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研究題目：Comparison of the effects produced by 3 different antioxidants on human gingival fibroblasts under oxidative stress

目的（0bjective）：

Periodontitis is a chronic inflammatory disease that affects the immune system. The overproduction of reactive oxygen species（ROS）to protect the host from bacterial invasion generates collateral tissue damage like bone loss, affecting oral health. It is possible that antioxidant（AO）agents would prevent or reduce harmful effects of ROS on periodontal tissues. The aim of this study was to compare the protective effects of three AO agents：resveratrol, quercetin and N-acetyl cysteine（NAC）, on human gingival fibroblasts（HGFs）after induction of ROS by exposure to hydrogen peroxide（H_2O_2）reproducing the conditions viewed in tissues affected by periodontitis.

対象および方法（Materials and methods）：

Cells culture：HGF-1 cell line was from ATCC®（CRL-2014™）. Cells were suspended in Dulbecco's Modified Eagle Medium（DMEM）supplemented with 10% heat-inactivated fetal bovine serum（FBS）, 100 U/ml penicillin and 0.1 mg/ml streptomycin and kept in a humidified incubator at 37°C, with an atmosphere of 5% CO_2. Before cells reach confluence, they were subcultured according to the design of the forward experiments.

Cell viability：24 hrs of incubation after subculture（10^4 cells/well）, HGFs were stimulated with H_2O_2（Final concentration：0.23 mM）during 48 hrs to induce oxidative stress. Also, designated groups were treated with resveratrol（Final concentrations：75 μM, 50 μM and 25 μM）, quercetin（Final concentrations：15 μM, 10 μM and 5 μM）and NAC（Final concentrations：2 mM, 1.5 mM and 1 mM）. Cellular viability was monitored in real-time using xCELLigence System（Roche®）by measuring the levels of electrical impedance on the bottom of every individual well and expressed as Cell Index（CI）.

ROS Staining：HGFs were dyed with ROX-Green®（Invitrogen）according to the manufacturer's instructions to observe ROS production after stimulation with H_2O_2（0.23 mM）. Designated groups were treated with resveratrol（50 μM）, quercetin（15 μM）and NAC（1.5 mM）and viewed under confocal laser microscope（Nikon®）.

Type I Collagen gene expression：HGFs were subcultured in polystyrene 96-wells plates（10^4 cells/well）and incubated for 24 -48 hrs. Then, they were stimulated with H_2O_2（1 mM）in the presence or absence of resveratrol（50 μM）, quercetin（15 μM）and NAC（1.5 mM）during 3 hrs. Later, the reaction was stopped and cells were washed with PBS. DMEM was

Table 1　Cell viability（expressed in %）of 3 antioxidants against oxidative stress induced by H_2O_2 on HGFs.

Antioxidant	Concentration	Incubation Tima				
		6 hrs	12 hrs	24 hrs	36 hrs	48 hrs
Resveratrol	75 μM	103	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

replaced and the plates were incubated at 37°C and 5% CO_2 for 3 and 24 hrs, respectively. The changes on the gene expression were submitted for quantification of total mRNA extracted from HGFs by quantitative RT-PCR.

Cellular respiration changes：HGFs were subcultured in XF24 TC plate（10^4 cells/well）and incubated for 24 -48 hrs. After incubation, the medium of each well was changed to modified DMEM XF assay medium（containing 1% of serum and sodium carbonate free）. According to the experiment design, the injection ports in the XF sensor cartridge hydration were filled with H_2O_2（1 mM）and XF Cell Mito Stress Test kit（Seahorse Bioscience®）reagents, which include olygomicin A, carbonyl cyanide 4 -（trifluoromethoxy）phenylhydrazone（FCCP）, antimycin A and rotenone. The selected wells received AO treatment with resveratrol and NAC respectively. According to the manufacturer's direction, oxygen consumption rate（OCR）was measured by XF^e 24 Flux Analyzer（Seahorse Bioscience®）to determine the changes produced by ROS in presence or absence of AO agents.

結果（Results）：

Cellular viability of resveratrol, quercetin and NAC against oxidative stress：

After the AO treatment, it was found that resveratrol（50 uM）was the most effective（85%）to control the oxidative stress induced by H_2O_2 after 48 hrs. Also, increased the CI（results not shown）when it was compared to the control group. It is followed by quercetin（15 uM）with 41% of effectiveness. NAC（1.5 mM）showed 81% of effectiveness after 6 hrs, but steadily decreased till 27%（Table 1）.

ROS production in HGFs induced by oxidative stress：

Differences in the levels of ROS production between the three AO agents were observed. In accordance with the previous results, resveratrol reduced the amount of ROS induced by H_2O_2, followed by quercetin and NAC（Fig. 1）.

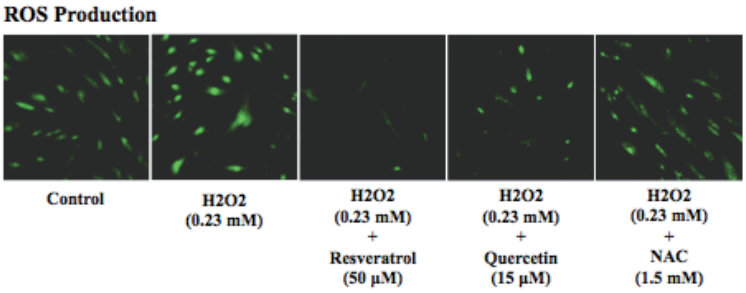

Fig. 1 HGFs-1 cells under oxidative stress induced by H_2O_2 (0.23 mM) and treated with AO agents

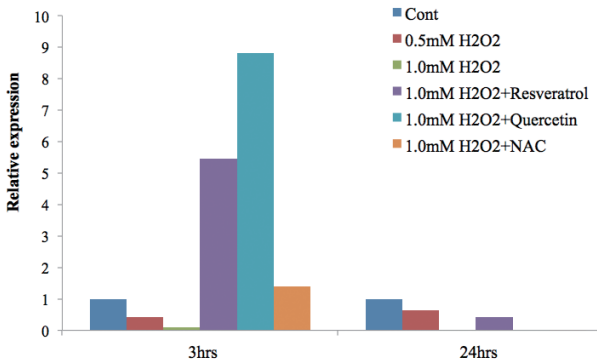

Fig. 2 Relative Type 1 collagen gene expression on HGFs-1 cells under oxidative stress

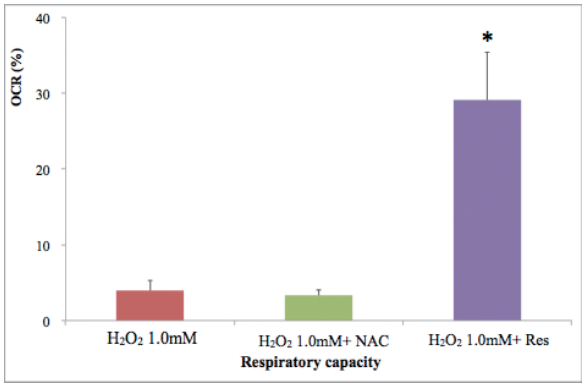

Fig. 3 Oxygen consumption rate (OCR) of HGFs under oxidative stress treated with AO agents

Type I Collagen gene expression after oxidative stress :

After 3 hrs post oxidative stress induction, the expression of type I collagen in cells treated with resveratrol and quercetin was enhanced 5.5 and 9 times, respectively compared with the control group. But, after 24 hrs post oxidative stress induction, the gene expression was nearly abolished and scarcely rescued by resveratrol (Fig. 2).

Recovery of mitochondrial respiratory capacity after oxidative stress :

HGFs cells under oxidative stress but treated with resveratrol had the highest OCR, which means that resveratrol would stimulate mitochondrial activity to counteract the oxidative damage, while NAC didn't increase its OCR. This indicates that NAC has extracellular effects not related to mitochondrial activity (Fig. 3).

考察（Discussion）：

The use of AO agents has been spreading because of their benefits to protect from the detrimental effects produced by oxidative stress and their easy availability in different natural sources. Periodontal disease is directly related to other systemic diseases like atherosclerosis, diabetes and rheumatoid arthritis. The continuous overproduction of ROS is the key factor that leads to the destruction of the periodontal support and systemic decay. For this reason, the use of AOs in the prevention or treatment of periodontitis arises as a promising possibility, in order to improve the quality of life. However, the mechanisms of AO agents are not well understood yet. In our study, we found that each AO had different antioxidant capacity and different cellular targets to improve the negative effects derived from oxidative stress. Among the three AO agents studied, resveratrol was likely to be the most effective AO that reduced the production of ROS, increased the expression of Type I collagen gene and enhanced mitochondrial activity under oxidative stress conditions leading to the recovery of the affected cellular functions.

成果発表（Research Achievement）：

1）Rita C. Orihuela-Campos, Naofumi Tamaki, Makoto Fukui, Kaname Miki, Sapta A. Mulyatno, Hiro-O Ito Real Time xCELLigence Analysis of Antioxidant Agents on Human Gingival Fibroblast Cells Viability 第 62 回日本口腔衛生学会・総会
2013 年 9 月 19 日（松本，Oral presentation）

2）Rita C. Orihuela-Campos, Naofumi Tamaki, Makoto Fukui, Kaname Miki, Hiro-O Ito Effects of different antioxidants on the biological properties of human gingival fibroblasts response towards ROS ヘルスバイオサイエンス研究部・5 教育部 2013 Tokushima Bioscience Retreat
2013 年 9 月 19 日（小豆島，Oral Presentation）

3）Rita C. Orihuela-Campos, Naofumi Tamaki, Makoto Fukui, Kaname Miki, Hiro-O Ito Effects of different antioxidants on human gingival fibroblasts under oxidative stress 第 5 回心・血管クラスターミニリトリート
2014 年 1 月 11 日（高松，Oral Presentation）

4）Rita C. Orihuela-Campos, Naofumi Tamaki, Makoto Fukui, Kaname Miki, Hiro-O Ito Effects of resveratrol, quercetin and N-acetyl cysteine on human gingival fibroblasts under oxidative stress 第 63 回日本口腔衛生学会・総会
2014 年 5 月 29 日～31 日発表予定（熊本，Oral Presentation）