Biological impacts of resveratrol, quercetin, and *N*-acetylcysteine on oxidative stress in human gingival fibroblasts

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In periodontitis, production of reactive oxygen species (ROS) by neutrophils induces oxidative stress and deteriorates surrounding tissues. Antioxidants reduce damage caused by ROS and are used to treat diseases involving oxidative stress. This study summarizes the different effects of resveratrol, quercetin, and *N*-acetylcysteine (NAC) on human gingival fibroblasts (HGFs) under oxidative stress induced by hydrogen peroxide. Real-time cytotoxicity analyses reveals that resveratrol and quercetin enhanced cell proliferation even under oxidative stress. Of the antioxidants tested, resveratrol is the most effective at inhibiting ROS production. HGFs incubated with resveratrol and quercetin up-regulate the transcription of type I collagen gene after 3 h, but only resveratrol sustained this up-regulation for 24 h. A measurement of the oxygen consumption rate (OCR, mitochondrial respiration) shows that resveratrol generates the highest maximal respiratory capacity, followed by quercetin and NAC. Simultaneous measurement of OCR and the extracellular acidification rate (non-mitochondrial respiration) reveals that resveratrol and quercetin induce an increase in mitochondrial respiration when compared with untreated cells. NAC treatment consumes less oxygen and enhances more non-mitochondrial respiration. In conclusion, resveratrol is the most effective antioxidant in terms of real-time cytotoxicity analysis, reduction of ROS production, and enhancement of type I collagen synthesis and mitochondrial respiration in HGFs.

Key Words: human gingival fibroblasts, periodontitis, resveratrol, quercetin, *N*-acetylcysteine

Chronic periodontitis is an infectious disease associated with Gram-negative anaerobic or facultative anaerobic bacteria. After bacterial infection, the initial inflammatory response activates the immune system through cytokines and chemokines. Additionally, lipid mediators of inflammation are released by macrophages, which recruit neutrophils and polymorphonuclear cells. Neutrophils are the first line of defense and produce a variety of toxic products at the site of infection including reactive oxygen species (ROS).[1,2] ROS are rapidly generated, short-lived, and diffusible. ROS have been proposed as second messengers to mediate cellular responses, making them essential to many normal biologic processes. At low concentrations, ROS stimulate the growth of gingival fibroblasts and epithelial cells in culture, but at higher concentrations they induce tissue damage by initiating free radical chain reaction.[3–6] Under normal conditions, protective antioxidants (enzymes and redox molecules) rapidly repair the damage induced by ROS.[3,7] Antioxidants can be categorized according to their mode of action (preventative or scavenging),

location (intra-, extracellular, or membrane associated), solubility (water or lipid soluble), and their origin/source (natural or synthetic).[8] An imbalance between ROS and antioxidants may be a key factor in the initiation and development of periodontal disease.[9] The enhanced and continuous production of ROS and/or impaired function of antioxidants leads to oxidative stress, which progressively induces the destruction of periodontal structure, alveolar bone, and connective tissue.[1,2,4,9,10] In current treatments, antioxidants are used as an alternative to complement the traditional therapies to treat diseases involving oxidative stress, yet their mechanism of action are not clearly understood.[11]

Polyphenols are reactive metabolites abundant in plant-derived foods, particularly fruits, seeds, and leaves. In the tissues of the digestive tract, particularly the oral mucosa, active polyphenols reach their highest concentration.[9] Polyphenols are now attracting attention as potential sources of agents capable of inhibiting, reversing, or retarding the progression of diseases caused by oxidative stress and inflammatory processes.[12–17]

Among the wide range of polyphenols, resveratrol (trans-3,4',5-trihyrodoxystilbene) is widely available and found in red wine, among other sources.[18] Resveratrol is thought to have cardio-protective, anti-inflammatory, and anti-aging properties in animal model systems.[19] The mechanism of action has been widely debated and attributed to many targets, including SIRT1, cyclo-oxygenase 1, and AMP-activated protein kinase.[20,21] Resveratrol induces enzymes such as superoxide dismutase and glutathione S-transferase.[22] Quercetin is another polyphenol universally present in many natural sources such as onions, apples, broccoli, berries, and capers.[23] Quercetin represents a major polyphenol in the human diet. *In vitro* studies indicate that quercetin has diverse biological effects, including induction of apoptosis, anti-mutagenesis properties, and modulation of the cell cycle. Also, quercetin inhibits the activation of protein kinase C (PKC) and the release of histamine. [14] Another antioxidant is the thiol *N*-acetylcysteine (NAC), a glutathione (GSH) precursor.[17] NAC has a wide range of protective effects against DNA damage and carcinogenesis. These effects are related to the ability of NACs to modulate metabolism, gene expression, and signal transduction pathways and to regulate antioxidants and anti-inflammatory activities.[24,25]

Human gingival fibroblasts (HGFs) are the major constituents of gingival tissues and are responsible to maintain homeostasis by regulating collagen metabolism. Therefore, the promotion of collagen synthesis and suppression of ROS may contribute to the

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integrity of gingival tissues and prevention of periodontitis.[26] Few *in vitro* studies have identified a way to avoid or delay the establishment of periodontitis at early stages, when the clinical outcomes are still not evident. Most *in vivo* studies are focused on bone loss, which is the latest clinical outcome of periodontitis. We hypothesize that the use of antioxidants will lessen the damage caused by ROS in response to oxidative stress and retard the initiation of periodontitis. The aim of our study is to compare the biological effects of three antioxidants: resveratrol, quercetin, and NAC on cultured HGFs under oxidative stress induced by exposure to H_2O_2. We investigated whether the antioxidants induced cellular proliferation or cytotoxicity through dynamic monitoring of HGF cells. Further, we verified the ability of each antioxidant to limit the production of ROS in HGFs following oxidative stress. Real-time quantitative reverse transcription-PCR (qRT-PCR) assays were performed to determine if the antioxidants were capable of maintaining or restoring the functioning of HGFs during short and long periods of oxidative stress. Finally, to confirm whether the antioxidants had a direct effect on HGFs' cellular machinery, we measured oxygen consumption rates (OCR, mitochondrial respiration) and extracellular acidification rates (ECAR, non-mitochondrial respiration) during oxidative stress.

Materials and Methods

Cell culture. The HGF-1 cell line was purchased from ATCC® (CRL-2014™; Manassas, VA). Cells were plated in cell culture flasks (Cole Parmer International, Vernon Hills, IL), and suspended in Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals LLC, Santa Ana, CA), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma Aldrich, St. Louis, MO) and kept in a humidified incubator at 37°C, with an atmosphere of 5% CO_2. Subculture was performed before cells reached confluence. Cells were washed with phosphate-buffered saline and briefly trypsinized (0.25% trypsin-0.2% EDTA; Life Technologies, Carlsbad, CA) to detach the cells from the flasks. HGF-1 cells were used for assays from the 6th to the 12th passage.[27]

Dynamic monitoring of cell viability and proliferation assay. The xCELLigence system (ACEA Biosciences, San Diego, CA) was used following the manufacturer's protocol. The data representing the cell status are based on the measured relative change in electrode impedance and are expressed as cell index (CI), a unit-less parameter. The presence of more cells on the top of the electrodes affects the local ionic environment at the electrode/solution interface, increasing the CI. The CI also can vary based on the quality of the cell interaction with the electrodes. If the cells are more attached or covering a larger area of the electrodes, they will induce a greater change in the CI. HGF-1 cells were suspended in cell culture medium and adjusted to 10,000 cells per well and seeded into E-plate 16. The plates were connected to the RTCA single plate station. HGFs were incubated until reaching the stationary growth phase for 24 h, and CI values were recorded, normalized, and termed "Base value". After that, HGF-1 cells were stimulated with H_2O_2 (final concentration: 0.23 mM) for 48 h to induce oxidative stress. At the same time, designated groups were treated with various concentrations of resveratrol (Yamada bee Co., Okayama, Japan), quercetin (Extrasynthese, Genay, France), and NAC (Sigma Aldrich). Controls were treated with medium only. The CI value of each group was monitored and recorded every 15 min for 48 h.[28]

The effectiveness (as percentage value) of each antioxidant was calculated using the following formula: Effectiveness (%) = (Sample CI_t – H_2O_2 CI_t) / (Control CI_t – H_2O_2 CI_t) × 100, where Effectiveness is antioxidants' effectiveness, CI corresponds to Cell Index, and t represents a time point during the incubation period.

ROS production. HGF-1 cells were subcultured onto a glass-bottomed dish (Matsunami Glass Ind. Ltd., Osaka, Japan) with 5,000 cells per well. H_2O_2 (0.23 mM) was added to induce oxidative stress. Simultaneously, designated groups were treated with resveratrol (50 μM), quercetin (15 μM), or NAC (1.5 mM). The cells were incubated for 30 min at 37°C. CellROX® Green Reagent (Life Technologies) was used to identify intracytoplasmic ROS and NucBlue™ Live Cell Stain (Life Technologies) was used as a nuclear counterstain. ROS production by HGF-1 cells was viewed under a confocal laser microscope (Nikon, Tokyo, Japan).[29]

qRT-PCR. To observe how the cell cycle and functioning are arrested during oxidative stress, HGFs were subcultured (10,000 cells/well) and stimulated with H_2O_2 (1 mM) to induce oxidative stress in the presence or absence of resveratrol (50 μM), quercetin (15 μM), or NAC (1.5 mM) and incubated at 37°C, with an atmosphere of 5% CO_2 for 3 h. Cells were then washed with phosphate-buffered saline, fresh culture medium was added to the wells, and the plates were incubated for 3, 12, 24, 48, or 72 h. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using RevertTra Ace (Toyobo, Osaka, Japan). Real-time PCR was performed using SYBR Green (Bio-Rad Laboratories, Foster City, CA) and a real-time PCR system (Bio-Rad Laboratories) according to the following cycling parameters: initial denaturation at 95°C for 1 min, 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The following primers were used for mRNA amplification: type I collagen (forward): 5'-GTGGAAATGATGGTGCTACT-3', (reverse): 5'-TTAGCACCAGTGTCTCCTTT-3'. GAPDH (forward): 5'-GTATTGGGCGCCTGGTCACC-3', (reverse): 5'-CGCTCCTGGAAGATGGTGATGG-3'. mRNA levels were calculated by determining the relative copy number compared with GAPDH and the Ct of the control group was normalized to 1.[26,30]

Mitochondrial oxygen consumption. OCR and ECAR were measured in human gingival fibroblasts with a XF 24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). HGF cells were seeded in every well of a XF 24-well cell culture microplate (Seahorse Bioscience) at a density of 10,000 cells/well in 200 μl of DMEM and incubated for 24 h at 37°C in 5% CO_2. After replacing the growth medium with bicarbonate-free DMEM XF assay medium (containing 1% serum) supplemented with 5.5 mM glucose and 4 mM L-glutamine and pre-warmed at 37°C, selected wells were treated with resveratrol (50 μM), quercetin (15 μM), or NAC (1.5 mM). HGFs were pre-incubated at 37°C without CO_2 for 30 min before starting the assay.

The detailed protocol is as follows. 1) Basal OCR reading, 2) addition of 75 μl H_2O_2 through port A to each well, 3) addition of 75 μl oligomycin (ATP synthase inhibitor) through port B to each well, 4) addition of 75 μl of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; mitochondrial uncoupler) through port C and, 5) addition of 75 μl of antimycin A with rotenone (complex I and III inhibitors, respectively) through port D. Each step had three cycles with cycles consisting of 3 min mixing, 2 min waiting and, 3 min measurement.[31]

Statistical analysis. All data were expressed as mean ± SEM. For statistical analysis, differences between the groups were analyzed with one-way analysis of variance using SPSS 19.0 (SPSS Inc., Tokyo, Japan).

Results

Dynamic monitoring of antioxidant effects on cell viability and proliferation. We compared the effects of three anti-oxidants against ROS by recurrent measurements with xCELLigence in three independent experiments. In comparison with control cells, after 48 h of incubation, resveratrol stimulated the
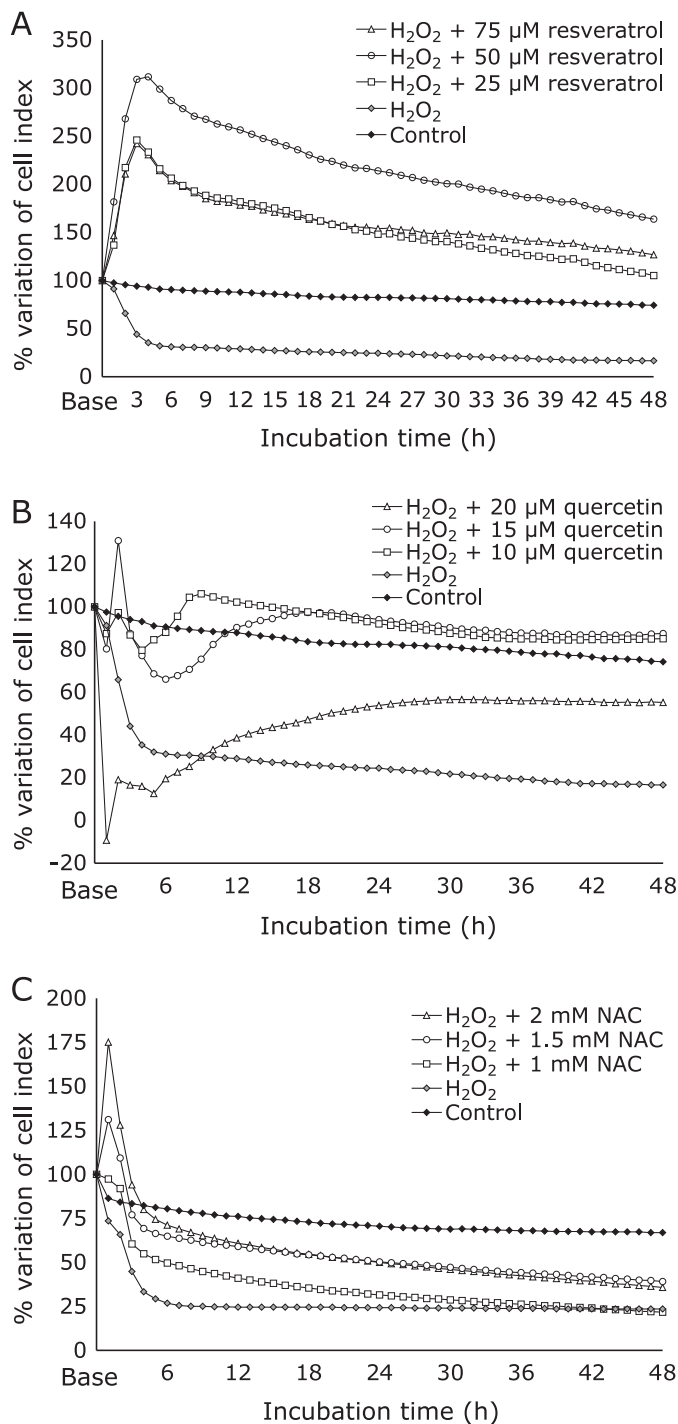
Fig. 1. Dynamic monitoring of cell viability and proliferation expressed as a cell index (CI) by xCELLigence system. HGFs (10,000 cells/well) were exposed to H_2O_2 (0.23 mM) to induce oxidative stress and treated with different concentrations of (A) resveratrol, (B) quercetin, and (C) NAC for 48 h. The percentage of variation of CI represents the changes recorded in CI values compared to the Base value. The graph shown is the result of the mean of three independent experiments (*n* = 6).

highest percentage of cell growth and proliferation, expressed as CI variation (Fig. 1A, Table 1). Quercetin also stimulated cell growth and proliferation, but levels were close to control group (Fig. 1B). NAC-treated cells did not stimulate cell growth or proliferation, reaching 40% of CI variation (Fig. 1C).

Table 1 shows that resveratrol was most effective at a concentration of 50 µM after 6, 12, 24, 36, and 48 h. The effectiveness of resveratrol declined progressively from 152% at 6 h to 85% at 48 h. Concentrations at 75 µM and 25 µM showed poorer results. Quercetin induced cytotoxicity at 20 µM (–7%) after 6 h of treatment, but this toxicity was gradually reverted and quercetin reached 22% of effectiveness after 48 h of incubation. At 15 µM, quercetin had 21% effectiveness compared with controls after 6 h of incubation. The effectiveness increased in a sustained manner and reached 41% after 48 h of incubation. NAC (1.5 mM) had an effectiveness of 86% after 6 h of incubation, which steadily decreased to 27% after 48 h. This was also observed with 2 mM (81%) and 1 mM (81%) following 6 h of incubation. After 48 h, 2 mM of NAC lost its effectiveness (1%).

Resveratrol and quercetin reduce ROS production during oxidative stress. Fig. 2 shows cell images produced by transmitted light after H_2O_2 stimulation with or without antioxidants. The strength of the green intracellular fluorescence corresponds to intracellular production of ROS. In the H_2O_2 group, ROS production was remarkably increased compared with the control group. The group treated with resveratrol (50 µM) greatly diminished ROS production, followed by quercetin (15 µM). NAC (1.5 mM) showed similar fluorescence intensity to the untreated group.

Resveratrol and quercetin promote upregulation of type 1 collagen gene transcription following oxidative stress. To investigate whether antioxidants alter gene expression levels of type I collagen following oxidative stress, we measured the amount of type I collagen mRNA expression in HGF-1 cells. Cells treated with resveratrol and quercetin showed significant upregulation of type I collagen, 1.70-fold ($p<0.05$) and 1.97-fold ($p<0.01$), respectively, following 3 h of ROS exposure. Collagen synthesis was downregulated in resveratrol-treated groups after 12 h, but was upregulated after 24 h ($p<0.05$). NAC did not stimulate the synthesis of type I collagen and rather decreased it in a constant manner from 0.30-fold (3 h) to 0.12-fold (72 h). The untreated group showed a sustained increase in gene expression, which reached its maximum (1.45-fold) at 24 h, and then progressively decreased in the following 48 (0.73-fold) and 72 (0.63-fold) h. The group exposed to 1 mM of H_2O_2 showed minimum gene expression, and its maximum value was 0.50-fold at 48 h of incubation. There was no significant difference found among the other groups (Fig. 3).

Resveratrol and quercetin enhance more mitochondrial respiration during oxidative stress. Because the mitochondria play a key role in controlling oxidative stress, mitochondrial respiratory profiles were examined following antioxidant treatment. The OCR was measured in HGFs following treatment with H_2O_2 and the mitochondrial respiratory capacity was observed in antioxidant-treated cells using the Seahorse XF^{24} flux analyzer. Compared with untreated cells, antioxidant-treated cells showed higher maximal respiratory capacity after 45 min (Fig. 4A). HGFs treated with resveratrol exhibited the highest maximal respiratory capacity, followed by quercetin and finally NAC.

After measuring OCR (mitochondrial respiration) and ECAR (non-mitochondrial respiration) simultaneously, and in real time, HGFs treated with resveratrol and quercetin stimulated more mitochondrial respiration than untreated cells. HGFs treated with NAC consumed less oxygen and enhanced more non-mitochondrial respiration (Fig. 4B). Finally, when we analyzed the spare respiratory capacity induced by each antioxidant, we found that HGFs treated with resveratrol or quercetin significantly increased the percentage of oxygen consumption to 28 and 20% more, respectively, compared with H_2O_2 treated group. There was no significant difference found between HGFs treated with NAC and H_2O_2 treated group (Fig. 4C).

Table 1. Effectiveness derived from real-time analysis of three antioxidants at different concentrations under oxidative stress induced by H_2O_2 (0.23 mM) on HGFs

	Concentration	Incubation Time				
		6 h	12 h	24 h	36 h	48 h
Resveratrol	75 μM	103[a]	88	75	73	63
	50 μM	152	134	110	100	85
	25 μM	104	90	72	65	51
Quercetin	20 μM	−7	6	17	22	22
	15 μM	21	36	40	41	41
	10 μM	34	43	39	39	39
NAC	2 mM	81	57	25	11	1
	1.5 mM	86	69	46	34	27
	1 mM	81	63	42	31	23

[a]Effectiveness is expressed as percentage value.
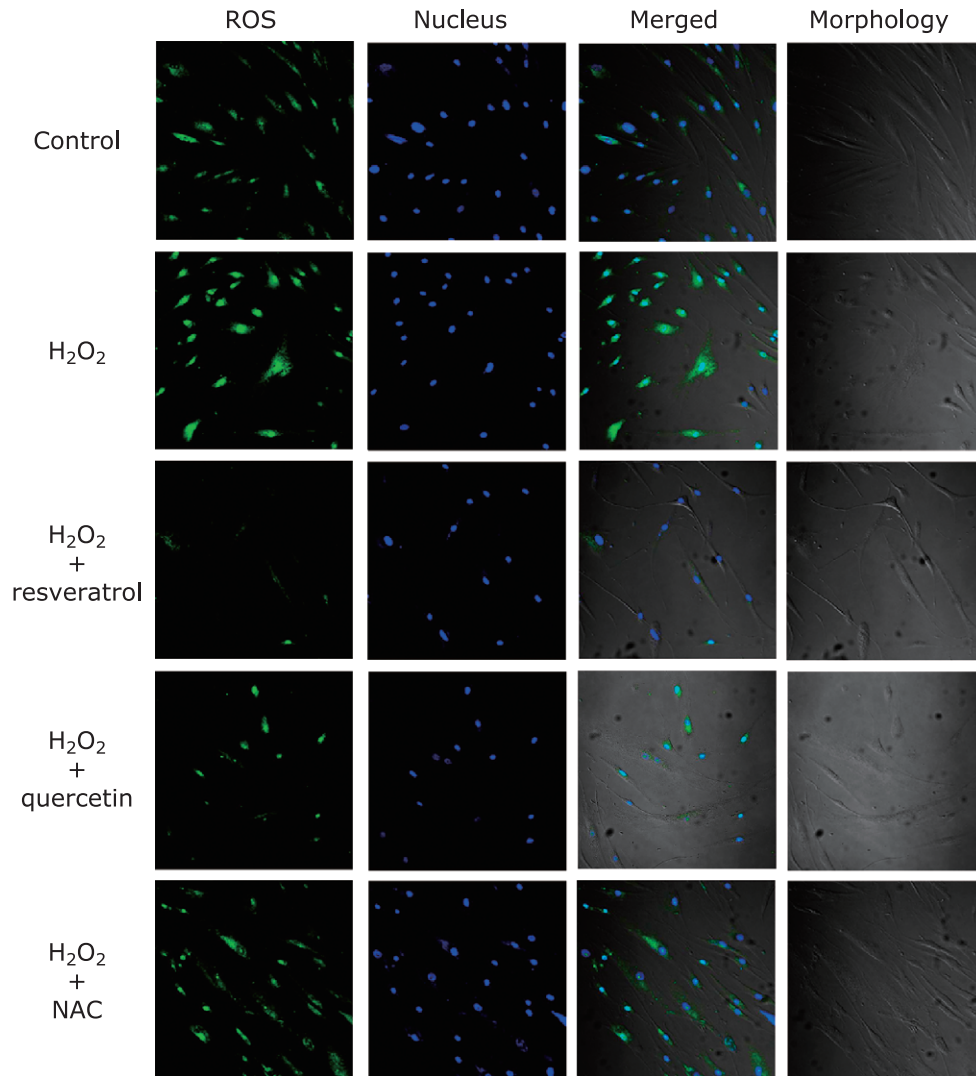
Fig. 2. Intracellular ROS scavenging activity of antioxidants in HGFs on oxidative stress. Representative double-stained, confocal laser microscopic images depicting ROS production induced by 0.23 mM H_2O_2 on HGFs (40×) with or without antioxidants after 30 min of incubation. Green fluorescence intensity corresponds to intracellular ROS production, and DAPI (blue) staining to viable cells. H_2O_2 affected the morphology of HGFs, which is typically large, flat, and elongated (spindle-shaped).
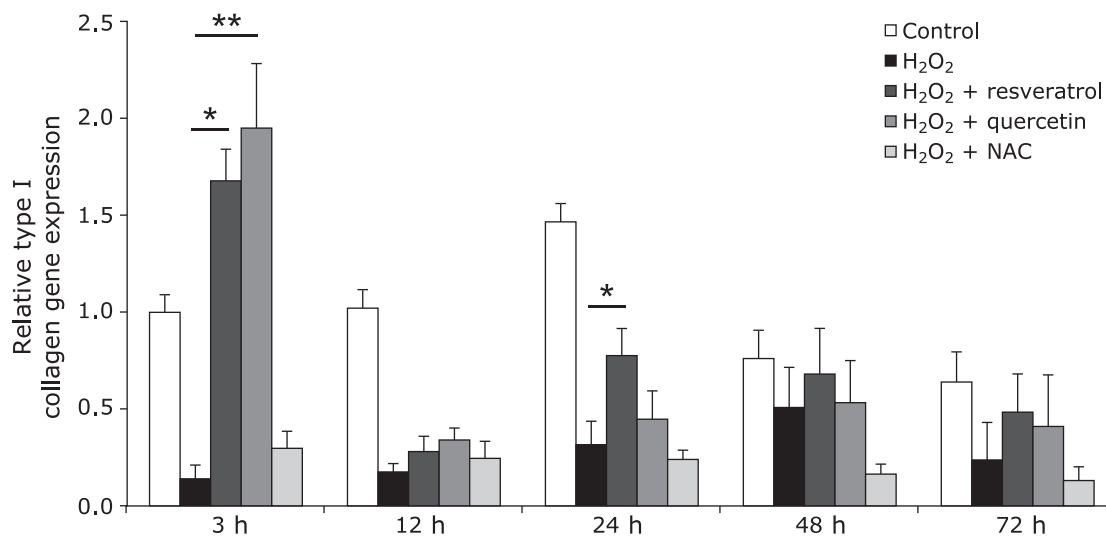
Fig. 3. Effect of resveratrol, quercetin, and NAC on type I collagen gene expression in HGFs under oxidative stress induced by H_2O_2 after different incubation periods. Relative mRNA of Type I collagen was detected by qRT-PCR. Bars represent mRNA expression normalized to GAPDH and relative to the control group. Data are expressed as mean ± SEM based on four independent experiments ($n = 9$). *$p<0.05$, **$p<0.01$: significantly different from H_2O_2 group.

Discussion

The effects of three different antioxidants were compared during oxidative stress induced by H_2O_2 in HGFs. Oxidative stress led to an increase in ROS production, which affected cellular viability, metabolism, and function. These findings demonstrate that antioxidants helped to control the detrimental effects produced by oxidative stress. In this study, the protective effects of resveratrol, quercetin, and NAC were examined. Resveratrol and quercetin enhanced cellular proliferation in oxidative stress conditions. The production of ROS was nearly inhibited by resveratrol, and greatly reduced by quercetin. Resveratrol and quercetin upregulated the synthesis of type I collagen gene after 3 h of incubation post-induction of oxidative stress, but only resveratrol kept it after 24 h. When the OCR was measured, resveratrol generated the highest maximal respiratory capacity, followed by quercetin and NAC, respectively. Further, the OCR and ECAR were evaluated and it was found that resveratrol and quercetin stimulated more mitochondrial respiration than cells without treatment. NAC treatment enhanced more non-mitochondrial respiration. Taken together, these results indicate that resveratrol was the most effective antioxidant to protect HGFs from damage under oxidative stress induced by H_2O_2.

Free radicals and other reactive small molecules have emerged as important regulators of many physiological and pathological processes.[32,33] In periodontitis, ROS serve as signaling messengers to induce immune responses.[5,33] Whether the effects of ROS are beneficial or harmful depends on the site, type, amount of ROS production, and the activity of the organism's antioxidant defense system.[33,34] As with other diseases related to ROS overproduction, periodontitis shows impairment in gingival fibroblasts' functioning owing to the toxic effects of ROS, resulting in reduced collagen synthesis, activation of metalloproteinases that break down type I collagen,[35] necrosis, and apoptosis of HGFs.[36]

In vitro and *in vivo* studies suggest that antioxidants can be used to supplement an organism's defense system and delay or prevent the onset of periodontitis.[13,15,17,37] Until now, most of these *in vitro* studies have only described the final outcomes of antioxidant treatments, but the effects of antioxidants during all the treatment and the changes within cells have not been reported, yet. In the current study, two real-time systems were used. First, the xCELLigence system (ACEA Biosciences) that monitors and detects any cellular physiologic change by measuring the impedance variation. Changes such as ion concentration or whether the cells are attached or not to the electrodes are monitored in individual wells.[38–40] Second, the XF Extracellular Flux Analyzer (Seahorse Bioscience), which measures both OCR (mitochondrial respiration) and ECAR (non-mitochondrial respiration) in real time. It is possible to observe three important factors of mitochondrial performance: ATP turnover, proton leak, and maximal respiration.[41]

The antioxidants studied showed different effects on cellular viability and mitochondrial respiration. A possible explanation for this is that the antioxidants have different mechanisms of action. Previous studies also indicate that resveratrol is able to provide protection against oxidative stress induced by metals owing to its antioxidant capacity and can enhance the cellular viability of fibroblasts.[42] Studies also show that quercetin enhances wound healing potential *in vitro* yet an explanation for this result is not fully explained.[43]

Evidence suggests that resveratrol modulates the mitochondrial respiratory chain through complexes I and III, which are known to be the major source of ROS in normal respiring mitochondria.[12] Resveratrol modulation of complex I is sirtuin dependent, which augments the mitochondrial substrate supply pathways (i.e., the tricarboxylic acid cycle and fatty acid oxidation) raising the mitochondrial $NAD^+/NADH$ ratio.[44] Resveratrol decreases complex III activity by competing with coenzyme Q. This property is especially interesting as this complex is the site where ROS are generated. By decreasing the activity of complex III, resveratrol not only opposes the production of ROS but can also scavenge them.[45,46]

Quercetin, the most represented polyphenol in the human diet, is usually present as a glucoside-conjugate or as a methyl-glucoside-conjugate.[23] In addition to antioxidant activity, *in vitro* studies show that quercetin has a variety of actions including anti-mutagenic and anti-tumorigenic activities,[47] long-lasting anti-inflammatory activity,[48] protein kinase C inhibition, superoxide dismutase-like activity, and modulation of the cell cycle.[14] Despite all these benefits, quercetin has low solubility in water resulting in poor absorption and low bioavailability, limiting its potential clinical application.[49,50] Quercetin glucosides need to be
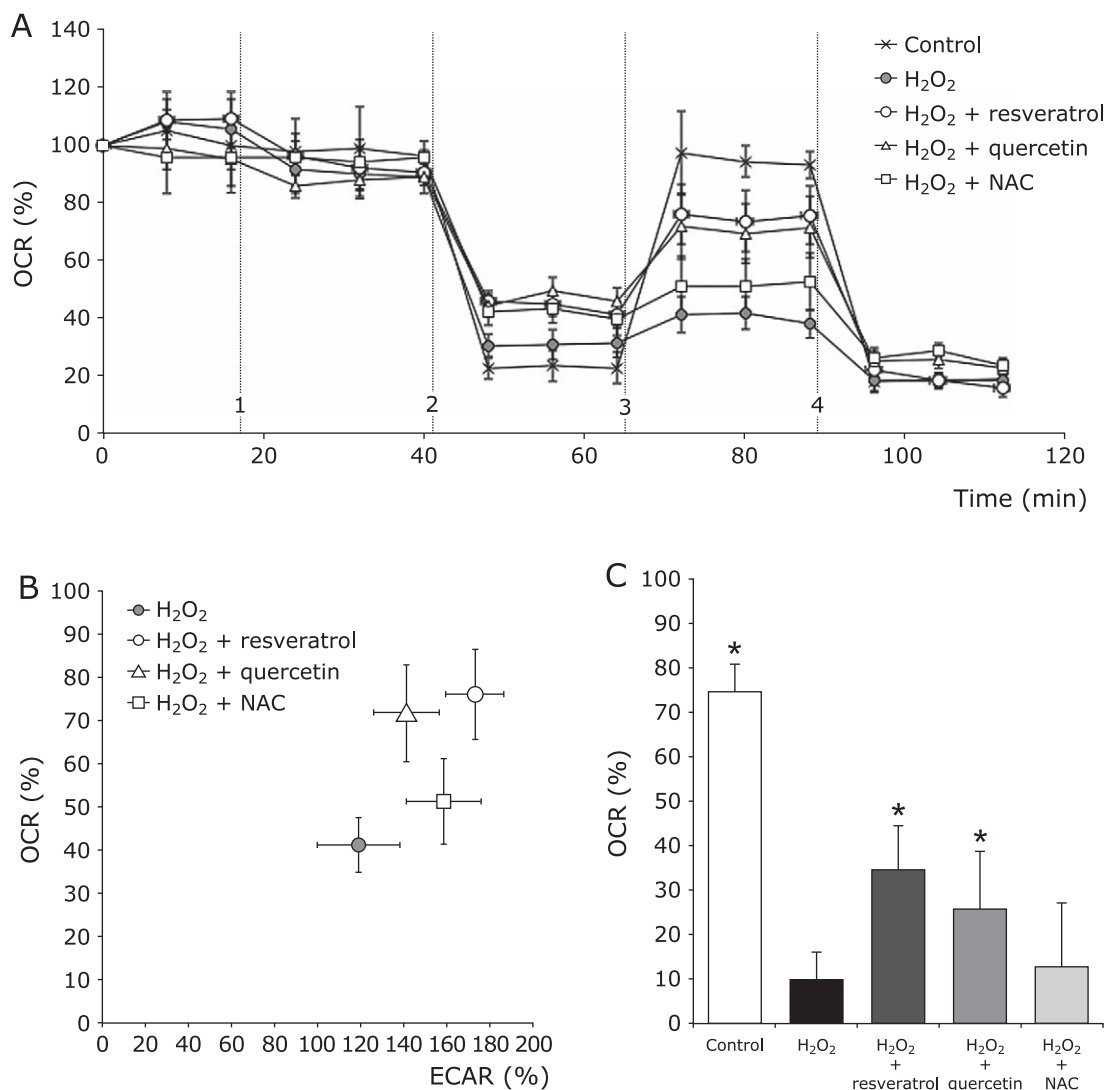
Fig. 4. Effect of antioxidants on mitochondrial respiratory profiles in HGFs under oxidative stress. (A) Changes in HGFs' cellular respiration under oxidative stress. OCR was measured in HGFs cells after 30 min of treatment with resveratrol (50 μM), quercetin (15 μM), and NAC (1.5 mM). Seahorse OCR protocol (mitochondrial stress test) included (1) H_2O_2 (1 mM) and three mitochondrial ETC complex inhibitors: (2) oligomycin, (3) FCCP, and (4) antimycin A with rotenone. OCR measured before (1) represents basal respiration, between (3) and (4) represents maximal respiratory capacity. Plots shown are means ± SEM ($n = 6$). (B) Simultaneous comparison of OCR versus ECAR in HGFs stressed by H_2O_2 (1 mM) and treated with resveratrol (50 μM), quercetin (15 μM), and NAC (1.5 mM). A higher OCR value indicates predominance of mitochondrial respiration. Similarly, a high ECAR value indicates predominance of non-mitochondrial respiration. Plots indicated the SEM ($n = 6$). (C) Antioxidant treatment enhances OCR rate in oxidative stress conditions. The bars show the comparison of the total amount of oxygen consumed by mitochondrial respiration in HGFs stressed by H_2O_2 (1 mM) following treatment with resveratrol (50 μM), quercetin (15 μM), and NAC (1.5 mM). Data are expressed as mean ± SEM ($n = 6$). *$p < 0.05$: significantly different from the H_2O_2 group.

converted to conjugate metabolites (aglycone) by deglycosylation followed by glucoronide/sulfate conjugation.[14] As a result, quercetin aglycone appears to be more accessible to cellular membranes because of its lipophilicity.[51] Unfortunately, the aglycone can function as a pro-oxidant, causing biological damage.[14] Moreover, it seems that quercetin might induce cytotoxicity at high concentrations (~40 μM).[48] Low solubility, conversion to another molecule, and the pro-oxidant activity of quercetin aglycone can explain the temporal drop of the CI value in the viability assay after 6 h of incubation (Fig. 1B). Shirai *et al.*[52] demonstrated that quercetin is more effective than its aglycone in preventing intracellular ROS production induced by H_2O_2 in mouse fibroblast-cultured cells. Additionally, it was not necessary that a large amount of quercetin reach the cytoplasm or cell

nucleus compartment to observe this effect. The study concluded that quercetin metabolites play a role in the modulation of cellular redox regulation without invasion into the cytosol.

The antioxidant mechanism of NAC is associated with its ability to stimulate GSH synthesis. It is rapidly deacetylated to cysteine and increases intracellular GSH levels, providing a substrate for GSH synthesis and ROS scavenging.[53] In the current study, NAC had the lowest antioxidant activity. A possible explanation is that in addition to its activity as a GSH precursor, NAC is, *per se*, responsible for protective effects in the extracellular environment.[24] NAC directly scavenged ROS, and reduced the oxidative burst.[17] Sueishi *et al.*[54] proposed that the scavenging rates depends on the type of ROS being scavenged, yet it is a common practice to determine scavenging rates against a single

ROS. Therefore, one should be cautious in judging the effectiveness of antioxidants.

In conclusion, our findings support the idea that antioxidants could enhance biological cellular functions during oxidative stress. Additionally, our results show that not all antioxidants have the same protective effects. We propose that this discrepancy can be explained by differing mechanisms of action and differing targets within cells. Given the fact that resveratrol enhanced cell proliferation, almost abolished ROS production, stimulated the synthesis of type I collagen gene, and stimulated mitochondrial respiration better than quercetin and NAC, we propose that resveratrol antioxidant is a beneficial supplement during the treatment of oxidative stress disorders. However, further studies using *in vivo* models are necessary to support the clinical use of antioxidants as a supplement to reduce oxidative stress and prevent periodontitis in humans.

Acknowledgments

Abbreviations

ECAR	extracellular acidification rate
HGF	human gingival fibroblast
NAC	*N*-acetylcysteine
OCR	oxygen consumption rate
ROS	reactive oxygen species

Conflict of Interest

Naofumi Tamaki was supported by a Yamada research grant in 2012. Resveratrol has been purchased from the Yamada Bee Farm (Okayama, Japan).

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