**Original**

The Study of Ozone Ointment on Human Gingival Fibroblasts Cell Proliferation
**Ability and Anti-Inflammatory**

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Abstract: Ozone is currently being considered as a possible oral antiseptic agent because it is strongly antimicrobial and does not induce microbial resistance. Here, we examined the effects of ozone exposure on the production of collagen type-I and inflammatory cytokines in primary human gingival fibroblasts (HGFs) in vitro using enzyme-linked immunosorbent assays. In this study, we demonstrated that ozone ointment increased type I collagen production and hindered pro-inflammatory cytokine secretion from primary HGFs in vitro. HGFs were isolated from a 65-year-old patient who had undergone surgery due to chronic periodontitis. The cells were exposed to media with or without 0.05, 0.5 and 5 ppm ozone ointment for 24 hours 2 min. No cytotoxic effect of the ozone ointment was observed up to the concentration of 0.5 ppm, cell viability was attenuated at the dose of 5 ppm. When ozone ointment was used at the non-cytotoxic concentration of 0.5 ppm, it significantly enhanced type I collagen production by HGFs within 24 hours. Secretion of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 by HGFs treated with lipopolysaccharide (LPS) decreased when ozone ointment was present in the medium. These results suggest that the therapeutic effect of ozone ointment against periodontal disease is partially due to modulation of the function of HGFs.

Key words: Ozone ointment, Cell proliferation, Anti-inflammatory, Periodontal disease, Human gingival fibroblasts

**Introduction**

Periodontal diseases, indicated as lifestyle-related diseases, are biological reactions caused by periodontal pathogenic bacteria inhabiting the oral cavity, and occur via complex interactions between mechanical stress and the host. In general, the destruction and progression of periodontal tissue are caused by the stimulation of bacterial mass (plaque), which induces the cells of the periodontal tissue to release various cytokines, resulting in the destruction of the periodontal tissue. According to these findings, HGFs shows its ability to function in vitro in various disease conditions. The following materials and antibodies were purchased: 100 ppm Ozone ointment (VMC Co., Ltd., Tokyo, Japan), lipopolysaccharide (LPS) (Sigma-Aldrich Inc., St. Louis, MO, USA) at 37ºC in a 5% CO₂ atmosphere. The following reagents were used in this study: antimicrobial agents, in the production of collagen type-I and inflammatory cytokines by HGFs and attempted to elucidate the mechanism of action of the ozone ointment on periodontal disease.

**Materials and Methods**

**Cell cultures**

HGF cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS: Equitech-Bio, Inc., Kerrville, TX, USA) 100 units/ml penicillin G, and 100 μg/ml streptomycin (Sigma-Aldrich Inc., St. Louis, MO, USA) at 37°C in a 5% CO₂ and 95% air humidified incubator in cell culture dish (Greiner Bio-One Int., Kremsmünster, Austria). HGFs used in this study were obtained from one volunteer after appropriate informed consent was obtained. The Ethics Committee of Osaka Dental University approved the study (protocol 110778). HGFs isolated from adherent gingival tissue on the extracted teeth of patients with chronic periodontal disease were cultured on collagen-coated plates in medium.

**Reagents**

The following materials and antibodies were purchased: 100 ppm Ozone ointment (VMC Co., Ltd. Tokyo, Japan), lipopolysaccharide (LPS)
from Porphyromonas gingivalis (P. gingivalis) (InvivoGen, San Diego, CA, USA), anti-Interleukin (IL)-6 and biotinylated anti-IL-6 antibodies (eBiosciences, San Diego, CA, USA), anti-IL-8 and biotinylated anti-IL-8 antibodies (R&D Systems, Minneapolis, MN, USA), biotinylated anti-collagen type I antibody (Rockland, Limerick, PA, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA), BrdU (5-bromo-2′-deoxyuridine) Cell Proliferation Assay kit (Millipore, Billerica, MA, USA).

**DNA synthesis and MTT assays**

For analysis of DNA synthesis, HGFs (1 x 10^4/cm^2) were cultured in DMEM containing 0.5% FBS (0.5% DMEM) for 24 hours. The cells were then cultured with diluted ozone ointment at 0.05, 0.5, and 5 ppm for 2 min, and the culture medium was removed. Then, the cells were washed with 0.5% DMEM and were cultured with 0.5% DMEM containing BrdU for 24 hours. The level of DNA synthesis in the cells was determined by measuring BrdU-incorporation using the BrdU Cell Proliferation Assay kit. For MTT assay, cells were cultured with the ozone ointment at the above concentrations in DMEM containing 10% FBS (10% DMEM) for 2 min, and the culture medium was removed. Then the cells were cultured with 10% DMEM for 24 hours after washing with 10% DMEM. The subsequent procedures were performed as described elsewhere.

**Enzyme-linked immunosorbent assays (ELISAs)**

To detect cytokine production, HGFs (1 x 10^4/cm^2) were cultured with the ozone ointment (0.5 ppm) for 2 min, and the culture medium was removed. And immediately the cells were cultured with P. gingivalis LPS (100 ng/ml) for 24 hours after washing with 10% DMEM. Then, the culture media were collected, and the cytokine levels were measured using the anti-IL-6 (1 μg/ml) and biotinylated anti-IL-6 (0.6 μg/ml), or anti-IL-8 (2.5 μg/ml) and biotinylated anti-IL-8 (0.2 μg/ml) antibodies. For collagen production, HGFs (1 x 10^4/cm^2) were cultured in DMEM containing 1% FBS (1% DMEM) with the ozone ointment (0.5 ppm) for 2 min. The cells were then washed and cultured with 1% DMEM. Levels of collagen type I were measured using the biotinylated anti-collagen type I antibody (0.2 μg/ml). ELISAs was performed as described in the user manual of the CytoSet kits (Biosource International, Camarillo, CA, USA).

**Figure 1.** Effects of ozone ointment on the viability of human gingival fibroblasts. Human gingival fibroblasts were exposed to media containing 0.05, 0.5, or 5 ppm ozone ointment (designated as +, ++, and +++). All data were compared with those for cells treated with control medium without ozone ointment. Data have been provided as means ± S.D. (n = 5). **p < 0.01 and ***p < 0.001, analyzed with ANOVA with a Dunnett’s test (vs. controls).

**Figure 2.** Effects of ozone ointment on collagen type-1 production by human gingival fibroblasts. Human gingival fibroblasts were exposed to media containing 0.5 ppm ozone (+). Data have been provided as means ± S.D. (n = 5). **p < 0.01, analyzed with a Student’s t-test (vs. controls).
were measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Collagen production was normalized to the total protein content of the cell lysates.

**Statistical analysis**
Quantitative data were statistically analyzed using either one-way analysis of variance (ANOVA) followed by a Tukey’s test or a Student’s t test. Differences were considered to be significant at p < 0.05.

**Results**

The effects of ozone ointment on the cell viability of HGFs were examined using an MTT assay (Fig.1). Up to 0.5 ppm dosage, the ozone showed no marked cytotoxicity against HGFs, but at 5 ppm, it caused a decrease in cell viability by approximately 20% in comparison with untreated controls. These findings suggested that ozone at this level might be cytotoxic against HGFs. Therefore, the conditions of this study were narrowed down to a range within which the ozone did not exhibit cytotoxicity against HGFs, and the following experiments were carried out using ozone ointment at 0.5 ppm. The substrate was no effect for cell proliferation (data not shown).

We next evaluated the effects of ozone ointment on the type-I collagen production ability of HGFs, which is directly linked to the regenerative capacity of periodontal tissues (Fig. 2). This level of ozone, which promoted cell proliferation in comparison with the controls during the evaluation of cell viability, also enhanced the production of type-I collagen by HGFs by approximately 1.4-fold.

The effects of ozone ointment on the secretion of inflammatory cytokines by HGFs are shown in Fig. 3. The production of IL-6 and IL-8 by HGFs was markedly promoted when the latter were placed under the stimulation of LPS from the periodontal pathogenic bacterium *P. gingivalis*, which was known to have a strong inflammation-inducing effect. In contrast, the production of interleukins was markedly suppressed when the stimulation was carried out in the presence of ozone ointment.

**Discussion**

In this study, we examined the effect of ozone ointment on collagen type-I and inflammatory cytokine production in HGFs. The concentration of cytokines such as interleukins IL-6 and IL-8 are known to increase in inflammatory periodontal tissues\(^6\). IL-6 has multiple functions; in addition to its regulatory function on the differentiation and proliferation of immune cells\(^6\), it also aggravates periodontal diseases by inducing bone resorption\(^6\). IL-8 is believed to be involved in the destruction of periodontal tissues by inducing neutrophils\(^6, 30\). Furthermore, various cells, including macrophages and lymphocytes, are known to be intricately involved in the aggravation of periodontal diseases. Among these, HGFs are the most abundantly present cells in gingival connective tissues. HGFs assume the production of collagen, the construction of the structure of periodontal tissues, and the maintenance of homeostasis\(^7\). When they are exposed to pathogenic bacteria or to their components such as LPS, they secrete inflammatory cytokines including IL-6 and IL-8 and thus induce the destruction of periodontal tissues\(^7\). In addition, the secretion of these cytokines has also been reported to be strongly involved in chronic inflammation\(^7\).

Our *in vitro* study has confirmed that ozone ointment enhanced the production of type-I collagen by HGFs, and inhibited the LPS-induced production of inflammatory cytokines (IL-6 and IL-8) therefrom. Furthermore, HGFs have previously been reported to express the genes for Toll-like receptors (TLR) 1–5 and 9, which are widely known as receptors for pathogens; in particular, TLR-2,3,4 and 5 have been reported to be specifically involved in the expression of IL8\(^30\), and TLR-2 is known to be involved in the expression of IL-6\(^7\). These results support the finding of enhanced production of these cytokines following pathogen stimulation. Thus, we demonstrated that ozone increased collagen type-I production and hindered pro-inflammatory cytokine secretion from primary HGFs *in vitro*. In HGFs, cell growth and DNA synthesis were promoted by 0.5 ppm ozone, as was type-I collagen production. In contrast, this ozone dosage inhibited the production of IL-6 and IL-8 by HGFs that was induced by stimulation using *P. gingivalis* LPS.

Ozone has an unpleasant smell and a short half-life of about 40 min\(^5\). Ozone also has low water solubility and thus, aqueous ozone formulations provide no long-term sterilization effect. On the other hand, ozone ointment, which consists of a polyethylene glycol solution containing ozone, does have a long-term sterilization effect. The advantages of ozone ointment include a 6-month-long sterilization effect, the lack of an unpleasant smell, and no development of bacterial strains manifesting ozone-resistance\(^31\). In the course of our studies, we have reported the safety evaluation of ozone for the skin and eye\(^6\), as well as its antimicrobial effects\(^8, 9\) and hemostasis\(^11\) using ozone gel. In addition, a number of reports have shown that ozone could cause improvements in periodontal diseases\(^27-29\). On the other hand, mechanism of ozone sterilization is destruction for cell membrane of bacteria\(^27\). We infer that the ozone ointment has also same activity. Also the ozone ointment has an accelerate effect in wound healing on animal experiment\(^32\).

However, the effects of ozone on the functions of the cells involved in periodontal disease have yet to be elucidated\(^10\). Periodontal diseases are caused by a number of cells and cytokine networks, and the contributing factors are complex, therefore, the collection of data from basic research studies using cultured cells is an important tool for understanding disease development and progression. Thus, in this study, we examined the effects of ozone on the production of inflammatory cytokines and type I collagen in HGFs *in vitro*, and attempted to elucidate the mechanism of action of ozone on periodontal disease. Together, these results suggest that clinical ozone ointment use would facilitate the positive balance between HGFs-mediated periodontal tissue maintenance and repair and the stimulation of inflammation and tissue degeneration following exposure to microbial pathogens.

**Conflict of Interest**
The authors have declared that no COI exists.

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