



Article

Subchronic Toxicity Study of Nitric Oxide Nanobubble Injection in Sprague Dawley Rats

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Abstract

Objective: The development of nanobubble technology has expanded to various gas molecules, such as nitric oxide (NO). NO donors have been frequently used for both therapeutic and diagnostic purposes. However, the use of NO in nanoformulations has received little attention thus far. This research aimed to assess the subchronic toxicity of NO nanobubbles in a preclinical study design. **Methods:** Ninety days of subchronic toxicity testing based on modified OECD guidelines in Sprague Dawley rats was used in this study to assess potential adverse effects of intravenous administration of NO nanobubbles (NONBs). Hematological factors, serum biochemistry, and histology of the liver, kidney, heart, lung, and spleen were investigated. **Results:** The intravenous NONB injection trial using graded doses of 0.01 mL, 0.04 mL, and 0.06 mL resulted in no deaths during the 90-day treatment period. However, liver disturbance and electrolyte imbalance emerged. This condition was supported by histological findings in the liver and kidney, which showed potential reversible damage, and persistent hemorrhage of the spleen. **Conclusions:** Intravenous administration of NONBs at a dose of up to 0.06 mL in this subchronic toxicity test remains safe. Further studies with adjustments to the test formulation are highly encouraged before the clinical trial stage in humans.

Keywords: nitric oxide; nanobubble; toxicity study; nanotechnology

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1. Introduction

Nanobubble technology has been extensively developed for medical and pharmaceutical applications. The existing research demonstrates promising results, especially regarding its stability and permeability [1,2]. These bubbles, due to their small size, can pass through cell membranes more easily, making them suitable for targeted therapies [3]. Hydrogen nanobubble technology is an example of cutting-edge innovation that harnesses hydrogen at the nanoscale. These tiny bubbles possess unique properties that make them

highly effective across a range of applications, from energy storage to medical treatments [4]. This technology enables the distribution of hydrogen to multiple systems with greater stability and efficiency by encapsulating the gas in tiny bubbles [5].

Building on the success of hydrogen nanobubble technology, development has expanded to similar products using other gases, including nitric oxide (NO). NO is a simple gas composed of two atomic elements: oxygen (O) and nitrogen (N). It is colorless and odorless, dissolving rapidly in both organic and aqueous solvents. The enzyme nitric oxide synthase (NOS) catalyzes the production of NO in various organisms, including bacteria, fungi, plants, and animals, as part of their cellular metabolic processes [6].

NO donors were commonly used in earlier research for both medicinal and diagnostic purposes. Microbubbles containing NO have been shown to enhance vasodilation, decrease platelet aggregation, and lower the likelihood of inflammatory cell infiltration and thrombus formation. Myocardial infarction and stroke management and prevention greatly benefit from this ability [7–9]. However, the application of NO in nanoformulations has not been extensively studied to date. Thus, this work used subchronic toxicity testing in Sprague Dawley rats to evaluate the potential health risks associated with the injection of NO nanobubbles (NONBs).

2. Materials and Methods

2.1. Injection Material

The injection material was obtained from the Indonesia Molecular Innovation (IMI) Foundation as an infusion fluid containing NONBs. The NONB solution was prepared in reverse osmosis water. A buffer containing 1% ascorbic acid and 1% sodium bicarbonate was used to maintain pH stability. All materials were prepared according to laboratory protocols, and all preparations were conducted aseptically to minimize contamination, as previously done for generating hydrogen nanobubbles [10]. Nanoparticle tracking analysis (NTA) was performed using Nanosight Pro (Malvern Panalytical, Malvern, UK).

2.2. Animal Material

This study was conducted on male and female Sprague Dawley rats aged 8–12 weeks and weighing 150–250 g at the start of the experiment. Female (nulliparous and not pregnant) and male rats were separated in cages during the study. The animals were acclimated for one week before the start of the experiment. The animals had free access to tap water for drinking and to standard laboratory feed. The rats were housed in groups of 6 per cage at room temperature (25 ± 3 °C), with sufficient ventilation and a natural light cycle (12 h/12 h). All procedures and protocols involving animals and their care were conducted in accordance with the guidelines of the Research Laboratory of Faculty of Medicine, Universitas Jenderal Soedirman and were approved by the Ethics Committee of the Faculty of Medicine, Universitas Jenderal Soedirman (065/KEPK/PE/VIII/2024).

2.3. Evaluation of Subchronic Toxicity

The subchronic toxicity of NONB injection was evaluated according to the modified OECD guideline 408 [11]. Six groups of 12 animals each (6 males and 6 females) were used for each dose. Group 1 served as a healthy control, and groups 2, 3, and 4 received intravenous NONB injections into the tail vein (0.01 mL, 0.04 mL, and 0.06 mL, respectively). Group 5 was a healthy satellite control. Group 6 was the satellite group, which received an intravenous injection of 0.06 mL NONBs. Those two groups were observed for an additional four weeks after the other groups. During treatment (90 days), the animals' body weights were assessed weekly, and signs of toxicity were carefully examined.

2.4. Blood and Organ Collection

Blood samples were collected once at the end of the experiment, just before termination. The animals' blood samples were collected after they had fasted overnight (10 h). They were extracted from the retro-orbital vein and stored in non-EDTA tubes for biochemical analysis and in EDTA tubes for hematological studies. Then, the rats were euthanized immediately. After dissecting the rats, organs such as the heart, liver, lungs, kidneys, and spleen were removed, cleaned in saline (0.9%), blotted on absorbent paper, and weighed to determine relative organ weight. The organs were then fixed in buffered formalin for histological examination.

2.5. Blood Analysis

White blood cells (WBCs), red blood cells (RBCs), lymphocytes (LYM), monocytes (MON), neutrophils (NEUT), eosinophils (EO), basophils (BASO), hemoglobin (HB), hematocrit (HCT), and platelet count (PLT) were all measured using an automated hematology analyzer (ABX Micros Horiba, Japan). Serum biochemical analysis was performed for glucose, total cholesterol, triglycerides, ALT, AST, urea, creatinine, sodium, and potassium using an automated biochemistry analyzer (KENZA MAX Biochemistry analyzer, manufactured by BIOLABO, Maizy, France).

2.6. Histological Analysis

The sample organs were dried and fixed in paraffin for microscopic inspection using standard pathology laboratory techniques. Histological investigation was performed using paraffin sections stained with hematoxylin and eosin (H&E). Histological scoring was performed in 10 fields of view per organ sample at 100× magnification. Liver histology was assessed according to the following criteria: normal liver (score 1); focal hydropic/fatty degeneration/necrosis (score 2); multifocal hydropic/fatty degeneration/necrosis (score 3); and diffuse hydropic/fatty degeneration/necrosis throughout the entire field of view (score 4). Kidney histology was assessed according to the following criteria: normal kidney (score 1); lesions such as nuclear necrosis, tubular degeneration, and proximal tubular dilation found in some fields of view (score 2); and lesions such as nuclear necrosis, tubular degeneration, and proximal tubular dilation found in every field of view (score 3). Heart histology was assessed according to the following criteria: normal heart (score 1); mild lesions (inflammation/fibrosis/vacuolization < 3 foci) (score 2); moderate lesions (inflammation/fibrosis/vacuolization > 3 foci) (score 3); and severe lesions (diffuse inflammation/fibrosis/vacuolization) (score 4). Lung histology was assessed based on the following criteria: normal lung (score 1); alveolar wall thickening without lung structure damage (score 2); alveolar wall thickening/fibrosis with lung architecture damage (score 3); extensive fibrosis with a honeycomb appearance (score 4); and total lung damage (score 5). Spleen histology was evaluated according to the following criteria: normal spleen (score 1); focal hemorrhage/necrosis (score 2); multifocal hemorrhage/necrosis (score 3); and diffuse hemorrhage/necrosis (score 4).

2.7. Statistical Analysis

Continuous data are presented as means $\pm$ SD, while categorical data are presented as numbers (percentages). ANOVA and Kruskal–Wallis tests followed by appropriate post hoc tests were used in the statistical analysis. A *p*-value < 0.05 indicates a significant difference in the variable's mean.

3. Results and Discussion

3.1. Particle Analysis of NONBs

Based on NTA analysis (Figure 1), NONB samples showed a particle concentration of 2.44×10^7 particles/mL. The particle size distribution showed a mode of 95 nm, a median (D50) of 126 nm, and a mean of 215 nm, with a standard deviation of 247 nm. Most particles were in the nanometer range, particularly around 72–126 nm. However, the distribution was broad, with a fraction of larger particles up to D90 of 458 nm, indicating that the sample was polydispersed.

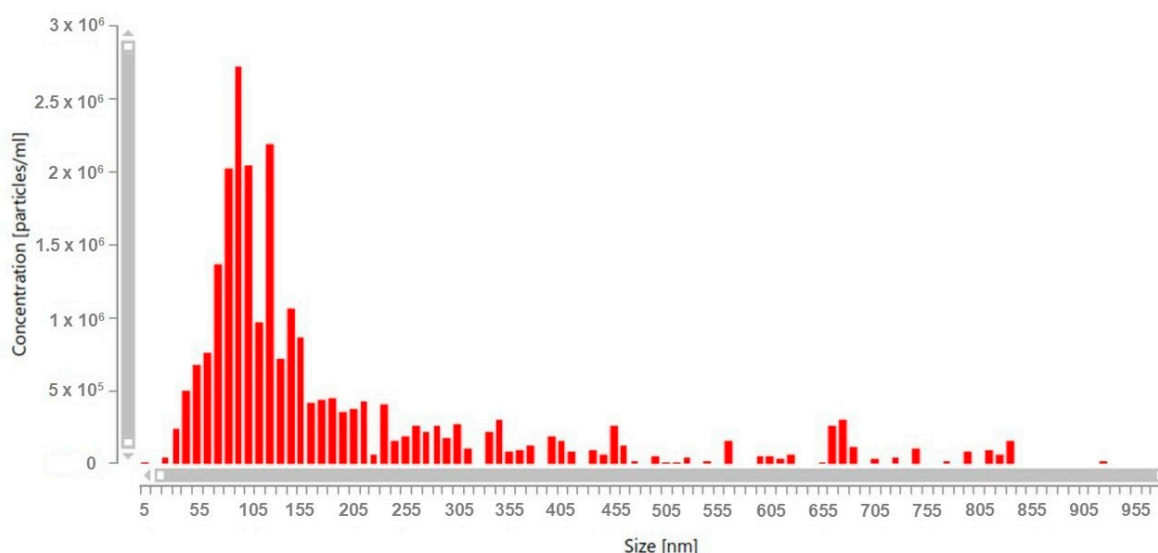


Figure 1. Concentration and distribution of NONBs.

3.2. Effects of NONBs on the Morbidity, Mortality, Body Weight, and Relative Organ Weight of Rats

Nitric oxide (NO) is an endogenous free radical and a signaling molecule involved in various roles in the nervous, cardiovascular, reproductive, and immune systems [12]. Because of its relative lipophilicity, neutral charge, and small size, NO can readily diffuse through the cell membrane without the need for receptors or channels [13]. NO is known for its paracrine effects on the vascular wall and for its short biological half-life of 1–10 s, with a short diffusion distance of 50–1000 μ m [14]. Relevant studies have demonstrated the unique biological activities and chemical properties of specific gaseous signaling molecules in the human body, including NO, carbon monoxide, and hydrogen sulfide [15]. Among the many gaseous molecular transmitters, NO exhibits highly prominent physiological and pathological effects in studies on various cells [16]. Besides its important role in various physiological regulatory systems, many studies have confirmed the close link between NO and the incidence and pathogenesis of multiple diseases [17]. Therefore, the use of NO for treatment has attracted widespread attention from scientists.

Researchers have attempted to develop NO donor delivery systems, including nanodelivery systems, which are expected to overcome NO's short half-life [18], including those from IMI. IMI is developing NO nanobubble (NONB) technology that enables the encapsulation and controlled release of NO. This technology is expected to be an innovative solution for NO delivery, improving efficacy while minimizing side effects. Based on the observations made, the NONB preparation administered at various doses did not cause the death of the experimental rats during the 90-day treatment period. The rats' activity was also monitored and was normal, with no physical changes, signs, or symptoms of illness. Body weight increased proportionally with age (Figure 2), and the average

weights of the liver, kidneys, heart, lungs, and spleen at the end of the study were also proportional to body weight (Table 1). This indicates that the highest dose administered, 0.06 mL of NONBs per day for 90 days, is still far below the lethal dose 50 (LD50).

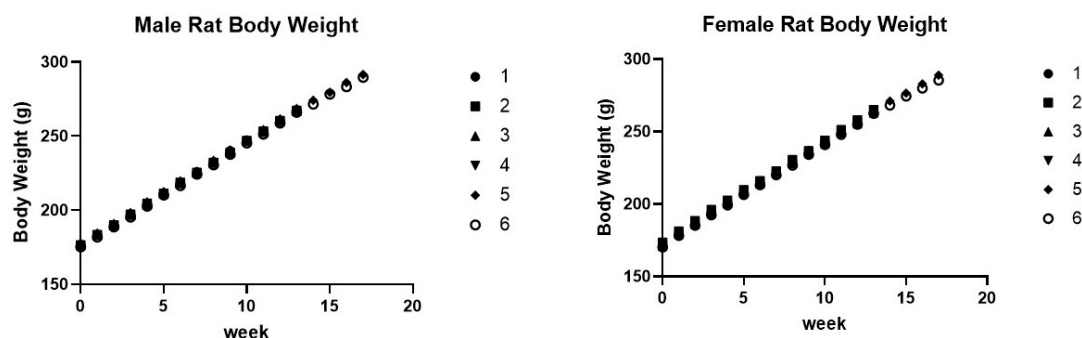


Figure 2. Body weight increased normally with age in male and female rats. The average weight of the rats at the beginning of the study was approximately 180 g, whereas by the end it had risen to nearly 290 g.

Table 1. Relative organ weights of rats.

Groups	Organ Weight (g)				
	Liver	Kidney	Heart	Lung	Spleen
Male					
1	10.71	2.41	1.21	1.87	0.94
2	10.67	2.41	1.2	1.87	0.93
3	10.75	2.42	1.21	1.88	0.94
4	10.69	2.41	1.2	1.87	0.94
5	11.66	2.41	1.21	1.88	0.94
6	11.59	2.39	1.2	1.86	0.93
Female					
1	10.52	2.37	1.18	1.84	0.92
2	10.61	2.39	1.19	1.86	0.93
3	10.53	2.37	1.18	1.84	0.92
4	10.52	2.37	1.18	1.84	0.92
5	11.56	2.6	1.3	2.02	1.01
6	11.42	2.57	1.29	1.99	0.99

3.3. Effects of NONBs on Hematological Parameters

The effects of NONBs on some of the hematological parameters are recorded in Table 2 (male rats) and Table 3 (female rats). Data in those tables show that NONB administration did not affect blood parameters in male or female rats. NO is an important regulator of hematology. It acts as a vasodilator, prevents platelet aggregation, and modifies erythrocyte function [19]. Hemoglobin (Hb) carries NO, which dilates the microvasculature, boosting local blood flow and oxygen delivery. NO bioactivity is maintained in red blood cells by its conjugation with Cys thiols in Hb, even though free NO cannot persist in significant quantities in the blood due to its quick sequestration by hemes and elimination in interactions with oxygenated Hb [20]. Nanoparticles can be assembled to control drug release precisely, and they exhibit good biocompatibility. Previous studies have shown the positive effects of NO nanoparticles on cardiovascular diseases and aortic endothelial cells [21]. In this study, NONBs maintained blood homeostasis, as indicated by normal hematological parameters.

Table 2. Hematological parameters of male rats.

Parameters	Groups						<i>p</i>
	1	2	3	4	5	6	
Number of erythrocytes (10 ⁶ /mm ³)	8.39 ± 0.37	8.50 ± 0.46	8.04 ± 0.30	8.00 ± 0.18	8.21 ± 0.31	8.66 ± 0.45	0.74
Hematocrit (%)	41.71 ± 1.48	43.16 ± 2.03	47.49 ± 1.98	41.77 ± 2.19	43.12 ± 1.38	43.93 ± 2.62	0.36
Hemoglobin (g/dL)	15.55 ± 0.45	15.18 ± 0.37	15.68 ± 0.28	15.71 ± 0.30	14.87 ± 0.33	15.57 ± 0.32	0.44
Number of leukocytes (10 ³ /mm ³)	11.41 ± 1.91	14.51 ± 1.42	12.04 ± 1.69	12.14 ± 1.77	12.38 ± 1.57	13.25 ± 1.74	0.82
MCH (pg)	17.18 ± 0.38	18.01 ± 0.59	18.27 ± 0.35	17.73 ± 0.46	17.38 ± 0.47	17.98 ± 0.52	0.57
MCHC (g/dL)	34.72 ± 0.97	34.66 ± 0.89	35.48 ± 0.72	35.03 ± 1.04	32.81 ± 0.85	34.58 ± 0.64	0.52
MCV (fl)	54.49 ± 1.09	50.84 ± 1.87	54.73 ± 0.99	50.11 ± 1.71	55.31 ± 2.45	54.73 ± 1.91	0.22
Methemoglobin (% Hb)	0.87 ± 0.98	0.79 ± 0.11	0.89 ± 0.76	0.99 ± 0.06	0.96 ± 0.63	0.68 ± 0.74	0.12
Number of platelets (10 ³ /mm ³)	941.50 ± 50.94	955.83 ± 46.71	1042.67 ± 50.61	994.50 ± 137.51	998.00 ± 56.74	970.00 ± 36.57	0.33

Table 3. Hematological parameters of female rats.

Parameters	Groups						<i>p</i>
	1	2	3	4	5	6	
Number of erythrocytes (10 ⁶ /mm ³)	7.81 ± 0.23	7.63 ± 0.34	7.39 ± 0.27	8.29 ± 0.12	7.65 ± 0.22	7.48 ± 1.60	0.132
Hematocrit (%)	45.33 ± 2.03	44.85 ± 1.98	44.58 ± 1.51	44.57 ± 1.36	45.28 ± 1.70	43.35 ± 2.15	0.975
Hemoglobin (g/dL)	15.34 ± 0.35	15.40 ± 0.27	15.44 ± 0.41	15.88 ± 0.30	15.16 ± 0.23	15.31 ± 0.37	0.731
Number of leukocytes (10 ³ /mm ³)	9.36 ± 1.05	9.47 ± 1.18	10.72 ± 1.25	10.20 ± 0.95	9.74 ± 1.32	9.24 ± 1.03	0.937
MCH (pg)	55.92 ± 1.20	54.38 ± 1.14	55.67 ± 0.54	55.03 ± 1.06	55.88 ± 0.45	54.3.0 ± 1.31	0.750
MCHC (g/dL)	17.98 ± 0.48	17.99 ± 0.55	17.48 ± 0.46	17.95 ± 0.57	17.91 ± 0.52	17.78 ± 0.39	0.976
MCV (fl)	34.43 ± 0.99	34.65 ± 0.98	33.64 ± 0.85	34.09 ± 0.98	33.82 ± 0.99	35.15 ± 0.86	0.876
Methemoglobin (% Hb)	0.91 ± 0.94	0.67 ± 0.12	0.60 ± 0.03	0.59 ± 0.72	0.72 ± 0.12	0.86 ± 0.13	0.140
Number of Platelets (10 ³ /mm ³)	1083.67 ± 42.61	1032.67 ± 59.14	994.50 ± 35.45	981.83 ± 33.80	1042.33 ± 44.89	977.50 ± 51.96	0.537

3.4. Effects of NONBs on Biochemical Parameters

The effects of NONBs on some of the serum biochemical parameters are summarized in Figure 3 (male rats) and Figure 4 (female rats). Blood glucose levels, total cholesterol, triglycerides, AST, ALT, urea, creatinine, and blood potassium levels in both male and female rats rose as the test preparation dosage increased. Nevertheless, blood sodium levels decreased and were inversely correlated with the test preparation's dosage. In both male and female rat groups, bivariate analysis revealed significant differences across all parameters except urea levels ($p < 0.05$). Detailed statistical analysis of serum biochemical parameters is provided in Appendix A.

The blood glucose levels of rats increased linearly with increasing doses of the test preparation in both male and female rats. Significant differences in blood glucose levels were found in male rats in group 1 compared to groups 4 and 6; group 2 compared to groups 4 and 6; and group 5 compared to groups 4 and 6 (Figure 3a). In female rats, significant differences in blood glucose levels were observed between groups 1, 2, and 5 compared with group 6 and between group 4 and group 5 (Figure 4a). The highest blood glucose level in the male rat group was 76.25 ± 1.11 mg/dL, while in the female rat group, it

was 73.48 ± 2.47 mg/dL. These values are still within the normal range for blood glucose in both sexes [22].

The blood glucose levels of male and female rats in this study increased in direct proportion to the increase in the dose of NONBs injected. This result is consistent with previous research showing that NO donors efficiently increase blood glucose levels that had been effectively decreased by nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor [23]. On the other hand, previous studies have shown the opposite effect of NO on glucose metabolism. NO is the primary regulator of insulin secretion by the Langerhans islets, playing an important role in biphasic insulin secretion and exerting an insulin-stimulating effect [24]. NO is involved in carbohydrate metabolism, and inhibition of the NO pathway leads to insulin resistance and type 2 diabetes mellitus [25].

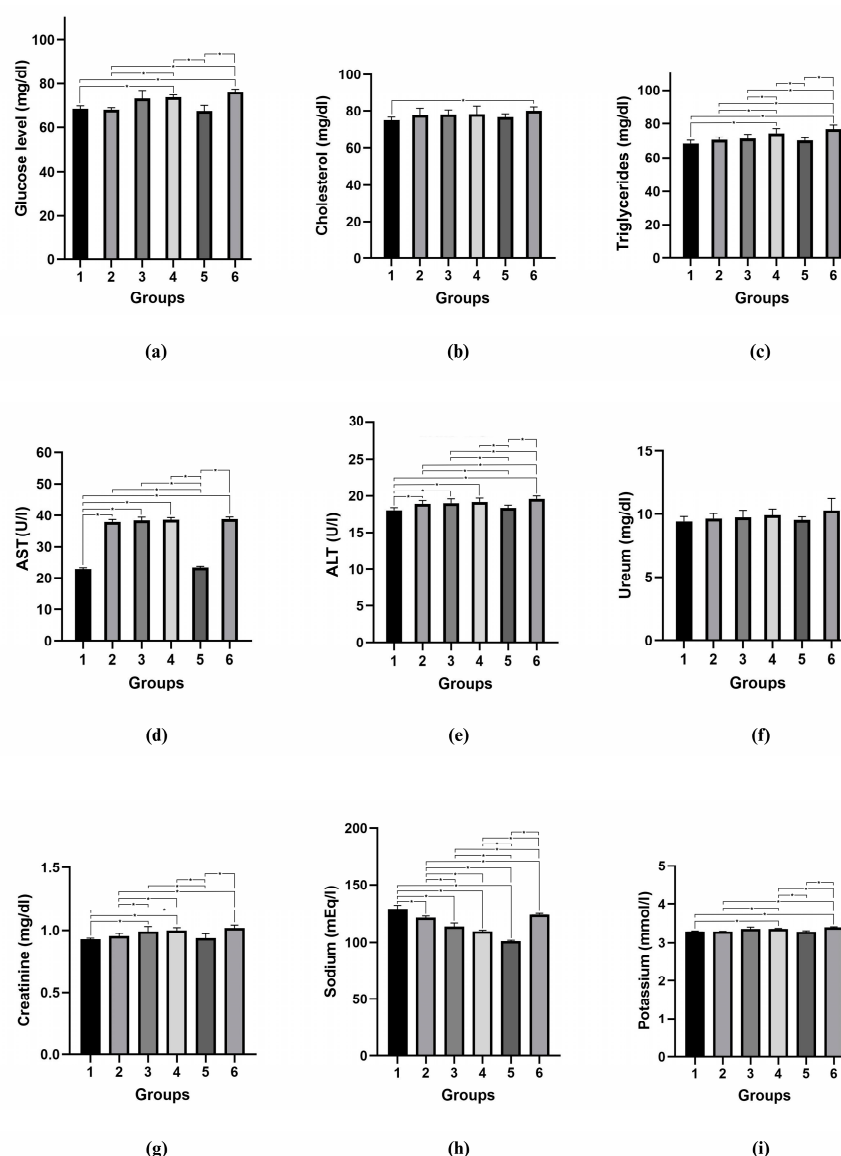


Figure 3. Results of male rat serum biochemistry between groups. (a) glucose level; (b) cholesterol level; (c) triglycerides level; (d) AST level; (e) ALT level; (f) urea level; (g) creatinine level; (h) sodium level; (i) potassium level. (*—statistically significant).

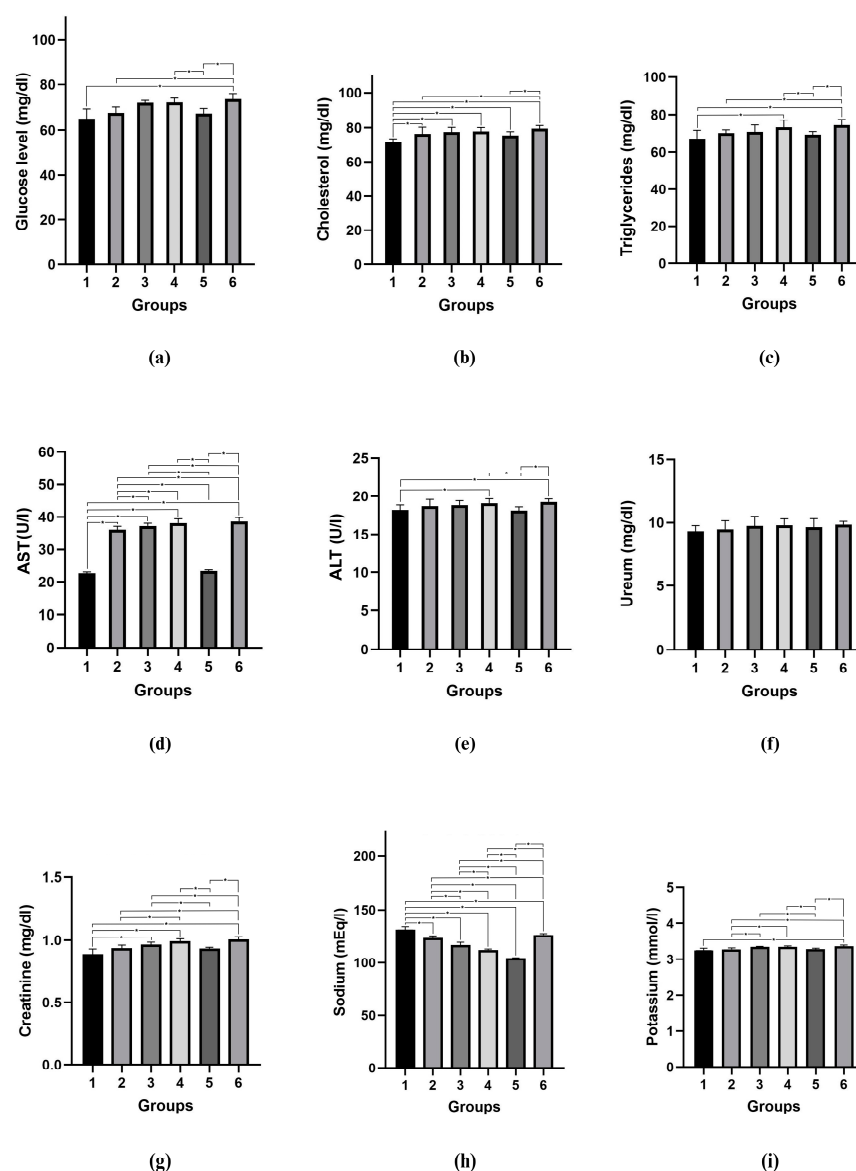


Figure 4. Results of female rat serum biochemistry between groups. (a) glucose level; (b) cholesterol level; (c) triglycerides level; (d) AST level; (e) ALT level; (f) urea level; (g) creatinine level; (h) sodium level; (i) potassium level. (*—statistically significant).

Controversy concerning the role of NO in glucose metabolism may arise from its dual effects on glucose-stimulated insulin secretion. NO can act as both a regulator and a potential disruptor of glucose metabolism, depending on the concentration of exposure. Low-to-moderate concentrations can increase glucose uptake and utilization. In contrast, high concentrations can inhibit glucose oxidation, reduce insulin secretion, and decrease glucose uptake [24–26], as evidenced by elevated blood glucose levels. In this study, the increase in blood glucose levels in both male and female rats following NO injection did not return to baseline within 4 weeks of the last injection. Instead, there was a slight, non-significant increase. These findings indicate that the effect of the highest NO injection dose (0.06 mL) persisted even after exposure has been discontinued. However, in general, intravenous NO injection in this study was relatively safe for glucose metabolism, as blood glucose levels in all experimental rats remained within normal limits by the end of the study.

The total cholesterol levels of the rats increased linearly with increasing doses of the test preparation in both male and female rats. Significant differences in cholesterol levels

were observed between groups 1 and 6 in male rats (Figure 3b). In female rats, significant differences were observed between group 1 and groups 2–6 and between group 6 and groups 2 and 5 (Figure 4b). The highest total cholesterol level in the male rat group was 80.05 ± 0.87 mg/dL, while in the female rat group, it was 79.54 ± 1.79 mg/dL. These values are still within the normal range for total cholesterol in both male and female rats [22,27].

Similarly to total cholesterol levels, triglyceride levels in male and female rats also increased in direct proportion to the increase in the dose of the test preparation. In male rats, significant differences were found between group 1 and groups 4 and 6; between group 2 and groups 4 and 6; between group 3 and groups 4 and 6; and between group 5 and groups 4 and 6 (Figure 3c). In female rats, significant differences were found between group 1 and groups 4 and 6; between group 2 and group 6; and between group 5 and groups 4 and 6 (Figure 4c). The highest triglyceride level in the male rat group was 76.75 ± 2.38 mg/dL, while in the female rat group, it was 74.54 ± 0.09 mg/dL. These values were within the normal range for rat triglycerides in both sexes [27].

Total cholesterol and triglyceride levels in both male and female rats also increased in this study, in direct proportion to the increase in NONB dose. This condition differs from previous research findings that demonstrated the important role of NO in lipid metabolism regulation. NO has a beneficial effect on lipid metabolism by activating sterol regulatory element-binding protein (SREBP)-2, and reduced NO bioavailability is associated with impaired lipid metabolism, increased levels of cholesterol, triglycerides, and LDL, and decreased levels of HDL [28–30]. However, in vitro studies on AML-12 hepatocytes exposed to NO donors support our findings. Exposure of hepatocytes to NO donors increases lipid accumulation depending on dose and duration. Donor NO causes an increase in the production of oxygen reactive species, particularly peroxides. At the same time, NO also increases GSH levels and activates antioxidant transcription factors such as hypoxia-inducible factor 1 α (HIF1 α) and nuclear factor erythroid 2-related factor 2 (Nrf-2) [31].

Similar to its effect on glucose metabolism, NO also has a dual effect on lipid metabolism. NO can act as a potential inhibitor of lipid peroxidation by scavenging peroxyl radicals and inhibiting peroxidase enzymes. However, NO can also be a potent oxidant through its reaction with superoxide to form peroxynitrite, a strong oxidant that can promote lipid peroxidation and oxidize lipid-soluble antioxidants [32]. However, overall, the intravenous administration of the NONB test preparation up to the highest dose (0.06 mL) was still relatively safe, as the total cholesterol and triglyceride levels of all experimental rats remained within the normal range throughout the study.

Liver function, assessed by AST and ALT levels, increased directly with test preparation dose. In male rats, a significant difference in AST levels was found between group 1 and group 5 compared to all treatment groups (Figure 3d). Similarly, significant differences in ALT levels were observed between group 1 and group 5 compared with all treatment groups and between group 3 and group 6 (Figure 3e). The highest AST level was 38.76 ± 0.29 U/L, while the highest ALT level was 19.58 ± 0.4 U/L, found in group 6. In female rats, there was also a linear increase in AST and ALT levels with increasing doses of the test preparation. Significant differences in AST levels were found between group 1 and group 5 compared with all treatment groups; between group 2 and groups 3 and 4; between group 4 and group 5; and between group 6 and groups 2, 3, and 5 (Figure 4d). A significant increase in ALT levels was observed in group 1 compared to groups 4 and 6 and in group 5 compared to groups 4 and 6 (Figure 4e). The highest AST and ALT levels were found in group 6, with values of 38.6 ± 1.23 U/L and 19.26 ± 0.4 U/L, respectively.

AST and ALT levels are commonly used to evaluate liver function and detect liver damage or disorders. In this study, an increase in AST and ALT levels in rats was observed with increasing doses of NONBs administered. These findings indicate liver disturbance,

confirmed by fatty degeneration of hepatocytes in the group that received NONB injections. The results of this study are consistent with previous research, both in vitro on AML-12 hepatocytes exposed to the NO donor diethylenetriamine-NONOate (DETA-NO) [31], and in vivo in experimental rats exposed to sodium nitroprusside (SNP), an antihypertensive drug that induces NO production [33]. However, other studies have shown that NO has a protective effect on the liver. Research on CCl₄-induced rats has demonstrated the hepatoprotective effect of the NO donor isosorbide-5-mononitrate [34]. Another study using NO V-PYRRO/NO donors also showed beneficial hepatoprotective effects on liver anatomy and physiology [35]. The controversy regarding existing research findings proves the complexity of NO's role in hepatotoxicity. The source of NO production, the dose and duration of exposure, and microenvironmental conditions are factors that influence NO's role in liver condition and function. In this study, intravenous exposure to NONBs caused liver function disturbances that persisted for up to 4 weeks after the cessation of injections. This indicates toxic effects of NONBs on liver function, which require longer recovery time.

In the kidney function test, a significant difference between groups was observed only for creatinine. Creatinine levels increased in male and female rats in direct proportion to the increase in dose of the test preparation. Significant increases were found in male rats between group 1 compared to groups 3, 4, and 6; group 2 compared to groups 4 and 6; group 3 compared to groups 5 and 6; and group 6 compared to groups 4 and 5 (Figure 3g). The highest creatinine level was found in group 6 at 1.02 ± 0.02 mg/dL (male rats) and 1 ± 0.01 mg/dL (female rats). The creatinine levels of all rats were within the normal range in both male and female rats [22,36].

In this study, kidney function was evaluated by examining urea and creatinine levels. No significant difference in urea levels was observed between the control group and the NONB-injected group. However, creatinine levels increased significantly in direct proportion to the increase in NONB dosage. The creatinine levels in the rats in this study remained within the normal range, indicating that the highest NONB dose (0.06 mL) did not cause kidney dysfunction. The results of this study are consistent with previous research. NO is a vasodilator that plays an important role in renal hemodynamic homeostasis in both normotensive and hypertensive conditions [37]. Research in rats has shown that both exogenous and endogenous NO exposure protect against ischemia–reperfusion-induced kidney tissue damage and dysfunction by suppressing endothelin 1 overproduction in post-ischemic kidneys [38]. Additionally, clinical studies in patients undergoing coronary angiography (CAG) and percutaneous coronary intervention (PCI) have demonstrated that supplemental NO donor therapy reduces the incidence of contrast-induced nephropathy and decreases serum creatinine levels, regardless of administration orally or intravenously [39].

In this study, serum electrolyte levels of sodium (Na) and potassium (K) were examined, which play an important role in maintaining body fluid balance, nerve and muscle function, and blood pressure regulation. The Na level in male and female rats decreased significantly, with the decrease inversely proportional to the dose of the test preparation. In male rats, significant differences were found between group 1 and groups 2–5; between group 2 and groups 4, 5, and 6; between group 5 and groups 3 and 4; and between group 6 and groups 3, 4, and 5. However, Na levels in group 6 did not differ significantly from those in group 1 (Figure 3h). In female rats, a significant decrease in Na levels was found in all comparisons between groups (Figure 4h). The highest Na levels were found in group 1 male and female rats, at 128.74 ± 3.25 mEq/L and 131.39 ± 2.62 mEq/L, respectively.

Conversely, the K value increased linearly with increasing dose of the test preparation. In male rats, significant increases were found in group 1 compared to groups 4 and 6; group 2 compared to groups 4 and 6; group 4 compared to groups 5 and 6; and group

5 compared to group 6 (Figure 3i). In female rats, significant differences were found between group 1 and group 6; between group 2 and groups 3, 4, and 6; and between group 5 and groups 3, 4, and 6 (Figure 4i). The highest K level was 3.4 ± 0.01 in male rats and 3.37 ± 0.03 mEq/L in female rats.

In this study, the Na and K levels in all rats were below the normal range based on previously published standards [22,40,41]. This condition can occur due to various factors, especially genetic and local conditions [22]. In the context of our research, the lack of data on the local normal reference for the rats used limits the ability to determine Na and K status. However, a control group can serve as a standard for assessing the intervention's effects [42]. This study reveals a temporary hyponatremia effect from NONB injections in male rats, as evidenced by Na levels in the satellite group not significantly different from those in the control group. Conversely, in female rats, low Na level persisted for 4 weeks after the cessation of NONB injections. Compared with the control group as the standard value, NONBs increased K levels in both male and female rats, and this effect persisted for 4 weeks after injection cessation.

NO plays an important role in regulating sodium and potassium balance. NO affects transport and reabsorption and both their effects on blood vessels and overall cardiovascular function [43,44]. Decreased serum sodium levels in the NONB-injected group may have occurred due to NO's role in regulating renal blood pressure and sodium excretion, as well as hormonal regulation by aldosterone, vasopressin, angiotensin II, and endothelin [45]. Donor NO, such as SNP, has been shown to increase renal sodium and water excretion, thereby decreasing serum sodium levels [46]. In this study, different effects of NONB injection on serum sodium levels between male and female rats may indicate a gender factor influencing susceptibility to the toxic effects of NONBs.

An increase in serum potassium level in the NONB-injected-group supports previous research. Our results are consistent with previous in vitro and in vivo studies demonstrating that NO donors activate KATP channels. This activation leads to increased potassium efflux, which contributes to elevated serum potassium levels [47,48]. Although serum sodium levels decreased and potassium levels increased, all rats remained clinically healthy until the end of the study, and kidney histology was normal.

3.5. Effects of NONBs on the Histology of Liver, Kidney, Heart, Lung, and Spleen

In this study, histological observations were made on several vital organs (liver, kidney, heart, lung, and spleen), as shown in Figures 5–9. Bivariate analysis of histological scores showed significant differences between groups in the liver, kidney, and spleen of male rats (Table 4). In female rats, significant differences were observed in the liver and spleen (Table 5). The results of the post hoc analysis of histological scoring with significant differences between groups are presented in Table 6. Significant differences in liver histology scores in male rats were found between group 1 compared to groups 2, 3, and 4; group 5 compared to groups 3 and 4; and group 4 compared to group 6. In female rats, significant differences in liver histology scores were observed between group 1 and groups 3 and 4 and between group 4 and groups 2, 5, and 6. Significant kidney histology scores were observed only in male rats in group 4 compared with groups 1, 2, 3, and 5. Significant spleen histology differences in male rats were found between group 1 compared to groups 2, 3, 4, and 6; group 2 compared to groups 4 and 5; and between group 5 compared to groups 3, 4, and 6. In female rats, significant differences in spleen histology scores were observed between group 1 and groups 2, 3, 4, and 6; between group 2 and groups 4 and 5; and between group 5 and groups 3, 4, and 6.

In this study, the liver histology of rats was consistent with serum biochemical results: increases in total cholesterol, triglyceride, AST, and ALT levels followed by fatty degeneration of hepatocytes. This histological finding is in line with the increasing dose of

NONBs administered. However, we found that NONB-induced liver damage was reversible: in the satellite group, 50% of male and female rat livers returned to normal histology, whereas the other 50% still showed mild-to-moderate damage. If correlated with the persistently high levels of AST and ALT until the end of the study, it can be concluded that the toxic effects of NONB injection on the liver require a recovery period longer than 4 weeks. The relatively normal histology of the kidneys, heart, and lungs in this study support the notion that NONB injections generally do not affect kidney function significantly.

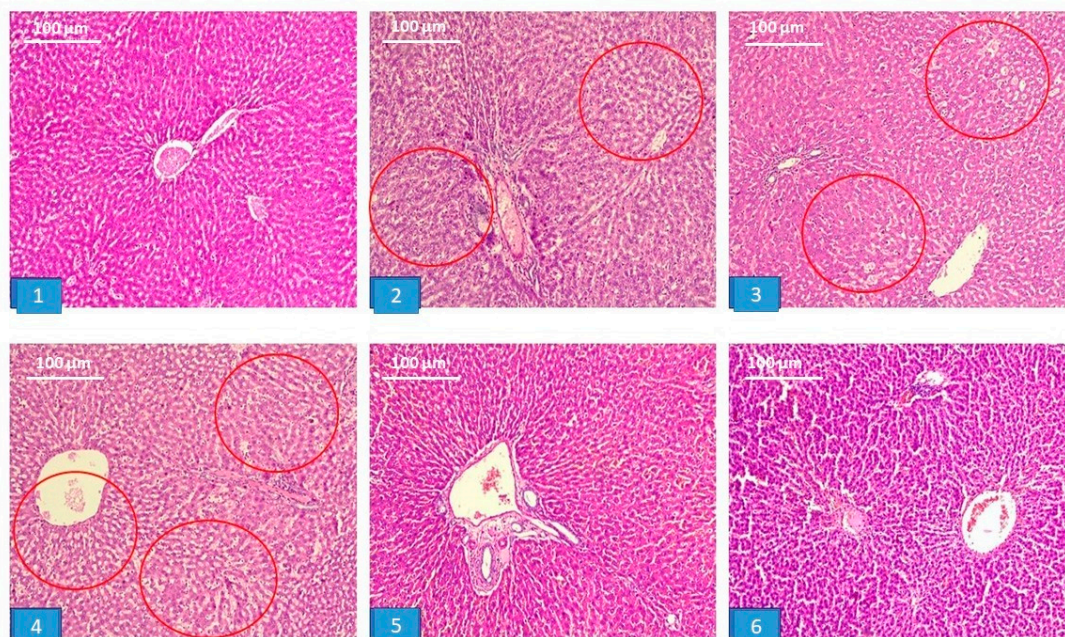


Figure 5. Histology of the liver. Normal liver histology (score 1) of rats in groups 1, 5, and 6; liver histology with multiple areas of fatty degeneration (red circle) in groups 2, 3, and 4 (score 3) (H&E, 100×).

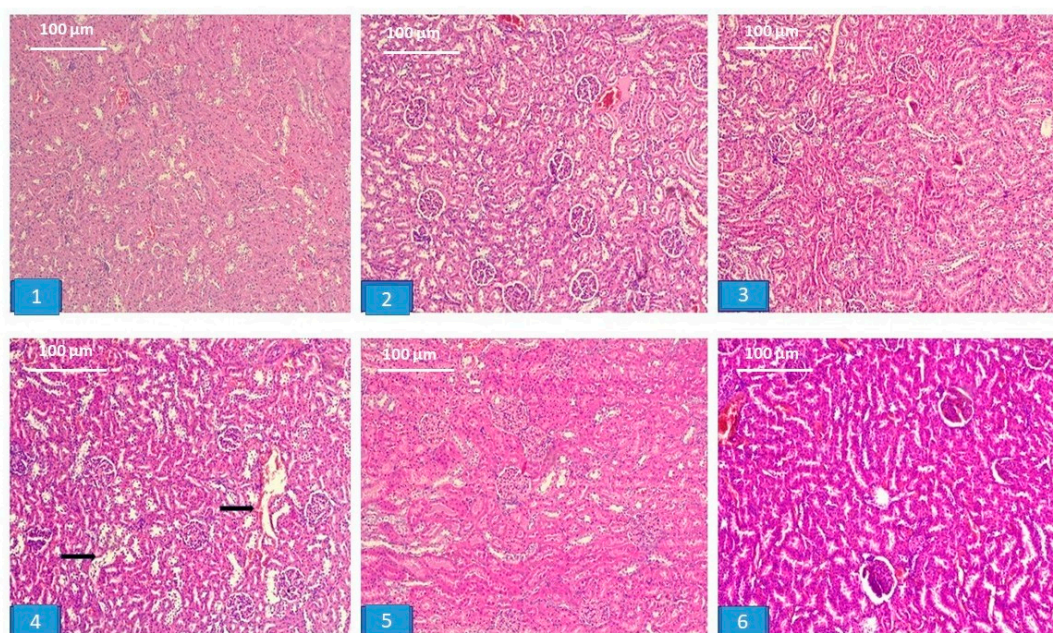


Figure 6. Histology of the kidney. Normal histology of the kidney (score 1) of rats in groups 1, 2, 3, 5, and 6; proximal tubular dilation with epithelial degeneration (black arrow/score 2) in group 4 (H&E, 100×).

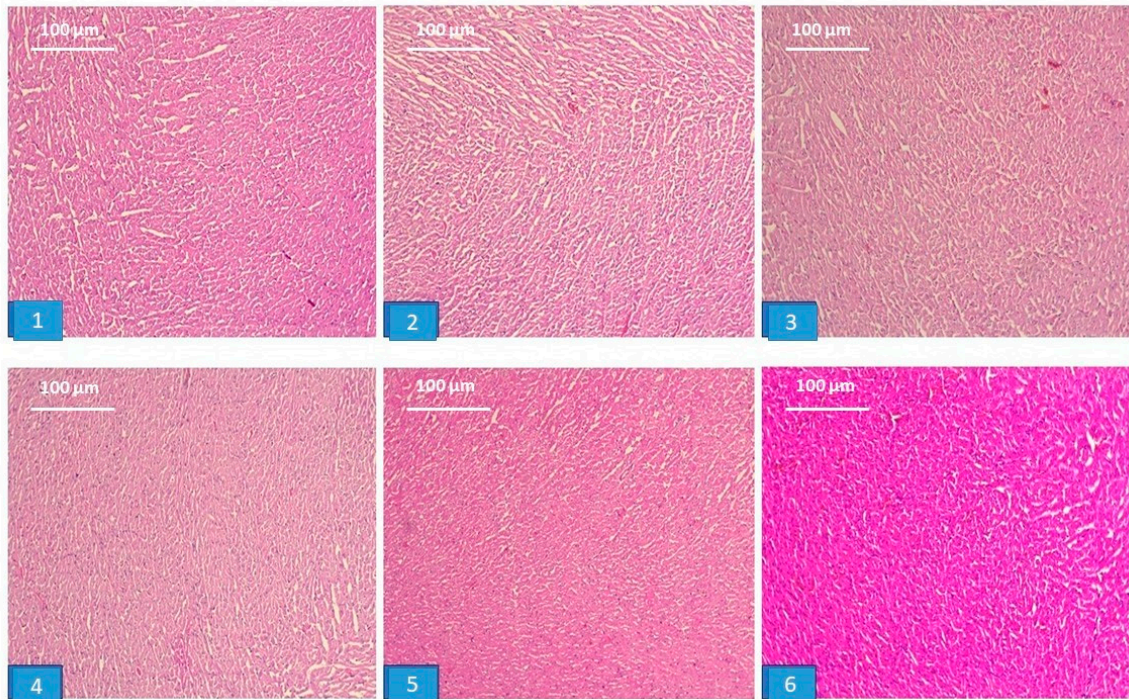


Figure 7. Histology of the heart. Normal histology of the heart (score 1) of rats in all groups (H&E, 100×).

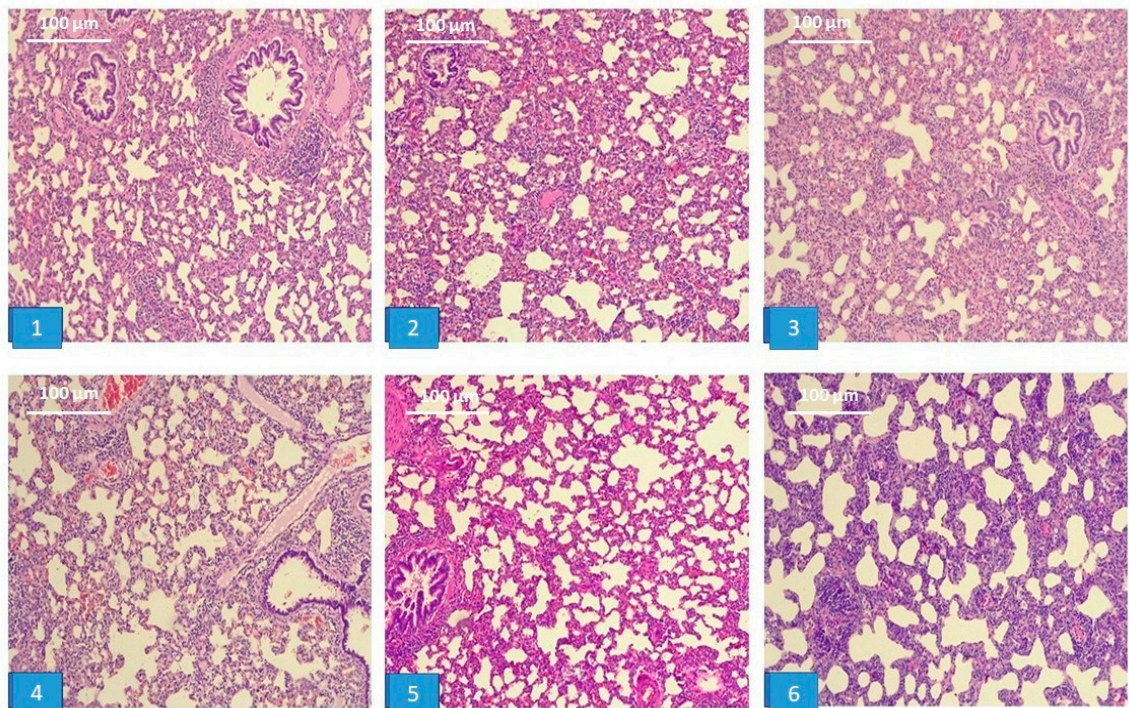


Figure 8. Histology of the lung. Normal histology of the lung (score 1) of rats in all groups (H&E, 100×).

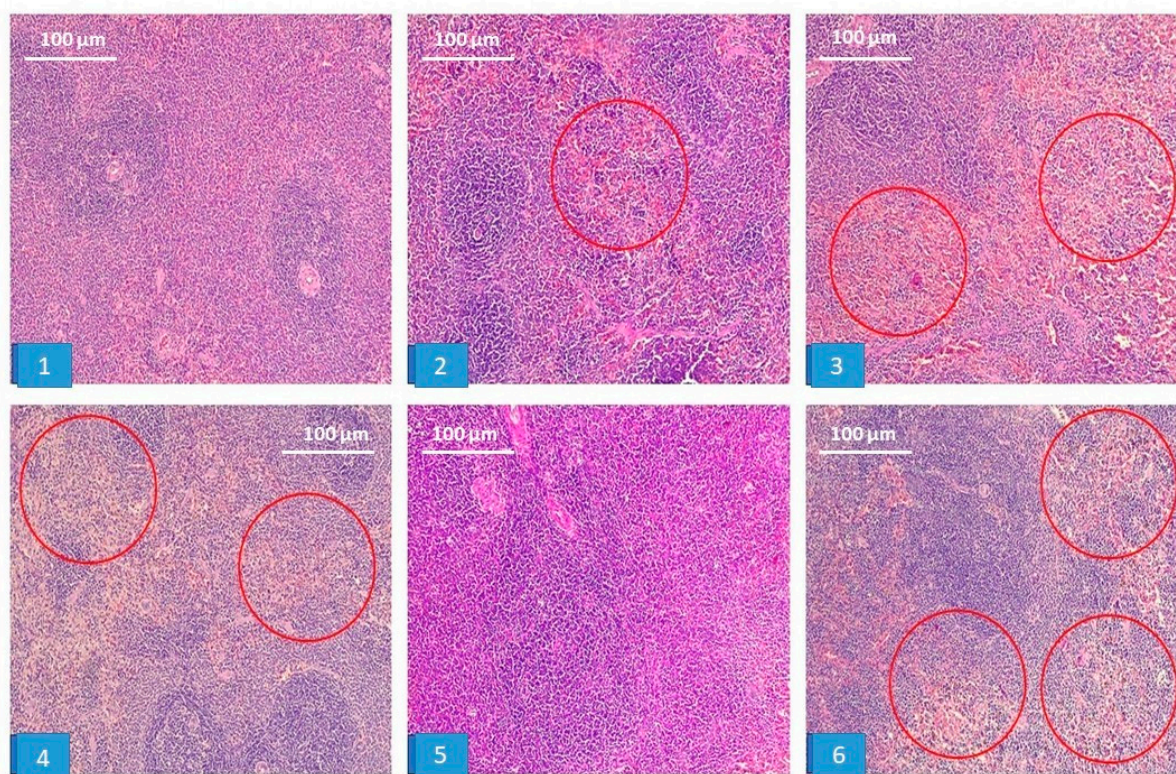


Figure 9. Histology of the spleen. Normal spleen histology (score 1) of rats in groups 1 and 5; focal hemorrhage found in group 2; multifocal hemorrhage (red circle/score 3) with hemosiderophages found in groups 3, 4, and 6 (H&E, 100×).

Persistent pathological conditions were found in the spleens of rats injected IV with NONBs linearly with increasing doses of the test preparation. Spleen histology showed bleeding with an infiltration of hemosiderin-laden macrophages. Hemosiderin is a form of iron storage, primarily derived from the breakdown of erythrocytes, which typically occurs in the red pulp of the spleen. Under normal conditions, hemosiderin can be found in the spleen in varying amounts depending on the species. Excessive amounts of splenic hemosiderin can be found in conditions of reduced erythropoiesis or rapid erythrocyte destruction in hemolytic anemia. Excessive hemosiderin in the spleen can also occur in chronic heart failure, iron dextran injections, focal accumulation of hematomas, infarcts, and bleeding due to trauma [49].

The increased amount of splenic hemosiderin in this study was likely due to focal accumulation of hematoma or bleeding resulting from hemodynamic instability induced by NONB injection. Excessive NO can increase intrasplenic microvascular pressure by relaxing arteries more than intrasplenic veins. This condition causes extravasation of intrasplenic fluid into the lymphatic system, which can contribute to splenic bleeding [50,51]. In addition, overproduction of NO can lead to loss of lymphoproliferative response in the spleen, particularly in T lymphocytes, resulting in cell death through apoptosis or necrosis [52]. In this study, no histopathologic evidence of cell death or necrosis was found in splenic tissue. However, further research is needed to determine the cause and mechanism of the multifocal hemorrhage and hemosiderosis observed.

Table 4. Histological organ scores in male rats

Groups	Liver Score, n (%)					<i>p</i>	Kidney Score, n (%)				<i>p</i>	Heart Score, n (%)					<i>p</i>	Lung Score, n (%)					<i>p</i>	Spleen Score, n (%)				<i>p</i>
	1	2	3	4	1		2	3	1	2		3	4	1	2	3		4	5	1	2	3		4				
1	6 (100)	0 (0)	0 (0)	0 (0)	0.001 *	6 (100)	0 (0)	0 (0)	0.01 *	6 (100)	0 (0)	0 (0)	0 (0)	1	6 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1	6 (100)	0 (0)	0 (0)	0 (0)	0.000 *			
2	2 (33.3)	4 (66.7)	0 (0)	0 (0)		5 (83.3)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		5 (83.3)	1 (16.7)	0 (0)	0 (0)				
3	0 (0)	1 (16.7)	5 (83.3)	0 (0)		5 (83.3)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	3 (50)	3 (50)	0 (0)				
4	0 (0)	1 (16.7)	5 (83.3)	0 (0)		1 (16.7)	5 (83.3)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	6 (100)	0 (0)				
5	5 (83.3)	0 (0)	1 (16.7)	0 (0)		5 (83.3)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)				
6	3 (50)	2 (33.3)	1 (16.7)	0 (0)		4 (66.7)	2 (33.3)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	2 (33.3)	4 (66.7)	0 (0)				
Total	16 (44.5)	8 (22.2)	12 (33.3)	0 (0)	36 (100)	26 (72.2)	10 (27.8)	0 (0)	36 (100)	36 (100)	0 (0)	0 (0)	0 (0)	36 (100)	36 (100)	0(0)	0(0)	0 (0)	0 (0)	36 (100)	17 (47.2)	6 (16.7)	13 (36.1)	0 (0)	36 (100)			

* Statistically significant using the Kruskal–Wallis test.

Table 5. Histological organ scores in female rats

Groups	Liver Score, n (%)					<i>p</i>	Kidney Score, n (%)				<i>p</i>	Heart Score, n (%)					<i>p</i>	Lung Score, n (%)					<i>p</i>	Spleen Score, n (%)				<i>p</i>
	1	2	3	4	1		2	3	1	2		3	4	1	2	3		4	5	1	2	3		4				
1	6 (100)	0 (0)	0 (0)	0 (0)	0.005 *	6 (100)	0 (0)	0 (0)	0.458	6 (100)	0 (0)	0 (0)	0 (0)	1	6 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1	6 (100)	0 (0)	0 (0)	0 (0)	0.000 *			
2	4 (66.6)	1 (16.7)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		5 (83.3)	1 (16.7)	0 (0)	0 (0)				
3	1 (16.7)	1 (16.7)	4 (66.7)	0 (0)		5 (83.3)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	3 (50)	3 (50)	0 (0)				
4	0 (0)	6 (100)	0 (0)	0 (0)		4 (66.7)	2 (33.3)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	6 (100)	0 (0)				
5	4 (66.7)	2 (33.3)	0 (0)	0 (0)		5 (83.3)	1 (16.7)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)				
6	3 (50)	1 (16.7)	2 (33.3)	0 (0)		4 (66.7)	2 (33.3)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)		6 (100)	0 (0)	0 (0)	0 (0)	0 (0)		0 (0)	2 (33.3)	4 (66.7)	0 (0)				
Total	18 (50)	11 (30.6)	7 (19.4)	0 (0)		30 (83.3)	6 (16.7)	0 (0)	36 (100)	36 (100)	0 (0)	0 (0)	0 (0)	36 (100)	36 (100)	0 (0)	0 (0)	0 (0)	0 (0)	36 (100)	17 (47.2)	6 (16.7)	13 (36.1)	0 (0)	36 (100)			

* Statistically significant using the Kruskal–Wallis test.

Table 6. Post hoc analysis of the histological organ scores in rats

Groups	<i>p</i> -Value				
	Male			Female	
	Liver	Kidney	Spleen	Liver	Spleen
1–2	0.019 *	0.317	0.001 *	0.140	0.001 *
1–3	0.001 *	0.317	0.002 *	0.006 *	0.002 *
1–4	0.001 *	0.005 *	0.001 *	0.001 *	0.001 *
1–5	0.317	1.000	1.000	0.138	1.000
1–6	0.058	0.138	0.002 *	0.058	0.002 *
2–3	0.399	1.000	0.241	0.069	0.241
2–4	0.399	0.027 *	0.005 *	0.006 *	0.005 *
2–5	0.093	0.317	0.001 *	0.847	0.001 *
2–6	0.226	0.523	0.093	0.527	0.241
3–4	1.000	0.027 *	0.056	0.140	0.056
3–5	0.01 *	0.317	0.002 *	0.154	0.002 *
3–6	0.019	0.523	0.575	0.221	1.000
4–5	0.01 *	0.005 *	0.001 *	0.019 *	0.001 *
4–6	0.019 *	0.093	0.138	0.021 *	0.056
5–6	0.338	0.138	0.002 *	0.715	0.002 *

* Statistically significant using the Mann–Whitney test.

The final results for the variables examined indicate the potential toxicity of intravenous NONB injections in this investigation. Electrolyte imbalance, spleen hemorrhage, and liver malfunction have grown to be serious problems that require attention. Pharmacokinetics, tissue distribution, and clearance are all known to be impacted by the size of the particles delivered. Particle size has a major impact on several physiological processes, including hepatic absorption and accumulation, tissue diffusion, tissue extravasation, and kidney excretion [53]. The NONBs used in this study, mostly 72–126 nm in size, were likely the cause of the abnormalities observed in the liver and spleen. Our data are supported by previous research, which shows that only particles ≤ 150 nm can enter and exit through the gaps in the liver's endothelial capillaries [54]. Smaller particles between 20 and 100 nm may disperse into the sinusoids of the liver, spleen, and bone marrow and partially exit the bloodstream through these organs' leaky capillaries [53].

In addition to particle size, particle distribution also affects the safety, stability, and effectiveness of nanoparticles. In this study, the NONBs used were typically polydispersed, indicating quite a wide range of particle sizes in the test material. This condition can also influence the systemic response to NONBs. Phagocytes can phagocytose particles larger than 100–150 nm or remain in these tissues for a long time after extravasation from blood vessels (primarily via discontinuous capillaries in the bone marrow, liver, and spleen, and to a lesser extent in the lungs). Most of these phagocytes accumulate in the spleen and liver for later elimination [55]. This may explain why the negative impact of NONBs on the liver and spleen persisted in the satellite group. Intoxication of the liver and spleen ultimately also affects electrolyte imbalance [56]. The relatively normal function and histology of the kidneys, heart, and lungs in this study were likely due to the relatively large dimensions of the NONB particles, which could not cross the capillary barriers of these organs.

This study has several limitations, including the determination of dosage using absolute volume rather than per unit body weight. This modification was due to the limited injection volume available for experimental rats. In addition, the absence of NO level measurement in the body is also a significant limitation that is highly recommended for future research to address. Measuring NO levels in the body is important, considering

that NO has a very short half-life: only a few seconds in the body and about 2 ms in the vascular lumen [57]. Measuring NO levels can more specifically address the phenomenon of damage that occurs, whether due to high residual NO levels in the body or to irreversible tissue damage resulting from metabolic reactions to NO itself.

4. Conclusions

Upon receiving escalating doses of 0.01 mL, 0.04 mL, and 0.06 mL throughout the 90-day treatment period, the rats in the intravenous NONB injection study did not perish. However, electrolyte imbalance and liver disturbance were detected biochemically. These abnormalities were further supported by histological findings in the liver and kidneys, which showed potential reversible damage, and splenic hemorrhage that persisted until the end of the study. At the same time, the heart and lungs remained normal. The results of this subchronic toxicity test indicate that intravenous administration of NONBs at doses up to 0.06 mL is still deemed safe. Nonetheless, the pathological signs that emerged call for cautious attention in future NONB development.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto, Indonesia (065/KEPK/PE/VIII/20242025—date of approval was 14 August 2024).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data reported in this paper are available from the corresponding author upon reasonable request.

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

Appendix A. Serum Biochemical Parameters

Table A1. Serum biochemical parameters of male rats

Groups	Glucose (mg/dL)		Total Cholesterol (mg/dL)		Triglycerides (mg/dL)		AST (U/L)		ALT (U/L)		Urea (mg/dL)		Creatinine (mg/dL)		Sodium (mEq/L)		Potassium (mEq/L)	
	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean Rank	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>
1	68.36 ± 1.41		74.87 ± 0.72		68.14 ± 1.91		4.50		18.04 ± 0.36		9.42 ± 0.38		0.92 ± 0.01		128.74 ± 3.25		3.3 ± 0.02	
2	68.02 ± 0.96		77.78 ± 1.49		70.36 ± 1.59		20.75		18.93 ± 0.43		9.64 ± 0.40		0.95 ± 0.02		121 ± 1.73		3.29 ± 0.01	
3	73.15 ± 3.62	<0.001 *	78.16 ± 0.97	<0.001 *	70.97 ± 2.61	<0.001 *	24.24	<0.001 *	19.02 ± 0.57	<0.001 *	9.75 ± 0.56	0.116	0.99 ± 0.04	<0.001 *	113.71 ± 3.24	<0.001 *	3.36 ± 0.05	<0.001 *
4	73.68 ± 1.02		78.29 ± 1.76		74.17 ± 2.83		26.08		19.18 ± 0.51		9.91 ± 0.49		0.99 ± 0.02		109.68 ± 1.16		3.37 ± 0.01	
5	67.48 ± 2.45		76.52 ± 0.81		69.99 ± 1.50		8.50		18.37 ± 0.36		9.53 ± 0.24		0.94 ± 0.04		101.06 ± 0.9		3.28 ± 0.04	
6	76.25 ± 1.11		80.05 ± 0.87		76.75 ± 2.38		26.92		19.58 ± 0.40		10.29 ± 0.94		1.02 ± 0.02		124.01 ± 0.96		3.4 ± 0.01	

* Statistically significant results; the ANOVA test was used for all variables except AST, for which the Kruskal–Wallis test was used.

Table A2. Serum biochemical parameters of female rats

Groups	Glucose (mg/dL)		Total Cholesterol (mg/dL)		Triglycerides (mg/dL)		AST (U/L)		ALT (U/L)		Urea (mg/dL)		Creatinine (mg/dL)		Sodium (mEq/L)		Potassium (mEq/L)	
	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>	Mean Rank	<i>p</i>	Mean ± SD	<i>p</i>	Mean ± SD	<i>p</i>
1	65.05 ± 4.28		71.59 ± 1.84		67.28 ± 4.51		22.65 ± 0.4		18.2 ± 0.67		18.2 ± 0.67		5.83		131.39 ± 2.62		3.25 ± 0.06	
2	65.74 ± 2.6		76.39 ± 3.96		70.23 ± 1.83		36.07 ± 1.04		18.69 ± 0.9		18.69 ± 0.9		13.25		123.47 ± 1.86		3.28 ± 0.04	
3	71.93 ± 1.04	<0.001 *	77.52 ± 2.69	<0.001 *	70.84 ± 3.85	0.008 *	37.22 ± 0.87	<0.001 *	18.85 ± 0.57	0.018 *	18.85 ± 0.57	0.571	21	<0.001 *	116.14 ± 3.71	<0.001 *	3.34 ± 0.02	<0.001 *
4	72.13 ± 1.81		77.77 ± 2.43		73.42 ± 3.87		38.11 ± 1.33		19.1 ± 0.59		19.1 ± 0.59		28		111.85 ± 1.04		3.35 ± 0.03	
5	67.34 ± 2.21		75.38 ± 2.33		69.37 ± 1.74		23.3 ± 0.43		18.13 ± 0.5		18.13 ± 0.5		11.67		103.71 ± 0.91		3.28 ± 0.03	
6	73.48 ± 2.47		79.54 ± 1.79		74.54 ± 0.09		38.6 ± 1.23		19.26 ± 0.4		19.26 ± 0.4		31.25		126.35 ± 1.06		3.37 ± 0.03	

* Statistically significant results; ANOVA test for all variables except creatinine using the Kruskal–Wallis test.

Table A3. Post hoc analysis of serum biochemical parameters of rats.

Groups	<i>p</i> -Value															
	Male								Female							
	Glucose ^α	Total Cholesterol ^α	Triglycerides ^β	AST ^δ	ALT ^β	Creatinine ^β	Sodium ^α	Potassium ^α	Glucose ^α	Total Cholesterol ^β	Triglycerides ^β	AST ^β	ALT ^β	Creatinine ^δ	Sodium ^α	Potassium ^α
1–2	0.99	0.55	0.091	0.003 *	0.002 *	0.09	0.009 *	0.97	0.82	0.003 *	0.13	0.00 *	0.19	0.052	0.00 *	0.87
1–3	0.13	0.17	0.33	0.003 *	0.001 *	0.00 *	0.00 *	0.17	0.65	0.00 *	0.072	0.00 *	0.08	0.008 *	0.00 *	0.07
1–4	0.00 *	0.52	0.00 *	0.004 *	0.00 *	0.00 *	0.00 *	0.00 *	0.56	0.00 *	0.003 *	0.00 *	0.02 *	0.004 *	0.00 *	0.06
1–5	0.96	0.66	0.16	0.05	0.22	0.54	0.00 *	0.94	0.84	0.01 *	0.283	0.25	0.82	0.059	0.00 *	0.13
1–6	0.00 *	0.01 *	0.00 *	0.004 *	0.00 *	0.00 *	0.095	0.00 *	0.025 *	0.00 *	0.001 *	0.00 *	0.01 *	0.004 *	0.00 *	0.03 *
2–3	0.10	1	0.63	0.42	0.75	0.032 *	0.012 *	0.12	0.052	0.46	0.75	0.046 *	0.66	0.066	0.00 *	0.048 *
2–4	0.00 *	1	0.005 *	0.14	0.35	0.01 *	0.00 *	0.00 *	0.051	0.36	0.11	0.00 *	0.27	0.006 *	0.00 *	0.048 *

2–5	0.99	1	0.77	0.003 *	0.036 *	0.27	0.00 *	0.99	1	0.51	0.66	0.00 *	0.12	0.677	0.00 *	1
2–6	0.00 *	0.77	0.00 *	0.143	0.017 *	0.00 *	0.047 *	0.00 *	0.021 *	0.04 *	0.032 *	0.00 *	0.128	0.004 *	0.017 *	0.016 *
3–4	0.99	1	0.017 *	0.62	0.54	0.61	0.166	0.99	1	0.86	0.19	0.117	0.50	0.07	0.001 *	1
3–5	0.89	0.78	0.44	0.003 *	0.01 *	0.002 *	0.001 *	0.10	0.19	0.16	0.45	0.00 *	0.53	0.022 *	0.00 *	0.02 *
3–6	0.43	0.70	0.00 *	0.62	0.036 *	0.092	0.003 *	0.46	0.72	0.25	0.064	0.018 *	0.27	0.012 *	0.00 *	0.088
4–5	0.00 *	0.93	0.003 *	0.004 *	0.00 *	0.001 *	0.00 *	0.01 *	0.021 *	0.12	0.043 *	0.00 *	0.01 *	0.004 *	0.00 *	0.024 *
4–6	0.18	0.93	0.051	0.68	0.13	0.23	0.00 *	0.009 *	0.87	0.25	0.57	0.38	0.66	0.254	0.00 *	0.88
5–6	0.00 *	0.10	0.00 *	0.004 *	0.00 *	0.00 *	0.00 *	0.002 *	0.01 *	0.01 *	0.01 *	0.00 *	0.00 *	0.004 *	0.00 *	0.01 *

* Statistically significant results; ^α Games–Howell test; ^β LSD test; ^δ Mann–Whitney test.

References

1. Kato, S.; Saitoh, Y.; Miwa, N. Hydrogen-bubbled platinum-colloid suppresses human esophagus- or tongue-carcinoma cells with intracellular platinum-uptake and the diminished normal-cell mortality. *Hum. Cell* **2020**, *33*, 1294–1301.
2. Xiao, L.; Miwa, N. 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* **2021**, *10*, 626.
3. Hansen, H.H.W.B.; Cha, H.; Ouyang, L.; Zhang, J.; Jin, B.; Stratton, H.; Nguyen, N.T.; An, H. Nanobubble technologies: Applications in therapy from molecular to cellular level. *Biotechnol. Adv.* **2023**, *63*, 108091.
4. Lohse, D. Bubble puzzles: From fundamentals to applications. *Phys. Rev. Fluids* **2018**, *3*, 110504.
5. Li, C.; Cao, Y.; Kohei, F.; Hao, H.; Peng, G.; Cheng, C.; Ye, J. Nano-bubble hydrogen water: An effective therapeutic agent against inflammation related disease caused by viral infection in zebrafish model. *Virol. Sin.* **2022**, *37*, 277–283.
6. Nowaczyk, A.; Kowalska, M.; Nowaczyk, J.; Grześk, G. Carbon monoxide and nitric oxide as examples of the youngest class of transmitters. *Int. J. Mol. Sci.* **2021**, *22*, 6029.
7. Huang, B.; Yang, L.; Yu, W.; Li, Y.; Li, L.; Gu, N. Advances of therapeutic microbubbles and nanobubbles. *Natl. Sci. Open* **2023**, *2*, 20220062.
8. Liao, T.; Li, Q.; Zhang, Y.; Yang, Z.; Huang, Z.; Han, F.; Chen, X.; Yin, T.; Ren, J.; Sun, Q. Precise treatment of acute antibody-mediated cardiac allograft rejection in rats using C4d-targeted microbubbles loaded with nitric oxide. *J. Heart Lung Transplant.* **2020**, *39*, 481–490.
9. Yang, F.; Li, M.; Liu, Y.; Wang, T.; Feng, Z.; Cui, H.; Gu, N. Glucose and magnetic-responsive approach toward in situ nitric oxide bubbles controlled generation for hyperglycemia theranostics. *J. Control. Release* **2016**, *228*, 87–95.
10. Agustin, V.; Hernowo, A. Stability evaluation of oxyhydrogen and hydrogen nanobubbles under thermal and pH stress conditions. *Sci. Rep.* **2025**, *15*, 44273.
11. OECD. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. In *OECD Guidelines for the Testing of Chemicals, Section 4*; OECD Publishing: Paris, France, 2018.
12. B. Seabra, A.; Duran, N. Nanoparticulated Nitric Oxide Donors and their Biomedical Applications. *Mini-Rev. Med. Chem.* **2016**, *17*, 216–223.
13. Dioguardi, F.S. To give or not to give? Lessons from the arginine paradox. *J. Nutr. Nutr.* **2011**, *4*, 90–98.
14. Radi, R. Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 5839–5848.
15. Verbeure, W.; van Goor, H.; Mori, H.; van Beek, A.P.; Tack, J.; van Dijk, P.R. The Role of Gasotransmitters in Gut Peptide Actions. *Front. Pharmacol.* **2021**, *12*, 720703.
16. Paul, S.; Pan, S.; Mukherjee, A.; De, P. Nitric Oxide Releasing Delivery Platforms: Design, Detection, Biomedical Applications, and Future Possibilities. *Mol. Pharm.* **2021**, *18*, 3181–3205.
17. Carpenter, A.W.; Schoenfisch, M.H. Nitric oxide release: Part II. Therapeutic applications. *Chem. Soc. Rev.* **2012**, *41*, 3742–3752.
18. Gao, D.; Asghar, S.; Hu, R.; Chen, S.; Niu, R.; Liu, J.; Chen, Z.; Xiao, Y. Recent advances in diverse nanosystems for nitric oxide delivery in cancer therapy. *Acta Pharm. Sin. B* **2023**, *13*, 1498–1521.
19. Kim-Shapiro, D.B.; Gladwin, M.T. Nitric oxide pathology and therapeutics in sickle cell disease. *Clin. Hemorheol. Microcirc.* **2018**, *68*, 223–237.
20. Premont, R.T.; Reynolds, J.D.; Zhang, R.; Stamler, J.S. Role of Nitric Oxide Carried by Hemoglobin in Cardiovascular Physiology. *Circ. Res.* **2020**, *126*, 129–158.
21. Tabish, T.A.; Crabtree, M.J.; Townley, H.E.; Winyard, P.G.; Lygate, C.A. Nitric Oxide Releasing Nanomaterials for Cardiovascular Applications. *JACC Basic Transl. Sci.* **2024**, *9*, 691–709.
22. Delwatta, S.L.; Gunatilake, M.; Baumans, V.; Seneviratne, M.D.; Dissanayaka, M.L.B.; Batagoda, S.S.; Udagedara, A.H.; Walpola, P.B. Reference values for selected hematological, biochemical and physiological parameters of Sprague-Dawley rats at the Animal House, Faculty of Medicine, University of Colombo, Sri Lanka. *Anim. Model. Exp. Med.* **2018**, *1*, 250–254.
23. Aluko, E.O.; Nna, V.U.; Fasanmade, A.A. Angiotensin converting enzyme inhibitor potentiates the hypoglycaemic effect of NG-nitro-L-arginine methyl ester (L-NAME) in rats. *Arch. Physiol. Biochem.* **2022**, *128*, e0308338.
24. Ataie, Z.; Dastjerdi, M.; Farrokhfall, K.; Ghiravani, Z. The Effect of Cinnamaldehyde on iNOS Activity and NO-Induced Islet Insulin Secretion in High-Fat-Diet Rats. *Evid.-Based Complement. Altern. Med.* **2021**, *2021*, 9970678.
25. Bahadoran, Z.; Mirmiran, P.; Ghasemi, A. Role of Nitric Oxide in Insulin Secretion and Glucose Metabolism. *Trends Endocrinol. Metab.* **2020**, *31*, 118–130.

26. Pappas, G.; Wilkinson, M.L.; Gow, A.J. Nitric oxide regulation of cellular metabolism: Adaptive tuning of cellular energy. *Nitric Oxide-Biol. Chem.* **2023**, *131*, 8–17.
27. Ihedioha, J.I.; Noel-Uneke, O.A.; Ihedioha, T.E. Reference values for the serum lipid profile of albino rats (*Rattus norvegicus*) of varied ages and sexes. *Comp. Clin. Pathol.* **2013**, *22*, 93–99.
28. Aluko, E.O.; David, U.E.; Ojetola, A.A.; Fasanmade, A.A. Aqueous extract of *Peristrophe bivalvis* (L.) Merr. leaf reversed the detrimental effects of nitric oxide synthase inhibitor on blood lipid profile and glucose level. *PLoS ONE* **2024**, *19*, e0308338.
29. Aluko, E.O.; Omobowale, T.O.; Oyagbemi, A.A.; Adejumbi, O.A.; Ajibade, T.O.; Fasanmade, A.A. Reduction in nitric oxide bioavailability shifts serum lipid content towards atherogenic lipoprotein in rats. *Biomed. Pharmacother.* **2018**, *101*, 792–797.
30. Chen, K.; Ma, Z.; Yan, X.; Liu, J.; Xu, W.; Li, Y.; Dai, Y.; Zhang, Y.; Xiao, H. Investigation of the Lipid-Lowering Mechanisms and Active Ingredients of Danhe Granule on Hyperlipidemia Based on Systems Pharmacology. *Front. Pharmacol.* **2020**, *11*, 528.
31. Aharoni-Simon, M.; Anavi, S.; Beifuss, U.; Madar, Z.; Tirosh, O. Nitric oxide, can it be only good? Increasing the antioxidant properties of nitric oxide in hepatocytes by YC-1 compound. *Nitric Oxide-Biol. Chem.* **2012**, *27*, 248–256.
32. Hogg, N.; Kalyanaraman, B. Nitric oxide and lipid peroxidation. *Biochim. Biophys. Acta-Bioenerg.* **1999**, *1411*, 378–384.
33. Khan, M.W.; Priyamvada, S.; Khan, S.A.; Khan, S.; Gangopadhyay, A.; Yusufi, A.N.K. Fish/flaxseed oil protect against nitric oxide-induced hepatotoxicity and cell death in the rat liver. *Hum. Exp. Toxicol.* **2016**, *35*, 302–311.
34. Abdel Salam, O.M.E.; Sleem, A.A.; Shafee, N. Hepatoprotective effects of the nitric oxide donor isosorbide-5-mononitrate alone and in combination with the natural hepatoprotectant, silymarin, on carbon tetrachloride-induced hepatic injury in rats. *Inflammopharmacology* **2010**, *18*, 87–94.
35. Liu, J.; Waalkes, M.P. Nitric oxide and chemically induced hepatotoxicity: Beneficial effects of the liver-selective nitric oxide donor, V-PYRRO/NO. *Toxicology* **2005**, *208*, 289–297.
36. He, Q.; Su, G.; Liu, K.; Zhang, F.; Jiang, Y.; Gao, J.; Liu, L.; Jiang, Z.; Jin, M.; Xie, H. Sex-specific reference intervals of hematologic and biochemical analytes in Sprague-Dawley rats using the nonparametric rank percentile method. *PLoS ONE* **2017**, *12*, e0189837.
37. Ahmad, A.; Dempsey, S.K.; Daneva, Z.; Azam, M.; Li, N.; Li, P.L.; Ritter, J.K. Role of nitric oxide in the cardiovascular and renal systems. *Int. J. Mol. Sci.* **2018**, *19*, 2605.
38. Kurata, H.; Takaoka, M.; Kubo, Y.; Katayama, T.; Tsutsui, H.; Takayama, J.; Matsumura, Y. Nitric oxide protects against ischemic acute renal failure through the suppression of renal endothelin-1 overproduction. *J. Cardiovasc. Pharmacol.* **2004**, *44*, S455–S458.
39. Ibrahim, A.A.; Tabassum, S.; Abuelazm, M.; Amin, A.M.; Rakab, M.S.; Rifai, M.; Manasrah, A.; Mahmoud, A.; Emara, A.G.; Abdelazeem, B.; et al. Preventive effects of nitric oxide donors in contrast-induced nephropathy in patients undergoing coronary artery angiography: An updated systematic review and meta-analysis of 13 randomized controlled trials. *Int. Urol. Nephrol.* **2025**, *57*, 1207–1232.
40. Han, Z.-Z.; Xu, H.-D.; Kim, K.-H.; Ahn, T.-H.; Bae, J.-S.; Lee, J.-Y.; Gil, K.-H.; Lee, J.-Y.; Woo, S.-J.; Yoo, H.-J.; et al. Reference Data of the Main Physiological Parameters in Control Sprague-Dawley Rats from Pre-clinical Toxicity Studies. *Lab. Anim. Res.* **2010**, *26*, 153–164.
41. Seibel, J.; Bodié, K.; Weber, S.; Bury, D.; Kron, M.; Blaich, G. Comparison of haematology, coagulation and clinical chemistry parameters in blood samples from the sublingual vein and vena cava in Sprague-Dawley rats. *Lab. Anim.* **2010**, *44*, 344–351.
42. Moser, P. Out of Control? Managing Baseline Variability in Experimental Studies with Control Groups. *Handb. Exp. Pharmacol.* **2020**, *257*, 101–117.
43. Laycock, S.K.; Vogel, T.; Forfia, P.R.; Tuzman, J.; Xu, X.; Ochoa, M.; Thompson, C.I.; Nasjletti, A.; Hintze, T.H. Role of nitric oxide in the control of renal oxygen consumption and the regulation of chemical work in the kidney. *Circ. Res.* **1998**, *82*, 1263–1271.
44. Macdonald, J.E.; Struthers, A.D. What is the optimal serum potassium level in cardiovascular patients? *J. Am. Coll. Cardiol.* **2004**, *43*, 155–161.
45. Carlström, M. Nitric oxide signalling in kidney regulation and cardiometabolic health. *Nat. Rev. Nephrol.* **2021**, *17*, 575–590.
46. Henstridge, D.C.; Kingwell, B.A.; Formosa, M.F.; Drew, B.G.; McConell, G.K.; Duffy, S.J. Effects of the nitric oxide donor, sodium nitroprusside, on resting leg glucose uptake in patients with type 2 diabetes. *Diabetologia* **2005**, *48*, 2602–2608.
47. Afzal, M.Z.; Reiter, M.; Gastonguay, C.; McGivern, J.V.; Guan, X.; Ge, Z.D.; Mack, D.L.; Childers, M.K.; Ebert, A.D.; Strande, J.L. Nicorandil, a Nitric Oxide Donor and ATP-Sensitive Potassium Channel Opener, Protects Against Dystrophin-Deficient Cardiomyopathy. *J. Cardiovasc. Pharmacol. Ther.* **2016**, *21*, 549–562.
48. Kim, J.-E.; Hwang, S.D.; Lee, S.W.; Song, J.H.; Kim, K. Potassium Channel Syndrome Caused by Nicorandil in Chronic Kidney Disease: A Case Report and Literature Review. *Electrolyte Blood Press.* **2022**, *20*, 17–20.

49. Boes, K.M.; Durham, A.C. Bone Marrow, Blood Cells, and the Lymphoid/Lymphatic System. In *Pathologic Basis of Veterinary Disease*, 6th ed.; Mosby: St. Louis, MO, USA, 2017; pp. 724–804.e2.
50. Andrew, P.S.; Deng, Y.; Sultanian, R.; Kaufman, S. Nitric oxide increases fluid extravasation from the splenic circulation of the rat. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2001**, *280*, R959–R967.
51. Andrew, P.; Deng, Y.; Kaufman, S. Fluid extravasation from spleen reduces blood volume in endotoxemia. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2000**, *278*, R60–R65.
52. Valenti, L.; Mathieu, J.; Chancerelle, Y.; Levacher, M.; Chanaud, B.; De Sousa, M.; Strzalko, S.; Dinh-Xuan, A.T.; Giroud, J.P.; Florentin, I. Nitric oxide inhibits spleen cell proliferative response after burn injury by inducing cytostasis, apoptosis, and necrosis of activated T lymphocytes: Role of the guanylate cyclase. *Cell. Immunol.* **2003**, *221*, 50–63.
53. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Khorasani, S.; Mozafari, M.R. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. *Pharmaceutics* **2018**, *10*, 57.
54. Bertrand, N.; Leroux, J.C. The journey of a drug-carrier in the body: An anatomo-physiological perspective. *J. Control. Release* **2012**, *161*, 152–163.
55. Kraft, J.C.; Freeling, J.P.; Wang, Z.; Ho, R.J.Y. Emerging research and clinical development trends of liposome and lipid nanoparticle drug delivery systems. *J. Pharm. Sci.* **2014**, *103*, 29–52.
56. Jiménez, J.V.; Carrillo-Pérez, D.L.; Rosado-Canto, R.; García-Juárez, I.; Torre, A.; Kershenovich, D.; Carrillo-Maravilla, E. Electrolyte and Acid-Base Disturbances in End-Stage Liver Disease: A Physiopathological Approach. *Dig. Dis. Sci.* **2017**, *62*, 1855–1871.
57. Thomas, D.D.; Liu, X.; Kantrow, S.P.; Lancaster, J.R. The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O₂. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 355–360.

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