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Research Article

Reactive oxygen species are crucial for hydroxychavicol toxicity toward KB epithelial cells

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Abstract. Betel quid (BQ) chewing shows a strong correlation to the incidence of oral submucous fibrosis (OSF), leukoplakia and oral cancer. BQ contains mainly areca nut, lime, *Piper betle* leaf (PBL) and the inflorescence of *P. betle* (IPB). Hydroxychavicol (4-allyl-catechol, HC), as a major phenolic compound in PBL and IPB, is shown to induce oxidative stress, glutathione (GSH) depletion and cell cycle deregulation. Using bivariate BrdU/PI flow cytometry, KB cells in DNA synthesis (S phase) are shown to be sensitive to the toxic effect of HC and show cell cycle arrest and apoptosis following exposure to 0.1 and 0.3 mM HC. HC-induced apoptosis and cell cycle arrest are associated with mitochondrial membrane potential ($\Delta\Psi$m) depolarization as revealed by a decrease in rhodamine fluorescence. N-acetyl-L-cysteine (1 mM), superoxide dismutase (100 U/ml) and catalase (1000 U/ml) were effective in prevention of HC-induced GSH depletion (as indicated by chloromethylfluorescein fluorescence), reactive oxygen species (ROS) production (by dichlorofluorescein fluorescence), cell cycle arrest and apoptosis. However, dimethylthiourea (2 mM), neocuproine (1 mM), 1,10-phenanthroline (200 µM) and desferrioxamine (0.5 mM) showed little effect on HC-induced cell changes. HC elevated the cellular and mitochondrial GSH levels at moderate concentrations (0.05–0.1 mM), whereas at a concentration of 0.3 mM, inhibitory effects were noted. These results indicate that HC consumption may be associated with BQ-chewing-related oral mucosal diseases via GSH depletion, ROS production, mitochondrial dysfunction, cell cycle disturbance and the induction of apoptosis. These events are related to the production of superoxide radicals and hydrogen peroxide.

Key words. Apoptosis; betel quid; cell cycle; glutathione; hydroxychavicol; mitochondrial membrane potential; reactive oxygen species.

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In India, Taiwan, South Africa, Papua New Guinea and numerous Southeast Asian countries, betel quid (BQ) chewing is a relatively popular oral habit [1–5]. There are an estimated 200–600 million habitual BQ chewers in the world [6], and more than 2 million BQ chewers in Taiwan [2]. BQ chewing is the major etiological factor for oropharyngeal cancer, oral submucous fibrosis (OSF) and oral leukoplakia in these countries. BQ usually contains areca nut (AN), betel leaf (PBL, *Piper betle* leaf) and lime with or without a piece of the inflorescence of *P. betle* (IPB) [3, 5]. A number of BQ components such as arecoline, AN-specific nitrosamines, safrole and hydroxychavicol (HC) are suggested to be the contributing factors of chemical carcinogenesis [3, 5–7]. However, the precise toxic mechanisms of BQ-chewing-related oral mucosal lesions are still not well understood.

PBL extract, as one major BQ component, has been shown to be a potent scavenger of reactive oxygen species (ROS) such as H_2O_2, superoxide and hydroxyl radicals, and may inhibit arachidonic-acid-induced platelet aggregation [8] and the oxidation of low-density lipoprotein [9]. When given to Swiss mice by intragastric intubation, PBL extract is not able to induce tumors [10]. Moreover, PBL decreases the acetoxymethyl nitrosamine-induced hamster oral tumor and tumor burden [11] as well as BQ-induced hamster cheek pouch tumors [12]. PBL extract also inhibits the AN-induced tumors of Swiss mice in vivo and the mutations of *Salmonella typhimurium* TA100 strain in vitro [13]. PBL contains effective components that may prevent the mutagenesis induced by various environmental mutagens, carcinogens and two tobacco-specific nitrosamines [14, 15]. Accordingly, an epidemiological study also found that chewing BQ containing PBL but without IPB is less risky for oral cancer occurrence than chewing BQ that contains IPB [2]. However, PBL extract inhibits O^6-methyl-guanine-DNA methyltransferase activity [16], a crucial enzyme for DNA repair, and induces chromatid aberrations on human white blood cells [17]. Possibly, different PBL ingredients have a differential effect, and thus the toxicities of each PBL component and its roles in the BQ-related oral mucosal lesions warrant further elucidation.

Eugenol, HC (4-allyl-catechol, 1-allyl-3,4-dihydroxybenzene) and safrole are three major organic compounds in PBL and IPB [18]. IPB contains 9.74 mg/g wet weight of HC [18]. O-dealkylation of safrole may produce HC, and subsequent quinoid formation has been considered for the genotoxic and cytotoxic effect of safrole [19]. Previous studies have suggested that HC exerts antioxidant, antinitrosation, antimutagenic and anticarcinogenic properties [14, 20–22] against various mutagens and carcinogens. HC prevents bone marrow micronucleated cell formation by tobacco-specific nitrosamines and dimethylbenzanthracene (DMBA) in Swiss male mice and mutations in the Ames *Salmonella*/microsome assay [21, 23, 24]. HC (25 μM) inhibits the benzopyrene-DNA interactions activated by mouse and rat liver S9 homogenates [20], and suppresses the nitrosation of methylurea via scavenging nitrite ions [14]. In contrast, HC may induce mutations in the Ames test (TA97, TA98, TA100, TA102 strains) in the presence of metabolic activation, copper-mediated plasmid DNA breaks and chromosomal aberrations of Chinese hamster ovary cells [7]. We have also found that HC shows differential scavenging properties toward H_2O_2, superoxide and hydroxyl radicals, with effective concentrations ranging from 0.02 to 50 μM [22]. But at concentrations higher than 0.1 mM, HC induces glutathione (GSH) depletion, reactive oxygen species (ROS) production, cell cycle arrest and apoptosis of oral KB epithelial cells [22]. Therefore, HC is possibly an antioxidant at low concentrations whereas at higher concentrations it may induce cell death mediated by oxidative stress. The salivary concentrations of HC, safrole and eugenol during BQ chewing are reported to be about 4.6 mM, 5.2 mM and 1.1 mM, respectively [25], which is dramatically higher than the reported toxic concentrations [22]. This indicates that HC may potentially induce oxidative stress on oral mucosal cells and play a role in BQ-chewing-related oral mucosal diseases. However, which types of ROS are responsible for HC-induced cellular events need to be further addressed.

Recently, oxidative stress has been shown to be crucial for the pathogenesis of carcinogenesis, atherosclerosis and immunodeficiency syndrome [26]. Oxidative stress may further induce cellular apoptosis via induction of GSH depletion, mitochondrial dysfunction and apoptosis [27]. We therefore tested whether HC may induce changes in mitochondrial membrane potential that is linked to cellular apoptosis. The precise reactive toxic species responsible for GSH depletion, mitochondrial changes, cell cycle arrest and apoptosis of KB cells were also tested using various antioxidants and detoxifying enzymes.

Materials and methods

Materials

Propidium iodide, dimethyl-sulfoxide (DMSO), dichlorofluorescein-diacetate (DCFH-DA), phenylmethylsulfonyl fluoride (PMSF), dithionitrobenzoic acid (DTNB) and dithiothreitol (DTT) were purchased from Sigma (St Louis, Mo.). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS) and trypsin/EDTA were from Gibco (Life Technologies, Grand Island, N. Y.). 5-Chloromethylfluorescein diacetate (CMF-DA) and rhodamine were obtained from Molecular Probes (Eugene, Ore.). Bromodeoxyuridine (BrdU), anti-BrdU antibody and reagents for flow cytometry were obtained from Becton Dickinson, Pharmingen, Worldwide Inc. (San-

Jose, Calif.). HC was synthesized as described previously [22]. The purity of HC was confirmed by infrared spectroscopy and nuclear magnetic resonance as about 94%. Oral KB epithelial cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM containing 10% FCS and antibiotics.

Bivariate BrdU/DNA content analysis of HC in KB cells

Bivariate BrdU/DNA content analysis of HC in KB cells was performed as described by Chen et al. [28]. Briefly, 5×10^5 KB cells were inoculated into 100-mm culture dishes. After 24 h, cells were exposed to fresh medium containing DMSO or HC (0.01–0.3 mM) for 24 h. Finally, cells were pulse-labeled with 10 mM BrdU at 37 °C for 30 min. Cells labeled with FITC-conjugated mouse IgG were used as isotype control. Floating cells in the culture medium were collected into 15-ml centrifuge tubes. Attached cells were then washed with PBS, trypsinized and also collected into the same 15-ml tube. After centrifugation (1500 rpm) for 5 min, the supernatant was decanted. The cell pellet was resuspended and rinsed in 1 ml of 1% bovine serum albumin (BSA)/PBS and centrifuged at 1500 rpm for 5 min. The supernatant was decanted and 5 ml of ice-cold 70% ethanol was added dropwise and vortexed. Cells were stored at –20 °C overnight. Then, cells were centrifuged at 1500 rpm for 5 min again, and the ethanol was removed completely. One milliliter of 2 N HCl/0.5% Triton X-100 was added to the cells, resuspended, and incubated at room temperature for 30 min to denature the double-strand DNA to form single-strand DNA. Then, 1 ml of 0.1 M $Na_2B_4O_7$ (pH 8.5) was added, mixed to neutralize the acidity, followed by adding 2 ml 1% BSA/PBS. Cells were centrifuged at 1500 rpm for 5 min again and the supernatant was decanted. Then, 1 ml of 0.5% Tween 20/1% BSA/PBS was added to resuspend the cells, the cells were transferred to 5-ml polystyrene round-bottom tubes, spun down as before and the supernatant was decanted. Ten microliters of FITC-conjugate anti-BrdU antibody was added and incubated at room temperature in the dark with slow shaking for 60 min. Cells were washed twice with 2 ml 0.5% Tween20/1% BSA/PBS and the supernatant was decanted after centrifugation. Finally, cells were resuspended in 0.6 ml PBS containing 5 µl 2 mg/ml RNase (DNase free), and 5 µl of propidium iodide (PI, 1 mg/ml in PBS) for 20 min and subjected to flow cytometric analysis of BrdU content (FITC) and DNA content (PI). The PI- and BrdU-elicited fluorescence of individual KB cells was measured by a FACSCalibur Flow Cytometer (Becton Dickinson) supplemented with an Argon ion laser. The wavelength of laser excitation was set at 488 nm and the emission was collected at FL3 fluorescence (PI) in a linear/log scale fashion. The wavelength of laser excitation for BrdU was set at 488 nm and the emission was collected at FL1 (525 nm). A total of 10,000 cells were analyzed for the control and experimental samples. For the BrdU incorporation assay, cells not labeled with BrdU were used as a negative control and for subtraction in quantitative analysis. The percentage of cells in G0/G1 phase, S phase, G2/M and sub-G0/G1 phase were determined using standard ModiFit software and the CellQuest program [22, 28, 29]. In some experiments, KB cells were labeled with 10 mM BrdU for 30 min, washed and then exposed to fresh medium containing DMSO (control) and HC (0.1 and 0.3 mM) for 6 and 24 h to elucidate the fate of S phase cells

Effects of antioxidants on the HC-induced cell cycle arrest and apoptosis

Various antioxidants, antioxidative enzymes or metal chelators were added 30 min prior to the addition of HC. Cells were collected 24 h later and analyzed as described above [22, 29] to elucidate the mechanism of HC-induced cell cycle deregulation and apoptosis.

Effect of antioxidants on HC-induced changes in mitochondrial membrane potential, GSH levels and the generation of ROS

Briefly, 5×10^5 KB cells in DMEM containing 10% FCS were exposed to various antioxidants, antioxidative enzymes or metal chelators for 30 min and then to HC (0.01–0.3 mM) or DMSO (as control) for a further 24 h. To evaluate whether HC may generate ROS intracellularly and its inhibition, cells were stained with 10 µM DCFH-DA for 30 min at 37 °C, detached with trypsin/EDTA, washed with PBS, resuspended in PBS and subjected immediately for flow cytometry [22]. For determining the intracellular level of reduced GSH, KB cells were treated as described above and then stained with 25 µM CMF-DA for 30 min at 37 °C, trypsinized, resuspended in PBS, and immediately used for flow cytometric analysis. The changes in mitochondrial membrane potential ($\Delta\Psi m$) were measured by flow cytometry [30] using the fluorescent dye rhodamine. Cells were stained with 10 µg/ml of rhodamine for 30 min at 37 °C, collected and then immediately submitted for flow analysis (Becton Dickinson).

Measurement of mitochondrial GSH levels

Mitochondria were isolated as described by Ferri et al. [31] with slight modification. Briefly, 3×10^6 KB cells were inoculated into 10-cm culture dishes. After exposure to DMSO (control) or HC (0.05, 0.1, 0.2, 0.3 mM) for 24 h, cells were collected from three identical culture dishes for each treatment, washed with PBS and centrifuged at 2000 rpm for 3 min. Supernatant was decanted and cells were then resuspended in hypotonic buffer (42 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 5 mg/ml aprotinin, 1 mg/ml

of both leupeptin and pepstatin A, 10 mM HEPES, pH 7.4). After centrifugation at 2000 rpm for 3 min, cells were resuspended in PT buffer (125 mM KCl, 2.5 mM potassium phosphate, 20 mM HEPES, pH 7.4) and homogenized at 4000 rpm to disrupt the cell membrane. Homogenates were centrifuged at 1200 g for 5 min at 4 °C and supernatants were collected and centrifuged again at 10,000 g for 10 min at 4 °C to isolate the heavy membrane pellet enriched in mitochondria.

Reduced GSH levels were measured by reaction with DTNB (Ellman's reagent) as described previously [32]. In short, the mitochondria pellet was incubated in 100 µl of 3% ice-cold sulfosalicylic acid, vortexed, and incubated on ice for 15 min to precipitate protein. After centrifugation at 12000 rpm for 15 min at 4 °C, 20 µl of the supernatant was reacted with 180 µl of 0.6 mM DTNB (in 0.2 M phosphate buffer, pH 8.0) and the optical density values (OD_{410}) were read using a Dynatech Microplate Reader. A known serial concentration of GSH was also reacted with DTNB and used for calibrating GSH levels in samples. Protein precipitates were dissolved in 250 µl of 1 N NaOH and an aliquot was used for protein concentration determination with Bio-Rad protein assay kits. Finally, the results were expressed as nmol GSH/mg protein.

Statistical analysis

Four or more separate experiments were done for each case. Results are expressed as the mean ± SE. Statistical analysis was conducted using paired Student's t test. A p value < 0.05 was regarded to constitute differences between groups.

Results

Induction of cell cycle arrest and apoptosis of KB cells by HC

HC has been shown to induce cell cycle deregulation of KB cells [22]. For further evaluation of whether S phase cells are sensitive to the toxic effect of HC, experiments were performed by pulse labeling KB cells with BrdU and then exposing these cells to HC (0.1 and 0.3 mM) for 6 and 24 h for clarifying the fate of DNA synthesis in cells following exposure to HC. As shown in figure 1b, untreated KB cells exhibit normal cell cycle progression. Most of the BrdU-labeled cells have progressed through the cell cycle entering into the G2/M and G0/G1 phase 6 h later. However, KB cells treated with 0.1 and 0.3 mM HC showed an S phase arrest (fig. 1c, d), as indicated by the arrest of these BrdU-labeled cells in S phase. After exposure to 0.1 mM HC for 24 h, some KB cells progressed into G2/M and G0/G1 phase (fig. 1f). But most of the KB cells were arrested at S phase or induced into apoptosis (as revealed by the presence of a sub-G0/G1 peak) after exposure to 0.3 mM HC for 24 h (fig. 1g).

Cells in G1 and G2/M phases have been shown to have low BrdU-linked fluorescence, whereas S phase cells have high BrdU-linked fluorescence. BrdU is a thymidine analog that is incorporated into DNA during DNA synthesis and repair. The intensity of fluorescence is generally proportional to the amount of incorporated BrdU during DNA synthesis in S phase or DNA repair induced by genotoxic agents. Bivariate analysis of BrdU incorporation/DNA content may discriminate between actively synthesizing and quiescent cells with an S phase DNA content [33]. Using a similar method, exposure of KB cells to HC for 24 h markedly elevated the percentage of cells incorporating BrdU (table 1). The percentage of cells incorporating BrdU rose from 13.4 to 33.2–85.8%, with 0.1–0.3 mM HC. The DNA content was shown as PI fluorescence, and exposure of KB cells to HC led to G2/M and S phase arrest as described recently [22]. Accordingly, exposure of KB cells to HC at concentrations higher than 0.05 mM induced evident S phase and G2/M arrest (0.2 mM HC). Maximal accumulation of KB cells in S phase was induced by 0.1 mM HC. At concentrations of 0.2 and 0.3 mM, apoptosis of KB cells can be detected as revealed by the presence of a sub-G0/G1 peak [22; data not shown] leading to a slight decrease in cells arrested in S phase.

Effect of HC on mitochondrial membrane potential ($\Delta\Psi m$)

Recently, HC has been shown to induce GSH depletion and ROS production by KB and HepG2 cells [22, 34]. The presence of GSH in mitochondria provides protection against ROS and exposure to oxidative stress may impair mitochondrial functions [27, 35–37]. Moreover, a fall in mitochondrial membrane potential (membrane depolarization) is usually associated with apoptotic cell death [38, 39] and HC has been found to induce KB cell apoptosis [22], thus, evaluating whether disturbance of mitochondria mediates the HC-induced toxicity and cell cycle deregulation in oral epithelial cells is critical. We evaluated mitochondrial membrane potential using the cationic fluorescent dye rhodamine 123, which selectively partitions into mitochondria based on the highly negative mitochondrial membrane potential [40]. The amount of rhodamine retained in the mitochondria is proportional to the mitochondrial membrane potential. As shown in figure 2, the mean rhodamine fluorescence decreased from 120 (DMSO control) to 97 and 73 with 0.1 and 0.2 mM of HC, respectively, for 24 h, indicating dissipation of the fluorescent dye and resulting $\Delta\Psi m$ depolarization.

Effect of NAC on HC-induced changes of KB cells

HC has been shown to induce oxidative stress in KB cells [22, 34]. However, which types of ROS were induced and their correlation to the GSH depletion, mitochondrial depolarization and cell cycle deregulation was not clear. We therefore used various antioxidants and detoxifying en-

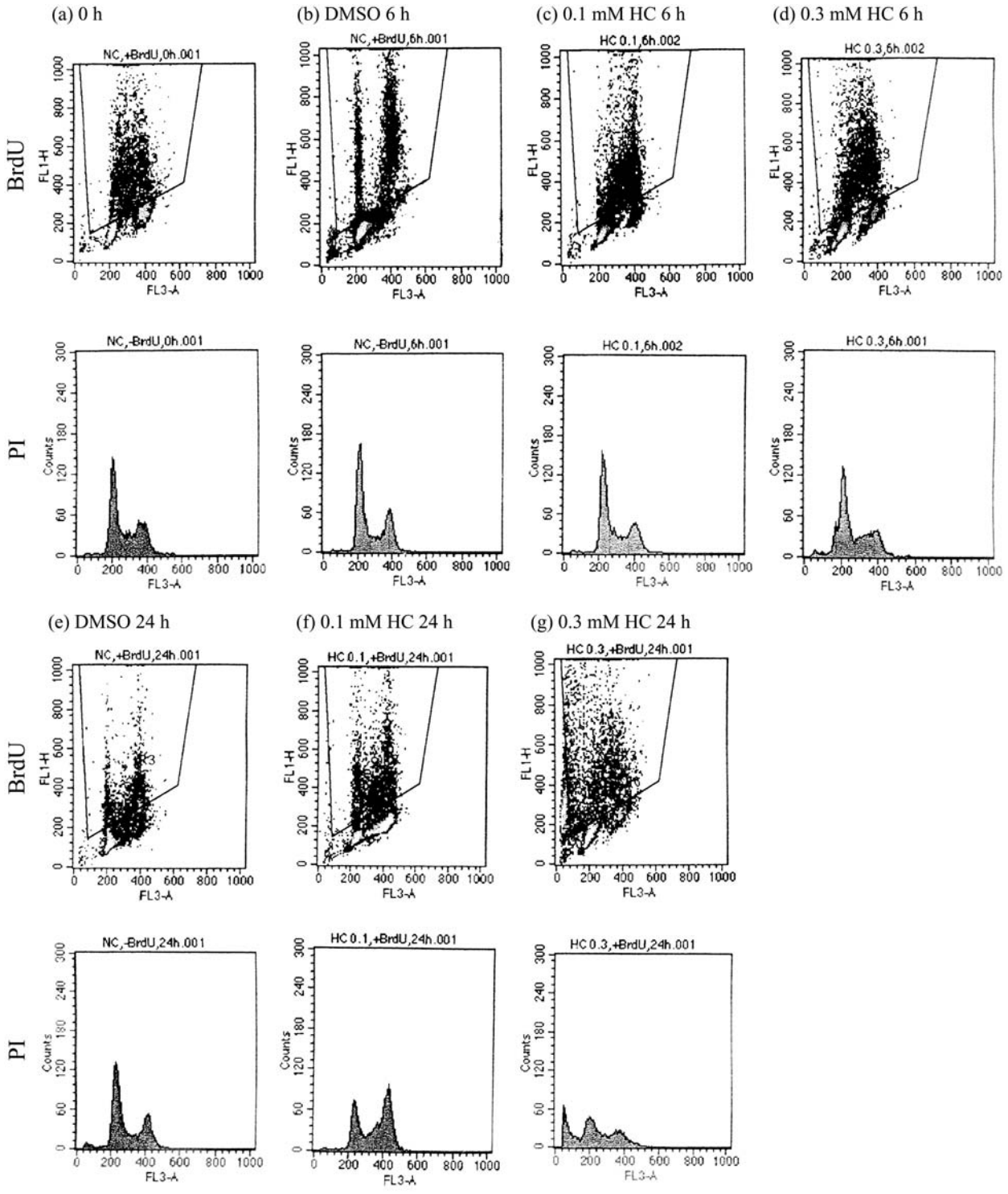

Figure 1. Inducing cell cycle arrest and apoptosis of KB cells by HC. KB cells were first labeled with BrdU for 30 min, washed and then exposed to fresh medium with DMSO (control) or 0.1 and 0.3 mM HC for 6 and 24 h. Cells were harvested, stained with PI and subjected to flow cytometry analysis. Baseline data (*a*) and KB cells exposed to DMSO (solvent control) for 6 h (*b*), 0.1 mM HC for 6 h (*c*), 0.3 mM HC for 6 h (*d*), DMSO for 24 h (*e*), 0.1 mM HC for 24 h (*f*) and 0.3 mM HC for 24 h (*g*). The gated region indicated BrdU(+) cells. The fate of BrdU(+) cells (cells in S phase during treatment) changed after exposure to HC.

Table 1. Effect of HC on BrdU incorporation and cell cycle distribution of KB cells. KB cells were exposed to DMSO (control) or 0.01–0.3 mM HC for 24 h. Cells were harvested for measurement of BrdU incorporation (n = 6) and cell cycle distribution (n = 5).

Chemical	BrdU-stained cells (% of cells)	Cell cycle distribution		
		G0/G1	S	late S +G2/M
Control	13.4 ± 2.1	72 ± 0.6	9.2 ± 0.7	18.6 ± 0.9
0.01 mM HC	12.4 ± 2.1	75 ± 0.9	9.6 ± 1.0	15.6 ± 0.8
0.05 mM HC	17.9 ± 2.1	67 ± 1.5*	16 ± 0.9*	16.2 ± 0.7
0.1 mM HC	33.2 ± 8.3*	45 ± 2.2*	35 ± 1.8*	19.8 ± 0.6
0.2 mM HC	56.2 ± 9.6*	46 ± 2.6*	28 ± 2.1*	25.6 ± 1.2*
0.3 mM HC	85.8 ± 2.9*	56 ± 1.6*	27 ± 3.3*	16.8 ± 4.2

*Denotes marked difference ($p < 0.05$) when compared with the control group.

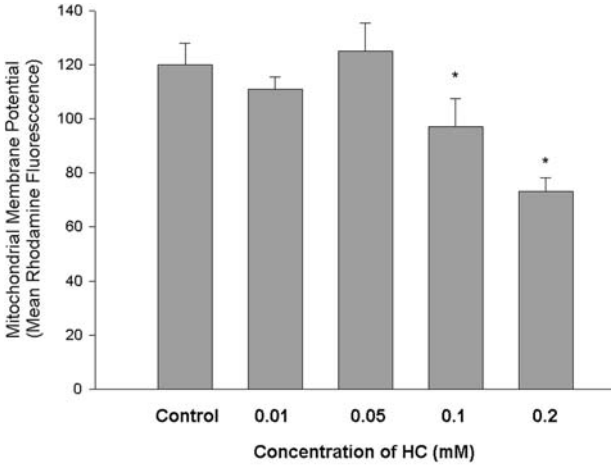

Figure 2. Inducing mitochondrial membrane depolarization by HC. KB cells were exposed to HC (0.01–0.2 mM) for 24 h. At the final 30 min, cells were stained with rhodamine, detached and immediately subjected to flow cytometry. Results are expressed as mean rhodamine fluorescence (mean ± SE). *Denotes significant difference ($p < 0.05$) when compared with control (n = 11).

zymes to clarify the properties of toxic ROS. Cleavage of DCFH-DA by cellular esterase generates DCFH, which when oxidized by H_2O_2, superoxide radical and peroxynitrite gives rise to fluorescent DCF. An increase in cellular fluorescence therefore reveals the elevated levels of intracellular ROS or nitrogen species [40]. NAC (1 mM), a general antioxidant, effectively decreased the 0.3 mM HC-induced DCF fluorescence from 630 to 258 (fig. 3a). Previous experiments have found that most of the KB cells have a high GSH content with an average CMF fluorescence of about 100. After exposure to HC, depletion of cellular GSH content was noted as revealed by the presence of an M1 population [22]. Similarly, as indicated by figure 3b, exposure of KB cells to 0.3 mM HC led to a decrease in GSH content as indicated by an increase in the cells in the M1 population (low GSH content) [22]. After exposure to 0.3 mM HC, the M1 population was about 69.8%. In the presence of 1 mM NAC, the HC-induced GSH depletion was effectively prevented, as revealed by a decreasing in the M1 population to 2.8%, comparable to the untreated control (low GSH content) (n = 6) (fig. 3b). NAC also prevented the HC-induced mitochondrial membrane depolarization, as indicated by reversal of decreased rhodamine fluorescence in HC-treated cells (scale = 39) to about that of untreated cells (mean scale = 108) (fig. 3c). Similarly, NAC (1 mM) prevented the HC-induced apoptosis of KB cells (data not shown) and partly decreased the percentage of cells arresting at S phase (fig. 3d).

Specific ROS scavengers on HC-induced effects on KB cells

Because NAC was effective in prevention of HC-induced ROS production, GSH depletion and apoptosis, we further explored the types of ROS by using specific ROS scavengers and detoxifying enzymes. Interestingly, we noted that superoxide dismutase (SOD, 100 U/ml) almost completely prevented the HC-induced increase in DCF fluorescence (fig. 4a). SOD also markedly prevented the HC-induced GSH depletion, as revealed by a decrease in the cell population with low CMF fluorescence (M1 region) from 69.8 to 9% (fig. 4b). HC-induced mitochondrial depolarization was also completely reversed by 100 U/ml SOD (fig. 4c). Intriguingly, SOD (100 U/ml) also effectively prevented the HC-induced apoptosis (data not shown), and partially decreased the percentage of HC-treated cells arrested at S phase (fig. 4d).

Interestingly, catalase, an H_2O_2-degrading enzyme, is also able to prevent HC-induced DCF fluorescence. As shown in figure 5a, catalase (1000 U/ml) decreased the 0.3 mM HC-induced DCF fluorescence value from 701 to 301. Catalase (1000 U/ml) also decreased the HC (0.3 mM)-induced GSH depletion, as revealed by increase in the population of cells in the M2 region (high CMF fluorescence) (fig. 5b). Catalase (500 U/ml) showed little effect on HC-induced mitochondrial membrane depolarization (fig. 5c), but was slightly effective at a concentration of 1000 U/ml (data not shown). Catalase also decreased the population of HC-treated KB cells arresting at S phase, as indicated by a decrease in the cells

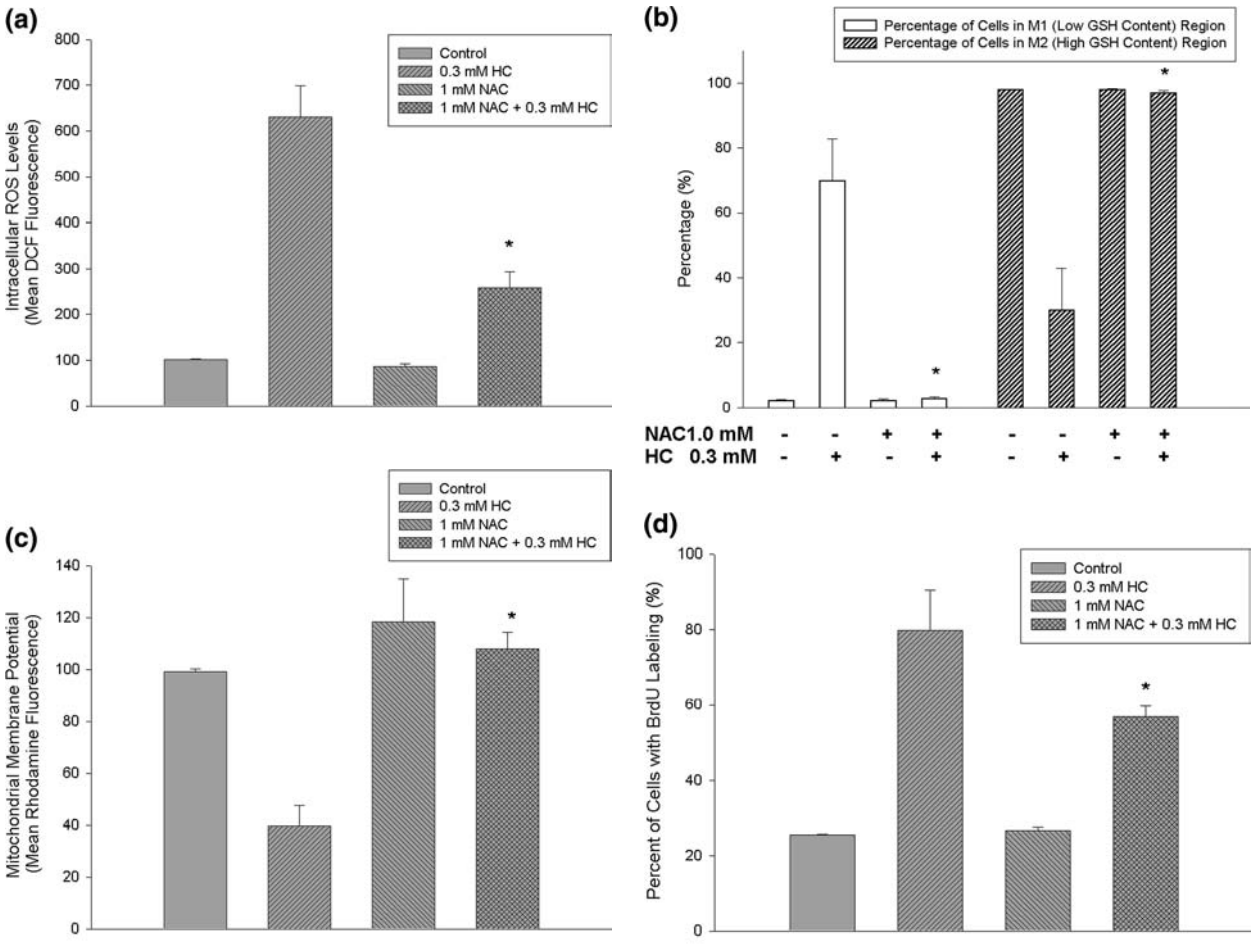

Figure 3. Effects of NAC on HC-induced oxidative stress, GSH depletion, mitochondrial membrane depolarization and BrdU incorporation. KB cells were exposed to 1 mM NAC 30 min prior to the addition of HC (0.3 mM) and incubated for 24 h. At the final 30 min, cells were stained with DCF (*a*), CMF (*b*), rhodamine (*c*) and BrdU (*d*), detached and subjected to flow cytometry. Results are expressed as mean fluorescence (mean ± SE). * Denotes significant difference ($p < 0.05$) when compared with HC-treated group ($n = 6$).

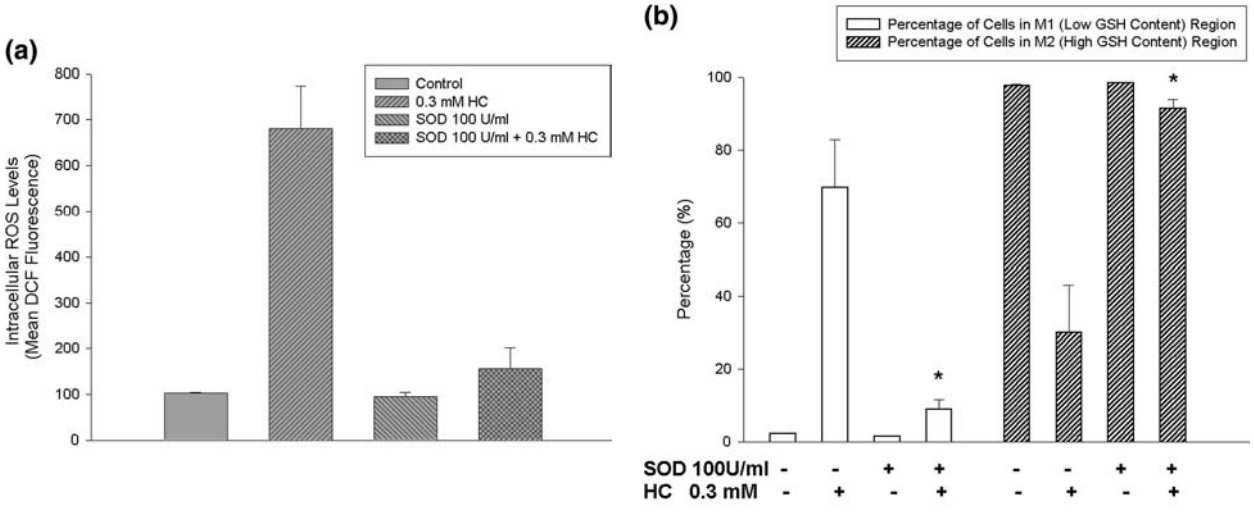

Figure 4. Effects of SOD on HC-induced oxidative stress, GSH depletion, mitochondrial membrane depolarization and BrdU incorporation. KB cells were exposed to SOD (100 U/ml) 30 min prior to the addition of HC (0.3 mM) and incubated for 24 h. At the final 30 min, cells were stained with DCF (*a*), CMF (*b*), rhodamine (*c*) and BrdU (*d*), detached and subjected to flow cytometry. Results are expressed as mean fluorescence (mean ± SE). * Denotes significant difference ($p < 0.05$) when compared with control ($n = 4$).

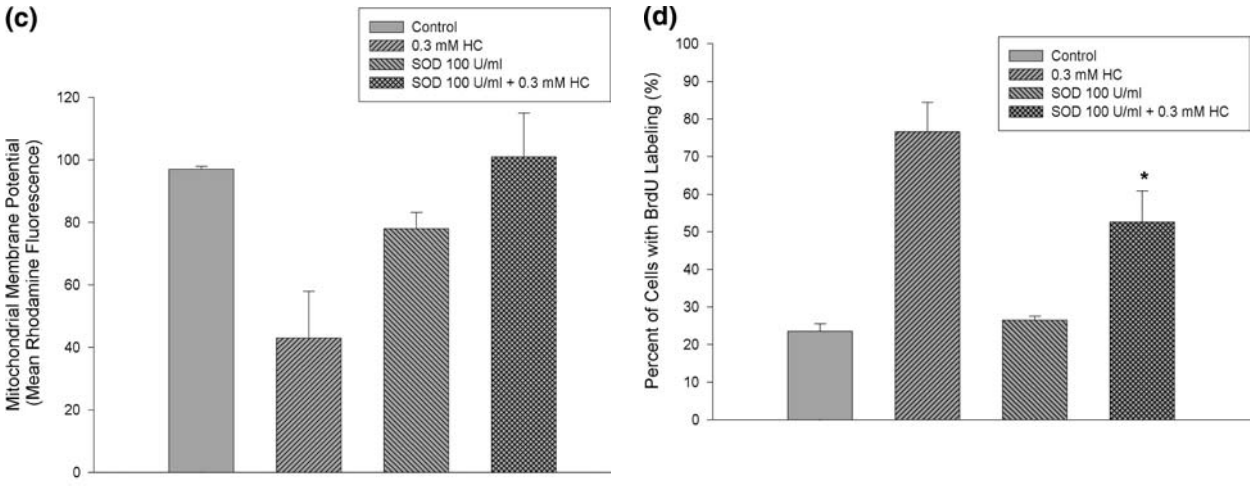

Figure 4. (continued)

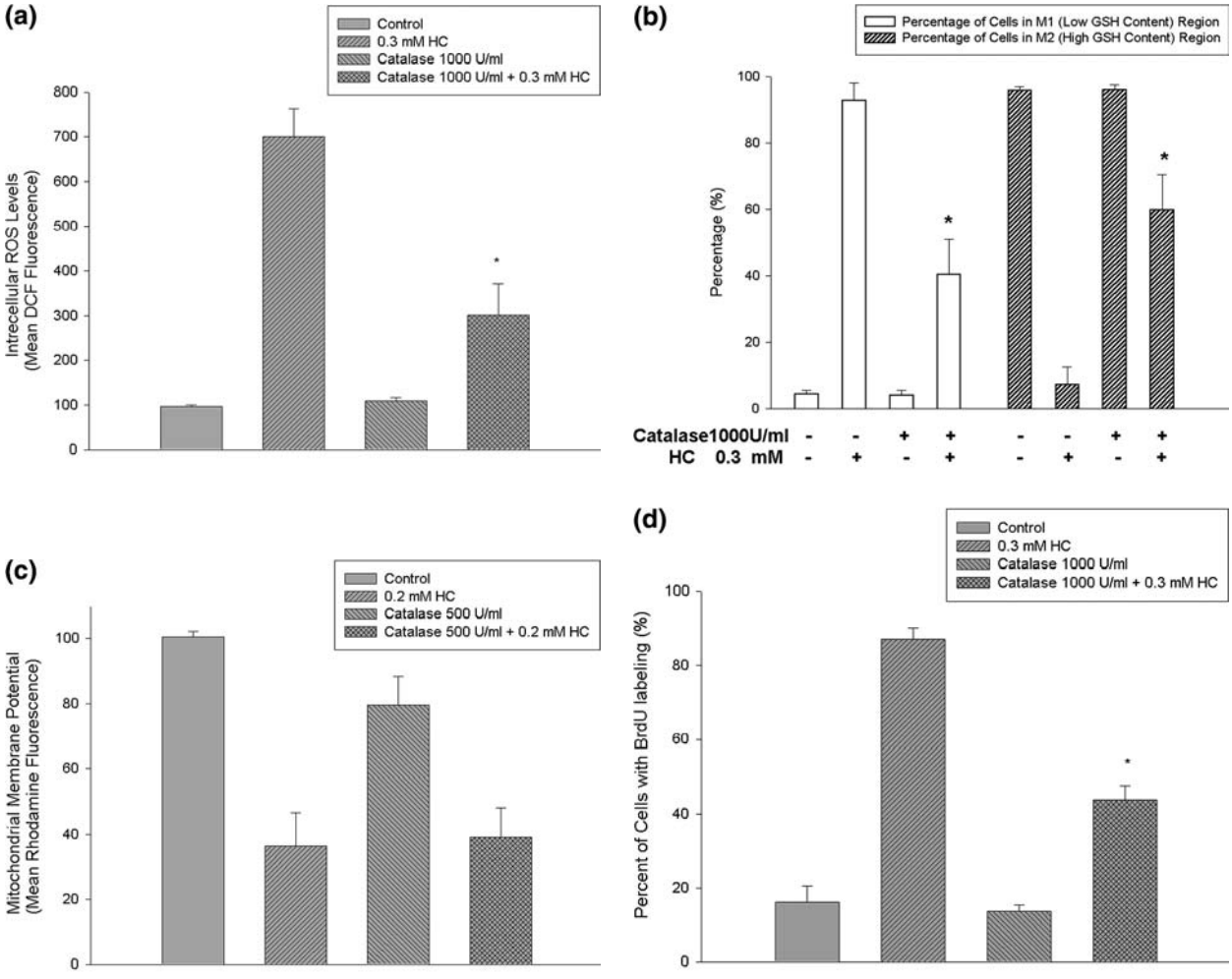

Figure 5. Effects of catalase on HC-induced oxidative stress, GSH depletion, mitochondrial membrane depolarization and BrdU incorporation. KB cells were exposed to catalase (500 or 1000 U/ml) 30 min prior to the addition of HC (0.2 or 0.3 mM) and incubated for 24 h. At the final 30 min, cells were stained with DCF (*a*), CMF (*b*), rhodamine (*c*) and BrdU (*d*), detached and subjected to flow cytometry. Results are expressed as mean fluorescence (mean ± SE). * Denotes significant difference ($p < 0.05$) when compared with HC-treated group ($n = 4$).

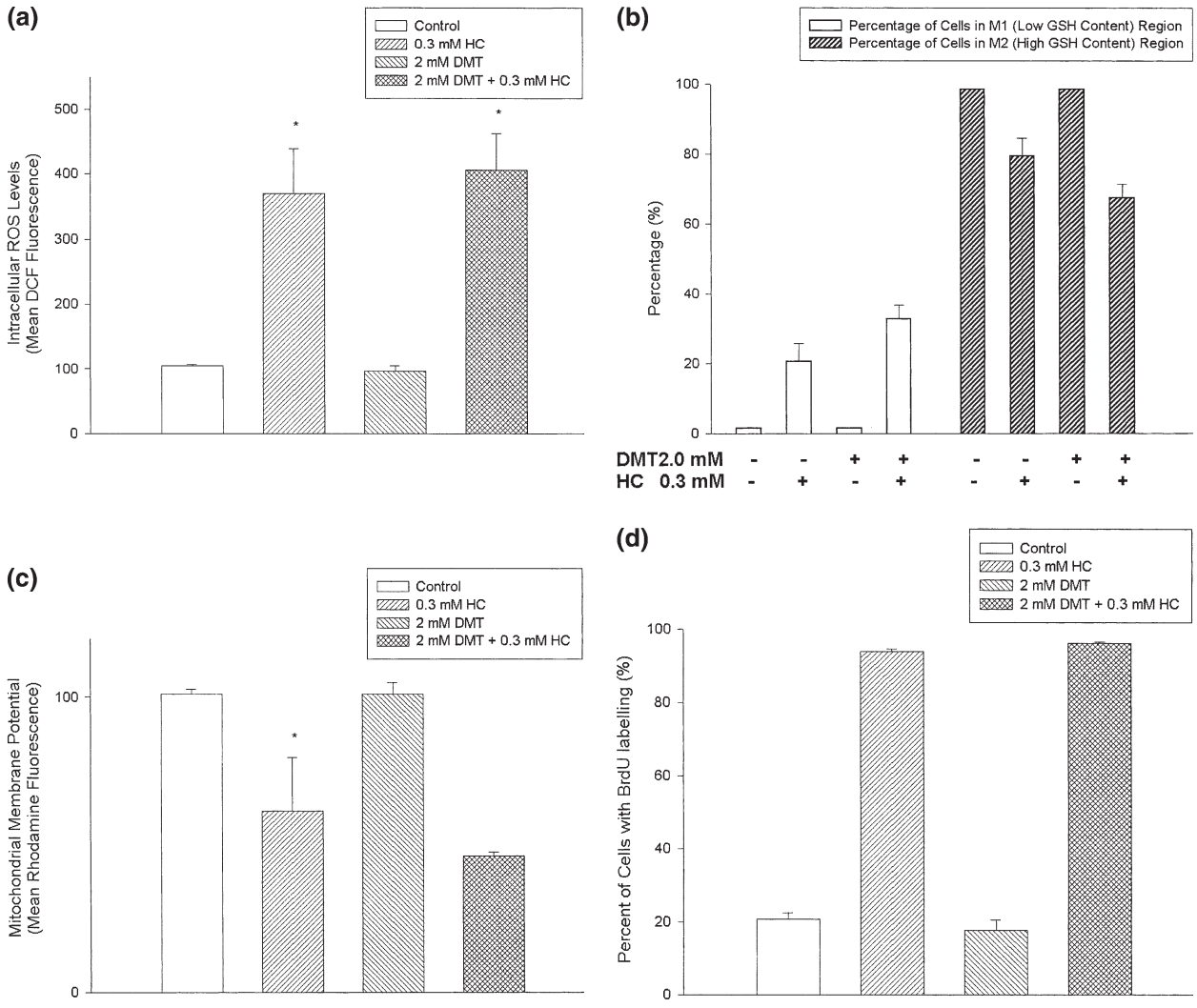

Figure 6. Effects of DMT on HC-induced oxidative stress, GSH depletion, mitochondrial membrane depolarization and BrdU incorporation. KB cells were exposed to 2 mM DMT 30 min prior to the addition of HC (0.3 mM) and incubated for 24 h. At the final 30 min, cells were stained with DCF (n = 4) (*a*), CMF (n = 4) (*b*), rhodamine (n = 3) (*c*) and BrdU (n = 3) (*d*), detached and subjected to flow cytometry. Results were expressed as mean fluorescence (mean ± SE). *Denotes marked difference when compared with control.

stained by BrdU (fig. 5d) and prevented the HC-induced apoptosis (data not shown).

Dimethylthiourea (DMT) (2 mM), a hydroxyl radical scavenger, is not able to prevent 0.3 mM HC-induced ROS production. Moreover, DMT in combination with HC contributed to an increase in DCF fluorescence (fig. 6a). DMT showed little prevention of HC-induced GSH depletion (fig. 6b). Furthermore, DMT showed little preventive effect on HC-induced mitochondrial depolarization (fig. 6c). DMT also showed little effect on HC-induced S phase arrest (fig. 6d) and apoptosis (data not shown).

Effects of metal chelators on HC-induced effects in KB cells

Neocuproine (1 mM), a copper chelator, showed little effect on HC-induced ROS production, mitochondrial membrane depolarization, BrdU incorporation (fig. 7a–c) and GSH depletion (n = 2) (data not shown). Neocuproine (1 mM) by itself decreased the $\Delta\Psi m$. Similarly, desferrioxamine (0.5 mM) and phenanthroline (100 and 200 μM), two iron chelators, also lack of preventive effects on these HC-induced cellular events (data not shown).

Effects of HC on cellular GSH and mitochondrial GSH levels in KB cells

The percentage of KB cells with low GSH levels (M1 region) increases after exposure to higher concentrations of HC [22]. In the present study, we further noted that exposure to 0.05 and 0.1 mM HC for 24 h markedly elevated the CMF fluorescence of KB cells residing in the M2 (high GSH) region. The mean CMF fluorescence of control cells was about 102, whereas exposure to 0.1 and 0.2 mM HC elevated the fluorescence to 116.7 and 121.8,

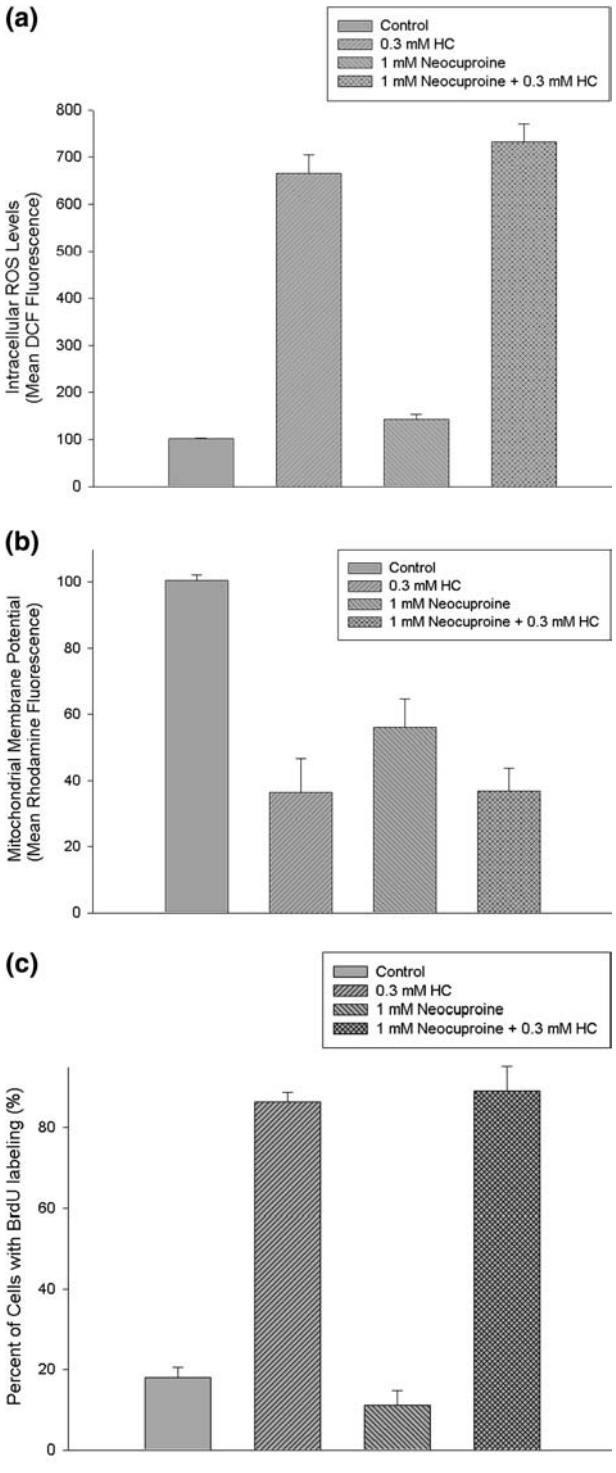

Figure 7. Effects of neocuproine on HC-induced oxidative stress, mitochondrial membrane depolarization and BrdU incorporation. KB cells were exposed to 1 mM neocuproine 30 min prior to the addition of HC (0.3 mM) and incubated for 24 h. At the final 30 min, cells were stained with DCF (*a*), rhodamine (*b*) and BrdU (*c*), detached and subjected to flow cytometry. Results are expressed as mean fluorescence (mean ± SE) (n = 4).

respectively. At concentrations of 0.3 mM, CMF fluorescence decreased to 87 (table 2). Since mitochondrial GSH levels are shown to be crucial for its functions, we therefore tested whether HC affects the mitochondrial GSH levels. As shown in table 2, HC stimulated the mitochondrial GSH level from 8.2 to 11.1 and 10.4 nmol/mg protein, at concentrations of 0.05 and 0.1 mM, respectively. But at concentrations higher than 0.2 mM, HC partly lowered the mitochondrial GSH level by 19%.

Discussion

Elucidation of the mechanisms responsible for BQ carcinogenesis is urgently required for future chemoprevention and treatment of oral cancer. In our previous experiment, HC was found to be an effective scavenger of various ROS such as H_2O_2, superoxide and hydroxyl radicals with differential potency at low concentrations (<0.05 mM) [22]. But at concentrations higher than 0.1 mM, HC induces marked oxidative stress, GSH depletion, S and G2/M cell cycle arrest and apoptosis of KB epithelial cells [22]. These results indicate that HC may be an antioxidant or prooxidant depending on exposure concentrations. This may partly explain why some of the previous reports suggest that HC exhibits antimutagenicity, anticarcinogenicity and can be an antioxidant [15, 20, 24, 41], whereas some reports suggest the potential induction of mutagenicity, DNA-breaking capacity and oxidative damage by HC [7, 34]. Since the HC content of IPB is high (9.74 mg/g wet weight) and the salivary concentration of HC during BQ chewing is reported to be 4.6 mM, that is, 15-fold higher than the present tested concentrations, induction of oxidative stress and depletion of GSH by HC, eugenol and other AN components [22, 29, 42–44] may partially justify why OSF tissues from BQ chewers show a decrease in GSH content [45]. Since cellular GSH content is important for cell growth, enzymatic reactions, cellular biosynthesis, signal transduction and protection against oxidative stress [46, 47], inducing GSH depletion, cell cycle arrest and cell growth retardation by BQ components may explain why mucosal atrophy was usually noted in BQ chewers' mucosa [3, 4]. This can be explained by the induction of cell cycle arrest and apoptosis by HC and other BQ ingredients [29].

A decrease in $\Delta\Psi$m (membrane depolarization) is associated with dysfunction of mitochondria and subsequent apoptotic cell death [38, 39]. Since oxidative stress and GSH depletion may impair mitochondrial function leading to cytochrome c release that is central to cellular apoptosis [27], we therefore tested whether HC has an impact on the mitochondrial membrane potential ($\Delta\Psi$m). Consistently, HC induced significant mitochondrial depolarization of KB cells, as revealed by a decrease in rhodamine uptake. This indicates that inducing the $\Delta\Psi$m de-

Table 2. Effects of HC on γ-glutamylcysteine ligase activity and cellular and mitochondrial GSH levels of KB epithelial cells.

Chemicals	Cells in M2 region		Mitochondrial GSH (nmol/mg protein)
	% of cells	mean CMF fluorescence	
DMSO (control)	98.3 ± 0.3	101.6 ± 1.7	8.2 ± 1.2
HC 0.05 mM	98.2 ± 0.3	94.8 ± 4.2	11.1 ± 1.6*
HC 0.1 mM	97.0 ± 0.1	116.7 ± 5.0*	10.4 ± 1.6*
HC 0.2 mM	79.8 ± 3.4*	121.8 ± 7.8*	7.9 ± 1.1
HC 0.3 mM	32.7 ± 6.4*	87.0 ± 8.4	6.7 ± 1.5

* Denotes marked difference ($p < 0.05$) when compared with the control group.
KB cells were exposed to DMSO or various concentrations of HC. Cells were collected after incubation for 24 h and used for measurement of the cellular GSH level (n = 5) and mitochondrial GSH (n = 8).

polarization by HC may be a major factor leading to cell apoptosis. Interestingly, although SOD, catalase and DMT decreased $\Delta\Psi$m mildly, because NAC lacked a similar effect, we cannot conclude that a minimal amount of ROS are required for maintenance of $\Delta\Psi$m. Altered mitochondrial morphology, changes in mitochondrial enzyme patterns and membrane transport are found in several tumor types including oral carcinoma [48, 49]. Disruption of $\Delta\Psi$m and elevation of cytosolic calcium is also reported to induce stress signaling in rhabdomyoblasts [50]. Further studies are necessary to clarify whether depolarization of mitochondria by HC was due to elevation of cytosolic calcium and accumulation of calcium in mitochondria as described by Duchen [51]. Furthermore, when cells were labeled with BrdU and then exposed to HC (0.1 and 0.3 mM), most of the treated cells arrested in S phase in the first 6 h of exposure, whereas untreated cells progressed through the cell cycle into the G2/M and G0/G1 phase (fig. 1). This indicates that cells in S phase (shown as BrdU-positive cells) are sensitive to the toxic effect of HC. Further exposure of S phase cells to HC (0.3 mM) for 24 h evidently induced apoptosis of KB cells, showing that prolonged arrest of cells in S phase may promote apoptotic cell death. Because HC may induce oxidative stress and consequent DNA damage, arrest of cells in S phase may offer a greater opportunity for the repair of damaged DNA [52–54]. However, additional studies are needed to extend this hypothesis.

HC has been shown to induce oxidative stress, leading to plasmid DNA breaks and 8-OH dG formation in CHO cells, especially in the presence of transition metal ions [7]. In addition, oxidative metabolism of HC may deplete cellular GSH by conjugation with other electrophilic HC metabolites such as quinones, quinone methide and imine methide which are responsible for its cytotoxicity [19, 55–57] or by glutathione peroxidase-mediated oxidation of GSH to produce GSSG [58]. Consistently, HC induced notable GSH depletion and intracellular ROS production in this study. But at concentrations of 0.1 and 0.2 mM, HC elevated the GSH levels of cells in the M2 region, as indicated by an increase in mean CMF fluorescence. However, at a concentration of 0.3 mM, HC increased the percentage of cells in the low GSH region (M1 region) and decreased the mean CMF fluorescence in cells residing in the M2 region. This suggests the presence of a cellular adaptive response to oxidative stress and GSH depletion. A similar adaptive reaction of human HepG2 cells and primary rat hepatocytes in response to moderate concentrations of exogenous quercetin and diallyl sulfide has been reported. But excessively high concentrations of both agents lead to marked GSH consumption that may overwhelm any concomitant GSH de novo synthesis [59–61]. Cellular GSH is regulated by the transport or extrusion of cysteine, the activity of γ-glutamyltranspeptidase and γ-glutamylcysteine ligase (γ-GCL), two rate-limiting enzymes for GSH degradation and synthesis, respectively. Cellular γ-GCL activity is generally low [62]. Some antioxidants, short-lived oxidants or GSH-depleting agents have been shown to induce γ-GCL that results in an overall chemoprotective effect [61]. Plating cell density also effectively regulates the γ-GCL activity and GSH synthesis of primary rat hepatocytes, which modulate cell proliferation [63, 64]. Further sequential studies on the effect of HC toward γ-GCL mRNA and protein expression and γ-GCL activity of oral mucosal cells will highlight our understanding of whether HC is carcinogenic or anticarcinogenic in the oral cavity.

GSH is crucial in attenuating the oxidative stress-induced disruption of the mitochondrial membrane potential [62]. Accordingly, GSH and NAC were also effective in prevention of HC-induced mitochondrial depolarization. Recently, depletion of GSH in PC12 cells results in selective inhibition of mitochondrial complex I activity and mitochondrial function [65]. Mitochondrial GSH is transported from the cytosol via a multicomponent transport system, which retains GSH under conditions where the cytosolic level might fall. [66]. In the present study, 0.05 and 0.1 mM HC elevated the mitochondrial GSH pools of KB cells. This was generally associated with an increase in cytosolic GSH levels of KB cells residing in the M2 region, which can be a cellular adaptive response. But at concentrations of 0.2 and 0.3 mM, HC partly decreased

the mitochondrial GSH levels of KB cells, possibly due to an increase of the cell population in the M1 (low GSH content) region. This depletion of mitochondrial GSH may partly explain mitochondrial disruption of KB cells by HC. However, the presence of additional factors should be further evaluated.

Because DCFH can be oxidized by H_2O_2, lipid hydroperoxide, peroxynitrite and superoxide radicals to produce DCF fluorescence [67–71], an intriguing question is to clarify the nature of ROS produced when KB cells are exposed to HC in vitro. NAC, as a general antioxidant, prevented the HC-induced GSH depletion, ROS production, cell cycle arrest and apoptosis, indicating that these events are mediated by ROS. We found that SOD and catalase, but not DMT, may completely prevent HC-induced GSH depletion, ROS production, cell cycle arrest and apoptosis. This indicates that HC-induced toxic events are mediated by extracellular production of superoxide radicals and H_2O_2 or possibly via enzyme-catalyzed phenoxy radical formation or o-quinone formation and the subsequent redox cycling during HC metabolism [55, 57]. But why hydroxyl radical scavengers and metal chelators showed little preventive effect needs further investigation to clarify whether hydroxyl radicals are produced but not directly involved in these toxic events or are produced just adjacent to target sites [72]. The induction of ROS by HC is not unexpected, because many phenolic antioxidants also display prooxidant properties and cause cell injury in different experimental conditions such as pH changes, in the presence of absence of transition metals, or metabolic enzyme activation [73, 74]. In the present study, we tried to use neocuproine (a cell-permeable copper chelator), desferrioxamine (a ferric ion chelator) and 1,10-phenanthroline (a cell-permeable ferrous, cupric and cuprous ion chelator) [75] to protect against the HC-induced cellular alterations. However, these ion chelators showed little effect on HC-induced GSH depletion, ROS production and cell cycle deregulation, indicating that these events are mediated by target-site-specific production of ROS [72] or enzyme-catalyzed production of reactive intermediates, but not by copper- or iron-catalyzed hydroxyl radical formation.

Because the salivary concentration of HC during BQ chewing is about 4.6 mM, HC should be considered to be associated with BQ-chewing-related oral mucosal alterations via GSH depletion, ROS production, mitochondrial deregulation, cell cycle disturbance and induction of apoptosis. These events are possibly related to the production of superoxide radicals and hydrogen peroxide, but not directly mediated by metal-catalyzed hydroxyl radical production. But additional in vivo animal experiments should be conducted to elucidate whether HC is carcinogenic or anticarcinogenic and may induce DNA damage in vitro. Using a specific dihydroethidium method [76] or Amplex red assay [77] to directly estimate the superoxide radical and H_2O_2 production of KB cells induced by HC may improve our knowledge of mechanisms of HC-induced oxidative stress. Recently, dietary intake of antioxidants such as GSH and vitamin C has been shown to reduce the oral and pharyngeal cancer risk [78, 79]. Protection against AN-induced DNA damage, arecoline-induced cytotoxicity and HC-induced oxidative stress by antioxidants [43, 44, 80] suggests that a dietary supplement of antioxidants might protect against and prevent BQ-chewing-related oral mucosal lesions.

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