) suggested that cancer cells may be more subject to electron transfer between coppers and polyphenols to generate ROS responsible for DNA damage. They proposed that such pro-oxidant action could be a common mechanism for anticancer and chemopreventive properties of plant polyphenols. Many previous studies have reported that the cytotoxicity of catechins was relatively specific to tumor cells when compared with normal cells (Ahmad et al., 1997, 2000; Chen et al., 1998; Vergote et al., 2002). Such a mechanism would better explain the anticancer effects of polyphenols as it accounts for the preferential cytotoxicity against cancer cells. We provide the strong evidence that the GABA-tea extract generate oxidative stress in the presence of copper ions. In addition, GABA-tea extract alone was capable of inducing DNA breakage at high dosages (1 and 2 mg/mL) in lymphocytes (Fig. 1B). Moreover, our data showed that the background level of Cu²⁺ was approximately 0.27 µM at the dosage of 1 mg/mL tea extract in the experiments (data not shown). It is far below than 10 µM used in the reaction, suggesting that the GABA-tea extract was not naturally contaminated by copper. These results indicated that GABA tea might be able to mobilize endogenous copper to degrade cellular DNA in the nucleus. Based on our data, we propose that GABA tea may exhibit anticancer and apoptosis properties through its pro-oxidant action. Further evidence will be needed to perform to support our hypothesis.
In summary, Cu²⁺ and GABA tea in this study conferred a synergistic DNA oxidative damage. This might be related to the redox activities of Cu²⁺ ions in the reaction and the generation of free radicals. In crude form of GABA-tea extract, a cascade of radical reactions might be mediated by plant polyphenols to generate DNA damage in the reaction system. These findings have implications for the potential of GABA-tea extract in anticancer properties, which might involve copper ions and the consequent pro-oxidant action.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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