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Bioactive antioxidant mixtures promote proliferation and migration on human oral fibroblasts

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ABSTRACT

Objective: Antioxidants (AOs) are the first line of defence against free radical damage and are critical for maintaining optimum health and well being. The need for AOs becomes even more critical with increased exposure to free radicals generated by pollution, cigarette smoke, drugs, illness, stress and exercise. Antioxidant supplementation is an excellent way of improving free radical protection. The aim of this study was to provide cytotoxicity, proliferation and migration data on the *in vitro* effects of bioactive AO mixtures on human oral fibroblasts.

Methods: Human oral fibroblasts were obtained from human gingival (HGF) and periodontal (HPDL) tissues. Each of these oral fibroblasts was cultured separately in three concentrations of the bioactive pure polyphenol and turmeric derivative mixtures; resveratrol (R), ferulic acid (F), phloretin (P) and tetrahydrocurcuminoids (T); [(RFT), (PFR), and (PFT)]. Cell viability, proliferation, morphology and migratory behaviour were analysed *in vitro* using high throughput *in vitro* 96 well plate wound assay.

Results: RFT decreased (10^{-3} M) and increased (10^{-5} M) cell number in HGF cells. Three concentrations (10^{-3}, 10^{-4}, and 10^{-5} M) of PFR and PFT increased DNA synthesis in HGF cells. PFT promoted cell migration but PFR and RFT had no significant change in HGF wound healing rates in a 96 well plate assay monolayer wound. In the HPDL cells, the 10^{-4} M concentration of both RFT and PFT increased cell number at 72 h and 96 h whereas the lower concentration 10^{-5} M of RFT significantly stimulated cell number at 96 h. PFR (10^{-3} M and 10^{-5} M) and PFT (10^{-3} M) increased DNA synthesis after 48 h treatment in HPDL cells.

Conclusions: High and low concentrations (10^{-3}–10^{-5} M) of these AOs (RFT, PFR) may have beneficial effects on functional mechanisms regulating fibroblast migration and proliferation during gingival healing or periodontal repair.

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1. Introduction

The role of antioxidants (AOs) in the oral and dental tissues is becoming an item of growing interest. Recent scientific studies have indicated the need for AO supplements for prevention and successful treatment of disorders associated with gingival tissues and other supporting structures of the teeth.[1–3] Multiple reports on resveratrol (R), ferulic acid (F), phloretin (P) and tetrahydrocucurminoids (T) have shown potential benefits in preventing or counteracting cellular damage, cancer, ageing, and other diseases.[4–7] Beneficial effects attributed to these polyphenol and turmeric derivative compounds include antioxidant, anti-angiogenic, anti-inflammatory, antiviral, and antitumor properties.[5–7]

One of the most potent polyphenolic phytoalexins is R (3,5,4′-trihydroxystilbene) that is found in green vegetables, citrus fruits and red grape wines. It is a stilbenoid, a derivate of stilbene, and is produced in plants by the enzyme stilbene synthase.[8] *In vitro*, R induced vascular endothelial growth factor production and increased vasculogenesis in endothelial progenitor cells from human peripheral blood.[9] The physiological concentrations of R may increase survival of human B cells.[10] Resveratrol has a dual-directional regulation effect on cell proliferation and cell cycle on MCF-7 cells (estradiol receptor-positive cells).[11] The incorporation of R in liposomes increased the rate of proliferation and survival of cells under stress conditions caused by UV-B light.[12] A recent study showed that R enhanced the ability of human bone marrow-derived mesenchymal stem cell (HBMSC) cultures to proliferate and undergo osteoblastic maturation.[13] In addition, R has a modulatory effect on cell cycle control and differentiation in cardiomyoblasts.[4] However, some studies showed an inhibitory effect of R on the proliferative potential of endometrial stromal cells[14] and in foetal calf serum (FCS) or platelet derived growth factor (PDGF)-induced 3T6 fibroblast.[15]

A component of raspberries, F, plays a regulatory role in cell proliferation of neural stem/progenitor cells (NSC/NPCs) cultured from the telencephalon of embryonic day-14 rats, with an increasing number and size of secondary formed neurospheres.[16] It is also involved in DNA synthesis and proliferation of nerve cells of retinas *in vitro* that can possibly be used as an alternative agent for the prevention and treatment of some degenerative retinal diseases.[17] F acts as an AO because it is a scavenger of free radicals such as reactive oxygen species (ROS) that are implicated in DNA damage, cancer, and accelerated cell ageing.[5,18] If F is added to a topical preparation of ascorbic acid and vitamin E, it may reduce oxidative stress and formation of thymine dimers in skin.[19] F exhibited potent anti-lipid peroxidative effects as well as the ability to modulate the status of carcinogen-detoxifying agents and AOs in 7,12 dimethyl benz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.[20,21] The inhibitory effect of F on rat tongue carcinogenesis was due to modifying the effects of a novel geranylated derivative, ethyl 3-(4′-geranyloxy-3′-methoxyphenyl)-2-propenoate (EGMP), synthesized from F, and initiated with 4-nitroquinoline 1-oxide.[22] F significantly reduced nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells (Raw 264.7 cells) compared to eugenol.[23] This reduction of NO production may be useful for preventing cell damage caused by superoxide O_2^-, and in particular by hydroxyl radical OH^- and NO, in living systems.[23]

One of the most important flavonoids derived from apples,[6] strawberries,[24] and tomatoes[25] is P. P possesses both AO and anti-inflammatory activities *in vitro* by acting as a transcription-based inhibitor of proinflammatory gene expression in chronic inflammatory bowel disease.[6] Recent studies made use of various parameters to show that P has anti-inflammatory and immunosuppressive effects by decreasing the proliferation-related index (PI) and NO production of LPS + Interferon gamma (IFN-γ) group of macrophages in T lymphocytes as well as its capacity to stimulate a cell cycle arrest at G_0/G_1 phase.[26] Previous work showed that P could promote 3T3-L1 adipocyte differentiation and adiponectin expression through peroxisome proliferator-activated receptor gamma (PPAR-γ) activation.[27] Moreover, microarray analysis demonstrated that several genes associated with lipogenesis, triglyceride storage, and insulin signal transduction were increased suggesting that P may be essential for preventing obesity associated inflammation, and insulin resistance.[27]

The antioxidant T originated from curcuminoids extracted from the roots of *Curcuma longa*, known as turmeric root. The functional role of T on wound healing and repair were observed on normal and diabetic mice.[7,28] Previous work assessed the efficacy of T treatment by utilizing a full thickness punch wound model in diabetic mice. The wounds of animals treated with T showed earlier re-epithelialization, improved neovascularization, increased migration rates of the involved cells such as dermal myofibroblasts, fibroblasts, and macrophages into the wound bed, and a higher collagen content.[7] In addition, earlier work using a cutaneous wound healing model established a close association amongst T, transforming growth factor-beta (TGF-β) and inducible nitric oxide synthase (iNOS).[29] It showed that topical application of T in wound beds led to an increased expression of TGF-β1 and iNOS in dexamethasone impaired-and unimpaired wounds.[29]

In this study the four AOs R, P, F and T in three combinations (RFT, PFR, PFT) were used. The aim of this study was to provide cytotoxicity, proliferation and migration data on the *in vitro* effects of these bioactive AO mixtures on gingival and periodontal cells. It was hypothesized that the use of these AOs may stimulate the HGF and HPDL cells to proliferate, migrate and undergo a variety of favourable cellular changes that occur during *in vitro* wound healing.

2. Materials and methods

2.1. Cell culture preparations

Human oral fibroblasts were obtained from human gingival and periodontal tissues of healthy nonsmokers with approval by Texas A&M Health Science Centre, Baylor College of Dentistry institutional review board. Each of these types of oral fibroblasts was separately cultured in

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high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO™ Invitrogen Corporation, Carlsbad, CA), 10% FBS, and 1% antimycotic/antibiotic (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B; Cellgro, Mediatech Inc.,VA). Cells from each tissue type were 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. Cells were passaged using a 0.25% trypsin solution (GIBCO™ Invitrogen, Corporation, Carlsbad, CA) and plated in new tissue culture flasks (FALCON™ BD Biosciences Discovery Labware, Bedford, MA). Cells were passaged when confluent. Passages 3–8 were used in all of the experiments. Human periodontal ligament fibroblasts (HPDL) isolated from freshly extracted human teeth as previously described[30] were used in parallel experiments as described for HGF.

2.2. Bioactive AO compounds used for testing

The bioactive AO compounds used included P (98.6%; MW-274; Kaden, Biochemicals, Hillsborough, NJ), F (99%; MW-194; Sigma–Aldrich, St. Louis, MO), T-CG (96.93%; MW-372; Sabinsa Corporation, UT), and R (98%; MW-228; Lalilab Durham, NC). Each combination used was a mixture of appropriate antioxidants (1/1/1) by weight so that the 40% solution contained 400 mg of the sum of components in 1 ml DMSO (99%; (400 mg/ml); Spectrum, Gardena, CA. A serial dilution (10^{-3} M, 10^{-4} M and 10^{-5} M) in molar concentrations of three AO compounds was used consisting of PFT; PFR; RFT. A vehicle control group with different concentrations of DMSO was included in all experiments.

2.3. AO treatment

Briefly, 5×10^3 cells were seeded into 96-well flat-bottomed microtiter plates (FALCON™ BD Biosciences Discovery Labware, Bedford, MA) and grown in 0.1 ml DMEM containing 10% FBS in 5% CO_2. Cells attached overnight at 80% confluency. Cells were serum starved (0.1% FBS) for 2 h prior to AO treatment. The media were replaced with DMEM containing 0.1% FBS with various concentrations of AOs (10^{-3} M, 10^{-4} M and 10^{-5} M) and incubated for 24 h, 48 h, 72 h and 96 h without changing the medium. Three to four replicate wells per AO concentration were used for all control (0.1% FBS, DMSO vehicle) and test samples.

2.4. Cell culture viability assay

Cell viability was assessed using a Cell Titre 96-Aqueous One Solution cell proliferation assay (MTS) kit (Promega, Madison, WI) after 24 h, 48 h, 72 h, and 96 h of AO exposure. The assay contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS and an electron-coupling reagent (phenazine ethosulfate; PES). Briefly, 20 μl of MTS solution was added to each well of the 96-well assay plate containing the samples in 100 μl of culture medium. The plates were incubated for 4 h at 37 °C in a humidified, 5% CO_2 atmosphere. The absorbance was recorded at 490 nm using a 96 well-plate reader. Three independent experiments were completed with three or four samples per treatment.

2.5. DNA synthesis

Briefly, cells were seeded to 96-well flat-bottomed microtiter plates and grown in 0.1 ml DMEM containing 10% FBS per well at 5×10^3 cells in 5% CO_2. Cells attached overnight at 80% confluency. Serum starvation was done before the cells were incubated in DMEM containing 0.1% FBS with various concentrations of AOs (10^{-3}–10^{-5} M, 10^{-7} M) for 48 h, without changing medium. After labelling with BrdU for 24 h, its incorporation into DNA was determined using a commercial kit (Roche Diagnostics, GmbH, Mannheim, Germany), according to the manufacturer's instructions. Five independent experiments were done with two or three samples per condition.

2.6. Apoptosis assays

2.6.1. Caspase-3 activity assay

Caspases (cysteine-requiring aspartate protease) are a family of proteases that mediate cell death and are important in the process of apoptosis. Caspase-3 activity was measured by using caspase-3 colorimeteric assay kit (Promega, Madison, WI), which is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the pNA moiety. Briefly, HGF/HPDLF cells (2.0×10^4 cells/well) were grown in 24 well plates and exposed to the different concentrations of bioactive AO mixtures for 48 h. The cells were washed with PBS at the endpoint and lysis buffer (50 μl) was added to each well. The plates were incubated on ice for 15–20 min. Cells were centrifuged at 4000 × *g* for 10–15 min. Cells in the plates were re-suspended by adding 40 μl of the assay buffer and 10 μl of the caspase substrate for incubation overnight at 37 °C. Absorbance was set at 405 nm. In addition to the DMSO control group, cells were also exposed to a known apoptotic inducer (Staurosporine, ST) and its inhibitor (Ac-DEVD-CHO) as experimental positive control groups. Three independent experiments were done in duplicate samples.

2.6.2. Image-iT™ LIVE Green Caspase Detection

HGF/HPDL cells in 24-well plates were cultured for 48 h with the highest concentrations of bioactive AO mixtures. In addition to the DMSO control group, cells were also exposed to a known apoptotic inducer (ST) and its inhibitor (Ac-DEVD-CHO) as experimental positive control groups. The cells were labelled with caspase (green) and propidium iodide (red) in a sufficient amount to cover the cells according to the manufacturer's instructions (Image-iT™ LIVE Green Caspase Detection; Molecular Probes, Invitrogen, USA). Cells were evaluated by fluorescence microscopy (Nikon wide field). Three independent experiments were done in duplicate samples.

2.7. In vitro static assay of wound healing

A high throughput format *in vitro* wound assay was used to analyse the effect of different concentrations of AOs on HGF and HPDL cell migration. Briefly, cells were seeded to 96-well microtiter plates at 1×10^4 cells per well. HGF and HPDL cell

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cultures were divided into two groups. Both HGF and HPDL groups were serum-starved in 0.1% FBS for 2 h.[31,32] A 96 well floating pin transfer device (VP Scientific VP-408FH, San Diego, CA) was used to create and standardize the wound areas for each well. Foam backing was inserted between the plates to provide resistance for the pins once it was placed into the well plates. The array was positioned in the top centre of each well and gently pulled across the plate. The plates were analysed for wound size. At the end time point, the wells were rinsed in PBS and fixation solution (3.7% formaldehyde, 0.2% Triton-X 100, 1× TBS) was added for 30 min. The cells were stained (0.25% Coomassie blue, 50% methanol, 10% acetic acid) at 0 h, 7 h, 14 h, and 24 h and air dried. Plates were imaged with a light scanner at 1200 dpi. Wound areas were measured using Nikon-Elements Software and NIH-Image J analyses, and expressed as mm.[2] Three separate experiments were done with three or five samples per condition.

2.8. *Statistical analysis*

Data was analysed using a SPSS statistical package for Mann–Whitney test of comparisons, with post hoc Bonferroni correction. The viability assay was repeated 3× with 4 wells/group. The p value was set at $p < 0.004$. For cell migration, the data were presented as the mean ± S.D. with $n = 3$–5 per treatment from two separate experiments. The p value was set at $p < 0.05$. For DNA synthesis, all experiments were repeated 5× with 2–3 wells/group. The p value was set at $p < 0.05$.

3. Results

3.1. *Morphology, viability and DNA synthesis*

Standard Giemsa staining was used to monitor the cell growth and determine morphological features. The cells were mainly spindle-shaped with red cytoplasm, a round purple nucleus observed at the centre of cells or at the margins of a cell from 24 h to 96 h after exposure to the antioxidants, with no differences in morphology between the groups (data not shown).

Cell viability after treatment with the various AO combinations (RFT, PFR, PFT) was analysed by a sensitive assay using the titre 96® Aqueous One Solution Reagent). In both HGF and HPDL cells, the DMSO vehicle did not adversely affect cell number compared to controls (Fig. 1A and B). RFT significantly increased HGF cell number at 24 h (10^{-4} M) and both RFT and PFR did so at 96 h (10^{-5} M). But the highest concentrations of RFT (10^{-3} M) and PFR (10^{-3} M) significantly decreased HGF cell numbers at all time points. PFT (10^{-5} M) also significantly decreased HGF cell numbers at 48 and 72 h (Fig. 1A).

HPDL cells reacted in a similar pattern to the AOs (Fig. 1B). RFT (10^{-4} M and 10^{-5} M) and PFT (10^{-4} M) significantly increased cell numbers compared to control at 72 and 96 h.

To investigate the effect of AOs on DNA synthesis in HGF and HPDL cultures, a AO dose–response test was completed (Fig. 2A and B) with only one time point (48 h). AOs stimulated BrdU incorporation into newly synthesized DNA in HGF cells

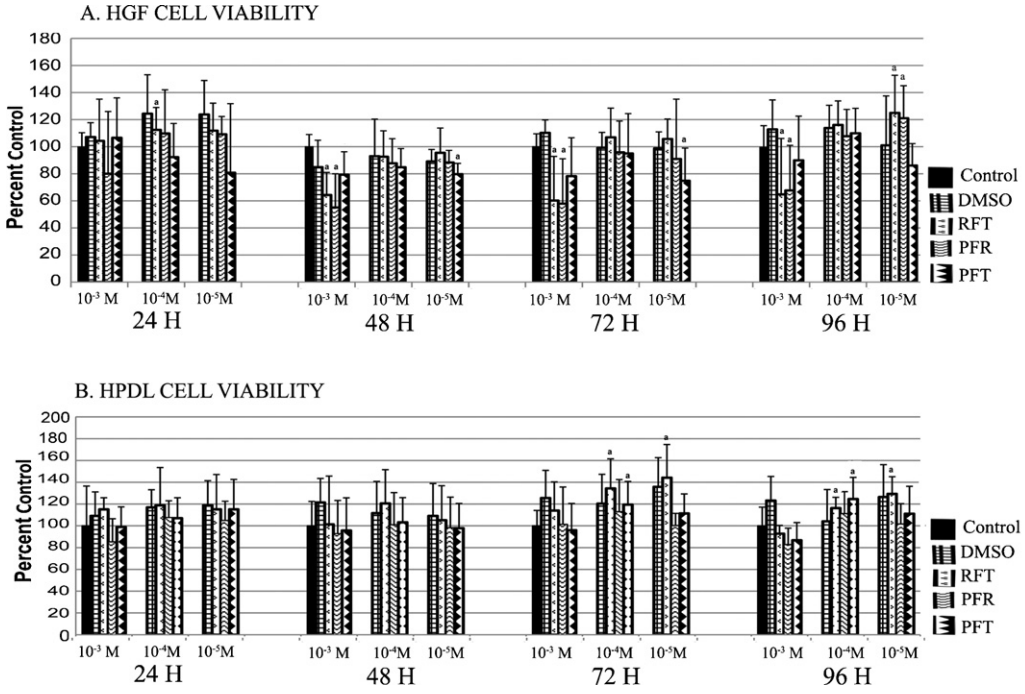

Fig. 1 – High concentration of AO mixtures decreased viability but low AO concentration increased viability. HGF (A) and HPDL (B) cells were incubated with control (0.1% FBS), DMSO vehicle or AOs for 24–96 h. The cell viability was determined by MTS assay. Controls (0.1% FBS) were set as 100% (black bars) and in other treatments were compared to controls. Higher AO concentrations (10^{-3} M) RFT and PFR significantly decreased HGF (A), but had no significant effect on HPDL cell viability (B). In contrast, low AO concentrations (10^{-5} M) significantly increased cell viability in HGF (RFT, PFR) and HPDL (RFT). Data are mean ± S.D. obtained from three independent experiments. $^{a}p < 0.004$ versus control.

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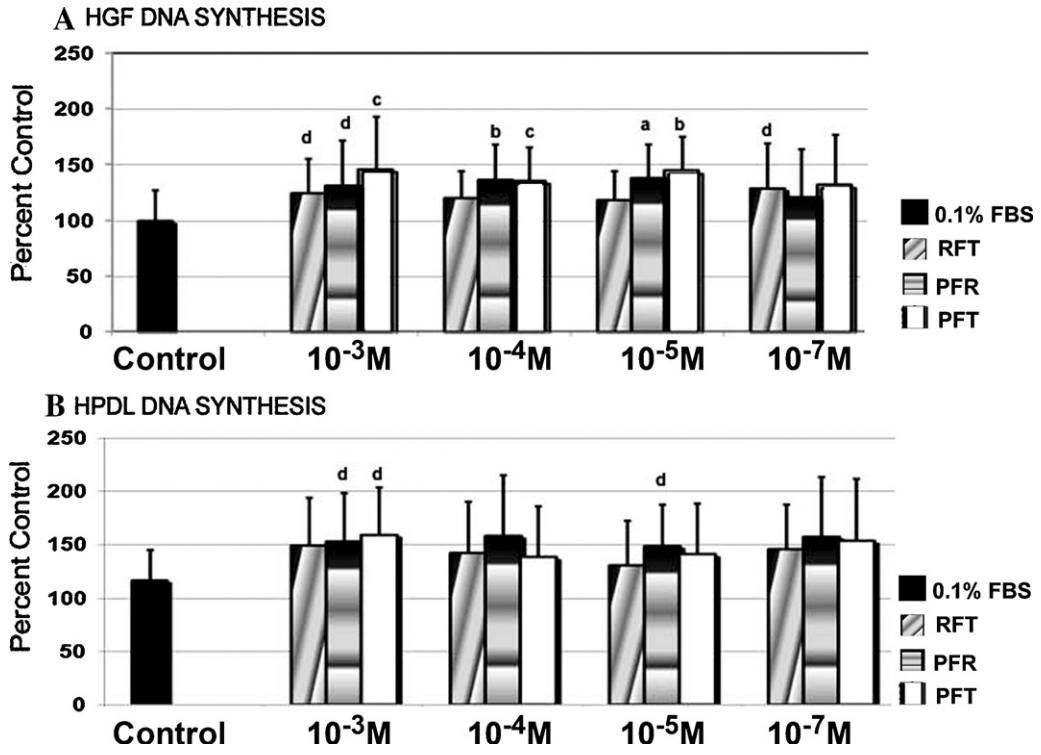

Fig. 2 – AO treatment induced stimulation of DNA synthesis in HGF and HPDL. Cells were incubated for 48 h with controls (black bars, 0.1% FBS) or the indicated AOs concentration and then analysed for BrdU incorporation. A, HGF cells responded to high concentration (10^{-3} M) by significantly increasing BrdU incorporation in RFT, PFR and PFT. B, The PFR at 10^{-3} M and 10^{-5} M as well as with 10^{-3} M PFT increased BrdU incorporation in HPDL cells. Data are mean ± S.D. obtained from 5 independent experiments. [a]$p < 0.001$; [b]$p < 0.005$; [c]$p < 0.01$; [d]$p < 0.05$ versus control.

with a significant effect of RFT at the highest and lowest concentrations (10^{-3} M and 10^{-7} M) whereas PFR and PFT stimulated DNA synthesis at a wide range of concentration 10^{-3}–10^{-5} M in HGF cells (Fig. 2A).

HPDL cells did not respond to RFT, but increased DNA synthesis in response to PFR (10^{-3} M and 10^{-5} M) and PFT (10^{-3} M) with 50–60% increase in BrDU incorporation (Fig. 2B).

3.2. *Apoptosis*

The AO molecules did not induce apoptosis at any concentration tested in assays using the activated caspase 3 as an outcome measurement (Fig. 3A and B). In a separate assay using Live Green caspase, there were no significant differences in apoptosis between groups (Fig. 4A and B). These experiments led to the conclusion that the AOs tested did not increase apoptosis in HGF or HPDL cells under these culture conditions.

3.3. *In vitro wound healing*

An established *in vitro* static assay model[33] was used to quantitatively define human oral fibroblast migration in a monolayer cell model by using NIH Image J software analysis (Fig. 5). The Coomassie stained wells were scanned (Fig. 5A) and then, images were converted into grayscale (Fig. 5B). An outline was delineated (Fig. 5C) to calculate the analysed wound areas expressed in mm^2 depending on the calibration setting. After 7–14 h of *in vitro* wound healing, a majority of the wounded areas showed differences between the HGF cells treated with high, and low concentrations (10^{-3}–10^{-5} M) of pure AOs and the 0.1% FBS (control group) in the rate of wound healing (Fig. 6A). However, only the PFT (10^{-5} M) group had a significant difference in wound healing rates compared with 0.1% FBS. RFT at 10^{-3} M and 10^{-5} M had a similar wound healing rate as the DMSO and 0.1% FBS controls. In the case of HPDL cells, none of the AO treatments had any effect on wound healing rates using this static assay (Fig. 6B).

4. Discussion

The purpose of this study was to test the hypothesis that AOs stimulate HGF and HPDL cells to undergo favourable cellular changes during wound healing in an *in vitro* model. The effects of three bioactive AO mixtures on HGF and HPDL cell viability, proliferation, apoptosis and migration were evaluated. These studies support several other investigations that reported AOs increased the number of endothelial cells[34] and modulated the growth of endometrial stromal cells.[35] However, similar to the findings reported here, higher AO concentrations inhibited cell proliferation in endometrial stromal cells.[35] Although this work used combination of AOs, studies using the individual AO, R, demonstrated an inhibitory effect on the foetal calf serum (FCS) and platelet derived growth factor (PDGF)-induced 3T6 fibroblast proliferation and DNA synthesis.[15] R has also

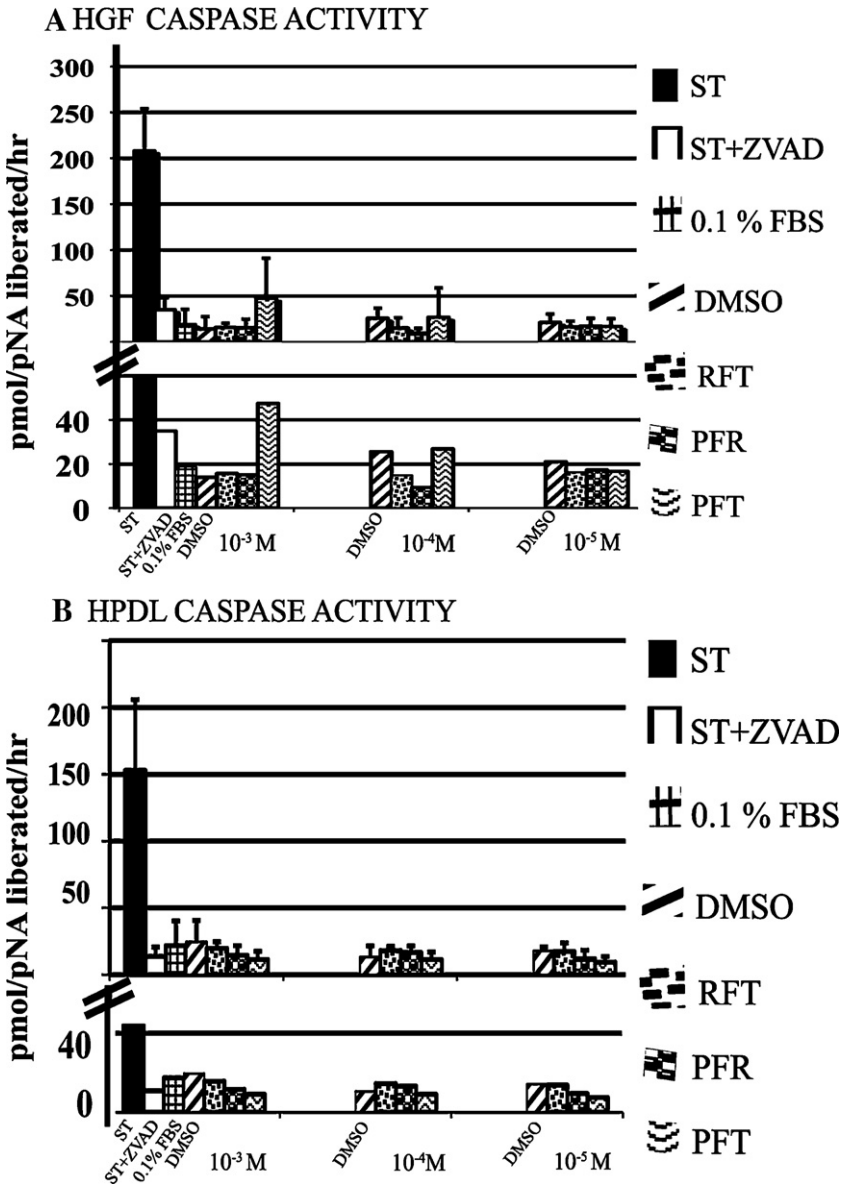

Fig. 3 – AO concentrations did not have a significant effect on apoptosis (caspase activity) in HGF and HPDL treated cells. HGF (A) and HPDL (B) cells were treated for 48 h with 0.1% FBS (control, notched bars), diluent [DMSO (0.1%, 0.01%, 0.001%, diagonal line bars)], caspase inhibitor z-VAD-FMK (50 μM; z-VAD, white bars) alone, Staurosporin (ST; 1 μM, black bars), or 1 h with z-VAD, white bars. The assay controls (ST-treated cells) indicated the results were valid. Data are mean ± S.D. obtained from three independent experiments using duplicate wells/treatment group.

been shown to directly stimulate cell proliferation and differentiation in osteoblastic MC3T3-E1 cells.[36] In carcinogenesis, R or a combination of R and quercetin, in concentrations equivalent to that present in red wines, are effective inhibitors of oral squamous cell carcinoma (SCC-25) growth and proliferation.[37] R at 10–100 μM may act via modulations in cyclin-dependent kinase (cdk) inhibitory machinery that result in a G_1-phase arrest of the cell cycle followed by apoptosis of human epidermoid carcinoma (A431) cells.[38] R induced WAF1/p21 that inhibits cyclin D_1/D_2-cdk6, cyclin D_1/D_2-cdk4, and cyclin E-cdk2 complexes, thereby imposing an artificial checkpoint at the G_1-S transition of the cell cycle.[38] Thus, it is not surprising to see that combinations of RFT and PFR at the highest concentration (10^{-3} M) decreased cell viability in the HGF cultures without increase apoptosis.

One of the major compounds in Si-Wu-Tang (SWT) is F. SWT is the most popular Traditional Chinese Medicine formula for woman's health. It also consists of paeoniflorin, paeonol, gallic acid, Z-ligustilide, ligustrazine, butylphthalide, senkyunolide A and catalpol. SWT not only promoted cell proliferation of various types of human breast carcinoma cell lines (MCF7, BT474, MDAMB231, and SKBR3), but also increased the phosphorylation of human epidermal growth factor receptor 2 (HER-2), protein kinase B (AKT), and extracellular regulated kinase 1/2 (ERK1/2), as well as overexpression of HER-2, on MCF7 cells.[39] In the present study, the

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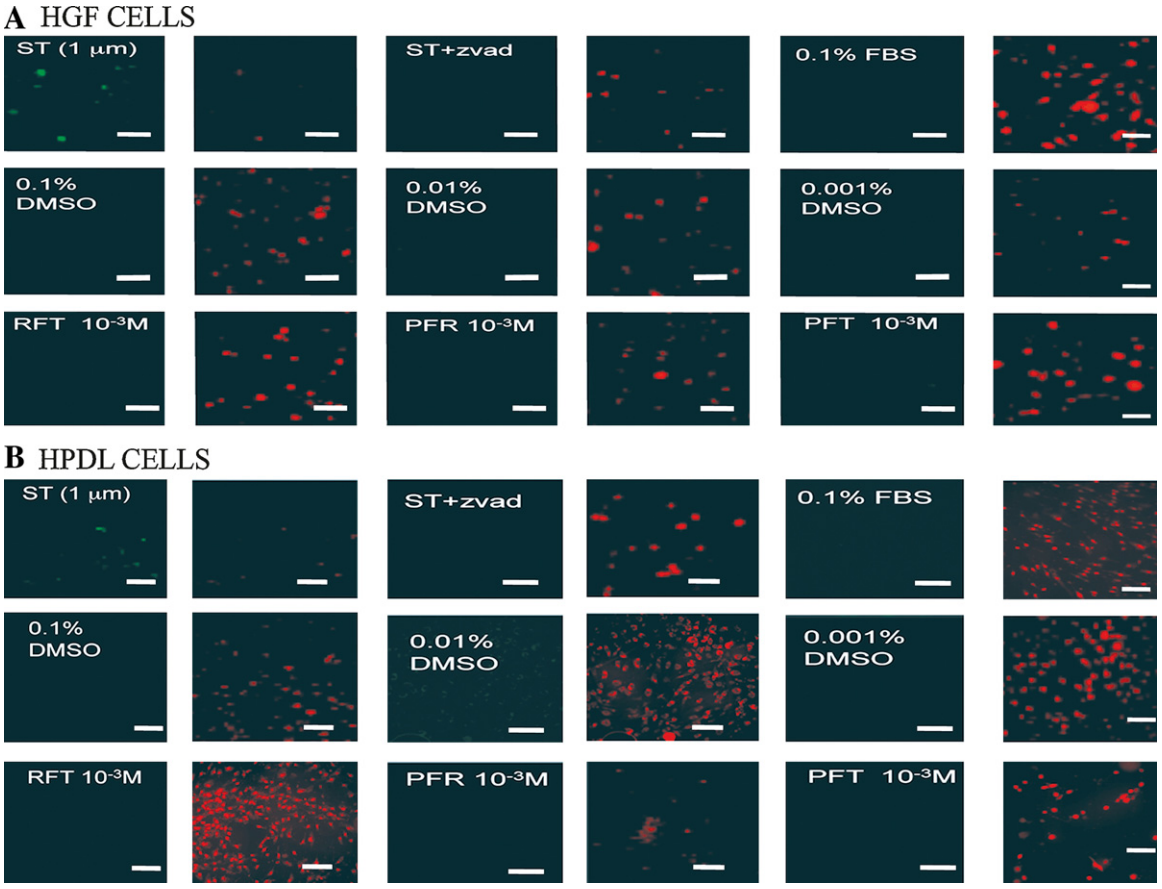

Fig. 4 – Image-iT ™ LIVE Green Caspase Detection. HGF (A) and HPDL (B) cells in 24-well plates were cultured for 48 h with the highest concentrations (10^{-3} M) of bioactive AO mixtures (PFT, PFR, PFT). In addition to the DMSO control groups, cells were also exposed to a known apoptotic inducer, Staurosporine (ST; 1 μM) and its inhibitor (Ac-DEVD-CHO) as experimental control groups. The cells were labelled with caspase (green) and propidium iodide (red) (Image-iT™ LIVE Green Caspase Detection; Molecular Probes, Invitrogen, USA). All fields had many nuclei (red) and all AO treatment groups had very low or no caspase staining in both HGF and HPDL cells. The ST treatment group had the highest caspase staining that was decreased in the zVAD controls. Cells were evaluated by fluorescence microscopy (Nikon wide field). Scale bar= 200 μm.

presence of increased numbers of viable cells in treatment groups can be largely attributed to a modest increase in the growth rate of RFT- and PFR-treated HGF cells. The decrease in viable HGF cells in groups treated with high concentrations of AOs may be attributed to a cytostatic effect of the compounds, since cells were resistant to necrosis-inducing effects of these AOs (data not shown). In HPDL cells, there was no inhibitory effect of all AO concentrations from 24 to 96 h but here again, a modest increase of HPDL cell number was noted at 10^{-4} M and 10^{-5} M RFT as well as with 10^{-4} M PFT.

The findings in HGF are in agreement with the proposal[40] that the dose–response to AOs is biphasic, implying that the moderate/low dose of AOs induced cell growth as opposed to higher doses that have cytotoxic[40,41] or cytostatic[42] effects. These investigators also evaluated the AO quercetin used for the colon cell lines HCT-116 and HT29 and the mammary adenocarcinoma cell line MCF-7, and demonstrated a significant biphasic effect of quercetin on cell proliferation in the colon cell lines. However, only a stimulating effect on cell proliferation was observed for the MCF-7 cell line.[40,41] The decreased cell numbers noted for the higher concentrations of AOs reported here might also fit with the antiproliferative effects of R reported with human endometrial adenocarcinoma termed Ishikawa cells, where growth inhibition results from a cytostatic mechanism, as indicated by colony formation assays, and the cells experience a significant prolongation of the S phase with a corresponding decrease in the G_1 phase.[43] For the HPDL cells, the modest increase or unchanged cell numbers can perhaps be attributed to a cytoprotective response to the different AO combinations, limiting cell death or apoptosis. However, further investigation of the mechanisms involved should be of great interest for future studies.

There is an apparent discrepancy between increased BrdU incorporation and decreased cell numbers seen at some of the concentrations and time points examined. The increased BrdU incorporation suggests increased DNA synthesis during the S-phase of the cell cycle over the 48 h incubation period. In the presence of fewer cells, it is likely that there is a heterogenous pool of cells, with the cells less sensitive to the AOs not progressing through the sensitive S phase into full cell division.[44] This idea is in agreement with the observed effect of R on Ishikawa cells, and the explanation that such an

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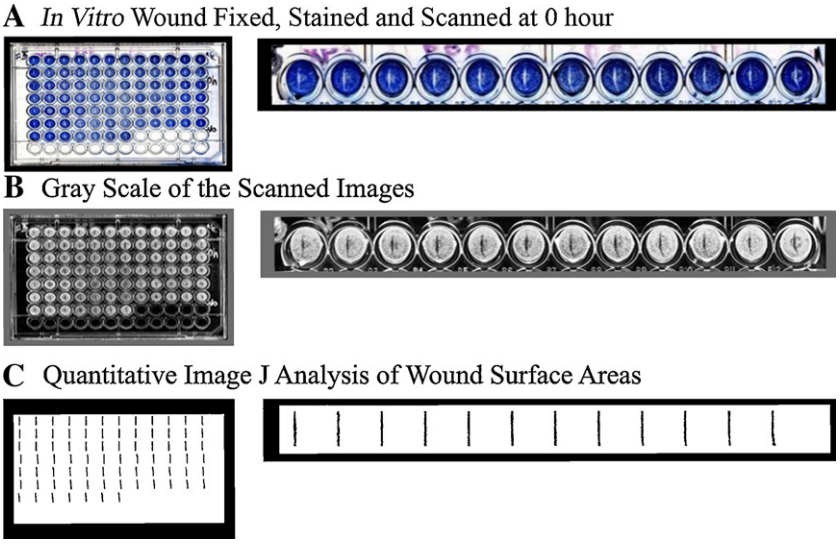

Fig. 5 – 96 well pin array *in vitro* wounds. (A) *In vitro* wound was fixed, stained and scanned at 0 h, (B) Grey scale of the scanned images and (C) Quantitative Image J analysis of wound surface areas.

increase in cell cycling in the S phase is suggestive of an arrest or slowing of the cell cycle machinery in S phase.[43] A similar mechanism was also observed amongst normal fibroblastic cells where it was shown that they possess normal mechanisms of growth regulation, until they are exposed to certain agents (antimetabolites) that perturb intracellular nucleotide pools causing growth arrest of these cells.[44] These researchers also established an association between the inhibitory effect

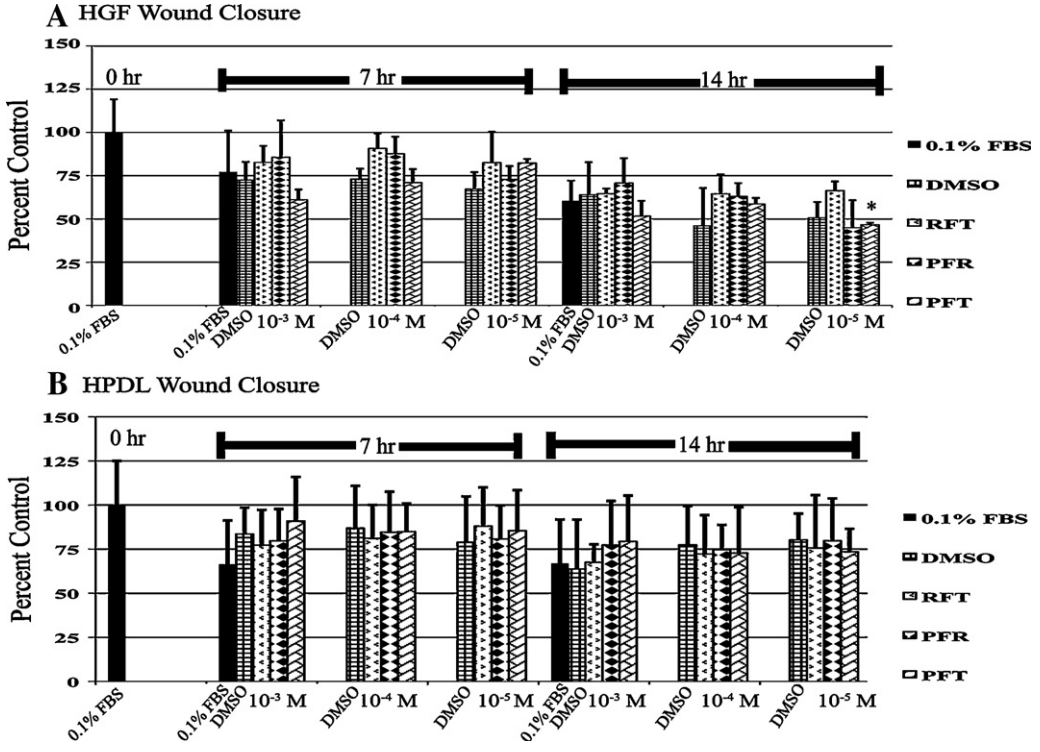

Fig. 6 – Wound area at 7 and 14 h AO treatment.HGF (A) and HPDL (B) cells were untreated (0.1% FBS), DMSO vehicle controls (0.1–0.001%) treated with AOs 2 h after *in vitro* wounds. At 7, and 14 h after wounding, the cells were fixed and stained with Coomassie blue to visualize the cells that had migrated into the *in vitro* wound area. Wound sizes were measured 7 and 14 h by tracing the wound margin and calculating the area using (NIH Image J). The wound areas were computed using a double blind system. The percentage of wound closure was calculated as: (area of original wound − area of actual wound)/area of original wound × 100. The data were presented as the mean ± S.D. n = 3–5 per treatment from two separate experiments. $^{*}p < 0.05$ versus control.

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of R and cell cycle progression, proposing a relationship between an increase in the percentage of cells in the S phase cycle with reduction of cells in G_0/G_1 and G_2/M phases in most cell lines.[45] The use of F at higher concentrations (1500 μM) did not cause strong toxic effects on human colonic cell line (Caco-2 cells), but it can affect the distribution of cells in the S phase. F also has a protective role against H_2O_2-induced cell damage in human dermal fibroblasts by reversing the loss of cell viability.[46] There are several studies that substantiate claims of cell growth induction through the use of pure AO compounds. The use of silymarin, a polyphenolic flavonoid with powerful AO activity stimulated cell growth in normal liver, kidney, and peripheral blood mononuclear cells.[47–50] To show the relationship between a known AO and glutathione precursor, N-acetylcysteine (NAC), and muscular cell proliferation, an *in vitro* culture model was established to test the effect of NAC on the growth of a rat skeletal muscle cell line (L6). NAC treatment stimulated concentration-dependent increased viability, cell number, and DNA incorporation in L6 cells from 12 to 24 h compared to control growth conditions.[51] Other studies also documented that F has its protective effects on neural progenitor cell proliferation,[16] and in a noise-induced ototoxicity.[52]

AOs are shown to prevent the free radical damage that is associated with normal and cancer cell lines. The mode of action of each AO varies, whether it is used singly, doubly or triply. In this study, there appears a possibility that these triple AO combinations of RFT, PFR, and PFT acted synergistically with each other to maintain cell viability but this will be a focus for future studies. Other factors that must be taken into consideration include; type of culture media used, sensitivity of cells, mode of action of toxic agents used. In the culture media alone, there are reagents that might mask possible features of inducing or toxic agents due to the presence of electrophilic biomolecules (amino acids, vitamins, etc.).[53]

To date, this is the first study to determine that triple AO combinations showed no toxic effects on cell viability. In addition, the tested concentration range (10^{-3}–10^{-5} M, and 10^{-7} M) for DNA synthesis is a prelude to further study on cell cycle regulatory effects in HGF and HPDL cells *in vitro* and *in vivo* experimental models. Since these AO combinations also did not cause apoptotic effects, it appeared that AOs may have direct effects on cell cycle. Some AOs inhibited normal cells in their growth, but protected them from apoptosis.[54] However, the apoptotic effects of AOs on oral fibroblasts following *in vitro* AO treatment had no effect on caspase-3 activation when compared to control cells. Therefore, AO treatment had no effect on the background level of apoptosis in the HGF and HPDL cells.

The results of this study have some implications for cell migration, which is a major process involved in wound healing. This *in vitro* wound assay typically involves culturing a confluent cell monolayer and then creating a scratch in the monolayer of cells. Areas of open wounds were visualized through a light microscope or a live cell imaging device over period of time as the cells from the leading edge of the wound move to fill the gap. The wound healing process can last from a few hours to days, which is dependent on cell type, conditions, and the wound surface area.

We hypothesized that the AOs tested may promote faster wound healing rate, but only one triple combination (PFT) showed a significant effect in HGF cells with little effect on HPDL wound healing using this assay (Fig. 6A and B). One of the disadvantages of the high throughput assay in a 96 well plate using a pin array to create the wounds is the lack of a well-defined wound surface area. The amount of force exerted in using the pin arrays created wound areas with varying sizes and widths with high variation from well to well. These variations likely influenced and slowed down cell migration into the wound surface area. We used two modes of measurement provided by software programs from Nikon Elements and NIH Image J. The representative images (Fig. 5) were all taken from Image J analysis detailing the method that was used for final measurements. The difference between these two modes of measurement is that in Nikon Elements the stained plates were not converted into grey scale mode. If the cells were not uniformly stained with Coomassie blue, artefacts may remain unnoticed and interfered with measurement values. In the NIH image J program, the images were converted into grey scale to delineate the extent of the wound. However, variations existed that could not be avoided by setting the baseline. When a live cell imaging station with an inverted phase/fluorescent microscope in an incubator with computer controls to record specific regions of interest (ROI) over time was used,[55] combinations of double and triple AOs showed greater effects than single AOs on individual cell migration rates. Triple combinations, PFR and RFT clearly and unambiguously counteracted the effects of nicotine and significantly increased migration rates in both HGF and HPDL cells.[55]

The present study is the first to demonstrate that combinations of pure bioactive AO mixtures were not cytotoxic to HGF and HPDL cells. In addition, proliferation and migration using various dose concentrations, did not cause alterations in apoptosis. Future studies are necessary to examine the effects of these AO mixtures on gingival and periodontal wound healing both *in vitro* and *in vivo*.

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Competing interests

The authors report no conflicts of interest related to this study, except LAO who has an ownership interest in PerioSciences.

Ethical approval

Not required.

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