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Original Article

Cytotoxic and proapoptotic activities of gallic acid to human oral cancer HSC-2 cells

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Key Words
Gallic acid; Nutraceuticals; Oral cancer; Oxidative stress; Polyphenols; Prooxidants

Abstract
Human carcinoma HSC-2 cells were more sensitive to a 24 h exposure to gallic acid than were normal human gingival fibroblasts. Acting as a prooxidant, gallic acid generated hydrogen peroxide in cell culture medium. The potency of gallic acid to HSC-2 cells was significantly lessened in the presence of scavengers of hydrogen peroxide, including catalase, pyruvate, and divalent cobalt cations, and was potentiated in the presence of the intracellular glutathione depleters, 1-chloro-2,4-dinitrobenzene, bis(2-chloroethyl)-N-nitrosourea, and DL-buthionine-[S,R]-sulfoximine. Exposure of HSC-2 cells to gallic acid decreased the level of intracellular reduced glutathione, caused lipid peroxidation, and increased the level of intracellular reactive oxygen species. Flow cytometric analyses of gallic acid-treated HSC-2 cells indicated a concentration-dependent response for the induction of apoptosis, which was reversed in the presence of divalent cobalt. Immunoblot analyses of gallic acid-treated cells showed proteolytic inactivation of poly(ADP-ribose) polymerase, an indication of apoptosis, which was also reversed in the presence of divalent cobalt cations. These studies demonstrated that the cytotoxic activities of gallic acid to HSC-2 cells were mediated through autooxidation of the polyphenol, leading to the induction of oxidative stress and thereby apoptotic cell death.

INTRODUCTION

Epidemiological evidence has identified an association between dietary habits and cancer risk. Consumption of natural plant products, such as vegetables, fruits, and nuts, has been linked to a low incidence of cancer, as well as to promoting an overall state of well being when regularly consumed. This chemopreventive effect has been associated with the consumption of high levels of nonnutritive phytochemicals, termed nutraceuticals, in these foods [1]. Many of the health benefits resulting from the consumption of plant-derived foods have been attributed to their phenolic compounds. One such polyphenol with anticancer properties, at least as studied with cells in culture, is gallic acid (3,4,5-trihydroxybenzoic acid; Fig.1). Gallic acid is widely distributed in plant-derived foods, occurring either in free form or in complex with tannins (gallotannins). Natural products rich in gallic acid include blackberry, raspberry, mango, tea leaves, walnut, gallnuts, grape seed, red wine, and sumac, amongst others [2].

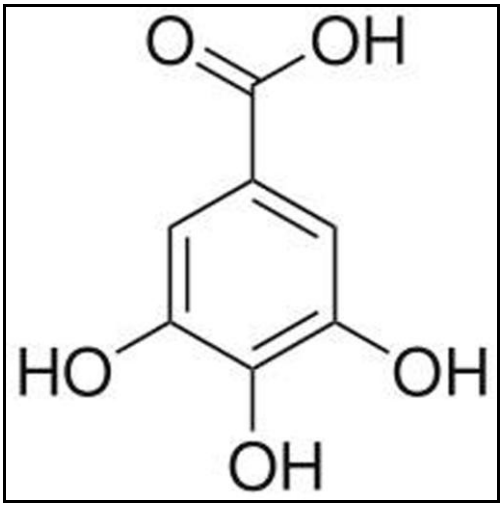

Figure 1. Molecular structure of gallic acid

Polyphenols, such as gallic acid, are interesting in that they exhibit both antioxidant and prooxidant properties [3], each having potential biologic significance. For example, Erol-Dayi *et al* [4] showed that gallic acid, acting as an antioxidant, protected cervical carcinoma HeLa cells against the toxic effects of exogenous hydrogen peroxide (H_2O_2), whereas Lee *et al* [5] observed that gallic acid, acting as a prooxidant, underwent autooxidation and generated H_2O_2 at quantities sufficient to inhibit growth of human colon Caco-2 cells.

The *in vitro* responses of cancer cells treated with gallic acid include the inhibition of cell growth and the induction of apoptosis and/or necrosis. In addition to overt cytotoxic effects, gallic acid blocked cells at G2/M phase of the cell cycle [6], affected molecular signaling pathways [7], and generated markers of apoptosis [2, 7, 8, 9]. The intent of the studies herein was to demonstrate a direct correlation between the prooxidant property of gallic acid and its antiproliferative and proapoptotic properties towards cancer cells.

Human carcinoma HSC-2 cells, derived from tissues of the oral cavity, were used in the studies herein. These cells are responsive to oxidative stress and were utilized in prior studies delineating the prooxidant nature of pomegranate extract [10], *Gingko biloba* leaf extract [11], and black tea theaflavins [12]. Head and neck squamous cell carcinoma (HNSCC) is one of the leading malignancies in the USA, with > 49,000 new cases diagnosed each year and with 10,000 HNSCC-associated deaths in 2010. Radiation and chemotherapy are the conventional treatment options for HNSCC patients at both early and late stage malignancy [13]. Our approach was to investigate the mechanistic action of gallic acid towards human oral cancer cells, with the overall intent of providing data potentially useful for chemotherapeutic protocols for HNSCC.

MATERIALS AND METHODS

Cell lines

Human squamous carcinoma HSC-2 cells derived from the oral cavity were obtained from H. Sakagami, Department of Dental Pharmacy, Meikai University School of Dentistry, Saitama, Japan, and normal human HF-1 gingival fibroblasts from P. Sacks, New York University School of Dentistry, New York, NY, USA. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 110 mg/l pyruvate (Cat. No. 11995-080), supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, and 1.25 µg/ml amphotericin B, termed the growth medium, and maintained in a humidified atmosphere with 5.5% CO_2 at 37ºC. Cell dissociation was achieved with 0.1% trypsin-0.04% EDTA for the carcinoma cells and 0.05% trypsin-0.02% EDTA for the fibroblasts.

Chemicals

Gallic acid stock solutions were prepared fresh daily by solubilizing gallic acid in dimethyl sulfoxide (DMSO) and sonicating for 3 min. Stock solutions were made at high enough concentration so that when diluted prior to use, the residual concentration of DMSO (0.4%) was not cytotoxic. Stock solutions of cobalt chloride ($CoCl_2 \cdot 6H_2O$) was prepared in water and of 1-chloro-2,4-dinitrobenzene (CDNB) and bis(2-chloroethyl)-*N*-nitrosourea (BCNU) in ethanol. Catalase (bovine liver), L-ascorbic acid, and DL-buthionine-[S,R]-sulfoximine (BSO) were directly introduced in exposure medium (DMEM lacking pyruvate (Cat. No. 11995-065) amended with 10% Serum Plus (JRH Biosciences, Lenexa, KS, USA), 2% FBS, and antibiotics by vortexing; fresh solutions were made for each experiment. All chemicals were from Sigma Chemicals (St. Louis, MO, USA).

Cell proliferation assay

Individual wells of a 96-well microtiter tissue culture plate were inoculated with 0.2 ml of the growth medium containing 2 x 10^4 cells/well for the carcinoma cells and 1.5 x 10^4 cells/well for the fibroblasts. After a day of incubation, the growth medium was removed and replaced with exposure medium, with or without varied concentrations of gallic acid. In some studies, cells were co-exposed to gallic acid in the presence of 200 U/ml catalase, 110 mg/l pyruvate, 250 µM Co^{2+}, or 5 mM BSO. In other experiments, the cells were pretreated for 15 min with 0.5 mM BCNU (in phosphate buffered saline, PBS), or for 20 min with 20 µM CDNB (in PBS) prior to exposure to gallic acid. One complete row, *i.e.* 8 wells, was used for each concentration of test agent, with and without the accompanying variable. After exposure to the test agents, viability was assessed with the neutral red (NR) assay, which is based on the uptake and accumulation of the supravital dye, NR.

The protocol for the NR assay was as follows. A foil-wrapped 4 mg/ml stock suspension of NR (in PBS) was stored at room temperature. The stock was diluted to a working concentration of 0.04 mg/ml NR in exposure medium and incubated overnight at 37ºC. After a 24 h exposure of the cells to the test agents, the medium was removed, 0.2 ml of NR-containing medium was added per well, and incubation was continued for 1 h at 37ºC. Cells were then rapidly washed and fixed with a 0.2 ml solution of 0.5% formalin-1% $CaCl_2$ (vol/vol) and the NR incorporated into the viable cells was released into the supernatant with 0.2 ml solution of 1% acetic acid-50% ethanol. Absorbance was recorded at 540 nm with a microtiter plate spectrophotometer.

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Intracellular glutathione assay

Measurements of intracellular reduced glutathione (GSH) were performed according to the procedures of Weisburg *et al* [14]. Cells, maintained in growth medium and grown to about 80% confluence in 60 mm tissue culture plates, were washed with PBS and treated for 4 h in serum-free DMEM amended with 25 to 150 µM gallic acid. In some studies, the cells were exposed to gallic acid in the presence of 250 µM Co^{2+}. Three plates of cells were used per concentration of test agent. Thereafter, the cells were washed with PBS, lysed with 0.06 ml of 0.2% Triton X-100, and proteins were precipitated with 0.06 ml of 5% sulfosalicyclic acid. After cell harvesting by scraping and centrifugation at 12,000g for 5 min, GSH was analyzed in 0.1 ml aliquots of the acid-soluble extract by determining the oxidation of GSH by a 6 mM solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), prepared in a phosphate buffer/EDTA (pH 7.5), to glutathione disulfide (GSSG), with a stoichiometric formation of 5-thio-2-nitrobenzoic acid, a yellow chromagen measured spectrophotometrically at 412 nm. With each assay, a standard curve was generated with known amounts of GSH and the data were recorded as GSH, in nmoles/10^6 cells.

Apoptosis assay: flow cytometry

HSC-2 cells, grown to ~80% confluence in 60 mm plates, were untreated or treated for 24 h with varying concentrations of gallic acid. Afterwards, the cells were washed with PBS, trypsinized, and diluted to a concentration just under 5×10^5 cells/ml. A 20 µl sample of cells, added to 380 µl of Guava ViaCount Reagent (Millipore, Billierica, MA, USA), was placed on ice in the dark for 5 min. Cell viability, apoptosis, and cell death were determined with a Guava Easycyte Miniflow Cytometer (Millipore). The Guava Viacount Reagent distinguishes between viable, apoptotic, and nonviable cells based on the differential permeability of its DNA binding dyes. The fluorescence of each dye is resolved operationally to allow for the quantitative assessment of viable, apoptotic, and non-viable cells present in a suspension.

Apoptosis assay: western blotting

HSC-2 cells grown to ~80% confluence were untreated or treated for 24 h with gallic acid in exposure medium. Cells lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA), containing complete EDTA-free protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN, USA), were then centrifuged (12,000g, 10 min) to remove cellular debris. Protein concentrations of the cell lysates were quantified with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). Equal concentrations of total protein from each sample were separated by SDS-PAGE (4-20% Pierce Precise Gels, Thermo Scientific), electroblotted to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA), and blocked with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were then probed with polyclonal anti-poly(ADP-ribose) polymerase (anti-PARP) antibody (rabbit antiserum, diluted 1:5,000 (Abcam, Cambridge, MA, USA)) to detect both full-length and cleaved PARP. The portion of the membrane corresponding to the molecular weight of actin was probed with a rabbit anti-actin antibody (rabbit antiserum, diluted 1:7,500 (Santa-Cruz Biotechnology, Santa Cruz, CA, USA)) to ensure equal protein loading. Following incubation with the appropriate peroxidase-conjugated secondary antibodies (1:5,000 dilution), membranes were developed using the ECL detection kit (GE Healthcare, Pittsburgh, PA, USA). Western blot analyses were performed a minimum of three times.

H_2O_2 assay

Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX) method using the PeroXOquant Quantitative Peroxide Assay Kit, lipid-soluble formulation (Pierce Biotechnology, Rockford, IL, USA). The principle of the FOX assay is based on H_2O_2 oxidation of Fe^{2+} to Fe^{3+}, which reacts with xylenol orange to yield a xylenol orange-Fe^{3+} complex which is measured spectrophotometrically. The protocol was as follows. In initial studies, gallic acid, from 0 to 200 µM, was incubated at room temperature for 4 h in exposure medium. In other studies, the reaction tubes contained 100 µM gallic acid with/without 110 mg/l pyruvate, 200 U/ml catalase, or 250 µM $CoCl_2$ in exposure medium. In all experiments, the FOX assay was performed after a 4 h incubation. At the end of the incubation, 90 µl samples were mixed with 10 µl of methanol and 0.9 ml of FOX reagent, followed by vortexing and a 20-min incubation at room temperature. The solutions were centrifuged at 12,000g for 5 min and absorbance of the supernatant was read at 595 nm against a blank consisting of 90 µl of exposure medium, 10 µl of methanol, and 0.9 ml of FOX reagent. A standard curve of H_2O_2 from 0 to 200 µmol/l was used to quantify the level of peroxide generated by gallic acid.

Fluorescent microscopy: detection of intracellular reactive oxygen species

HSC-2 cells were grown on coverslips until ~80% confluent, washed with PBS and treated with exposure medium (serum-free DMEM, without pyruvate and phenol red) amended with various concentrations of gallic acid test agent for 4 hr at 37ºC. A 10 mM stock solution of 2',7'-dichlorofluorescin diacetate (DCFDA) was prepared by dissolving the powder in DMSO, then diluting it with warm exposure medium to 20 µM. HSC-2 cells were washed with PBS and treated with

DCFDA for 30 min at 37ºC. Thereafter, the extracellular DCFDA was removed and the cells were incubated for an additional 30 min at 37ºC in the presence of warm exposure medium. During this time, the internalized dye was hydrolyzed by intracellular esterases and the resultant dichlorofluorescein was oxidized by intracellular ROS generated from the autooxidation of gallic acid, to produce a fluorescent product. Following this incubation, the cells were washed with PBS, fixed with 3.7% formaldehyde in PBS, air dried, and attached to slides. The slides were observed with a Zeiss microscope equipped with a filter set capable of detecting the fluorescent oxidized product with an absorption maximum of 492 nm and a fluorescent emission maximum of 517 nm.

Lipid peroxidation

Lipid peroxidation was determined by measuring the production of malondialdehyde in culture supernatants as described previously [14]. Confluent monolayers of cells in 60 mm dishes were washed twice with PBS and treated for 3 h with 4 ml PBS (control), 2 mM Fe^{2+} (as $FeCl_2 \cdot 4H_2O$) in PBS, 100 µM gallic acid in PBS, and a combination of Fe^{2+} with gallic acid. Thereafter, the agents were aspirated and 1 ml of 24% trichloroacetic acid was added, the cells were scraped, and the resulting suspensions were centrifuged at 13,000g for 10 min to remove the precipitated proteins. Then, 1 ml of the supernatant, to which 0.5 ml of 1% thiobarbituric acid in 0.05 M NaOH was added, was heated at 95°C for 20 min. After cooling in a refrigerator, the samples were centrifuged at 13,000g for 10 min. Upon heating, the malondialdehyde formed in the peroxidizing lipid system reacted with thiobarbituric acid to produce a pink chromagen, which was measured at 532 nm.

Brightfield microscopy

HSC-2 cells, grown on coverslips until ~80% confluent in 35 mm cell culture plates, were exposed for 24 h to gallic acid, in the absence or presence of 110 mg/l pyruvate or 250 µM $CoCl_2$. Thereafter the cells were fixed in methanol, stained with Giemsa solution, and subsequently observed with brightfield microscopy for cell morphologies indicative of apoptosis.

Statistics

All experiments were performed a minimum of three times. Data for the FOX assays were presented as the arithmetic mean ± standard errors of the mean (SEM) and for the cytotoxicity data were presented as the mean arithmetic percentages of control ± SEM. Experimental data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant differences. The P value of the effect had to be < 0.05 to be considered significant.

RESULTS

Cytotoxic effects of gallic acid toward normal and cancer cell lines

Preliminary studies defined the concentration range of the cytotoxic potency of gallic acid towards HSC-2 carcinoma cells and HF-1 fibroblasts. Once defined, this allowed for the selection of appropriate concentrations of gallic acid for use in subsequent studies of oxidative stress. The carcinoma HSC-2 cells were more sensitive to gallic acid than were the HF-1 fibroblasts. The midpoint cytotoxicity value (NR_{50}) for a 24 h exposure to gallic acid was 80 µM for the HSC-2 cells and 175 µM for HF-1 fibroblasts (Fig.2). As the goal of this research was to evaluate gallic acid as an anticarcinogenic agent, additional studies focused on the HSC-2 cells.

Prooxidant property of gallic acid

The generation of H_2O_2 in cell culture exposure medium maintained at room temperature and amended with gallic acid for 4 h was determined with the FOX assay. Gallic acid was a strong generator of H_2O_2 (Fig.3). DCFDA was used to monitor intracellular ROS in HSC-2 cells exposed for 4 h to 100 and 200 µM gallic acid, and, as an internal control, to 200 µM H_2O_2. Minimal fluorescence, indicating background intracellular ROS, was detected in control cells. Fluorescence, in a concentration-dependent mode, was noted in HSC-2 cells treated for 4 h with 100 and 200 µM gallic acid, and also was noted with 200 µM H_2O_2. Cells treated for 3.5 h with 200 µM gallic acid in the presence ascorbic acid, an antioxidant, showed a reduced level of intracellular ROS as compared to cells treated with gallic acid alone (Fig.4). A 3 h exposure to 100 µM gallic acid induced lipid peroxidation, which was moderately potentiated in the presence of Fe^{2+} (Fig.5).

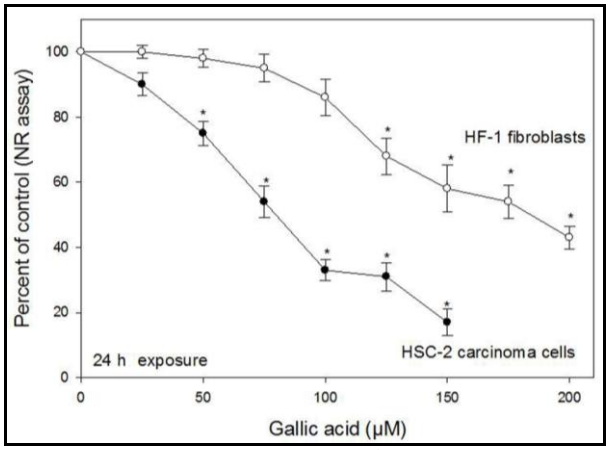

Figure 2. Comparative 24 h cytotoxicity of gallic acid to human oral carcinoma HSC-2 cells and to human normal gingival HF-1 fibroblasts. Data are expressed as the arithmetic mean percent of control ± SEM. *P < 0.1 as compared to control, untreated cells; n = 3-4.

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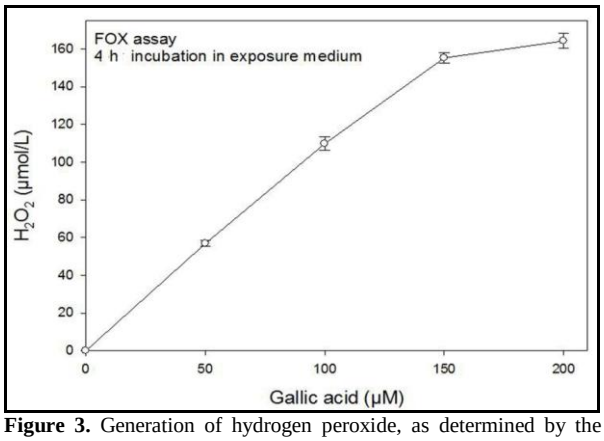

Figure 3. Generation of hydrogen peroxide, as determined by the FOX assay, in cell culture medium amended with varying concentrations of gallic acid. Hydrogen peroxide was the reference peroxide used to construct the standard curve. Data are expressed as the arithmetic mean ± SEM; n = 3.

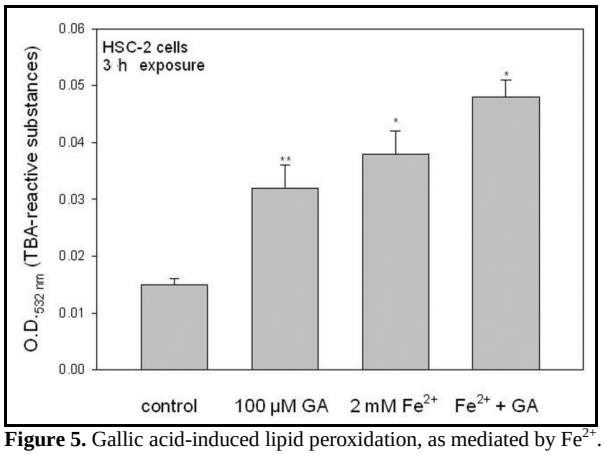

Figure 5. Gallic acid-induced lipid peroxidation, as mediated by Fe^{2+}. The data are presented as the arithmetic mean ± SEM. ******P < 0.05, *****P < 0.01.

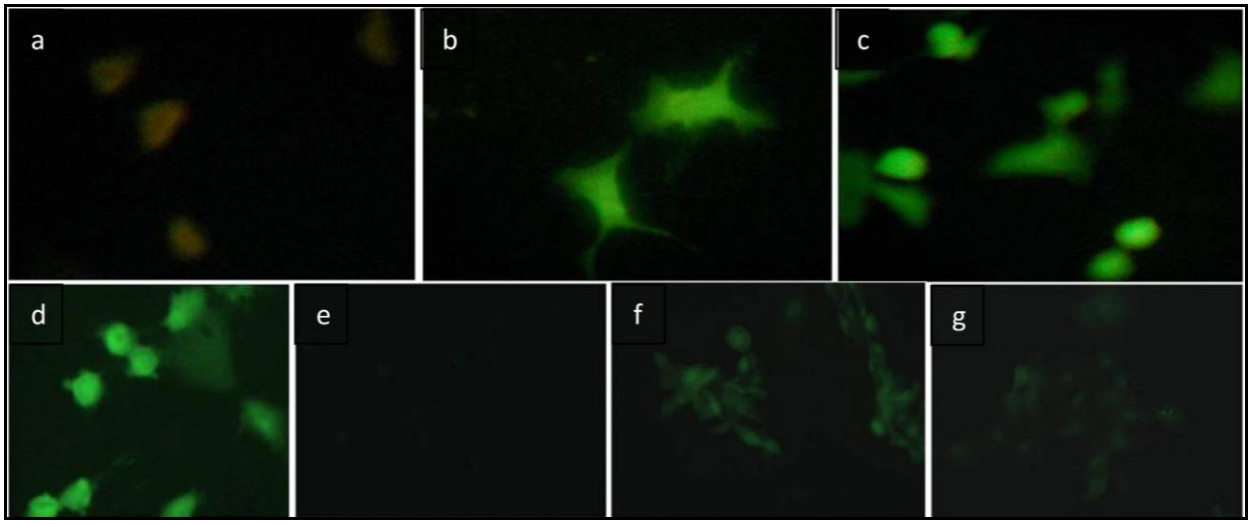

Figure 4. HSC-2 cells were untreated and treated with gallic acid, without or with ascorbic acid, or with hydrogen peroxide, stained with DCFDA, and viewed with a fluorescent microscope: **(a)** untreated control; **(b)** 4 h treatment with 100 µM gallic acid; **(c)** 4 h treatment with 200 µM gallic acid; **(d)** 4 h treatment with 33 µM hydrogen peroxide (positive oxidant control); **(e)** 3.5 h treatment with 200 µM ascorbic acid; **(f)** 3.5 h treatment with 200 µM gallic acid; and **(g**) 3.5 h co-treatment with 200 µM gallic acid and 200 µM ascorbic acid.

HSC-2 cells were treated with cytotoxic concentrations of gallic acid in the presence of scavengers of H_2O_2, including catalase, pyruvate, and divalent cobalt (Co^{2+}). The cytotoxicity of gallic acid to HSC-2 cells was greatly reduced upon coexposure with these scavengers of H_2O_2 (Fig.6). In a cell free system using the FOX assay, the detection of H_2O_2 in cell culture medium maintained at room temperature and amended with gallic acid was reduced in the presence of scavengers of H_2O_2 (Table 1).

The intracellular level of GSH was monitored in HSC-2 cells treated for 4 h with gallic acid. Increasing the concentration of gallic acid from 25 to 150 µM lessened the level of intracellular GSH (Fig.7). In the presence of Co^{2+}, a scavenger of H_2O_2, the level of intracellular GSH was unaffected by gallic acid (Table 2).

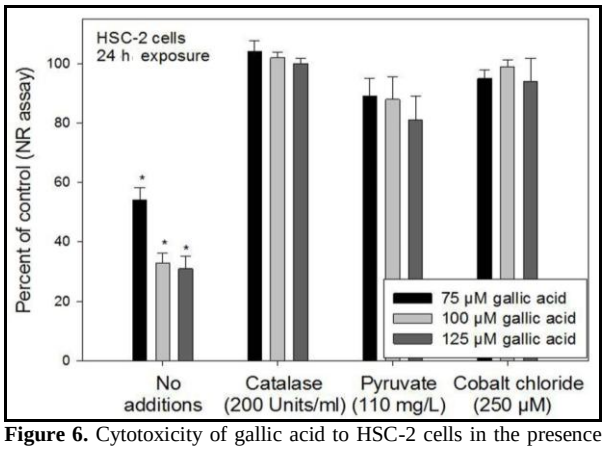

Figure 6. Cytotoxicity of gallic acid to HSC-2 cells in the presence of scavengers of hydrogen peroxide as compared to control (*i.e.*, no additions to gallic acid). Data are expressed as the arithmetic mean percent of control ± SEM. *****P < 0.1 as compared to control cells not treated with gallic acid; n = 5.

Table 1. Detection of hydrogen peroxide in cell culture exposure medium after a 4 h incubation of 100 µM gallic acid, in the absence and in the presence of scavengers of hydrogen peroxide

Test conditions	Hydrogen peroxide (µmol/L)
Gallic acid (alone)	108 ± 3.6
Gallic acid + 200 Units/ml catalase	2 ± 1.2*****
Gallic acid + 110 mg/l pyruvate	10 ± 2.3*****
Gallic acid + 250 µM $CoCl_2$	25 ± 3.5*****

Hydrogen peroxide was determined with the FOX assay, using hydrogen peroxide as the reference peroxide. Data are expressed as the arithmetic mean ± SEM. *****P < 0.01 as compared to gallic acid alone; n = 4.

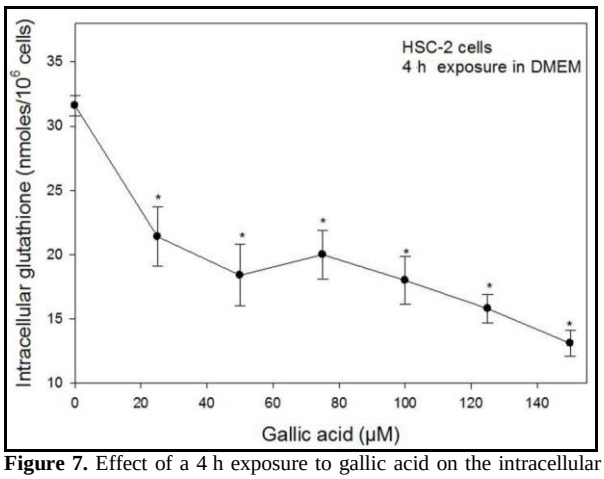

Figure 7. Effect of a 4 h exposure to gallic acid on the intracellular levels of reduced glutathione in HSC-2 cells. Data are expressed as the arithmetic mean ± SEM. *P < 0.1 as compared to control, untreated cells; n = 4.

Table 2. Decreases in intracellular glutathione in HSC-2 cells following a 4 h incubation in cell culture medium in the absence and presence of 250 µM $CoCl_2$

Test conditions	Reduced glutathione (nmoles/10^6 cells)
Gallic acid alone	
0 µM (control)	33 ± 1.7
75 µM	25 ± 0.9*****
125 µM	16 ± 1.1*****
Gallic acid + $CoCl_2$	
0 µM (control)	28 ± 2
75 µM	31 ± 2.5
125 µM	27 ± 1.6

Data are expressed as the arithmetic mean ± SEM. *****P < 0.01 as compared to gallic acid alone; n = 4.

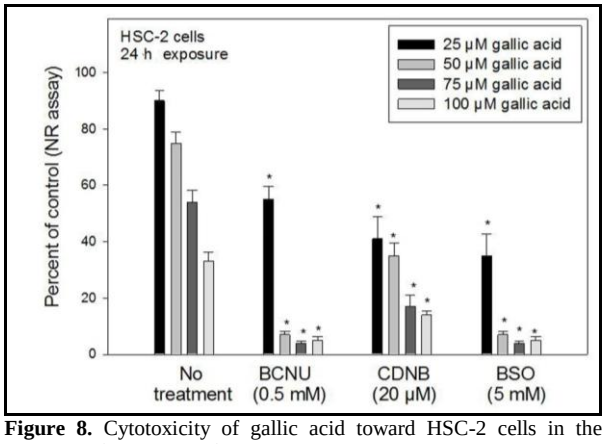

Figure 8. Cytotoxicity of gallic acid toward HSC-2 cells in the presence of depleters of intracellular reduced glutathione. Data are expressed as the arithmetic mean percent of control ± SEM. *P < 0.1 as compared to cells not treated with a depleter of glutathione; n = 3.

To further confirm that the prooxidant nature of gallic acid was responsible for its antiproliferative effects, HSC-2 cells were pre- or co-treated with various intracellular GSH depleters, including CDNB, BSO, and BCNU. Pretreatment with CDNB or BCNU and coexposure with BSO potentiated the cytotoxicity of gallic acid to the HSC-2 cells (Fig. 8).

Gallic acid as an inducer of apoptosis via oxidative stress

As shown by flow cytometry, gallic acid induced apoptosis in a concentration-dependent manner (Fig.9). The population of apoptotic cells was greatly reduced in the presence of $CoCl_2$ (Fig.10). Immunoblot analysis corroborated the results obtained by flow cytometric analysis. PARP cleavage products were detected in protein lysates of HSC-2 cells exposed for 1 day to 100 to 150 µM of gallic acid, and in the presence of $CoCl_2$, gallic acid failed to trigger apoptosis (Fig.11). Induction of apoptosis by gallic acid and its reversal in the presence of pyruvate and Co^{2+} were also visualized by microscopy after a 24-hr treatment. Untreated HSC-2 cells appeared as cobblestones, each with a large cytoplasm and nucleus. In the presence of gallic acid, the cytoplasmic mass was reduced, the nuclei were hypercondensed, and cell blebbing was apparent, all characteristic of apoptotic cell death. However, when gallic acid treatment was coamended with pyruvate and $CoCl_2$, the proapoptotic effects of gallic acid were eliminated (Fig.12).

DISCUSSION

Substantial amounts of H_2O_2 were detected in a concentration-dependent manner in cell culture medium amended with gallic acid. The autooxidation of gallic acid in cell culture medium to generate H_2O_2 has been recognized [15]. H_2O_2 was not detected in cell culture medium co-amended with gallic acid in the presence of the H_2O_2 scavengers, catalase, pyruvate, and Co^{2+}. Catalase enzymatically decomposes H_2O_2 to H_2O and molecular oxygen; pyruvate is oxidatively decarboxylated by H_2O_2 to

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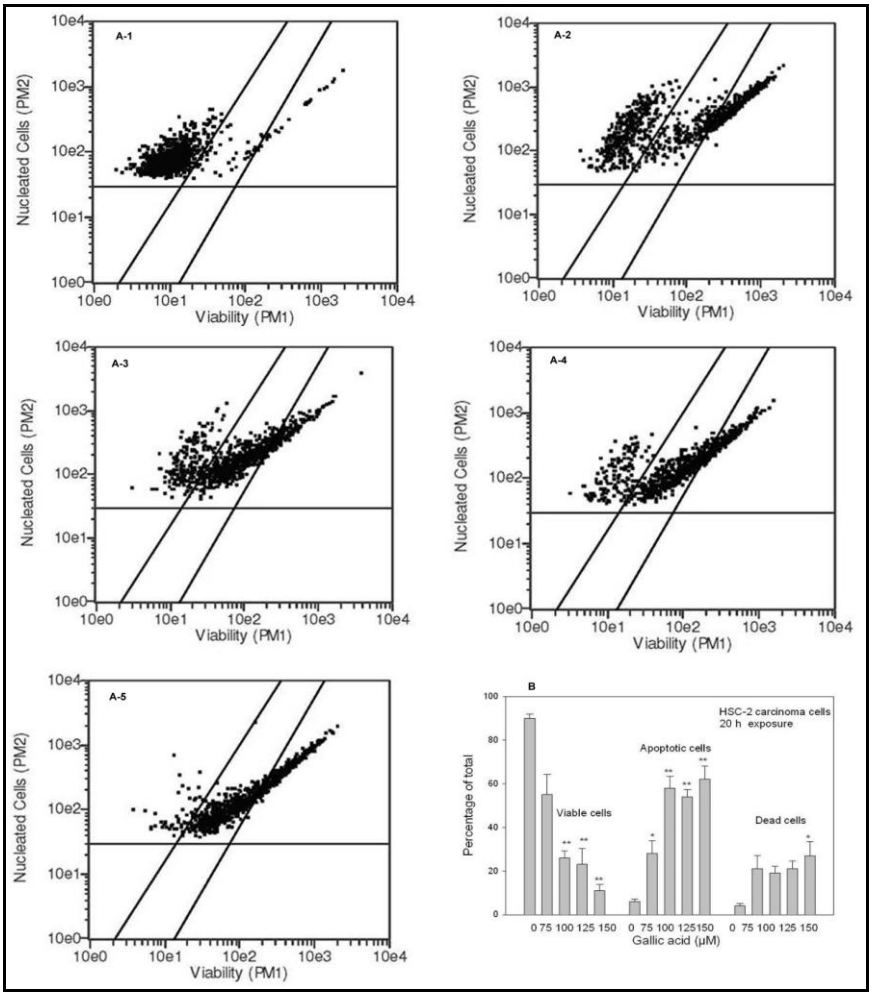

Figure 9.
(A) Representative FACS profiles of the viability and apoptosis of HSC-2 cells after a 20 h treatment with varying concentrations of gallic acid. Viable cells are located on the left side of each panel; apoptotic cells between the two lines; and dead cells on the right side of the panel:
Panel 1 is untreated cells,
Panel 2 is cells treated with 75 μM gallic acid;
Panel 3 is cells treated with 100 μM gallic acid;
Panel 4 is cells treated with 125 μM gallic acid;
and **Panel 5** is cells treated with 150 μM gallic acid.
(B) Cytometric analysis, using Guava ViaCount Reagent, of a 20 h exposure of HSC-2 cells to increasing concentrations of gallic acid. Percentage of viable, apoptotic, and dead cells were quantified by the flow cytometer. The data are expressed as the arithmetic mean percent cells as viable, apoptotic, and dead ± SEM. *P < 0.05 and **P < 0.01 as compared to control, untreated cells; n = 3.

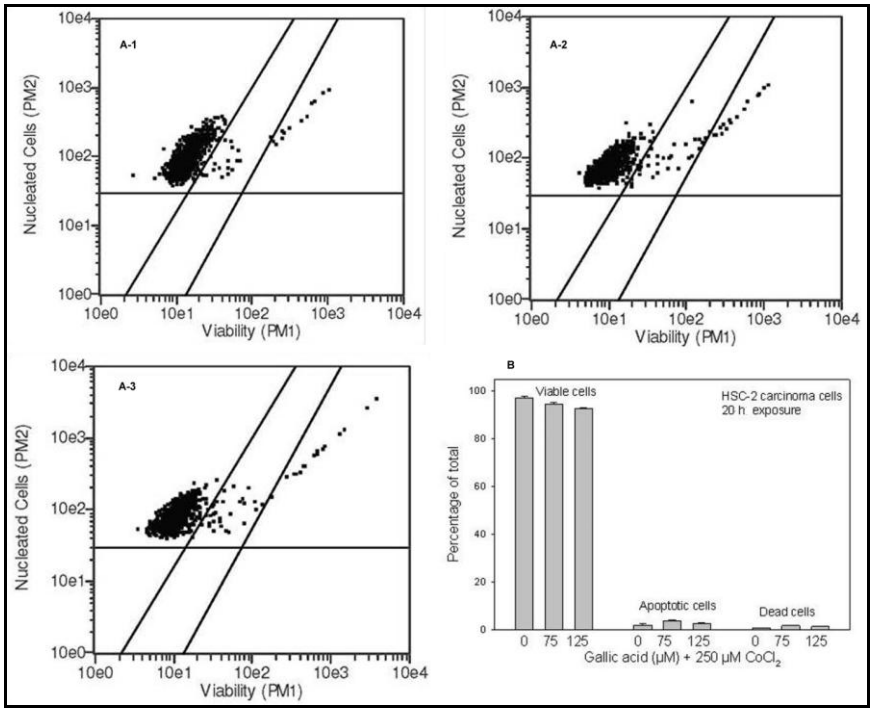

Figure 10.
(A) Representative FACS profiles of viabile and apoptotic HSC-2 cells after a 20 h treatment with 250 μM $CoCl_2$, alone (control) and in the presence of 75 and 125 μM gallic acid.
Viable cells are located on the left side of each panel; apoptotic cells between the two lines; and dead cells on the right side of the panel:
Panel 1 is control cells;
Panel 2 is cells treated with $CoCl_2$ and 75 μM gallic acid;
and **Panel 3** is cells treated with $CoCl_2$ and 125 μM gallic acid.
(B) Cytometric analysis, using Guava ViaCount Reagent, of a 20 h exposure of HSC-2 cells to 250 μM $CoCl_2$ alone (control), and in the presence of 75 and 125 μM gallic acid.
Percentage of viable, apoptotic, and dead cells were quantified by the flow cytometer.
The data are expressed as the arithmetic mean percent cells as viable, apoptotic, and dead ± SEM; n = 3.

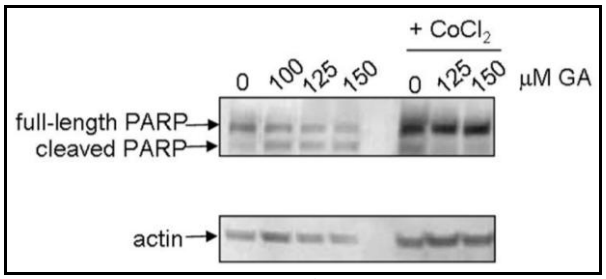

Figure 11. Immunoblot analysis of PARP cleavage in HSC-2 cells untreated and treated with increasing concentrations of gallic acid (GA) after a 20 h exposure in exposure medium without and with 250 µM $CoCl_2$. Cellular proteins, separated by 10% SDS-PAGE, were transferred to nitrocellulose and probed with an antibody specific for full length PARP and one specific for the ~85 kD C-terminal cleaved fragment of PARP, which were identified by comparison to a standard molecular weight marker. An anti-actin antibody was the loading control.

yield acetate and CO_2 and H_2O; Co^{2+} catalytically decomposes H_2O_2 to H_2O and molecular oxygen [16]. The effect of catalase, in particular, clearly demonstrated that H_2O_2, rather than another ROS, was generated in cell culture medium amended with gallic acid.

Gallic acid was a more potent cytotoxic agent toward carcinoma HSC-2 cells than toward normal gingival HF-1 fibroblasts. Others have noted the hypersensitivities of various cancer cells, as compared to normal cells to gallic acid [5, 17]. In fact, this differential sensitivity of cancer and normal cells to gallic acid, apparently, is a characteristic cellular response to polyphenols, in general [18]. Isuzugawa *et al* [19] observed the greater resistance of normal cells than of cancer cells to gallic acid and suggested that this was related to the higher intracellular content of catalase in normal cells, thereby accounting for their greater protective defense against oxidative stress. Furthermore, cancer cells, in general, have greater metabolic activities than quiescent fibroblasts, and thus may have greater basal levels of mitochondrial-derived ROS. Additional extraneous ROS, such as those generated from the autooxidation of gallic acid, could readily overwhelm cellular defenses against oxidative stress [20].

The prooxidant nature of gallic acid was noted with HSC-2 cells exposed to gallic acid and treated with DCFDA, a colorless, nonfluorescent, nonpolar molecule that passively diffuses into cells and is used as an intracellular indicator of ROS. Once in the cell, esterases cleave the two acetates to form DCF, a nonpermeable, polar molecule, which upon oxidation yields a fluorescent product. Minimal, or background, fluorescence was observed in untreated HSC-2 cells. As expected, strong fluorescence was noted in cells treated with H_2O_2. Increased levels of intracellular ROS, as indicated by the degree of fluorescence, were noted in a concentration-dependent manner upon treatment of the HSC-2 cells with 200 µM gallic acid. The lessening of fluorescence in HSC-2 cells co-treated with gallic acid and ascorbic acid, as compared to gallic acid alone, further confirmed the prooxidant nature of gallic acid. Similarly, using DCFDA as the indicator, intracellular ROS were detected in human lung adenocarcinoma A549 cells [21], and in human prostate cancer LNCaP cells [22] exposed to gallic acid. Lipid peroxidation induced by gallic acid was moderately potentiated in the presence of Fe^{2+}. Similar findings were noted with oral carcinoma cells treated with epigallocatechin gallate [14].

The 24 h cytotoxicity of gallic acid to HSC-2 cells was greatly lessened in the presence of scavengers of H_2O_2, *i.e.*, catalase, pyruvate, and Co^{2+}, indicating that gallic acid-induced cytotoxicity was mediated by the generation of H_2O_2. These studies confirmed the findings of Chen *et al* [6], noting that catalase lessened the toxicity of gallic acid to human prostate DU145 cancer cells.

Reduced glutathione (GSH), a tripeptide and the most prevalent intracellular free thiol, plays an essential role in preserving an optimal intracellular redox environment and serves as the main cellular defense against damage by oxidative stress [23]. The intracellular level of GSH in HSC-2 cells was progressively lessened in the presence of increasing concentrations of gallic acid. Depletion of

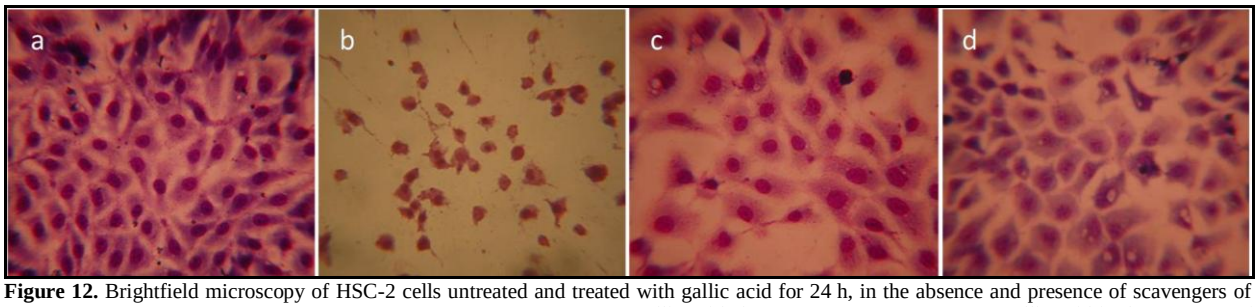

Figure 12. Brightfield microscopy of HSC-2 cells untreated and treated with gallic acid for 24 h, in the absence and presence of scavengers of hydrogen peroxide: **(a)** control, untreated cells; **(b)** treated with 125 µM gallic acid alone; **(c)** treated with 125 µM gallic acid in the presence of 110 mg/l pyruvate; and **(d)** treated with 125 µM gallic acid in the presence of 250 µM $CoCl_2$. (Giemsa staining, 320x)

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intracellular GSH upon exposure to gallic acid was noted also in human primary umbilical vein endothelial cells [24] and in human pulmonary adenocarcinoma Calu-6 cells [25]. The lessening of GSH intracellular levels upon exposure of HSC-2 cells to gallic acid was almost completely eliminated in the presence of Co^{2+}.

Cells depleted of their stores of GSH are hypersensitive to oxidative stress. To further confirm that the prooxidant nature of gallic acid was responsible for the growth inhibitory effects, HSC-2 cells were pre- or co-treated with various GSH depleters, each interfering with a different aspect of GSH-GSSG cycling. The GSH depleters included CDNB, which inactivates glutathione S-transferase, BSO, an inhibitor of glutamylcysteine synthetase, and BCNU, an inhibitor of glutathione reductase [16]. The antiproliferative effect of gallic acid toward the HSC-2 cells was potentiated by treatment with these GSH depleters. Similarly, the growth inhibitory effect of gallic acid to human primary umbilical vein endothelial cells [24] and to human lung fibroblasts [9] was enhanced in the presence of BSO.

A marker of irreversible apoptotic cell death is the proteolytic cleavage and, thereby, inactivation of poly(ADP-ribose) polymerase (PARP) by caspases. PARP cleavage products were detected in protein lysates of HSC-2 cells exposed to gallic acid. Others noted that apoptosis induced by gallic acid was associated with activation of caspase-3 [22] and caspase-9 [21], accompanied by caspase proteolytic cleavage of PARP to low molecular weight fragments. Flow cytometric analyses supported the induction of gallic acid-mediated apoptosis, in a concentration-dependent mode, for HSC-2 cells. As shown both by flow cytometry and by immunoblot analysis, induction of apoptosis by gallic acid was nearly eliminated in the presence of Co^{2+}, indicating that oxidative stress was the molecular trigger of apoptosis in gallic acid-treated HSC-2 cells.

The research herein with HSC-2 cells clearly showed that the cytotoxic and proapoptotic effects of gallic acid correlated with its autooxidation, generating substantial levels of H_2O_2 to induce oxidative stress. Induction of oxidative stress in gallic acid-treated HSC-2 cells was noted by the lessening of toxicity in the presence of H_2O_2 scavengers, by the potentiation of toxicity in the presence of GSH depleters, and by the lowering of intracellular GSH. Oxidative stress appeared to trigger molecular signaling pathways leading to apoptosis. These studies may direct further research to develop the usage of nutraceuticals, such as gallic acid, as adjuncts to chemotherapeutic options in the mediation of oral carcinogenesis.

ABBREVIATIONS

BCNU; bis(2-chloroethyl)-*N*-nitrosourea

BSO; DL-buthionine-[S,R]-sulfoximine

CDNB; 1-chloro-2,4-dinitrobenzene

DCFDA; 2',7'-dichlorofluorescin diacetate

DMEM; Dulbecco's modified Eagle's medium

DMSO; dimethyl sulfoxide

FBS; fetal bovine serum

FOX; ferrous ion oxidation xylenol orange

GSH; glutathione

HNSCC; head and neck squamous cell carcinoma

HSC-2; human squamous carcinoma

NR; neutral red

PARP; poly(ADP-ribose) polymerase

ROS; reactive oxygen species

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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