

Brief Report

Effects of Nitrogen, Oxygen, and Hydrogen Nanobubbles on Human Lung Fibroblast Viability

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Abstract

Nanobubbles (NBs) (<1 μm) exhibit unique physicochemical properties and high stability, which have potential applications in biological systems, including microbial metabolism enhancement, fibroblast proliferation, and tumor inhibition. However, the influence of different gaseous components on cell viability remains unclear. This study examined the effects of nitrogen, oxygen, and hydrogen NBs on human lung fibroblast cell (MRC-5) viability. NBs were generated in a Dulbecco's modified Eagle's medium using a gas–liquid mixing method and characterized by a nanoparticle tracking analysis. After 48 h of culture, cell viability increased 1.34-, 1.30-, and 1.29-fold for nitrogen, oxygen, and hydrogen NBs, respectively, with only nitrogen NBs showing a significant increase ($p < 0.05$). Flow cytometry with carboxyfluorescein succinimidyl ester analysis showed no difference in proliferation among groups, indicating that enhanced viability might result from gas-specific effects rather than direct stimulation of cell division. These findings highlight the potential biomedical applications of nanobubbles and their constituent gases.

Keywords: nanobubbles (NBs); gas effect; fibroblast cell; cell viability

1. Introduction

Nanobubbles (NBs) are bubbles trapped in liquids, which are <1 μm in size [1,2]. NBs have unique properties compared with macrosized bubbles, including a long lifespan ranging from days to months. Thus, NBs have been used in various fields, and several attempts have been made to apply them in cell culture. Air and oxygen NBs promote the metabolism and growth of plants and animals [3]. *Lactobacillus* produces lactic acid faster in a nitrogen NB environment than in a normal group that consumes the same amount of glucose; this happens because of the zeta potential generated by NBs [4]. WI-38 lung fibroblasts cultured in a medium containing oxygen NBs promote cell division and have a wound-healing effect [5], and the viability of MRC-5 cells increases in a nitrogen NB environment [6]. NBs containing oxygen and nitrogen inhibit the growth of HL-60 leukemia cells and positively affect the culture of L929 fibroblasts [7]. These studies suggest that NBs have considerable potential for various cell biological applications. However, few studies have investigated the direct effects of the gaseous components of NBs on cells.

Cells are sensitive to their surroundings and exhibit various responses. Changes in the composition of the gas surrounding a culture medium can affect cell differentiation, viability, and other factors [8–10]. Hypoxia promotes the expression of fibroblast growth



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factor 2 and the proliferation of human bone marrow stromal cells [11]. Argon and xenon prevent apoptosis in human osteosarcoma U2OS cell cultures in which nitrogen is replaced with inert gases [12]. However, the influence of individual gaseous components within NBs on cell viability has not been thoroughly investigated.

This study hypothesized that the gases contained in NBs influence cell function and growth. To verify and quantify the effects of NBs, NBs containing three different gases (nitrogen, oxygen, and hydrogen) were directly generated in a Dulbecco's modified Eagle's medium (DMEM, Gibco®, Thermo Fisher Scientific, Waltham, MA, USA). Human lung fibroblast cells (MRC-5) were cultured, and cell viability was assessed. In summary, the findings of this study may provide a basis for further research on the effects of NBs and their component gases on various cell types and potential biomedical applications of NBs.

2. Materials and Methods

2.1. NB Generation and Measurement

NBs were fabricated using a gas–liquid mixing (agitation) method using a linear actuator [13]. Two holes with a diameter of 0.5 mm were drilled into the lid of a sterilized disposable conical tube. The tube was filled with DMEM and sealed with a double-holed lid. Nitrogen, oxygen, and hydrogen were supplied through one of the two holes using a tube, while the other hole allowed DMEM to be displaced by the injected air and discharged. After filling half of the tube with gas, the holes and conical tube lid were sealed with Parafilm to maintain sterility and prevent gas leakage. The sealed conical tube was mounted on an actuator and agitated at 117 rpm. An NB-containing cell culture medium was prepared by mixing the NB-containing DMEM with 10% fetal bovine serum (FBS, Gibco®, Thermo Fisher Scientific, MA, USA) and 1% antibiotic–antimycotic (Anti-Anti, 100X, Gibco®, Thermo Fisher Scientific). The size and density of NBs formed in DMEM were measured using a nanoparticle tracking analysis (NTA) instrument (NanoSight LM10-HSBFT14; Malvern, UK), a charge-coupled device camera, and a 642 nm red laser. The generation of NBs in DMEM was repeated seven times on different days.

2.2. Cell Culture

MRC-5 cells obtained from the Korean Cell Line Bank (Korean Cell Line Research Foundation, Seoul, Republic of Korea) were used in experiments. A standard culture medium consisting of 89% DMEM was used without NBs (control group) and with NBs (experimental group), in addition to 10% FBS and 1% antibiotic–antimycotic. Cells were cultured in an incubator maintained at 37 °C, 95% humidity, and 5% CO₂. To ensure cell attachment, the cells were cultured in a standard culture medium for 24 h after seeding. After the cells were attached to the culture dish, the culture medium of the experimental group was replaced with the culture medium containing DMEM with NBs, and the culture medium of the control group was replaced with pure DMEM. Subsequently, the cells were cultured for an additional 48 h to test the effect of the gases contained in the NBs on the cells. In each experiment, the control group was cultured in 15 wells of a 24-well plate, whereas each NB group was cultured in 10 wells per gas type, for a total of 30 NB wells. This experimental setup was repeated seven times.

2.3. Nucleus Staining

To measure cell viability, cell nuclei were stained using the NucBlue® Live ReadyProbes™ reagent (NucBlue; Invitrogen™, Thermo Fisher Scientific) and then counted.

Cells were seeded in a 24-well plate at a concentration of 5000 cells/well. The dye was prepared by diluting NucBlue to a concentration of two drops (approximately 60 µL) per mL in a standard culture medium. After removing the culture medium from each

well, 0.5 mL of the prepared dye was added, and the cells were incubated for 20 min. Staining was performed after cell attachment and after 48 h of culture in the medium containing NBs. Stained cells were imaged at the center of the well using an inverted fluorescence microscope (CKX41; Olympus, Tokyo, Japan). The images were postprocessed using ImageJ 1.52s software (US National Institutes of Health, Bethesda, MD, USA) to count the number of cells.

2.4. Flow Cytometry Analysis

Flow cytometry was performed to measure cell proliferation. A carboxyfluorescein succinimidyl ester (CFSE) cell proliferation analysis was performed using FACS Canto™II (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For the analyses, cells were seeded in $\Phi 150$ mm culture dishes at a concentration of 1×10^6 cells/dish.

A dye solution was prepared for the CFSE analysis. The CFSE dye was dissolved in dimethyl sulfoxide at a concentration of 5 mM, and the solution was diluted to 1000:1 in Dulbecco's phosphate-buffered saline. Before seeding cells, 1 mL of the prepared dye solution was added to the cell suspension of 1×10^6 cells and cultured in an incubator at 37 °C for 20 min. Cell proliferation was analyzed via flow cytometry 24 and 72 h after seeding.

2.5. Statistical Analysis

All data are presented as the mean $\pm$ standard deviation (SD). Statistical analyses were performed using SPSS Statistics 24.0.0 (IBM Corporation, Armonk, NY, USA). Comparisons between the two groups were conducted using a two-tailed Student's *t*-test. For multiple group comparisons, a one-way analysis of variance followed by post hoc test and Tukey's multiple comparison test were used. A *p*-value of < 0.05 was considered statistically significant. The number of independent biological replicates (*n*) was 93 for the control group, and 63, 57, and 68 for the oxygen-, hydrogen-, and nitrogen-NB groups, respectively.

3. Results and Discussion

3.1. NB Generation

The concentration and size of the NBs generated in DMEM were measured using an NTA instrument (Figure 1). Seven independent experiments were performed on different days; in each experiment, 15 samples were analyzed for the control group and 10 samples for each NB-treated group, with all NB-containing media prepared on the same day having the same NB concentration. The concentrations of NBs used in these experiments were designated as Case 1–7 according to the order in which the measurements were performed. Figure 1a–c show the concentration of NBs generated in DMEM for different gases. The measured concentrations (mean $\pm$ SD) of the nitrogen, oxygen, and hydrogen NBs ranged from $4.025 \pm 0.72 \times 10^8$ to $6.07 \pm 0.75 \times 10^8$ NBs/mL (Figure 1a), $3.17 \pm 0.28 \times 10^8$ to $4.56 \pm 1.02 \times 10^8$ NBs/mL (Figure 1b), and $1.55 \pm 0.30 \times 10^8$ to $9.04 \pm 0.49 \times 10^8$ NBs/mL (Figure 1c), respectively. Nitrogen and oxygen, which had similar molecular weights, formed NBs with similar average concentrations of $4.71 \pm 0.70 \times 10^8$ and $4.22 \pm 0.46 \times 10^8$ NBs/mL, respectively. However, hydrogen, which had a lower molecular weight, formed NBs with an average concentration of $4.24 \pm 2.20 \times 10^8$ NBs/mL. This was similar to the concentrations of the other NBs but with a large deviation. Notably, hydrogen, owing to its low molecular weight and high diffusivity, may have partially permeated through the Parafilm sealing, leading to greater variability in NB concentration. Therefore, we presumed that the high mobility and low impermeability of hydrogen during NB formation via gas–liquid mixing (stirring) method contributed to the observed variation in NB concentration.

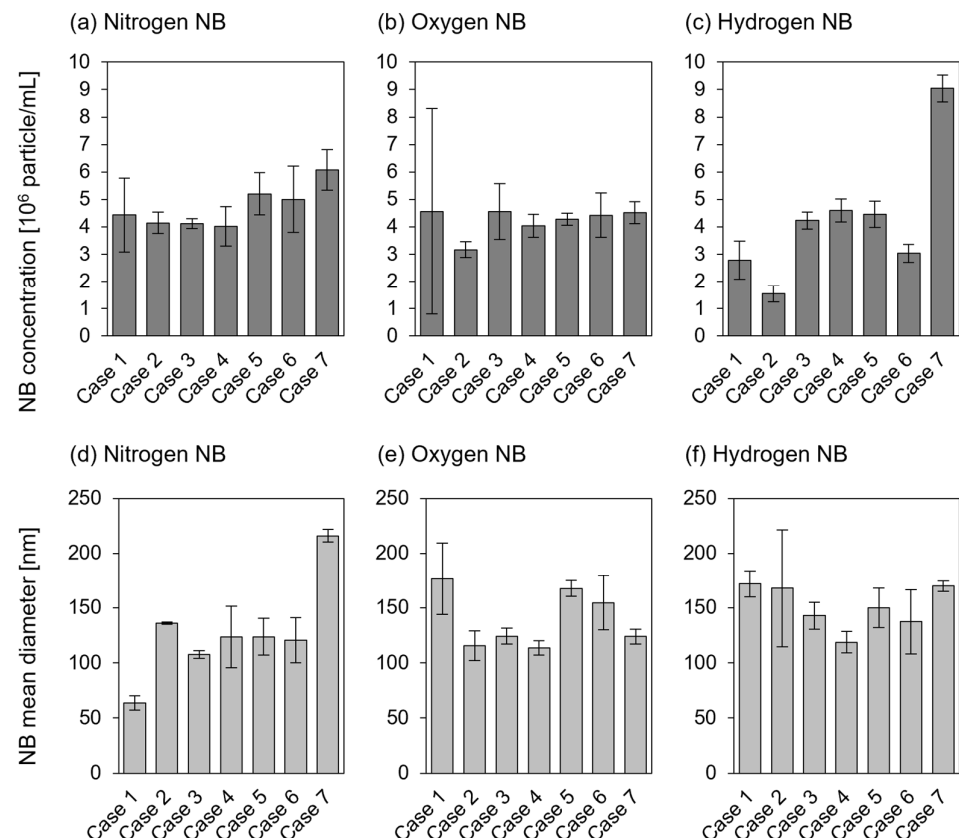


Figure 1. Concentration and average diameter of nanobubbles (NBs) for different experimental cases. (a) Average concentration of nitrogen NBs. (b) Average concentration of oxygen NBs. (c) Average concentration of hydrogen NBs. (d) Average diameter of nitrogen NBs. (e) Average diameter of oxygen NBs. (f) Average diameter of hydrogen NBs.

Figure 1d–f show the sizes of the NBs formed in DMEM for different gases. The average diameters of the nitrogen, oxygen, and hydrogen NBs were 63.5 ± 6.5 to 216 ± 5.7 nm (Figure 1d), 113.67 ± 6.6 to 176.67 ± 32.71 nm (Figure 1e), and 118.67 ± 9.74 to 172 ± 12 nm (Figure 1f), respectively. As DMEM contained various components with heterogeneous properties, the size distribution and concentration range of the NBs were presumed to be broader than those in pure water. The heterogeneity of the constituents of DMEM and differences in the mobility of gas molecules likely contributed to the observed variations in NB diameter and concentration.

The NB concentration over time was measured for each gas. Although slight differences were observed depending on the gas type, the NB concentration stabilized at approximately 43.0% of the initial value after 48 h (Figure 2). NBs have been reported to be stabilized by several factors, including strong hydrogen bonding with water, high initial gas concentration, diffusion barriers formed by NB clusters, and electrically charged gas–liquid interfaces [14–16]. Even in the case of hydrogen, which had high diffusivity and explosivity, the NB concentration was maintained at approximately 48.0% compared with the initial concentration. This suggested that hydrogen loss from the culture medium during the experimental period was limited and hydrogen NBs were sufficiently stable under the applied culture conditions. Furthermore, in comparison with conventional approaches that involve directly filling the culture environment with a specific gas to study gas–cell interactions, the use of NBs offers an additional advantage by reducing safety concerns, such as explosion risk, because only a very small amount of hydrogen (under 1 nL) is present in the culture medium as NB at initial condition.

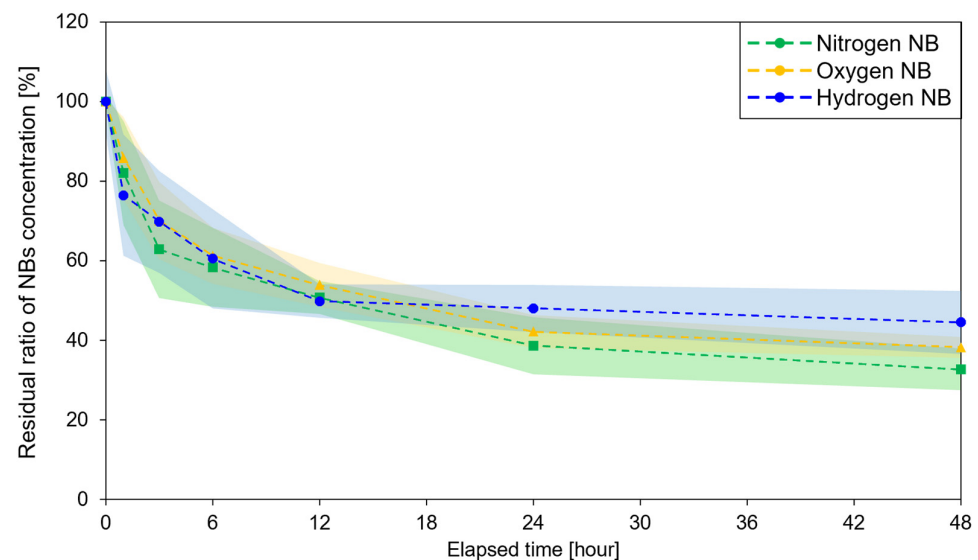


Figure 2. Residual ratio of NB concentration for each constituent gas over time in DMEM. The NB concentration in DMEM decreased rapidly during the first 3 h, followed by a gradual decline to approximately 43% of the initial concentration at 24 h. This level was maintained throughout the 48 h cell culture period. The shaded areas represent the SD.

Although dissolved gas concentrations were not directly measured in this study, previous reports have shown that NBs did not induce a significant increase in bulk dissolved gas levels in aqueous solutions [17,18]. Therefore, the observed cellular responses are unlikely to be solely attributed to hypoxic or hyperoxic conditions.

3.2. Cell Population and Counting

MRC-5 cells were cultured in a medium containing NBs, which were composed of nitrogen, oxygen, and hydrogen, to evaluate cell viability. MRC-5 is human lung fibroblast cell. Fibroblasts are suitable for comparing fundamental cellular responses to changes in culture conditions owing to their high culture stability and reproducibility. Furthermore, because the lungs perform gas exchange, lung cells interact directly with gases. Therefore, in this experiment, human lung fibroblasts were used as a primary evaluation model to qualitatively examine the effects of a culture medium with NBs containing three gases on cell viability. This study is limited to measuring cell viability, and interpretation of the NB mechanism of action, oxidative stress, and toxic effects is limited based on these results.

Cell viability was measured by staining and counting cell nuclei using ImageJ. The fluorescence image (Figure A1a) was converted to black and white image such that the fluorescently stained nuclei were white (Figure A1b). Subsequently, the image level was adjusted to clearly separate the background from the nuclei. The brightness was adjusted to minimize noise while ensuring that the nucleus image was not affected. To prevent noise from being counted, we used the noise reduction function to remove dots with a size of ≤ 5 pixels (Figure A1c) and counted stained cells (white dot, Figure A1d).

In the control group, after cell attachment, the medium was replaced with a medium without NBs, and the number of cells increased by 1.27 times after 48 h. When the medium was replaced with the medium containing nitrogen, oxygen, and hydrogen NBs, the number of cells increased by 1.34, 1.30, and 1.29 times, respectively. This indicated that the NB-containing medium positively affected cell viability, and the effect depended on the gas species (Figure 3).

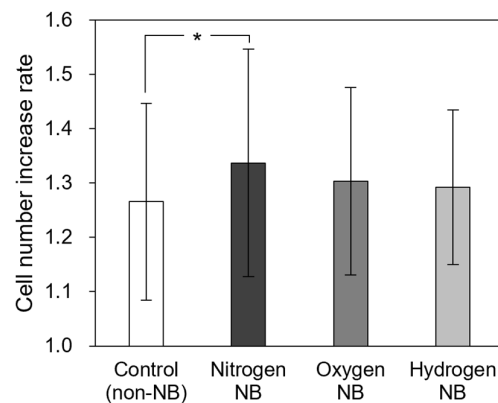


Figure 3. Comparison of cell nuclei counts obtained using ImageJ. Medium containing nitrogen NBs showing a significant increase in nuclei count (5.5%) compared with the control group. Medium containing oxygen and hydrogen NBs showing increases of 2.4% and 1.6%, respectively; however, these differences were not statistically significant. * indicates statistical significance at $p < 0.05$ compared with the control group.

No significant differences ($p > 0.05$) were observed between the control and oxygen ($p = 0.390$) or hydrogen ($p = 0.683$) NB groups. By contrast, a significant difference ($p < 0.05$) was observed between the control and nitrogen NB groups ($p = 0.045$). Notably, cell viability was consistently the lowest in the control group and increased in the order of the hydrogen, oxygen, and nitrogen NB groups. Although the culture period was short and the statistical significance was limited, the repeated observation of this trend across a sufficient sample size suggested that the type of gas contained in the NBs influenced cell growth.

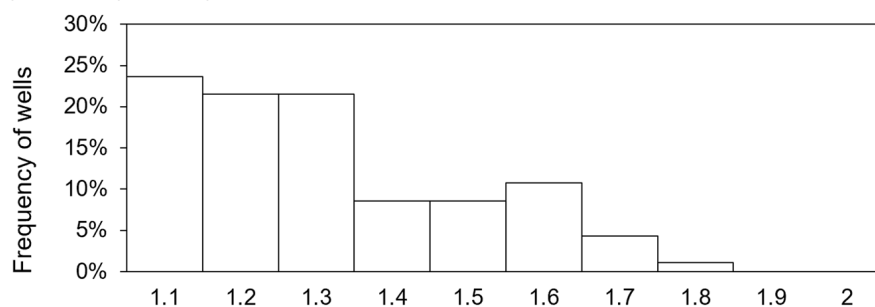
The distribution of cell viability for different gases in the NBs was monitored well-by-well (Figure 4). In the control group ($n = 93$), the highest proportion (23.66%) of wells showed a 1.0–1.1-fold increase in cell viability, and 1.08% of the wells showed a 1.7–1.8-fold increase (Figure 4a). In the nitrogen NB group ($n = 68$), the highest proportion (26.47%) of wells showed a 1.2–1.3-fold increase in cell viability, and cell viability increased by up to 1.9–2.0 times (Figure 4b). In the oxygen NB group ($n = 63$), the highest proportion (26.98%) of wells showed a 1.1–1.2-fold increase in cell viability, and a relatively high proportion (23.81%) of wells showed a 1.3–1.4-fold increase in cell viability. The maximum increase (1.7–1.8 times) was observed for 4.76% wells (Figure 4c). In the hydrogen NB group ($n = 57$), the highest proportion (29.82%) of wells showed a 1.3–1.4-fold increase in cell viability, and the maximum increase (1.6–1.7 times) was observed for 3.51% wells. Notably, in the hydrogen NB group, 96.49% wells showed a 1.0–1.5-fold increase in cell viability, resulting in the lowest cell viability of 1.29 (Figure 4d).

A histogram analysis revealed a significant difference in the proportion of wells with a 1.0–1.1-fold increase in cell viability when NBs were present. NBs exerted a positive effect on cell viability, with the modal value of viability shifting to 1.2–1.3-times, 1.1–1.2-times, and 1.3–1.4-times for nitrogen, oxygen, and hydrogen NBs, respectively.

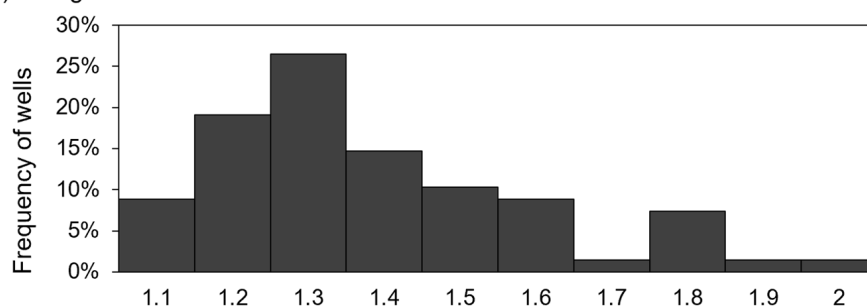
As the generation of NBs in DMEM was unstable compared with that in distilled water, the concentration of NBs used in the experiment was not uniform. Therefore, the number of NBs generated in 1 mL of DMEM was normalized and compared by dividing it by the number of cells (Figure 5). Although no clear trend was observed between the normalized NB concentration and cell viability, the relationship was described using a simple linear model for intuitive comparison. The coefficients of determination (R^2) were 0.029, 0.048, and 0.064 for nitrogen, oxygen, and hydrogen, respectively, indicating weak linear correlations. Nevertheless, a slight increase in cell viability was observed with increasing numbers of NBs per cell for all gas types. The slopes of the linear correlation were 1.1599×10^{-8} , 1.5343×10^{-8} , and 1.6928×10^{-8} for nitrogen, oxygen, and hydrogen,

respectively. These correlations suggested differences in the sensitivity of cell viability to NB concentration among the gases; specifically, cell viability appeared to be approximately 32% and 46% more sensitive to oxygen and hydrogen NBs, respectively, compared with nitrogen NB. In addition, the y-intercepts of the linear fits were 1.2726, 1.2133, and 1.1917 for nitrogen, oxygen, and hydrogen, respectively, indicating that oxygen and hydrogen NBs had a relatively weaker baseline influence on cell viability.

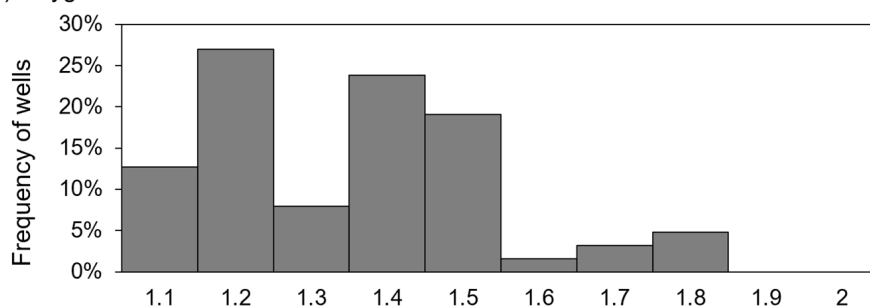
(a) Control (non-NB)



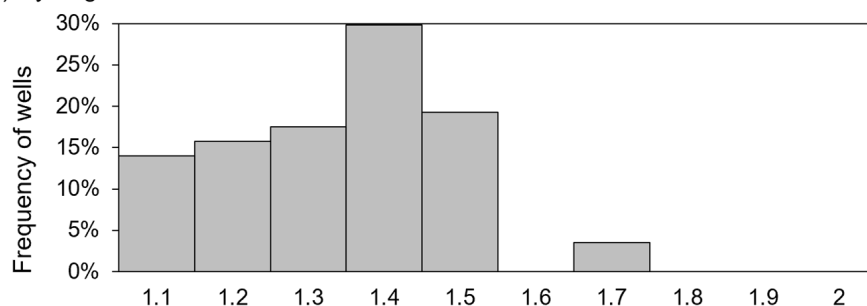
(b) Nitrogen NB



(c) Oxygen NB



(d) Hydrogen NB



Cell number increase rate

Figure 4. Distribution of cell counts according to cell increase rate. (a) Control case without NBs in the medium. (b) Medium containing nitrogen NBs. (c) Medium containing oxygen NBs. (d) Medium containing hydrogen NBs.

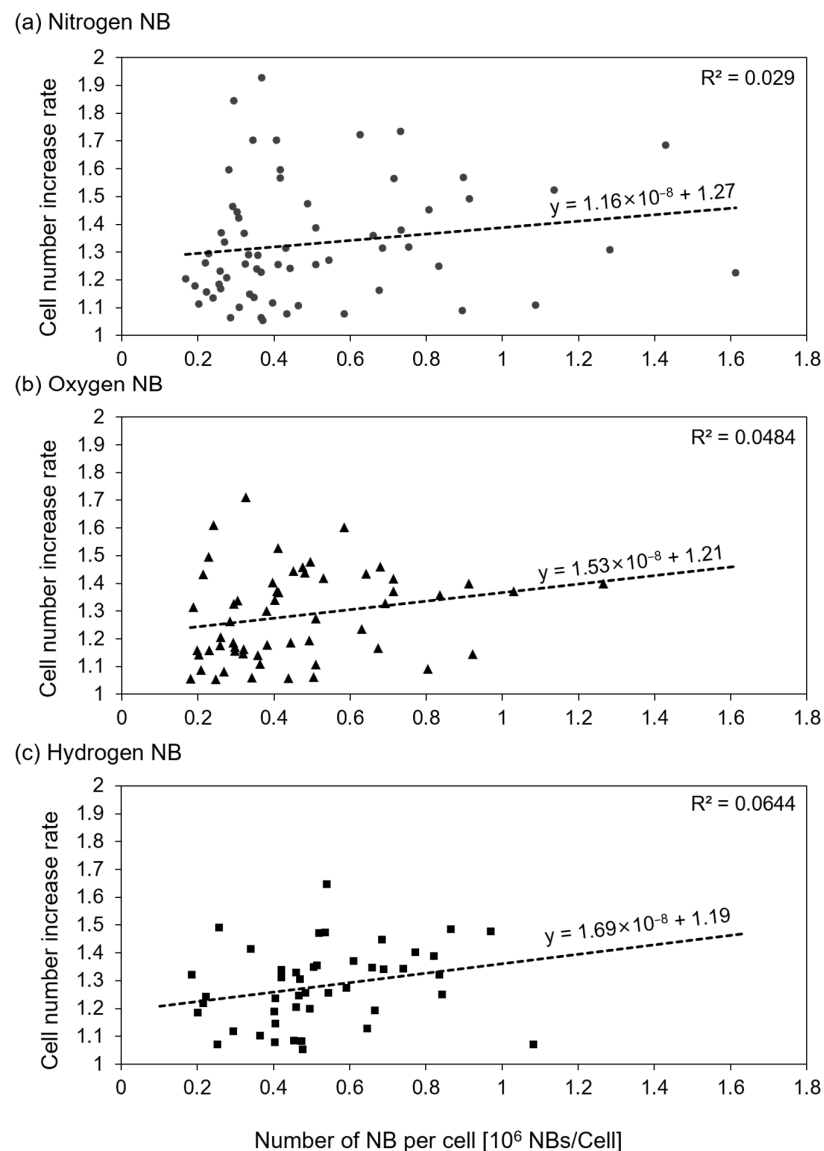


Figure 5. Cell increase rates according to the number of NBs per cell. (a) Nitrogen NBs. (b) Oxygen NBs. (c) Hydrogen NBs.

Although the reactive oxygen species (ROS) levels are not directly measured in this study, possible interpretations based on the previous literature are discussed with caution. In the case of nitrogen NBs, the observed increase in cell viability may be related to indirect modulation of the cellular redox environment associated with reduced oxygen availability [5]. Similarly, for hydrogen NBs, the higher cell viability observed may be consistent with previously reported ROS-scavenging effects; however, excessive suppression of ROS, which are known to play roles in intercellular signaling, may potentially limit further increases in cell viability [19]. By contrast, oxygen NBs may be associated with relatively higher ROS levels, which can partially account for the smaller average increase in cell viability observed in this group. Nevertheless, the overall presence of NBs appeared to shift the cell population toward higher viability ranges. These interpretations remain speculative and require direct experimental validation through dedicated ROS and apoptosis assays in future studies.

3.3. Cell Division Through CFSE Assay

Cell proliferation was quantitatively confirmed through CFSE analysis (Figure 6). The average fluorescence concentration on day 1 was 32,828. The average fluorescence concen-

tations of the control, nitrogen NB, oxygen NB, and hydrogen NB groups on day 3 were 14,427, 14,156, 14,083, and 15,609, respectively, and cell proliferation increased by 1.14, 1.16, 1.17, and 1.05 times at 48 h, respectively. Although no significant differences were observed in the CFSE results, repeated cell count experiments consistently showed similar trends. This suggests that differences in cell number may be more closely related to variations in cell death rather than changes in cell proliferation; however, this interpretation remains speculative. Further studies incorporating direct analyses of apoptosis and proliferation are required to clarify the underlying mechanisms.

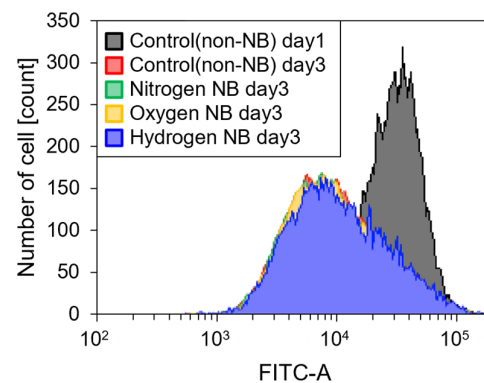


Figure 6. Flow cytometry analysis for cell proliferation. In all cases, approximately 1.1-fold proliferation occurred after 48 h, with no significant difference.

4. Conclusions

In this study, we investigated the effect of nitrogen, oxygen, and hydrogen NBs on the viability of MRC-5 cells. Cells were cultured for 48 h in a medium containing the NBs of each gas. In comparison with the control group, cell viability increased by 1.34-, 1.30-, and 1.29-fold in the nitrogen, oxygen, and hydrogen NB groups, respectively. Among them, only the cells that were cultured in the nitrogen NB medium showed a significant increase in viability. These findings indicate that the type of gas encapsulated in NBs can differentially influence fibroblast viability, with nitrogen exerting the strongest positive effect. The difference is modest, but the same trend is observed in repeated experiments, and the difference may become more pronounced in long-term culture.

Although the precise mechanisms remain unclear, potential factors include the electrochemical properties of each gas, altered ROS dynamics, and physicochemical characteristics of NBs. Repeated experiments demonstrated that nitrogen NBs might enhance cell survival by modulating the cellular microenvironment, whereas oxygen and hydrogen NBs exhibited weaker effects.

This study provides new evidence that the gas composition of NBs is a substantial determinant of their biological activity. However, the limited scope of biological analyses restricts a comprehensive elucidation of the underlying mechanisms. Therefore, further studies measuring the ROS levels, apoptosis-related markers, and involvement of intracellular mitochondrial function and cellular energy metabolism are needed [20]. In addition, investigations using diverse cell types will be necessary to better define the biological effects and explore the potential biomedical applications of gas-specific NBs. Future research must also address the technical challenges associated with the direct formation of NBs in culture media for long-term experiments, such as the time-consuming production process, increased media consumption (3- to 4-fold), and risk of potential contamination.

Author Contributions: Conceptualization, J.P. and H.H.; methodology, J.P. and H.H.; software, J.P. and H.H.; validation, J.P. and H.H.; formal analysis, J.P. and H.H.; investigation, J.P. and H.H.; resources, J.P. and H.H.; data curation, J.P. and H.H.; writing—original draft preparation, J.P.; writing—

review and editing, J.P., J.-M.K. and J.Y.P.; visualization, J.P.; supervision, J.Y.P.; project administration, J.-M.K. and J.Y.P.; funding acquisition, J.Y.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data are available upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NB	Nanobubble
DMEM	Dulbecco's modified eagle's medium
MRC-5	Human lung fibroblast cells
FBS	Fetal bovine serum
Anti-Anti	Antibiotic-antimycotic
NTA	Nanoparticle tracking analysis
NucBlue	NucBlue [®] Live ReadyProbes [™] reagent
CFSE	Carboxyfluorescein succinimidyl ester
ROS	Reactive oxygen species

Appendix A

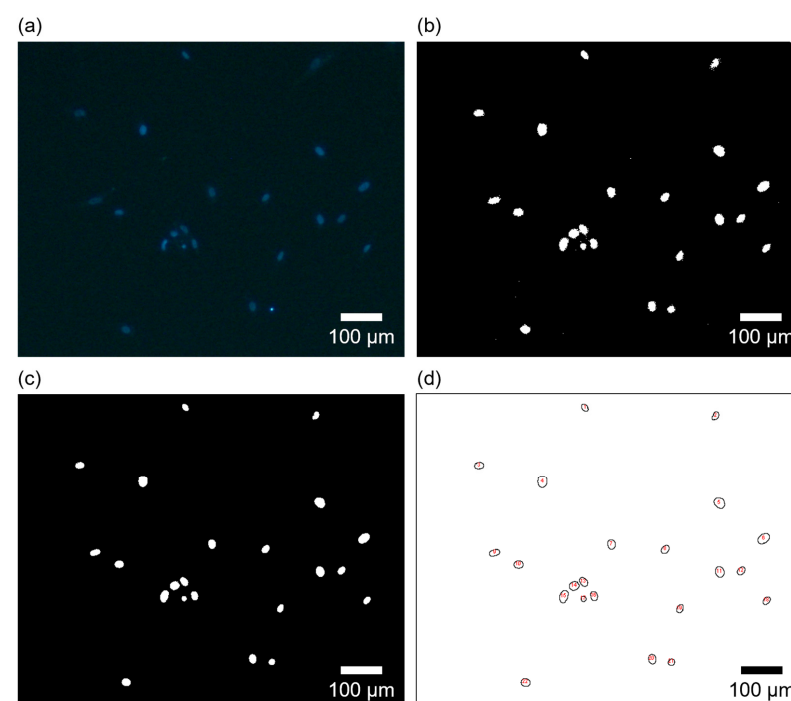


Figure A1. Nuclei quantification process of MRC-5 cells stained with NucBlue using ImageJ. (a) Fluorescent image of MRC-5 cells. (b) Conversion to black-and-white image with nuclei displayed in white. (c) Noise removal to eliminate artifacts. (d) Final step of cell counting.

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