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Oxyhydrogen Nanobubbles Suppress FoxP3 and Ki-67 Expression in a Wistar Rat Model of Hepatocellular Carcinoma

Natasya Adiba Zahrah[1], Sri Widyarti[1], Dyah Kinasih Wuragil[2], Wibi Riawan[3], Olly Indrajani[4], Ahmad Lubab[5], Aditya Tri Hernowo[4,6], Sutiman Bambang Sumitro[1,4], Aulanni'am[7*]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.
[2] Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, Indonesia.
[3] Department of Biochemistry, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia.
[4] Indonesian Molecule Institute, Malang, Indonesia.
[5] Department of Mathematics, Faculty of Science and Technology, Universitas Islam Negeri Sunan Ampel Surabaya, Indonesia.
[6] Faculty of Medicine, Universitas Islam Indonesia, Sleman, Special Region of Yogyakarta, Indonesia.
[7] Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Malang, Indonesia.

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Corresponding Author:
Aulanni'am
aulani@ub.ac.id

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Abstract: HCC is difficult to treat due to its complex tumor microenvironment and the ineffectiveness of available therapies. Gas-based anticancer approaches are promising, but are hampered by instability and difficulties in targeted delivery. HHOnbs (containing hydrogen (H_2), oxygen (O_2), and low-dose hydrogen peroxide (H_2O_2) at the nanoscale) have been proposed to improve the stability and precision of gas-based therapies. This study aimed to investigate the effects of HHOnbs on the immunoregulatory marker FoxP3 and the proliferation marker Ki-67 in a Wistar rat model of HCC induced by diethylnitrosamine (DEN)/carbon tetrachloride (CCl_4). The design used was a true experimental design. HHOnbs were administered intravenously. Liver tissue was assessed using immunohistochemistry to measure FoxP3 and Ki-67 expression. HHOnbs treatment significantly reduced FoxP3 and Ki-67 expression ($p<0.05$). FoxP3 is a marker of regulatory T cells (Tregs), which often inhibit antitumor immune responses. Its decrease indicates an increased immune response potential. Ki-67 is a marker of cell proliferation. Its decrease indicates the suppression of cancer cell growth. Further investigations are warranted to compare its efficacy with standard therapies, assess different treatment durations, and elucidate the underlying molecular signaling pathways.

Keywords: Forkhead box P3; In Vivo; Ki-67; Liver cancer; Nanobubbles; Oxyhydrogen; Tumor microenvironment index

Introduction

Cancer is one of the main focuses of the Sustainable Development Goals (SDGs), particularly the third goal, which aims to reduce premature deaths from non-communicable diseases (Melyda et al., 2022). However, previous research confirms that there will be more than 35 million new cancer cases by 2050, an increase of approximately 77% from the 20 million cases estimated in 2022 (Bray et al., 2024). For example, the incidence of liver cancer is projected to rise by approximately 54% by 2040 (Guo et al., 2024a), and over one million deaths are expected by 2030, according to the WHO (Villanueva, 2019). Hepatocellular carcinoma (HCC) is the most prevalent type, representing 75–85% of cases (Masuzaki, 2023). Currently, HCC ranks as the sixth most common cancer and the fourth leading cause of cancer-related mortality worldwide (Sun et al., 2021). This has resulted in the SDGs not being optimally achieved.

The tumor microenvironment (TME) of HCC comprises tumor cells, non-parenchymal liver cells,

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tumor-associated fibroblasts, and immune cells (Chen et al., 2023). Regulatory T cells (Tregs), which express the transcription factor FoxP3, maintain immune homeostasis within the tumor microenvironment (Wang et al., 2023b). However, increased infiltration of FoxP3-expressing Tregs is associated with higher tumor grade and poorer prognosis in HCC (Tu et al., 2016). Additionally, Ki-67 expression is also crucial in HCC progression, as this protein directly regulates tumor cell proliferation (Yang et al., 2023).

The progression of HCC can be suppressed with chemotherapy, radiotherapy, and surgery. However, these treatments often cause adverse effects (Anggi et al., 2023), including gastrointestinal disturbances such as nausea, vomiting, diarrhea, constipation, mucositis, and appetite loss (Choulli et al., 2024), alopecia (Guan et al., 2023), cardiotoxicity such as left ventricular dysfunction, heart failure, arrhythmias, and hypertension (Kourek et al., 2022), and reduced bone mineral density (Bedatsova & Drake, 2019). Such toxicities can diminish patient quality of life and limit treatment tolerance (Haukland et al., 2020). These challenges underscore the need for more effective therapies that inhibit tumor growth while minimizing toxicity.

Gas-based therapeutic approaches have shown promising anticancer effects. Hydrogen gas inhibits cell proliferation, induces apoptosis, and suppresses tumor growth in lung cancer (Wang et al., 2018a). Oxygen therapy has also been reported to reduce cancer progression in a cohort of 45 patients without increasing the risk of tumor development, recurrence, or metastasis (Demir et al., 2025). Moreover, low-dose hydrogen peroxide, ranging from 50 to 200 μM, can induce cell cycle arrest through the modulation of oxidative stress-related genes in MCF7 breast cancer cells (Chua et al., 2009) and in A549 lung cancer cells by reducing the expression of cyclin D1 and cyclin E (Upadhyay et al., 2007).

Gases encounter several barriers during transport to tumor tissues, including abnormal vascular structures and unstable distribution within the tumor environment (Jing et al., 2021). These limitations can reduce gas levels at the target site (Meng et al., 2025b), while increasing accumulation in other tissues, which may lead to systemic toxicity and adverse effects (Jing et al., 2021). This concern is heightened because some therapeutic gases can become toxic at high concentrations (He, 2017). Therefore, optimized drug delivery systems are essential to ensure that therapeutic gases reach the intended organ or site with precise timing and concentration.

Nanobubbles (NBs) have gained considerable interest as drug delivery agents due to their noninvasive and targeted properties (Terlikowska et al., 2024). NBs are gas-filled bubbles with diameters below 1 μm (Guo et al., 2025c), a size that allows for safe extravasation and passage through blood capillaries and the blood-brain barrier (Cavalli et al., 2016). NBs can also avoid recognition by the reticuloendothelial system, which may extend their circulation time and increase drug accumulation at the target site (Jin et al., 2022). These features support their broad use as drug delivery systems in cancer therapy.

Although hydrogen, oxygen, and hydrogen peroxide have each shown anticancer potential in many studies (Demir et al., 2025; Kehr, 2024; Wang et al., 2018a; Meng et al., 2024a; Chua et al., 2009; Upadhyay et al., 2007), no studies have combined the use of hydrogen, oxygen, and hydrogen peroxide as a cancer therapy, particularly for HCC, and the use of NBs as a delivery system for this combination is still limited. Therefore, this study aimed to investigate the potential combination of these three compounds in the form of oxyhydrogen nanobubbles (HHOnbs) by evaluating their effects on FoxP3 and Ki-67 expression in an in vivo study (DEN- and CCl4-induced Wistar rat model of HCC). This study is expected to provide a novel NBs-based therapeutic approach that can enhance antitumor responses in HCC.

Method

Animal Subjects and Ethical Approval

This study was conducted from February to September 2025 and was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Brawijaya (Approval No. 188-KEP-UB-2024). Male Wistar rats (R. norvegicus), aged 2–3 weeks with an initial body weight of 45-125 g were obtained from PT Kemuning Resource Management, Karanganyar, Central Java, and maintained under controlled conditions (23 ± 1°C, 55 ± 5% humidity, 12 h light/dark cycle) and fed a standard pellet diet with unlimited access to drinking water at the Laboratory of Experimental Animal Science, Faculty of Veterinary Medicine, Universitas Brawijaya. Rats showing illness or deemed unfit for treatment were excluded to avoid bias.

Experimental Design

A true experimental in vivo design was applied. The study comprised three experimental groups, including two control groups and one treatment group. The control groups consisted of a negative control and positive control, in which the negative control included healthy rats without HCC induction, whereas the positive control consisted of rats with HCC. The treatment group comprised HCC-induced rats that received HHOnbs therapy. The sample size was determined using Federer's formula (1).

$$(t-1)(n-1) \geq 15 \quad (1)$$

t : Number of groups
n : Number of rats

HCC Induction

HCC was induced using a two-stage DEN–CCl_4 protocol (Raissa et al., 2022). In the initiation stage, 2–3-week-old rats received a single intraperitoneal injection of DEN (Sigma-Aldrich, Cat. No. N0258) at 50 mg/kg BW in 2 mL of vehicle. In the promotion stage, at 4–5 weeks of age, rats were administered CCl_4 (Merck, Cat. No. 1.02222.2500) mixed with corn oil (Sigma-Aldrich, Cat. No. C8267) (1:9, v/v) at a dose of 0.15 mL per dose, 24 times at 2-day intervals. This protocol reliably induces liver fibrosis and inflammation resembling human HCC pathology (Zhang et al., 2024a).

Oxyhydrogen Nanobubbles (HHOnbs) Treatment

The HHOnbs solution utilized in this study was provided in pharmaceutical-grade quality by a licensed manufacturer of intravenous solutions, namely Institute Molekul Indonesia (IMI). NBs were dispersed in sterile injectable water and generated via an exclusive electrolytic gas infusion method, in which medical-grade hydrogen and oxygen were combined at a 2:1 molar ratio, resulting in the intrinsic formation of low-dose hydrogen peroxide during NB generation (Indrajani et al., 2025). The dose of HHOnbs used in this study was 200 million per milliliter. A volume of 0.06 mL per injection was administered and rounded to 0.1 mL for practical application. The injections were performed 12 times at two-day intervals intravenously. Based on this regimen, each administration delivered approximately 20 million HHOnbs, resulting in a cumulative total dose of 240 million HHOnbs over the entire experimental period.

Immunohistochemistry

Liver tissues were fixed in 10% buffered formalin, embedded in paraffin, and sectioned at 4 µm. Sections were deparaffinized by placing them in an oven overnight, followed by placing the sections in xylene (I–III, 15 min each) and rehydrated using graded ethanol (absolute I–III and 80%, 15 min each). The slides were then stored at 4°C overnight. The immunohistochemical analysis was performed using a kit from ScyTek. Liver tissue sections were treated with 3% H_2O_2 for 40 minutes, followed by incubation in blocking serum in a humidity chamber for 30 minutes and three PBS washes. Primary antibodies against FoxP3 (1:100; Santa Cruz Biotechnology, Cat. No. sc-53876) and Ki-67 (1:100; Santa Cruz Biotechnology, Cat. No. sc-23900) were applied for one hour, followed by three PBS washes, incubation with secondary antibody for one hour, and three additional PBS washes. HRP was applied for 40 minutes, sections were stained with DAB for 10–40 minutes and washed three times with PBS. Mayer's hematoxylin was applied for 1–2 minutes, followed by three PBS washes, treatment with bluing reagent or alkaline water, and air-drying for 2–3 days. Sections were mounted with entellan and left to dry for approximately five days.

FoxP3 scoring was performed by evaluating both staining intensity and the proportion of positive cells, adapted from a previously described method (Liu et al., 2021a). Fifteen random fields per slide were examined at 200× magnification. Positive cells were quantified using ImageJ software. Ki-67-positive nuclei were also counted using ImageJ and expressed as the number of positive cells per field. Staining intensity was categorized as 0 (no staining), 1 (light yellow), 2 (yellowish brown), and 3 (dark brown). The proportion of positive cells was scored as 1 (0–10%), 2 (11–25%), 3 (26–50%), and 4 (51–75%). Total score categories were defined as negative (0), weak (1–2), moderate (3–4), and strong (5–7). For interpretative purposes, FoxP3 expression levels were categorized into relative TME immunoregulatory states based on the semi-quantitative FoxP3 score, with weak expression representing low immunosuppressive activity, moderate expression indicating intermediate immunosuppression, and strong expression reflecting a highly immunosuppressive TME.

Research Data Analysis Techniques

The number of positive cells of each group was analyzed using IBM SPSS Statistics 26. Normality and homogeneity were assessed, followed by one-way ANOVA or the Kruskal–Wallis test, depending on the data distribution. A p-value < 0.05 was considered significant, and post hoc tests were performed to identify pairwise group differences.

Result and Discussion

The Overview of FoxP3 Expression

HHOnbs therapy significantly reduced FoxP3 expression compared with the positive control group (Figure 1). Based on the FoxP3 scoring, the TME index was categorized as weak in the negative control group, strong in the positive control group, and moderate in the HHOnbs-treated group. Post hoc analysis confirmed a marked increase in FoxP3 expression in the positive control compared with the negative control ($p < 0.0001$), validating successful HCC induction. Although FoxP3 expression in the HHOnbs-treated group remained significantly higher than in the negative control ($p < 0.01$), it was significantly lower than in the positive control group. This suggests that HHOnbs therapy attenuated the increase in FoxP3 associated with HCC, although it did not completely restore physiological FoxP3 levels.

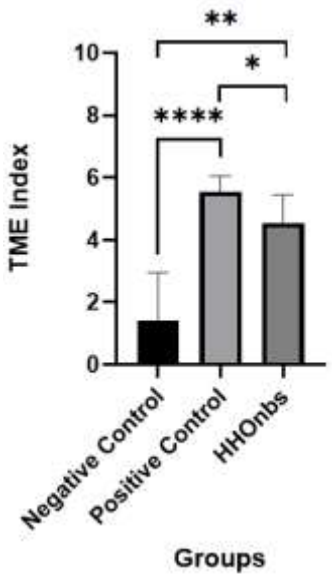

Figure 1. Post-hoc analysis results for each treatment group. *: $p < 0.05$; **: $p < 0.01$; ****: $p < 0.0001$

The reduction in FoxP3 expression suggests that HHOnbs may attenuate Treg cell activity, which is elevated in the tumor microenvironment and contributes to immunosuppression. FoxP3 is a transcription factor in Treg cells that maintains immune homeostasis (Zhang et al., 2023b). However, when overexpressed, it promotes HCC progression, invasiveness, and metastasis (Granito et al., 2021) by suppressing the functions of other immune cells (Teng et al., 2021). FoxP3 is initially expressed in the cytoplasm of activated effector T cells, then translocates to the nucleus in Treg cells, where it regulates genes that mediate immunosuppressive activity (Alfaar et al., 2022) (Figure 2).

The decrease in FoxP3 expression indicates reduced Treg activity or infiltration in tumor tissue, supporting a stronger antitumor immune response (González-Navajas et al., 2021). This reduction may result from decreased immunosuppressive cytokines, such as IL-10 and TGF-β (Muth et al., 2022), as well as lowered activation of cell-to-cell contact pathways, including CTLA-4 and PD-1 (Contreras-Kallens et al., 2022). Additionally, metabolic competition can limit the proliferation and function of effector T cells (Hooda et al., 2024). Furthermore, cytolytic-mediated apoptosis, which is mediated by perforin and granzyme B, further reduces Treg-mediated suppression (Rueda et al., 2016).

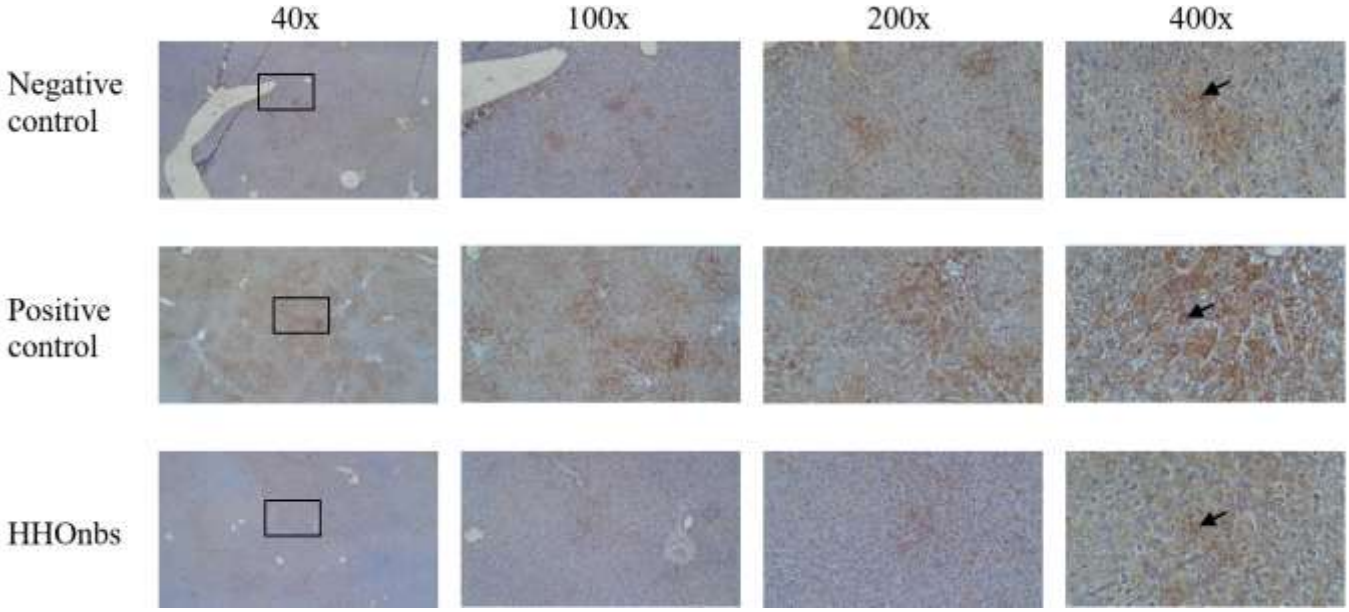

Figure 2. Immunohistochemical staining of FoxP3 in liver tissue at magnifications of 400×. Representative images from the negative control, positive control, and HHOnbs-treated groups. Arrows indicate the $FoxP3^+$

The Overview of Ki-67 Expression

This study primarily evaluated tumor proliferation at the cellular level using Ki-67 immunoreactivity. Although macroscopic tumor burden parameters such as tumor volume or weight were not measured, Ki-67 is a well-established prognostic marker that strongly correlates with tumor growth and aggressiveness in HCC. The results demonstrated a significant decrease in Ki-67 expression in the HHOnbs-treated group compared with the positive control group (Figure 3). Tukey's post-hoc test revealed a highly significant difference between the negative and positive control groups ($p < 0.0001$). A significant difference was likewise observed between the negative control and HHOnbs groups ($p < 0.01$). Furthermore, Ki-67 expression was significantly reduced in the HHOnbs-treated group compared with the positive control group ($p < 0.01$).

The decrease in Ki-67 expression in the HHOnbs-treated group indicates suppressed HCC cell proliferation (Figure 4). As a marker of all active cell-cycle phases, Ki-67 reflects tumor growth dynamics (Lashen et al., 2023). This finding is clinically relevant because high Ki-67 expression in HCC has been consistently associated with poorer overall survival and more aggressive tumor phenotypes (Yang et al., 2023). Therefore, HHOnbs-mediated Ki-67 attenuation underscores its potential to limit tumor growth and malignant progression.

Ki-67 is a key prognostic marker in HCC, with higher expression correlating with increased tumor cell proliferation (Ramos-Santillan et al., 2024). It regulates nucleolar organization, chromatin accessibility, and gene expression programs that enable tumor adaptation to stress (Andrés-Sánchez et al., 2022). Elevated levels are associated with larger and more advanced tumors (Alghezi et al., 2025). Therefore, reduced Ki-67 expression indicates suppressed tumor proliferation and contributes to the inhibition of HCC progression.

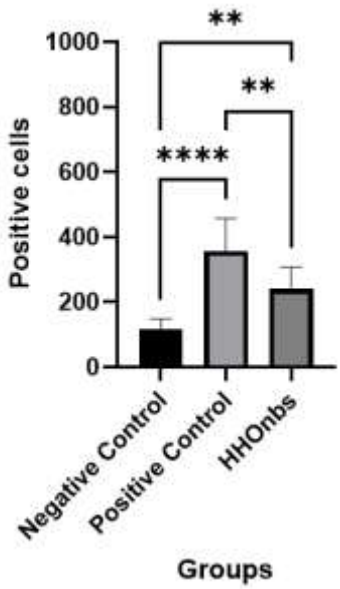

Figure 3. Tukey's post-hoc test results for each treatment group. **: $p < 0.01$; ****: $p < 0.0001$

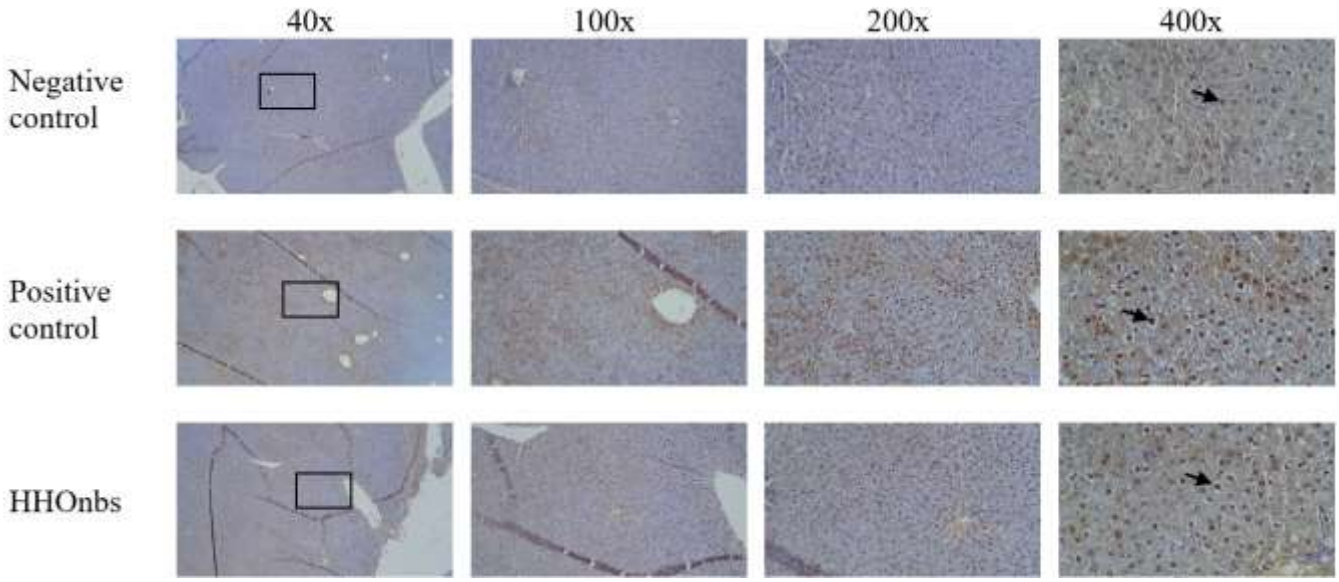

Figure 4. Immunohistochemical staining of Ki-67 in liver tissue at magnifications of 400×. Representative images from the negative control, positive control, and HHOnbs-treated groups. Arrows indicate the Ki-67+

The Therapeutic Potential of HHOnbs in Cancer

This study demonstrates that HHOnbs effectively reduce HCC progression in Wistar rats, as shown by decreased FoxP3+ and Ki-67+ expression. HHOnbs are composed of hydrogen, oxygen, and low-dose hydrogen peroxide (Indrajani et al., 2025). As supported by an in vitro study, molecular hydrogen has been shown to induce the expression of antioxidant genes and enhance the activity of antioxidant enzymes (Murakami et al., 2017). Consistently, hydrogen-based therapy modulates immune responses, enhances T-cell antitumor function, inhibits T-cell dysfunction, and suppresses ROS-dependent signaling in cancer cells (Zhou et al., 2024).

Hypoxia is a hallmark of tumor cells, creating a microenvironment that favors the proliferation of immunosuppressive cells while impairing the development of cytotoxic T cells (Feldman, 2024). However, an in vitro study has shown that oxygen administration improves the survival of normal cells more than that of cancer cells under hypoxic conditions (Kehr, 2024). Oxygen-based therapy is thus proposed to increase tumor oxygenation, suppress tumor cell proliferation, and potentially overcome hypoxia-associated treatment resistance (Qin et al., 2022).

Although hydrogen peroxide is a type of ROS, low concentrations confer significant physiological benefits.

Consistent with previous studies, low levels of hydrogen peroxide can reduce ROS production, enhance antioxidant enzyme activity such as SOD and CAT, and lower oxidative stress markers, including MDA (Wang et al., 2020c). Additionally, low doses of hydrogen peroxide can induce cell-cycle arrest by modulating oxidative stress–related genes (Chua et al., 2009) and downregulate cyclin D1 and cyclin E in cancer cells (Upadhyay et al., 2007).

The combination of hydrogen, oxygen, and hydrogen peroxide within a single therapeutic system may generate synergistic effects. This combination has been shown to alleviate oxidative stress and exhibit potent antioxidant and anti-inflammatory activities, as observed in patients with chronic kidney disease (CKD) (Indrajani et al., 2025). In cancer, antioxidants can reduce ROS (Lim et al., 2023) and support GSH synthesis (Hipólito et al., 2020), while anti-inflammatory effects disrupt the tumor microenvironment, inhibit migration, enhance apoptosis, and increase therapeutic sensitivity (Zappavigna et al., 2020).

The observed reduction in FoxP3 and Ki-67 expression may reflect a combined modulation of oxidative stress, hypoxia, and inflammatory signaling within the tumor microenvironment. While hydrogen may attenuate ROS-dependent immunosuppressive signaling, oxygen delivery could alleviate tumor hypoxia, a condition known to promote Treg accumulation and tumor proliferation. Low-dose hydrogen peroxide may further influence cell-cycle regulatory pathways.

In addition to the advantages of each component, the therapeutic potential of HHOnbs is further supported by the superior characteristics of NBs. NBs possess the ability to encapsulate and coat specific therapeutic agents, enabling more efficient and targeted delivery of drugs directly to tumor cells (Wu et al., 2021). Moreover, their nanoscale size allows NBs to penetrate through inter-endothelial gaps within the tumor vasculature, ensuring more precise targeting and minimizing undesired side effects (Sarkar et al., 2025).

Consistent with previous studies, NBs have demonstrated significant therapeutic potential in the treatment of HCC. Chitosan-based NBs loaded with idarubicin effectively deliver drugs while reducing side effects in HUH7 cells (Mossenta et al., 2025). Lipid-based NBs modified with PD-L1 and chlorin e6 inhibited tumor growth in H22 and HepG2 cells by regulating ROS, inducing apoptosis, and modulating NK cells and lymphocyte functions (Liu et al., 2022b), while lipid-based NBs with curcumin and doxorubicin enhanced drug accumulation and promoted apoptosis in Hepa 1-6 cells (Guo et al., 2024b).

Limitations and Future Research Recommendation

Although this study proves that intravenous administration of HHOnbs in HCC model rats has provided optimal benefits, this study still has several limitations. First, this study has not made a direct comparison between HHOnbs therapy and conventional therapy based on gold standard drugs. Such a comparison is important to determine the extent of the effectiveness of HHOnbs compared with conventional therapy in general. Second, the duration of HHOnbs therapy administration is still limited to 12 intravenous injections. Therefore, further studies are needed using a longer therapy duration to determine the relationship between dose and response. Third, this study did not evaluate molecular signaling pathways associated with FoxP3 or Ki-67 regulation. Therefore, future studies should incorporate other molecular assays to assess immune checkpoint pathways and cell-cycle regulators.

Conclusion

This study demonstrates that intravenous administration of HHOnbs significantly reduces the expression of FoxP3 and Ki-67 in a DEN- and CCl4-induced Wistar rat model of HCC. The attenuation of FoxP3 expression indicates a reduction in HCC-associated immunosuppressive features within the tumor microenvironment, while decreased Ki-67 expression reflects suppressed tumor cell proliferation. Although FoxP3 levels were not fully restored to those of healthy controls, the observed downregulation relative to untreated HCC suggests a measurable therapeutic effect. Collectively, these findings support the potential of HHOnbs as a nano-enabled gas-based strategy for modulating immune regulation and proliferative activity in solid tumors characterized by hypoxia and immune suppression. From a practical perspective, this work provides a preclinical foundation for further optimization of HHOnbs dosage, treatment duration, and molecular signaling pathways, thereby informing the development of adjunctive or alternative therapeutic approaches for HCC.

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Author Contributions

N. A. Z: Writing—original draft preparation, data curation, investigation, methodology. S. W: Supervision, writing—

review and editing, conceptualization. D. K. W: Methodology, resources. W. R: Methodology, resources, investigation. O. I: Funding acquisition, conceptualization, resources. A. L: Software, data curation. A. T. H: Funding acquisition, conceptualization, resources. S. B. S: Funding acquisition, conceptualization, resources. A. A: Supervision, writing—review and editing, conceptualization.

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Conflicts of Interest
The authors declare no conflict of interest.

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