Berkala **PENELITIAN HAYATI**

ISSN: 08526834 | E-ISSN:2337-389X

Volume 30 | No. 1 | **April** | 2024

Original Article

Evaluating the safety and therapeutic efficacy of intravenous hydrogen nanobubble infusions in a hypercholesterolemic rat model

Aditya Tri Hernowo [1,2*], Sri Widyarti [1,3], Sherry Aristyani [1]

[1] Indonesian Molecule Institute, Elpico Mall 2nd floor No. 23, Malang, Indonesia
[2] Faculty of Medicine, Universitas Islam Indonesia, Jl. Kaliurang No.KM. 14,5, Krawitan, Umbulmartani, Ngemplak, Sleman, Indonesia
[3] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya, Jl. Veteran No.10-11, Ketawanggede, Lowokwaru, Malang, Indonesia

Abstract

Hydrogen has emerged as a promising candidate due to its antioxidant properties and potential to modulate electron flow, enhancing mitochondrial energy metabolism and reducing oxidative stress. In this study, hypercholesterolemia male Wistar rats were utilized to investigate the safety and efficacy of intravenous hydrogen nanobubble solution (HNB). The study assessed serum lipid levels, complete blood count, and organ health following HNB administration compared to positive (simvastatin) and negative controls. Bayesian statistics were used to validate the findings. The administration of HNB did not adversely affect the immediate survival of the rats on a high-fat diet (HFD), whereas simvastatin significantly reduced survival rates. It showed a dose-dependent immunomodulatory effect, reducing white blood cell, lymphocyte, and monocyte counts. It also influenced erythropoiesis, as indicated by increased red blood cell counts and hemoglobin levels. A significant elevation in platelet counts was observed, suggesting potential effects on platelet production or lifespan. The study also noted changes in liver enzyme levels, with HNB indicating potential hepatoprotective effects. Furthermore, HNB treatment decrease in mean fat droplet count, suggesting a mitigation of diet-induced hepatic steatosis. The findings suggest that HNB may serve as a protective agent against the deleterious effects of a high-fat diet, offering immunomodulatory and hepatoprotective benefits. This study provides a foundation for considering HNB as a potential therapeutic intervention for hypercholesterolemia and warrants further investigation into the long-term impacts of nanobubble treatment on diet-induced pathologies. The consistency of results across traditional statistical significance and Bayesian evidence strengthens the validity of the observed treatment-specific effects.

Keywords: Bayesian statistics, hydrogen, hypercholesterolemia, nanobubble, statin, steatosis

Received: Jan 01, 2024 Revised: March 1, 2024 Accepted: April 21, 2024

Introduction

Hypercholesterolemia, characterized by elevated cholesterol levels, is a subset of hyperlipidemia or dyslipidemia that encompasses lipid imbalances in the bloodstream. Excessive plasma lipoproteins, key lipid transporters, underscore this condition which is intimately linked with the onset of atherosclerosis and, if unmanaged, escalates the risk of heart disease and other cardiovascular disorders (Suckling, 2014; Bayly, 2014). Globally, cardiovascular diseases account for approximately 17.9 million deaths annually, comprising 32% of all global deaths (WHO, 2021). Familial hypercholesterolemia (FH), a genetic anomaly associated with mutations in the LDL receptor gene, exacerbates LDL cholesterol levels in the bloodstream (Admira & Sayuti, 2018). Dietary habits, particularly those rich in saturated fats, also serve as environmental contributors to elevated cholesterol levels (Ibrahim et al., 2023).

Statin medications are the cornerstone for cholesterol management, primarily by inhibiting the HMG-CoA reductase enzyme. This enzyme plays a pivotal role in synthesizing not just cholesterol but also critical biomolecules like coenzyme Q10 and vitamin D (Zaleski et al., 2018). Hence, statins' influence extends beyond cholesterol regulation to potentially affect essential cellular functions, sometimes resulting in adverse effects, particularly on muscle and liver health (Golomb & Evans, 2008). However, H_2 therapy has emerged as a safe alternative, exhibiting cholesterol-lowering and antioxidant properties (Song et al., 2013; Iketani et al., 2018).

H_2, the simplest and lightest molecule, diffuses with ease across cell membranes, facilitating the transport of hydrophilic compounds into mitochondria and nuclei. Its therapeutic applications, particularly for its antioxidant and antiapoptotic properties, were highlighted when inhaled hydrogen gas was found to protect against ischemic-reperfusion brain injury (Ohsawa et al., 2007). It selectively neutralizes harmful reactive oxygen species (ROS) and reactive nitrogen species (RNS), potentially boosting cellular antioxidant capacity to curb oxidative damage (Ohta et al., 2015).

Within the cellular context, mitochondria, recognized as significant ROS producers, may undergo dysfunction under prolonged oxidative stress. Here, H_2 can modulate electron flow within mitochondria to diminish ROS production, thereby bolstering mitochondrial energy metabolism and possibly restoring cellular integrity (Tian et al., 2021). H_2 further induces antioxidant enzymes like superoxide dismutase (SOD) and catalase, enhancing redox homeostasis and lessening oxidative stress, which positions H_2 as a viable adjunct therapy in cancer treatments to alleviate the side effects of radiation

*Corresponding Author:
Aditya Tri Hernowo
Indonesian Molecule Institute, Malang, Indonesia
E-mail: dr.hernowo@uii.ac.id

http://dx.doi.org/10.23869/bphjbr.30.1.20247
Published by © PBI East Java. Open Access www.berkalahayati.org

and chemotherapy (Meng et al., 2015; Li et al., 2015; Dong et al., 2018).

Moreover, the anti-inflammatory and anti-apoptotic capabilities of H_2 have been documented. It impedes chronic inflammation via mitochondrial oxidation inhibition and impedes the NLRP3 inflammasome, which plays a pivotal role in inflammation (Shao et al., 2016; Chen et al., 2019). H_2 affects the balance of pro-apoptotic and anti-apoptotic factors, regulating signaling pathways crucial for cell survival, thereby suggesting its neuroprotective potential (Hong et al., 2014; Ge et al., 2017).

Exploiting these properties, H_2 has been incorporated into therapeutic strategies for degenerative diseases, including cancer and cardiovascular conditions, administered via inhalation or hydrogen-rich water (Li et al., 2019; Ramanathan et al., 2023; Saengsin et al., 2023). Although clinical trials have noted some side effects of hydrogen water, the advent of H_2 nanobubbles offers enhanced stability and solubility, suggesting improved therapeutic efficacy (Nakao et al., 2010; Ono et al., 2011; Iida et al., 2016; Kato et al., 2015; Li et al., 2022; Tanaka et al., 2022). In light of these findings, the present study aims to explore the safety and multifaceted impacts of administering a hydrogen nanobubble solution intravenously in a hypercholesterolemic rat model, pursuing an innovative and safe treatment avenue for hypercholesterolemia.

Methods

This investigation was conducted as an experimental study employing a pre- and post-test randomized controlled group design to explore the effects of treatments on specified variables. The independent variable is the administration of hydrogen nanobubble solution (HNB). The dependent variables are the serum level of LDL, HDL, triglycerides, total cholesterol, complete blood count, as well as the conditions of the adipose tissue, liver, kidneys, heart, and lungs. The control variables are age, sex, strain, and body weight of the Wistar rats. The HNB was procured from the Reverse Aging and Homeostasis Club, available as water for injection in different dosages (low dose - 5mL; and high dose - 25mL). The male wistar rats normal diet (lab chow) is formulated so that it contains proteins (20-25%), carbohydrates (60-70%), fats (4-5%), and vitamins and minerals (added to ensure a balanced diet and support various bodily functions).

Fiber: Helps in digestion and is included in the diet at about 4-5%. Male Wistar rats with hypercholesterolemia were utilized as the experimental subjects and sourced from the Faculty of Pharmacy, Gadjah Mada University, Yogyakarta.

The study was conducted from October to December 2022. All treatments, including injections, behavioral observations, and animal care, were performed in the molecular biology laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Brawijaya University. Hematological assessments were carried out at the Veterinary Hospital of Brawijaya University, while the histopathological examinations of the liver and kidneys were conducted at the Brawijaya University, Malang (068-KEP-UB-2022).

Research procedure

A one-week acclimatization period for the rats was implemented prior to the commencement of the experiment. The rats were randomly assigned into seven groups, each consisting of five subjects (Table 1).

Table 1. Group names and treatment modalities. Abbreviations: HNB - hydrogen nanobubbles; NS - normal saline.

Group	Treatment
HFDnoTx	High-fat diet without any treatment (control)
HFDSimv	High-fat diet with the administration of simvastatin
HFDhdNB	High-fat diet with the administration of intravenous high-dose HNB 2mL
HFDldNB	High-fat diet with the administration of intravenous low-dose HNB 2mL
NDhdNB	Normal diet with the administration of intravenous high-dose HNB 2mL
NDldNB	Normal diet with the administration of intravenous low-dose HNB 2mL
HFD + NaCl	High-fat diet with the administration of intravenous 0.9% NS 2mL (control)

The saline buffer and HNB solutions were administered intravenously, while simvastatin was given orally at a dosage of 0.18 mg/day per 200 grams of body weight. The saline buffer and HNB were administered for 16 days every other day, while simvastatin was given daily. Body weights were measured daily before the experiment, throughout the treatment period, and at the study's conclusion. Cholesterol check was done using a blood cholesterol rapid diagnostic kit. It was taken prior to the commencement of the experiment, then on the sixth day, or after the third intravenous treatment. Upon completion of all treatments, the final assessments include triglyceride and total cholesterol levels, and the histopathological analysis of the liver tissue.

Statistical analysis

As we expected the number of data will not be of normal distribution, we opt to use nonparametric statistical testing. As we want to compare for more than twou groups of data, nonparametric analysis utilizing Kruskal-Wallis test with subsequent post hoc tests if necessary. Any finding with $p<0.05$ is considered significant. Bayesian statistics is deployed where relevant to measure the evidential strength of any significant finding.

Results

The study investigated the effects of varying dietary regimens and treatments on hematological and biochemical parameters in an animal model. Data were grouped into treatments with no treatment, low dose HNB, and high dose HNB, compared against a normal range for each parameter.

In groups exposed to a high-fat diet without any additional treatment or supplemented with NaCl or HNB, the survival rate was uniformly high, with 100% of the subjects surviving for the duration of the study. The group treated with simvastatin exhibited a significantly lower survival rate of only 40%. These findings are illustrated in Figure 1, which provides a clear comparison of survival rates across the different treatment groups.

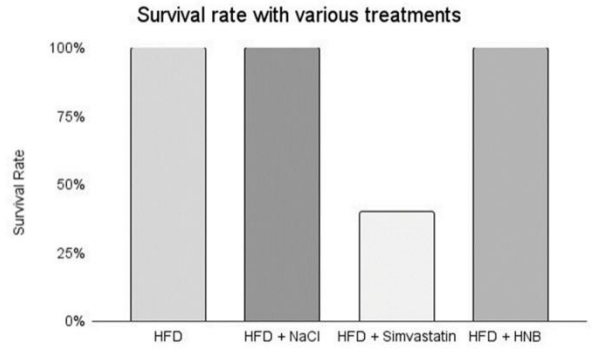

Figure 1. Rats survival rate with various treatments. (HFD - high-fat diet; NaCl - sodium chloride; HNB - hydrogen nanobubble).

The study evaluated the impact of various treatments on hematological and biochemical parameters in a controlled experiment. In the normal diet group, the leucocyte counts showed a decrease from the control group (6.70±0.00) to low dose HNB treatment (3.33±0.42), with a partial recovery in high dose HNB (5.00±0.26). Lymphocyte counts increased significantly in both low and high dose HNB treatment (3.03±0.42 and 4.33±0.38, respectively), compared to the control, while monocyte and granulocyte counts decreased significantly in all HNB treatments compared to the control. It suggested a treatment-related modulation of immune cell profiles. There was a decrease in erythrocyte count and hemoglobin concentration without any treatmment, with a subsequent increase in treatments low and high dose HNB treatments toward normal levels. Mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) levels increased slightly across treatments, indicating changes in erythrocyte morphology, while red cell distribution width - coefficient variation (RDW-CV) and red cell distribution width - standard deviation (RDW-SD) varied less significantly. Platelet count increased with treatments, particularly noted in the high dose HNB (916.33±40.70), suggesting a possible effect on platelet production or lifespan. Mean platelet volume (MPV) and platelet distribution width (PDW) showed slight variations, indicating changes in platelet size and distribution. Regarding biochemical parameters, there was a decrease of glutamate oxalacetate transaminase (GOT) levels at low and high dose of HNB treatments (185 ± 35.36 and 166 ± 28, respectively) relative to the controls (199 ± 0.0). Glutamate pyruvate transaminase (GPT) levels increased significantly with low dose HNB (91 ± 18.38), indicating potential treatment impacts on liver function. The GOT/GPT ratio decreased with low dose HNB (1.87 ± 0.89), suggesting alterations in liver enzyme activity balance. Creatinine levels showed a general decrease across treatments, indicating potential effects on kidney function. These findings suggest that the administered treatments have diverse impacts on hematological and biochemical markers, reflecting their influence on immune cell distribution, red blood cell (RBC) morphology, platelet dynamics, and liver and kidney function. The variability across different parameters underscores the complexity of the treatment effects and warrants further investigation into their biological mechanisms and implications.

The analysis of the relative organ weights following various dietary treatments reveals significant alterations in the adipose tissue, liver, kidneys, and heart-lung weights across different groups. The relative organ weights measuring is one of the important parameters to assess the substance toxicity of substances or interventions used. The weight reduction has been linked to depression of cellular metabolism and growth (Akhigbe, 2014). The study aimed to compare the effects of a normal diet, a high-fat diet (HFD), HFD supplemented with NaCl, HFD supplemented with Simvastatin, and HFD supplemented HNB on the relative organ weights in Rats. Rats on a normal diet showed a mean adipose tissue weight of 0.69 ± 0.22. This weight substantially increased to 3.82 ± 0.62 under HFD, indicating significant fat accumulation. Supplementation with NaCl (3.03 ± 0.18), simvastatin (2.79 ± 1.51), and HNB (2.56 ± 0.27) alongside HFD showed a reduction in adipose tissue weight compared to HFD alone, with HNB showing the most pronounced effect. The liver weight in the normal diet group was 2.67 ± 0.06, which increased to 3.34 ± 0.06 in the HFD group. Supplementation with NaCl, Simvastatin, and HNB alongside HFD resulted in liver weights of 3.31 ± 0.15, 3.43 ± 0.27, and 2.93 ± 0.29, respectively. Notably, the liver weight was highest in the Simvastatin group, suggesting potential hepatomegaly or lipid accumulation. The left kidney weight was relatively stable across treatments, with the normal diet group at 0.32 ± 0.01 and minimal variations in the other groups. The right kidney followed a similar pattern, with the normal diet group at 0.34 ± 0.00 and slight reductions observed in the HFD group, which were somewhat mitigated by the supplementary treatments. The heart and lung weight remained fairly consistent across all dietary treatments, with a slight increase observed in the simvastatin group (1.24 ± 0.34) compared to the normal diet group (1.20 ± 0.22). This suggests that the treatments did not significantly affect the combined heart and lung weights relative to the normal diet. Figure 2 summarizes and illustrates these findings.

These findings suggest that while HFD leads to increased fat accumulation and liver weight, the supplementary treatments, especially HNB, can mitigate these effects to some extent. The relative stability of kidney and heart-lung weights across treatments indicates that these organs were less affected by the dietary conditions and supplementary treatments applied in this study.

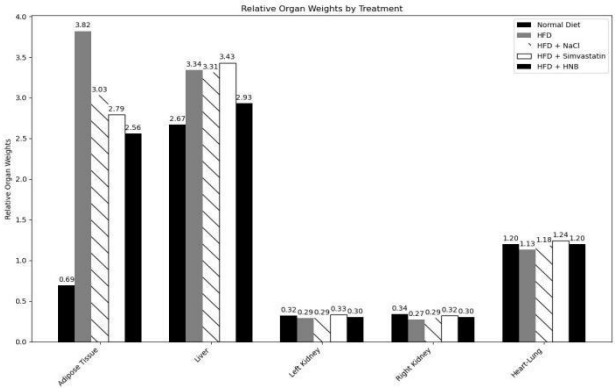

Figure 2. Relative organ weights with various treatments. (HFD - high fat diet; NaCl sodium chloride; HNB - hydrogen nanobubble)

The analysis of blood cholesterol and triglyceride levels under various dietary treatments provides insight into the metabolic effects of a high-fat diet and its modifications with sodium chloride, simvastatin, and hydrogen nanobubble treatment. The baseline cholesterol levels in rats subjected to an HFD were measured at 207.75 ± 2.89 mg/dL. The introduction of NaCl to the HFD marginally increased these levels to 213.25 ± 8.5 mg/dL, indicating that NaCl supplementation has a slightly elevating effect on cholesterol levels. Conversely, treatments with simvastatin and HNB dramatically reduced cholesterol levels to 99 ± 0 mg/dL for both treatments. This demonstrates the effectiveness of both simvastatin and HNB in lowering cholesterol levels in the context of an HFD.

Triglyceride measurements further confirmed the impact of dietary modifications on lipid metabolism. Rats on an HFD exhibited high triglyceride levels of 560.3 ± 68.8 mg/dL. Supplementation with NaCl resulted in a decrease to 377.7 ± 84.1 mg/dL, suggesting a moderate mitigating effect on triglyceride accumulation. More substantial reductions were observed with simvastatin and HNB treatments, where triglyceride levels were reduced to 270 ± 63.1 mg/dL and 204.7 ± 46.2 mg/dL, respectively. These results highlight the significant lipid-lowering potential of simvastatin and HNB in the setting of a high-fat dietary regimen. Figure 3 summarizes and illustrates these findings.

While an HFD elevates cholesterol and triglyceride levels, the introduction of NaCl, simvastatin, and particularly HNB can mitigate these effects to varying degrees. The lipid-lowering effects of simvastatin and HNB are especially notable, demonstrating their potential utility in managing hyperlipidemia induced by high-fat diets.

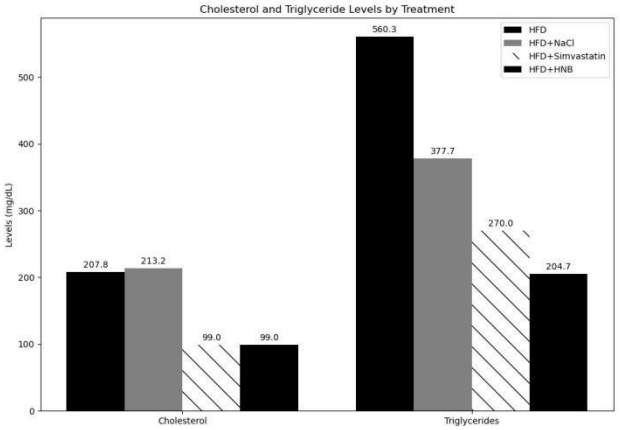

Figure 3. Lipid profile with various treatments. (HFD - high fat diet; NaCl - sodium chloride; HNB - hydrogen nanobubble).

We also performed histological analysis to see the status of steatosis in various treatments. Each group's fat droplet count was recorded, and the data's validity was ensured by accounting for missing values. The group receiving a high-fat diet without any treatment or receiving simvastatin had the maximum fat droplet counts; the presence of the hydrogen nanobubble yielded lower counts despite the high-fat diet. Obviously lower mean fat droplet counts were observed in the high-fat diet group treated with high-dose nanobubble when compared to the high-fat diet group treated with simvastatin or none. Subsequent statistical test revealed significant difference of the fat droplet counts among the groups.

($Kruskal - Wallis\ H$ = 36. 22, p <. 001); with Bayesian statistics (BFM= 3.9 X 10^{15}) indicates that this finding is backed up extremely strongly by the data.

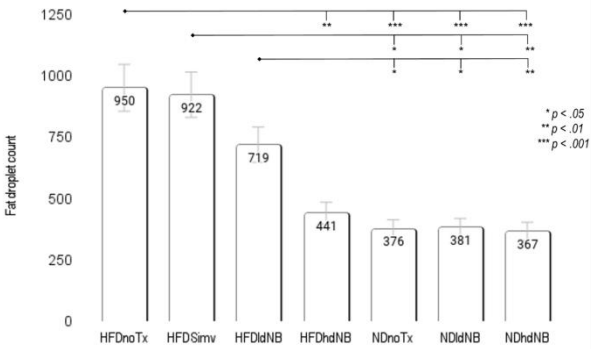

Figure 4. Fat droplet counts with various treatments. The fat droplets were counted on the HE-stained histological section at 40x magnification. Statistical difference between groups are stated as *p<0.05(*), p<0.01(**),* and *p<0.001(***)*. Abbreviations: HFD - high-fat diet; Simv - simvastatin; hd - high dose; ld - low dose; NB - hydrogen nanobubble; Tx treatment.

The figure 4 illustrates the fat droplet counts and how the groups differ with each other. Bayesian statistics indicates an extremely strong evidence supporting the fact that the high-fat diet group treated with high dose

nanobubble is indeed exhibiting lower hepatic fat droplet counts than if without any treatment ($BF10 = 236.68$).

Discussion

The survival rates observed in this study highlight significant differences between the control groups and various treatments administered to rats subjected to a high-fat diet (HFD). The purpose was to assess the impact of HNB treatment and the cholesterol-lowering drug simvastatin on survival rates under conditions of dietary stress. The high-fat diet alone, or in combination with NaCl or HNB, did not adversely affect the immediate survival of the rats. Conversely, the group treated with simvastatin exhibited a significantly lower survival rate of only 40%. This stark contrast underscores the potential adverse effects of simvastatin on the health and viability of rats, a well-established model for Duchenne Muscular Dystrophy (DMD). Research conducted by Mucha et al. (2021) and Verhaart et al. (2021) consistently demonstrated that simvastatin fails to yield any improvement in muscle function, histology, or gene expression associated with fibrosis, regeneration, and oxidative stress in this model. In stark contrast, simvastatin treatment has been associated with a detrimental increase in muscle tissue necrosis, failing to enhance muscle function, grip strength, or the specific force of single muscles.

Furthermore, Braga et al. (2018) uncovered that simvastatin significantly impairs muscle regeneration by inhibiting critical processes such as myoblast proliferation and myotube formation. Collectively, these findings robustly suggest that simvastatin treatment, especially in the context of a high-fat diet, can indeed exacerbate health conditions leading to a lowered survival rate in rat. The convergence of evidence across independent laboratories underscores the necessity of re-evaluating the use of simvastatin in models of muscular dystrophy, particularly concerning its potential to compromise survival by adversely affecting muscle health and regeneration.

The results demonstrate that dietary regimens and supplementation profoundly affect hematological parameters and organ health in the studied animal model. The decrease in WBC and lymphocyte counts in the A25 group suggests a suppressive effect on the immune system, potentially beneficial in conditions characterized by immune hyperactivity. Following nanobubble treatment with hydrogen, a significant decrease in WBC counts was observed, indicating a dose-dependent immunomodulatory effect (Hashemi et al., 2023; Nghia et al., 2022). This decrease in WBC counts was mirrored in the lymphocyte and monocyte populations, suggesting an altered immune response post-treatment (Youmin et al., 2022; Liu et al., 2022). The immunomodulatory effects of nanobubbles, particularly in the context of immune cell counts, highlight the potential of nanobubble technology to influence immune responses. The observed changes in WBC, lymphocyte, and monocyte counts post-nanobubble treatment underscore the intricate interplay between nanobubbles and the immune system, showcasing the promising immunomodulatory capabilities of nanobubble therapy using hydrogen (Karol et al, 2022).

The increase in RBC counts and hemoglobin levels in the same group may reflect an improved erythropoietic function or a response to increased physiological demand. The nanobubble treatments containing hydrogen influenced erythropoiesis by affecting red blood cell counts and hemoglobin levels. Studies on nano-encapsulated forms of erythropoietin showed enhanced erythropoietic activity with specific polymers like pectin (Khasenbekova et al., 2014). Additionally, nanobubbles containing hydrogen, oxygen, and nitrogen were found to activate mitochondria and promote cell growth, which could impact erythropoiesis (Kamei, 2019). Furthermore, research on oxygen nanobubbles demonstrated significant effects on tumor growth inhibition and epigenetic regulation, including hypermethylation of specific genes, which could influence erythropoiesis (Han, 2022). These findings collectively suggest that nanobubble treatments, particularly those containing hydrogen, have the potential to modulate erythropoiesis through various mechanisms, highlighting their impact on red blood cell production and hemoglobin levels.

The significant elevation in platelet counts in the A25 group could indicate a pro-thrombotic state or an adaptive response to inflammation. Elevated platelet counts have been linked to adverse outcomes in conditions like ischemic stroke when combined with high levels of C-reactive protein (HS-CRP) (Liu et al., 2023). In cancer patients, higher platelet levels before and after diagnosis were associated with poorer survival rates, suggesting a potential prognostic value (Vasiliy, 2022). Moreover, in individuals with comorbid Type 2 Diabetes Mellitus (T2DM) and Major Depressive Disorder (MDD), enhanced platelet hyperactivation and inflammation were observed, increasing the risk of vascular complications (Saharia et al., 2022). Additionally, in T2DM patients post-NSTE-ACS, platelet-dependent thrombus burden was associated with mortality, highlighting the interplay between thrombosis and inflammation (Viswanathan et al., 2022). Therefore, elevated platelet counts may indeed reflect a pro-thrombotic state and an adaptive response to inflammation in various clinical scenarios. However, the minimal changes in MPV and PDW suggest that while platelet production is affected, the size and variability of platelets remain stable. The study on nanobubble treatment involving hydrogen in rats demonstrated significant effects on platelet production (Karol et al., 2022; Li et al., 2018; Takeuchi et al., 2012). Specifically, the introduction of oxygen and air-nanobubble water led to notable enhancements in the weight and length of rats, indicating a positive impact on growth (Ebina et al., 2013). Moreover, hydrogen was found to inhibit collagen-induced platelet aggregation, suggesting a potential influence on platelet production (Bećirović, 2023). However, the study did not directly address minimal changes in mean platelet volume (MPV) and platelet distribution width (PDW) as indicators of platelet production. While the data supports the overall benefits

of nanobubble treatments on growth and platelet aggregation, further research specifically focusing on MPV and PDW alterations would be necessary to conclude their direct impact on platelet production.

The variations in liver enzyme levels across treatments highlight the potential hepatoprotective or hepatotoxic effects of the diets and treatments. Specifically, normalizing these enzymes in the A25 group may indicate a protective effect against diet-induced hepatic damage. Hydrogen nanobubbles have been found to possess anti-inflammatory properties and can improve hepatic inflammation and metabolic dysfunction, making them beneficial for chronic liver diseases (Liu et al., 2022). On the other hand, oxygen-sufficient nanobubbles have been designed to enhance sonodynamic therapy efficacy by providing oxygen to overcome the hypoxic tumor microenvironment, effectively inhibiting tumor growth (Tan et al., 2021). Additionally, nanobubbles loaded with a sono`sensitizer and a ferroptosis promoter have shown enhanced sonodynamic therapy and potent ferroptosis induction, offering a novel approach for treating hepatocellular carcinoma (Chen et al., 2022). These findings collectively suggest that hydrogen nanobubbles hold hepatoprotective potential by targeting inflammation, improving therapy efficacy, and inducing cell death in liver diseases.

The relative organ weight findings, particularly the increase in liver weight under high-fat conditions, underscore the metabolic challenges posed by such diets. The increase suggests lipid accumulation or liver hypertrophy, common responses to excessive dietary fat intake. Studies have shown that high-fat diets lead to hepatic lipid accumulation (Soares et al., 2018; Nagumalli et al., 2022), alterations in fatty acid composition (Honma et al., 2012), and increased susceptibility to hepatic steatosis and injury (Graham et al., 2023).

Furthermore, multi-generational exposure to high-fat diets has been linked to increased lipid accumulation in adipose tissue, elevated serum lipid and glucose levels, and changes in enzyme activities associated with fatty acid metabolism (Takasaki et al., 2012). These findings collectively highlight the detrimental effects of excessive dietary fat intake on lipid metabolism, liver health, and overall metabolic homeostasis in rats.

The lipid profile changes observed under different dietary conditions and treatments emphasize the importance of diet in managing lipid metabolism. The increased triglycerides in the HFD groups underscore the risk of dyslipidemia associated with high-fat diets. Hydrogen and oxygen nanobubble treatments have shown promising effects on lipid levels in rats. Studies have demonstrated that hydrogen nano-bubble water (HW) can significantly reduce lipid accumulation in adipocytes and adipose tissue equivalents, potentially inhibiting adipogenesis and inflammation associated with obesity-related metabolic disorders (Xiao & Miwa, 2021). On the other hand, oxygen nanobubbles have been explored for their impact on tumor growth in breast cancer-bearing rats, showing a significant decrease in tumor size and weight, along with alterations in gene expression related to tumor growth and angiogenesis (Black et al., 2012). These findings suggest that both hydrogen nanobubbles may have beneficial effects on lipid levels in rats, with hydrogen nanobubbles potentially targeting adipose tissue metabolism and oxygen nanobubbles affecting tumor growth and related gene expression. The effects of treatments on lipid levels, particularly the potential for certain treatments to mitigate these effects, warrant further investigation into their mechanisms of action and long-term implications on cardiovascular health.

High-fat diets affect the liver health directly, by inducing steatosis or fat deposition in the liver (fatty liver). Our study showed that the rats receiving high fat diet show the highest fat droplet counts. The administration of simvastatin did not change the situation, however, the administration of hydrogen nanobuble resulted in a substantial decrease in mean fat droplet count. These results imply that the intervention of nanobubble have a statistically significant effect on reducing fat droplet counts, particularly when compared to the high-fat diet groups without treatment. We included Bayesian statistical testings to measure the strength of the evidence supporting the finding. The presence of statistical significance, as well as the Bayes factor, indicates that these are not random variations but potentially meaningful differences attributable to the interventions applied.

Conclusion

The compilation of results presented in this study forms a robust matrix of evidence regarding the deleterious effects of a high-fat diet on rats, particularly when paired with certain treatments such as simvastatin. The high fat droplet counts and decreased survival rates in rats treated with simvastatin underscore the need for a careful re-evaluation of this treatment in the context of high-fat dietary conditions. On the contrary, the administration of hydrogen nanobubbles appears to attenuate the adverse effects typically associated with a high-fat diet, as evidenced by a substantial decrease in mean fat droplet counts and altered hematological and liver enzyme levels, suggesting a protective immunomodulatory and hepatoprotective role. Our findings, supported by Bayesian statistical testing, provide a compelling argument for the consideration of nanobubble technology as a potential therapeutic intervention in conditions exacerbated by high-fat diets. Future studies are warranted to further elucidate the underlying mechanisms and long-term impacts of nanobubble treatment in the modulation of diet-induced pathologies. The convergence of traditional statistical significance with Bayesian evidence strengths adds a rigorous dimension to our conclusions, reinforcing the validity of the observed differences as treatment-specific effects rather than mere statistical anomalies.

Acknowledgement

The authors gratefully acknowledge the financial support provided by the Reverse Aging & Homeostasis Club, which was instrumental in the completion of this research.

References

Admira P, Sayuti K. (2018). Xantelasma dan arkus kornea juvenilis sebagai manifestasi familial hypercholesterolemia. *Jurnal Kesehatan Andalas 7*: 125-130.

Akhigbe RE. 2014. (2014). Discordant Results in Plant Toxicity Studies in Africa: Attempt of Standardization. *In: Victor K. Toxicological Survey of African Medicinal Plants*. USA: Elsevier. p. 53-61

Bayly GR. (2014). Lipids and disorders of lipoprotein metabolism. *In: Clinical Biochemistry: Metabolic and Clinical Aspects. 3th ed.* USA: Elsevier.

Bećirović E, Ljuca K, Bećirović M, Ljuca N, Bajrić M, Brkić A, Ljuca F. (2023). Prognostic value of a decrease in mean platelet volume, platelet distribution width, and platelet-large cell ratio for major adverse cardiovascular events after myocardial infarction without ST-segment elevation: An observational study. *Biomolecules and Biomedicine 23*: 866.

Black KJ, Lock AT, Thomson LM, Cole AR, Tang X, Polizzotti BD, Kheir JN. (2017). Hemodynamic effects of lipid-based oxygen microbubbles via rapid intravenous injection in rodents. *Pharmaceutical Research 34:* 2156-2162.

Braga FJAF, Abreu AG, Rios CEP, Trovão LO, Silva DLF, Cysne DN, Nascimento JR, Fortes TS, Silva LA, Guerra RNM, Maciel MCG, Serezani CH, Nascimento FRF. (2018). Prophylactic treatment with simvastatin modulates the immune response and increases animal survival following lethal sepsis infection. *Frontiers in Immunology 9:* 390293.

Cai J, Kang Z, Liu WW, Luo X, Qiang S, Zhang JH, Ohta S, Sun X, Xu W, Tao H, Li R. (2008). Hydrogen therapy reduces apoptosis in neonatal hypoxia-ischemia rat model. *Neuroscience Letters 441:* 167–172.

Chen H, Mao X, Meng X, Li Y, Feng J, Zhang L, Zhang Y, Wang Y, Yu Y, Xie K. (2019). Hydrogen alleviates mitochondrial dysfunction and organ damage via autophagy-mediated NLRP3 inflammasome inactivation in sepsis. *International Journal of Molecular Medicine 44:* 1309–1324.

Chen Y, Shang H, Wang C, Zeng J, Zhang S, Wu B, Cheng W. (2022). RNA-seq explores the mechanism of oxygen-boosted sonodynamic therapy based on all-in-one nanobubbles to enhance ferroptosis for the treatment of HCC. *International Journal of Nanomedicine,* 105-123.

Dong A, Yu Y, Wang Y, Li C, Chen H, Bian Y, Zhang P, Zhao Y, Yu Y, Xie K. (2018). Protective effects of hydrogen gas against sepsis-induced acute lung injury via regulation of mitochondrial function and dynamics. *International Immunopharmacology 65:* 366–372.

Ebina K, Shi K, Hirao M, Hashimoto J, Kawato Y, Kaneshiro S, Morimoto T, Koizumi K, Yoshikawa H. (2013). Oxygen and air nanobubble water solution promote the growth of plants, fishes, and mice. *PLoS One 8*: e65339.

Ge L, Yang M, Yang NN, Yin XX, Song WG. (2017). Molecular hydrogen: a preventive and therapeutic medical gas for various diseases. *Oncotarget 8:* 102653-102673

Golomb BA, Evans MA. (2008). Statin adverse effects: a review of the literature and evidence for a mitochondrial mechanism. *American journal of cardiovascular drugs: drugs, devices, and other interventions 8:* 373–418.

Graham DS, Liu G, Arasteh A, Yin XM, Yan S. (2023). Ability of high fat diet to induce liver pathology correlates with the level of linoleic acid and Vitamin E in the diet. *PLoS One 18*: e0286726.

Han X, Ji P. (2022). Carbon dots for the treatment of cancer-related anemia. *Blood Science 4:* 174-175.

Hashemi S, Aghamiri SM, Siavashpour Z, Kahani M, Zaidi H, Jaberi R. (2023). Hydrogen nanobubbles: A novel approach toward radio-sensitization agents. *Medical Physics 50:* 6589–6599.

Hong Y, Shao A, Wang J, Chen S, Wu H, McBride DW, Wu Q, Sun X, Zhang J. (2014). Neuroprotective effect of hydrogen-rich saline against neurologic damage and apoptosis in early brain injury following subarachnoid hemorrhage: possible role of the Akt/GSK3β signaling pathway. *PloS One 9*: e96212.

Honma T, Shinohara N, Ito J, Kijima R, Sugawara S, Arai T, Tsuduki T, Ikeda I. (2012). High-fat diet intake accelerates aging, increases expression of Hsd11b1, and promotes lipid accumulation in liver of SAMP10 mouse. *Biogerontology 13:* 93-103.

Ibrahim MA, Asuka E, Jialal I. (2023). Hypercholesterolemia.In: Stat Pearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK459188

Iida A, Nosaka N, Yumoto T, Knaup E, Naito H, Nishiyama C, Yamakawa Y, Tsukahara K, Terado M, Sato K, Ugawa T, Nakao A. (2016). *The clinical application of hydrogen as a medical treatment. Acta medica Okayama 70:* 331–337.

Iketani M, Sekimoto K, Igarashi T, Takahashi M, Komatsu M, Sakane I, Takahashi H, Kawaguchi H, Ohtani-Kaneko R, Ohsawa I. (2018). Administration of hydrogen-rich water prevents vascular aging of the aorta in LDL receptor-deficient mice. *Scientific Reports 8:* 16822.

Kamei I. (2019). U.S. Patent Application No. 16/161,192.

Kawamura T, Huang CS, Tochigi N, Lee S, Shigemura N, Billiar TR, Okumura M, Nakao A, Toyoda Y. (2010). Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats. *Transplantation 90:*1344–1351.

Kato S, Matsuoka D, Miwa N. (2015). Antioxidant activities of nano-bubble hydrogen-dissolved water assessed by ESR and 2,2'-bipyridyl methods. *Materials science & engineering. C, Materials for biological applications 53:* 7–10.

Khasenbekova Z, Gulayev A, Nechayeva E, Nurgozhin T, Zhumadilov Z. (2014). Study of the erythropoiesis activity of nano-encapsulated forms of erythropoietin. *Central Asian Journal of Global Health 2:*110.

Li C, Cao Y, Kohei F, Hao H, Peng G, Cheng C, Ye J. (2022). Nano-bubble hydrogen water: An effective therapeutic agent against inflammation-related disease caused by viral infection in zebrafish model. *Virologica Sinica 37:* 277–283.

Li M, Liu Y, Chen J, Liu T, Gu Z, Zhang J, Gu X, Teng G, Yang F, Gu N. (2018). Platelet bio-nanobubbles as microvascular recanalization nanoformulation for acute ischemic stroke lesion theranostics. *Theranostics 8:* 4870-4883.

Li S, Liao R, Sheng X, Luo X, Zhang X, Wen X, Zhou J, Peng K. (2019). Hydrogen gas in cancer treatment. *Frontiers in Oncology 9:* 696.

Li Y, Li Q, Chen H, Wang T, Liu L, Wang G, Xie K, Yu Y. (2015). Hydrogen gas alleviates the intestinal injury caused by severe sepsis in mice by increasing the expression of heme oxygenase-1. *Shock (Augusta, Ga.) 44:* 90–98.

Liu F, Yang P, Wang Y, Shi M, Wang R, Xu Q, Peng Y, Chen J, Zhang J, Wang A, Xu T, Zhang Y, He J. (2023). HS-CRP modifies the prognostic value of platelet count for clinical outcomes after ischemic stroke. *Journal of the American Heart Association 12:* e030007.

Liu Y, Yang S, Zhou Q, Zhou J, Li J, Ma Y, Hu B, Liu C, Zhao Y. (2022). Nanobubble-based anti-hepatocellular carcinoma therapy combining immune check inhibitors and sonodynamic therapy. *Nanoscale Advances 4:* 4847-4862.

Meng X, Chen H, Wang G, Yu Y, Xie K. (2015). Hydrogen-rich saline attenuates chemotherapy-induced ovarian injury via regulation of oxidative stress. *Experimental and Therapeutic Medicine 10:* 2277–2282.

Mucha O, Podkalicka P, Kaziród K, Samborowska E, Dulak J, Łoboda A. (2021). Simvastatin does not alleviate muscle pathology in a mouse model of Duchenne muscular dystrophy. *Skeletal Muscle 11:* 1-16.

Nakao A, Toyoda Y, Sharma P, Evans M, Guthrie N. (2010). Effectiveness of hydrogen-rich water on antioxidant status of subjects with potential metabolic syndrome-an open label pilot study. *Journal of Clinical Biochemistry and Nutrition 46:* 140–149.

Nghia NH, Nguyen NT, Binh PT, May LT, Huy TT, Giang PT, St-Hilaire S, Van PT (2022). Effect of nanobubbles (oxygen, ozone) on the Pacific white shrimp (Penaeus vannamei), Vibrio

parahaemolyticus, and water quality under lab conditions. *Fisheries and Aquatic Sciences 25:*429-440

Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. (2007). Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nature Medicine 13:* 688–694.

Ohta, S. (2015). Molecular hydrogen as a novel antioxidant: overview of the advantages of hydrogen for medical applications. *Methods in enzymology 555:* 289–317.

Ono H, Nishijima Y, Adachi N, Tachibana S, Chitoku S, Mukaihara S, Sakamoto M, Kudo Y, Nakazawa J, Kaneko K, Nawashiro H. (2011). Improved brain MRI indices in the acute brain stem infarct sites treated with hydroxyl radical scavengers, Edaravone and hydrogen, as compared to Edaravone alone. A non-controlled study. *Medical Gas Research 1:*12.

Perveen I, Bukhari B, Najeeb M, Nazir S, Faridi TA, Farooq M, Ahmad QU, Abusalah MAHA, ALjaraedah TY, Alraei WY, Rabaan AA, Singh KKB, Abusalah MAHA. (2023). Hydrogen therapy and its future prospects for ameliorating COVID-19: Clinical applications, efficacy, and modality. *Biomedicines 11:* 1892.

Ramanathan D, Huang L, Wilson T, Boling W. (2023). Molecular hydrogen therapy for neurological diseases: a review of current evidence. *Medical Gas Research 13:* 94–98.

Saengsin K, Sittiwangkul R, Chattipakorn SC, Chattipakorn N. (2023). Hydrogen therapy as a potential therapeutic intervention in heart disease: from the past evidence to future application. *Cellular and molecular life sciences 80:* 174.

Shao A, Wu H, Hong Y, Tu S, Sun X, Wu Q, Zhao Q, Zhang J, Sheng J. (2016). Hydrogen-Rich Saline Attenuated Subarachnoid Hemorrhage-Induced Early Brain Injury in Rats by Suppressing Inflammatory Response: Possible Involvement of NF-κB Pathway and NLRP3 Inflammasome. *Molecular neurobiology 53:*3462–3476.

Song G, Li M, Sang H, Zhang L, Li X, Yao S, Yu Y, Zong C, Xue Y, Qin S. (2013). Hydrogen-rich water decreases serum LDL-cholesterol levels and improves HDL function in patients with potential metabolic syndrome. *Journal of Lipid Research, 54:* 1884–1893.

Suckling K. (2014). Atherosclerosis. *Reference Module in Biomedical Science*. USA: Elsevier

Tanaka Y, Xiao L, Miwa N. (2022). Hydrogen-rich bath with nano-sized bubbles improves antioxidant capacity based on oxygen radical absorbing and inflammation levels in human serum. *Medical Gas Research 12*: 91–99.

Tian Y, Zhang Y, Wang Y, Chen Y, Fan W, Zhou J, Qiao J, Wei Y. (2021). Hydrogen, a novel therapeutic molecule, regulates oxidative stress, inflammation, and apoptosis. *Frontiers in Physiology 12*: 789507.

Takeuchi S, Wada K, Nagatani K, Osada H, Otani N, Nawashiro H. (2012). Hydrogen may inhibit collagen-induced platelet aggregation: an ex vivo and in vivo study. *Internal Medicine 51*: 1309-1313.

Verhaart IEC, Cappellari O, Tanganyika-de Winter CL, Plomp JJ, Nnorom S, Wells KE, Hildyard JCW, Bull D, Aartsma-Rus A, Wells DJ. (2021). Simvastatin treatment does not ameliorate muscle pathophysiology in a mouse model for Duchenne muscular dystrophy. *Journal of Neuromuscular Diseases 8*: 845-863.

Viswanathan GN, Harper AR, Badimon JJ, Marshall SM, Zaman AG. (2022). Platelet dependent thrombogenicity, inflammation and five year mortality in patients with non-st elevation acute coronary syndrome and type 2 diabetes mellitus: An observational study. MedRxiv 2022.03.04.22271893.

WHO (2021). Cardiovascular diseases (CVDs). https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds). Accessed 16 October 2023

Xiao L, Miwa N. (2021). Hydrogen nano-bubble water suppresses ROS generation, adipogenesis, and interleukin-6 secretion in hydrogen-peroxide-or PMA-stimulated adipocytes and three-dimensional subcutaneous adipose equivalents. *Cells 10*: 626.

Zaleski AL, Taylor BA, Thompson PD. (2018). Coenzyme Q10 as Treatment for Statin-Associated Muscle Symptoms-A Good Idea, but…. *Advances in nutrition (Bethesda, Md.) 9*: 519S–523S.

Zhang Y, Fan W, Li X, Wang WX, Liu S. (2022). Enhanced removal of free radicals by aqueous hydrogen nanobubbles and their role in oxidative stress. *Environmental Science & Technology 56:*15096-15107.