**Original**

**The Effect of Ozone Gel on Bone Matrix Production by Human Osteosarcoma Cell Line Saos-2**

Pao-Li Wang\(^1\), Yoiichi Tachi\(^2\), Kazuya Masuno\(^3\), Nobutaka Okusa\(^4\) and Yasuhiro Imamura\(^5\)

\(^1\) Department of Innovation in Dental Education, Osaka Dental University, Osaka, Japan  
\(^2\) Laboratory of Nutritional Physiology, Tokyo Kasei University, Tokyo, Japan  
\(^3\) Department of Forensic Dentistry, Osaka Dental University, Osaka, Japan  
\(^4\) Department of Dental Pharmacology, Matsumoto Dental University, Shiojiri, Japan

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Abstract: Ozone is currently being considered as a potential oral antiseptic agent because it is highly antimicrobial and does not induce microbial resistance. In this study, we demonstrated that an optimal dosage of ozone gel enhanced the proliferation, type 1 collagen production, and alkaline phosphatase (ALP) secretion of Saos-2 cells in vitro. Proliferation of Saos-2 cells was assessed by MTT and DNA synthesis assays. Type 1 collagen production and ALP secretion were evaluated using enzyme-linked immunosorbent assay (ELISA) and ALP assays. The cells were treated with/without 0.05, 0.5, 5 ppm ozone gel for 24 h. Ozone gel (0.5 ppm) significantly induced the proliferation of Saos-2 cells. At this concentration, ozone gel enhanced type 1 collagen production and ALP secretion. The results indicated that ozone gel controls the cellular metabolism of osteoblasts, resulting in the secretion of early bone-related biomarkers.

Key words: Ozone gel, ALP, Collagen, Osteoblast, Saos-2 cells

**Introduction**

Ozone is currently being considered in dentistry as a potential alternative oral antiseptic agent. Its strong antimicrobial effect without the development of drug resistance has been previously noted in water purification and food preservation techniques\(^4\,5\). In dentistry, ozone has been used either in gaseous or aqueous forms for the elimination of pathogens causing caries, in the disinfection of root canals, and as a rinse for avulsed teeth\(^6\,9\). However, ozone has an unpleasant smell and a short half-life of approximately 40 min\(^1\). Ozone also has low water solubility, and therefore, aqueous ozone formulations provide no long-term sterilization effect. On the contrary, ozone gel, which consists of a glycerin solution containing ozone, has a long-term sterilization effect. The advantages of ozone gel include a 6-month-long sterilization effect, the lack of an unpleasant smell, and no development of bacterial strains manifesting ozone-resistance. Previously, we reported the safety evaluation of ozone for the skin and eye, as well as its antimicrobial effects and role in hemostasis using ozone gel\(^10\,11\). In addition, a number of reports have shown that ozone can ameliorate periodontal diseases\(^13\,15\). However, the effects of ozone on the functions of cells involved in periodontal disease are yet to be elucidated\(^16\,19\). Recently, we reported the effects of ozone gel on the production of inflammatory cytokines and type I collagen in human gingival fibroblasts (HGFs) \textit{in vitro}, and attempted to elucidate the mechanism of action of ozone on periodontal disease\(^20\). In this study, we examined effects of ozone gel on type I collagen production and alkaline phosphatase (ALP) secretion in the human osteosarcoma cell line Saos-2.

**Materials and Methods**

**Cell cultures**

Saos-2 cells (RIKEN BRC Cell Bank, Tokyo, Japan) were cultured in Dulbecco’s modified Eagle's medium (DMEM, Nissui pharmaceutical Co. Ltd., Tokyo, Japan) with 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 µg/ml streptomycin at 37°C in a 5% CO\(_2\) and 95% air humidified incubator.

**DNA synthesis and MTT assays**

For DNA synthesis, Saos-2 cells (1x10\(^4\)) were cultured in DMEM containing 0.5% FBS (0.5% DMEM) for 24 h. The cells were cultured with the ozone gel (VCM Co. Ltd., Tokyo, Japan) or 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 bromodeoxy uridine (BrdU) for 24 h. The level of DNA synthesis was determined by measuring BrdU-incorporation using the BrdU cell proliferation assay kit (Millipore Tokyo, Japan). For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich) assay, cells were cultured with the ozone gel at 0.05, 0.5, and 5 ppm in DMEM containing 10% FBS (10% DMEM) for 2 min, and the culture medium was removed. After washing with 10% DMEM, the cells were cultured with 10% DMEM for 24 h. The subsequent procedures were performed as described elsewhere\(^21\).

**Enzyme-linked immunosorbent assay (ELISA)**

For collagen production, Saos-2 cells (1x10\(^4\)) were cultured in DMEM containing 1% FBS (1% DMEM) with the ozone gel (0.5 ppm) for 2 min. The cells were washed and cultured with 1% DMEM. Levels of type I collagen in the media were measured using the biotinylated anti-type I collagen antibody (0.2 µg/ml, Rockland). ELISA was performed as described in the user manual of CytoSet kits (Biosource, Tokyo, Japan)\(^22\). The cells for collagen production were lysed with 0.5% Triton X-100, and the protein concentration of the cell lysates was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Tokyo, Japan). Collagen production was normalized to the protein content of the cell lysates.

**ALP activity**

Saos-2 cells (1x10\(^4\)) were cultured with the ozone gel (0.5 ppm) for 2 min, and the culture medium was removed. The cells were washed...
with 0.5% DMEM and cultured with 10% DMEM for 24 h. The cells were lysed with 0.05% TritonX-100 and ALP activity of the lysates was measured using a LabAssay ALP kit (Wako Pure Chemicals Industries, Ltd., Osaka, Japan). The protein concentrations of the cell lysates were also measured using a BCA protein assay kit. ALP activity was normalized to the protein content of the cell lysates.

**Statistical analysis**

Quantitative data were statistically analyzed using either one-way analysis of variance (ANOVA) followed by Tukey’s test (DNA synthesis and MTT assays) or Student’s t-test (measurement of ALP activity and ELISA for collagen production) using the StatMate software (ATMS). Differences were considered to be significant at p < 0.05.

**Results**

**Effect of ozone gel on proliferation of Saos-2 cells**

After 24 h of incubation, the ozone gel treatment increased Saos-2 proliferation in a dose-dependent manner (Fig. 1). Ozone gel at 0.5 ppm significantly induced cell growth compared to the other concentrations, whereas no obvious change was observed with 0.5 ppm ozone gel treatment. Similar to the results of the MTT assay, 0.5 ppm ozone gel effectively elevated DNA synthesis in Saos-2 cells, indicating the optimal concentration of ozone gel required for facilitating the proliferation of Saos-2 cells.

**Effect of ozone gel on type 1 collagen production and ALP secretion by Saos-2 cells**

Ozone gel at 0.5 ppm significantly induced cell growth. This, considering the results of the proliferation assays, we further evaluated the effect of ozone gel on collagen 1 production and ALP secretion from Saos-2 cells using 0.5 ppm ozone gel. Addition of ozone gel in the media effectively increased type 1 collagen production (Fig. 2) and ALP secretion (Fig. 3) from Saos-2 cells compared to that without ozone gel treatment.

**Discussion**

The effect of ozone gel on bone matrix production by osteoblasts is unclear. The present study showed that while high concentrations of the ozone gel decreased the cell viability index, low concentrations of the gel enhanced cell proliferation. Furthermore, 0.5 ppm ozone gel promoted the secretion of type 1 collagen and ALP, which are highly related to bone formation.

Previously, Oda et al. reported that 10 ppm ozone gel suppresses the proliferation of human gingival fibroblasts. The present study showed that while cell proliferation was significantly suppressed at high concentrations (5 ppm) of ozone gel, 0.5 ppm ozone gel promoted cell proliferation. In a study on non-gel forms of ozone, Tsujue et al. reported that ozone water dissolved in phosphate-buffered saline (PBS) induced cell damage at 3 ppm as visualized by hematoxylin and eosin staining. A study using alveolar macrophages reported that 0.5 ppm ozone gas causes cellular damage to the monocyte strain THP-1. Ozone gel has the advantage of providing sustained release of ozone, which is contained.
6. Huth KC, Paschos E, Brand K and Hickel R. Effect of ozone on non-viable tissue. J Dent Res 99: 75-84, 2000
7. Bocci V. Mechanism of action on ozone. In: Oxygen-ozone therapy. A critical evaluation, ed by Bocci V, Maruzen Publishing, Inc., Tokyo, 2012, pp 17-25. (in Japanese)
8. Wang PL, Shiota G and Shiba A. Safety evaluation of ozone gel for skin and eye on animal experiments. J Hard Tissue Biol 20: 313-318, 2011
9. Fukui T, Masuno K, Makita Y, Fujiwara S and Shiota G. Antimicrobial effects of ozone gel against periodontal bacteria. J Hard Tissue Biol 23: 445-448, 2014
10. Sakai D, Makita Y, Masuno K, Fujiwara S and Okazaki J. Local hemostatic effect of aqueous ozone in cutting wound surface. J Hard Tissue Biol 23: 245-248, 2014
11. Srikanth A, Sathish M and Sri Harsha AV. Application of ozone in the treatment of periodontal disease. J Pharm Bioallied Sci 5: S89-94, 2013
12. Saini R. Ozone therapy in dentistry: A strategic review. J Nat Sci Biol Med 2: 151-153, 2011
13. Arpita R, Swetha JL, Babu MR and Sudhir R. Recent trends in non-surgical periodontal care for the general dentist-a review. Bangi J Dent Res Edu 4: 78-82, 2014
14. Wang G, Umstead TM, Phelps DS, Al-Mondhiry H and Flores J. The effect of ozone exposure on the ability of human surfactant protein A variants to stimulate cytokine production. Environ Health Perspect 110: 79-84, 2002
15. Li Z, Tighe RM, Feng F, Leford JG and Hollingsworth JW. Genes of innate immunity and the biological response to inhaled ozone. J Biochem Mol Toxicol 27: 3-16, 2013
16. Huang W, Wang G, Phelps DS, Al-Mondhiry H and Flores J. Human SP-A genetic variants and bleomycin-induced cytokine production by THP-1 cells: effect of ozone-induced SP-A oxidation. Am J Physiol-Lung Cell Mol Physiol 286: 546-553, 2004
17. Mikerov AN, Umstead TM, Gan X, Huang W, Guo X, Wang G, Phelps DS and Flores J. Impact of ozone exposure on the phagocytic activity of human surfactant protein A (SP-A) and SP-A variants. Am J Physiol-Lung Cell Mol Physiol 294: 121-130, 2008
18. Makita Y, Imamury Y, Masuno K, Fujitama, Shiota G, Shiba A and Wang PL. The effect of Ozone on Collagen Type-1 and Inflammatory Cytokine Production in Human Gingival Fibroblasts. Dentistry 5:339. doi:10.4172/2161-1122.1000339, 2015
19. Imamury Y, Fujitama Y, Oomori Y, Usui S and Wang PL. Cooperation of salivary protein histatin 3 with heat shock cognate protein 70 relative to the G1/S transition in human gingival fibroblasts. J Biol Chem 284: 14316-14325, 2009
20. Imamury Y and Wang PL. Salivary histatin 3 inhibits heat shock cognate protein 70-mediated inflammatory cytokine production through toll-like receptors in human gingival fibroblasts. J Inflamm (Lond) 11: 4, 2014
21. Oda H, Manyuama, K, Tsubokawa M, Sioida G, Kamoi H, Nagahiro K and Sato S. Effect of ozone gel on oral pathogens and human gingival and periodontal ligament fibroblasts. Jpn J Conserv Dent 57: 369-376, 2014
22. Tsujigami H. Study of cytotoxic and bacterial effects of ozonized water against periodontium-derived cells and periodontopathic bacteria. J Jpn Assoc Periodontol 44: 46-54, 2002
23. Kleskard D1, Laval-Gilly P and Falla J. Ozone-mediated cytotoxicity after short-term exposure and its relation to the production of cellular metabolites (NO, H2O2). Cell Biol Toxicol 18: 259-269, 2002
26. Miron RJ and Zhang YF. Osteoinduction: a review of old concepts with new standards. J Dent Res 91: 736-744, 2012
27. Zhang J, Guan M, Xie C, Luo X, Zhang Q and Xue Y. Increased growth factors play a role in wound healing promoted by noninvasive oxygen-ozone therapy in diabetic patients with foot ulcers. Oxid Med Cell Longev. 273475. doi: 10.1155/2014/273475, 2014
28. Rei L, martínez-sánchez G, perez-davison G and Sirito M. Role of ozone/oxygen in fibroblast growth factor activation. Discovering the Facts Int J Ozone Therapy 9: 55-58, 2010
29. Tan YY, Yang YQ, Chai L, Wong RW and Rabie AB. Effects of vascular endothelial growth factor (VEGF) on MC3T3-E1. Orthod Craniofac Res 13: 223-228, 2010
30. Srikanth A1, Sathish M and Sri Harsha AV. Application of ozone in the treatment of periodontal disease. J Phar Bioallied Sci 5: 89-94, 2013