Proliferative Effects of Nitrogen Nanobubbles on Fibroblasts

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Research Article

Keywords: nanobubble, gas-liquid mixing, cell cycle, cell proliferation

Posted Date: July 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-701270/v1

Abstract

In recent years, the potential of nanobubbles (NBs) for biological activation has been actively investigated. In this study, we investigated the proliferative effects of nitrogen nanobubbles (N-NBs) on fibroblast cells using cell assays with image analysis and flow cytometry. A high concentration of N-NBs (more than 4×10^8 NBs/mL) was generated in Dulbecco's modified Eagle's medium (DMEM) using a gas-liquid mixing method. In image analysis, the cells were counted and compared, which showed an 11% improvement in proliferation in the culture medium with N-NBs. In flow cytometry, the decrease in the fluorescence intensity was analyzed, which revealed a 1.5% improvement in proliferation in the culture medium with N-NBs. This study represents the first successful attempt of directly generating quantified NBs in a culture medium for cell culture. The findings suggest that the N-NBs in the culture medium can facilitate cell proliferation.

1. Introduction

Cells are controlled by a variety of stimuli such as biological growth factors, hormones, electrical fields, and mechanical forces[11]. Proliferation is a factor affected by external stimulation, which can be easily and quickly observed and analyzed. In tissue engineering and regenerative medicine, the enhancement of cell proliferation is of great significance. Various stimuli including not only biological factors but also engineered conditions have been investigated to improve cell proliferation. Hypoxic conditions and fibroblast growth factor-2 improved the proliferation of human bone marrow stromal cells[22]. Gelatin microparticles loaded with ephedra extract improved the proliferation of human lung epithelial cells[3]. The mouse fibroblast cell line L929 was found to be positively responsive to electric stimulation in terms of cell division[4]. Recently, nanobubbles (NBs) are being investigated for their potential application to improve cell proliferation. NBs refer to gas-filled cavities less than 1 μm in size in liquid. From the perspective of classical thermodynamics, NBs should diffuse and disappear in water within the order of microseconds due to high internal pressure[5,6]; however, various studies have reported results (theoretical and experimental approaches) demonstrating that NBs can exist in a stable state[7,8]. NBs have attracted much attention in several areas, including biomedical applications, due to their unique physicochemical properties[9–12]. There are several studies on the biological effect of NBs on living organisms[13–16]. The overall effect of NBs on the growth of plants, fishes, and mice was investigated in 2013, which revealed that NBs accelerated the growth of plants, fishes, and mice and increased their (fishes and mice) weight[12]. Although the mechanism of NBs in growth promotion remains unclear, NBs are known to play an important role in accelerating the growth of living organisms. More recently, the effect of NBs was assessed by comparing seed germination in NB water with seed germination in distilled water and H_2O_2 solutions through the measurement of superoxide radicals ($O_2^{\bullet -}$) in seeds by nitroblue tetrazolium (NBT) staining[16]. The levels of superoxide radicals in NB water and 0.3 mM H_2O_2 solution were similar and considerably higher than in distilled water. The production of reactive oxygen species (ROS, $^{\bullet}OH$) by NBs might contribute to the physiological induction of seed germination. In addition, NBs adsorbed on

polystyrene films were used as a scaffold for the cell culture of mouse fibroblast L929 cells to show that the NBs could promote the proliferation of fibroblast cells[17]. However, there may be a suppressive/negative effect from the use of NBs. The proliferation of dental follicle stem cells in a culture medium containing air and oxygen NBs was inhibited due to the high oxygen content of NBs[18]. The above-mentioned studies suggest that NBs may have different (positive and negative) effects on biological activation depending on the type of organism or type of gas in the NBs. Moreover, there are limited studies on cell proliferation using a cell medium containing NBs.

In this study, we propose a new cell culture technology using nitrogen NBs (N-NBs) to investigate the effect of NBs on cell proliferation capability. The N-NBs were directly generated in Dulbecco's modified Eagle's medium (DMEM), and MRC-5 cells were cultured with DMEM containing N-NBs. For NB generation, a gas-liquid mixing method was used, and the concentration and size of the N-NBs were examined. We also performed image analysis and flow cytometry to evaluate cell proliferation and demonstrated that cell proliferation in the culture medium with N-NBs was enhanced. The findings of this study could be used to expand the scope of application for various cell types and gaseous NBs and could contribute to further research on the biological applications of NBs in the near future.

2. Materials And Methods

2.1. Materials

For cell culture, MRC-5 (Medical Research Council cell strain 5), which is a fibroblast cell line, was purchased from the Korean Cell Line Bank (Korean Cell Line Research Foundation, Republic of Korea). DMEM (Gibco®, Thermo Fisher Scientific, USA) was used as a basal medium to culture MRC-5 cells. Fetal bovine serum (FBS; Gibco®, Thermo Fisher Scientific, USA) and Antibiotic-Antimycotic (Anti-Anti, 100X; Gibco®, Thermo Fisher Scientific, USA) were used as supplements. For NB generation in DMEM, highly-purity nitrogen gas (99.999%; Shinyoung Gas Co., Republic of Korea) was used.

2.2. Preparation of cell medium with N-NBs

In this study, a gas-liquid mixing (agitation) method with a linear actuator was used[19] to generate a large number of N-NBs in DMEM in a short time. A sterilized disposable conical tube was dipped in DMEM vertically, and nitrogen gas was supplied to fill half of the tube. The tube was then capped and sealed with parafilm to prevent the influx of contaminants such as particles, oil, and microbes, which can negatively affect the cell culture. The sealed tube was mounted onto an actuator and agitated with the set cycle (117 strokes/min). After N-NB generation in DMEM, the cell medium with N-NBs was prepared by mixing the DMEM containing N-NBs with 10% FBS and 1% Anti-Anti.

2.3. Measurements of concentration and size of N-NBs in DMEM

For the analysis of N-NBs in DMEM, a nanoparticle tracking analysis (NTA) method was used with a NTA instrument (NanoSight LM10-HSBFT14; Malvern, UK), which is widely used in the NB research field[20,21]. It is a NB visualization technique that provides size, count and concentration measurements. The NTA instrument was equipped with a charge-coupled device (CCD) camera to capture the dispersed light and a red laser light source with a wavelength of 642 nm to excite fluorescence. The DMEM containing N-NBs was placed in the sample chamber, which had a volume of 0.3 mL. With laser light illumination, the N-NBs appeared individually as fast-moving dispersed dots of light (white dots) under Brownian motion, which were automatically tracked and captured by the CCD camera. Subsequently, the NTA image analysis program (i.e., the NTA software) determined the concentration and size of the N-NBs in DMEM. In addition, changes in the concentration and size of the N-NBs in DMEM was observed for 48 h to ensure the presence and stability of the N-NBs during the cell culture process.

2.4. Cell culture experiment

We used MRC-5 cells between passage 10 and 15 in the experiment. The cells were cultured in an incubator at 37°C with 5% CO_2 and 95% humidity using a culture medium containing DMEM supplemented with 10% FBS and 1% Anti-Anti. There was a slight difference in the cell culture process due to the difference in the analysis method as follows.

First, cell proliferation was analyzed by image analysis. To obtain reliable data, cell culture experiments were performed 5 times independently. To attach the cells, we loaded 1 mL of non-NB culture medium including MRC-5 cells (5×10^3 / mL) in 24-well plates. After 24 h of cell attachment, NucBlue® Live ReadyProbes™ reagent (NucBlue; Invitrogen™, Thermo Fisher Scientific, USA) was added (1 drop per well) and incubated at 37°C for 30–40 min to stain the cells. Images at the center of the well were taken using an inverted fluorescence microscope (CKX41; Olympus, Japan). Then, half of the 24 well was replaced with the culture medium with N-NBs (experimental group), and the remaining half of the 24well was replaced with the fresh culture medium without N-NBs (control group). After incubation for 48 h, NucBlue staining was performed, and images of the cells were taken. To count the number of cells per image, image analysis was performed using imageJ. (US National Institutes of Health, USA)

Second, cell proliferation was analyzed by flowcytometry. The cells were stained using CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Kit (Invitrogen™, Thermo Fisher Scientific, USA). CFSE dye was diluted in dimethyl sulfoxide (DMSO) at a concentration of 5 mM to make a stock solution, and the stock solution was diluted in PBS at a ratio of 1000:1 to prepare a PBS-dye solution. The cells were stained by adding 1 mL of the PBS-dye solution to 1×10^6 cells in suspension and incubated at 37°C for 20 min. After adding the culture medium without N-NBs to the stained cell solution (5×) with incubation for 5 min, the cell was pelleted using centrifuge, and the supernatant was removed. The stained cells were seeded in Φ150-culture dishes (1×10^6 cells each). After 24 h of cell attachment in culture medium without N-NBs, half of culture dishes was replaced with the culture medium with N-NBs (experimental group), and the remaining half of culture dishes was replaced with the fresh culture medium without N-NBs (control group), followed by incubation for 48 h. Subsequently, the cells were

detached from the dish using trypsin (Gibco®, Thermo Fisher Scientific, USA), and flow cytometry analysis was performed using FACS Canto™II (Becton, Dickinson and Company, USA).

3. Results And Discussion

3.1. NB generation in DMEM

The generated N-NBs in DMEM were examined, and their concentration and size were measured (Fig. 1). Figure 1 (a)-(b) shows the images captured using a CCD camera with a NTA instrument. The black background and white dots represent DMEM and the generated NBs, respectively. In the DMEM without NBs (control), only a black background was observed (Fig. 1 (a)). A large number of white dots (NBs) were observed in the DMEM containing N-NBs (Fig. 1 (b)). Based on the analysis of the concentration and size of the generated N-NBs in DMEM (Fig. 1 (c)), polydispersed N-NBs were generated in the range of 10 to 500 nm. The concentration and average size of the N-NBs are shown in Fig. 1 (d). There was some variation in the number of N-NBs generated in DMEM; nevertheless, more than 400 million NBs were generated (case 1: $4.06 \pm 0.15 \times 10^8$ NBs/mL, case 2: $4.02 \pm 0.71 \times 10^8$ NBs/mL, case 3: $5.20 \pm 0.76 \times 10^8$ NBs/mL, case 4: $6.07 \pm 0.74 \times 10^8$ NBs/mL, case 5: $5.31 \pm 0.50 \times 10^8$ NBs/mL). The mean diameter of the N-NBs was also slightly different; nevertheless, the size of the N-NBs generated in DMEM using a gas-liquid mixing method was approximately 100 ~ 300 nm. The change in the N-NB concentration over time is shown in Fig. 2. Overall, the N-NB concentration was decreased with time and was stably maintained at more than 31% after 48 h (around $1.71 \pm 0.07 \times 10^8$ NBs/mL). These results indicated that the concentration of the N-NBs in the cell medium was sufficiently remained to evaluate their effect on the cell culture.

3.2. Cell proliferation; NucBlue staining

To evaluate the effect of N-NBs on cells, cell proliferation was examined by image analysis using imageJ. The fluorescent image (Fig. 3 (a)) was converted black and white image and the brightness was adjusted to the extent that the nucleus of cells was not affected to minimize noise (Fig. 3 (b)). The stained cell nucleus and noises, that were bright region of fluorescent imange, were converted to white dot. To remove noise, white dots with a diameter of 5 pixels or less have been removed (Fig. 3 (c)). Then, white dots with an area of 10 pixels^2 or more were counted (Fig. 3 (d)). The results of cell counting using ImageJ showed that in the control group, cell proliferation was increased on average of 1.18 times between day 1 and day 3, and in the experimental group, the average value was 1.31 times, which was 11% higher than that in the control group (Fig. 3 (e)). Although the cells were seeded at a density of 5,000 cells/mL, there was a difference in the cells attached to the bottom of the well (Fig. 3 (f)), and the number of NBs generated in DMEM was also different within a certain range (Fig. 1 (d)). Considering the influence of cell attachment and NB concentration, the normalization method was used. The number of NBs (N) was divided by the number of cells normally attached to the floor on day 1 (M) to normalize as the number of NBs per cell (K = N/M), and the cell proliferation rate was evaluated. The results showed that cell proliferation tended to be proportional to the number of NBs per cell (K). The trend can be expressed with a linear equation: y =

4.48×10^{-8} x + 1.12 (Fig. 3 (g)). The slope was very small, y = 4.48×10^{-8}; that means, a weak association was observed between the number of N-NBs per cell and the proliferation rate. In statistical analysis using SPSS Statistics (IBM Co., USA), a statistical significance was observed in cell proliferation (Fig. 3 (e)). Although there was no large difference in the cell proliferation rate for 3 days, the effect of N-NBs on cell proliferation could be greater in a long-term culture environment for 1 week or longer.

3.3. Cell proliferation; CFSE staining

CFSE staining was performed to assess cell proliferation. CFSE will stain the cell nucleus, and the fluorescence intensity is reduced by half per division. Therefore, it is possible to determine the number of proliferating cells based on the fluorescence intensity. In the control group and the experimental group, the fluorescence intensity on day 3 (the degree of shifting to the left side of the graph) was compared with the fluorescence intensity on day 1. The results showed that the average CFSE fluorescence intensity of the control group and the experimental group on day 3 was decreased from 32,828 to 14,427 and 14,156, respectively (Fig. 4). The CFSE fluorescence intensity was lower by around 1.5% in the experimental group compared with the control group. The results were consistent with the cell proliferation results obtained by NucBlue staining. The findings suggest that if cells are exposed to NBs for an extended period, there may be a notable difference in cell proliferation.

3.4. Propidium Iodide-Ribonuclease (PI-RNase) staining

Analysis of cell proliferation by image analysis and flowcytometry demonstrated that the culture medium with N-NB group promote the cell proliferation 11% and 1.5% more than without N-NB group, respectively. Assuming that the difference in proliferation was due to the cell cycle, PI-RNase staining was performed to confirm the difference in the number of cells according to the phase of the cell cycle. PI-RNase staining analyzed by flow cytometry (Fig. 5). Area 1 is the sub-G1 phase with dead cells, and the cells have less than 2n chromosomes (Fig. 5 (a)). Area 2 is the G1 phase in which cells with 2n chromosomes prepare for replication. Area 3 is the S phase in which chromosomes replicate and become chromatids; in this step, the number of chromosomes increases from 2n to 4n. Lastly, area 4 is the G2/M phase in which the chromatids are ready for separation by the kinetochore microtubules, and the nucleus and cytoplasm are divided; in this phase, the number of chromosomes is 4n. A comparison of the population rate of cells in each phase between the control group and experimental group revealed that it was 1.93% lower in the G1 phase and 0.42% and 1.57% higher in the S phase and G2/M phase, respectively, in the experimental group (Fig. 5 (b)). The higher population rate of cells in the S phase and G2/M phase suggests that there are many dividing cells in the experimental group. The results from cell cycle analysis are in agreement with the results of image analysis and flowcytometry (differences of 11% and 1.5%, respectively), indicating that the N-NB environment may have a positive effect on cell division.

4. Conclusions

In this study, the proliferative effect of N-NBs on MRC-5 cells was investigated. For MRC-5 cell culture, N-NBs were generated in DMEM using a gas-liquid mixing method at a high concentration (more than 4 ×

10^8 NBs/mL). In addition, the N-NBs were stably maintained at more than 31% (around 1.71 ± 0.07 × 10^8 NBs/mL) after 48 h. To evaluate cell proliferation, image analysis with NucBlue staining and flow cytometry with CFSE staining were performed. The results of image analysis and flow cytometry revealed that proliferation in the culture medium with N-NBs was increased by 11% and 1.5%, respectively, compared with that in the culture medium without N-NBs. In addition, cell cycle analysis with flow cytometry was performed to examine the improvement in proliferation. The population rate of cells in the G1 phase was 1.93% lower in the culture medium with N-NBs than in the culture medium without N-NBs. In addition, the rate in the S phase and G2/M phase was 0.42% and 1.57% higher, respectively, in the culture medium with N-NBs than in the culture medium without N-NBs. Taken together, the findings suggest that the presence of the culture medium with N-NBs may stimulate the proliferation of MRC-5 cells. Further studies on the proliferative effects of various gaseous NBs on cell cultures will be required for a more comprehensive understanding of the biological interaction between NBs and cells. To the best of our knowledge, this study is the first to investigate the effect of N-NBs (directly generated in DMEM) on a fibroblast cell culture. Our study could serve as a reference for future studies on the biological effect of NBs on living organisms.

Declarations

Acknowledgements

This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIT(2018M3A9H1023141). This research was also supported by the Chung-Ang University Graduate Research Scholarship in 2018 (H.H.). The authors would like to thank Dr. Sung-Hwan Kim from Cell-Smith, Co. for helpful experimental tips.

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Figures

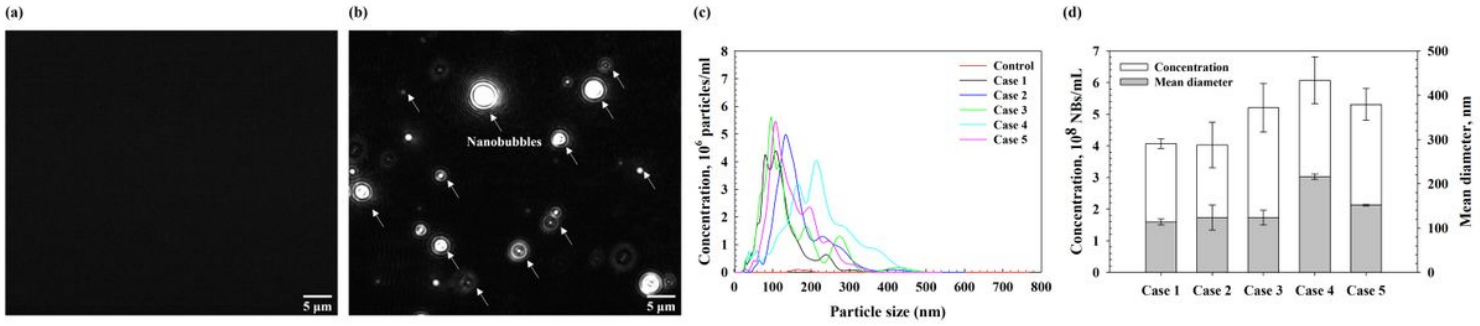

Figure 1

Results of NB analysis performed using the NTA apparatus. (a) Before NB generation (control); no particles are present in the DMEM. (b) After NB generation (case 1); a number of NBs (white dots) are detected. The difference in the size of the NBs can be attributed to the phase difference in the z-axis direction of the NBs in the DMEM. The NBs appear larger because they are scattered by the laser. The actual size of the NBs is determined using the NTA software by using the recorded image. (c) Size distribution and (d) concentration and mean diameter according to the results of the NB generation test, independently conducted five times (cases 1–5) to verify the reliability of the gas–liquid mixing method. The size of the generated NBs in the DMEM is polydisperse between 10 to 500 nm, and the mean diameter is confirmed to converge to less than 300 nm.

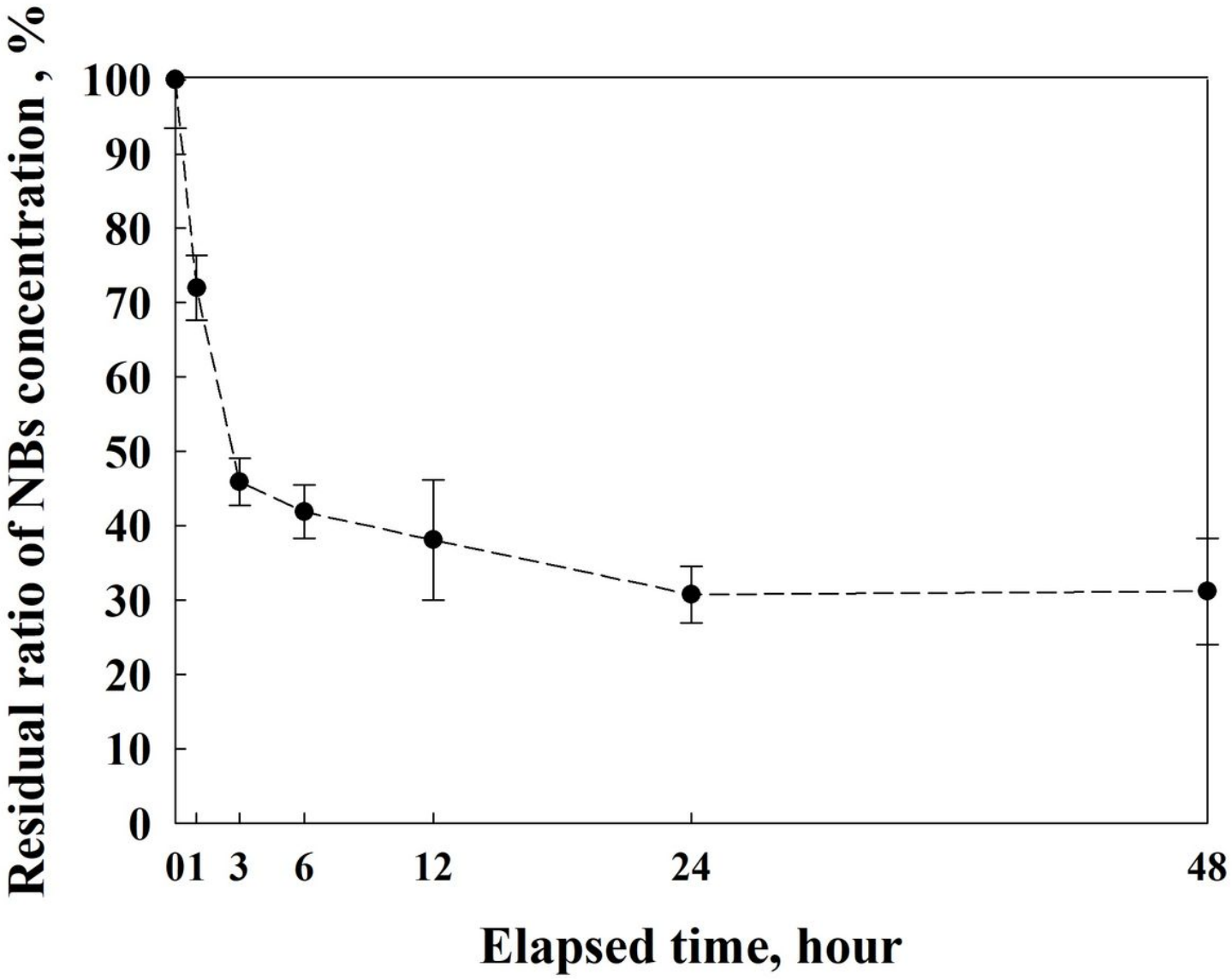

Figure 2

Residual ratio of the NB concentration over time in the DMEM. The concentration of the NBs in the DMEM decreased sharply for 3 h and later exhibited a gradual decrease to 31% of the initial concentration after 24 h. This value was maintained for the 48 h in which the cell culture was performed.

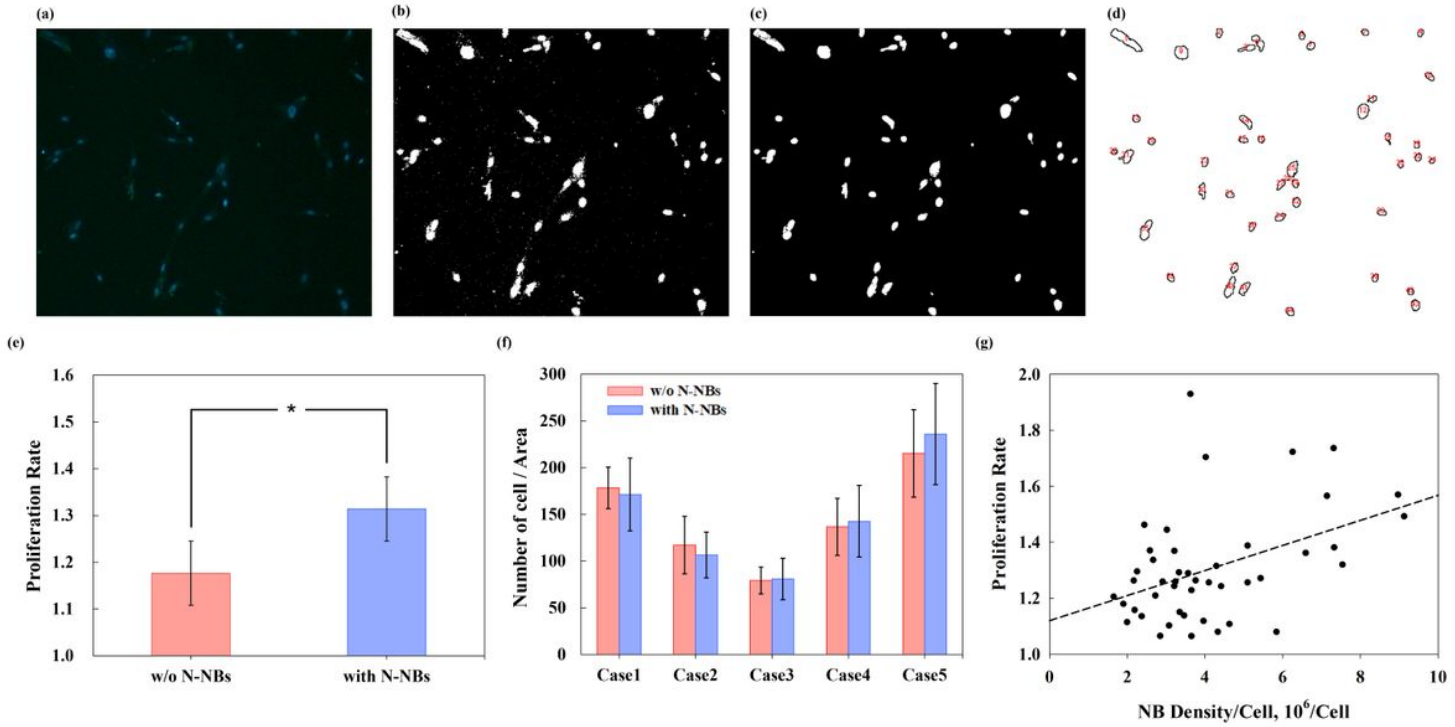

Figure 3

Result of the ImageJ-based image analysis for the MRC-5 cell nucleus stained with NucBlue. (a) Original fluorescence image. (b) Simplified black and white image. (c) Image with noise removed. (d) Quantification of the number of cells. (e) Proliferation rate between days 1 and 3. (f) Difference in the number of cells on day 1 for the different cases. The number of cells attached to the bottom varies among the cases. (g) Cell proliferation rate normalized by the number of NB density per cell. The proliferation rate increases with the NB density.

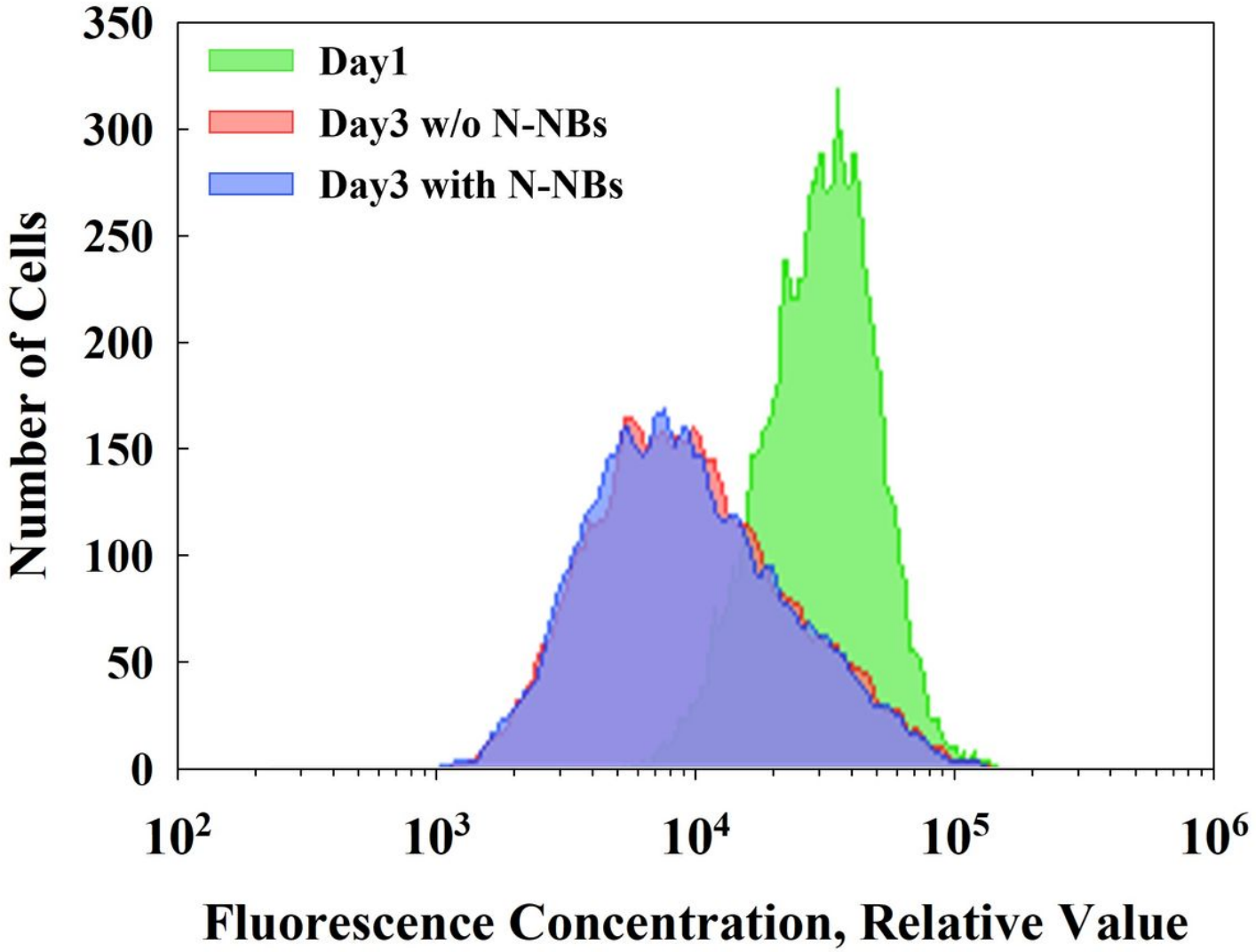

Figure 4

Result of flowcytometry analysis. On day 1, the mean fluorescence intensity is 32,828, similar for the cases without and with N-NBs. On day 3, the average fluorescence intensity is 14,427 and 14,156 in the culture medium without N-NBs and with N-NBs, respectively. A lower mean fluorescence concentration corresponds to a larger number of proliferated cells. Although the difference in the fluorescence concentration is small, the difference is likely to be more pronounced in the long-term culture.

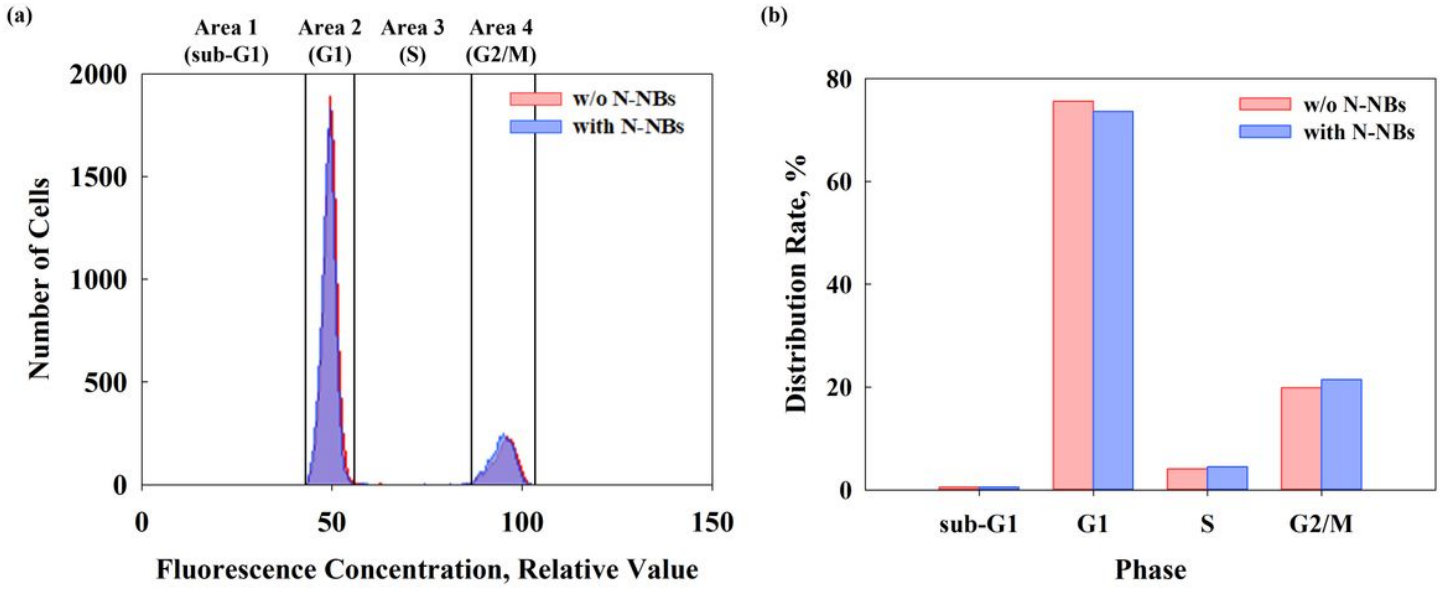

Figure 5

Cell cycle analysis. (a) Flow cytometry histogram. The histogram shows the distribution of cells in each area in the culture medium with and without N-NBs. Area 1 (sub-G1 phase) includes dead cells with less than n chromosomes. In Area 2 (G1 phase), cells have 2n chromosomes and are prepared for chromosome replication. In Area 3 (S phase), the cells replicate the chromosomes and have 2n–4n chromosomes. In Area 4 (G2/M phase), the cell is divided in two and has 4n chromosomes. (b) Cell distribution rate in each phase. Compared with that in the culture medium without N-NBs, the population of cells in the G1 phase in the culture medium with N-NBs is smaller and that in the S phase and G2/M phase is larger. The culture medium with N-NBs promotes cell proliferation, which increases the cell distribution in the S phase and G2/M phase. The total number of cells is 10,000.