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Antiproliferative Effects of Dietary Phenolic Substances and Hydrogen Peroxide

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There has been controversy as to whether the antiproliferative activity of dietary phenolic substances on cancer cells is due to the bioactivities of phenolics or the generation of hydrogen peroxide (H_2O_2) in media as an artifact. This study was to investigate whether the formation of H_2O_2 by different phenolics induces acute toxicity and carcinogenicity in normal rat liver epithelial cells. Gallic acid, one of the major antioxidants present in fruits and vegetables, dose-dependently generated considerably more H_2O_2 in DMEM media without cells than did quercetin. Gallic acid exerted stronger antiproliferative activity than quercetin on both Caco-2 human colon cancer cells (Caco-2 cells) and WB-F344 normal rat liver epithelial cells (WB cells) cultured in DMEM media, and the effect was partially reduced by catalase. Furthermore, gallic acid (but not quercetin) also inhibited gap-junction intercellular communication (GJIC; a carcinogenic phenomenon), which was in part protected by the addition of catalase. Exogenous H_2O_2 addition also inhibited the proliferation of both Caco-2 cells and WB cells and inhibited GJIC in a dose-dependent manner, but these effects were almost abolished by the treatment with catalase. From these results it is concluded that the antiproliferative effects of some antioxidants on cancer cells are partially due to their prooxidant actions.

KEYWORDS: Antioxidants; antiproliferation; gallic acid; quercetin; hydrogen peroxide; gap-junction intercellular communication

INTRODUCTION

Some phytochemicals are potential cancer chemopreventive agents, partly due to their antiproliferative activity on cancer cells. Contradictory results on the antiproliferative effects of dietary phenolic substances on cancer cells in relation to the formation of phenolics-induced hydrogen peoroxide (H_2O_2) in the media have been reported in this journal. Lapidot et al. (*1*) reported that antiproliferative activity of apple extracts is associated with the generation of H_2O_2 by phenolics. They also showed that gallic acid and quercetin generate substantial amounts of H_2O_2, which can inhibit the proliferation of cancer cells. However, Liu and Sun (*2*) responded that apple extracts did not induce H_2O_2 generation based on the phenol red assay. They stressed that addition of catalase to apple phenolics in the media did not reduce the antiproliferative activity, but rather slightly increased the activity. Furthermore, H_2O_2 up to 100 μM did not inhibit the proliferation of cancer cells and was completely decomposed within 1 h.

Most phenolic phytochemicals are generally recognized as antioxidants, but some phenolic substances can exert prooxidant activities under certain conditions, such as in the presence of transition metal ions or alkalis. Several studies have shown that metal-mediated autoxidation of some phenolic phytochemicals generates semiquinone radicals, resulting in the enhancement of redox activity to produce reactive oxygen species (ROS) including H_2O_2 (*3*, *4*). Thus, some phenolic phytochemicals may also exert antimutagenic and antitumor-promoting activities at relatively low doses, whereas some excess antioxidant remaining in a free form may instead exhibit acute toxicity or carcinogenicity. Epidemiological and human studies also support the idea that a low risk of cancer is more strongly related to antioxidant-rich diets than to individual dietary supplement antioxidants (*5*, *6*).

H_2O_2 is a major molecule that induces many human diseases including cancer, heart disease, and cerebrovascular disease through diverse cellular processes, although high doses can reduce the growth of tumor cells (*5*, *7*, *8*). The involvement of H_2O_2 in carcinogenesis has been shown in both in vivo and in vitro studies (*5*−*7*). Gap-junction intercellular communication (GJIC) is essential for maintaining the homeostatic balance by modulating cell proliferation and differentiation in multicellular organisms (*9*). Inhibition of GJIC is strongly related to carcinogenicity, particularly to tumor promotion (*10*). A recent study (*7*) suggests that carcinogenicity of H_2O_2 is attributable to the inhibition of GJIC.

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The present study investigated whether the antiproliferative activity of some dietary phenolic substances on cancer cells is due to the generation of H_2O_2 in media as an artifact and, if so, whether the H_2O_2 generated by some phenolic phytochemicals induces acute toxicity and inhibits GJIC in normal rat liver epithelial cells.

MATERIALS AND METHODS

Chemicals. Gallic acid, quercetin, H_2O_2, catalase (bovine liver, EC 1.11.1.6), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade (Fisher, Springfield, NJ). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), glutamate, penicillin, and streptomycin were obtained from GIBCO BRL (Grand Island, NY).

Measurement of Hydrogen Peroxide. The concentration of H_2O_2 was determined using ferrous iron oxidation in xylenol orange (the FOX assay) according to the procedure described by Nourooz-Zadeh (*11*). Gallic acid and quercetin dissolved in distilled deionized water (ddH$_2$O) and dimethyl sulfoxide (20 mg/mL, respectively) were used as sample stock solution. Twenty microliters of diverse concentration of each sample was mixed with 180 μL of FOX working reagent [100 μM xylenol orange, 4.4 mM butylated hydroxytoluene, 250 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, and 25 mM H_2SO_4 in 90% (v/v) methanol] for 1 h at 37°C, and the absorbance at 560 nm was measured using a microplate reader (Emax, Molecular Devices, CA). The H_2O_2 concentration was calculated from a standard curve with authentic H_2O_2.

Cell Culture. Caco-2 human colon cancer cells (Caco-2 cells) were obtained from Korean Cell Line Bank, and WB-F344 normal rat liver epithelial cells (WB cells) were kindly provided by Dr. J. E. Trosko at Michigan State University. Both cell lines (passages 8−15) were cultured in DMEM media supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin in a humidified incubator maintained 37 °C and supplied with 5% CO_2 and 95% air.

Cytotoxicity. The cytotoxicity was measured by the MTT assay as previously described (*12*). Briefly, cells were cultured in 96-well plates at 10^4 cells/well in media for 4 h. Each well was then filled with fresh DMEM containing various doses of phenolic phytochemicals, and the cells were incubated for another 48 h at 37 °C. Each well was then incubated with MTT for 4 h. The liquid was removed, and DMSO was added to dissolve the solid residue. The optical density at 570 nm of each well was then determined by using a microplate reader. The cytotoxicity was determined by absorbance reductions of the samples at various concentrations with or without catalase (100 Sigma units/mL) when compared with untreated control. All data are presented as mean ± standard deviation (SD) for at least three replications for each prepared sample.

Bioassay of GJIC. The GJIC was measured by the scrape-loading/dye-transfer technique as described previously (*13*). Briefly, WB cells were treated with various concentrations of gallic acid or with 300 μM H_2O_2 for 1 h. The GJIC assay was conducted at non-cytotoxic doses of the samples, as determined by the MTT assay. Following incubation, the cells were washed twice with 2 mL of phosphate buffer solution (PBS). Lucifer yellow was added to the washed cells, and three scrapes were made with a surgical steel-bladed scalpel at low light intensities. Each scrape was performed so as to ensure that it traversed a large group of confluent cells. After an incubation period of 3 min, the cells were washed four times with 2 mL of PBS and then fixed with 2 mL of a 4% formalin solution. The number of communicating cells as indicated by the dye was counted under an inverted fluorescence microscope (Olympus Ix70, Okaya, Japan).

RESULTS AND DISCUSSION

Both gallic acid and quercetin at concentrations of 25 μg/mL or more can generate H_2O_2 in a dose-dependent manner in DMEM media without cells (**Figure 1**). Gallic acid generated 269 μM H_2O_2 at the concentration of 100 μg/mL for 1 h, whereas quercetin generated 59 μM H_2O_2 under the same condition. Gallic acid generated much higher amounts of H_2O_2 than quercetin, but the generation of H_2O_2 was diminished in both cases by the addition of catalase.

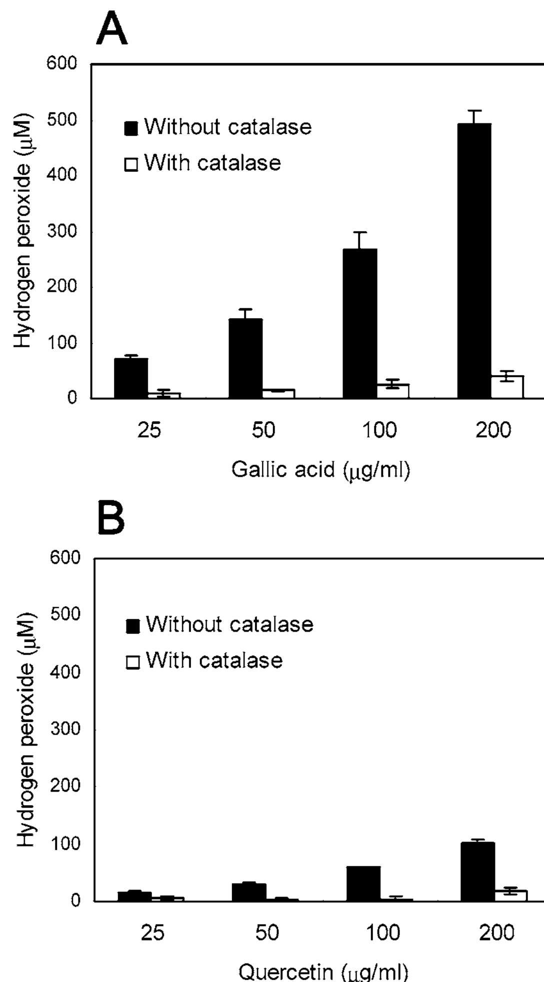

Figure 1. Generation of H_2O_2 by gallic acid (**A**) and quercetin (**B**) in the absence or presence of catalase in DMEM including 10% FBS for 1 h. H_2O_2 was measured by the FOX assay. Error bar = SD, $n \geq 6$.

We also found that gallic acid and quercetin at concentrations of 12.5 μg/mL or more inhibited the proliferation of both WB and Caco-2 cells in a dose-dependent manner (**Figure 2**). Gallic acid exhibited cytotoxicities of 75 and 54% at the concentration of 25 μg/mL in both Caco-2 and WB cells, respectively, whereas quercetin showed cytotoxicities of 34 and 22% in both cells under the same condition. The cytotoxicity of gallic acid and quercetin was in part abolished by the addition of catalase. Addition of exogenous H_2O_2 at a concentration of 100 μM or more also inhibited the proliferation of both Caco-2 and WB cells in a dose-dependent manner, but these effects were completely diminished by catalase (**Figure 3**).

On the contrary, Liu and Sun (*2*) reported that the cytotoxicity of apple phenolics on Caco-2 cells was not diminished by catalase. One unit of catalase (Sigma Chemical Co.) was defined as the concentration of enzyme decomposing 1.0 μM H_2O_2 per minute at pH 7.0 at 25 °C. Because 1 Sigma unit of catalase used by Liu and Sun (*2*) did not decompose H_2O_2 in 1 min (**Figure 4**), the generation of H_2O_2 by apple phenolics may in part induce activation of intracellular signal pathways leading to cell death. Liu and Sun (*2*) used DMEM media including 5% FBS to culture the Caco-2 cancer cells, whereas both Lapidot et al. (*1*) and the present study used DMEM media including 10% FBS. Other studies used DMEM media including 15 or

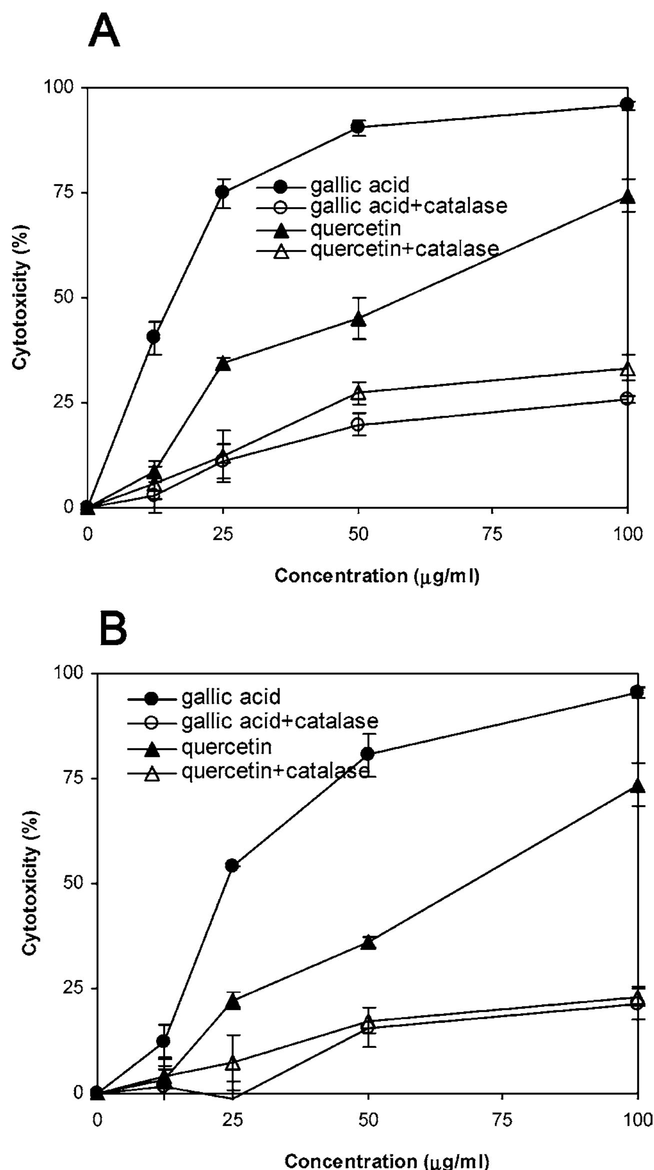

Figure 2. Cytotoxicity of gallic acid and quercetin in the absence or presence of catalase on Caco-2 human colon cancer cells (**A**) and WB-F344 rat liver epithelial cells (**B**) in DMEM including 10% FBS. Cytotoxicity was determined by the MTT assay. Error bar = SD, $n \geq 6$.

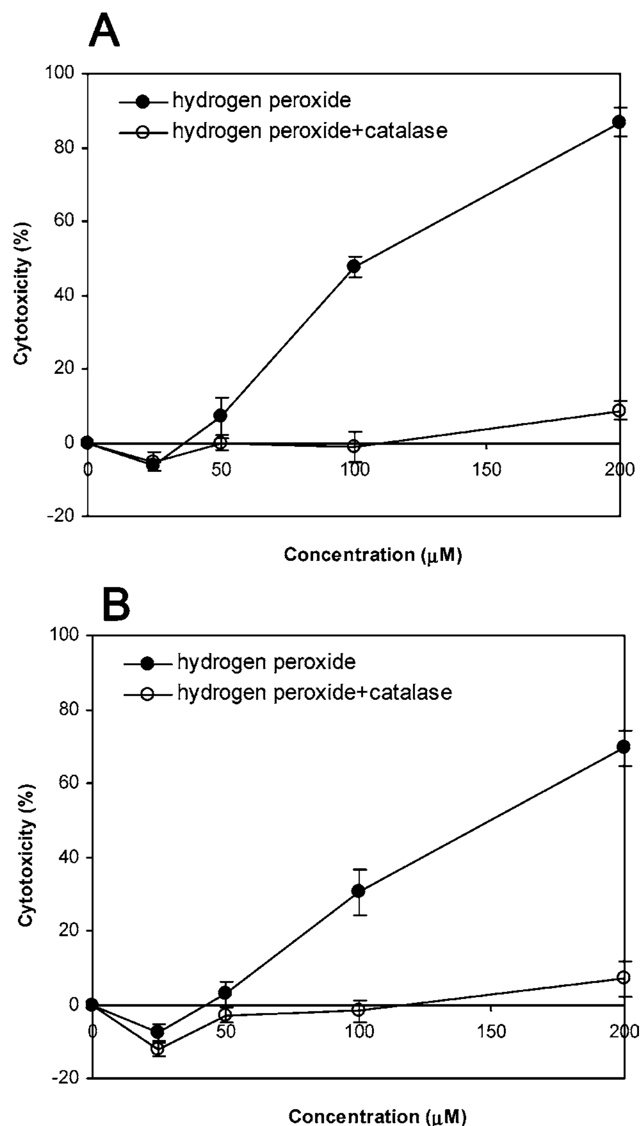

Figure 3. Cytotoxicity of authentic H_2O_2 in the absence or presence of catalase on Caco-2 (**A**) and WB (**B**) cells in DMEM including 10% FBS. Cytotoxicity was determined by the MTT assay. Error bar = SD, $n \geq 6$.

20% FBS for culturing Caco-2 cells (*14*, *15*). As shown in **Figure 5**, the generation of H_2O_2 by gallic acid was much higher in DMEM than in PBS. Gallic acid also generated 2 times higher amounts H_2O_2 in DMEM media including 20% FBS than in DMEM including 5% FBS (**Figure 5**). Other previous reports (*3*, *4*) support the assumption that the generation of H_2O_2 by gallic acid is mediated by metal ions present in the media and FBS. Thus, the discrepancy of the results between Liu and Sun (*2*) and Lapidot et al. (*1*) may in part due to the difference in the concentration of catalase and FBS as well as apple extracts used. Our results indicate that the inhibitory effects of phenolic phytochemicals on the proliferation of both cancer and normal cells are partially due to their prooxidant activity.

Liu and Sun (*2*) suggested that the ferrous iron oxidation in xylenol orange (FOX) assay used by Lapidot et al. (*1*) is not acceptable for measuring H_2O_2 content. They also reported that H_2O_2 was decomposed within 1 h and that H_2O_2 at concentrations up to 100 μM did not inhibit the proliferation of cancer cells. The FOX assay has been used to detect H_2O_2 by many scientists. The basic principle of this method is oxidation of ferrous ions by the sample oxidizing agents to ferric ions, which bind with xylenol orange to give a colored complex (*11*). The validity of the FOX assay for the measurement of phenolics-induced H_2O_2 was confirmed in several previous studies (*16*−*19*). The present study also shows that authentic H_2O_2 showed a linear dose−response curve and that catalase completely abolished the H_2O_2 when it was measured using the FOX assay (**Figure 6**), thereby verifying the specificity of the FOX assay and indicating that it is appropriate for precise measurements of the H_2O_2 content. Our results also show that H_2O_2 in DMEM media was decomposed slightly after 1 h (**Figure 7**). These results were in good agreement with those of Lapidot et al. (*1*), Long et al. (*3*), Watanabe et al. (*17*), and Nakagawa (*16*). The H_2O_2 generated by phenolic phytochemicals can induce diverse cellular response because it activates intracellular signaling pathway in a few minutes.

Yang et al. (*20*) used a flow cytometry assay with 2′,7′-dichlorofluorescein diacetate to show that epigallocatechin gallate (EGCG) generates substantial amounts of H_2O_2 in a dose-dependent manner, which induces apoptosis of H661 human

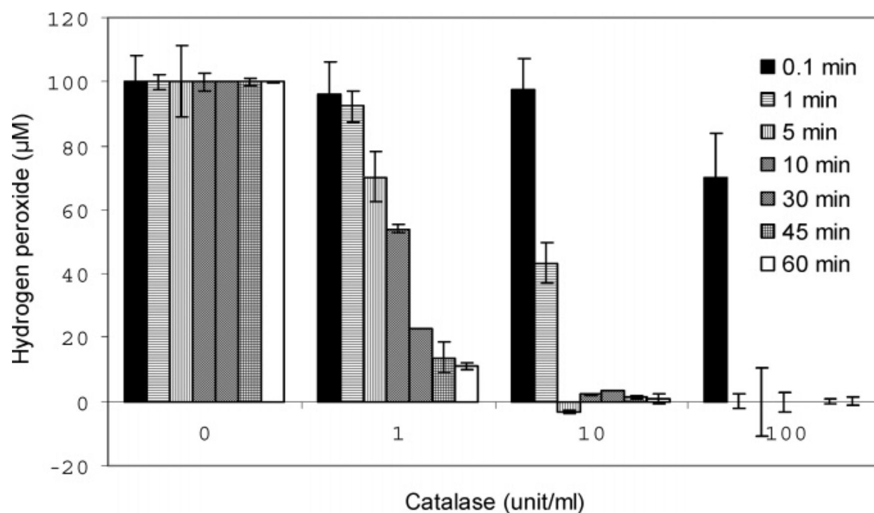

Figure 4. Time response curve of the abrogation of H_2O_2 by the addition of the indicated concentrations of catalase in DMEM including 10% FBS. H_2O_2 was measured by the FOX assay. Error bar = SD, $n \geq 6$.

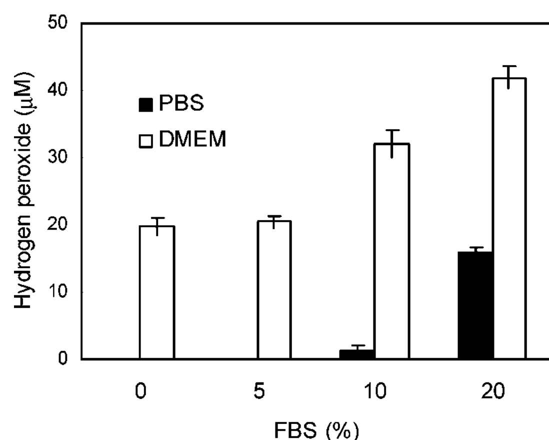

Figure 5. Generation of H_2O_2 by the addition of 10 μg/mL gallic acid in PBS or DMEM including the indicated concentrations of FBS for 1 h. H_2O_2 was measured by the FOX assay. Error bar = SD, $n \geq 6$.

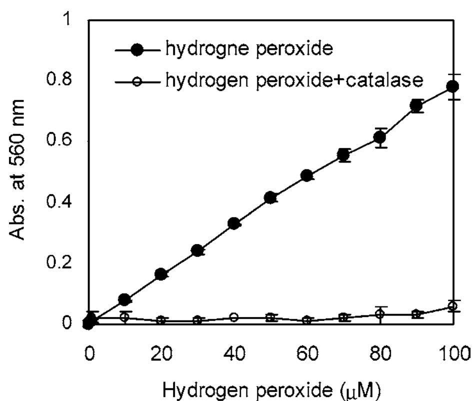

Figure 6. Dose–response curves of authentic H_2O_2 in the absence or presence of catalase in DMEM including 10% FBS. H_2O_2 was measured by the FOX assay. Error bar = SD, $n \geq 6$.

lung cancer cells in a manner similar to that caused directly by H_2O_2; both of these effects were abolished by exogenously added catalase. Similarly, a recent study shows that the induction of apoptosis in leukemia cells by EGCG was suppressed by the treatment of catalase and the Fe(II)-chelating reagent *o*-phenanthroline (*16*). Liu and Sun (*2*) did not address the reports cited by Lapidot (*1*) that H_2O_2 and EGCG inhibited cancer cell proliferation in a dose-dependent manner (*20*, *21*).

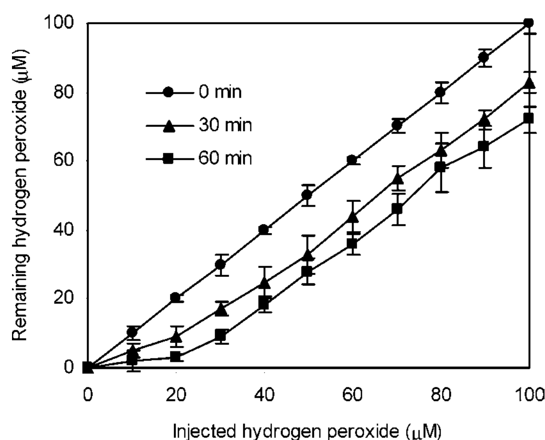

Figure 7. Time course curves of the decomposition of H_2O_2 added to DMEM. Error bar = SD, $n \geq 6$.

In addition, they did not examine the effect of H_2O_2 at concentrations of 100 μM or more on the proliferation of Caco-2 cells.

There are multiple lines of evidence that the inhibition of GJIC is a carcinogenic process. Most normal cells have functional GJIC, whereas most cancer cells have dysfunctional GJIC (*9*). Moreover, a consistent finding is that tumor promoters inhibit GJIC, whereas antitumor-promoting agents and anticancer drugs can reverse the down-regulation of GJIC. H_2O_2 causes the DNA damage that is associated with tumor initiation. It was reported that H_2O_2 also promotes cancer through the inhibition of GJIC (*22*, *23*).

We investigated whether the formation of H_2O_2 by some phenolic phytochemicals results in the inhibition of GJIC in normal rat liver epithelial cells. Authentic H_2O_2 (**Figure 8B**) markedly inhibited GJIC compared to the untreated negative control (**Figure 8A**). The addition of gallic acid (**Figure 8C**), but not quercetin (**Figure 8D**), to the cells also completely inhibited GJIC. The inhibition of GJIC by H_2O_2 and gallic acid was partially abolished by the addition of catalase (**Figure 9**). A recent study shows that gallic acid (500 mg/kg) caused a 4-fold increase in plasma ALT levels after 24 h when injected intraperitoneally into CD-1 mice (*24*), indicating potential hepatic damage of gallic acid in vivo in rodent models. These results indicate that a high dose of gallic acid may cause potential toxicity and carcinogenicity.

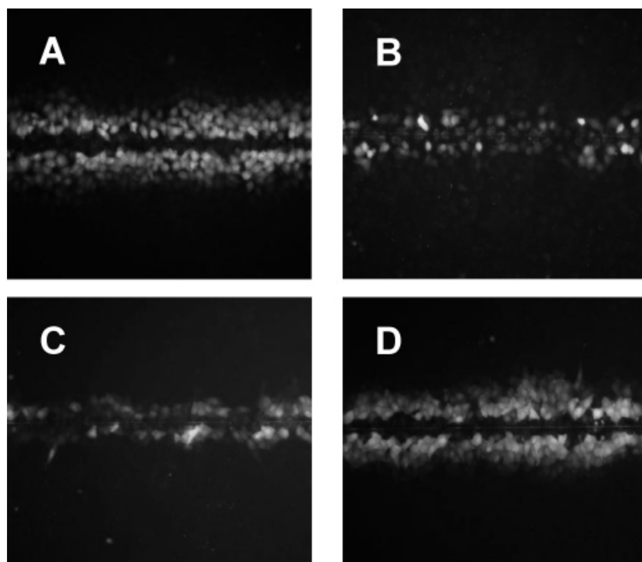

Figure 8. Effects of H_2O_2, gallic acid, and quercetin on GJIC in WB cells: (**A**) control cells; (**B**–**D**) cells were exposed to 300 μM H_2O_2 (**B**), 100 μg/mL gallic acid (**C**), or 100 μg/mL quercetin (**D**) for 1 h, respectively. GJIC was assessed using the scrape-loading/dye-transfer method, and data are representative of six separate experiments.

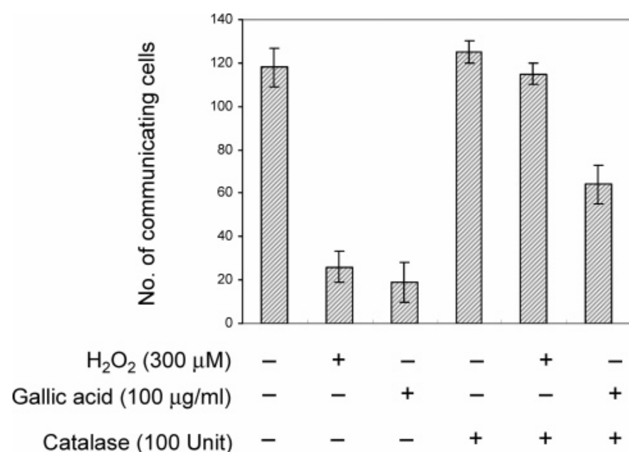

Figure 9. Effects of catalase on H_2O_2 and gallic acid-induced inhibition of GJIC in WB cells. Cells were exposed to 300 μM H_2O_2 and 100 μg/mL gallic acid for 1 h in the absence or presence of catalase, respectively. GJIC was assessed using the scrape-loading/dye-transfer method, and the number of communicating cells visualized with the dye was counted under an inverted fluorescence microscope. Error bar = SD, $n \geq 6$.

Phenolics are generally recognized as antioxidants, but they can exert prooxidant activities under certain conditions, such as in the presence of transition metal ions or alkalis. The prooxidant activity is thought to be directly proportional to the total number of hydroxyl groups; gallic acid and EGCG have multiple hydroxyl groups in their chemical structures, particularly in the B-ring, and they significantly increased the production of hydroxyl radicals in a Fenton system (*25*). More importantly, some antioxidants may have prooxidant activity regardless of the conditions. Several studies suggest that high doses of gallic acid and EGCG induce DNA damage (*26*−*28*) and that the damaging effects of EGCG are probably due to the generated H_2O_2. Thus, some phenolics, particularly higher concentrations of gallic acid and EGCG, can induce cancer cell death or DNA damage, due in part to the generation of reactive oxygen species (ROS) including H_2O_2 in media as artifacts.

Some other dietary phenolic substances can block cell proliferation through the inhibition of signal transduction, that is, not via the generation of ROS. Unlike EGCG, the addition of catalase to theaflavin digallate (TFdiG), a black tea polyphenol, did not reduce antiproliferative activity (*21*), which suggests that TFdiG does not suppress cancer cell proliferation via the generation of H_2O_2. Yang et al. (*21*) suggested that the antiproliferative effects of TFdiG are associated with the inhibition of c-Jun phosphorylation, which results in lowered activator protein-1 (AP-1) activity. The inhibition of AP-1 activity by TFdiG may be a key mechanism underlying the inhibition of cell growth (*21*, *29*). Importantly, Yang et al. (*21*) showed that the antiproliferative activity of EGCG is involved in the inhibition of AP-1 activity as well as H_2O_2 generation.

Kang et al. (*30*) also showed that capsaicin in red pepper induces apoptosis of H-*ras*-transformed human breast epithelial cells but not normal human epithelial cells. They propose that capsaicin can induce apoptosis through the activation of c-Jun N-terminal protein kinase and p38 mitogen-activated protein kinase. A recent study also found that NADPH oxidase-mediated generation of ROS may be essential to capsaicin-induced apoptosis in HepG2 cells (*31*). We also found that capsaicin can suppress the proliferation of HepG-2 human hepatoma cells without the generation of H_2O_2 in media (data not shown).

H_2O_2 exhibited toxicity and inhibition of GJIC in normal rat liver epithelial cells, and these effects were almost nullified by the addition of catalase (**Figures 3** and **9**). However, the cytotoxicity and inhibition of GJIC by gallic acid were not completely diminished by the addition of catalase (**Figures 2** and **9**). These results indicate that the cytotoxicity or inhibition of GJIC by some dietary phenolic phytochemicals may be associated with the activation of signal transduction as well as ROS generation in media.

The effect of dietary phenolic phytochemicals on carcinogenesis varies with the structure of the individual compounds and their dose. The major phenolic substances in apples are quercetin, epicatechin, procyanidin, chlorogenic acid, and phloretin, and not gallic acid and EGCG (*32*, *33*). Importantly, the effects of quercetin on the generation of ROS are much lower than those of gallic acid and EGCG, as shown by Lapidot et al. (*1*), Long et al. (*3*), and the present study. Unlike gallic acid and EGCG, quercetin showed a protective effect on H_2O_2-induced DNA damage in an ex vivo cellular assay (*34*). Furthermore, quercetin, epicatechin, and vitamin C, major antioxidants in apple, also exerted protective effects against the inhibition of GJIC by H_2O_2 (*13*, *35*). Quercetin and phloretin significantly inhibited TPA-induced COX-2 expression in mouse skin and mouse epidermal cells (K. W. Lee et al., unpublished observation). Apple extracts also inhibit tumor promoter-induced carcinogenesis and associated cell signaling in vivo (*36*). Epidemiological studies indicate that a high consumption of fruits can reduce the risk of cancer. Therefore, cancer chemopreventive effects of apples may be attributable to antitumor-promoting activities through the inhibition of inflammation and modulation of GJIC at non-cytotoxic dose.

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