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Protective Effects of Antioxidants against Smokeless Tobacco-induced Oxidative Stress and Modulation of *Bcl-2* and *p53* Genes in Human Oral Keratinocytes

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The oral use of chewing tobacco has greatly increased in recent years, and this usage is associated with cancers of the mouth, lip, nasal cavities, esophagus and gut. Oral cancer accounts for 3% of all cancers in U.S.A. and is the seventh most common cancer. Previous studies in our laboratory have demonstrated the protective abilities of a novel IH636 grape seed proanthocyanidin extract (GSPE) against reactive oxygen species both *in vitro* and *in vivo* models, and provided significantly better protection as compared to vitamins C, E and β-carotene. In the recent past, we have demonstrated smokeless tobacco (STE)-induced oxidative stress, apoptotic cell death in a primary culture of normal human oral keratinocytes (NHOK), and have compared the protective abilities of vitamins C and E, singly and in combination, and GSPE in this pathobiology [*Free Rad. Biol. Med.*, **26**, 992–1000 (1999)]. In the present study, we have assessed the protective role of vitamins C and E, and GSPE against STE-induced modulation of intracellular oxidized states in NHOK cells as demonstrated by laser scanning confocal microscopy. Approximately 11%, 26%, 28% and 50% protection were observed following incubation with vitamin C, vitamin E, a combination of vitamins C plus E, and GSPE, respectively. DNA fragmentation was assessed as an index of oxidative DNA damage and similar results were observed. Furthermore, the cellular viability and functional roles of *Bcl-2*, *p53* and *c-myc* genes were assessed in STE-induced oxidative stress in NHOK cells. NHOK cells were treated with STE (0–200 μg/ml) for 24 h and changes in the expression of *Bcl-2*, *p53* and *c-myc* genes were measured by reverse transcriptase–polymerase chain reaction (RT–PCR), and the protective effect of GSPE was assessed. Approximately a 2.0-fold increase in *p53* gene expression was observed following incubation of the oral keratinocytes with 100 μg/ml of STE, beyond which the expression of *p53* decreased, confirming increased apoptotic cell death with a higher concentration of STE as reported earlier. GSPE significantly modulated STE-induced changes in *p53*. The expression of antiapoptotic *Bcl-2* gene decreased with STE treatment and the expression of *Bcl-2* gene increased significantly following preincubation with GSPE. No significant change in the expression of transcription factor *c-myc* gene responsible for cell cycle growth was observed following incubation with STE and/or GSPE. Thus, *c-myc* may not be involved in STE-induced cytotoxicity towards NHOK cells. These results suggest that antioxidant

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protection of STE-induced cellular injury is associated with alterations in *Bcl-2* and *p53* expression.

Keywords: Oxidative stress, cultured human oral keratinocytes, smokeless tobacco extract, laser scanning confocal microscopy, free radicals, proanthocyanidins, vitamin C, vitamin E, *Bcl-2*, *p53* and *c-myc* genes

INTRODUCTION

Epidemiological studies have demonstrated that the oral use of smokeless tobacco products (snuff) has increased greatly in recent years. Johansson *et al.*[1] have shown that oral use of the moist smokeless tobacco products is casually associated with cancers of the mouth, lip, nasal cavities, esophagus and gut, and is indirectly related to many other forms of cancer. Studies in our laboratories have showed that acute and chronic administration of smokeless tobacco extract (STE) to rats increases hepatic mitochondrial and microsomal lipid peroxidation, hepatic nuclear DNA-single strand breaks, production of reactive oxygen species (ROS) and nitric oxide (NO) in the peritoneal macrophages, and enhanced excretion of urinary lipid metabolites.[2–5] NO is a highly reactive endogenous chemical that is produced by activated macrophages and demonstrates cytotoxic activity.[6]

We have established a primary culture of normal human oral keratinocytes (NHOK) in our laboratory, and assessed the concentration-dependent cytotoxic effect of STE.[7] Concentration-dependent increases occurred in the production of ROS, DNA fragmentation and protein kinase C activity in these cells following incubation with STE.[7] In another related study, concentration-dependent apoptotic cell death was determined by flow cytometry using the fluorescent dye, propidium iodide. Approximately 9%, 29% and 35% increases in apoptotic cell death, were observed following treatment with 100, 200 and 300 μg STE/ml, respectively. Approximately 51–85% decreases in apoptotic cell death were observed in NHOK cells following incubation with vitamins C and E, singly and in combination, and GSPE, while GSPE exhibited better protection.[8]

STE-mediated oxidative stress and cellular injury, as well as apoptotic cell death in NHOK cells might occur due to alterations in the expression of certain cellular regulatory genes. The key gene families regulating apoptotic cell death are *p53*, *c-myc* and *Bcl-2*.[9–13] *p53* is a nuclear phosphoprotein involved in cellular functions including differentiation, response to DNA damage, regulation of cell cycle check points and DNA repair, and induction of apoptosis.[14–16] The protooncogene *Bcl-2* delays the onset of apoptosis induced by a variety of agents including exposure to chemotherapeutic drugs. It is reported that *Bcl-2* can also block cell death caused by chemotherapeutic drugs, UV radiation, heat-shock/stress, and free radicals.[17] Another protooncogene *c-myc* plays a positive role in cell death and apoptosis.[18] Several studies have suggested a role for *p53* in *c-myc*-induced apoptosis upon serum withdrawal in fibroblasts.[19,20]

Proanthocyanidins are naturally occurring polyphenolic antioxidants widely available in fruits, vegetables, nuts, seeds, flowers, and bark. They are known to exhibit a wide range of pharmacological and chemopreventive properties against free radicals, oxidative stress and apoptotic cell death.[21,22] Previous studies in our laboratory have demonstrated the protective abilities of GSPE against biochemically generated ROS,[23] H_2O_2-induced modulation of intracellular oxidized states in cultured J774A.1 macrophage and neuroactive PC-12 cells,[24] and TPA-induced oxidative damage in liver and brain tissues, and peritoneal macrophage activation in mice.[25] GSPE also protected against myocardial ischemia-reperfusion injury and infarction in rats,[26] acetaminophen-induced hepatotoxicity and Bcl-X_L inactivation in mice,[27] and acute and chronic stress-induced oxidative gastrointestinal mucosal injury *in vivo*.[28] GSPE also demonstrated selective cytotoxicity towards

human MCF-7 breast cancer, A-427 lung cancer and CRL-1739 gastric adenocarcinoma cells, while enhancing the growth and viability of murine macrophage J774A.1 and normal human gastric mucosal cells.[29] GSPE significantly protected against drug- and chemical-induced multiorgan toxicity *in vivo*.[30] GSPE also provided protection against idarubicin- and 4-hydroxyperoxycyclophosphamide-induced cytotoxicity towards cultured human Chang liver cells.[31]

The present study was designed to assess STE-induced oxidative stress and DNA damage in NHOK cells and the roles of regulatory genes including *Bcl-2*, *p53* and *c-myc* in understanding the mechanistic pathways of GSPE cytoprotection. STE-induced modulation of overall intracellular oxidized states in a primary culture of NHOK cells were determined by laser scanning confocal microscopy using 2,7-dichlorofluorescein diacetate (DCFH) as the fluorescent probe, and the protective abilities of vitamins C (75 μM) and E (75 μM), singly and in combination, and GSPE (100 μg/ml) were also assessed. Respiratory effects of these antioxidants on STE exposed NHOK cells were assessed by determining the ability of these cells to reduce the tetrazolium dye MTT, based on the activation of the enzyme succinate dehydrogenase. Furthermore, both concentration- and time-dependent effects of STE on cellular viability in NHOK cells were determined by Trypan blue exclusion technique. Changes in mRNA expression of *p53*, *Bcl-2* and *c-myc* genes were determined by RT–PCR.

MATERIALS AND METHODS

Chemicals

Standardized smokeless tobacco (moist snuff) was purchased from the University of Kentucky Tobacco and Health Research Institute, Lexington, KY. A novel IH636 grape seed proanthocyanidin extract (GSPE) (commercially known as ActiVin, batch # AV609016) was obtained from InterHealth Nutraceuticals, Inc. (Benicia, CA), which is a natural extract of approximately 54% dimeric-, 13% trimeric- and 7% tetrameric proanthocyanidins, and approximately 6% monomeric bioflavonoids. Keratinocyte growth medium (KGM) was obtained from Clonetics for the growth and maintenance of the primary culture (La Jolla, CA). All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or GIBCO (Grand Island, NY) and were of analytical grade or the highest grade available.

Preparation of Smokeless Tobacco Extract (STE)

Quantities of smokeless tobacco were mixed with five volumes (5 ml/gm) of 0.10 M phosphate buffer, pH 7.0, and stirred at room temperature for 24 h. The pH of the extracts was readjusted to pH 7.0 after 1 h of stirring to ensure a physiological pH, and the extracts were centrifuged at 40,000 *g* for 1 h. The supernatant fractions were filtered through a Millipore filter (0.45 μm) (Millipore Corporation, Boston, MA), lyophilized and stored at − 70 °C. STE were used within 6–8 weeks of preparation, although storage for up to six months did not produce any changes in the ability to induce oxidative stress and membrane damage. The extracts were reconstituted in phosphate buffer at a concentration of 1.0 mg freeze-dried material per ml.[2–4,7] STE was standardized from a batch to batch by quantitating the nicotine content in a Perkin Elmer 200 gas chromatograph (Perkin Elmer Corporation, Norwalk, CT),[7] equipped with a hydrogen flame ionization detector and a fused silica capillary column, 15 × 0.32 mm inside diameter. The instruments were operated in a split mode and the injector and detector temperatures were maintained at 225 °C and 227 °C, respectively. The column temperature was initially programmed at 80 °C for 1 min, and changed at 30 °C/min up to 285 °C and held for 5 min. Helium was used as the carrier gas at 25 psi.[7,8]

Primary Culture of Normal Human Oral Keratinocytes (NHOK) and STE Treatment

Retromolar tissues, obtained from the oral cavity of healthy female and male volunteers [Creighton University IRB approval #M-1117] were washed with PBS and calcium magnesium free Hank's balanced salt solution. The tissues were digested with collagenase and dispase for 60 min at 37 °C, and the cells were grown and maintained in KGM media (Clonetics, La Jolla, CA) as described by Bagchi *et al.*[7,8] and plated on 60 mm petri dishes coated with fibronectins and laminin (Biocoat Cell Environments, Bedford, MA). Confluent normal keratinocytes were successfully obtained after 7–10 days as observed under microscope. Cells were used when they reached 50–60% confluency in all studies. These NHOK cells were preincubated for 4 h with vitamins C, E and grape seed proanthocyanidin extract (GSPE) prior to STE incubations (0–300 μg/ml).

Determination of Cell Viability by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyl tetrazolium Bromide) Assay and Trypan Blue Exclusion Technique

The cleavage of the tetrazolium salt MTT into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase was used for assaying cell survival and proliferation.[32,33] This assay is extensively used for measuring cell survival and proliferation. Percent specific cytotoxicity is calculated as follows:

$$\% \text{ Cell viability} = [\text{O.D. of effectors} + \text{targets}] - [\text{O.D. of effectors}]/[\text{O.D. of targets}]$$

$$\text{O.D.} = \text{optical density}$$

There is a direct proportionality between the formazan produced (expressed as O.D.) and the number of viable cells. However, it depends on the cell type, cellular metabolism and incubation time with MTT.[34] Briefly the method is based on the capacity of mitochondrial enzymes of viable cells to reduce the yellow soluble salt MTT to a purple blue insoluble formazan precipitate which, is quantified spectrophotometrically after dissolving in an organic solvent. Earlier studies demonstrated a linear relationship between formazan formation and the number of viable cells. This colorimetric method has several advantages over radioactive methods, requiring fewer effector cells and is very sensitive for assessing cellular viability. A 5 mg/ml stock solution of MTT was prepared in sterile phosphate buffered saline (PBS) and filtered through a 0.22 μm filter to sterilize and remove any small amount of insoluble residue or blue formazan product. The stock solution was stored in the dark at 4 °C for no more than 3–4 days. Respiratory effects of selected antioxidants on STE (0–300 μg/ml) exposed NHOK cells (24 h) were assessed by determining the ability of the cells to reduce the tetrazolium dye MTT based on the activation of the enzyme succinate dehydrogenase as described by Mossman[32,33] and by us.[31] Cells were plated at a density of 1.0×10^6 cells. After treatment, 100 μl of dye (4 mg/ml in Dulbecco's phosphate buffered saline) was added to each well and incubated for 2 h at 37 °C. The optical density of each well was read at 570 nm.

Both concentration- and time-dependent effects of STE on cellular viability of NHOK were also assessed by the Trypan blue exclusion technique as described earlier by us.[35] The control and STE treated NHOK cells were trypsinized, centrifuged and resuspended in culture medium. Each cell suspension (0.10 ml) was mixed with 0.1 ml Trypan blue solution (0.2% PBS). Live and dead cells were counted using a hemocytometer and blue stained cells were counted as nonviable. The percentage viability was calculated based on the percentage of unstained cells.

Laser Scanning Confocal Microscopy

Approximately 50% confluent NHOK cells grown in six well plates were treated with different concentrations of STE (0–200 μg/ml) and

incubated at 37 °C in an atmosphere of 95% air and 5% CO_2 for 24 h. The overall intracellular oxidized states of cells were measured using a Meridian ACS 570 laser scanning confocal microscope (Okemor, MI) using a flourescent dye 2,7-dichlorofluoroscein diacetate (DCFH) as the fluorescent probe (Molecular Probes, Inc., Eugene, OR). For assays, media are replaced with Hank's solution containing 5 mM DCFH. After 5 min of incubation at room temperature, the fluorescence intensity for each point was measured according to the method described earlier.[7] To assess protective ability of vitamins C and E, and GSPE, these cells were also pre-incubated for 4 h with either vitamins C and E, a combination of vitamins C plus E, or GSPE prior to STE treatment, and relative fluorescence intensities were measured. The excitation wavelength of 513 nm was used. Relative fluorescence intensity was calculated using untreated control cells as standard. Approximately 20 cells were used for each individual group.[7]

DNA Fragmentation

DNA fragmentation in samples is expressed as percent of total DNA appearing in the supernatant fractions. Effects of STE treatment on keratinocytes are reported as percent of fragmentation relative to untreated cells.[7,8] Treated and untreated cells were lysed and homogenized in lysis buffer (5 mM Tris HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000 g for 20 minutes to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 M perchloric acid and 5.5 M perchloric acid was added to the supernatant samples to reach a concentration of 0.5 M. Samples were heated at 90 °C for 15 min and centrifuged at 1,500 g for 10 min to remove protein. Resulting supernatant fractions were reacted with diphenylamine reagent (1.5 g diphenylamine + 1 ml conc. H_2SO_4 + 100 ml glacial acetic acid + 0.50 ml 2%), and allowed to stand at room temperature for 16–20 h.[7] The absorbances of acetaldehyde were measured spectrophotometrically at 600 nm. The data are expressed as the micrograms of fragmented DNA in the supernatant fraction divided by the total DNA recovered (fragmented DNA in the supernatant plus intact DNA in the pellet).[7,8]

RT–PCR Analysis for the Expression of *Bcl-2*, *p53* and *c-myc* Genes in STE-treated Activated NHOK Cells

To determine *p53* gene mutations in NHOK cells, reverse transcriptase polymerase chain reaction (RT–PCR) was carried out in the DNA thermocycler (Perkin Elmer Cetus Corporation, Norwalk, CT). NHOK cells were treated with (0–200 μg/ml) of STE. Keratinocytes were also preincubated with GSPE (0.50 μg/ml and 100 μg/ml) before treatment with STE. RNA was isolated from treated and untreated cells using a commercially available reagent, Tri reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA degradation was checked semiquantitatively by subjecting RNA samples to non-denaturing agarose gel electrophoresis. The intact appearance of 28s and 18s ribosomal bands was considered an indication that total RNA degradation was minimal.[36] A reverse transcription polymerase chain reaction (RT–PCR) technique was used to determine the expression of *p53*, *Bcl-2* and *c-myc* genes in NHOK cells treated with STE and GSPE as described previously.[36] Briefly, 1 μg of RNA was reverse transcribed using 150 mg of random primers following standard procedures with the exception of the addition of DMSO (1% final concentration) and incubated at 44 °C for 1 h. PCR was then performed on 10 μl of total RNA as a template with 0.05 OD_{260} units of random primer for reverse transcriptase. The temperature conditions were as follows: 5 min at 95 °C followed by 2 min at 62 °C followed by 25 cycles of 20 sec at 72 °C, 45 sec at 94 °C and 20 sec at 62 °C. PCR products

were visualized by electrophoresis in ethidium bromide stained, 2% agarose gel. As a housekeeping gene control, β-tubulin gene expression was measured using the same procedure and appropriate primers 5′(AAGAAATCCAAGCTGGAGTTC)3′ and 5′(GTTGGTCTGGAATTCTGTGAG)3′. All primer sets were designed to span introns to eliminate any potential signal from contaminating DNA in the RNA preparation.[36] The differences in the levels of expression of *p53*, *Bcl-2* and *c-myc* in STE treated with and without GSPE was determined using densitometric analysis of the PCR product bands. The results of the PCR product was digitized using BioRad's Gel Analysis System. The density of each band was then determined using Image Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software and compared to the control values.[36]

Statistical Analyses

Statistical significance of the difference between the results was analyzed by using variance (ANOVA) with Scheff's method as the post hoc test. Differences were considered significant when the p-value was $< .05$. Each value is the mean ± SD from 4 to 6 experiments.

RESULTS

STE-induced Modulation of Intracellular Oxidized States by Confocal Microscopy and Protection by Antioxidants

Figure 1 demonstrates the protective abilities of vitamins C and E, singly and in combination, and GSPE against STE-induced modulation of intracellular oxidized states in NHOK cells as determined by laser scanning confocal microscopy using 2,7-DCFH as the fluorescent probe. Previous studies in our laboratory have demonstrated concentration-dependent changes in fluorescent intensity following incubation of the NHOK cells with STE.[7] When cells were incubated with increasing concentrations of STE, the color and fluorescent intensity of the nuclei changed, possibly because of a change in the redox states of the cells and accumulation of the dye in the nuclear fractions. The dye is incorporated into the cells and is converted to a fluorescent metabolite by oxidation.[7,37] The relative fluorescence intensities of NHOK cells treated with STE (200 μg/ml) and/or antioxidants are presented in Table I. Fluorescence intensity increased 3.7-fold following incubation of the NHOK cells with 200 μg/ml of STE for 24 h, as compared to STE-untreated cells. Thus, STE modulated intracellular oxidized states in NHOK cells. No significant changes in fluorescent intensity were observed following incubation with vitamins C, E and GSPE alone (Table I). Preincubation of the NHOK cells with vitamin C (75 μM), vitamin E (75 μM), a combination of vitamins C plus E (75 μM each), and GSPE (100 μg/ml) for 4 h decreased STE-induced increase in fluorescence intensity by 11%, 26%, 28% and 50%, respectively, as compared to STE-treated NHOK cells (Table I).

Measurement of Cellular Viability on STE-treated NHOK Cells by MTT Assay and Trypan Blue Exclusion Technique

Concentration-dependent effects of STE were studied on the reduction of the dye MTT in NHOK cells (Table II). Incubation of the NHOK cells with 100, 200 and 300 μg/ml of STE for 24 h at 37 °C resulted in 15%, 21% and 46% decreases in succinate dehydrogenase activities, respectively, as compared to untreated cells (Table II). To assess the protective effects of antioxidants, NHOK cells were preincubated with vitamin C (75 μM), vitamin E (75 μM), a combination of vitamins C plus E (75 μM each), and GSPE (100 μg/ml) for 4 h prior to STE treatment. Approximately 10%, 37%, 57% and 78% protection were observed following preincubation of the NHOK cells with vitamin C (75 μM), vitamin E (75 μM), a combination of vitamins C plus E

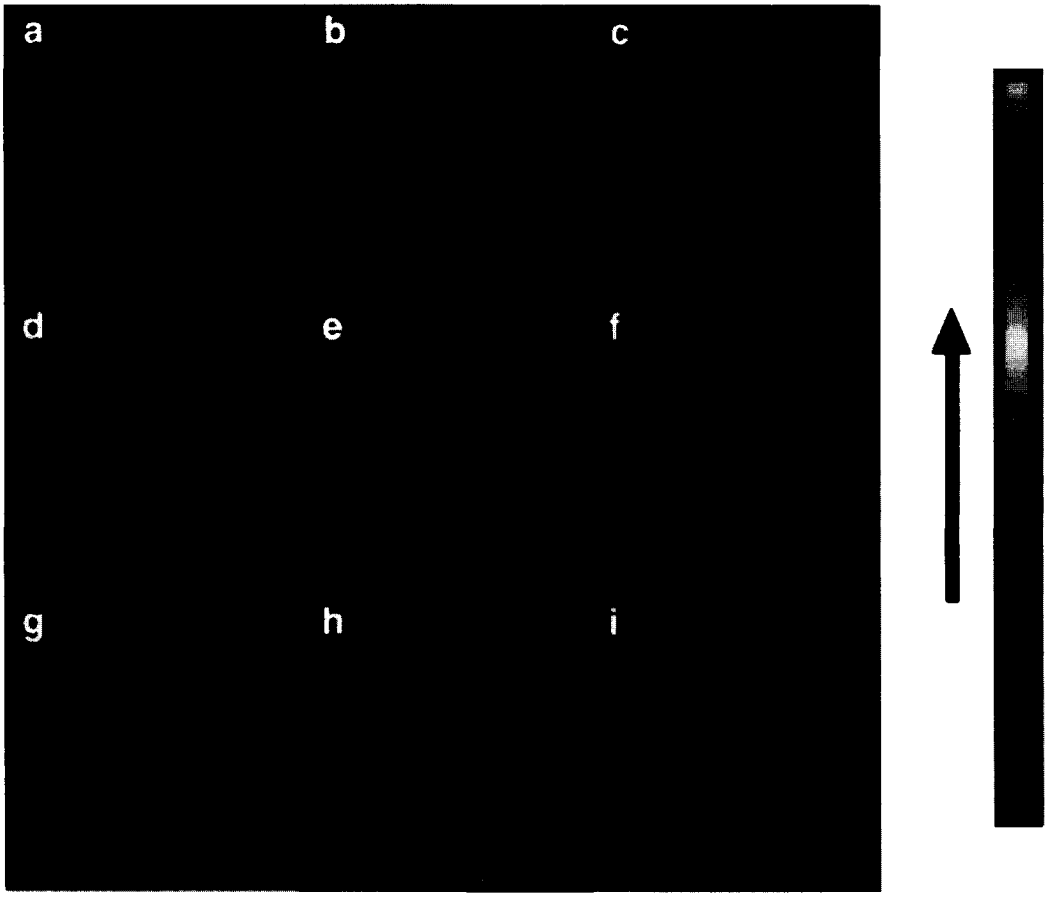
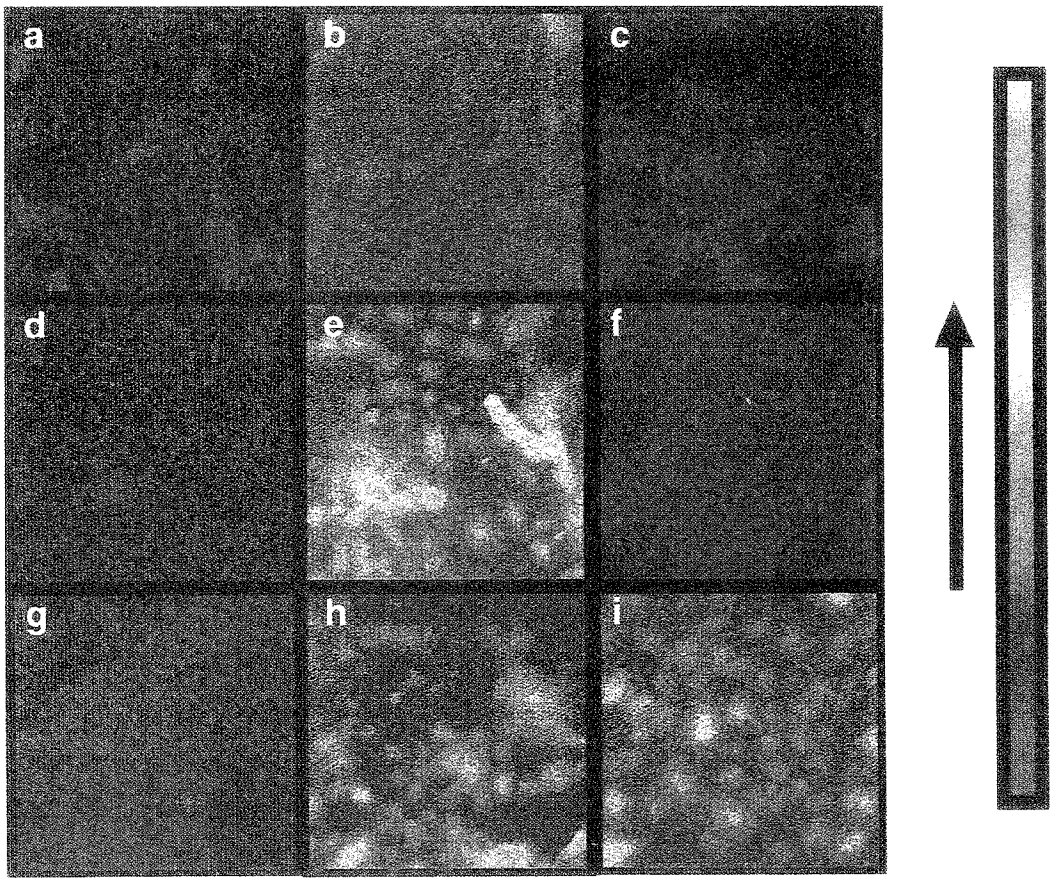


FIGURE 1 Changes in intracellular redox states of human oral keratinocytes (NHOK) following treatment with STE and/or antioxidants. (a) Control; (b) GSPE (100 μg/ml); (c) Vitamin C (75 μM); (d) Vitamin E (75 μM); (e) STE (200 μg/ml); (f) STE (200 μg/ml) + GSPE (100 μg/ml); (g) STE (200 μg/ml) + Vitamin C (75 μM) + Vitamin E (75 μM); (h) STE (200 μg/ml) + Vitamin C (75 μM); (i) STE (200 μg/ml) + Vitamin E (75 μM). (See Color plate I at the end of this issue.)

TABLE I Changes in intracellular oxidized states of human oral keratinocytes (NHOK) following treatment with STE for 24 h

Treatment	Fluorescence intensity	Percentage control
Control group	112 ± 30^{a}	100
GSPE (100 μg/ml)	146 ± 12^{b}	130
Vitamin C (75 μM)	$130 \pm 32^{a,b}$	116
Vitamin E (75 μM)	125 ± 20^{a}	112
STE (200 μg/ml)	412 ± 76^{c}	368
STE (200 μg/ml) + vitamin C (75 μM)	364 ± 22^{c}	325
STE (200 μg/ml) + vitamin E (75 μM)	302 ± 30^{d}	270
STE (200 μg/ml) + vitamin C (75 μM) + vitamin E (75 μM)	296 ± 26^{d}	264
STE (200 μg/ml) + GSPE (100 μg/ml)	208 ± 51^{e}	186

NHOK cells were incubated with STE for 24 h in the presence and absence of vitamins C, vitamin E and a grape seed proanthocyanidin extract (GSPE) at the indicated concentration. Relative fluorescence intensity of each cell was calculated relative to untreated control cells. Data are expressed as the mean values of 20 cells. SD values with non-identical superscripts are significantly different ($p < .05$).



Color Plate I (see page 7, figure 1) Changes in intracellular redox states of human oral keratinocytes (NHOK) following treatment with STE and/or antioxidants. (a) Control; (b) GSPE (100 μg/ml); (c) Vitamin C (75 μM); (d) Vitamin E (75 μM); (e) STE (200 μg/ml); (f) STE (200 μg/ml) + GSPE (100 μg/ml); (g) STE (200 μg/ml) + Vitamin C (75 μM) + Vitamin E (75 μM); (h) STE (200 μg/ml) + Vitamin C (75 μM); (i) STE (200 μg/ml) + Vitamin E (75 μM).

TABLE II Reduction of tetrazolium dye MTT and DNA fragmentation in normal human oral keratinocytes (NHOK) cells following treatment with STE and preincubation with antioxidants vitamins C and vitamins E and grape seed proanthocyanidin extract (GSPE), singly and in combination

Treatment	Cell viability (percentage control)	DNA fragmentation (percentage control)
Control group	2.30 ± 0.56^{a} (100)	5.82 ± 0.42^{a} (100)
STE (100 μg/ml)	1.97 ± 0.35^{b} (85)	8.95 ± 0.64^{b} (154)
STE (200 μg/ml)	1.81 ± 0.44^{b} (78)	9.55 ± 0.32^{b} (164)
STE (300 μg/ml)	1.26 ± 0.26^{c} (54)	16.67 ± 0.93^{c} (286)
STE (200 μg/ml) + vitamin C (75 μM)	1.86 ± 0.38^{b} (81)	8.69 ± 0.49^{b} (149)
STE (200 μg/ml) + vitamin E (75 μM)	1.99 ± 0.35^{b} (86)	7.58 ± 0.64^{b} (130)
STE (200 μg/ml) + vitamin C (75 μM) + vitamin E (75 μM)	$2.09 \pm 0.44^{a,b}$ (90)	7.14 ± 0.56^{b} (123)
STE (200 μg/ml) + GSPE (100 μg/ml)	2.19 ± 0.14^{a} (95)	6.73 ± 0.38^{a} (116)

Cultured human oral keratinocytes (1×10^{6}) cells treated with (0–200 μg/ml) of STE for 24 h. The activity of the succinate dehydrogenase was assessed based on the reduction of the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). DNA fragmentation was performed as described in the Materials and Methods section. Data are expressed as the mean values of 4–6 individual incubations. Values with nonidentical superscripts are significantly different ($p < .05$).

(75 μM each), and GSPE (100 μg/ml), respectively, as compared to STE-treated NHOK cells.

Both concentration- and time-dependent effects of STE on cellular viability of NHOK were assessed by Trypan blue exclusion technique (Figure 2). A concentration-dependent effect on cell death was observed. Most cells lose their adhesiveness by 24 h when incubated with high concentrations of STE. However, 55–70% of the cells were still viable (Figure 2).

DNA Fragmentation

DNA fragmentation is an index of oxidative DNA and cellular damage. The cytotoxicity of STE was examined by determining DNA frag-

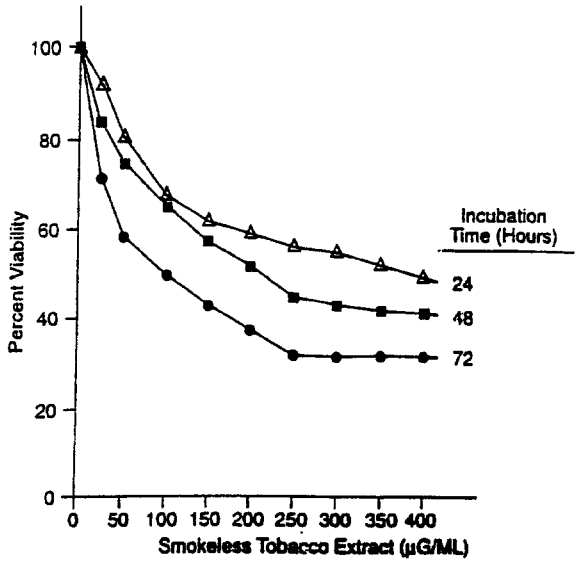


FIGURE 2 Effects of various concentrations of STE (0–400 μg/ml) on the percent viability of cultured human oral keratinocytes. Viability assays were performed using Trypan blue exclusion method after 24, 48 and 72 h of incubation. Each value is the mean of 4–6 individual incubation at each time point.

mentation 24 h after treatment of keratinocytes in culture with 100, 200, and 300 μg/ml of STE. Table II demonstrates the concentration-dependent effects of STE on DNA fragmentation in NHOK cells. STE induced significant DNA damage. Approximately 1.5-, 1.6-, and 2.9-fold increases in DNA fragmentation were observed following exposure of the NHOK cells with 100, 200, and 300 μg/ml of STE, respectively, as compared to the control untreated cells. Pretreatment of the NHOK cells with vitamin C (75 μM), vitamin E (75 μM), a combination of vitamins C plus E (75 μM) each and GSPE (100 μg/ml) for 4 h decreased STE (200 μg/ml) induced DNA fragmentation by 9%, 21%, 25% and 29% respectively, as compared to the STE-treated cells.

RT–PCR Analysis for the Expression of *Bcl-2*, *p53* and *c-myc* Genes in STE-treated NHOK Cells and Modulation by GSPE

Figure 3 shows the expression levels of cell cycle and apoptosis related genes, *Bcl-2* and *p53*, in NHOK cells treated with STE (200 μg/ml) and/or GSPE (0 μg/ml, 50 μg/ml and 100 μg/ml) using RT–PCR techniques. The RT–PCR analysis of STE-treated NHOK cells showed a remarkable decrease in *Bcl-2* antiapoptotic gene expression following treatment with STE (200 μg/ml)

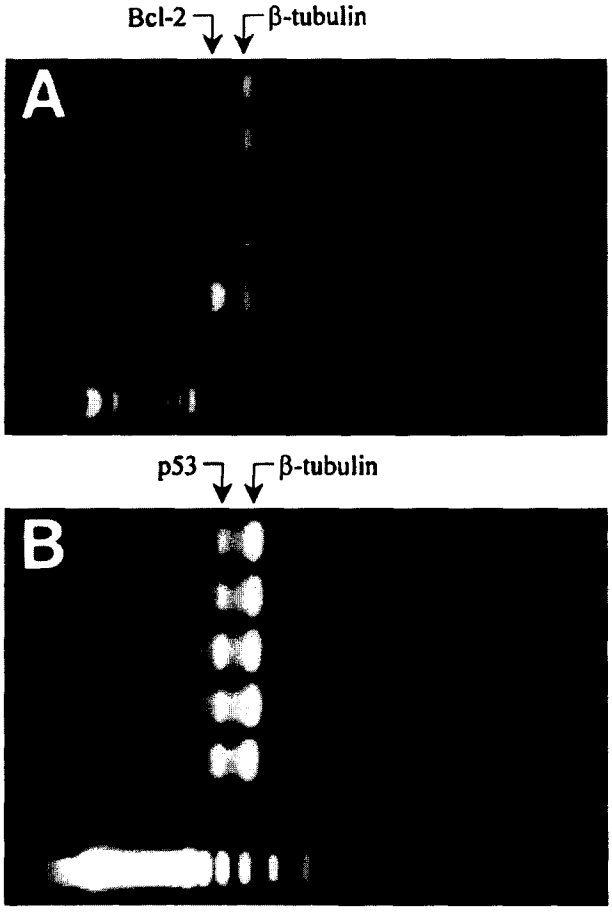


FIGURE 3 Modulation of *Bcl-2*, *p53* mRNA expression in normal human oral keratinocytes (NHOK) following treatment with STE and/or GSPE [GSPE-1: 50 μg/ml and GSPE-2: 100 μg/ml]. (A) *Bcl-2* mRNA expression; (B) *p53* mRNA expression.

for 24 h. Following preincubation of NHOK cells with GSPE (50 μg/ml and 100 μg/ml) for 4 h prior to STE treatment showed an increase in *Bcl-2* mRNA expression (Figure 3A) indicating GSPE ameliorates the toxic effects of STE. A concentration-dependent increase in *Bcl-2* mRNA expression was observed following preincubation with GSPE. At 100 μg/ml concentration of GSPE preincubation completely reversed the cytotoxic effect of GSPE as demonstrated by near complete restoration of *Bcl-2* mRNA expression in these cells. Thus, NHOK cells preincubated with GSPE for 4 h followed by STE treatment showed an increase in *Bcl-2* mRNA expression, which corresponds to our previous apoptosis study.[8]

The RT–PCR analysis of the STE-treated NHOK cells for the expression of proapoptotic gene *p53* revealed that there was an increase/alteration of 2.0-fold in *p53* mRNA expression (Figure 3B) following STE (200 μg/ml) treatment. The 2-fold increase of the band was quantified using Image Pro Plus (Media Cybergenetics, Inc., Springfield, MD), as described in detail in the Method section. The expression of *p53* reduces dramatically beyond 200 μg/ml STE, confirming increased apoptotic cell death with higher concentration of STE.[8] Thus, expression of *p53* gene in NHOK cells is affected following incubation with STE. These alterations were not seen in the NHOK cells that were treated with GSPE alone (data not shown). Treatment of keratinocytes with STE and GSPE (50 μg/ml and 100 μg/ml) decreased the *p53* expression levels to that of control untreated cells (Figure 3B). The toxic effect of STE on *p53* gene expression was ameliorated following GSPE (50 μg/ml and 100 μg/ml) preincubation. No concentration-dependent effect of GSPE was observed. We have also assessed the protective effects of vitamins C and E preincubation on the expression of these genes in STE-treated NHOK cells using RT–PCR. Since, there was no significant differences, for the sake of brevity the data are not included in the figure.

Furthermore, we determined the mRNA expression levels of *c-myc* gene, a cell cycle growth and differentiation related gene in these keratinocytes, using RT–PCR techniques. The expression levels of *c-myc* were not significantly affected following treatment with STE with or without GSPE (data not shown) indicating that *c-myc* is not involved in STE associated cytotoxicity in oral keratinocytes.

DISCUSSION

Previous studies have demonstrated dose- and time-dependent increases in the production of free radicals, DNA damage, lipid peroxidation and apoptotic cell death of human oral keratinocytes after STE treatment.[8] The results of our present study clearly demonstrate that incubation of human oral keratinocytes with STE produces significant increases in intracellular oxidized states of the cells, which can be ameliorated with the use of antioxidants including vitamins C and E, and GSPE. Furthermore, STE can significantly modulate *Bcl-2* and *p53* mRNA expression in NHOK cells and these cytotoxic effects can be reversed by GSPE. The mechanism associated with the cytotoxicity and carcinogenicity of smokeless tobacco are not clear.

In the present study, laser scanning confocal microscopic technique was used to assess the modulation of intracellular oxidized states as an index of oxidative damage to cultured cells at a single cell level. The overall intracellular oxidized states of these NHOK cells were measured,[7,37] and a dramatic increase in fluorescence intensity was observed following incubation with STE for 24 h. Pretreatment of the cells with vitamins C and E, singly and in combination, and GSPE decreased STE-induced fluorescence intensities significantly (Figure 1 and Table I). These results indicate that antioxidants used in this study can significantly protect against STE-induced oxidative damage in these cells, while GSPE provided signific-

antly better protection than vitamins C and E, singly and in combination.[8] DNA fragmentation was assessed to determine STE-induced oxidative DNA damage to the NHOK cells, and the protective abilities of these antioxidants were determined. All the antioxidants significantly protected DNA fragmentation in the cultured NHOK cells, while GSPE provided the best protection as compared to vitamins C and E, singly and in combination (Table II). The cleavage of the tetrazolium salt MTT into a blue colored formazan by the mitochondrial enzyme succinate dehydrogenase is potentially very useful and sensitive for assaying cell survival and proliferation. Succinate dehydrogenase is considered as a marker of the mitochondrial electron transport chain.[32,33] The cell viability measured by MTT assay is a very sensitive colorimetric assay and only viable cells with intact mitochondria can reduce the tetrazolium dye, MTT. The conversion takes place only in living cells and the amount of formazan produced is proportional to the number of viable cells present. Current results indicate that antioxidants and GSPE preincubation significantly increases the cellular viability of STE treated NHOK cells (Table II and Figure 2).

Earlier studies in our laboratory have demonstrated concentration-dependent increases in the release of lactate dehydrogenase (LDH) in human oral cells following incubation with STE. Approximately 2.3-fold increases in LDH leakage was observed following 24 h incubation with 250 μg STE/ml.[38] Similarly, Trypan blue exclusion technique demonstrated concentration- and time-dependent effects of STE on cellular viability in NHOK cells, the data is clearly shown in Figure 2. Most cells lost their adhesiveness by 24 h following incubation with high concentrations of STE, however, 55–70% of the human oral cells were still viable.

The tumor suppressor protein *p53* is a transcription factor regulating cell cycle progression, cell survival and DNA repair in cells exposed to environmental stimuli.[39] In the present study, we observed modulation of *p53* mRNA expression in NHOK cells with STE treatment. This modulation of *p53* expression by STE in NHOK cells was reversible when cells were preincubated with GSPE for 4 h. Antioxidants have been demonstrated to increase *Bcl-2* expression *in vitro*. However, *Bcl-2* does not inhibit formation of free radicals but blocks their damaging effects. *Bcl-2* further acts as a free radical scavenger.[10] In our present study, we observed increases in *Bcl-2* mRNA expression when cells were preincubated using GSPE before STE treatment (Figure 3A). Protective effects of GSPE were also observed using laser scanning confocal microscopy technique (Figure 1). Present results in conjunction with our previous study suggest an increase in *Bcl-2* mRNA expression may interfere with cellular signaling events and protect NHOK cells from undergoing apoptosis.

We have previously demonstrated that GSPE pre-exposure increases expression of a cell death inhibitory gene Bcl-X_L in the hepatic tissue and prevented acetaminophen-induced lethality, DNA fragmentation and apoptotic cell death in the liver tissue. GSPE pre-exposure prior to acetaminophen treatment completely blocked Ca^{+2}/Mg^{+2}-dependent endonuclease mediated ladder like fragmentation of genomic DNA. Acetaminophen causes severe liver damage in animals and humans. Hepatocellular leakage of an enzyme alanine aminotransferase (ALT), primarily reflects the degree of liver injury and has been commonly used as a marker for hepatoxicity. GSPE pre-exposure prior to acetaminophen administration (500 mg/kg i.p.) significantly protected hepatotoxicity as demonstrated by ALT activity [control 45 ± 2 U/L, acetaminophen $29{,}813 \pm 463$ U/L, GSPE 27 ± 2 U/L; GSPE + Acetaminophen 2792 ± 78 U /L].[27]

Chemotherapeutic agents are known to induce apoptosis in the target cells. In an earlier study in our laboratory we have demonstrated that preincubation of Chang liver cells with GSPE decreased the number of apoptotic cell population induced by chemotherapeutic agents

Idarubicin (Ida) and 4-hydroxyperoxycyclophosphamide (4-HC). GSPE pretreatment decreased the number of apoptotic cell population induced by these chemotherapeutic drugs and increased the expression of apoptotic regulatory gene *Bcl-2* in GSPE treated cells as measured by RT–PCR. The above study showed one of the cellular mechanisms of chemoprevention of GSPE appears to be via upregulation of *Bcl-2* gene.[40] Similarly, like the present study, there was a decrease in *p53* expression when cells were preincubated with GSPE. There was an increased expression of Bcl-2 in the cells treated with GSPE. The above results demonstrated that GSPE can ameliorate the toxic effects associated with chemoptheraputic drugs used in the treatment of cancer by inhibiting apoptosis.

In another recent *in vitro study*, GSPE at low concentrations (1–5 μg/ml) downregulated TNFα-induced VCAM-1 expression in primary human umbilical vein endothelial cells (HUVEC).[41] Such regulation of inducible VCAM-1 by GSPE was also observed at the mRNA expression level. A cell–cell co-culture assay was performed to verify whether the inhibitory effect of GSPE on the expression of VCAM-1 was also effective in downregulating actual endothelial cell/leukocyte interaction. GSPE treatment significantly decreased – induced adherence of T cells to HUVEC, and this may be another cytoprotective mechanism attributed by GSPE in addition to its potent antioxidant activity.

The protooncogene *c-myc* has been shown to be important in apoptosis regulation and to be amplified and overexpressed in human and rodent liver neoplasia.[12] In our present study we observe that expression of *c-myc* as measured by RT–PCR, did not alter significantly, indicating that *c-myc* may not be involved in STE associated toxicity in NHOK cells (data not shown).

In summary, the results of this study strongly suggests that oxidative stress and reactive oxygen species play a significant role in the cytotoxic effects of smokeless tobacco on cultured normal human oral keratinocyte cells and the modulation of *Bcl-2* and *p53* genes by STE. Furthermore, our results clearly demonstrate production of ROS oxidative DNA damage and enhanced intracellular oxidized states in NHOK cells in response to STE treatment. Alterations in the expression of *p53* and *Bcl-2* in the presence of STE may constitute the defining stages of carcinogenesis in the development of oral cancer. Furthermore, the chemoprotection by GSPE against STE-induced cellular injury may involve modulation of cellular regulatory genes as *Bcl-2* and *p53*.

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