Antioxidant effects of antioxidant biofactor on reactive oxygen species in human gingival fibroblasts

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The purpose of this study was to investigate the effects of anti-oxidant biofactor (AOB) on reactive oxygen species (ROS). Generation of superoxide radical ($O_2{}^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) was determined using an electron spin resonance (ESR) spin-trapping method. AOB was added at different concentrations to these free radical generating systems. The generation of both $O_2{}^{\bullet-}$ and $^{\bullet}OH$ was scavenged by the addition of AOB in a dose-dependent manner. These results indicate that AOB has strong antioxidant properties against these radicals. We further investigated the anti-oxidative effect of AOB on human gingival fibroblasts (HGFs). HGFs were treated for 3 h with α-MEM containing a combination of AOB and H_2O_2 (AOB + H_2O_2 group), containing H_2O_2 (H_2O_2 group), or containing AOB alone (AOB group). Non-stimulated HGFs were used as a control group. The number of surviving cells was in the order of the AOB group > control group > AOB + H_2O_2 group > H_2O_2 group. The level of expression of type I collagen mRNA and production of collagen were also in the order of the AOB group > control group > AOB + H_2O_2 group > H_2O_2 group. In conclusion, our results suggest that AOB may protect HGFs against oxidative stress by reducing stress-induced ROS.

Key Words: free radical, antioxidant baiofactor, electron spin resonance spin-trapping method, periodontal disease, collagen

Reactive oxygen species (ROS) include superoxide anion ($O_2{}^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) that are generated from an oxidative burst by enzymatic pathways of inflammatory cells.[1] One well-known process of free radical production in the body is the transition from $O_2{}^{\bullet-}$ to H_2O_2, which generates $^{\bullet}OH$.[2] Excess production of ROS is considered to be the cause of various diseases. It has been reported that ROS is involved in apoptosis, inflammation, DNA damage and aging.[3–6] ROS is also considered to be an inflammation factor in periodontal disease.[7] ROS generated by bacteria and/or metal ions in the oral cavity may attack gingival tissue, periodontal ligaments and osteoblasts.[8–10] Moreover, periodontal disease is a life style disease.[11] Therefore, development of a technique to eliminate ROS in the oral cavity is necessary for the prevention of periodontal disease, and antioxidant treatment is beneficial for the treatment of periodontal disease.

Recently, foods have been developed with the aim of ROS elimination. One such food is a processed grain food termed antioxidant biofactor (AOB), which has strong antioxidant properties and inhibits cellular oxidant stress.[12] AOB is a unique processed grain food. In addition, AOB is known to contain a variety of substances that have antioxidant activity, including flavonoids, α-tocopherol, vitamins, tannins, superoxide dismutase and catechins. Minamiyama *et al.*[13] reported that AOB strongly inhibited the auto-oxidation of rat brain homogenates *in vitro* in a dose-dependent manner. They suggested that AOB has strong antioxidant properties and further additional biological effects, which might be of importance in the context of the prevention of degenerative diseases. Therefore, it is considered that AOB have a powerful mixture of scavenging activity on ROS of various kinds and improve immunity. However, there have been few studies of the anti-oxidative effects of the various components of AOB in periodontal disease. The present study was conducted to investigate the ability of AOB to scavenge both $O_2{}^{\bullet-}$ and $^{\bullet}OH$ radicals by using the electron spin resonance (ESR) spin-trapping method with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) *in vitro*. We further investigated cell viability, Type I collagen mRNA expression and the production of collagen following oxidative stress in the presence or absence of AOB in human gingival fibroblasts (HGFs).

Materials and Methods

Chemicals. AOB was obtained from AOA Japan (Kobe, Japan). H_2O_2 and TiO_2 were obtained from Wako Pure Chemicals (Osaka, Japan). DMPO (Dojin Chemicals, Kumamoto, Japan) was used as a spin-trapping agent for free radicals.

Adjustment of AOB. AOB (3 g) was mixed with pure water (PW: 27 ml) by vortexing and by exposure to a super sonic wave for 10 min. The solution was then allowed to stand, after which the supernatant fluid was collected and filtered as a 10% AOB solution. AOB was then diluted to the following concentrations 0, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00 or 10.00 w/v%.

Materials. The visible light irradiation device used was the xenon light device (APOLLO 95E, Dental/Medical Diagnostic Systems Inc., Woodland Hills, CA), which was used at 440–500 nm wavelength.

$O_2{}^{\bullet-}$ scavenging effects of AOB. $O_2{}^{\bullet-}$ was generated for this experiment by reacting H_2O_2 with TiO_2 under visible light irradiation.[14] The experimental protocol was as follows: 100 μl of a 6%-H_2O_2 solution was mixed with 8 mg-TiO_2, 30 μl of PW, 50 μl of a 0–10%-AOB solution and 20 μl of an 890 mM-DMPO solution in 96-well plates. After 15 s incubation, the mixture was irradiated with visible light for 6 s. After 1 min, the ESR spectrum was recorded using a JES FA200 (JEOL, Tokyo, Japan). The conditions for ESR were as follows: microwave power, 8 mW;

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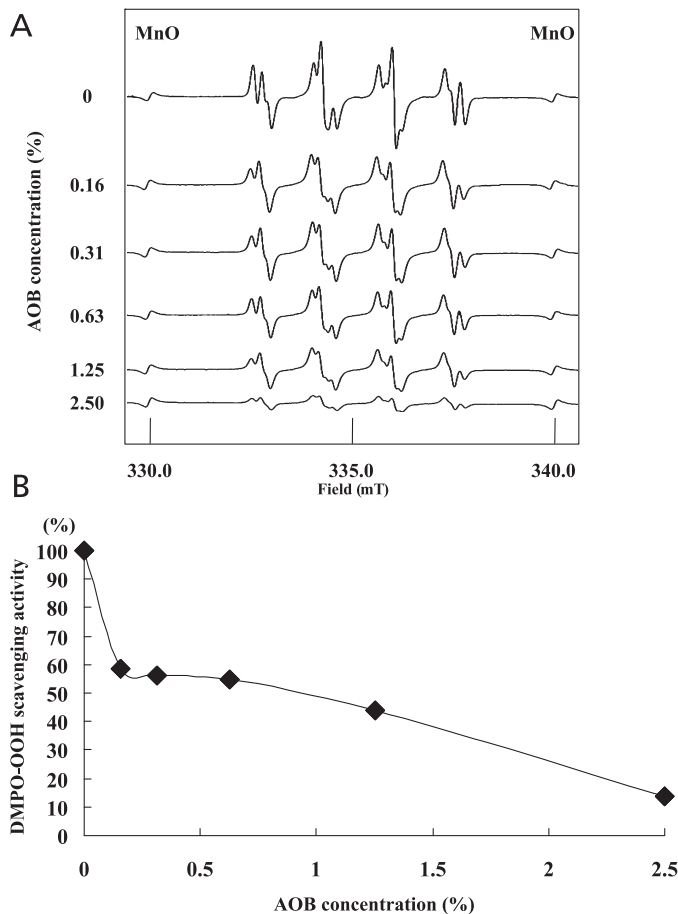E-mail: matsui.satoshi@nihon-u.ac.jp

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A

MnO   MnO

AOB concentration (%)

0

0.16

0.31

0.63

1.25

2.50

330.0   335.0   340.0
Field (mT)

B

(%)

DMPO-OOH scavenging activity

100
90
80
70
60
50
40
30
20
10
0

0   0.5   1   1.5   2   2.5
AOB concentration (%)

Fig. 1. The $O_2^{\bullet-}$ scavenging effect of AOB.
$O_2^{\bullet-}$ was generated *in vitro* from the reaction of H_2O_2 with TiO_2 irradiated by visible light, in the presence of the indicated concentrations of AOB. A. The ESR spectra of DMPO-spin adducts of $O_2^{\bullet-}$ (DMPO-OOH) was then assayed. B. Representative plots of the relationship between the ESR signal intensity of DMPO-OOH and the concentration of AOB are shown.

magnetic field, 335.0 ± 5 mT; sweep time, 2 min; amplitude, 2×10^2; modulation, 0.1 mT; and time constant, 0.03 s.[15]

$\cdot$OH scavenging effects of AOB. $\cdot$OH was generated for this experiment by photolysis of H_2O_2 by visible light irradiation.[16] The experimental protocol was as follows: 100 μl of a 6% H_2O_2 solution was mixed with 30 μl of PW, 50 μl of a 0–10%-AOB solution and 20 μl of 890 mM DMPO solution in 96-well plates. After 15 s incubation, the mixture was irradiated with visible light for 6 s. After 1 min, the ESR spectrum was recorded using a JES FA200 (Fig. 1B). The conditions for ESR were as follows: microwave power, 8 mW; magnetic field, 335.0 ± 5 mT; sweep time, 2 min; amplitude, 2×10^2; modulation, 0.1 mT; and time constant, 0.03 s.[15]

Cell-culture conditions and treatment with H_2O_2 and/ or AOB. HGFs were obtained from four healthy 21- to 24-year-old patients, with informed consent. The experimental protocol was reviewed and approved by the Institutional Review Board (EC 03-025). Small samples of gingival tissues were dissected out and explanted into tissue culture dishes. The cells were grown in α-MEM (Gibco Laboratories, Grand Island, NY) supplemented with 100 μg/ml penicillin G (Banyu Pharmaceutical Co., Tokyo, Japan), 100 μg/ml kanamycin sulfate (Sigma Chemical Co., St. Louis, MO), 0.3 μg/ml amphotericin B (Flow Laboratories, McLean, VA), 5 mM HEPES buffer (pH 7.2) and 10% FBS, under 5% CO_2 in air, at 37°C. Cells between passage 6 and passage 8 were pooled and used for all experiments.[17] HGFs were treated

with the α-MEM growth medium supplemented with a combination of AOB (final concentration 0.0001%) and H_2O_2 (final concentration 1 mM), and were then cultured for 3 h (AOB + H_2O_2 group). Other HGF groups were stimulated with 1 mM H_2O_2 for 3 h (H_2O_2 group) or with 0.0001% AOB for 3 h (AOB group). Non-stimulated cells were used as the control group. All groups were then washed with fresh α-MEM to remove the treatment medium.

Assay of cell viability. For assay of cell viability, HGFs were seeded into 96-well plates at a density of 4×10^3 cells/well in 0.1 ml of the appropriate medium containing α-MEM with 1% FBS and were cultured at 37°C for 24 h. The cells were then stimulated with AOB and/or H_2O_2.

Cell viability was measured 24, 48 or 72 h later by using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). This assay is based on the conversion of the water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] to a water-soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases. The medium (100 μl) was incubated with 10 μl of the WST-8 solution for 2 h at 37°C. Absorbance was recorded at 450 nm on a microplate reader. Cell viability was expressed as a percent of the control culture value.

RNA extraction and RT-PCR technique. HGFs (1×10^6) were cultured in tissue culture dishes (10 cm) containing 10 ml of α-MEM supplemented with H_2O_2, AOB or AOB + H_2O_2 for 1 or 3 days. Total cellular RNA was extracted from the cells using an RNeasy mini kit (QIAGEN, Hilden, Germany). The RNA isolation procedure was in accordance with the protocol provided with the RNeasy mini kit. cDNA synthesis and amplification by RT-PCR were conducted using a One-Step RT-PCR kit. For the PCR mixture, RNA (200 ng) and oligonucleotide primers (500 nM) were used. The PCR primers used for PCR of Type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with reference to the reported sequences, these primers were as follows: GAPDH (forward) 5'-ATC ACC ATC TTC CAG GAG-3', (reverse) 5'-ATG GAC TGT GGT CAT GAG-3'; Type I collagen (forward) 5'-GTG GAA ATG ATG GTG CTA CT-3' (reverse) 5'-TTAG CAC CAG TGT CTC CTTT-3'.

The GeneAmp PCR system 9600 (PerkinElmer, Waltham, USA) was programmed for cDNA synthesis, and the procedure involved pre-denaturation for 30 min at 95°C, followed by 30 thermal cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C, chain elongation for 30 s at 72°C, and final extension for 10 min at 72°C. The PCR fragments were electrophoresed on 1.5% agarose gels and were subsequently stained with ethidium bromide.

Collagen production. The collagen content of samples was estimated on day 3 to day 9 following treatment using the method described by Walsh *et al.*[18] Briefly, type I collagen solution was diluted in either 50 mM/L of Tris-HCl, 100 mM/L of NaCl, or 10 mM/L of CaCl, together with 0.92% NaN 3 (pH 7.5), or in culture medium. The standards and cell culture supernatants were dispensed into individual wells of microtiter plates and the plates were incubated at 37°C for 16 h (humidified) and then at 37°C (dry) for 24 h. After the plates were rinsed in distilled water, the wells were filled with 0.21% Sirius Red F3BA (Chromagesellschafl Schmid GMBH Co., Stuttgart, Germany) in saturated picric acid (wt/vol), and the samples were stained for 1 h at room temperature. The plates were washed with 100 mM/L of NaOH. The eluted stain was then drawn up and down several times into a multichannel pipette and was then placed into a second plate. Absorbance was read at 540 nm using a model 2550 microplate reader (Bio-Rad Labs, Sunnyvale, CA), and the collagen content of the samples was estimated from the absorbance of the standards.

Statistical analysis. All values are presented as means ± SD, and the significance of differences was determined using the Bonferoni ($p<0.05$).

Results

$O_2^{•-}$ scavenging activity of AOB. To determine the anti-oxidant properties of AOB, we first tested if AOB can scavenge $O_2^{•-}$ species generated from the reaction of H_2O_2 with TiO_2 when irradiated by visible light. Fig. 1 shows representative ESR spectra of DMPO-spin adducts of $O_2^{•-}$ (DMPO-OOH) generated in this way in the presence of different concentrations of AOB. A plot of the DMPO-OOH signal intensity as a function of the concentration of AOB indicated that AOB can scavenge DMPO-OOH in a dose-dependent manner.

$^•$OH scavenging activity of AOB. We next analyzed the effect of different concentrations of AOB on the ESR spectra of DMPO-spin adducts of $^•$OH (DMPO-OH) generated from the photolysis of H_2O_2 when irradiated by visible light. A plot of the DMPO-OH signal intensity as a function of the concentration of AOB indicated that AOB can also scavenge DMPO-OH in a dose-dependent manner (Fig. 2).

Cell viability. To determine if the elimination of H_2O_2 by AOB enhances HGF cell viability, the viability of cells exposed to H_2O_2 and/or AOB for 3 h was assayed using a commercial kit. Non stimulated cells were used as the control group. The cell viability of these HGFs was in the order of the AOB group > control group > AOB + H_2O_2 group > H_2O_2 group. The cell viability of the AOB group was significantly increased compared with that of the control group after 24, 48 or 72 h of treatment. The cell viability of the AOB + H_2O_2 group was significantly greater than that of the H_2O_2 group after 24, 48 or 72 h of treatment (Table 1) (b: $p<0.05$) ($n = 6$).

mRNA expression of type I collagen. We then determined if AOB enhancement of cell viability in the presence of H_2O_2 involved an effect on the expression of Type I collagen mRNA. Collagen is the main component of gingival tissue and its production is inhibited following periodontal disease. The effect of exposure of HGF cells to H_2O_2 and/or AOB for 1 or 3 days on the mRNA expression of Type I collagen was analyzed using RT-PCR. Type I collagen mRNA expression was increased in cells treated only with AOB for 1 or 3 days compared to the control group. The level of expression of Type I collagen mRNA was in the order of the AOB group > control group > AOB + H_2O_2 group > H_2O_2 group (Fig. 3).

Collagen production. To confirm that the production of collagen correlated with collagen protein levels, the collagen content of the treated cells was assayed from day 3 to day 9 after treatment with AOB and/or H_2O_2 using a colorimetric assay. The production of collagen increased in all groups over this time period and peaked on day 9. The production of collagen was significantly greater in the AOB group than in the control group on
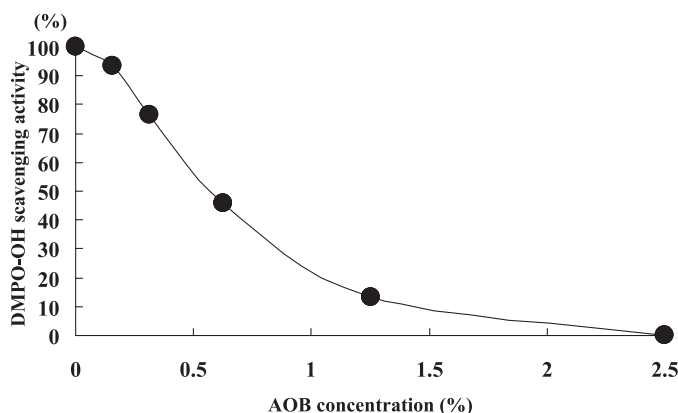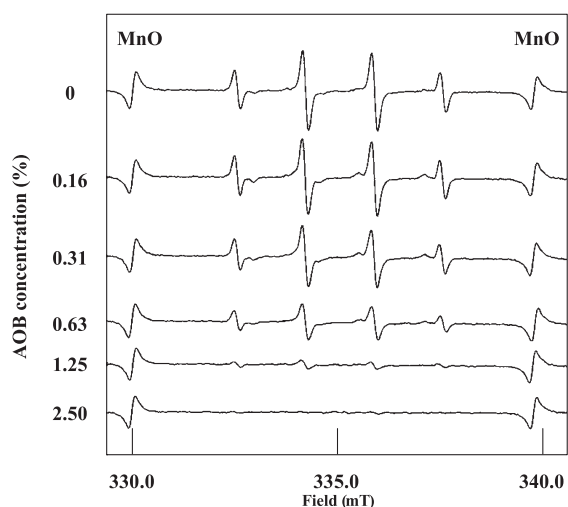

Fig. 2. The $^•$OH scavenging effect of AOB.
$^•$OH was generated *in vitro* by photolysis of H_2O_2 by visible light irradiation in the presence of the indicated concentrations of AOB. The ESR spectra of DMPO-spin adducts of $^•$OH (DMPO-$^•$OH) were then assayed. Representative plots of the relationship between the ESR signal intensity of DMPO-OH and the concentration of AOB are shown.

days 6 and 9, and was also significantly higher in the AOB + H_2O_2 group than in the H_2O_2 group on days 6 and 9 (Fig. 4) ($p<0.05$) ($n = 7$).

Discussion

Excess production of ROS is considered to be the cause of various diseases. Further, it has been reported that free radicals

Table 1. Effect of ROS on cell viability in the presence or absence of AOB

	24 h	48 h	72 h
control	100.00 ± 2.45	100.00 ± 4.16	100.00 ± 3.02
H_2O_2	86.53 ± 3.68[a,b]	79.98 ± 2.12[a,b]	73.68 ± 1.94[a,b]
AOB + H_2O_2	93.53 ± 2.74[b]	85.65 ± 4.69[b]	83.66 ± 2.74[b]
AOB	110.43 ± 3.19[a]	108.75 ± 1.98[a]	109.65 ± 1.62[a]

a: Significantly ($p<0.05$) control vs H_2O_2 or AOB.
b: Significantly ($p<0.05$) H_2O_2 vs H_2O_2 + AOB.
$n = 6$.
HGFs were incubated with AOB, H_2O_2, AOB + H_2O_2 or with buffer control for the indicated times. Cell viability was then assessed using cell counting kit-8. The viability of the AOB + H_2O_2 group was significantly greater than that of the H_2O_2 group, and the viability of the AOB group was significantly higher than the control group, at 24, 48 or 72 h after treatment (b: $p<0.05$).
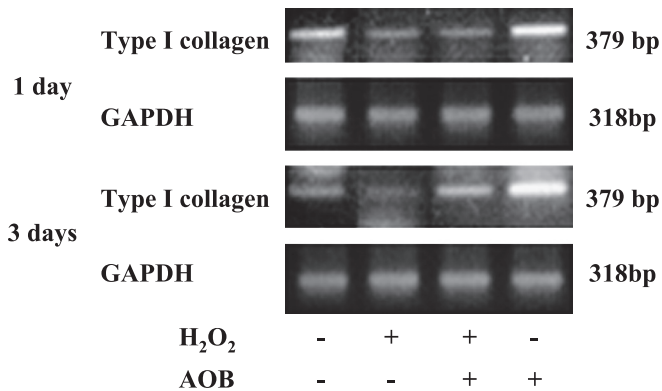
Fig. 3. mRNA expression of type I collagen after treatment with H_2O_2 in the presence or absence of AOB.
HGFs were incubated with AOB, H_2O_2, AOB + H_2O_2 or with buffer control for 1 or 3 days. Type I collagen mRNA expression was then assessed using RT-PCR. Type I collagen mRNA expression was much greatest in the AOB group than in other group at 1 or 3 days after treatment, and greater in the AOB + H_2O_2 group than in H_2O_2 group at 1 or 3 days after treatment.

(*$p<0.05$: control vs H_2O_2 or AOB)   (**$p<0.05$: H_2O_2 vs AOB+H_2O_2)
n=7

Fig. 4. Production of collagen after treatment with H_2O_2 in the presence or absence of AOB.
HGFs were incubated with AOB, H_2O_2, AOB + H_2O_2, with control for 3, 6, 9 days. Collagen production was then assessed using a colorimetric assay. Collagen production was significantly greater in the AOB + H_2O_2 group than in the H_2O_2 group on 6 or 9 days after treatment and was significantly greater in the AOB group than in the control group on 6 or 9 days (*$p<0.05$). However, collagen production was not significantly greater in the AOB + H_2O_2 group than in the control group on 3, 6 or 9 days.

are involved in tumorigenesis, apoptosis, inflammation, DNA damage, and aging.[3–6] Therefore, health food is taken into consideration for deletion of ROS. Since AOB has strong anti-oxidant properties and biological effectivity against ROS, AOB has recently received a lot of attention as a health food for the elimination of ROS.[13,19] However, there have been few studies of the anti-oxidation ability of the various ingredients contained in AOB. Furthermore, there have been few reports concerning the free radical scavenging ability of AOB. Therefore, an analysis of the ability of AOB to scavenge $O_2^{\cdot-}$ and $\cdot OH$ is required.

In this study, we demonstrated the ability of AOB to scavenge $O_2^{\cdot-}$ and $\cdot OH$ radicals by using an *in vitro* ESR spin-trapping method and we further showed that AOB can protect cells against H_2O_2 inhibition of cell viability and collagen production.

Using this ESR spin-trapping method, and *in vitro* $O_2^{\cdot-}$ and $\cdot OH$ generating systems, we showed that AOB can strongly suppress the generation of these free radicals by visible light irradiation *in vitro* in a dose-dependent manner. It is well known that the components of AOB include substances such as flavonoids, α-tocopherol, tannins, catechins and vitamins. Kashima has reported[20] that the generation of $O_2^{\cdot-}$ and $\cdot OH$ from the reaction of H_2O_2 with metal ions, which is similar to our *in vitro* method for generation of these radicals, was suppressed by catechin. Furthermore, it has been reported that ROS is eliminated by flavonoid and α-tocopherol.[13] These data suggest that the ability of AOB to scavenge $O_2^{\cdot-}$ and $\cdot OH$ that we observed in our study was most likely mediated by more than one component of AOB.

Periodontal disease is a bacterially induced inflammatory disease that is accompanied by gingival inflammation and alveolar bone loss.[21] The tissue damage induced by periodontal disease is directly caused by bacteria and is indirectly caused by inflammation and by an immune response against these bacteria. During the inflammatory process, activated phagocytes such as neutrophils and macrophages produce large quantities of ROS via NADPH oxidase.[22] Moreover, patients with periodontal disease display increased neutrophil numbers and activity.[23] In this respect it is of interest that our results showed that AOB can protect HGFs against damage as a result of oxidant stress. Thus, cells treated with AOB had a higher viability than control cells, and cells exposed to H_2O_2 had a significantly higher viability if AOB was present. Moreover, not only did AOB increase cell viability, but we showed that AOB also significantly enhances the
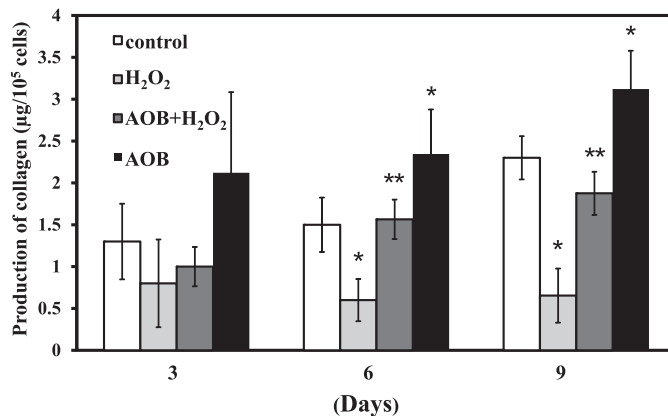
production of both type I collagen mRNA and collagen protein, and protects HGFs against the inhibition of collagen production induced by H_2O_2. Since collagen is the main component of gingival tissue, and the progress of periodontal disease is associated with decreased collagen production, our data further suggest that AOB may be useful for combating periodontal disease.[24] Our data further suggest that ROS may play a role in the breakdown of the collagen network in gingivitis. Tanaka *et al.* reported[25] that ROS is an important inflammatory factor for HGFs. Our data are also consistent with other studies that have shown that AOB can decrease cell damage and the destruction of collagen due to ROS in HGFs. The combined data suggest that AOB may collaborate with signals such as growth factors that modulate the synthesis of collagen in HGFs. However, in our study, we did not describe the relationship between expression of matrix metalloproteinases (MMPs) and ROS in HGFs. MMPs are main factor in destruction of the collagen in gingival tissue.[26] Therefore, we are investigating the inhibitory effect of AOB on expression of MMPs caused by ROS in HGFs. Further investigations into this are currently under way in our laboratory.

Acknowledgment

Abbreviations

AOB	antioxidant biofactor
ESR	electron spin resonance
$O_2^{\cdot-}$	superoxide anion
$\cdot OH$	hydroxyl radical
MMPs	matrix metalloproteinases

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