Bioactive polyphenol antioxidants protect oral fibroblasts from ROS-inducing agents

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ABSTRACT

Background: Oxidative damage to soft oral tissues may result from exposure to the chemicals or biochemicals found in teeth-whitening products, dental restorations, tobacco, and alcohol. Our working hypothesis is that oral tissues are susceptible to the toxic effects of stressors such as hydrogen peroxide (H_2O_2), ethanol (EtOH) and nicotine (Nic), which decrease cell viability/DNA synthesis and elevate reactive oxygen species (ROS). In this study, we investigated specific polyphenols and turmeric derivative antioxidants (AO) in combinations that counteracted the effects of these stressors on cultured oral fibroblast proliferation and ROS production.
Methods: Oral fibroblasts were exposed to stressors for 30 min and then treated with 10^{-5} M of bioactive AO mixtures [resveratrol, ferulic acid and tetrahydrocurcuminoid (RFT), phloretin, ferulic acid and resveratrol (PFR), phloretin, ferulic acid and tetrahydrocurcuminoid (PFT)] for 24 h. Cell viability and DNA synthesis were monitored using incorporated 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium (MTS) and 5-bromo-2-deoxyuridine (BrdU) assays, respectively. Total ROS was measured with dichlorodihydrofluorescein diacetate (H_2DCFDA).
Results: Incubation of oral fibroblasts in the stressors for 30 min resulted in a dose-dependent decrease of DNA synthesis and number of viable cells, and an increased total ROS activity. AO treatment counteracted the insults by restoring DNA synthesis levels and cell viability, and decreasing the total ROS activity.
Conclusion: The AO combinations of RFT, PFR and PFT protected the oral fibroblasts from the detrimental effects of H_2O_2, EtOH and Nic by decreasing total ROS and increasing cell viability and DNA synthesis.

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1. Introduction

Cellular reactive oxygen species (ROS) generation is counterbalanced by the action of AO enzymes and other redox molecules.[1] Because of potentially harmful effects, excessive ROS must be eliminated from the cells using a variety of AO defense mechanisms. Oxidative stressors affecting the inflammatory processes and wound-healing models have also been shown in numerous fibroblastic cell types obtained from

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human dermis, lung, cornea and heart.[2–7] On the other hand, ROS may mediate transforming growth factor-beta (TGF-β) signalling through the process of fibrogenesis. One study showed that TGF-β induced the expression of nicotinamide adenine dinucleotide phosphate-4 (NOX-4) and α-smooth muscle actin (α-SMA) in primary human cardiac fibroblasts.[8] Furthermore, the inhibition of NOX-4 in myofibroblastic foci/ mesenchymal cells from idiopathic pulmonary fibrosis (IPF) patient lungs blocked the TGF-β ROS production and myofibroblastic activation.[9,10] All these studies demonstrate that ROS is involved in the activation of some signalling molecules induced by cytokines and other AO combinations.

One of the most common stressors to the oral tissue is nicotine (Nic). Nic, a major component of cigarette smoke, promotes a high degree of ROS release, resulting in heightened oxidative damage of human gingival fibroblasts.[11] A previous study demonstrated that nicotine inhibits the attachment and growth of gingival and periodontal ligament fibroblasts.[12] In addition, it was observed that the effect of cigarette smoke extract (CSE) at high concentrations stimulates ROS extracellular ROS generation in neutrophils of periodontally healthy individuals. Consequently, CSE prevents ROS generation in neutrophils induced with F. nucleatum and IgG-opsonised S. aureus.[13]

Alcohol consumption can be considered a risk indicator for periodontitis.[14,15] Seven of the twelve studies on alcohol consumption and all of the four studies on alcohol dependence reported positive associations between alcohol intake and periodontitis.[14,15] An epidemiological data also showed that poor dental status may be associated with oral cancer risk due to an increased salivary acetaldehyde production from ethanol among heavy drinkers.[16] The effect of clinical concentrations of alcohol greater than 5% inhibited fibroblast proliferation and TGF-β-induced collagen synthesis which are important factors in mediating delayed healing in alcoholic patients.[17] Some alcohol-containing mouthrinses can affect the oral tissues due to a toxic compound, acetaldehyde that caused a dose- and time-dependent inhibition on cell adhesion and viability, leading to structural disruptions of cytoplasmic organelles in HGF.[18] In another in vitro study, EtOH inhibits fibroblast growth factor-mediated aortic smooth muscle cell proliferation by reducing phosphorlyation of downstream kinases and disrupting the cell cycle regulation.[14] Fibroblastic cells isolated from trauma patients caused impairments in re-epithelialisation, angiogenesis, and inflammation in wounds following acute EtOH exposure.[5]

The hydroxyl radical generated by H_2O_2 provokes biological reactions such as inflammation, carcinogenesis, ageing and mutations.[19,20] Most home tooth-whitening products contain 35% H_2O_2, but previous studies where 3% H_2O_2 were used increased injury to damaged tissue.[21,22] Moreover, in vitro studies revealed that low concentrations of H_2O_2 appeared to have cytotoxic effects on odontoblastic cell line (MDPC-23),[23] preosteoblastic MC3T3-E1 cells,[23] and HGFs.[24]

Each AO, such as resveratrol, ferulic acid, tetrahydrocurcuminoids, and phloretin, possesses anti-angiogenic, anti-inflammatory, antiviral, and/or anti-tumorigenic properties.[25–28] Our previous work showed that combinations of double and triple AOs had a greater effect than single AOs on

migration rates and Rac activation.[29] Therefore, we hypothesised that augmenting the natural AOs in the oral cavity will reduce ROS and improve oral health in patients with a higher risk of developing periodontal disease due to personal habits such as smoking, drinking alcohol, or using tooth-whitening substances. We utilised both HGF and HPDL because they are cells associated with the periodontium. Although they have specific area junctions, these two cell types do display functional differences in viability, proliferative rate and other biochemical processes.[30] In this study, we investigated the ability of specific AO combinations to counteract the effects of these stressors (H_2O_2, EtOH and Nic) on cultured oral fibroblast viability, DNA synthesis and ROS activity.

2. Materials and methods

2.1. Cell culture, pretreatments and AO exposure

Human gingival tissues from healthy nonsmokers were collected with institutional review board approval and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), 10% foetal bovine serum (FBS), and 1% antimycotic/ antibiotic. The tissues were then incubated at 37 °C in a humidified gas mixture (5% CO_2 and 95% air), and the medium was changed every 24–48 h. Human gingival fibroblasts (HGF) grew from the tissue after a week. The cells were passaged when confluent using 0.25% trypsin solutions and plated in new tissue culture flasks. Passages 3–8 were used in all experiments. Human periodontal ligament fibroblasts (HPDL) isolated from freshly extracted human teeth as previously described[30] were used in all experiments. The HPDL cells were cultured using the same conditions as for HGF. These cell types are collectively termed "oral fibroblasts".

A solution of H_2O_2 (3%, Walgreens Company, Deerfield, IL) was diluted at 0.00075% (223 μM) and 0.0005% (145 μM) for the experiments. The cells were exposed to H_2O_2 in 0.1% FBS for 30 min, washed with Dulbecco's phosphate-buffered saline (PBS, Gibco, Grand Island, NY) and treated with AO mixtures (RFT, PFR, PFT) in 0.1% FBS medium (high-glucose Dulbecco's modified Eagle's medium, DMEM, Hyclone, South Logan, UT) and 1% antimycotic/antibiotic (Gibco, Grand Island, NY) for 24 h.

Similar procedures were employed for EtOH or Nic exposure and AO treatments. Pure EtOH was obtained from US Industrial Chemicals Company (Tuscola, IL) and Nic (Sigma, St. Louis, MO). For the dose–response experiments, the cells were exposed to different EtOH concentrations (10% and 5%) and Nic concentrations (8 mM and 6 mM) for 30 min. The HPDL cells were also exposed to Nic for 60 min. After exposure, the cells were rinsed and treated with 10^{-5} M (0.4%, wt/v) AO mixture (RFT, PFR, PFT) for 24 h. All AO solutions were prepared as described below.[29]

The AOs tested were resveratrol (R, Lalilab Durham, NC), ferulic acid (F Sigma Aldrich, St. Louis, MO), tetrahydrocurcuminoid (T, Sabinsa Corporation, Piscataway, NJ), and phloretin (P, Kaden, Biochemicals, Hillsborough, NJ). The first composition tested, designated "RFT," was a 1:1:1 by weight ratio composition of resveratrol, ferulic acid, and tetrahydrocurcuminoids CG. The second composition tested, designated

"PFR," was a 1:1:1 by weight ratio composition of phloretin, ferulic acid, and resveratrol. The third composition tested, designated "PFT," was a 1:1:1 by weight ratio composition of phloretin, ferulic acid, and tetrahydrocurcuminoids CG. Compositions were prepared as 40% wt/v (total AOs) solutions in DMSO (e.g. a total of 400 mg AOs in 1 ml total volume) (1.6×10^{-3} M). Compositions were diluted with DMSO to achieve the lower concentrations of 4% wt/v (1.6×10^{-4} M) and 0.4% wt/v (1.6×10^{-5} M).

2.2. Cell viability assay

Cell viability was assessed by MTS colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sul-phophenyl]-2H-tetrazolium) (Promega Corporation, Madison, WI), which measures the activity of enzymes that reduce MTS to formazan dyes to distinguish the number of living cells. After each experiment (stressor exposure followed by AO incubation), the adherent cells were washed twice with PBS $1\times$ prior to the addition of 20 μL of MTS reagent to each well (96-well plate format) and incubated for 2.5 h at 37 °C. The absorbance at 490 nm was measured using a SpectraMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The controls included wells containing cells treated with 0.1% FBS. The treatments included wells containing cells with AOs only and also those exposed to both AO combinations and stressors. All HGF and HPDL experiments had 3–6 replicate wells/treatment group; the experiments were repeated at least 3 times.

2.3. Live/Dead® assay

A Live/Dead® assay (Invitrogen Molecular probes, Eugene, Oregon) was used to confirm the MTS viability tests. Adherent HGF and HPDL cells were grown overnight to confluence with a density of 25,000 cells/well on sterile glass cover slips. The cells were exposed to the stressors and AOs as described above. Staurosporine-treated cells were used as positive control samples. The cells were washed with PBS prior to the live–dead assay to remove or dilute the serum esterase activity generally present in serum-supplemented growth media to decrease any non-specific background. Live cells fluoresce green [excitation/emission (ex/em) ~495 nm/~515 nm] and dead cells fluoresce red (ex/em ~495 nm/~635 nm). The experiments were repeated at least three times.

2.4. DNA synthesis (5-bromo-2-deoxyuridine, BrdU) assay

A BrdU assay was used to determine whether the surviving cells could synthesize DNA. Briefly, 5×10^3 cells were seeded to 96-well, flat-bottomed microtiter plates and grown in 0.1-ml DMEM containing 10% FBS at 37 °C in 5% CO_2 in air. The cells attached overnight at 70% confluency. After each experiment (stressor exposure and AO treatment) as described above, the cells were labelled with BrdU for 24 h, and its incorporation into DNA was determined using a commercial kit (Roche Diagnostics, GmbH, Mannheim, Germany), according to the manufacturer's instructions. Three independent experiments were done with two or three samples per condition.

2.5. Reactive oxygen species assay

ROS was assessed using the dichlorodihydrofluorescein diacetate (H_2DCFDA) reagent (Invitrogen, Molecular Probes, Eugene, OR) according to the manufacturer's directions. The oral fibroblasts were cultured and seeded into 96-well plates as described above. In this assay, the cells were pretreated with the AO combinations (RFT, PFR, PFT) before exposure to the ROS-inducing stressors. After achieving 70% confluence, the cells were treated with the AO combinations for 24 h, then exposed to the stressors for 10 min and washed with PBS. The ROS detection reagent H_2DCFDA was added to each well for 30 min. All experiments included the following groups: control (0.1% FBS); stressors only; and RFT, PFR, and PFT combinations only. Cells treated with phorbol-12-myristate-13-acetate (PMA) were used as the positive control group. Detection for ROS was done at 485 and 525 nm. The experiments were repeated at least three times with 2–3 samples per treatment group.

2.6. Statistical analysis

The data were analysed using a SPSS statistical package [one-way analysis of variance-Fisher's least significant differences (LSD) for comparisons]. In the viability, DNA synthesis and ROS assays, all experiments were repeated at least three times with both cell types for each independent assay ($p = 0.05$).

3. Results

3.1. AOs increase cell viability after stressor treatment

The incubation of HGF cells in the presence of H_2O_2 for 30 min resulted in a dose-dependent decrease in the number of viable cells (Fig. 1A, diagonal and vertical line patterns in bars), with less than 25% viability at 0.00075% (Fig. 1A, vertical line pattern in bars). The cell viability at the lowest dose of H_2O_2 (0.0005%) was not significantly different from that of the control cell (0.1% FBS, Fig. 1A, black bar). The AO treatments significantly increased cell viability in the presence of 0.00075% H_2O_2 (Fig. 1A RFT, PFR, PFT; $p < 0.05$). Only PFR increased the percentage of viable cells in the 0.0005% H_2O_2 treatment group (Fig. 1A; $p < 0.05$). The cell viability in both RFT and PFT did not change in the 0.0005% H_2O_2 treatment group (Fig. 1A) and remained similar to that of the 0.1% FBS controls.

After 30 min exposure to H_2O_2, the HPDL cells also decreased at the highest dose concentration (0.00075%) in many viable cells (Supp. 1A, vertical line pattern). The viable cells in all AO combinations significantly increased; the PFT group had the highest percentage at 80% cell recovery, followed by the PFR and RFT groups [Supp. 1A, RFT, PFR, PFT compared to diagonal and vertical line patterns; ($p < 0.05$)].

EtOH exposure induced a dose-dependent decrease in HGF cell viability at 30 min (Fig. 1B, diagonal and vertical line patterns) compared to the controls (Fig. 1B, black bar; $p < 0.05$). A 50–60% loss of cells was observed from exposure to 5–10% EtOH. Treatment with the AO mixtures (RFT, PFR, PFT) significantly increased the viable HGF cell numbers to over
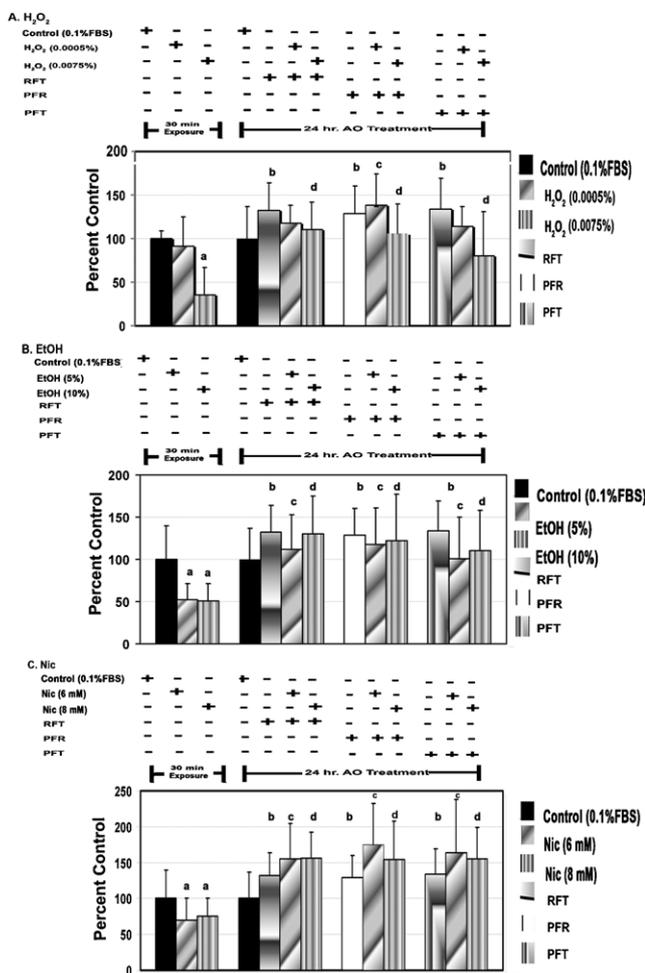
Fig. 1 – (A) All AO compounds tested rescued HGF cells exposed to 0.00075% hydrogen peroxide (H_2O_2) for 30 min. HGF cells responded to increasing concentrations of H_2O_2 in a dose-dependent manner (diagonal and vertical lines in bars). AOs (RFT, PFR and PFT) significantly increased cell survival at 0.00075% H_2O_2. Data are mean ± S.D. ($p < 0.05$) obtained in 2–3 culture wells per condition; [a]H_2O_2-exposed cells vs. the control (0.1% FBS-30 min, black bar), [b]AOs (RFT, PFR, and PFT) vs. the control (0.1% FBS-24 h, black bar), [c]AOs (RFT, PFR, PFT) vs. H_2O_2 (0.0005%), [d]AOs (RFT, PFR, PFT) vs. H_2O_2 (0.00075%). (B) All AO compounds tested rescued HGF cells exposed to ethanol (EtOH) for 30 min. HGF cells responded to increasing concentrations of EtOH in a dose-dependent manner (diagonal and vertical lines in bars). AOs (RFT, PFR and PFT) increased cell survival at all concentrations. Data are mean ± S.D. Obtained from 2 to 3 culture wells per condition. $p < 0.05$ was considered to be statistically significant. [a]EtOH exposed cells vs. the control (0.1% FBS-30 min, black bar), [b]AO-treated cells vs. control (0.1% FBS-24 h, black bar), [c]AOs (RFT, PFR, and PFT) vs. EtOH (5%), [d]AOs (RFT, PFR, PFT) vs. EtOH (10%) (C). All AO compounds tested rescued HGF cells exposed to nicotine (Nic) for 30 min. HGF cells responded to increasing concentrations of Nic in a dose-dependent manner (diagonal and vertical lines in bar). AOs (RFT, PFR and PFT) increased cell survival at all concentrations. Data are mean ± S.D.

100% of the controls after exposure to 5% and 10% EtOH (Fig. 1B; $p < 0.05$).

The HPDL cells responded in a dose-dependent manner to increasing concentrations of EtOH with decreased cell viability by 30 min (Supp. 1B, diagonal and vertical line patterns). The AO treatments (RFT, PFR, or PFT) increased the percentage of viable cells to nearly that of the control in the 10% EtOH group and 20–30% in the 5% group (Supp.1B; $p < 0.05$).

Nicotine (Nic) exposure led to a decrease in the number of viable HGF cells after 30 min (Fig. 1C, diagonal and vertical line patterns). The percent reduction was approximately 40% at all Nic concentrations tested compared to the controls (Fig. 1C, black bar; $p < 0.05$). AO treatment (RFT, PFR and PFT) significantly increased the numbers of viable cells at all Nic concentrations tested (Fig. 1C; $p < 0.05$). All AO combinations induced a 2.5–3-fold increase in the viable cells exposed to 8 mM and 6 mM Nic (Fig. 1C).

There was a modest change in the number of viable HPDL cells after 60 min exposure to Nic (Supp.1C, black bars). The decrease in viable cells was between 20 and 25% for all Nic concentrations (Supp. 1C, diagonal and vertical line patterns; $p < 0.05$). The number of viable cells in the AO combinations (RFT, PFR, PFT) significantly increased compared to the controls (Supp. 1C, 0 group; $p < 0.05$).

3.2. AOs decrease numbers of dead cells induced by stressors

The qualitative Live/Dead® fluorometric assay confirmed that the stressors reduced the number of live cells when the Live/Dead® reagent was added and the fluorescent responses measured. The increase in green fluorescence indicated a high percentage of viable HGF (Fig. 2C and D) and HPDL (Supp. 2C and D) cells in the control (0.1% FBS, Fig. 2A) compared to the stressor-exposed cells. The increased red fluorescence reflected the number of dead cells in the stressor-treated groups in a concentration-dependent manner (Fig. 2C and D). The stressor-exposed cells showed a substantial level of recovery 24 h after AO treatment with more intense green fluorescence staining, indicating that the HGF (Fig. 3D and E) and HPDL (Supp. 3D and E) were alive. These data confirmed the quantification of the MTS viability assay as shown in the previous figures (Fig. 1A–C) and supplemental figures (Supp. 1A–C).

3.3. AOs increase DNA synthesis in HGF and HPDL cells

To assess the effects of stressors and AOs on cell proliferation, the DNA synthesis of oral fibroblasts after stressor exposure, followed by AO treatment, was evaluated by BrdU incorporation assay (Fig. 4). Treatment with different concentrations of H_2O_2 induced a 20% reduction in proliferating HGF cells after 30 min (Fig. 4A). A 0.00075% concentration of H_2O_2 significantly

($p < 0.05$) obtained from 2 to 3 culture wells per condition; [a]Nic-exposed cells vs. the control (0.1% FBS-30 min), [b]AO-treated cells vs. control (0.1% FBS-24 h, black bar), [c]AOs (RFT, PFR, PFT) vs. Nic (6 mM), [d]AOs (RFT, PFR, and PFT) vs. Nic (8 mM).
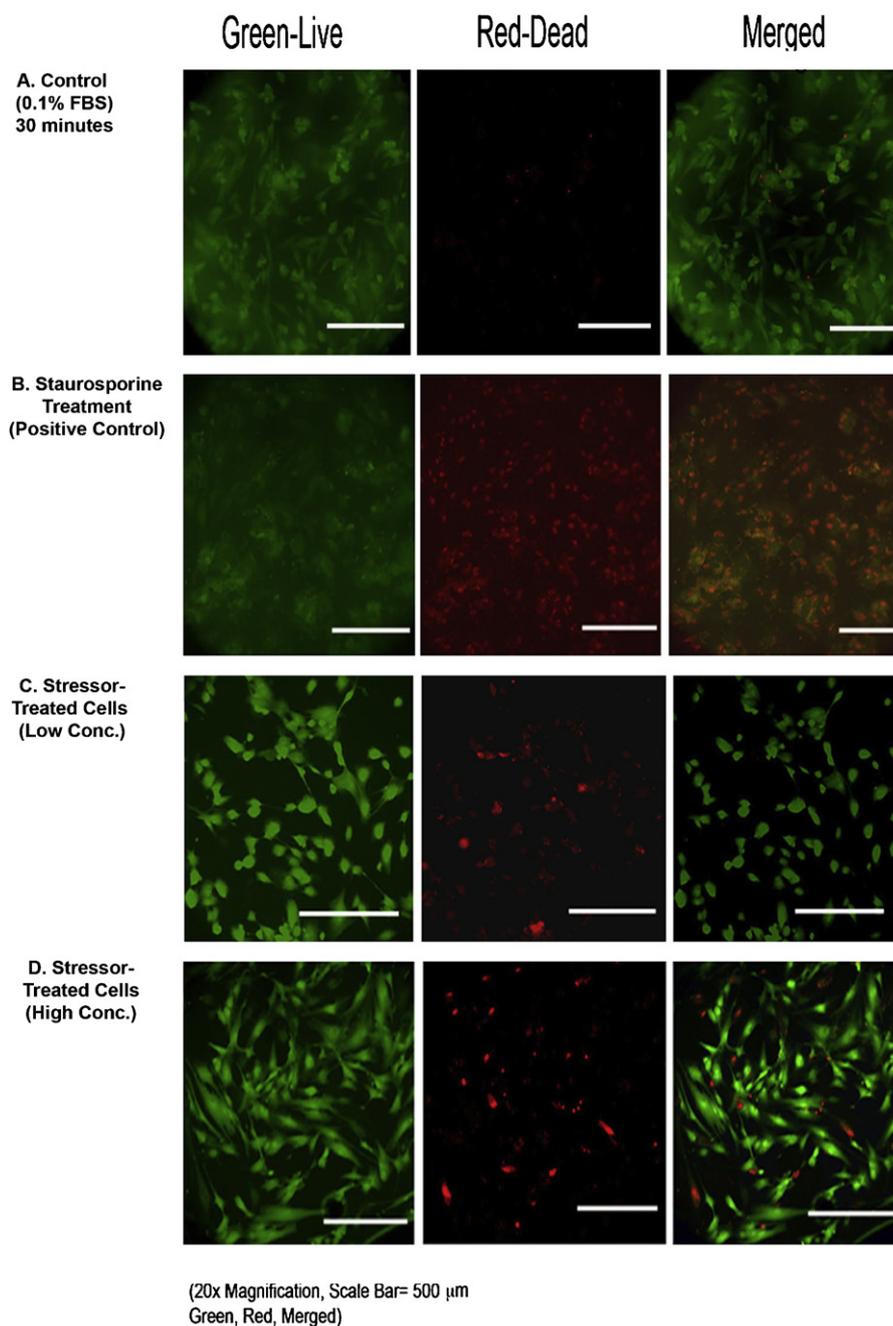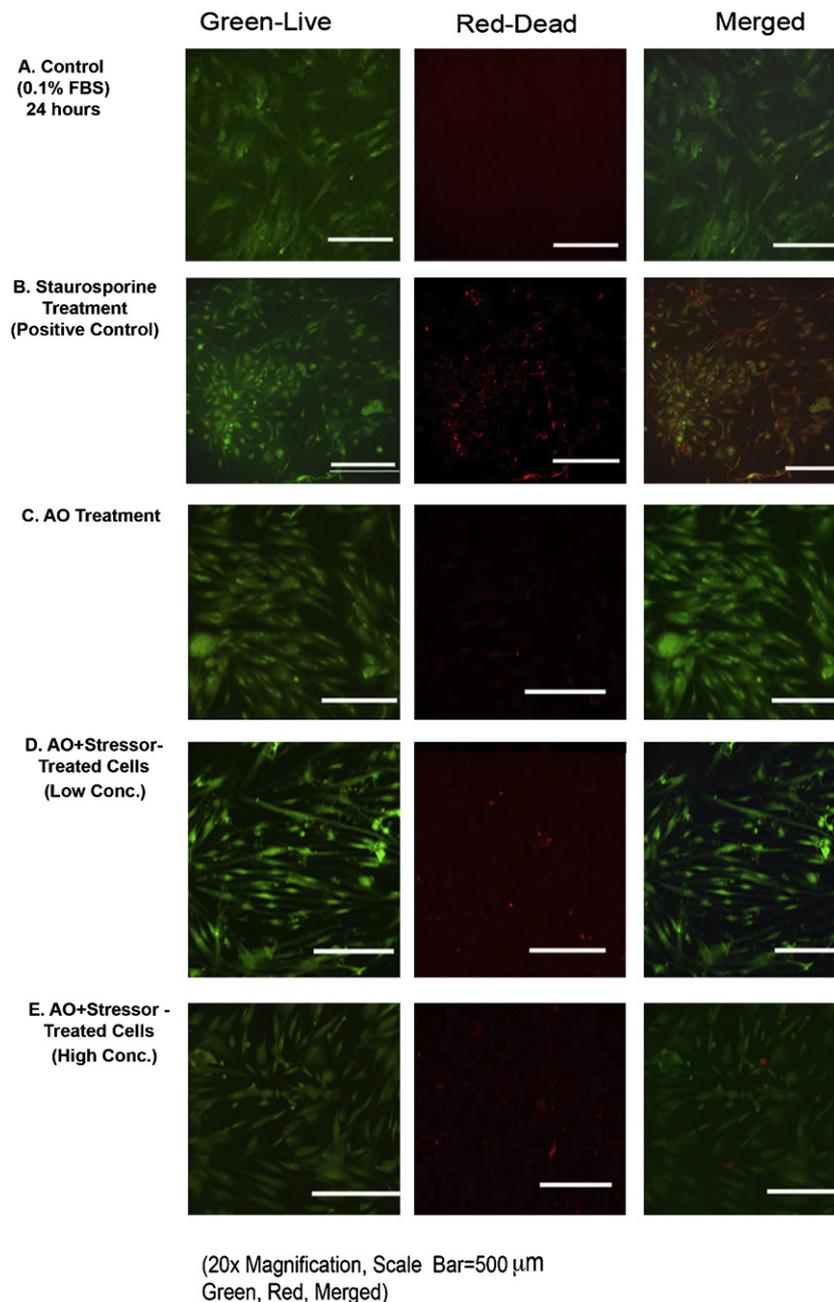
Fig. 2 – ROS related-stressors led to reduction of HGF cell numbers. Adherent HGF cells were grown to confluence with a density of 25,000 cells/well on sterile glass cover slips overnight. Shown here are representative images of all cells exposed to the low and high concentrations of stressors (C and D, H_2O_2, EtOH and Nic) for 30 min and then analysed using a Live-Dead® reagent, and the fluorescent responses were measured. Staurosporine-treated cells (B) were used as positive controls. Representative images obtained from at least three independent experiments. 20× Magnification, scale bar = 500 μm. (For interpretation of the references to colour in text, the reader is referred to the web version of the article.)

decreased DNA synthesis compared to the controls (Fig. 4A; diagonal line pattern; $p < 0.05$). After 24 h, the DNA synthesis of AO-treated oral fibroblasts was significantly increased by 20% with the addition of RFT and PFR compared to the control cells (0.1% FBS; Fig. 4A–C, black bar; $p < 0.05$). However, the results from PFT treatment were no different from those of the control. Only RFT significantly increased DNA synthesis

in the cells exposed to 0.00075% H_2O_2 (Fig. 4A; diagonal line pattern; $p < 0.05$).

Similar results (Supp. 4A–C) were observed in the HPDL cells exposed to AOs. Both the RFT and PFR treatments significantly enhanced HPDL cell proliferation compared to the control cells (0.1% FBS; Supp. 4A–C, black bar; $p < 0.05$). DNA synthesis in the HPDL cells was not significantly

(20x Magnification, Scale Bar=500 μm
Green, Red, Merged)

Fig. 3 – AOs decrease numbers of dead cells induced by stressors. Adherent HGF cells were grown to confluence with a density of 25,000 cells/well on sterile glass cover slips overnight. The cells were exposed to stressors and AOs as described above. Staurosporine-treated cells (B) were used as positive control samples. Cells were washed with PBS prior to the live–dead assay to remove or dilute serum esterase activity generally present in serum-supplemented growth media to decrease non-specific background. Live cells fluoresce green (excitation/emission (ex/em) $\sim$495 nm/$\sim$515 nm), and dead cells fluoresce red (ex/em $\sim$495 nm/$\sim$635 nm). Shown here are representative images of all stressor-exposed cells at low (D, 0.0005% H_2O_2, 5% EtOH, 6 mM Nic) and high concentrations (E, 0.00075% H_2O_2, 10% EtOH, 8 mM Nic). There was a substantial level of recovery after 24 h of AO treatment with more intense green fluorescence staining indicating that the HGF cells were alive. Representative images obtained from at least three independent experiments. 20$\times$ magnification, scale bar = 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

inhibited after exposure to different concentrations of H_2O_2. However, an increase in DNA synthesis was noted in both the PFR (0.00075%, 0.0005% H_2O_2) and PFT groups (0.00075% H_2O_2) (Supp. 4A, diagonal line and vertical line patterns; $p < 0.05$).

Two concentrations (10% and 5%) of EtOH inhibited DNA synthesis in the HGF cells (Fig. 4B, diagonal line and fine vertical line patterns; $p < 0.05$). Only PFR stimulated DNA synthesis in the cells exposed to 10% EtOH (Fig. 4B, diagonal line pattern; $p < 0.05$).
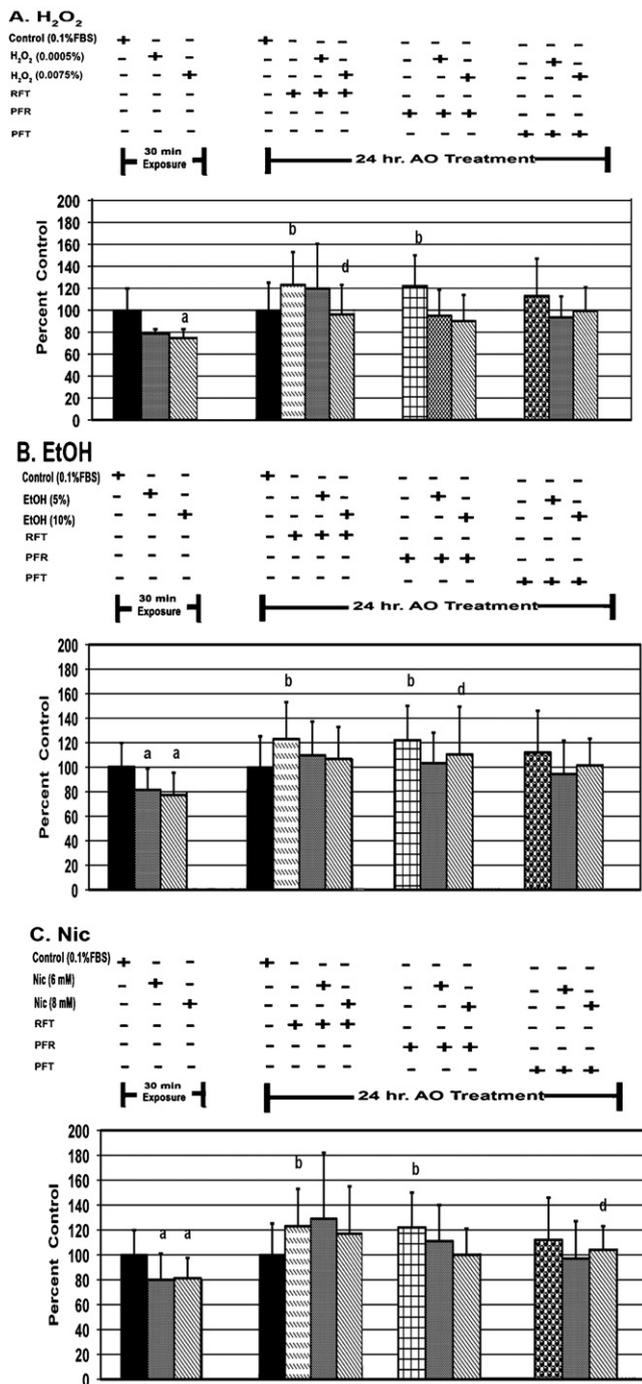
Fig. 4 – Post AO treatment induced a modest increase of proliferating HGF cells. HGF cells were exposed to different concentrations of stressors (A, H_2O_2; B, EtOH and C, Nic) for 30 min prior to 24-h AO treatment and then analysed for BrdU incorporation into DNA. Data are mean ± S.D. ($p < 0.05$) obtained from 2 to 3 culture wells per condition. [a]Stressor-exposed cells (H_2O_2, EtOH, Nic vs. control (0.1% FBS-30 min, black bar), [b]AO-treated cells (RFT, PFR, and PFT) vs. control (0.1% FBS-24 h), [c]AO + stressor-exposed cells (0.0005% H_2O_2/5%EtOH/6 mM Nic) vs. only stressor-exposed cells (0.0005% H_2O_2/5% EtOH/6 mM Nic), [d]AO + stressor-exposed cells (0.00075% H_2O_2/10% EtOH/8 mM Nic) vs. only stressor-exposed cells (0.00075% H_2O_2/10% EtOH/8 mM Nic).

The HPDL cells did not respond in a dose-dependent manner regarding the EtOH exposure; only RFT counteracted the effect of EtOH at one concentration (Supp. Fig. 4B; 5% EtOH, fine vertical line pattern; $p < 0.05$).

In the Nic-treated groups, DNA synthesis in all the HGF cells was significantly reduced compared with the control (0.1% FBS, Fig. 4C, black bar; $p < 0.05$). Only PFT increased the DNA synthesis in 8 mM Nic-exposed cells (Fig. 4C; 8 mM Nic, diagonal line pattern, $p < 0.05$).

Exposure of the HPDL cells to different concentrations of Nic did not change the DNA synthesis levels. PFR significantly stimulated DNA synthesis in the Nic-exposed cells although PFT or RFT did not (Supp. 4C; 8 mM Nic, diagonal line pattern, $p < 0.05$).

3.4. AOs suppress HGF and HPDL responses through generation of reactive oxygen species

The effect of oral stressors with and without AO combinations on ROS production by cells was measured against the fluorescence of a 2′,7′-dichlorofluorescein (DCF) standard (Fig. 5). In oral fibroblast cultures, H_2O_2, EtOH and Nic increased ROS production, and pretreatment with AOs reduced the levels of ROS in most experimental groups compared to the controls.

The response of HGF cells to different concentrations of H_2O_2 showed varying degrees of ROS activity. H_2O_2 (0.0005% and 0.00075%) increased ROS activity by 1.5 and 1.25 folds, respectively, compared with the control (0.1% FBS, Fig. 5A, black bar; $p < 0.05$). In HGF cells pretreated with triple AO combinations before H_2O_2 exposure, the ROS activity was lower than in the cells not pre-treated with AOs; all AO combinations significantly reduced ROS at lower concentrations of H_2O_2 (Fig. 5A, $p < 0.05$).

HPDL cells exposed to H_2O_2 alone produced increased ROS levels (Supp. 5A, NO AO group). When the AO pretreatments were completed, there was a significant ($p < 0.05$) reduction in ROS activity with RFT pretreatment of cells exposed to 0.00075% H_2O_2. There were no other significant differences noted among the groups of untreated, AO-treated and stressor-exposed cells.

The generation of ROS in EtOH-exposed HGF cells was significant in the 10% EtOH group (Fig. 5B; $p < 0.05$), which was counteracted by all AO treatments by 2.5 fold change (Fig. 5B; $p < 0.05$).

In the HPDL cells, both 5% and 10% EtOH concentrations elicited higher ROS activity than the levels produced by the control (0.1% FBS) cells (Supp. 5B; $p < 0.05$). Both RFT and PFR attenuated the ROS activity at these EtOH concentrations. However, only PFR induced significant reductions in ROS at 10% EtOH (Supp. 5B; $p < 0.05$).

ROS production by Nic-treated HGF cells was significantly greater than for the control cells. Six mM, and 8 mM Nic induced 2- and 1.5-fold increases, respectively, over the control cells (Fig. 5C; $p < 0.05$). When PFR and PFT pretreatment were applied to the Nic-treated cells, the ROS activity was significantly reduced (Fig. 5C; $p < 0.05$). However, RFT pretreatment only significantly decreased the ROS activity of 6 mM Nic-treated cells (Fig. 5C; $p < 0.05$). PFR pretreatment produced a greater decrease in ROS activity compared to RFT and PFT (Fig. 5C).
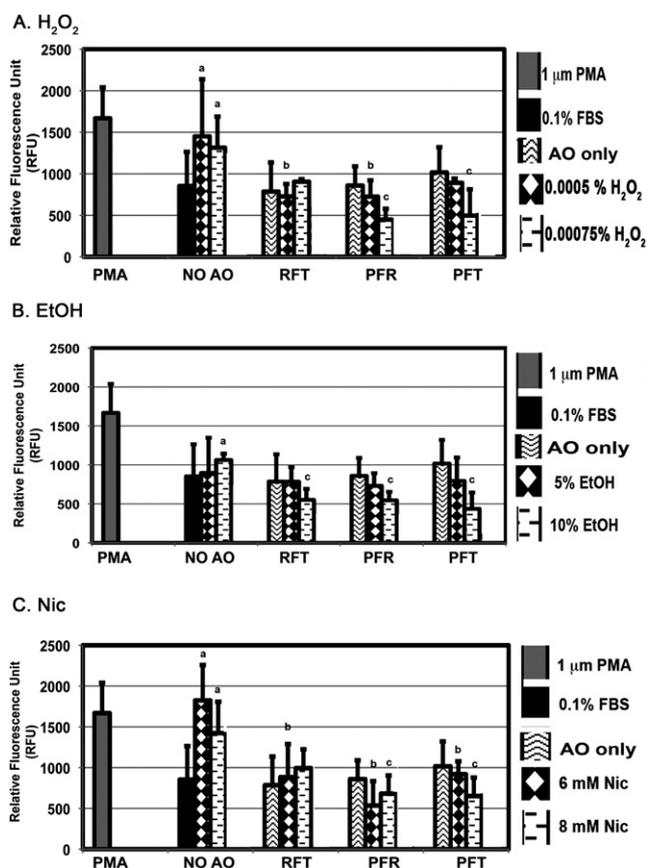
Fig. 5 – Specific AO combinations suppressed HGF responses through generation of reactive oxygen species. Cells were treated with the AO combinations (RFT, PFR, and PFT) before exposure to the ROS inducing stressors (H_2O_2, EtOH and Nic) for 10 min and then analysed by a ROS detection reagent, H_2DCFDA. Cells treated with phorbol-12-myristate-13-acetate (PMA) were used as positive control group. Data are mean ± S.D. ($p < 0.05$) obtained from 2 to 3 culture wells per condition. [a]Stressor-exposed cells (H_2O_2, EtOH, Nic vs. control (0.1% FBS, black bar), [b]AO + stressor-treated cells (0.0005% H_2O_2/5% EtOH/6 mM Nic) vs. only stressor-treated cells (0.0005% H_2O_2/5% EtOH/6 mM Nic), [c]AO + stressor-treated cells (0.00075% H_2O_2/10% EtOH/8 mM Nic) vs. only stressor-treated cells (0.00075% H_2O_2/10% EtOH/8 mM Nic).

For the HPDL cells, all Nic concentrations elevated the ROS activity in a dose-dependent manner with 8 mM Nic inducing the highest activity (Supp. 5C; $p < 0.05$). RFT pretreatment decreased ROS in the presence of 6 mM Nic (Supp. 5C). PFR and PFT pretreatment did not produce significant changes in ROS activity in these groups (Supp. 5C).

4. Discussion

This study tested the capacity of specific AO combinations to counteract the effects of the stressors H_2O_2, EtOH and Nic on cultured oral fibroblast viability, DNA synthesis and ROS activity. Individual AO compounds have been shown to have protective effects on cells. One of the bioactive compounds used, R, a polyphenol derived from red grapes, also protected human lens epithelial cells from H_2O_2-induced cell apoptosis and excessive ROS accumulation affecting the MAP Kinase p38 and c-Jun N-terminal kinases (JNK) pathways.[31] F and its derivative counteracted both H_2O_2-induced protein and lipid oxidation and loss in cell viability in human dermal fibroblasts.[32] In the neural system where glutamate created an imbalance of cellular redox homeostasis in a mouse hippocampal cell line (HT22), curcumin had a neuroprotective effect against oxidative glutamate toxicity by inhibiting MAP kinase signalling and influencing cell cycle regulation.[33] Another study demonstrated that esculetin (6,7-dihydroxy-coumarin), a plant-derived coumarin and immunomodulator, recovered cell viability when exposed to H_2O_2 in Chinese hamster lung fibroblast.[34] These studies indicate that H_2O_2 may reduce cell proliferation and viability through the MAP kinase pathways and that AOs restore the cell cycle. Corroborating this finding, Nic was shown to inhibit myofibroblast differentiation in HGF cells, which was mediated through the Rac and PAK 1/2 signalling pathways.[35]

In live cell migration studies, combinations of double and triple AOs promoted migration more than single AOs.[29] Triple combinations of PFR and RFT clearly counteracted the effects of Nic and significantly increased the migration rates in both HGF and HPDL cells.[29] The AO combinations used were a mixture of three single pure AOs that specifically catalyse the decomposition of reactive oxygen radicals produced by the tested stressors. Employing these three AOs in different combinations decreased ROS activity at various levels (2–3 folds) and also restored cell proliferation and increased viability. In general, the assays used in this study showed that HGF cells responded to the stressors and AO treatments with less variability than did HPDL cells. This difference in response and variability may be due to the more homogenous population of HGF cells compared to HPDL cells. Under these culture conditions, HGFs adhered, spread and proliferated well in the culture plates.[30] This lab and others have shown that HPDL cells are functionally different from HGFs.[30,36,37] HPDL cells contain a heterogeneous population and appear to be less sensitive to AOs with more variations.[37] These variations may also be influenced by apparent discrepancies in assays like MTS for cell viability, which may lead to false positive results when testing these AO compounds with reduction potential.[38,39] To prevent any direct interference of AOs with MTS formazan reduction, the adherent cells were washed with PBS (1×) prior to the addition of the MTS reagent. However, to rule out any inaccuracy, the MTS assay results obtained were also confirmed by conducting a live/dead assay for a qualitative determination of viable and dead cells.

Tooth whitening with H_2O_2-based systems has moved from the dental office to over-the-counter products. In addition, H_2O_2 is also used to decrease bacterial biofilms in patients with gingivitis. Normal oral soft tissues, including dental pulp fibroblasts, produce enzymes such as catalase and glutathione peroxidase, which detoxify H_2O_2 and superoxide dismutase (SOD).[40–42] If the exogenous level of H_2O_2 is too high, it might overwhelm the cellular protective mechanisms, which puts these oral fibroblasts at risk of cellular damage.[40,43,44] In this

study, however, we observed that cell viability was reduced markedly by H_2O_2, consistent with a previous investigation.[44] In another study, oral fibroblasts exposed to 100 μmol/l H_2O_2 (equivalent to 0.03%) underwent an 80% reduction in proliferating cells as assessed by [³H]-thymidine incorporation.[45] In the present study, AO treatment significantly counteracted the effects of H_2O_2 by increasing cell viability and survival and decreasing ROS production, demonstrating that these triple AO combinations protect oral fibroblast cells from H_2O_2-induced cytotoxicity and may be useful in patients with H_2O_2-induced irritation.

EtOH significantly reduced cell viability and increased ROS in oral fibroblasts. Recent studies have shown that oral tissue oxidative damage induced by ROS following EtOH consumption may be harmful to periodontal health.[1] In a rat study, EtOH consumption increased gingival oxidative damage and the production of TNF-α in periodontal ligament fibroblasts, thus increasing the severity of periodontal inflammation in this ligature model of periodontitis.[46] The counteractive effect of AOs seen in gingival cells was found in a study showing that R and its three analogues significantly protected against EtOH-induced oxidative DNA damage in human peripheral lymphocytes.[47] A similar study also evaluated the potential protective effects of phenolics AOs: (−)-epigallocatechin-3-gallate (EGCG), T, R, melatonin, and α-lipoic acid, by preventing EtOH-associated apoptosis in foetal rhombencephalic neurons.[48]

Similar to H_2O_2 and EtOH, Nic increased ROS activity in oral fibroblasts, as well as decreased cell viability. Tobacco smoke and its by-products can damage DNA and increase free radicals/ROS, thereby increasing oxidative stress in periodontium-associated cells. In addition, smoking may affect AO levels, since a dose-related reduction of salivary and gingival crevicular fluid SOD levels was found in both light and heavy smokers.[49] These effects contribute to the pathogenesis of gingival and periodontal disease.[49] A related study reported that Nic inhibited HPDL growth, proliferation and protein synthesis.[50,51] Since nicotine is a major chemical component in tobacco smoke, it may be closely linked to increased ROS production, culminating in heightened oxidative damage to gingival tissue, periodontal ligament, and alveolar bone.[52] Triple combinations of AOs significantly reversed the toxic effects of Nic, similar to the reversal of cell migration inhibition noted previously.[53]

The ROS activity in both control and AO-treated cells without stressors was similar, indicating that the cells were in a low ROS state and AO treatment alone did not decrease ROS. Moreover, cells were sensitised to ROS due to experimental techniques during the 24 h incubation with decreased FBS (0.1%). The increased ROS activity after stressor exposure may contribute to the decreased cell viability. Other underlying mechanisms may also contribute to the decreased cell viability and proliferation noted. It was observed that AO treatment decreased ROS production in stressor-treated cells. Most AO compounds possessed free radical scavenging effects and other actions necessary for maintaining and protecting the cell's redox state.[48] However, in the current experiments, it was not determined if the specific AO effect of these combinations was associated with a particular stressor because the assay measured total

ROS. Other studies using the same AOs have shown specific AO effects. For example, F treatment reduced the generation of ROS with a net decrease of protein oxidation in human melanocytes irradiated with UVB.[54] Triphlorethol-A forms adducts with DNA and proteins in formaldehyde-treated Chinese hamster lung fibroblast cells.[55] Also, polyphenolic apple juice extracts containing quercetin and P protected human colon cell lines against oxidative cell damage (DNA damage, cellular redox status, glutathione and AO capacity).[56]

In conclusion, these data clearly demonstrated that common oral stressors have the potential to increase gingival cellular damage by decreasing cell viability and proliferation while increasing ROS. Furthermore, these natural AO products applied complementary or synergistic mechanisms to counteract the damaging effects of the stressors to decrease ROS and increase cell viability.

Funding

Competing interests

Edward P. Allen has equity interest in PerioSciences, LLC. Lynne A. Opperman has equity interest in PerioSciences, LLC. Rest of authors have no conflict of interest.

Ethical approval

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2012.04.021.

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