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Original Contribution

# Oxidative damage in human gingival fibroblasts exposed to cigarette smoke

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ABSTRACT

Cigarette smoke, a complex mixture of over 7000 chemicals, contains many components capable of eliciting oxidative stress, which may induce smoking-related disorders, including oral cavity diseases. In this study, we investigated the effects of whole (mainstream) cigarette smoke on human gingival fibroblasts (HGFs). Cells were exposed to various puffs (0.5–12) of whole cigarette smoke and oxidative stress was assessed by 2′,7′-dichlorofluorescein fluorescence. The extent of protein carbonylation was determined by use of 2,4-dinitrophenylhydrazine with both immunocytochemical and Western immunoblotting assays. Cigarette smoke-induced protein carbonylation exhibited a puff-dependent increase. The main carbonylated proteins were identified by means of two-dimensional electrophoresis and MALDI–TOF mass spectrometry (redox proteomics). We demonstrated that exposure of HGFs to cigarette smoke decreased cellular protein thiols and rapidly depleted intracellular glutathione (GSH), with a minimal increase in the intracellular levels of glutathione disulfide and S-glutathionylated proteins, as well as total glutathione levels. Mass spectrometric analyses showed that total GSH consumption is due to the export by the cells of GSH–acrolein and GSH–crotonaldehyde adducts. GSH depletion could be a mechanism for cigarette smoke-induced cytotoxicity and could be correlated with the reduced reparative and regenerative activity of gingival and periodontal tissues previously reported in smokers.



Despite the large body of epidemiological evidence that exists today establishing a strong correlation between smoking and disease, such as lung respiratory [1] and cardiovascular disorders [2], various types of cancer [3], and oral cavity disorders [4,5], the molecular mechanisms of smoke-related disorders and how smoking initiates and/or enhances diseases often remain unclear.

There are many harmful components in both mainstream (i.e., the smoke inhaled by active smokers, emitted at the mouthpiece of a cigarette) and sidestream (i.e., the smoke emanating from the cigarette between puffs; it is the main component (85%) of second-hand, or environmental, tobacco smoke, also known as passive, or involuntary, smoking) cigarette smoke that can damage cellular molecules, eventually leading to cell death. Two major phases were identified in whole cigarette smoke, a complex mixture of over 7000 chemical compounds [6]: a tar phase and a gas phase. Both phases are rich in reactive oxygen species (ROS) and reactive nitrogen species [7,8]. It was estimated that a single cigarette puff contains approximately $10^{14}$ free radicals in the tar phase and $10^{15}$ radicals in the gas phase [9]. In agreement with the concept that oxidative stress, an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [10], is capable of causing tissue damage and disease states [10],

*Abbreviations:* biotin–HPDP, *N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DNPH, 2,4-dinitrophenylhydrazine; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH–ACR, GSH adduct with acrolein; GSH–CRO, GSH adduct with crotonaldehyde; HGF, human gingival fibroblasts; HSA, human serum albumin; mBrB, monobromobimane; NEM, *N*-ethylmaleimide; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; streptavidin–HRP, streptavidin–horseradish peroxidase conjugate; TCA, trichloroacetic acid; TPM, total particulate matter.

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oxidative stress seems to play a central role in cigarette smoke-mediated diseases [e.g., 11,12]. Cigarette smoke can also induce the production of endogenous oxidants and reactive species in an inflammatory response to smoke-induced irritation [13].

Additional harmful constituents of tobacco smoke are highly reactive, volatile aldehydes, including α,β-unsaturated chemicals such as acrolein (2,3-propenal) and crotonaldehyde (2-butenal) [7]. Acrolein is present in very high concentrations in the vapor phase of all cigarettes, its levels varying up to 10-fold between high-tar and ultra-low-tar cigarette smoke extracts. Several reports have estimated that between 100 and 600 μg of acrolein is generated per cigarette (50–70 ppm) and that acrolein constitutes 50–60% of total vapor-phase electrophiles [14]. Cigarette smoke extracts from commercial cigarettes containing the average tar content of 15 mg yielded 394 ± 29 μmol/L acrolein [15]. Extracts of different ultralights and light commercial cigarettes all yielded between 311 and 370 μmol/L acrolein and corresponding levels of other aldehydes, indicating a lack of correlation between the purported lightness of the tobacco and the level of acrolein [15]. Smoking one cigarette per cubic meter of air of room space in 10–13 min (10 puffs) generates acrolein levels up to 0.84 mg/m$^3$ [16]. The respiratory tract is commonly exposed to a range of α,β-unsaturated aldehydes from cigarette smoke exposure. It was estimated that, during cigarette smoking, acrolein concentrations at the airway surface may be as high as 80 μM [17]. α,β-Unsaturated aldehydes are present in saliva and airway secretions in low-micromolar concentrations in healthy subjects and are elevated up to 10-fold in heavy smokers [18,19]. The common feature of these α,β-unsaturated aldehydes is the presence of an unsaturated carbonyl group that confers them the capacity to form stable covalent adducts with nucleophilic amino acids (i.e., Cys, His, and Lys), often resulting in protein carbonylation, as well as with the thiol group in glutathione (GSH) [20–23].

Oral cavity tissues are the first exposed to mainstream cigarette smoke and their responses to harmful stimuli are critical in maintaining periodontal homeostasis. Cigarette smoke is a known modulator of various oral cavity pathologies, but the mechanism(s) by which cigarette smoke constituents affect gingival fibroblast integrity needs to be elucidated. In this study, we exposed cultured human gingival fibroblasts (HGFs) to increasing puffs of whole (mainstream) cigarette smoke and assessed changes in protein carbonylation and intracellular total glutathione as well as formation of both intracellular and extracellular GSH–α,β-unsaturated aldehyde adducts.

## Materials and methods

### *Materials*

An HPLC Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) was purchased from Agilent Technologies Italia S.p.A. (Cernusco sul Naviglio, Milan, Italy). HPLC-grade and analytical-grade organic solvents were purchased from Sigma–Aldrich (Milan, Italy) or from BDH (Poole, England). HPLC-grade water was prepared with a Milli-Q water purification system. EFBA (eptafluorobutyric acid), L-glutathione, and protease inhibitor cocktail (leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride) were purchased from Sigma–Aldrich. Acrolein and crotonaldehyde were purchased from Fluka (Buchs, Switzerland). H-Tyr-His-OH (TH) was a generous gift from Flamma S.p.A. (Chignolo d'Isola, Bergamo, Italy). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA). EZ-Link biotin–HPDP (*N*-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide) was obtained from Thermo Scientific (Rockford, IL, USA). Anti-dinitrophenyl–KLH (anti-DNP) antibodies, rabbit IgG fraction, goat anti-rabbit IgG, horseradish peroxidase conjugate, and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA). Monoclonal anti-GSH antibody was obtained from Virogen (Watertown, MA, USA). Goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Precision Plus Protein All Blue standards, ranging from 10 to 250 kDa, were obtained from Bio-Rad Laboratories s.r.l. (Segrate, Italy). Sheep anti-mouse IgG, horseradish peroxidase conjugate, Amersham ECL Plus Western blotting detection reagents, and streptavidin–horseradish peroxidase conjugate (streptavidin–HRP) were purchased from GE Healthcare Europe GmbH (Milan, Italy). Research-grade cigarettes (3R4F) were purchased from the College of Agriculture, Kentucky Tobacco Research and Development Center, University of Kentucky (Lexington, KY, USA). According to the analysis of 3R4F reference cigarettes preliminarily performed by the College of Agriculture Reference Cigarette Program, University of Kentucky, and further confirmed in a recent study [24], the average values (mean ± SD) for standard parameters for the smoke of 3R4F reference cigarettes are total particulate matter 11.0 ± 0.33 mg/cigarette, tar (nicotine-free dry particulate matter) 9.4 ± 0.56 mg/cigarette, nicotine 0.73 ± 0.04 mg/cigarette, and carbon monoxide 12.0 ± 0.6 mg/cigarette.

### *Cell culture and cell viability assay*

HGFs were obtained by a gingival biopsy from a young healthy subject, who had clinically normal gums, with no signs of inflammation or hyperplasia. Health, drugs, alcohol abuse, and smoking histories were considered as exclusion criteria. The donor gave his informed consent to the biopsy, which was obtained from adherent gums under local anesthesia during minor oral surgical procedures. The gingival tissue fragment was extensively washed with sterile phosphate-buffered saline (PBS), plated in T-25 flasks, and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% $CO_2$. When fibroblasts grew out from the explant, they were trypsinized (0.25% trypsin–0.2% EDTA) for secondary cultures. Viability was assessed by the trypan blue exclusion method. Confluent HGFs were used at the fifth passage.

### *Exposure of human gingival fibroblasts to cigarette smoke*

HGFs were exposed to mainstream cigarette smoke using a homemade smoking device, consisting in a system connected to the tissue culture flask, to the cigarette, and to a 60-ml syringe. By regulating the system with a valve, it is possible to aspirate the cigarette smoke using the syringe and then to deliver the smoke into the flask. The use of the syringe allows one to aspirate and to deliver a precise and fixed quantity of smoke into the flask, quantified as number of "puffs." One single puff corresponds to a 60-ml volume of cigarette smoke aspirated into the syringe; 12 puffs correspond to one cigarette. Before the connection to the smoking device, the HGFs were washed with sterile PBS. The washing PBS was replaced with 1 ml of sterile PBS and each flask was exposed to 0.5, 2, 5, or 12 puffs for 1 min or left untreated (control). Each treatment was performed in triplicate. In preliminary experiments, cell-free T-25 flasks containing 1 ml of sterile PBS were exposed to 0.5, 2, 5, or 12 puffs for 1 min; the smoke-exposed buffer was then recovered from each flask and the smoke delivery system was validated by measuring the absorbance at a wavelength of 340 nm. The absorbance measured at $A_{340}$ showed insignificant variation between flasks exposed to the same number of cigarette puffs.

### *Total particulate matter mass in mainstream smoke*

Total particulate matter (TPM) was collected by passing the mainstream smoke of two cigarettes through a 19-mm glass fiber filter pad and that of four cigarettes through cellulose/acetate filter pads (one cigarette per filter pad). The smoking protocol was the same as that used to expose HGFs to cigarette smoke. To estimate TPM mass,