Antioxidant combinations protect oral fibroblasts against metal-induced toxicity ☆

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ABSTRACT

Objective: In dentistry, the use of metals in fillings, braces, implants, bridges and other prosthodontic restorations is a common practice. Previous studies revealed that zinc (Zn) and copper (Cu) released from gold alloys, and nickel (Ni) released from nickel–chromium alloys, have a highly cytotoxic effect on fibroblast cell cultures. Our working hypothesis is that oral fibroblasts are susceptible to damage from metals because they elevate reaction oxygen species (ROS). In this study, we investigated specific antioxidant (AO) combinations to determine if they counteract the effects of Cu, Ni and Zn on cultured oral fibroblast proliferation and oxidative damage.

Methods: Oral fibroblasts were pretreated with Cu, Ni and Zn for 60 min. Thereafter, cells were treated with 10^{-5} M combinations of bioactive AO resveratrol (R), ferulic acid (F), phloretin (P) and tetrahydrocurcuminoids (T) (RFT, PFR, PFT) for 24 h. Cell viability and DNA synthesis were monitored by 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS) and 5-*bromo*-2-*deoxyuridine* (BrDU) assays. ROS was measured using the fluorescence response of dichlorodihydrofluorescein diacetate (DCF).

Results: AO compounds increased recovery of cells exposed to Cu and Zn. Moreover, AO treatment induced DNA synthesis in the presence of the metal stressors. Cu and Ni stimulated production of ROS. PFR treatment decreased ROS in the presence of Cu, Ni and Zn.

Significance: These data indicate that pure AOs counteracted the detrimental effects of Cu, Ni, Zn on oral fibroblasts in vitro by increasing cell viability, and DNA synthesis and decreasing ROS activity.

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1. Introduction

The accumulation and absorption of excessive amounts of metallic ions found in most fillings, braces, implants and bridges can lead to over productive salivation, metallic taste in the mouth, and gingivitis, and may also form free radicals.[1–6] Metal ions can affect the inflammatory process in cells and tissues associated with gingiva, bone, lung, endothelium, nerve and cancerous growths.[7,8] Cytotoxicity effects of ions

released from fixed metal restorations showed Zn, Cu and Ni significantly affect fibroblast cell cultures.[9,10] The molecular mechanisms of cytotoxic and cell death inducing activity of dental metallic ions in both fibroblasts and osteoblasts are irreversible.[8] Contact dermatitis is also a common allergic manifestation associated with Ni toxicity from orthodontic appliances.[11] Moreover, oxidative stress and mitochondrial dysfunction play important roles in the neurotoxicity of Ni in neuroblastoma cells.[12] Zn ions in low concentrations can stimulate leukotriene B4 secretion in polymorphonuclear cells causing endothelial damage.[13] Cu induces oxidative stress with the formation of stable lipid peroxidation by-products signalling a transcription process through activation of mitogen-activated protein kinase (MAPK) pathways in COS-7 cells.[14] These studies provide evidence that metal ions induce reactive oxygen species (ROS) that activate signalling molecules to change cellular responses.

Oral ROS generation is counterbalanced by the action of antioxidant (AO) enzymes and other redox molecules.[15] Excessive ROS needs to be neutralized in cells by a variety of AO defense mechanisms.[16] There is a therapeutic need for increasing the AO in the oral cavity. Several topical oral AO compositions are being developed to ameliorate disease prevention practices. These topical gel treatments can reduce soft oral tissue damage by locally reducing the effects of ROS from biochemical insults or ingestion of metals (Cu, Ni and Zn) from dental restorations and appliances. In this study, we investigated the ability of specific AO combinations to counteract the effects of metal ions on cultured oral fibroblast viability, DNA synthesis and ROS activity.

2. Materials and methods

2.1. Cell culture, (pretreatments) and AO exposure

All experiments were conducted on parallel cultures of human gingival fibroblasts (HGF) and human periodontal ligament cells (HPDL). Human gingival tissues from healthy nonsmokers were collected with institutional review board approval and tissues were cultured in high glucose Dulbecco's modified Eagles medium (DMEM), 10% foetal bovine serum (FBS), and 1% antimycotic/antibiotic. Tissues were incubated at 37 °C in a humidified gas mixture (5% CO_2 and 95% air) and medium changed every 24–48 h. HGF grew from the tissues after a week. 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.

HPDL were isolated from freshly extracted human teeth as previously described.[17] Culture conditions as described for HGF were similarly employed for HPDL cells. These cell types were collectively termed as oral fibroblasts and were previously characterized.[18]

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).

A solution of copper ($CuCl_2$; Chem Service, West Chester, PA, M.W. = 170.48) was diluted at (5×10^{-4} M, 4×10^{-4} M) for the experiments. Cells were exposed to $CuCl_2$ in 10% FBS for 60 min, and then 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 using Ni and Zn exposure and AO treatments. The metals used were pure nickel chloride 6-hydrate crystal ($NiCl_2$, J.T. Baker, Phillipsburg, NJ, M.W.= 237.75) and zinc ($ZnCl_2$, Sigma Aldrich, St. Louis, MO, M.W. = 136.30). For dose–response experiments, cells were exposed to different Ni concentrations (2×10^{-3} M and 1×10^{-3} M) and Zn concentrations (3×10^{-4} M and 2×10^{-4} M) for 60 min.[9,10] After exposure to metal stressors, 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 previously.[18] Specific controls were described for each assay, but generally included cells treated with 0.1% FBS only. Treatments included wells containing cells exposed to AOs only or to metal ion stressors and AO combinations.

2.2. Cell viability assay

Cell viability was assessed by MTS colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) (Promega Corporation, Madison, WI). This assay 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). Controls included wells containing cells treated with 0.1% FBS only. Treatments included wells containing cells exposed to AO combinations only, and to AO combinations and stressors. All HGF and HPDL experiments had 3–6 replicate wells/treatment group and the experiments were repeated at least three times.

2.3. Live/dead assay

A Live/Dead® assay (Invitrogen Molecular Probes, Eugene, OR) was used to confirm MTS viability tests. Adherent HGF and HPDL cells were grown to confluence with a density of 25,000 cells/well on sterile LabTek™ slides (Fisher Scientific, Pittsburgh, PA) overnight. The cells were exposed to stressors and

AOs as described for the MTS assay. 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 fluoresced green (calcein AM (ex/em) 494/517 nm) and dead cells fluoresced red (ethidium homodimer-1 ex/em 528/617 nm). Experiments were repeated at least three times.

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

The BrdU assay was used to determine the ability of surviving cells to 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. Cells attached overnight at 70% confluency. After each experiment (controls, stressor exposure with or without 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 completed with 4–5 samples per group.

2.5. Reactive oxygen species assay

ROS was assessed using the dichlorodihydrofluorescein diacetate (H_2DCFDA) reagent (Invitrogen, Molecular Probes, Eugene, OR) as per the manufacturer's directions. Oral fibroblasts were cultured and seeded into 96-well plates as described above. After achieving 70% confluence, the cells were pretreated with AO combinations for 24 h. Cells were then treated with stressors for 30 min and washed with PBS. The ROS detection reagent, H_2DCFDA was added to each well for 30 min. All experiments included the following controls: 0.1% FBS, positive control (PMA, 5 μM) metal ion stressor only, RFT, PFR, and PFT combinations only. ROS detection was recorded at 485 and 525 nm. The experiments were repeated at least three times with 2–3 samples per treatment group.

2.6. Statistical analysis

Data was analysed using a SPSS statistical package (one-way analysis of variance-Fisher's Least Significant Differences (LSD) for comparisons). In viability, DNA synthesis and ROS assays, all experiments were repeated at least three times with both cell types for each independent assay. The P value was set at 0.05.

3. Results

3.1. AOs increase cell viability after metal ion stressor treatment

Exposure to Cu for 60 min caused a dose-dependent decrease in viable cells (Fig. 1A), with 50–40% viability at 5×10^{-4} M and 4×10^{-4} M respectively compared to controls. Due to the variability, this was not significantly different from control cell viability (10% FBS, Fig. 1A, black bar). AO treatments significantly increased cell viability in the presence of

4×10^{-4} M Cu (Fig. 1A[c] RFT, PFR, PFT; $p < 0.05$). The AOs did not change cell viability in the 5×10^{-4} M Cu treatment group (Fig. 1A[d]) and viability remained similar to that of the 0.1% FBS controls.

HPDL cells also had a dose dependent decrease in viable cells of 60% and 70% to 5×10^{-4} M and 4×10^{-4} M respectively of Cu, after the 60 min exposure (Supplementary Fig. 1A). All AO combinations (RFT, PFR, PFT) significantly increased the number of viable cells by 40–50% in the presence of both Cu concentrations (Supplementary Fig. 1A, $p < 0.05$).

Ni exposure induced a significant dose dependent decrease in HGF cell viability at 60 min (Fig. 1B and A) compared to controls (Fig. 1B, black bar; $p < 0.05$). A 50–70% loss of cells was observed from exposure to 1×10^{-3} M and $2 \times 10^{-3} \times$ M Ni. Treatment with PFT of 2×10^{-3} M Ni exposed cells significantly increased viable HGF cell numbers over 50% percent of controls (Fig. 1B[d]; $p < 0.05$).

HPDL cell viability decreased in a dose dependent manner by 60 min in response to increasing concentrations of Ni (Supplementary Fig. 1B). AO treatments (RFT, PFR, or PFT) did not increase the percentage of viable cells in any Ni treated groups (Supplementary Fig.1B; $p < 0.05$).

Exposure to all Zn concentrations led to a modest decrease of 40–60% in numbers of viable HGF cells after 60 min (Fig. 1C). However, only the 3×10^{-4} M Zn significantly decreased HGF cell number when compared to controls (Fig. 1C[a], $p < 0.05$). AO treatment (RFT, PFR and PFT) significantly increased numbers of viable cells after exposure to 3×10^{-4} M Zn (Fig. 1C[d]; $p < 0.05$). All AO combinations also induced an increase in numbers of viable cells exposed to 2×10^{-4} M but not significant (Fig. 1C).

There was a decrease in numbers of viable HPDL cells of between 30 and 40% after exposure to all Zn concentrations (Supplementary Fig. 1C, $p < 0.05$). None of the AO combinations (RFT, PFR, PFT) increased the number of viable cells in any of the Zn treated groups (Supplementary Fig. 1C, $p < 0.05$).

3.2. Metal ion stressors decreased numbers of live cells

The qualitative Live/dead® fluorometric assay confirmed that the metal ion stressors caused a decrease in the number of live cells and an increase in numbers of dead cells. The increase in green fluorescence showed a high percentage of viable HGF (Fig. 2A–D) and HPDL (Supplementary Fig. 2A–D) cells in the control (10% FBS) cells compared to the metal ion exposed cells. The increased red fluorescence reflected the number of dead cells in metal ion treated groups in a concentration dependent manner. The metal ion exposed cells showed a substantial level of recovery after 24 h of AO treatment with more intense green fluorescence staining indicating that both the HGF (Fig. 3A–E) and HPDL (Supplementary Fig. 3A–E) cells were alive. These data confirmed the quantification of the MTS viability assay (Fig. 1A–C) and (Supplementary Fig.1A–C).
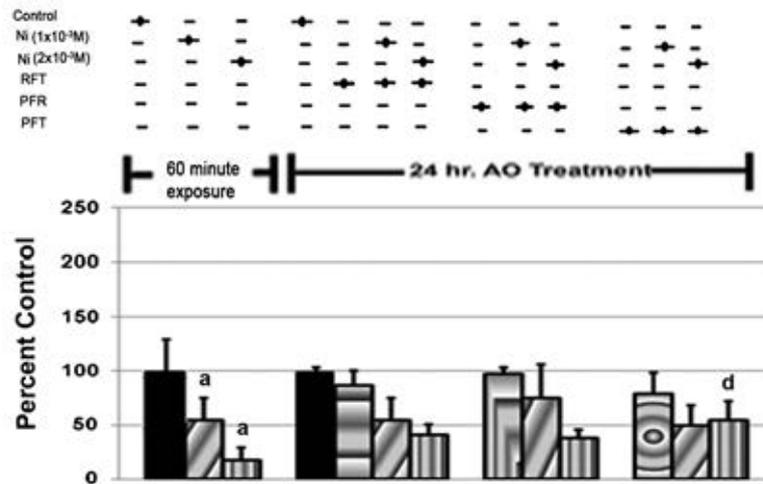
3.3. AOs increase DNA synthesis in HGF and HPDL cells

To assess the effects of metal ion stressors and AOs on cell proliferation, DNA synthesis of oral fibroblasts after stressor exposure followed by AO treatment was evaluated by a BrdU
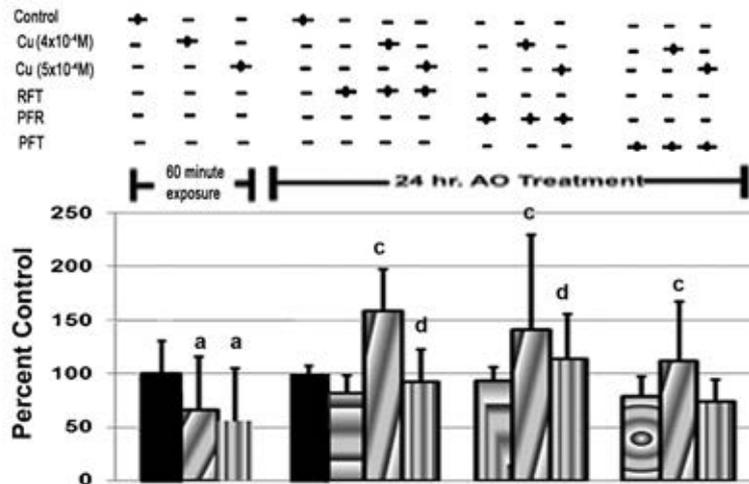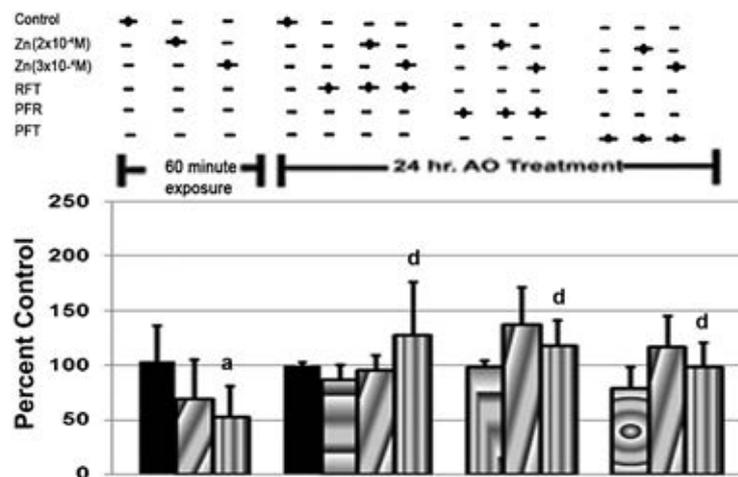
Fig. 1 – All AO compounds tested rescued HGF cells exposed to metal ion stressors (Cu, Ni, Zn) for 60 min. HGF cells responded to increasing concentrations of Cu (A), Ni (B), Zn (C) in a dose dependent manner (diagonal and vertical lines in bars). AOs (RFT, PFR and PFT) increased cell survival at all concentrations of Cu (A), Ni (B), Zn (C). Data are mean ± S.D. obtained in 3 and 6 culture wells per group. $p < 0.05$ was statistically significant.
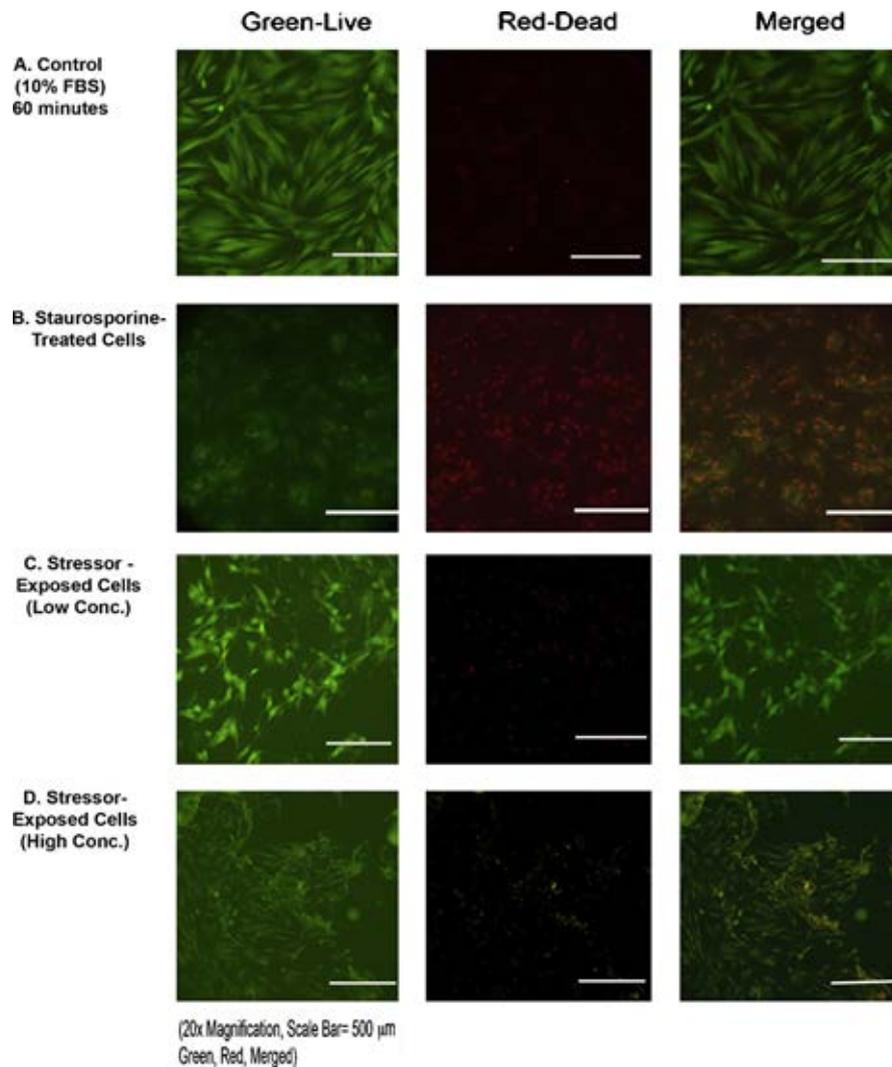
Fig. 2 – ROS related-stressors led to reduction of HGF cell numbers. Shown here are representative images of all cells exposed to low and high concentrations of stressors (C–D, Cu, Ni and Zn) for 30 min. Staurosporine-treated cells (B) were used as positive control samples. 20x Magnification, Scale Bar = 500 μm.

incorporation assay. Treatment with different concentrations of Cu reduced by 40–50% the proliferating HGF cells after 60 min (Fig. 4A). Cu significantly decreased DNA synthesis compared to controls (Fig. 4A[a], $p < 0.05$). After 24 h of PFR or PFT treatment, DNA synthesis of HGF cells exposed to Cu (4×10^{-4} M) was significantly increased when compared to HGF cells exposed to Cu (4×10^{-4} M) only (Fig. 4A[b], $p < 0.05$). One AO combination, PFT induced a significant improvement in cell proliferation in cells exposed to the high Cu concentration (5×10^{-4} M) (Fig. 4A[c]).

In parallel experiments, DNA synthesis of HPDL cells (Supplementary Fig. 4A) was reduced by 50–60% at all Cu concentrations tested. All AOs (RFT, PFR, PFT) significantly enhanced DNA synthesis of cells exposed to both Cu concentrations (4×10^{-4} M and 5×10^{-4} M) (Supplementary Fig. 3A, $p < 0.05$).

Both concentrations (1×10^{-3} M and 2×10^{-3} M) of Ni significantly inhibited DNA synthesis in HGF cells (Fig. 4B[a], $p < 0.05$). PFR and PFT rescued DNA synthesis in cells exposed to both concentrations of Ni (Fig. 4B[b,c] $p < 0.05$). However, PFT only increased DNA synthesis of cells exposed to 2×10^{-3} M Ni (Fig. 3B, $p < 0.05$).

After Ni exposure of HPDL cells, DNA synthesis was significantly decreased. RFT and PFT counteracted the effect of Ni at the high concentration (2×10^{-3} M) of Ni, rescuing DNA synthesis to nearly 100% (Supplementary Fig. 3B; $p < 0.05$). However, only PFT increased DNA synthesis above 100% in Ni (1×10^{-3} M) treated cells (Fig. 3B, $p < 0.05$).

In the Zn treated groups, DNA synthesis in all HGF cells was significantly reduced by 40% with both Zn concentrations when compared with control (10%FBS, Fig. 4C[a], $p < 0.05$). Two AO combinations (PFR and PFT) significantly rescued the DNA synthesis reduced by both concentrations of Zn (2×10^{-4} M; 3×10^{-4} M) (Fig. 4C[b,c], $p < 0.05$). RFT slightly increased DNA synthesis in Zn (3×10^{-4} M) treated cells, but this change was not significant (Fig. 4C[c], $p < 0.05$).

Exposure of HPDL cells to different concentrations of Zn reduced DNA synthesis levels by 50%. All AO combinations significantly rescued DNA synthesis in the Zn-exposed cells (Supplementary Fig. 4C, $p < 0.05$).
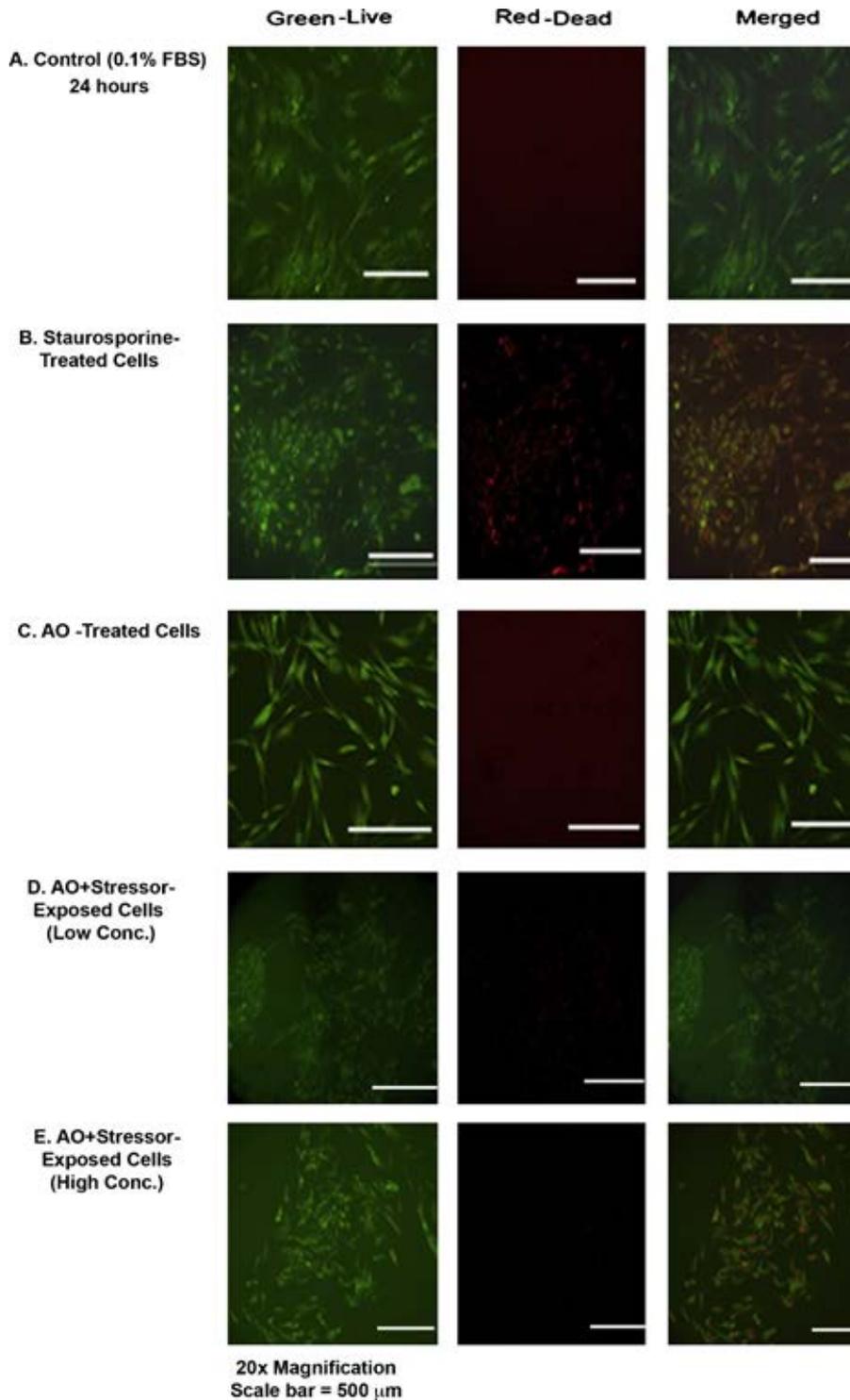
Fig. 3 – AOs decrease numbers of dead cells induced by stressors. Shown here are representative images of all stressor-exposed cells at low (D, 4×10^{-4} M Cu, 1×10^{-3} M Ni and 2×10^{-4} M Zn) and high concentrations (E, 5×10^{-4} M Cu, 2×10^{-3} M Ni and 3×10^{-4} M Zn) after AO treatment. Staurosporine-treated cells (B) were used as positive control samples. 20× Magnification, Scale Bar = 500 μm.

3.4.   AOs suppress ROS in metal ion treated HGF and HPDL cells

All previous experiments clearly demonstrated that the metal ions decreased oral fibroblast viability and that the three combinations of AOs tested could reverse the effects with post treatments (viability and DNA assays). This last set of experiments was designed to determine if the metal ions were directly changing ROS in the cells as a possible mechanism of action. In these experiments the cells were
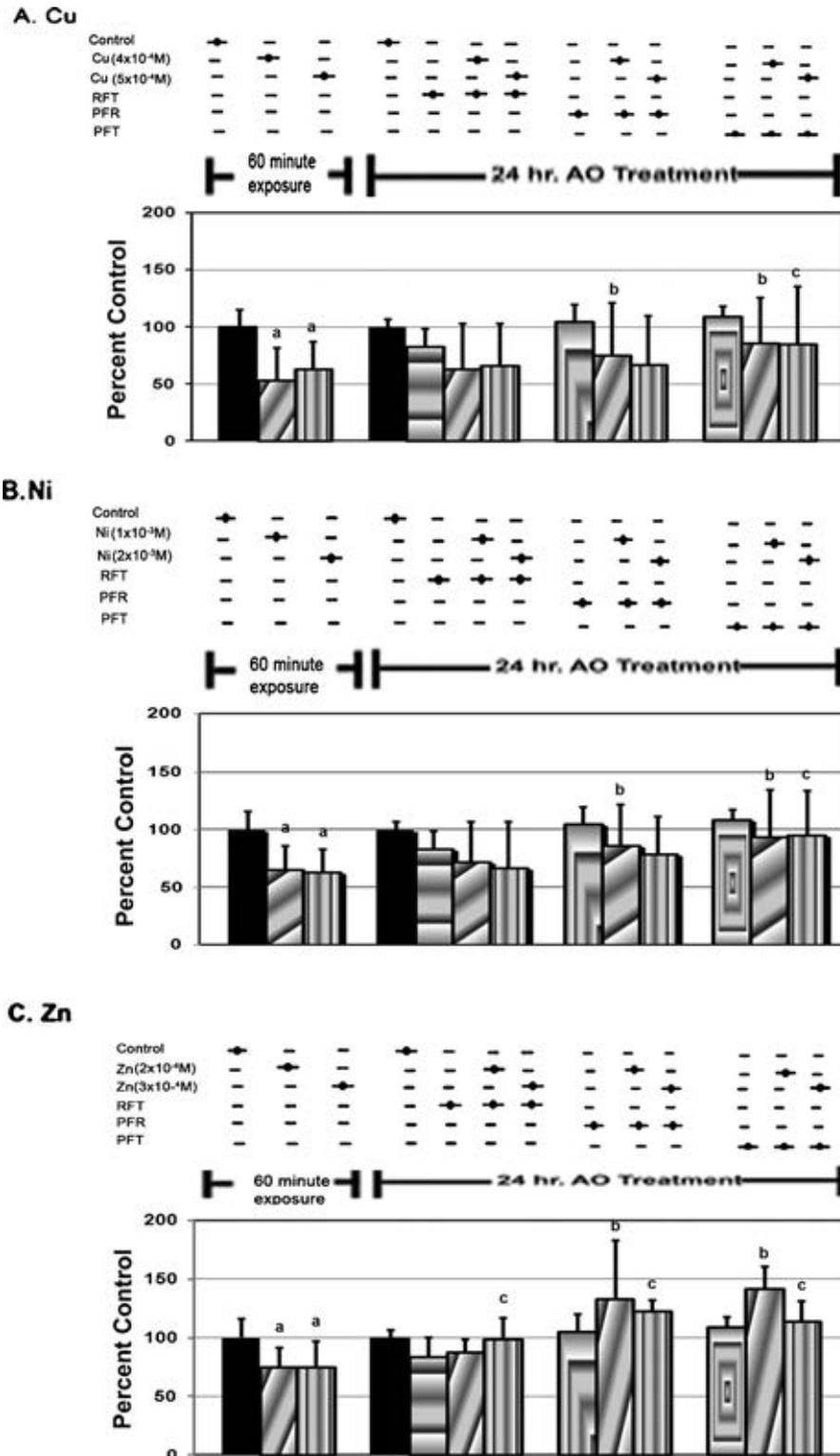
Fig. 4 – Post AO treatment induced a modest increase of proliferating HGF HGFcells. HGF cells were exposed to different concentrations of stressors (A, Cu, B, Ni and C, Zn) for 60 min prior to 24 h AO treatment and then analyzed for BrdU incorporation into DNA. Data are mean ± S.D. obtained from 4 and 5 culture wells per condition. $p < 0.05$ was considered to be statistically significant.

pretreated with or without AO combinations before exposure to the metal ions. ROS production was measured using 2′,7′-dichlorofluorescein (DCF) with the internal positive control,

PMA (Fig. 5). In cells that were not pretreated with AOs, the 0.1% FBS control induced moderate ROS levels that were significantly increased in cells exposed to Cu, or Ni, but not Zn
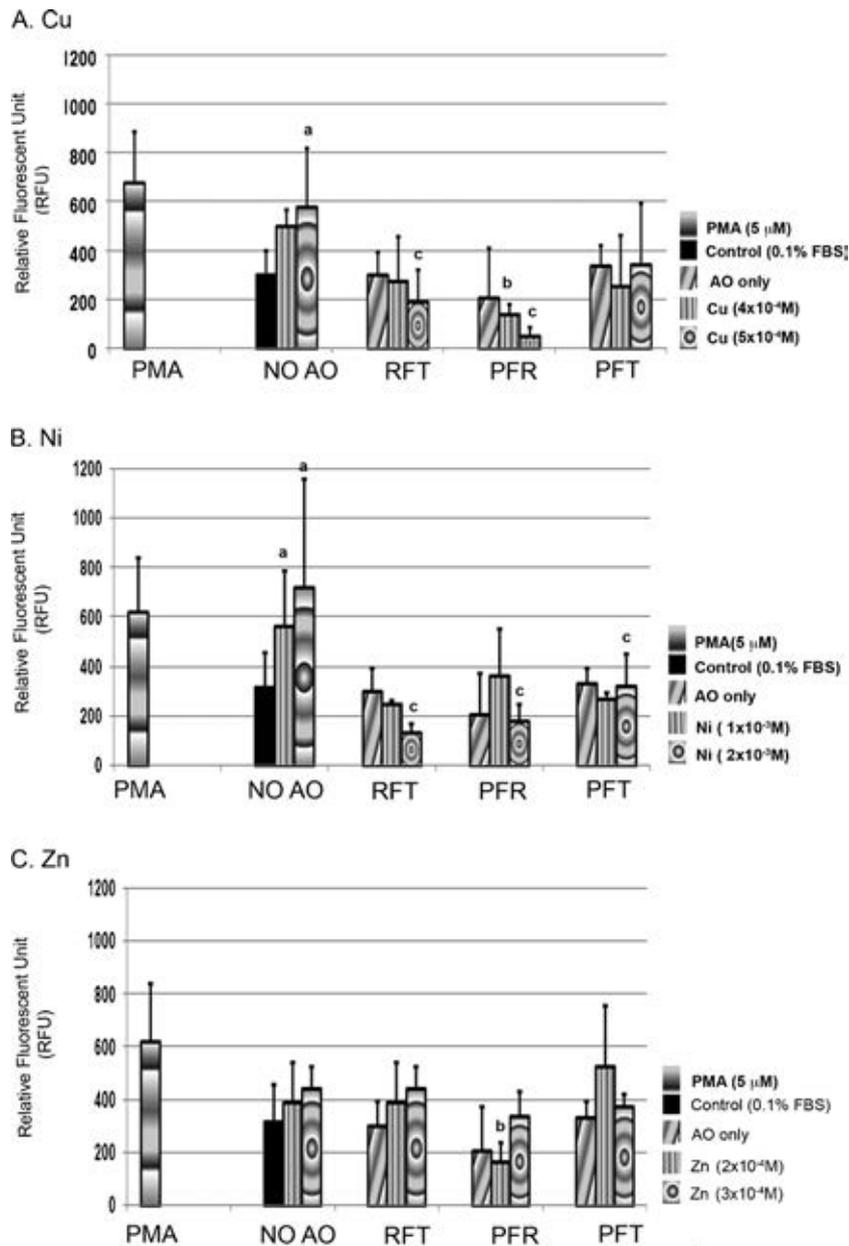
Fig. 5 – Specific AO combinations suppressed HGF responses through generation of reactive oxygen species. Cells were treated with the AO combinations (RFT, PFR, PFT) before exposure to the ROS inducing stressors (A, Cu, B, Ni and C, Zn) for 30 min and then analyzed by a ROS detection reagent, H_2DCFDA. Data are mean ± S.D. obtained from 2–3 culture wells per condition. $p < 0.05$ was considered to be statistically significant.

(Fig. 5A[a]). Cells that were pretreated with AOs had reduced ROS levels in most experimental groups compared to (Fig. 5B[b,c]). Cells treated with AOs only were not significantly different from the 0.1% control.

As expected, HGF cells responded to Cu with a dose mediated increase in ROS activity with increasing concentrations of Cu. Cu at 4×10^{-4} M and 5×10^{-4} M increased ROS activity by more than 1.5 fold when compared with the control (0.1% FBS, Fig. 5A[a], $p < 0.05$). In HGF cells pretreated with triple AO combinations, ROS activity was lower than cells not pretreated with AOs. PFR significantly reduced ROS induced at both Cu concentrations (Fig. 5A[b,c], $p < 0.05$). RFT significantly

decreased ROS activity in cells exposed to the high concentration of Cu (5×10^{-4} M) (Fig. 5A[c], $p < 0.05$).

HPDL cells exposed to Cu (4×10^{-4} M) alone had increased ROS levels (Supplementary Fig. 5A, no AO group). In cells treated with RFT, there was a significant ($p < 0.05$) reduction in ROS activity of cells exposed to Cu (4×10^{-4} M). There were no significant differences noted among groups of untreated, or PFR, and PFT pretreated and Cu ion exposed cells.

The generation of ROS in Ni exposed HGF cells was significant and dose dependent at both concentrations (Fig. 5B[a], $p < 0.05$). The increased ROS activity at the high Ni concentration (2×10^{-3} M) was counteracted significantly by

all AO pretreatments between a 2.5 and 3 fold change (Fig. 5B[c], $p < 0.05$).

In HPDL cells, both 1×10^{-3} M and 2×10^{-3} M Ni concentrations elicited slightly higher ROS activity compared to control (0.1%FBS) cells (Supplementary Fig. 5B). The AO pretreatments did not have a significant effect on ROS (Supplementary Fig. 5B).

ROS production in Zn treated HGF cells was not significantly different from the control cells (Fig. 5C). PFR was the only AO combination that changed ROS activity significantly (Fig. 5C[b], $p < 0.05$).

In HPDL cells, all Zn concentrations also had no effect on ROS activity (Supplementary Fig. 5C). The AO pretreatment also did not produce significant changes in ROS activity in these groups (Supplementary Fig. 5C).

4. Discussion

The purpose of this study was to test the ability of specific AO combinations to counteract the effects of the metal stressors Cu, Ni and Zn on cultured oral fibroblast viability, DNA synthesis and ROS activity. The disruption of metal ion homeostasis may lead to increased formation of reactive oxygen species (ROS), which can affect AO protection and promote DNA damage, lipid peroxidation, and protein modification.[1] There is, however, no study that has investigated the efficacy and potency of treatment of AO combinations on metal-induced toxicity and oxidative stress in different cellular types, due to the broad and complex nature of the AO combinations that were used in this study. Hence, we hypothesized that these AO combinations may protect the cells against metal-induced toxicity and oxidative damage.

One of the aims of this study was to provide evidence of the extent to which these metal ions caused cytotoxicity in oral fibroblast cultures. Some studies have relied on indirect measurements of free radical activity in biological fluids[19,20] and in cell culture models.[21,22] The exposure of oral fibroblasts to these metal ions (Cu, Ni and Zn) was attributed to cytotoxicity, which was supported by assays for viability, DNA synthesis and ROS activity.

It has been recognized that the cytotoxicity induced by Cu-stimulated redox cycling led to an increased intracellular levels of ROS in hepatocytes,[23] cancer cell lines,[24] and in lung fibroblasts.[25] A previous study revealed that apoptosis markers, DNA fragmentation and caspase 3 activation were the end result of Cu cytotoxicity in gingival fibroblasts.[8] In another study, Cu was one of the metals that triggered the induction of necrotic cell death in mouse osteoblasts.[26]

In the case of Ni, the concentrations of Ni ions released from orthodontic brackets reduced viable cell numbers and DNA synthesis of both periodontal and gingival fibroblasts more than 50%,[27] and also induced DNA damage in oral cells.[28] Under conditions of low toxic concentrations, Ni also caused alterations in various tissues and organs. The toxicity levels range from 0.00004 mM (4×10^{-5} M) and became lethal to humans at 0.06 mM (6×10^{-2} M) in whole blood.[29] It was also reported that Ni exposure led to decreased lipid peroxidation (LPO) in a concentration dependent manner in a human

placenta model.[30] A case study confirmed that nanoparticles of Ni were a toxicological hazard causing adult respiratory distress syndrome in an occupationally exposed worker.[31] In in vitro culture of mouse neuroblastoma cells, Ni at high concentrations significantly inhibited cell proliferative potential.[32] Oral administration of Ni sulphate for 35 days to mice/rats decreased reproductive tissue weights, sperm count and motility.[33] The exposure of female rats to Ni before copulation, during gestation and lactation also reduced the number of pregnancies and live pups born.[34]

Zn toxicity is associated with mitochondrial dysfunction, an elevation in the production of ROS,[1] and the inactivation of glutathione reductase (GR).[35–38] It is also possible that decreased oral fibroblast number following exogenous application of Zn was due to the dissolved Zn^{2+} in the culture medium or inside cells.[39] During the course of investigating the toxicity of Zn to these two types of cultured fibroblasts, we observed that HGF was more sensitive to the action of Zn ions.

Having demonstrated evidence for a dose-dependent growth inhibition effect of high and low concentrations of the metal ions (Cu, Ni and Zn) to oral fibroblasts, we administered an AO combination treatment approach to counteract the cytotoxic effects in this in vitro model. In our study, it appeared likely that there were some specific types of oxidation markers necessary for the oral fibroblast response to Cu, Ni, and Zn exposure, which could be associated with lipid, DNA, or protein oxidation. However, none of these traditional markers of ROS activity had been measured to rule out oxidation products after Cu, Ni and Zn exposures in oral fibroblasts. AO counteracted Cu, Ni and Zn exposure in oral fibroblasts, which may be associated with the basic redox status and AO status of the oral fibroblasts in culture. The results obtained from the present study suggest that AO combinations may have protective effects against metal-induced toxicity. Others have shown that AO protective mechanisms against the effects of metal ions may involve the induction of AO genes.[40–43] Cytotoxicity itself induces oxidative stress in oral fibroblasts or may affect some AO genes, which may have a role in the susceptibility to the effects of metal ions.[7,43–47] It is also interesting to note that one of the AOs, curcumin, showed a significant and concentration dependent effect on Cu-induced oxidative damage in primary rat cortical neurons.[48] A previous study revealed that other forms of individual AO compounds such as catalase, provided protection against the oxidative stress induced by Ni.[49] The mediated effects of AOs (glutathione (GSH)/mannitol) revealed that Ni-induced human lymphocyte toxicity may be influenced by oxygen radical intermediates through generation of OH.[49] The protective effect of a specific flavonoid known as naringin, attenuated the alterations in renal and urine markers, decreasing lipid peroxidation markers against Ni toxicity in kidney.[50] The grape seed procyanidin extract had potential benefits against Ni-induced toxicity in rat reproductive organs by enhancing sperm motility by down-regulating c-kit expression and reducing the levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), nitric oxide (NO), and Bax expression.[51] Many protective agents have reduced the toxicity of Zn in cultured brain cells, which include glutathione GSH and its precursor N-acetylcysteine,[52–54] pyruvate,[55,56] carnosine[57] and zinc chelators.[58] Our previous study showed

that the biochemical insults found in tooth whitening products, tobacco, and alcohol; (hydrogen peroxide (H_2O_2)), nicotine (Nic) and ethanol (EtOH) significantly affected cell viability, DNA synthesis and ROS activity.[59] The AO combinations of RFT, PFR and PFT protected oral fibroblasts from the damaging effects of H_2O_2, EtOH and Nic by decreasing total ROS and increasing cell viability and DNA synthesis.[59] Knowledge of the mechanisms of the effects of oxidative stress should in the future allow the development of potent AO therapies.

In conclusion, these data clearly indicate that metal stressors may increase gingival cellular damage by decreasing cell viability, and proliferation while increasing ROS. Moreover, these pure AO products may have complementary or synergistic mechanisms to counteract the detrimental effects of the metal stressors to decrease ROS and increase cell viability and DNA synthesis.

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.2012.05.013

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Competing interest

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 declared.

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