



Article

Smaller Oxygen Nanobubbles More Effectively Suppress Colitis and Colon Carcinogenesis than Larger Oxygen Nanobubbles

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Abstract

Oxygen nanobubble (NBO₂) water is reportedly a promising therapeutic and radiosensitizing agent against solid cancers. However, the significance of nanobubble size in inflammation-associated colorectal carcinogenesis in vivo remains elusive. We investigated whether small NBO₂ water exerts stronger preventive effects against colitis and colorectal carcinogenesis in an azoxymethane/dextran sulfate sodium-induced mouse model of colitis-associated cancer. Differences in particle size between the small and large NBO₂ water samples were confirmed by atomic force microscopy. The mice received drinking water containing either small or large sized NBO₂ throughout the experiment. Small NBO₂ water significantly reduced disease activity index scores, histopathological colitis scores, colonic shortening, CD68-positive inflammatory macrophage density, and tumor numbers. However, body weight, water intake, food intake, and spleen weight were unaffected. Immunohistochemistry revealed that small NBO₂ water reduced the percentage of Ki-67-positive tumor cells and the proportions of hypoxia-inducible factor-1 α -positive epithelial and stromal cells, whereas no significant differences were observed in CD8- or forkhead box P3-positive cells. We conclude that nanometer-sized oxygen bubbles prevent inflammation-associated colorectal carcinogenesis, and that particle size is a critical determinant of biological effects. Small amounts of NBO₂ water may help control colitis and tumor development by alleviating hypoxia in the tumor microenvironment.



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Keywords: nanobubble size; hypoxia; colitis-associated cancer; HIF-1 α ; AOM/DSS mouse model

1. Introduction

Nanobubbles (NBs) are ultrafine gas bubbles with distinctive physicochemical properties, characterized by long-term stability owing to strong hydrogen-bonding interactions at the gas–water interface and electrostatic repulsion from their negatively charged surfaces [1]. The small size of NBs contributes significantly to their stability because Brownian motion counteracts buoyant forces, allowing prolonged interactions with biological systems [2]. Studies have demonstrated that NB size critically influences their bioactivity, cellular uptake, and gas delivery efficiency. Smaller NBs have been reported to exhibit enhanced oxygen dissolution capacity, greater cellular permeability, and superior biological responses compared with larger counterparts [3]. Thus, understanding the relationship between NB size and physiological effects is essential for optimizing their therapeutic potential against a range of diseases.

Oxygen nanobubble (NBO₂) water has been reported to be a promising therapeutic and radiosensitizing agent in cancer treatment. In inflammatory bowel disease (IBD), macrophages, effector T cells, and regulatory T cells are key immune populations that orchestrate chronic inflammation and mucosal injury through dysregulated cytokine production and impaired immune tolerance [4–6]. Several preclinical studies have shown that NBO₂ water significantly suppresses hypoxia-induced resistance in tumor cells, thereby improving therapeutic outcomes [7,8]. Previously, our group developed a single-nanometer-range NBO₂ water, referred to as small NBO₂ water in this study. Our *in vitro* and *in vivo* investigations demonstrated that this ultrafine NBO₂ water suppresses hypoxia-induced radiation resistance and xenograft tumor growth by downregulating the accumulation of hypoxia-inducible factor 1- α (HIF-1 α) [9,10]. Importantly, long-term administration studies in animal models have confirmed its safety, with no adverse effects on body weight, biochemical parameters, or major organ histology. These findings suggest that our single-nanometer-range NBO₂ water may overcome cancer cell growth and therapeutic resistance while maintaining an excellent safety profile.

HIF-1 α is a central transcriptional regulator of inflammatory and metabolic responses in immune cells and plays a key role in the pathogenesis of IBD [11,12]. Recent studies have demonstrated that HIF-1 α expression in stromal immune cells exacerbates colitis by promoting pro-inflammatory cytokine production, thereby sustaining chronic intestinal inflammation [11,13]. Based on these findings, targeting HIF-1 α in stromal immune cells has been identified as a promising strategy for mitigating intestinal inflammation and preventing colitis-associated cancer. Importantly, pharmacological inhibition of HIF signaling has also been shown to suppress tumor progression in inflammation-associated colorectal cancer models. In particular, treatment with acriflavine, a potent inhibitor of HIF-1/2 dimerization, markedly reduced tumor development in azoxymethane/dextran sulfate sodium (AOM/DSS)-induced mice by blocking HIF activation [14]. These results suggest that therapeutic modulation of HIF activity may represent a promising approach for controlling colitis-associated colorectal carcinogenesis. However, to date, no study has directly evaluated the preventive effect of oxygen nanobubbles on inflammation-associated colorectal carcinogenesis. In this context, we hypothesized that NBO₂ water may reduce colonic inflammation and inhibit tumor development in inflammation-associated colorectal cancer by lowering HIF-1 α accumulation. Although NBO₂ water has shown therapeutic

potential, it remains unclear how its particle size influences HIF-1 α suppression, the overall progression of colitis, and inflammation-driven tumorigenesis.

No head-to-head size comparison of NBO₂ in the AOM/DSS colitis–cancer model exists. To address this gap, we employed an AOM/DSS-induced mouse model of colitis-associated cancer to systematically compare the effects of NBO₂ water with different bubble sizes (small versus large) on disease progression. Furthermore, to evaluate the size-dependent effects of NBO₂ water in this model, we performed immunohistochemical analyses of HIF-1 α (a hypoxia marker), Ki-67 (a proliferation marker), and immune cell markers, including CD68 (a macrophage marker), CD8 (a cytotoxic T-cell marker), and forkhead box P3 (Foxp3; a regulatory T-cell marker), in harvested colon tissues.

2. Results

2.1. Size Difference Between Small and Large NBO₂ Water in This Study

This study investigated the effect of NBO₂ particle size on colitis and colorectal carcinogenesis in an animal model. First, the particle sizes of the small and large NBO₂ water used in the experiments were evaluated by atomic force microscopy (AFM). AFM imaging revealed that small NBO₂ had a markedly smaller particle size than large NBO₂ water (Figure 1a). Furthermore, particle size distribution analysis demonstrated that small NBO₂ water molecules exhibited a peak in the single-nanometer range, whereas large NBO₂ water molecules showed a peak at approximately 30–50 nm (Figure 1b).

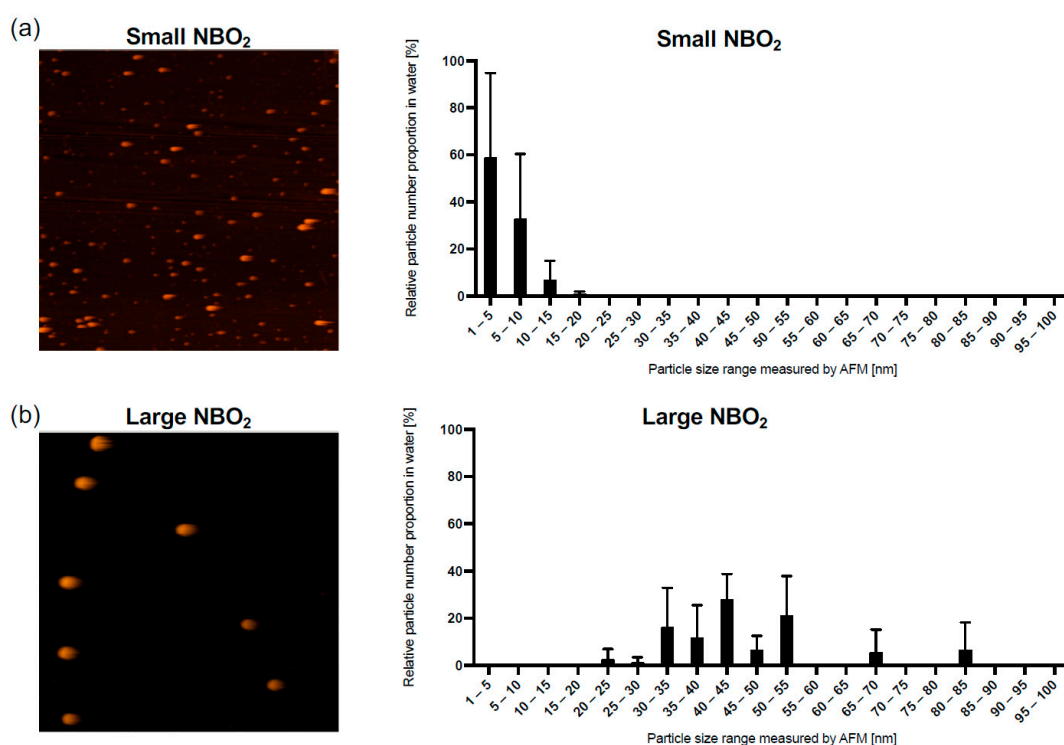


Figure 1. Characterization of particle size in small and large oxygen nanobubble (NBO₂) water using atomic force microscopy (AFM). (a) Representative AFM images and corresponding particle size distribution histogram of small NBO₂ water. (b) Representative AFM images and corresponding particle size distribution histogram of large NBO₂ water. AFM measurements were performed in three independent experiments.

2.2. Small NBO₂ Water Suppresses Colitis in a Colitis-Associated Cancer Model

To evaluate the effect of NBO₂ water particle size on colitis and colorectal carcinogenesis, we employed an AOM/DSS-induced colitis-associated cancer model (Figure 2a). A comparison of colitis severity among the groups showed that the disease activity index

(DAI) score was significantly lower in the small NBO₂ water group than in the water (control) and large NBO₂ water groups (Figure 2b). No significant differences in body weight, water intake, or food intake were observed among the water, small NBO₂, and large NBO₂ water groups throughout the experimental period (Figure S1a,b). During the experimental period, two mice in the large NBO₂ water group died due to exacerbation of colitis symptoms; therefore, subsequent analyses were conducted with the following sample sizes: (large NBO₂ water group, $n = 5$; other groups, $n = 7$).

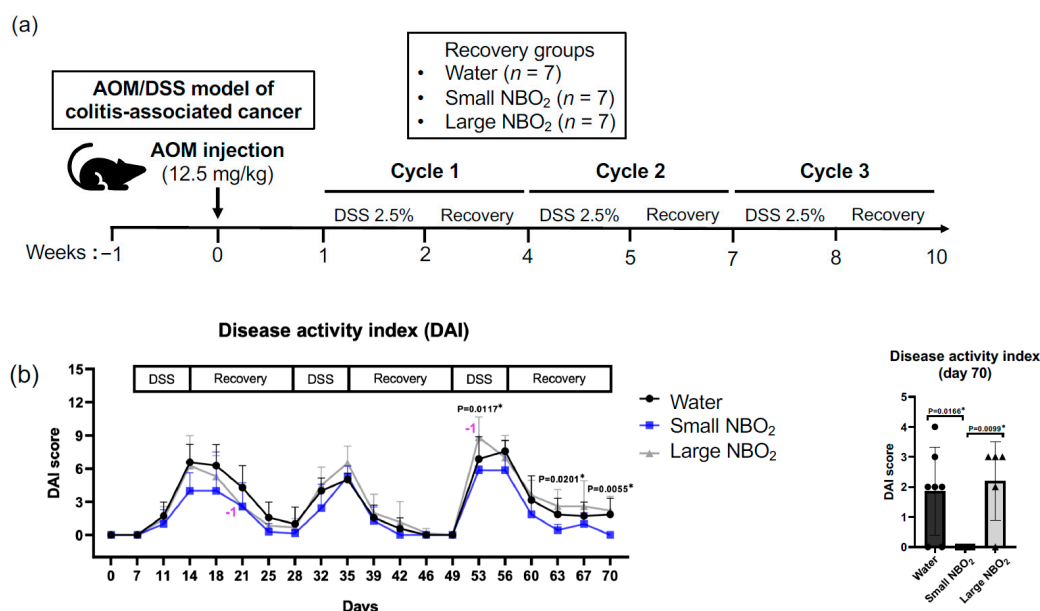


Figure 2. Effects of small and large oxygen nanobubble (NBO₂) water on colitis severity in an azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer model. (a) Experimental design. Mice were injected intraperitoneally with azoxymethane (AOM, 12.5 mg/kg) at week 0, followed by three cycles of 2.5% dextran sulfate sodium (DSS) administered in drinking water for 7 days, interspersed with recovery periods using normal drinking water or NBO₂ water. The mice were divided into three recovery groups: water as control ($n = 7$), small NBO₂ water ($n = 7$), and large NBO₂ water ($n = 7$). (b) Disease activity index (DAI) scores were recorded throughout the experiment. Left panel: time course of DAI scores in each group. The “−1” in the figure indicates that two mice in the large NBO₂ water group died during the experiment owing to severe colitis. Data are presented as mean $\pm$ standard deviation (SD). Right panel: DAI scores on day 70. Statistical analysis was performed using one-way analysis of variance followed by Tukey’s post hoc test. * $p < 0.05$ indicates a statistically significant difference between groups.

2.3. Macroscopic and Histopathological Assessment of Colons and Spleens in a Colitis-Associated Cancer Model

We compared colon length, number of colonic tumors, histopathological colitis scores, and spleen size among the experimental groups. Colon length was significantly greater in the small NBO₂ water group than in the water (control) and large NBO₂ water groups, indicating that small NBO₂ attenuated inflammation-associated colonic shortening (Figure 3a). The number of colonic tumors was significantly lower in the small NBO₂ water group than in the water group, whereas no significant difference was observed between the large NBO₂ water and water groups (Figure 3b). Histopathological colitis scores, assessed in H&E-stained sections, were significantly lower in the small NBO₂ water group than in both the water and large NBO₂ water groups (Figure 3c). Because splenomegaly is recognized as a marker of systemic inflammation, we compared spleen weights among the groups and found no significant differences between the small NBO₂ water group and either the water or the large NBO₂ water group (Figure 3d).

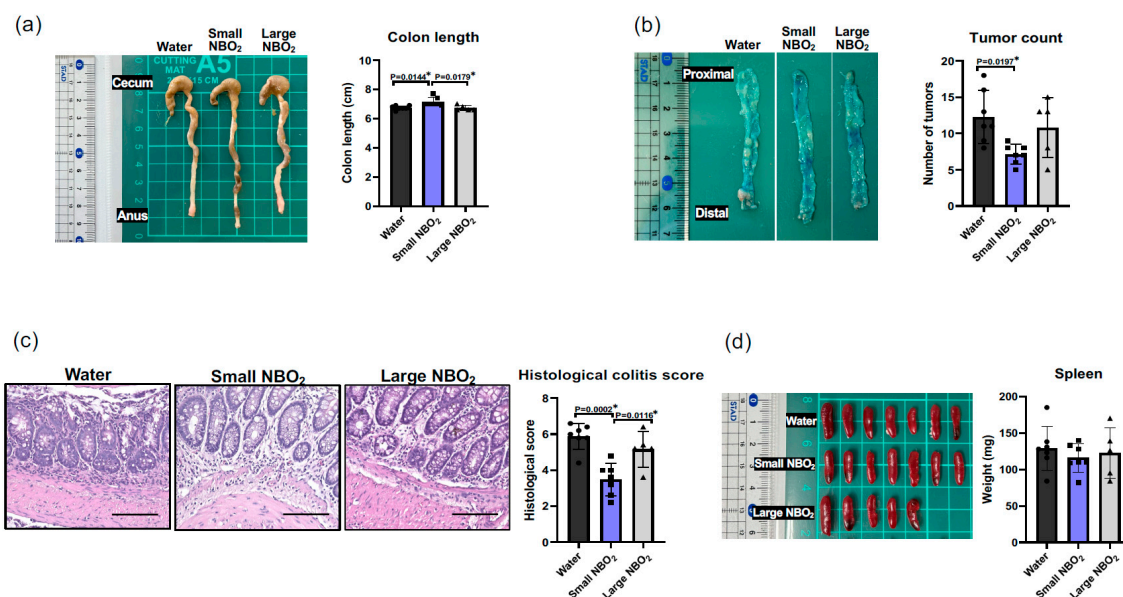


Figure 3. Effects of small and large oxygen nanobubble (NBO₂) water on colon length, tumor burden, histological colitis score, and spleen weight in an azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer model. (a) Representative images and quantitative analysis of colon length at sacrifice. The small NBO₂ water group ($n = 7$) showed significantly longer colons than the water control ($n = 7$) and large NBO₂ water groups ($n = 5$). (b) Representative images and quantitative analysis of colon tumor counts. Tumors were visualized after 0.2% indigo carmine staining, and the total number of tumors in each colon was recorded. The small NBO₂ water group exhibited significantly fewer tumors than the water control group. (c) Representative hematoxylin and eosin (H&E)-stained images of colon sections and histological colitis scores. Scale bars: 100 μ m. The small NBO₂ water group showed significantly lower histological colitis scores than the water control and large NBO₂ water groups. (d) Representative images and quantitative analysis of spleen weight at sacrifice. No significant differences were observed among the groups. Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using one-way analysis of variance, followed by Dunnett's multiple-comparison test (for comparisons with the control group) or Tukey's post hoc test (for pairwise comparisons). * $p < 0.05$ indicates a statistically significant difference.

2.4. Differential Expression of Proliferative, Hypoxic, and Immune Markers in Colon Tumor and Adjacent Non-Cancerous Tissues from AOM/DSS Model Mice

In colon tumor and non-cancerous colon tissues obtained from the AOM/DSS-induced colitis-associated cancer model, immunohistochemical staining for Ki-67, HIF-1 α , CD68, CD8, and Foxp3 was performed, and their expression levels were quantitatively analyzed (Figures 4, 5 and S2). In tumor tissues, the percentage of Ki-67-positive tumor cells, an indicator of proliferative activity, was significantly lower in the small NBO₂ water group than in the water group (Figure 4a). For the hypoxia marker HIF-1 α , separate evaluations of tumor epithelial cells and stromal cells in the mucosal layer showed that the percentages of HIF-1 α -positive epithelial cells and HIF-1 α -positive stromal cells were significantly lower in the small NBO₂ water group than in the water group (Figure 4b). The density of CD68-positive cells, a marker of pro-inflammatory macrophages, was significantly lower in the small NBO₂ water group than in both the water and large NBO₂ water groups (Figure 5a,b). In contrast, the densities of CD8-positive cytotoxic T lymphocytes and -positive regulatory T cells in tumor tissues did not differ significantly among the groups (Figure 5a,b). The same analyses were performed in non-cancerous colon tissues; however, no significant differences in the expression of Ki-67, HIF-1 α , CD68, CD8, or Foxp3 were observed among the experimental groups (Figure S2).

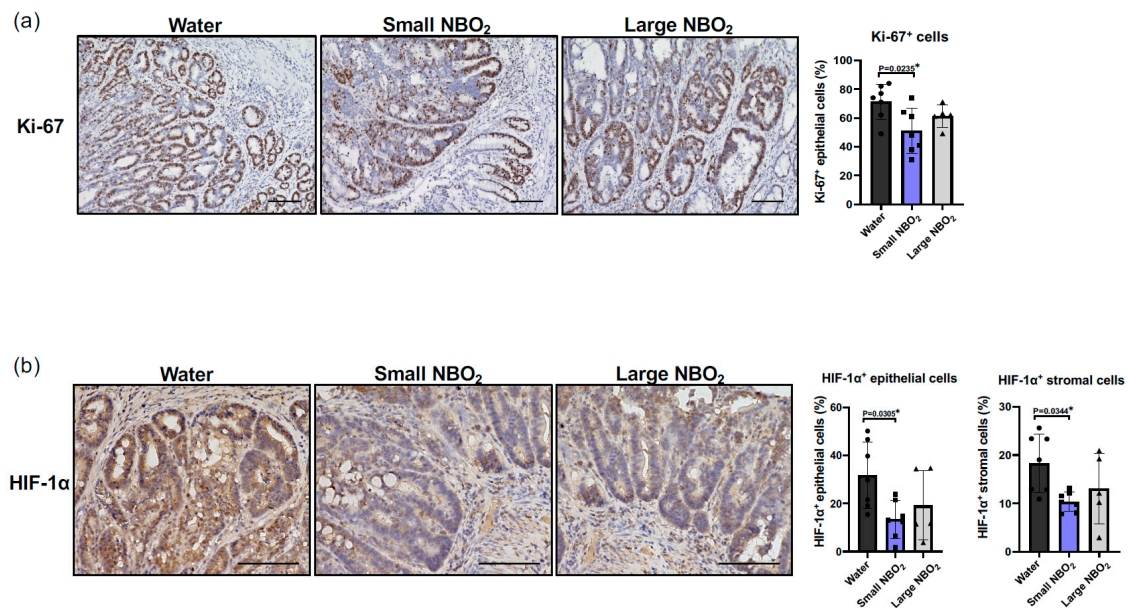


Figure 4. Effects of small and large oxygen nanobubble (NBO₂) water on tumor cell proliferation and hypoxia-inducible factor-1α (HIF-1α) expression in an azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer model. **(a)** Representative immunohistochemical images of Ki-67 staining in tumor tissues from each group (left) and quantification of Ki-67-positive epithelial cells (right). The percentage of Ki-67-positive epithelial cells was significantly lower in the small NBO₂ water group ($n = 7$) than in the water control group ($n = 7$). **(b)** Representative immunohistochemical images of HIF-1α staining in tumor tissues from each group (left) and quantification of HIF-1α-positive epithelial and stromal cells (right). The percentages of HIF-1α-positive epithelial and stromal cells were significantly lower in the small NBO₂ water group ($n = 7$) than in the water control group ($n = 7$). Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. * $p < 0.05$ indicates a statistically significant difference. Scale bars: 100 μ m.

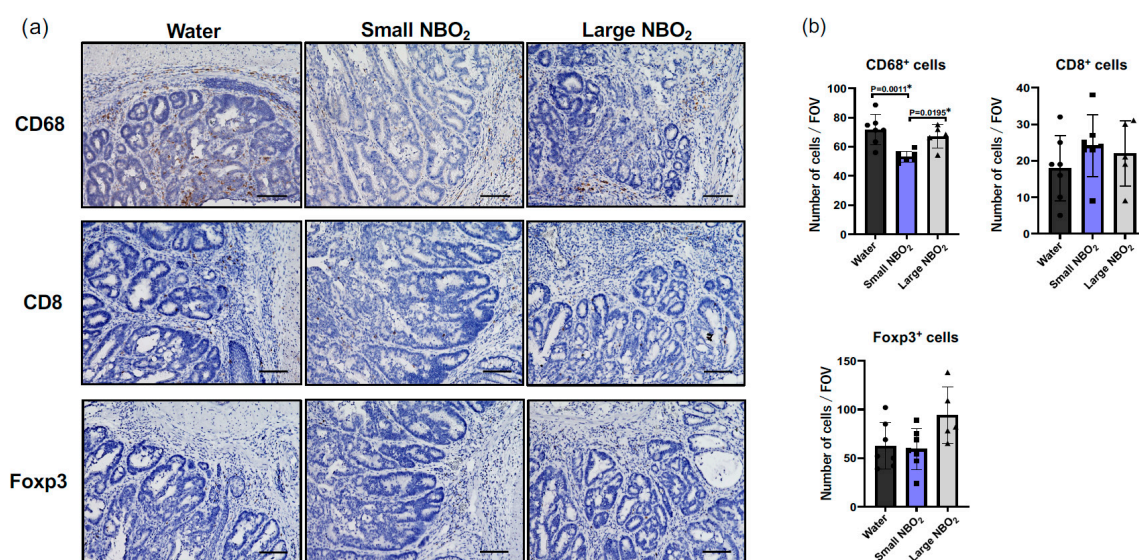


Figure 5. Effects of small and large oxygen nanobubble (NBO₂) water on immune cell infiltration in tumor tissues from an azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer model. **(a)** Representative immunohistochemical staining of CD68 (a marker of pro-inflammatory macrophages), CD8 (a marker of cytotoxic T lymphocytes), and forkhead box P3 (Foxp3; a marker of regulatory T cells) in tumor tissues from each group. Scale bars: 100 μ m. **(b)** Quantification of CD68⁺ cells (top left), CD8⁺ cells (top right), and Foxp3⁺ cells (bottom) per field

of view (FOV). The number of Cd68⁺ cells was significantly lower in the small NBO₂ water group ($n = 7$) than in the water control ($n = 7$) and large NBO₂ water groups ($n = 5$), whereas no significant differences were observed among groups for CD8⁺ or Foxp3⁺ cells. Data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. * $p < 0.05$ indicates a statistically significant difference.

3. Discussion

This study demonstrated that small NBO₂ water, with a smaller particle size than large NBO₂ water, significantly suppressed colitis and colorectal carcinogenesis compared to both water and large NBO₂ water in an AOM/DSS-induced colitis-associated cancer model. Specifically, administration of small NBO₂ water reduced the DAI score, alleviated colonic shortening, decreased histopathological colitis scores, and reduced the number of colonic tumors compared with the water control and large NBO₂ water groups, without affecting body weight, water intake, food intake, or spleen size, the latter serving as an indicator of systemic inflammation. Furthermore, immunohistochemical analyses revealed that small NBO₂ water significantly decreased the percentage of proliferating tumor cells, proportion of HIF-1 α -positive epithelial cells and stromal cells, and density of pro-inflammatory macrophage infiltration in tumor tissues. In contrast, no significant differences were observed among the groups in the densities of cytotoxic T cells and regulatory T cells within tumors, or in any evaluated markers in non-cancerous colon tissues. To the best of our knowledge, this is the first study to demonstrate that oxygen nanobubbles can modulate colitis and inflammation-associated colorectal carcinogenesis. Notably, we provide the first comparative evidence that nanobubble particle size is functionally significant, and that our previously validated small NBO₂ water, confirmed to be safe in animal studies, effectively suppresses colitis, colorectal tumorigenesis, HIF-1 α -expressing cells, and macrophage infiltration within intestinal tissue.

Oxygen nanobubbles are nanoscale gas bubbles that remain highly stable in liquids owing to their minimal buoyancy, allowing them to persist for extended periods. They possess exceptionally high oxygen solubility and gas transfer efficiency per surface area, enabling enhanced oxygen delivery to biological tissues and potentially improving hypoxic environments [15]. Previous studies have shown that the physical properties of NBs, particularly particle size, influence tissue infiltration and interactions with the tumor microenvironment. Smaller NBs have been reported to penetrate tissues more effectively than larger ones and to deliver oxygen more efficiently to hypoxic regions [3]. In the present study, the small NBO₂ water group exhibited significantly lower DAI and histological colitis scores compared with the large NBO₂ water group. Furthermore, the proportions of HIF-1 α -positive epithelial and stromal cells within tumors was significantly lower in the small NBO₂ water group than in the control group, suggesting that small NBO₂ water improved the intratumoral hypoxic environment. Previous reports have shown that stromal immune cells exposed to hypoxia increase pro-inflammatory cytokine production through HIF-1-dependent pathways, thereby amplifying inflammatory responses [11,13]. Moreover, elevated inflammatory cytokines in the tumor microenvironment have been implicated in the pathogenesis of colitis-associated cancer and in promoting tumor cell malignancy [16]. The present observation that reduced HIF-1 α levels in the small NBO₂ water group were accompanied by a lower percentage of Ki-67-positive tumor cells is consistent with these previous findings. As a plausible mechanism underlying the antitumor effects of low-concentration NBO₂ water, small sized NBO₂ water may suppress HIF-1 α stabilization in stromal and immune cells by improving the hypoxic microenvironment, thereby attenuating HIF-1 α -dependent inflammatory cytokine production [17]. Furthermore, this reduction in hypoxia-induced inflammatory signaling may consequently suppress tumor-promoting

inflammation and contribute to inhibition of development of colitis-associated colorectal cancer development [18]. Taken together, these results suggest that small amounts of NBO₂ water may suppress inflammation, and thereby colitis and colorectal carcinogenesis, by improving the hypoxic microenvironment within the tumor stroma.

The greatest advantage of small NBO₂ water is its high tissue penetration and oxygen delivery capacity. Nanoscale oxygen bubbles remain stable in liquid for extended periods and are expected to rapidly reach locally hypoxic areas, such as sites of inflammation. In our previous study, the small size of NBO₂ water used was shown to be safe in experimental animals, with no adverse effects on body weight, red blood cell count, blood biochemistry, or histology of major organs. Furthermore, small NBO₂ water suppressed intratumoral HIF-1 α accumulation, inhibited tumor growth, and enhanced radiosensitivity in a mouse tumor model [9]. For clinical application, small NBO₂ water may offer several advantages. First, it can deliver oxygen rapidly and safely to hypoxic areas, which could be beneficial in cancer therapy and inflammation control, where hypoxia-induced treatment resistance is a major challenge. Second, it has demonstrated a high safety profile in preclinical models, with no adverse effects or organ damage, suggesting its potential applicability even in elderly patients or those with comorbidities. Third, it is minimally invasive and suitable for long-term use because it can be administered orally as drinking water, allowing prolonged administration without impairing the quality of life. Fourth, it has the potential for synergistic effects with other treatments; enhancement of radiosensitivity has been reported, and it may be advantageous when combined with chemotherapy, immune checkpoint inhibitors, or existing anti-inflammatory drugs, such as 5-aminosalicylic acid (5-ASA), and molecular-targeted agents used in IBD. In general, a potential risk of gas embolism exists because nanobubble oxygen is delivered in a fluid form. Gas embolism is generally associated with intravascular delivery of gaseous agents and the presence of microbubbles large enough to obstruct blood vessels. Previous clinical and experimental studies indicate that embolic events occur primarily with gas bubbles in the tens to hundreds of micrometers range, which can occlude capillaries and small vessels [19,20]. In contrast, the oxygen nanobubbles used in the present study were ≤ 10 nm in diameter, as determined by AFM. This size is several orders of magnitude smaller than capillary diameters (approximately 5–10 μ m), making physical vascular obstruction highly unlikely. In addition, NBO₂ water was administered orally as drinking water, rather than via intravascular routes. Since gas embolism is typically caused by rapid intravascular gas entry, the route of administration used here further minimizes embolic risk. At the nanoscale, oxygen nanobubbles have been reported to exhibit physicochemical properties distinct from conventional gas bubbles, behaving more similarly to dissolved oxygen and undergoing rapid dissolution under physiological conditions [21,22]. Consistent with these considerations, no histopathological abnormalities were observed in the lung, brain, or kidney following NBO₂ water administration, as previously reported [9]. Collectively, these findings suggest that NBO₂ water poses a negligible risk of gas embolism under the experimental conditions used in this study.

However, this study also has limitations. In vitro AFM analysis enabled the visualization and quantification of the small NBO₂ particles used (Figure 1), however, no established method currently exists to visualize, detect, or quantify such small NBO₂ particles in vivo. Furthermore, we were unable to quantify the NBO₂ dosage or the surface area-to-volume ratio in in vivo experiments. Consequently, whether small NBO₂ water circulates systemically to directly modulate colonic inflammation or whether its primary mechanism involves the presence of small NBO₂ bubbles in the intestinal lumen after oral intake, improving local mucosal hypoxia remains unclear. In addition, although HIF-1 α immunohistochemistry was used as an indirect indicator of tissue hypoxia, direct assessment of intestinal hy-

poxia was not performed in the present AOM/DSS-induced colitis-associated cancer model. Specifically, established approaches such as pimonidazole-based immunofluorescence staining or direct tissue pO_2 measurements using oxygen-sensitive probes (e.g., OxyLite) were not employed, and this represents a limitation of the study. Although previous *in vivo* and *in vitro* studies have reported evidence that NBO_2 water can alleviate hypoxic conditions, the absence of direct hypoxia measurements in intestinal tissue limits the ability to definitively link the observed effects to local oxygenation changes in this model. In this study, the target markers were limited and they may be insufficient to elucidate the complexity of interactions between HIF-1 α and its downstream signaling pathways. Furthermore, the relatively small sample size in the present study may have limited the statistical power to detect subtle changes in immune cell populations, particularly $CD8^+$ T cells and $Foxp3^+$ regulatory T cells. Therefore, negative findings for these markers should be interpreted with caution, as the limited sample size may reduce the ability to detect biologically relevant differences. In addition, in this study, we did not establish a definitive translational dosing strategy or a regulatory pathway for human application. Simple volumetric extrapolation from mice to humans suggests that a direct conversion of daily fluid intake would result in an impractically large volume, highlighting that dose scaling based solely on oral intake is not appropriate for nanobubble-based formulations. Therefore, optimization of dosing, administration routes, and exposure profiles will need to be systematically evaluated in future clinical studies. Moreover, key translational steps, including good laboratory practice compliant toxicology studies, chemistry, manufacturing, and controls development, and alignment with a food and drug administration investigational new drug framework, remain to be addressed. Alternative routes of administration, such as intravenous delivery, may also provide important mechanistic and translational insights and should be explored in future investigations. For future clinical trials, developing methods to trace and quantify NBO_2 water *in vivo* is essential. In parallel, evaluating combination therapy using small size NBO_2 water, the safety of which has been confirmed in animal studies, with existing anti-inflammatory agents for IBD could further maximize its clinical potential.

4. Materials and Methods

4.1. Preparation of NBO_2 Water

Small NBO_2 water containing single-nanometer-sized oxygen bubbles was supplied by Sigma Technology, Inc. (Osaka, Japan) and produced using their proprietary patented device. Briefly, oxygen and purified water were mixed at 0.4 MPa and expelled through a nozzle. The oxygen-containing water collided at a high velocity, generating small NBO_2 water with single nanometer-sized oxygen bubbles, as described previously [9,10]. Large NBO_2 water was purchased from Naxnox.

4.2. AFM Analysis

The particle size of the oxygen nanobubbles in NBO_2 water was measured using an atomic force microscope (AFM; NP-NEX, Research Institute for Biomolecule Metrology Co., Ltd., Tsukuba, Japan). Because oxygen nanobubbles have a negatively charged surface, positively charged substrates were used for immobilization. Samples of NBO_2 water were placed on the substrate immediately before imaging, and high-speed scanning was performed at 6 s/frame to minimize changes in bubble size. AFM imaging was conducted in the tapping mode at room temperature. The scan size was set to 2000 nm with a resolution of 400×400 pixels. For each sample, three non-overlapping scan areas were randomly selected, and three distinct locations were analyzed within each area. Image analysis was performed using the Nano Particle Count Tool software ver 2.10 (Research Institute for Biomolecule Metrology Co., Ltd., Japan) with the following parameters: minimum particle

width of 4.5 pixels for small bubbles and 15.0 pixels for large bubbles, minimum circularity of 0.70, threshold height level = 1 (of 256 levels), and the median filter was disabled.

4.3. Murine Colitis-Associated Tumorigenesis Model (AOM/DSS Model)

Twenty-one female C57BL/6 mice aged 4 weeks were purchased from CLEA Japan, Inc. (Tokyo, Japan). Colitis-associated colorectal tumorigenesis was induced by intraperitoneal (i.p.) injection of AOM (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 12.5 mg/kg body weight, followed by oral administration of 2.5% DSS (MP Biomedicals, Santa Ana, CA, USA) in drinking water. Mice aged 5–6 weeks received a single i.p. injection of AOM and, 7 days later, were administered 2.5% DSS for 7 days, followed by 14 days of regular drinking water containing either distilled water, small NBO₂ water, or large NBO₂ water. This DSS treatment cycle was repeated three times. All mice were euthanized on day 70, as illustrated in Figure 2a. During the first experimental cycle, one mouse in the large NBO₂ water group died, and an additional mouse was euthanized after reaching the predefined humane endpoint, defined as a reduction in body weight to less than 80% of baseline. Consequently, the final sample size in the large NBO₂ water group was $n = 5$, whereas the other experimental groups consisted of $n = 7$ animals.

4.4. Treatment and Evaluation of Experimental Mice

AOM/DSS-treated mice were divided into three groups, with seven mice per group. Mice were monitored two to three times per week for body weight, stool consistency, and stool bleeding. The severity of colitis was scored using the disease activity index (DAI) [23], which was determined as follows: change in body weight (no weight loss or weight gain = 0; 1–5% weight loss = 1; 5–10% = 2; 10–15% = 3; and >15% = 4), stool consistency (normal and well-formed = 0; very soft and unformed = 2; watery stool = 4), and stool bleeding (normal color = 0; reddish = 2; bloody = 4). The DAI score was calculated as the sum of these scores and ranged from 0 (healthy) to 12 (severe colitis).

Whole colon and spleen samples from all mice were harvested on day 70. Colon length and spleen weight were measured, and the colon was cut longitudinally along its main axis. The number of tumors was then recorded. Colon tissues were fixed in 4% paraformaldehyde and paraffin-embedded using the Swiss roll technique [24]. For histologic evaluation of inflammation, paraffin-embedded colon sections were stained with hematoxylin and eosin (H&E) and evaluated. The histological colitis score was determined based on the following criteria [25]: degree of mucosal immune cell infiltration (0, normal; 1, mild; 2, modest; 3, severe), degree of submucosal immune cell infiltration (0, normal; 1, mild; 2, modest; 3, severe), degree of muscular immune cell infiltration (0, normal; 1, moderate to severe), epithelial damage (0, no change; 1, epithelial infiltrate; 2 crypt abscesses or erosion; 3, ulceration), and mucosal architecture (0, no change; 1, hyperplasia without dysplasia; 2, low- or high-grade dysplasia; 3, invasive carcinoma). The total histological colitis score ranged from 0 (healthy) to 12 (severe colitis).

4.5. Immunohistochemistry

Four-micrometer sections were prepared from paraffin-embedded colon tissues obtained from AOM/DSS model mice, including both tumor areas and non-cancerous areas, and mounted on silane-coated glass slides. Sections were deparaffinized and immersed in 0.3% hydrogen peroxide (H₂O₂)/methanol for 30 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed by heating the sections in boiling water using an Immunosaver (Nishin EM, Tokyo, Japan) for 45 min at 98 °C. Non-specific binding sites were blocked by incubation with Protein Block Serum-Free (DAKO, Santa Clara, CA, USA) for 30 min. The following primary antibodies were used: anti-HIF-1 α (rabbit polyclonal, Novus Biologicals, Centennial, CO, USA, NB100-479SS,

dilution 1:200), anti-Ki-67 (rabbit monoclonal, Cell Signaling Technology, Danvers, MA, USA, 12202, 1:200), anti-CD8 (rabbit monoclonal, Abcam, Cambridge, United Kingdom, ab209775, 1:1000), anti-FOXP3 (rabbit monoclonal, Cell Signaling Technology, 12653, 1:200), anti-CD163 (rabbit monoclonal, Abcam, ab182422, 1:500), and anti-CD68 (rabbit polyclonal, Abcam, ab125212, 1:500). Sections were incubated with primary antibodies for 24 h at 4 °C. Detection was performed using the Histofine Simple Stain Mouse MAX-PO (Rabbit) Kit (Nichirei, Tokyo, Japan), according to the manufacturer's instructions. Visualization was achieved using 0.02% 3,3'-diaminobenzidine tetrahydrochloride containing 0.005% H₂O₂ in 50 mM ammonium acetate–citrate buffer. The sections were lightly counterstained with Mayer's hematoxylin and mounted. Negative control specimens were processed without primary antibodies, and no specific staining was observed.

4.6. Immunohistochemical Evaluation

Quantification of HIF-1 α and Ki-67 expression was performed according to previously described methods [26] with minor modifications. Briefly, one representative field showing the highest level of expression (hotspot) in both cancerous and adjacent non-cancerous colon tissues was selected under $\times 200$ magnification. Within each hotspot, the total number of cells and the number of positively stained cells were counted separately in specific compartments (epithelial or intraepithelial stromal cells). The percentage of positive cells was calculated as follows: (number of positive cells $\div$ total number of cells) $\times$ 100. Immune cell infiltration (CD68, CD163, CD8, and Foxp3) was evaluated according to established methods [27,28]. Positively stained immune cells were manually counted in one hotspot field in both cancerous and adjacent non-cancerous tissues at $\times 200$ magnification, and cell density was expressed as the number of positive cells per field of view.

4.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, Boston, MA, USA). Differences among groups were assessed using one-way analysis of variance as appropriate. Tukey's post hoc test or Dunn's multiple-comparisons test was used for pairwise comparisons. A p value < 0.05 was considered statistically significant.

5. Conclusions

In conclusion, this study is the first to demonstrate that the particle size in NBO₂ water is a critical determinant of its biological effects in an animal model. Using small amounts of NBO₂ water, which has already been proven safe for long-term administration in experimental animals, we provide direct evidence that improving hypoxia in the colonic mucosa suppresses colitis and colorectal carcinogenesis. This demonstration that oxygen nanobubbles can prevent inflammation-associated colorectal carcinogenesis provides important foundational data for future clinical applications and strongly supports the further development of small NBO₂ water as a safe and promising therapeutic approach.

6. Patents

Tachibana Y., Tachibana K., Harada K., Sasajima S., Honma K., Matsumoto Y. Micro/nanobubble generating method, generating nozzle, and generating device. Sigma Technology, Inc., JP, issued 23 July 2014. JP5555892B2.

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Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: Nobutoshi Mutsuki was employed in Sigma Technology Inc. The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

AOM	Azoxymethane
DSS	Dextran sulfate sodium
DAI	Disease activity index
FOV	Field of view
HIF-1 α	Hypoxia-inducible factor 1-alpha
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
NBO ₂	Oxygen nanobubble
NBs	Nanobubbles

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