

Effects of methanolic extract from Fuzhuan brick-tea on hydrogen peroxide-induced oxidative stress in human intestinal epithelial adenocarcinoma Caco-2 cells

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Abstract. The present study investigated the protective effect of methanolic extract from Fuzhuan brick-tea (FME) on hydrogen peroxide (H₂O₂)-induced oxidative stress in the human intestinal epithelial adenocarcinoma cell line Caco-2. Caco-2 cells were pretreated with different concentrations (50, 100 and 200 µg/ml) of FME for 2 h and then exposed to H₂O₂ (1 mM) for 6 h. FME did not exhibit a significant cytotoxic effect and increased the cell viability following H₂O₂ treatment by decreasing lipid peroxidation in Caco-2 cells. To investigate the protective effect of FME on H₂O₂-induced oxidative stress in Caco-2 cells, the levels of intracellular glutathione (GSH) and the activity of the endogenous antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-px) and glutathione S-transferase (GST), were determined. FME significantly increased the level of GSH and the activity of antioxidant enzymes. The results from the present study demonstrated that FME has a protective effect on H₂O₂-induced oxidative damage in Caco-2 cells through the inhibition of lipid peroxidation and the increase in the activity of antioxidant enzymes. In addition, FME reduced the H₂O₂-induced expression of interleukin-8 at both the mRNA and protein levels in Caco-2 cells.

Introduction

Reactive oxygen species (ROS)-induced oxidative stress is caused by an imbalance between the antioxidant defense system and the generation of oxidants in the human body. It is associated with a number of human diseases, such as cardio-

vascular disease (CVD), diabetes, inflammatory disease, aging and cancer (1-3). It is well known that the intestinal epithelium plays an important role in nutrient absorption, and serves as a physical barrier separating the host from the external environment, thereby contributing to the defense against pathogens and xenobiotics mediated by the gut immune system (4). Overproduction of ROS results in lipid peroxidation, protein oxidation and DNA damage, and induces cell damage in intestinal epithelial cells (1). ROS-induced intestinal epithelial cell damage has been associated with the pathogenesis of inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (UC) (1). The endogenous antioxidant system, including glutathione (GSH) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-px) and glutathione S-transferase (GST) acts as a scavenger of the accumulated ROS, and thereby protects organs and cells from ROS-induced oxidative damage (3). Dietary intake of beverages, such as polyphenol-enriched tea products, may increase the levels of protective antioxidants in the body and improve the activity of the human antioxidant defense system to prevent ROS-induced colitis (5-8).

In response to pathogens, oxidative stress and pro-inflammatory cytokines, such as the tumor necrosis factor (TNF)-α, intestinal epithelial cells, increase the production of the chemokine interleukin-8 (IL-8), triggering an inflammatory reaction in the colonic mucosa that promotes IBD and colorectal carcinogenesis (9-11). Modulation of the IL-8 production in the intestinal epithelial cells is thus important to maintain intestinal health, and to attenuate the symptoms of IBD and colon cancer (12,13).

Fuzhuan brick-tea is a traditional fermented tea prepared by incubating leaves of *Camellia sinensis* var *sinensis* with *Eurotium* spp. fungi at 26-28°C for 12-15 days. Fuzhuan brick-tea is widely consumed by ethnic groups in the border regions of southern and western China (14). In China, Fuzhuan brick-tea is also used in folk medicine for its anti-dysenteric (14,15), anti-bacterial (15,16), anti-obesity and hypolipidemic activities (17).

The present study was designed to investigate the cytoprotective effects of methanolic extract from Fuzhuan brick-tea on H₂O₂-induced oxidative stress and to elucidate the underlying mechanisms in the human colon adenocarcinoma Caco-2 cell

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line. These cells are considered as a good model to study the function of the small intestine and exhibit typical features of healthy human intestinal epithelial cells, such as brush border microvilli, tight junctions and dome formation (18).

Materials and methods

Chemical reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, penicillin-streptomycin and 0.05% trypsin-0.53 mM EDTA were purchased from Gibco-BRL (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), TRIzol reagent, oligo(dT)₁₈ primers, murine maloney leukemia virus (MMLV) reverse transcriptase, RNase inhibitor, ethidium bromide (EtBr), and agarose were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Additional chemicals that were used were of standard analytical grade.

Fuzhuan brick-tea extract preparation. Fuzhuan brick-tea was purchased from Yiyang Fucha Tea Industry Development Co., Ltd. (Hunan, China). A total of 50 g of lyophilized Fuzhuan brick-tea was used for three extractions in 20-fold volume of methanol at room temperature and avoiding the light for 24 h. The methanol extracts were combined, filtered on filter paper (Whatman International Ltd., Maidstone, UK) and vacuum-concentrated at 50°C in a rotary evaporator (Büchi RE 111; Büchi Labortechnik, Flawil, Switzerland). The Fuzhuan brick-tea methanolic extract (FME) was dissolved in dimethyl sulfoxide (DMSO) and stored at -4°C until further analysis.

Cell culture. Human colon adenocarcinoma Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely maintained in DMEM medium supplemented with 20% (v/v) FBS, 1% penicillin-streptomycin, 1% glutamine and 1% non-essential amino acids in a humidified 5% CO₂ incubator (model 3110; Forma Scientific, Inc., Marietta, OH, USA) at 37°C.

Cell viability assay. Cell viability was assessed using the MTT assay. The cells were seeded in 96-well plates (Nalge Nunc Int. Corp., Rochester, NY, USA) at a density of 1x10⁴ cells/well. Following a 24-h incubation, the cells were primarily treated with different concentrations of FME (25, 100 and 200 µg/ml) for 2 h, and exposed to H₂O₂ (1 mM) for 6 h. Then, 100 µl MTT reagent (0.5 mg/ml) was added to each well and the cells were incubated in a humidified incubator at 37°C to allow MTT to be metabolized. After 4 h, 100 µl DMSO was added to each well to dissolve formazan deposits. The absorbance of the samples was measured at a 540 nm wavelength using a microplate reader (model 680; Bio-Rad, Hercules, CA, USA).

Quantification of lipid peroxidation. Lipid peroxidation was quantified using a thiobarbituric acid (TBA) reactive substance (TBARS) assay (19). First, the treated cells were washed with cooled phosphate-buffered saline (PBS) (pH 7.4, 0.1 M), scraped into trichloroacetic acid (TCA; 2.8%, w/v) and sonicated at 40 V 3 times at 10-sec intervals on ice. Total cell protein concentrations were determined using a bicinchoninic acid (BCA) assay kit (Bio-Rad). The suspension was mixed with 1 ml TBA (0.67%, w/v) and 1 ml TCA (25%, w/v),

heated (30 min at 95°C) and centrifuged (3,000 x g; 10 min at 4°C). TBA reacted with the products of oxidative degradation of lipids, producing red complexes, the absorbance of which was measured at 532 nm using a UV-2401PC spectrophotometer (Shimadzu, Kyoto, Japan).

Determination of intracellular glutathione (GSH) level. The intracellular GSH level was determined according to Ellman's method (20). The treated cells were washed with cooled PBS, collected and mixed with 10% sulfosalicylic acid solution to remove proteins, and centrifuged at 13,000 x g for 10 min at 4°C. The sample suspension (50 µl) was mixed with 200 µl Tris-HCl buffer (pH 8.9, 0.8 M) and 10 µl 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; 4 mg/ml) for 5 min at room temperature. The absorbance of the mixture was measured at a 412 nm wavelength using a UV-2401PC spectrophotometer (Shimadzu) for 5 min.

Antioxidant enzyme activity. Caco-2 cells grown in a 6-well cell culture plate (Nalge Nunc Int. Corp.) were incubated with different concentrations (25, 100 and 200 µg/ml) of FME for 2 h and then exposed to H₂O₂ (1 mM) for 6 h. The cells were washed with PBS, removed by scraping and centrifuged, and the resulting cell pellet was stored at -80°C. Cell pellets were thawed, resuspended in 300 µl cold lysis buffer (PBS and 1 mM EDTA), homogenized and centrifuged (12,000 x g; 10 min at 4°C). The supernatants were used for activity measurements. CAT activity was assessed according to the method described by Nelson and Kiesow (21), which is based on spectrophotometric measurement, at 240 nm, of the metabolized H₂O₂ substrate. SOD activity was assayed using a modified version of the method of auto-oxidation of pyrogallol (22). One unit of SOD activity was defined as the amount of enzyme that inhibited the auto-oxidation rate of pyrogallol by 50%. GSH-px activity was assayed according to the method described by Hafeman et al (23). GST activity was determined according to the method of Habig et al (24), by measuring the absorption of the formed 2,4-dinitrochlorobenzene (CDNB)-GSH conjugate at 345 nm. Protein contents were determined using a protein assay kit from Bio-Rad according to the manufacturer's instructions. All measured activities were expressed as units (U) of enzyme activity per mg protein.

IL-8 enzyme-linked immunosorbent assay (ELISA). Caco-2 cells grown in a 6-well cell culture plate were incubated with different concentrations of FME for 2 h and then exposed to H₂O₂ (1 mM) for 6 h. At the end of the experiment, 100-µl aliquots were collected from culture medium of each well, and IL-8 production was measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

mRNA expression of IL-8 determined by RT-PCR. Expression of IL-8 in the cells was measured by RT-PCR. Total RNA was isolated with the TRIzol reagent and centrifuged at 12,000 x g for 15 min at 25°C following the addition of chloroform. Isopropanol was added to the supernatant at a 1:1 ratio and the RNA was pelleted by centrifugation (12,000 x g for 15 min at 4°C). After washing with 70% ethanol, the RNA was solubilized in diethyl pyrocarbonate (DEPC)-treated RNase-free double-distilled water and quantified by measuring the

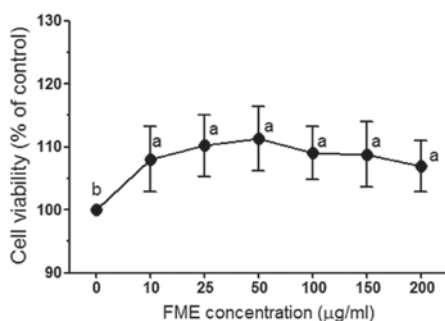


Figure 1. Effects of Fuzhuan brick-tea methanolic extract (FME) on cell viability in Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments. Different letters (a and b) on the data points designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test.

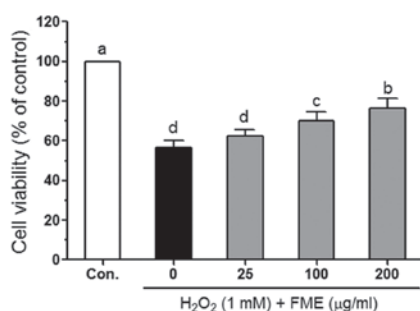


Figure 2. Effects of Fuzhuan brick-tea methanolic extract (FME) on cell viability in 1 mM hydrogen peroxide (H₂O₂)-treated Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments. Different letters (a-d) on the bars designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. Con, control (healthy) cells.

absorbance in a UV-2401PC spectrophotometer (Shimadzu) at 260 nm. Equal amounts of RNA (1 µg) were reverse transcribed by incubating in an AccuPower PCR PreMix (Bioneer Corp., Daejeon, South Korea) containing 1X reverse transcriptase buffer, 1 mM dNTPs, 500 ng of oligo(dT)₁₈ primers, 140 units of MMLV reverse transcriptase, and 40 units of RNase inhibitor, for 45 min at 42°C. PCR was then performed in an automatic thermocycler (Bioneer Corp.) as follows: 28 cycles (94°C for 60 sec, 57°C for 30 sec, and 72°C for 45 sec) and one cycle at 72°C for 5-min, as previously described (25). PCR products were separated in 2% agarose gels and visualized by EtBr staining. β -actin was used for normalization.

Statistical analysis. Data are presented as the mean \pm standard deviation (SD). Differences between mean values of individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant when $P < 0.05$. The SAS v9.1 statistical software package (SAS Institute Inc., Cary, NC, USA) was used for the analysis.

Results

Effects of FME on H₂O₂-induced cell damage in Caco-2 cells. To investigate FME-induced cytotoxicity, Caco-2 cells were incubated with different concentrations (10, 25, 50, 100 and

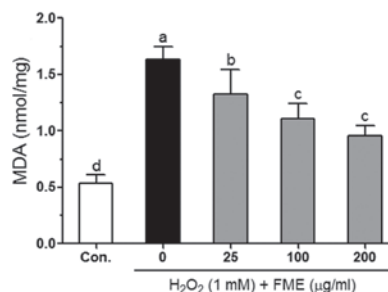


Figure 3. Effects of Fuzhuan brick-tea methanolic extract (FME) on the intracellular malondialdehyde (MDA) level in 1 mM hydrogen peroxide (H₂O₂)-treated Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments. Different letters on the bars (a-d) designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. Con, control (healthy) cells.

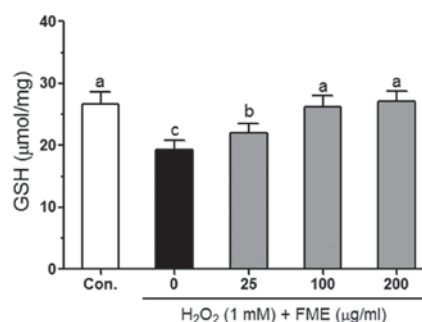


Figure 4. Effects of Fuzhuan brick-tea methanolic extract (FME) on the intracellular glutathione (GSH) level in 1 mM hydrogen peroxide (H₂O₂)-treated Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments. Different letters (a-c) on the bars designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. Con, control (healthy) cells.

200 µg/ml) of FME and the cell viability was determined by the MTT assay. After 24 h incubation, FME did not exert any significant cytotoxic effect in Caco-2 cells (Fig. 1). Therefore, the concentrations of 25, 100 and 200 µg/ml were selected for subsequent experiments. H₂O₂ (1 mM) significantly reduced viability of Caco-2 cells (Fig. 2). However, following treatment with FME, cell viability increased in a dose-dependent manner.

Effects of FME on H₂O₂-induced lipid peroxidation in Caco-2 cells. ROS-induced oxidative damage is related to lipid peroxidation in the cell membrane, and is thus accompanied by an increase in the production of malondialdehyde (MDA), a biomarker of cell membrane lipid peroxidation (26). The MDA level markedly increased (up to 3-fold) in 1 mM H₂O₂-treated cells, reaching 1.64 nmol/mg compared to 0.54 nmol/mg detected in control cells (Fig. 3). FME dose-dependently reduced the H₂O₂-induced MDA level in Caco-2 cells. At the concentration of 200 µg/ml, FME significantly reduced the MDA level (0.96 nmol/mg) by 58% compared to control cells (treated only with 1 mM H₂O₂).

Effect of FME on the GSH level in H₂O₂-treated Caco-2 cells. The level of GSH, a major and ubiquitous non-enzymatic antioxidant compound, is important for the activity of the antioxidant defense system that protects from oxidative stress-induced cell

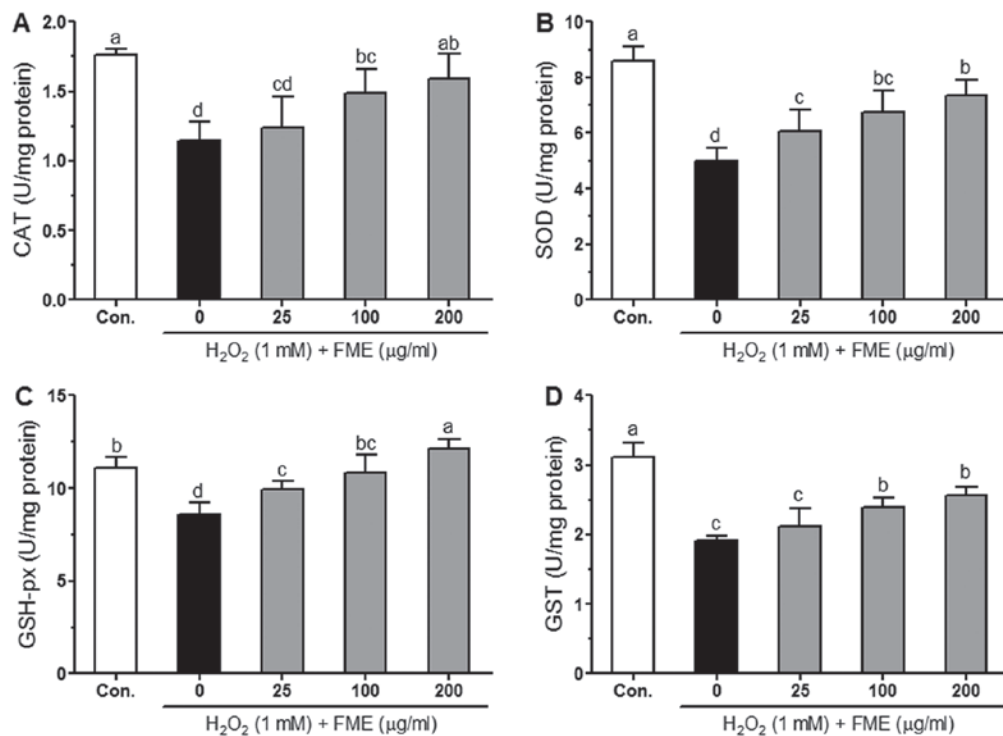


Figure 5. Effects of Fuzhuan brick-tea methanolic extract (FME) on the (A) activity of catalase (CAT), (B) superoxide dismutase (SOD), (C) glutathione peroxidase (GSH-px) and (D) glutathione S-transferase (GST) in 1 mM hydrogen peroxide (H_2O_2)-treated Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments. Different letters (a-d) on the bars designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. Con, control (healthy) cells.

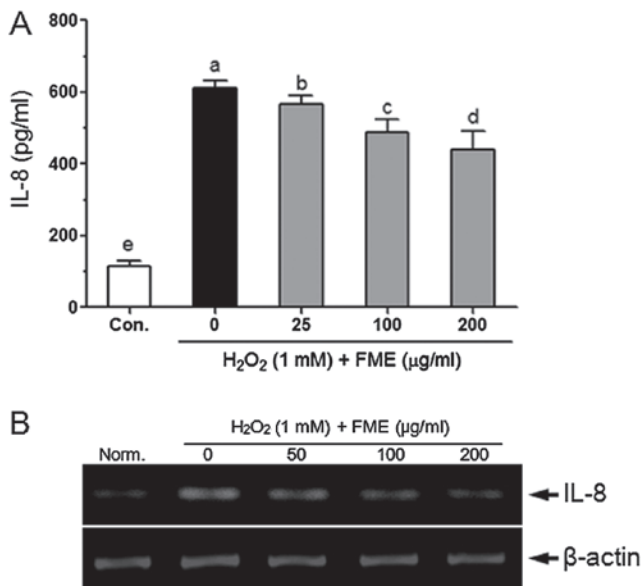


Figure 6. Effects of Fuzhuan brick-tea methanolic extract (FME) on (A) protein and (B) mRNA levels of IL-8 in 1 mM hydrogen peroxide (H_2O_2)-treated Caco-2 cells. Data are presented as mean \pm standard deviation (SD) of three independent experiments for (A) and as a representative blot with β -actin as the control for (B). Different letters (a-d) on the bars (A) designate statistically significant differences ($P < 0.05$) according to Duncan's multiple range test. Con, control (healthy) cells.

damage (27). Treatment with 1 mM H_2O_2 reduced the GSH level (19.35 nmol/mg) in the Caco-2 cells compared to control cells (26.64 nmol/mg) (Fig. 4). Pretreatment with different concentrations of FME significantly increased the intracellular

GSH level compared to control cells (treated only with 1 mM H_2O_2).

Effect of FME on CAT, SOD, GSH-px and GST activity in H_2O_2 -treated Caco-2 cells. It is well known that the activity of endogenous antioxidant enzymes such as CAT, SOD, GSH-px and GST protects cells from ROS-induced oxidative damage (28). The effects of FME on antioxidant enzyme activities in H_2O_2 -treated Caco-2 cells are shown in Fig. 5. H_2O_2 (1 mM) significantly ($P < 0.05$) decreased the CAT, SOD, GSH-px and GST activities compared to control cells. Pretreatment with FME increased the activity of these enzymes, most often in a significant manner as compared to H_2O_2 -treated cells. The increase in the activity of the enzymes was in general dose-dependent, with the most significant results observed for the GSH-px enzyme.

Effect of FME on the transcription and translation of IL-8 in H_2O_2 -treated Caco-2 cells. Oxidative stress was reported to induce IL-8 production in Caco-2 cells (25). The IL-8 level was significantly increased in response to treatment with 1 mM H_2O_2 for 6 h compared to control cells (Fig. 6A). Pretreatment with FME significantly and dose-dependently reduced the H_2O_2 -induced IL-8 production in Caco-2 cells. FME also reduced the H_2O_2 -induced mRNA level of IL-8 in Caco-2 cells (Fig. 6B).

Discussion

Fuzhuan brick-tea is a traditional Chinese fermented tea, rich in rutin, quercetin, gallic acid, catechin, epicatechin (EC),

epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and gallic catechin gallate (GCG) (17). The cytoprotective activity of Fuzhuan brick-tea has not been studied. Recent studies indicated that elevated ROS levels induce an inflammatory reaction, cause death of intestinal epithelial cells, and promote IBD and colon cancer (1,29). The present study was conducted in order to investigate the potential cytoprotective and anti-inflammatory effect of FME in H_2O_2 -stimulated human intestinal epithelial adenocarcinoma Caco-2 cells.

The intestinal epithelial cells are the major constituent of the mucosal barrier, and as such, play an important role in pathogenic microbe-induced infections, and in maintaining immune homeostasis in the colon (30). The human colon contains >1,000 microbial species with >10¹⁴ colony-forming units (CFU) per gram of feces (31,32). The intestinal microbial communities are closely associated with the pathogenesis of IBD (33). For example, *Enterococcus faecalis* produces extracellular superoxide ($O_2^{\cdot-}$) and H_2O_2 , and was shown to cause intestinal epithelial cell death (34). The reactive oxygen species H_2O_2 can easily cross cell membranes and react with Fe^{2+} to generate highly reactive $\cdot OH$ radicals through the so-called Fenton's reaction. $\cdot OH$ radicals attack a number of cellular compounds, such as DNA, proteins and membrane lipids, and thus cause cell damage (35). H_2O_2 was reported to significantly decrease the viability of Caco-2 cells and to increase the generation of MDA, a final product of lipid peroxidation (36). MDA is a cytotoxic product (37) that has been associated with the pathogenesis of colon diseases, in particular IBD and colon cancer (38). In the present study, we show that 1 mM H_2O_2 significantly increased the MDA level in Caco-2 cells. However, pretreatment with different concentrations (25, 100 and 200 $\mu g/ml$) of FME effectively reduced the H_2O_2 -induced increase in the MDA level. In addition, numerous studies have demonstrated that treatment with several plant-derived antioxidants such as rutin, quercetin, EGCG and polyphenols can ameliorate the H_2O_2 -induced production of MDA in Caco-2 cells (39-43).

GSH is a major non-enzymatic antioxidant, and protects Caco-2 cells from H_2O_2 -induced cell damage (36,44). We show that H_2O_2 significantly decreased the GSH level in Caco-2 cells. However, we found that pretreatment with FME dose-dependently inhibited the H_2O_2 -induced decrease in the GSH level. Aherne et al (45) reported that pretreatment with different plant extracts such as sage (*Salvia officinalis* L.), echinacea (*Echinacea purpurea* L.) and oregano (*Origanum vulgare* L.) increased the intracellular GSH levels, thereby protecting Caco-2 cells from H_2O_2 -induced cell damage. Our results indicate that an increased GSH level achieved by pretreatment with FME can also protect Caco-2 cells from H_2O_2 -induced oxidative stress.

In mammalian cells, accumulating free radicals and ROS are scavenged by the endogenous antioxidant system, comprising GSH and the antioxidant enzymes CAT, SOD, GSH-px and GST (3). SOD catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2 and H_2O_2 is further reduced to H_2O by CAT and GSH-px. A few studies have reported that lack of the endogenous antioxidant enzymes correlates to development of colitis and colon cancer, while increased activity of antioxidant enzymes in the colon effectively reduces oxidative stress-induced colonic

tissue damage (5-8). In the present study, we found that CAT and SOD activities are significantly decreased following exposure to H_2O_2 (1 mM), and this finding is in agreement with results from the study of Katayama et al (44). We also found that pretreatment with FME significantly increased the CAT and SOD activities in H_2O_2 -treated Caco-2 cells. Treatment with different dietary flavonoids (such as kaempferol and quercetin) increased the CAT activity in Caco-2 cells (46). Wijeratne and Cuppett (47) also reported that carnosol and carnosic acid significantly increased the SOD activity and protected Caco-2 cells from lipid hydroperoxide-mediated oxidative stress. However, treatment with rutin and quercetin did not significantly affect the activity of CAT and SOD in Caco-2 cells treated with H_2O_2 (40). GSH-px, the most important enzymatic scavenger of H_2O_2 , is involved in detoxification from lipid hydroperoxides (48). Increasing the activity of GSH-px prevented the transport of lipid hydroperoxides in Caco-2 cells (49). Pretreatment with FME elevated the intracellular GSH-px activity in cells treated with 1 mM H_2O_2 for 6 h compared to control cells (treated with H_2O_2 alone). Carrasco-Pozo et al (50) reported that treatment with quercetin, epicatechin and rutin protects Caco-2 cells from indometacin-induced oxidative damage, by increasing the ratio of GSH/oxidized glutathione (GSSG). In addition, quercetin, catechin and epicatechin were also shown to protect human astrocytoma U373 MG cells from H_2O_2 -induced cell damage by increasing the GSH-px activity (51). Treatment with other antioxidants, such as carnosol and carnosic acid, also increased the GSH-px activity, thereby protecting Caco-2 cells from lipid hydroperoxide-mediated oxidative stress (47). GST is a detoxification enzyme expressed in most mammalian cells, and catalyzes the conjugation of electrophilic compounds to glutathione (52), providing protection from H_2O_2 -induced cell death (53). Pretreatment with FME dose-dependently increased the GST activity in H_2O_2 -treated Caco-2 cells. Increasing the activity of GST was reported to cause a reduction in H_2O_2 -induced damage in Caco-2 cells (44,54). These results suggest that Fuzhuan brick-tea that is enriched in phytochemicals can act as a chemoprotective agent, protecting Caco-2 cells from H_2O_2 -induced oxidative stress by enhancing the activity of the endogenous antioxidant system.

In response to external stimuli, such as bacteria, toxins, chemicals and oxidative stress, intestinal epithelial cells overexpress and secrete the chemokine IL-8 (9-11). In IBD and colon cancer pathogenesis, IL-8 plays an important role in inducing the infiltration of neutrophils and T cells into the intestinal mucosa (55,56). A number of studies demonstrated that treatment with 5-caffeoylquinic acid, caffeic acid and isoflavones effectively reduces the H_2O_2 and TNF- α -induced IL-8 overproduction, as well as the overexpression of the *IL-8* gene (57,58). In this study, we found that pretreatment with FME effectively attenuated the H_2O_2 -induced IL-8 overproduction, and also reduced the mRNA expression of *IL-8* in Caco-2 cells exposed to H_2O_2 . In addition, Netsch et al (59) reported that treatment with 250 $\mu g/ml$ of green tea extract significantly reduces the production ($P<0.05$) and mRNA expression ($P<0.01$) of *IL-8* in IL-1 β -stimulated Caco-2 cells.

In conclusion, we demonstrated that FME can protect Caco-2 cells from H_2O_2 -induced oxidative stress. This is accomplished through an increase in the intracellular GSH

level, in the activity of endogenous antioxidant enzymes (CAT, SOD, GSH-px and GST), as well as through a reduction in H₂O₂-induced production of MDA. Our results also show that FME significantly reduced the protein and mRNA level of IL-8 in the H₂O₂-treated human colon adenocarcinoma cell line Caco-2. The results from the present study suggest that Fuzhuan brick-tea may serve as a preventive agent in the treatment of intestinal inflammations.

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