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Journal of Dental Health Oral Disorders & Therapy

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Oxygen and air nanobubbles in water inhibit proliferation of dental follicle stem cells *in vitro*

Abstract

Background: Nanobubbles (NBs) are small gas particles with diameters less than 200 nm, and have unique properties, such as long retention times and high gas content in liquids. Oxygen and air NBs are reportedly bioactive, promoting growth in animals; however, there are few reports on the mechanisms governing such effects. Accordingly, this study investigated the effects of oxygen and air NBs on the proliferation of dental follicle stem cells (DFSCs) in vitro.

Methods: DFSCs were obtained from an impacted supernumerary tooth. The cells were cultured with Dulbecco's modified Eagle's medium containing oxygen or air NBs water for 0, 2, 4, or 6 days. Each cell number was measured by a colorimetric assay using CCK-8.

Results and discussion: Oxygen and air NBs significantly inhibited cell proliferation in a dose-dependent manner. However, there was no significant difference between oxygen and air NBs media. High oxygen content may have inhibited cell proliferation.

Keywords: nanobubble, microbubble, dental follicle stem cells

Volume 9 Issue 6 - 2018

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Received: November 11, 2018 | **Published:** November 16, 2018

Introduction

Nanobubbles (NBs) water contains small gas particles with diameter of less than 200 nm and may have a gas content exceeding its solubility1. NBs water also has unique characteristics: its NBs possess high internal pressure and remain stable for a longer period than do microbubbles (MBs, or gas bubbles less than 50µm in diameter) because of the negatively charged NBs surface, and smaller buoyancy.[1] Oxygen and air NBs and MB water are reportedly bioactive and promote growth in animals and plants.[2–4] and have increased survival rates of fish in long-duration carbon dioxide anesthesia experiments.[5] Bactericidal effects of ozone NBs water have been demonstrated in the treatment of periodontitis.[6] Accordingly, NBs water is expected to be a promising technology for the medical field.[7] However, the mechanisms governing the effects of NBs water on living organisms at the cellular and molecular levels are still unclear. Thus, the evaluation of their effects at cellular and molecular levels will be required for better use of NBs water in medicine and dentistry. Human dental follicle stem cells (DFSCs) can be clinically obtained from impacted supernumerary teeth in pediatric dentistry, and are useful for bioassay.[8] The purpose of this study was to investigate the influence of oxygen and air NBs water on the proliferation of DFSCs *in vitro*.

Material and methods

All experiments were conducted with the approval of the research ethics committee of the Faculty of Dentistry of Tokyo Medical and Dental University (approval number D2016-038).

Cell culture of DFSCs

A 7-year-old male patient undergoing extraction of an impacted supernumerary tooth with no medical history and his guardian were informed about this research and provided written consent for participation. DFSCs were prepared from the impacted tooth as described previously.[8]

Production of oxygen and air NBs water

Oxygen and air NBs water were generated with a nanobubble generator (NanoEarth, Earthlink Co., Inc., Kanagawa, Japan). The generator was operated for approximately 30 min according to evaluation by an electron spin resonance spectrometer (JES-TE300, JEOL, Ltd., Tokyo, Japan) following the method of Takahashi et al.[9,10]

Preparation of NBs culture media

Powdered Dulbecco's modified Eagle medium (DMEM: Sigma-Aldrich Japan) and sodium hydrogen carbonate (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) were dissolved in air and oxygen NBs water and sterilized using 22µm filters. These air and oxygen NBs media were referred to "100% air and 100% oxygen NBs medium," respectively (A). We also prepared a control medium using Milli-Q water without NBs. This control medium was referred to 0% NBs medium (B). We produced working culture media by mixing media A and B at A ratios of 0 (control), 10, 50, and 100%.

Cell counting

The cultured DFSCs were seeded in 96-well plates at 5 x 103 per well in DMEM (n=6). On the next day, the media were changed to working culture media (air and oxygen NBs media, 0, 10, 50, or 100%), and the cells were cultured for 0, 2, 4, or 6 days under 5% CO2. Media were changed every other day. At the end of the incubation period, the culture media were replaced with 100µl of fresh media, and 10µl

of Cell Counting Kit-8 solution (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added to each well in accordance with the instructions. After 2 h of incubation for coloration, the optical density (OD) at 450/630 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, California, USA). For cell number determination in these media, suspensions in specific ratios of media A and B were inoculated into a 96-well plate, and OD was measured as mentioned above using CCK-8 to derive the conversion formulas for OD to cell number. All data are expressed as means±standard deviation (SD). Differences between cell numbers in NBs and control media at 6 days were assessed by two-way ANOVA. The level of significance was set at 0.05.

Results

NBs media reduced OD at all cell numbers compared with that of the control (Figure 1). In this study, air NBs media produced lower OD values than did oxygen. The conversion formulas were derived by a first-order approximation with correlation coefficients greater than 0.97. Both oxygen and air NBs media significantly inhibited cell proliferation in dose-dependent manners after 2 days (Figure 2), and the difference was very clear after 4 days. At 6 days, cell numbers in oxygen NBs media were 16–69% that in control medium, and cell numbers in air NBs media were 21–77% that in control medium, demonstrating the significant dose-dependent effect on proliferation by two-way ANOVA. Although lower cell numbers were observed in oxygen NBs media than in air NBs media at each time point, the differences were not significant.

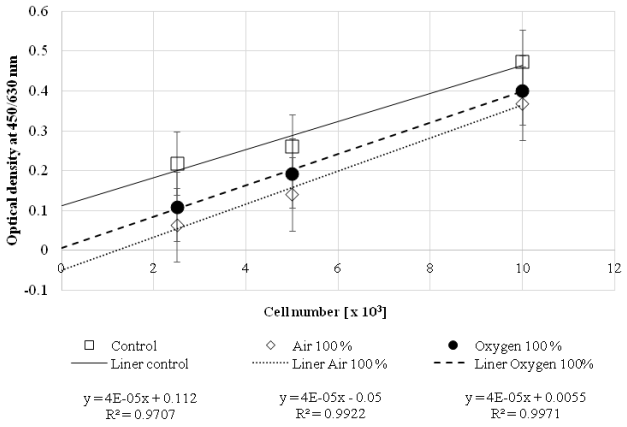

Figure 1 Derivation of formulas for conversion of optical density at 450/630 nm to cell number in NBs media. The optical density of the cells in 100% air NBs, 100% oxygen NBs, and control medium were measured at 2500, 5000, and 10000 cells. In each medium, a first-order approximation was adopted as a conversion formula.

Discussion

We measured proliferation of cells in culture with NBs media, and used human DFSCs to examine the clinical effects of NBs water as they relate to dentistry. To our knowledge, this is the first report of inhibitory effects of oxygen and air NBs media on cell proliferation; previous agricultural, fishery, and animal studies reported growth promotion.[2–4] The relationship between the inhibitory effects we observed and the growth promotion reported in living organisms is still unclear. It is possible that air and oxygen NBs affect cell proliferation in culture and whole organisms differently. Comprehensive elucidation of the effects of air and oxygen NBs water on oral tissue and living organisms will require further investigation both *in vitro* and *in vivo*. The color characteristics of control and air and oxygen NBs media were somewhat different. Notably, oxygen NBs may alter cellular redox states, causing a discrepancy between actual viable cell numbers and those determined using the CCK-8 assay kit. Therefore, we calculated cell numbers according to a first-order approximation based on the graph of OD vs cell number (Figure 1). The OD values of both NBs media were relatively lower than that of the control, suggesting that NBs themselves influence the optical density of water.

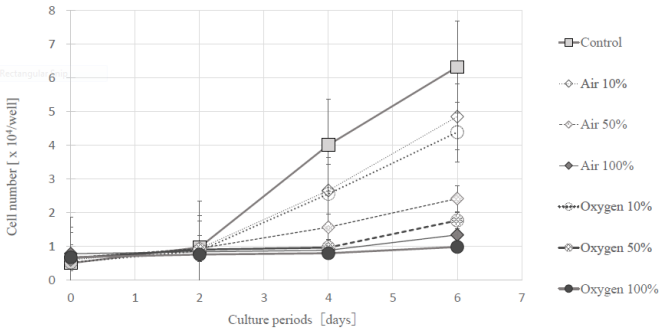

Figure 2 DFSCs proliferation in NBs media. Optical density of cultured cells in each NBs medium was converted to cell number by the conversion formulas described in Figure 1. OD values of the cultured cells in 10% and 50% air and oxygen NBs media were converted to cell numbers using the formulas for 100% air and oxygen NBs and control media adjusted according to the media mixing ratios.

After 4 days, the proliferation curves in all experimental groups were lower than that in the control. Cell numbers in the 100% oxygen medium changed little during the experimental period. Though there were no significant differences in growth inhibition between oxygen and air NBs, oxygen content may have induced oxygen toxicity conditions in the culture. On the other hand, the presence of the NBs themselves rather than the gas type might have affected the proliferation. Further experiments using a 100% nitrogen NBs medium might clarify the effects of oxygen in NBs.

Conclusion

High oxygen content in NBs media may inhibit cell proliferation in cell culture.

Acknowledgements

This work was supported by a JSPS KAKENHI Grant-in-Aid for Young Scientists (B) (grant no. 26870173).

Conflicts of interest

Authors declare that there is no conflict of interest.

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Citation: Oishi A, Shimokawa H, Sakaniwa E, et al. Oxygen and air nanobubbles in water inhibit proliferation of dental follicle stem cells *in vitro*. *J Dent Health Oral Disord Ther.* 2018;9(6):460–462. DOI: 10.15406/jdhodt.2018.09.00427