Anti-oxidative and anti-inflammatory effects of astaxanthin on H$_2$O$_2$-induced oxidative stress in human submandibular gland cells

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Abstract: Reactive oxygen species (ROS) have recently been reported to be one of the elements involved in the pathogenesis of decreased saliva secretion. In addition to the endogenous anti-oxidant systems pre-existing in vivo, exogenous anti-oxidants have attracted attention in the defense against ROS. Astaxanthin (AX) is a type of natural carotenoid, is widely distributed in various organisms, and has strong anti-oxidative and anti-inflammatory effects. In this study conducted on human submandibular gland cells, namely, cell lines derived from human salivary glands, we examined the in vitro effects of AX on H$_2$O$_2$-induced oxidative stress-related disorders. Our findings showed that the prophylactic addition of 10 µM AX significantly decreased the levels of 8-hydroxy-2'-deoxyguanosine and interleukins-6 and 8, as well inhibited the decrease in cell proliferation and apoptosis. In conclusion, the anti-oxidative and anti-inflammatory effects of AX protect salivary gland cells from oxidative stress-induced disorders.

Key words: xerostomia, oxidative stress, astaxanthin, antioxidant, anti-inflammation

Introduction

The decrease in salivary secretion in the elderly leads to a number of functional disorders of the oral cavity, such as gustatory abnormalities, inadaptation of dentures, oral mucosal diseases, and dysphagia. In addition, reduced salivary secretion affects not only the oral cavity, but also the entire body, and causes aspiration pneumonia, infections, as well as upper gastrointestinal disorders and this leads to a deterioration of the quality of life (QOL)\(^1\)-\(^3\). According to a systematic review, xerostomia has a prevalence of 5.5-39%, which increases to 17-40% in individuals aged 65 years or older\(^4\).

Reduced salivary secretion can be caused by various conditions: some are caused by conditions affecting the salivary glands themselves, such as in the case of aging, Sjogren's syndrome (SS), and radiation therapy of oral cancer. Others are associated with systemic diseases such as diabetes, medications, and psychiatric disorders. The latter has a high prevalence\(^5\)-\(^9\).

In addition, oxidative stress has recently been found to be a contributing factor to the decrease in salivary secretion\(^10\). Oxidative stress disrupts cell structure and function through the production of free radicals, particularly reactive oxygen species (ROS), and has been shown to act as an important factor in aging and various diseases\(^11\). Various types of ROS accumulate in the body with age, including endogenous ROS as well as exogenous ROS from pharmacological agents, ultraviolet light, and the environment. Meanwhile, with increasing age, the anti-oxidative potential of the body decreases and the control...
mechanisms in charge of maintaining the balance between oxidation and reduction reactions are no longer capable of functioning properly^{12, 13}. As a result, oxidative stress accumulates in the body and causes oxidative damage to the phospholipids and proteins of the cell membrane. In addition, oxidative stress has been reported to cause DNA damage, increase the expression of inflammatory cytokines, cause inflammatory reactions, and induce apoptosis^{14-16}.

The use of anti-oxidant therapy for the treatment of such oxidative stress-related disorders has recently been the focus of attention. Astaxanthin (AX; 3,3-dihydroxy-beta, beta-carotene-4,4-dione) is a type of carotenoid-like β-carotene and lycopene. It consists of a red-orange pigment that is classified as a xanthophyll. AX is widely distributed in nature, namely in fish species such as salmon and sea bream, as well as in crustaceans such as shrimps and crabs; however, it cannot be biosynthesized in the human body^{17}. The anti-oxidative effect of AX has been reported in a number of previous studies^{18, 19}. In addition, AX has also been reported to have various biological activities, such as anti-inflammatory effect, inhibitory effect on lipid peroxidation, anticancer, antidiabetic, immunomodulatory, and anti-aging effect^{20-23}. Particularly, with regard to oral dryness, a previous study on the administration of AX to patients with SS has shown that the salivary secretion tended to increase^{24}. However, no detailed study has thus far examined the anti-oxidative effect of AX in oxidative stress-related disorders affecting the salivary gland cells.

Therefore, in this in vitro study, we examined the oxidative stress-related disorder induced by H2O2 in human submandibular gland cells and the anti-oxidative and anti-inflammatory effects of the prophylactic administration of AX.

**Materials and Methods**

**Cell culture**

A human salivary gland cell line (HSG) was used (stock cell line distributed by the Department of Internal Medicine, Faculty of Medicine, University of Tsukuba). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO; Carlsbad, CA) containing 10% fetal bovine serum (FBS; Bio West; Nuaille, France) and 1% penicillin-streptomycin (GIBCO; Carlsbad, CA), at a temperature of 37°C in the presence of 5% CO2.

**Cell treatment**

The time course of the cell culture is shown in Fig. 1. First, HSG cells were seeded to a density of $1.0 \times 10^4$ cells/ml in a 48-well multi-plate (Corning Incorporated; CA, USA) and were cultured in DMEM for 48 hours. AX (AstaReal Co., Ltd.; Tokyo, Japan) was dissolved in 99% dimethyl sulfoxide (DMSO; Wako Pure Chem. Ind. Ltd.; Osaka, Japan) and was added to DMEM such that the final concentration of DMSO would reach 0.5%. Oxidative stress was induced by adding H2O2 (Nacalai Tesque; Kyoto, Japan) to the culture medium. The control group was cultured in DMEM only; a group whose culture medium was replaced 48 hours later with a culture medium supplemented with AX was termed the AX group; an oxidative stress stimulus group in which DMEM was later replaced with a culture medium containing H2O2 was termed the H2O2 group; and an AX prophylactic treatment group that was cultured in a medium supplemented with AX, which was replaced 24 hours later with a culture medium supplemented with H2O2, was termed the AX H2O2 group (Fig. 1).

**DNA assay**

The cells’ proliferative capacity was evaluated
by performing a DNA assay. The microplates used for the time-course described in Fig. 1 were washed twice with phosphate-buffered saline (Nacalai Tesque; Kyoto, Japan) and the cells were stored frozen at −80°C until the measurements were carried out. After freezing and thawing, a water-soluble solution containing 0.2 mg/ml sodium dodecyl sulfate, 9.0 mg/ml NaCl, and 4.4 mg/ml sodium citrate was added and the HSG cells were dissolved completely through incubation for 1 hour. The cell lysate was mixed with Hoechst 33258 dye solution (bisbenzimide H33258 fluorochrome trichloride dimethyl sulfoxide solution; Nacalai Tesque; Kyoto, Japan) containing 9.0 mg/ml NaCl and 4.4 mg/ml sodium citrate. The fluorescence intensity of the mixture was measured at an excitation wavelength of 355 nm and a fluorescence wavelength of 460 nm (n = 5) by using a microplate reader (SH-9000; Hitachi High-Technologies; Tokyo, Japan).

First, in order to examine the effect of AX containing 0.5% DMSO as a solvent for AX on HSG cells, the cells were cultured for 48 hours, then the culture medium was replaced with an- other cultured medium supplemented with 1, 5, or 10 μM of AX. The cell count was performed 24 hours later by performing a DNA assay.

In addition, in order to determine the optimal concentration of H2O2 for the induction of oxidative stress, the cells were cultured for 48 hours, then the cell counts at 24 hours in the 0.5, 0.75, 1, and 1.5 mM H2O2 groups were measured by performing a DNA assay.

Further, in order to examine the prophylactic effect of AX against the H2O2-induced oxidative stress-related disorders, the cell counts in the AX H2O2 group after 6, 12, 24, and 48 hours of culture were measured by performing a DNA assay.

8-Hydroxy-2’-deoxyguanosine (8-OHdG) measurement
The H2O2-induced oxidative stress-related disorder was examined by quantifying the oxidative DNA damage marker 8-OHdG by using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical; Michigan, USA). In accordance with the time course shown in Fig. 1, the cells were cultured for 6, 12, 24, and 48 hours in a culture medium supplemented with 1 mM H2O2, after which the culture supernatants
were collected and stored frozen at −80°C until the measurements were carried out. The absorbance was measured at 420 nm by using a microplate reader, and the concentrations of 8-OHdG contained in the samples were calculated (n=5).

Cytokine assay
The amounts of interleukin-6 (IL-6) and IL-8 were determined by using an ELISA kit (Bio-Legend, Inc.; San Diego, CA, USA). In accordance with the time course shown in Fig. 1, the cells were cultured for 6, 12, 24, and 48 hours in a culture medium supplemented with 1 mM H2O2, after which the culture supernatants were collected and stored frozen at −80°C until the measurements were carried out. The absorbance was measured at 450 nm by using a microplate reader, and the concentrations of IL-6 and IL-8 contained in the samples were calculated (n=5).

Assessment of apoptosis
Apoptosis was measured by determining the concentrations of a fragment (CK18-Asp396) of cytokeratin-18, which included the Asp396-neo-epitope by using an ELISA kit (PEVIVA AB; Bromma, Sweden). In accordance with the time course shown in Fig. 1, the cells were cultured for 6, 12, 24, and 48 hours in a culture medium supplemented with 1 mM H2O2, after which the culture supernatants were collected and stored frozen at −80°C until the measurements were carried out. The absorbance was measured at 450 nm by using a microplate reader, and the concentrations of CK18-Asp396 contained in the samples were calculated (n=5).

Statistical analysis
To test the statistical significances, a one-way analysis of variance was performed and was followed by multiple comparisons using Tukey's HSD test (honestly significant difference).

Results

DNA assay
The results of the DNA assay regarding the cellular proliferative potential are shown in Fig. 2. HSG cells were cultured for 24 hours in a culture medium supplemented with AX containing 0.5% DMSO, which was used as a solvent for AX, and differences in cellular proliferative potential due to differences in AX concentrations were evaluated. The findings showed no significant difference between the groups and none of the concentrations of AX was cytotoxic (Fig. 2a). In addition, HSG cells were cultured for 24 hours in a culture medium supplemented with H2O2 and the differences in cell growth according to the differences in H2O2 concentrations were evaluated. The findings showed that the cell counts decreased in a concentration-dependent manner and that the inhibition of cell growth was significantly greater at 1 mM and 1.5 mM than that at 0 µM. Thus, in this study, 1 mM was considered as the optimal concentration of H2O2 for the induction of oxidative stress in HSG cells (Fig. 2b). Further, the prophylactic efficacy of AX against H2O2-induced oxidative stress-related disorder was examined and the findings showed that at 6, 12, 24, and 48 hours, cell growth in the H2O2 group significantly reduced in comparison to that in the control group and the AX group, whereas in the AX H2O2 group, cell growth at 24 and 48 hours was significantly greater than that in the H2O2 group (Fig. 2c).

8-OHdG measurement
The results of the assessment of H2O2-induced oxidative stress in HSG cells based on the levels of 8-OHdG are shown in Fig. 3. The AX group and the control group showed no significant difference in the concentrations of 8-OHdG, indicating that AX did not cause any oxidative DNA
Fig. 2 DNA assay.

(a) Effect of astaxanthin (AX) on cell proliferation: The human salivary gland (HSG) cells were cultured for 24 hours in a culture medium supplemented with various concentrations of AX and later, the cell counts were calculated by performing a DNA assay. No significant difference in cell count was found between each of the groups (Tukey’s HSD test).

(b) Effect of H$_2$O$_2$ on cell proliferation: HSG cells were cultured for 24 hours in culture media supplemented with various concentrations of H$_2$O$_2$ and later, cell counts were calculated by performing a DNA assay. The cell counts were significantly lower in the 1 mM and 1.5 mM groups than in the 0 mM group. Statistical significance was determined by Tukey’s HSD test at *p < 0.05, **p < 0.01.

(c) Preventive effect of AX on H$_2$O$_2$-induced cell death: HSG cells were prophylactically treated with 10 µM AX for 24 hours, after which 1 mM H$_2$O$_2$ was added and cell culture was continued for 6-48 hours. Later, the cell counts were determined by performing a DNA assay. In the AX H$_2$O$_2$ group, the cell counts at 24 and 48 hours were significantly higher than those found in the H$_2$O$_2$. Statistical significance was determined by Tukey’s HSD test at *p < 0.05, **p < 0.01.
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damage. Meanwhile, after 6 hours of cell culture, the concentrations of 8-OHdG increased significantly in the H\textsubscript{2}O\textsubscript{2} group compared to that in the control group and the AX group, indicating that oxidative stress was induced by H\textsubscript{2}O\textsubscript{2}. However, the concentrations of 8-OHdG in the AX
H\textsubscript{2}O\textsubscript{2} group were lower than those found in the H\textsubscript{2}O\textsubscript{2} group and a significant difference was found at 12 hours.

**Cytokine assay**

The levels of IL-6 and IL-8 induced by H\textsubscript{2}O\textsubscript{2} in HSG cells are shown in Fig. 4. The amounts of IL-6 and IL-8 in the AX group did not significantly differ from their levels in the control group and AX showed no inflammatory effect. Meanwhile, at 48 hours, the amount of IL-6 in the H\textsubscript{2}O\textsubscript{2} group increased significantly compared to that in the control group and the AX group. However, at 48 hours, the amount of IL-6 in the AX H\textsubscript{2}O\textsubscript{2} group significantly decreased in comparison to that in the H\textsubscript{2}O\textsubscript{2} group (Fig. 4a).

**Assessment of apoptosis**

The H\textsubscript{2}O\textsubscript{2}-induced apoptosis of HSG is shown in Fig. 5. The quantity of CK18-Asp396 in the AX group did not significantly differ from that found in the control group and AX had no effect on apoptosis. Meanwhile, at 24 and 48 hours, the quantity of CK18-Asp396 in the H\textsubscript{2}O\textsubscript{2} group increased significantly in comparison to that in the control group and the AX group, indicating that apoptosis had been induced. However, at 24 and 48 hours, the quantity of CK18-Asp396 in the AX H\textsubscript{2}O\textsubscript{2} group decreased significantly in comparison to that in the H\textsubscript{2}O\textsubscript{2} group, indicating the inhibition of apoptosis.
A decrease in salivary secretion can be due to various causes and develops because of multiple factors. However, because most patients with xerostomia are elderly, aging is believed to have an influence on the development of the condition as a background factor. In addition, ROS have recently been reported to be involved in the pathogenesis of diseases causing salivary secretion disorders, and previous findings have also suggested that similar pathogenic mechanisms were involved in disturbances of salivary secretion resulting from SS, which is an autoimmune disease, or those occurring after radiation therapy. Therefore, accumulation of oxidative stress with age may affect the salivary glands themselves and may be involved in the

**Discussion**

Fig. 4 Effect of astaxanthin (AX) on interleukin (IL)-6 and IL-8 contents. Human salivary gland cells were pre-treated with 10 µM AX for 24 hours, after which 1 mM H2O2 was added and cell culture was continued for 6-48 hours. After that, the IL-6 and IL-8 contents were quantified by using the enzyme-linked immunosorbent assay method. (a) IL-6: At 48 hours, the IL-6 content in the AX H2O2 group was significantly lower than that found in the H2O2 group. (b) IL-8: At 6-48 hours, the IL-8 content in the AX H2O2 group was significantly lower than that found in the H2O2 group. Statistical significance was determined by Tukey’s HSD test at *p < 0.05, **p < 0.01.
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decrease in salivary secretory function in the elderly.

Meanwhile, AX has an extremely strong singlet oxygen-quenching ability26, 27) and a lipid peroxidation inhibitory effect20, 26). In addition, because AX also penetrates the cell membrane and accumulates specifically in mitochondria, which serve as a place for the production of energy, AX has also been reported to protect cells from ROS and to inhibit the development of a state of oxidative stress28). Further, AX inhibits the production of inflammatory cytokines mediated by nuclear factor kappa B (NF-κB), which is transcribed in response to oxidative stress, and previous reports have also suggested that AX had a combined anti-inflammatory effect29). Therefore, in this study, we examined the impact of the anti-oxidative and anti-inflammatory effects of AX on H2O2-induced oxidative stress in human salivary gland-derived cells.

The DNA assay showed that an H2O2 stimulus caused a significant decrease in the cells’ proliferative capacity. However, the findings also confirmed that a prophylactic administration of AX caused a significant inhibition of the decrease in cell growth at 24 and 48 hours. This suggested that AX may have protected the salivary gland cells from H2O2-induced oxidative stress, and that it may have prevented or ameliorated DNA damage, changes in histological structure, and functional decline of the salivary glands.

8-OHdG is an oxidative damage marker for DNAs with a hydroxylated deoxyguanosine (dG, a base from which DNA is composed of) at position 8. 8-OHdG levels are influenced by the balance between the generation and the elimination of ROS by anti-oxidants and are believed to be a biological indicator of oxidative stress affecting the human body30). For this reason, diabetes and aging have been reported to increase the urine and serum levels of 8-OHdG31). In addition, the salivary levels of 8-OHdG have been reported to be higher in patients with SS than
that in healthy subjects, and oxidative stress has also been reported to be involved in the pathogenesis of disturbances of salivary secretion\textsuperscript{24, 25}. Therefore, in our study, 8-OHdG, which was believed to reflect the effect of ROS on living organisms, was quantified by using the ELISA method. The results showed that H₂O₂ stimuli caused a significant increase in 8-OHdG. However, the findings confirmed that after 12 hours, the prophylactic administration of AX caused a significant inhibition of the increase in 8-OHdG. This suggested that H₂O₂-induced-oxidative stress was involved in DNA damage in HSG cells, and that the anti-oxidative effect of AX can protect cells against such DNA damage. Therefore, prophylactic treatment using AX may potentially reduce oxidative stress, which accumulates with age in the salivary glands, and may help prevent xerostomia in the elderly.

In addition, abnormalities of cytokine regulatory function are one of the mechanisms responsible for the age-related changes in salivary secretory function\textsuperscript{32, 33}. NF-κB, which is an intranuclear transcription factor, plays an important role in the expression of inflammatory cytokines such as tumor necrosis factor alpha, IL-6, and IL-8. ROS, such as H₂O₂, cause the intracellular balance between oxidation and reduction reactions to shift towards oxidation, activating the transcription of NF-κB, and inducing the expression of various genes involved in inflammation. As a result, inflammatory cytokines are expressed and inflammation occurs\textsuperscript{34, 35}. The salivary concentrations of cytokines such as IL-6 and IL-8 have been reported to be significantly higher in patients with SS than in healthy subjects\textsuperscript{36}. In our study as well, the addition of H₂O₂ to HSG cells significantly increased the amounts of IL-6 and IL-8. However, the prophylactic addition of AX led to a significant inhibition of the production of IL-6 at 48 hours and IL-8 at 6, 12, 24, and 48 hours. This suggested that the anti-oxidative effect of AX might have caused a reduction of ROS. This may have led to a reduction of the transcriptional activity of NF-κB, and inhibited the production of downstream inflammatory cytokines, thereby exerting an anti-inflammatory effect.

Meanwhile, oxidative stress is widely known to cause apoptosis in various cells\textsuperscript{37}. Apoptosis is the main protective response to oxidative damage, especially DNA damage, and it is essential to embryo development and morphogenesis as well as for a normal immune function. However, when a dysregulation of apoptosis signaling occurs, it triggers various cellular responses and causes diseases\textsuperscript{37}. The accumulation of oxidative stress in the salivary glands owing to aging is believed to cause apoptosis in acinar cells, trigger organic changes such as vacuolar degeneration and fatty degeneration, and decrease glandular parenchyma\textsuperscript{38-41}.

In addition, mitochondria play an important role in oxidative stress-induced apoptosis. Oxidative stress triggers changes in the mitochondrial membrane potential, and opens the membrane permeability transition pores, causing the leakage of cytochrome C into the cytoplasm. This activates the caspase cascade and induces apoptosis\textsuperscript{42}. In HSG cells, oxidative stress has been reported to induce a caspase cascade-dependent apoptosis\textsuperscript{43}. Meanwhile, AX has been reported to inhibit apoptosis by preventing this loss of mitochondrial membrane potential\textsuperscript{44}. In our study, fragments of CK18 cleaved by caspase-3 released from cells during apoptosis were detected using the ELISA method. The results showed that H₂O₂ stimuli caused a significant increase in apoptosis, but a prophylactic addition of AX allowed for significant inhibition of apoptosis at 24 and 48 hours. This suggested that AX may have decreased the apoptosis induced by H₂O₂ oxidative stress, and may have protected salivary gland cells.
The above has confirmed that AX provided salivary gland cells with adequate anti-oxidative and anti-inflammatory effects. The mechanism of action is believed to involve inhibition of NF-κB transcriptional activity by AX. In our previous study using human gingival epithelial cells, Miyachi et al reported that AX inhibited the transcriptional activity of NF-κB\(^23\). Therefore, the mechanism of inhibition of the transcriptional activity of NF-κB in salivary gland cells may also need to be examined in detail in the future.

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**Conflict of Interest**

No potential conflicts of interest were disclosed.

**References**


ヒト唾液腺由来細胞へのH₂O₂誘導による酸化ストレスに対するアスタキサンチンの抗酸化・抗炎症作用

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近年、唾液分泌低下を来す病態形成の一つに活性酸素種（reactive oxygen species：ROS）の関与が報告されている。ROSへの防御は生体内に備えられている内因性抗酸化システムに加えて、抗酸化物質に注目が集まっている。アスタキサンチン（astaxanthin：AX）は種々の生物に広く分布している天然カロテノイドの一種で、強力な抗酸化・抗炎症作用を有する。本研究ではヒト唾液腺由来の細胞株であるhuman submandibular gland cellにおいて、H₂O₂で誘導した酸化ストレス障害に対して、AXが及ぼす作用をin vitroで検討した。その結果、10 µMのAXを予防的に添加することで、8-Hydroxy-2'-deoxyguanosineとinterleukin-6の産生量が有意に低くなり、細胞増殖の減少とアポトーシスが有意に抑制された。以上、AXの持つ抗酸化および抗炎症作用が、酸化ストレス障害から唾液腺細胞を保護することが示唆された。

キーワード：口腔乾燥、酸化ストレス、アスタキサンチン、抗酸化、抗炎症