

Effect of Dietary Sodium Nitrite Supplementation on Spontaneous Intracerebral Hemorrhage in Stroke-Prone Spontaneously Hypertensive Rats

Running title: Nitrite reduces the incidence of intracerebral hemorrhage

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37 **Received 18 June 2025; Revised 9 September 2025; Accepted 18 September 2025; Published online**
38 **31 December 2025**

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ABSTRACT

Diet-derived nitrite can benefit cardiovascular function. However, the effects of nitrite supplementation on idiopathic intracerebral hemorrhage (spontaneous ICH) are unclear. This study, therefore, investigated the impacts of chronic nitrite supplementation on the survival rate and risk for spontaneous ICH in stroke-prone spontaneously hypertensive/Izumo strain (SHRSP/Izm) rats fed with a high-salt diet. Experimental study I- Six-week-old male rats were categorized into two groups: 1) SHRSP + salt, in which rats were administered saline drinking water, and 2) SHRSP + salt + nitrite, in which rats were administered nitrite-added saline drinking water for 14 weeks each. The survival curves during this period did not vary significantly between the groups. However, nitrite administration markedly reduced the incidence of ICH and the extent of cerebral hemorrhage. Experimental study II- The impacts of nitrite supplementation on blood pressure in salt-loaded 8-week-old male SHRSP/Izm rats were evaluated for >4 weeks. During the first week, systolic blood pressure was remarkably lower in the nitrite group than in the control (without nitrite feeding). Similarly, at week 4, cardiac mass and brain mass were significantly lower. In conclusion, nitrite treatment reduced the extent of cerebral hemorrhage caused by hypertension and administration of a high-salt diet.

Key words: intracerebral hemorrhage, nitrite, nitric oxide, and SHRSP

INTRODUCTION

Stroke is classified as ischemic or hemorrhagic. The incidence rate of ischemic stroke has decreased in recent decades, unlike that of hemorrhagic strokes, such as subarachnoid hemorrhage (SAH) and intracerebral hemorrhage (ICH), as well as the number of severe hospitalizations among the elderly (Rothwell et al., 2004; Islam et al., 2008, An et al., 2017). Hemorrhagic stroke accounts for 18%–24% of all cases and is more common in men; its risk enhances with age (Ojaghihaghighi et al., 2017). Hemorrhagic strokes, including ICH and SAH, are associated with high mortality rates (Qureshi et al., 2009, Keep et al., 2012) and have a much worse prognosis compared to ischemic stroke, with a 30-day mortality rate of $\leq 50\%$ (Broderick et al., 1992, Castello et al., 2022). Controlling the growth of a hematoma caused by vascular rupture is an important therapeutic strategy for hemorrhagic strokes; however, an effective treatment method for ICH has not yet been established (Davis et al., 2006, Fekadu et al., 2019).

Nitric oxide (NO) is a gas that demonstrates antihypertensive, antiplatelet aggregation, anti-inflammatory, and antioxidant effects (Loscalzo, 2001, Garcia and Stein, 2006). It also improves blood flow and is essential for maintaining *in vivo* cardiovascular homeostasis. Nitrate or nitrite supplementation promotes NO formation independent of nitric oxide synthase (NOS); NO can benefit against disorders of the cardiovascular system, kidneys, and other organs (Ohtake et al., 2010, Machha and Schechter, 2011, Bahadoran et al., 2015, Lundberg and Weitzberg, 2022, Bryan et al., 2023, Carlström et al., 2024, Celik et al., 2025). The therapeutic effects of nitrate or nitrite addition on ischemic stroke (Wang et al., 2022) and SAH (Ezra et al., 2024) have been previously reported, unlike its impacts on spontaneous ICH.

Nitrate from foods such as green leafy and root vegetables is nontoxic and promotes the formation of NO through an entero-salivary pathway (Hord et al., 2009, Bowles et al., 2024).

Approximately 75% of the nitrate ingested by humans is excreted by the kidneys, and the remaining 25% accumulates in the salivary glands, where it is secreted into the oral cavity via the saliva, which contains nitrate at concentrations ≤ 20 - times greater than those in the plasma (Spiegelhalder et al., 1976, Lundberg and Govoni, 2004, Montenegro et al., 2016). This nitrate secreted is reduced to nitrite by the oral cavity bacteria and is absorbed through a recycling pathway into the gut–saliva circulatory system. This nitrite is further converted to NO by enzymes (XOR and ALDH2), thiols (cysteine and glutathione), deoxy-hemoglobin, and deoxy-myoglobin under acidic and hypoxic conditions in the stomach and other tissues (Bender and Schwarz, 2018). Although dietary nitrate is inert with a minimal risk of toxicity, that derived from preserved meats and other sources is potentially toxic and can produce carcinogenic nitroso compounds under certain circumstances (Tang, 2009; Hord, 2017). However, the nitrite formed naturally *in vivo* after the ingestion of dietary nitrate has not yet been associated with adverse health effects (Hord, Tang, 2009, Hord NG, 2017, Bowles, Burleigh, 2024). The present study investigated the effects of chronic nitrite supplementation on hemorrhagic stroke and survival rates in stroke-prone spontaneously hypertensive rats (SHRSP) fed a high salt diet, a widely studied hypertension model associated with the development of ICH (Lee et al., 2007). Although rats orally supplemented with nitrate reduce it to nitrite, they concentrate less of it in the saliva; they generate reduced levels of salivary nitrate and nitrite compared to humans (Montenegro, Sundqvist, 2016). In the present study, rats were orally administered nitrite instead of nitrate to ensure its substantial oral availability.

MATERIALS AND METHODS

Animals

SHRSP/ Izumo strain (Izm) rats were purchased from Japan SLC, Inc., Shizuoka Prefecture, Japan. They were maintained by the Disease Model Cooperative Research Association. Such rats spontaneously develop ICH when on a high-salt intake (Smeda et al., 2018). In the present study, the animals were fed a standard rat diet containing 0.5% NaCl, 20.8% protein, 4.8% fat, 3.2% fiber, and 58.2% carbohydrate (Funabashi Farm, Chiba, Japan). The salt intake for all animals was enhanced by adding NaCl to their drinking water. At the start of the study, the rats were randomized into either the control group (C), given drinking water containing 85.5 mM NaCl, or the experimental group provided with drinking water containing 85.5 mM NaCl with/without 0.6 mM NaNO₂ (E1/E2) (Wako Pure Chemical Industries, LTD). At the end of the experiment, anesthesia was provided using a solution consisting of 250 mL isoflurane (099-06571) and 500 mL propylene glycol (164-04996), and the animals were euthanized by exsanguination through blood collection. All animal experiments were performed in compliance with the relevant guidelines proposed by Kinjo Gakuin University Animal Center, Kinjo Gakuin University, Aichi, Japan. The study protocol was approved by the Committee on Ethics of Animal Experiments of Kinjo Gakuin University Animal Center (Approval number: 181).

Experimental study I

The SHRSP/Izm rats, 6 weeks of age, were categorized into two groups as described above: rats provided with NaCl in drinking water (SHRSP + salt, n = 12; control group) and those given NaCl + NaNO₂ in drinking water (SHRSP + salt + nitrite group, n = 12; nitrite-treated group). Each solution was provided to the rats from 6 to 20 weeks of age. Changes in the body weight and survival rate for all rats during this salt-loading period were recorded. If a rat died during this period, the brain, heart, liver, and kidneys were promptly excised and weighed.

The excised brains were fixed in formalin at room temperature for >72 h. They were sliced along the coronal plane at equal intervals to obtain ten cross-sections (~2 mm). The dorsal and ventral views of each cross-section were photographed, and the digital images were stored on a PC. Thus, 20 photographs per rat brain were captured. The cross-sections were then evaluated to determine the ICH incidence in each group. For rats with ICH, the proportion of sections that contained ICH lesions (%) and the total area of the ICH lesions relative to the entire brain area (%) were also calculated. The image analysis software ImageJ version 1.52 (<https://imagej.net/ij/>) was used to determine these areas.

Experimental study II

As hypertension is a risk factor for ICH, we measured the effects of nitrite supplementation on blood pressure and on blood NO_x concentrations (nitrate, nitrite, and the primary NO metabolites). For this study, 7-week-old SHRSP/Izm rats were purchased, acclimatized for 1 week, and then divided into the two experimental groups: SHRSP + salt (control group) and SHRSP + salt + nitrite (nitrite-treatment group). Rat feeding with salt + nitrite (nitrite-treatment group; n = 5) or salt alone (control group; n = 5) was limited to 4 weeks to avoid dropout due to death, as observed in longer studies. Body weights were determined every other week. Systolic blood pressure (SBP) was measured weekly in conscious animals restrained in a plastic cage. Their tails were heated before measuring blood pressure with a BP-98A tail-cuff plethysmography system (Softron, Tokyo, Japan). SBP was measured thrice, and the average value was recorded. At the end of the experiment, the animals were anesthetized and exsanguinated; their livers, kidneys, hearts, and brains were weighed.

The blood samples were centrifuged at 700× g for 20 min at 4 °C, and the plasma samples were stored at –80 °C. The plasma concentrations of nitrate and nitrite—the main nitric

oxide metabolites (abbreviated NO_x)—were ascertained using an ENO-20 HPLC system specific to NO_x (Eicom Corp, Kyoto, Japan). This method is based on separating nitrate and nitrite by ion chromatography, followed by the online reduction of nitrate to nitrite, post-column derivatization with Griess reagent, and detection at 540 nm (Ohtake et al., 2007, Ohtake, Koga, 2010). Plasma levels of glucose, triglycerides (TGs), free fatty acids, blood urea nitrogen, creatinine, aspartate aminotransferase, and alanine aminotransferase were measured by outsourcing to the Specimen Research Laboratory Inc., Tokyo, Japan.

Total brain RNA (30 mg) was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The isolated RNA (1 µg) was reverse-transcribed with the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan). qPCR was performed using the PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific K.K., Tokyo, Japan) and a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific K.K.). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an internal standard. Table 1 shows the sequences of the primers used.

Statistical analyses

The data are expressed as means with 95% confidence intervals. Statistical significance testing of inter-group variations was performed using Fisher's exact test for the categorical variables and the Mann–Whitney U-test for the numerical variables. Statistical significance (two-tailed testing) was set at $P < 0.05$. Kaplan–Meier survival analysis and log-rank testing were employed to compare the survival curves. All statistical analyses were performed utilizing the MedCalc software version 23.2.1 (www.medcalc.org).

RESULTS

Experimental study I: survival rate after nitrite supplementation

The Kaplan–Meier survival curves spanning 14 weeks after the start of salt loading are presented in Figure 1. Although the survival rate appeared to decline earlier in the control than in the nitrite-supplemented group, the intergroup survival curves did not differ significantly ($P = 0.169$).

Experimental study (1): incidence of stroke after nitrite supplementation

The animals were dissected after death, and their brains were excised. These were immediately fixed in formalin to visually confirm the cerebral hemorrhage sites (Fig. 2a). Each brain was divided into approximately 10 sections to determine the extent of stroke development (Fig. 2b). Extensive and deep ICHs were observed in the control group, mainly in the neocortical region. In contrast, the administration of sodium nitrite markedly reduced such a risk. Quantitative analysis showed that 11/12 (91.7%) and 5/12 (41.7%) animals in the control and nitrite groups developed ICH, respectively. Thus, stroke incidence was significantly lower in the nitrite-treated rats (95% CI: 19.3%–68.0%) compared to control rats (95% CI: 64.6%–98.5% and $P = 0.027$; Fisher's exact test). The odds of stroke were 15.4-fold higher in the controls than in nitrite-treated animals (95% CI: 1.5–161.0). Among the control group animals with stroke, 63% (7/11) demonstrated multiple hemorrhagic lesions, whereas only 20% (1/5) in the nitrite-treated group had >1 lesion. Furthermore, the proportion of brain cross-sections with ICH lesions was 20% in the control group, whereas it was significantly lower (5%) in the treatment group ($P = 0.0029$) (Fig. 3a). In the animals with ICH, the ICH area % appeared smaller in the nitrite-treated than in the control group. However, the difference was insignificant, perhaps owing to the few

rats that experienced stroke in the nitrite-treated group (Fig. 3b). These results indicate that nitrite supplementation reduced the incidence rate and extent of ICH in the nitrite-treated group than in the control rats.

Experimental study (1): changes in body and organ weight after long-term nitrite supplementation

During the whole experiment, the body weights did not vary significantly between groups, except in weeks 13–15, when the nitrite-treated rats were heavier than the control rats (Table 2). There were no significant inter-group differences between absolute organ weight and organ weight adjusted for body weight (data not shown).

Experimental study (2): changes in body and organ weights after short-term nitrite supplementation

There were no deaths or loss of samples after short-term administration (4 weeks) of salt or salt + nitrite. Similarly, at this time point, the body, liver, and kidney weights did not differ markedly between the control and salt + nitrite groups (Table 3). In contrast, the heart and brain weights were markedly lower in the treated than in the control group.

Experimental study (2): effect of nitrite supplementation on SBP

SBP was measured weekly in rats from 8–12 weeks of age using the tail-cuff method (Table 4). After 1 week of high salt intake, SBP was markedly lower in the treated group than in the control group. However, there were no remarkable inter-group variations in blood pressure at 10–12 weeks of age.

Experimental study (2): plasma nitrate and nitrite concentrations after nitrite supplementation

The plasma nitrate levels were markedly greater in the treatment-group rats than in the control rats given for 4 weeks (Fig. 4a). In contrast, the plasma nitrite levels showed the opposite trend (Fig. 4b). The total plasma levels of NO metabolites (nitrate + nitrite) were greater in the nitrite-treated rats than in the controls. In our previous studies on the SHRSP5/Dmcr rat strain (an SHRSP substrain susceptible to arteriolipidosis induced by a high-fat high-cholesterol diet), we found that supplemental dietary nitrite was also associated with increases in plasma nitrate levels without any concomitant elevation in nitrite levels (Sonoda et al., 2022). It is possible that the orally administered nitrite did not remain in the plasma in its native form and was rapidly reduced to NO that was subsequently metabolized to nitrate. Additionally, the nitrite was ingested during the night when the animals were active and drinking water, whereas the blood samples were collected during the day; thus, the nitrite may have been removed from circulation by this time.

Experimental study (2): alterations in biochemical parameters after nitrite supplementation

No significant inter-group variations were observed in the plasma levels of glucose, free fatty acids, BUN, and alanine aminotransferase. In contrast, those of TGs and creatinine were markedly higher, whereas aspartate aminotransferase level was lower in the nitrite-treated rats compared to the control group (Table 5).

Experimental study (2): expression levels of inflammation-, NOS-, and oxidative stress-related genes in the brain

The mRNA expressions of the inflammation-inducing cytokines, TNF- α and NF- κ B, were significantly downregulated in the treated group compared to the control group animals (Fig. 5a, b). Those of the two NOS isoforms, iNOS and nNOS, were markedly suppressed by nitrite supplementation (Fig. 5d, e). In contrast, the mean levels of the eNOS mRNA increased slightly; however, it was statistically insignificant (Fig. 5c). The mRNA levels of the oxidative stress markers, HO-1 and Nrf2, were remarkably downregulated by nitrite (Fig. 5f, g).

DISCUSSION

The present study found that oral administration of sodium nitrite significantly reduced (by 50%) the incidence of ICH in salt-loaded SHRSP rats than in the controls. Treatment with sodium nitrite also increased the plasma NO_x concentration, consistent with the known ability of supplemental nitrite to enhance circulating NO levels in rats. Our findings fit well with those of previous studies, which demonstrated that orally or intravenously administered sodium nitrite can also demonstrate neuroprotective effects in rodents subjected to global cerebral ischemia and reperfusion (Fukuda et al., 2015). In addition, the results were in alignment with previous findings that sodium nitrite infusion can promote cerebral perfusion in humans with subarachnoid hemorrhage (Ezra, Franko, 2024).

The capacity of supplemental nitrite in reducing ICH risk may be related to its ability to suppress blood pressure, as nitrite, after reduction to NO, induces vasodilation, which in turn activates soluble guanylate cyclase activity in the smooth muscle cells, increasing cGMP levels and a subsequent vasodilation. Hypertension is a major risk factor for ICH (O'Donnell et al., 2016), with the risk enhanced by ~40% for every increase of 10 mmHg in SBP (Lawes et al., 2003). Intravenous or oral administration of nitrite can decrease blood pressure in normotensive and hypertensive strains of rats (Classen et al., 1990, Vleeming et al., 1997, Sonoda, Kono,

2022). We found that the SBP was significantly lower during the first week of nitrite treatment compared to the control rats. In the nitrite-treated animals, the slower rate of blood pressure rise during the first week of salt loading may have provided a certain degree of protection against hypertension-induced vascular damage. We did not detect any marked differences in SBP between the two groups after the first week. However, because the tail-cuff method used does not allow for 24-h continuous monitoring of blood pressure, it is possible that the nitrite-treated animals also had lower blood pressure at other time points of the study. The SHRSP strain is highly sensitive to the pressor effects of a high-salt diet (Griffin et al., 2001). Subnormal NO activity in response to increases in NaCl intake is a major pathogenetic determinant of salt sensitivity (Kurtz et al., 2022). Notably, supplementation with nitrite, an important NO precursor, reduced the incidence of ICH despite the administration of large amounts of salt. As discussed earlier, the antihypertensive effect of nitrite, mediated through enhanced NO activity, most likely contributed to protecting the salt-loaded SHRSP animals from ICH.

NO is a messenger molecule with diverse biological functions. It plays a vital physiological role in disorders of various organs, including blood vessels, brain, lungs, liver, kidneys, and stomach, as well as the immune, musculoskeletal, and reproductive systems. Nitrite and nitrate, which are precursors of NO, when supplemented, induce protective effects in these organs (Stuart-Smith, 2002). In the experimental study (1), no significant differences in the weights of the brain, heart, liver, and kidneys were observed because the animals died at different time points. However, in study (2), in which the NaCl and nitrite administration period (4 weeks) and the dissection time were the same for all animals, heart and brain hypertrophy were lower in the nitrite-treated rats than in controls (Table 3). The protective influence of NO replacement therapy on myocardial hypertrophy is well known; supplementation with L-arginine, a substrate

for all NOS isoforms, improves left ventricular hypertrophy in spontaneously hypertensive rats by increasing myocardial cGMP content without affecting the blood pressure (Matsuoka et al., 1996). However, reports on the usefulness of NO replacement therapy in ICH are limited. Spontaneous ICH has a multistep pathology, with blood extravasating into the brain parenchyma, followed by peri-clot bleeding, which causes hematoma expansion, peri-hemorrhage edema, and tissue swelling induced by permeability-enhancing compounds released from the acute hematoma. The mechanism underlying such an expansion of the hematoma remains unclear, but it is likely related to an upregulation of the inflammatory cascade. This enhancement causes an imbalance in the hemostatic mechanisms and elevates the expression of matrix metalloproteinases, leading to vascular congestion, secondary to destruction and stretching of the physical support provided by the endothelial basement membrane. This effect is associated with microscopic rupture of the surrounding blood vessels due to a mass effect of the thrombus and intracranial pressure (Yasemin and Adnan, 2021).

Secondary brain injury due to ICH is a prime therapeutic target to improve poor clinical outcomes. The complex cascade of secondary injury involves excitotoxicity, neuroinflammation, and profound oxidative stress, leading to edema and progressive neuronal death (Zheng et al., 2022). Central to these pathological processes is the dysregulation of NO bioavailability. We demonstrated that nitrite supplementation attenuates the expression of inflammatory cascades involving NF- κ B, iNOS, and TNF- α and reduces the expression of genes mediating oxidative damage (Fig. 5). In ICH, hemoglobin from the hematoma can scavenge the protective NO produced by eNOS. Such an acute NO deficiency activates the pro-inflammatory transcription factor NF- κ B, which in turn drives the expressions of TNF- α and iNOS. The subsequent enhanced output of NO from iNOS, in a highly reactive environment, forms cytotoxic

peroxynitrite. This entire cascade results in severe cellular damage and intense oxidative stress (Tschoe et al., 2020). Nitrite therapy offers a multipronged solution by exerting vasodilatory, anti-inflammatory, and antioxidant effects via its conversion into NO in the hypoxic perihematoma tissues. Its vasodilatory action counteracts ischemia. Its anti-inflammatory activity stems from restoring physiological NO levels, which re-establishes inhibitory control over the NF- κ B pathway, thereby suppressing the levels of inflammatory mediators like TNF- α and iNOS. Finally, by reducing inflammation and the enzyme-catalyzed production of free radicals, it exhibits a potent antioxidant effect, mitigating overall oxidative stress. While clinical trials using the NO donor glyceryl trinitrate have yielded conflicting results, they do highlight the potential importance of early treatment. Preclinical data also support the benefits of NO in ICH models (Bath, 2015, 2019). In conclusion, nitrite therapy represents a promising strategy for ICH, leveraging its vasodilatory, anti-inflammatory, and antioxidant properties to combat secondary brain injury.

The present investigation revealed that chronic nitrite supplementation reduced the incidence and severity of spontaneous ICH in SHRSP rats maintained on a high-salt diet, although it did not influence the overall survival rates. Our findings were consistent with those of an earlier study showing that chronic nitrate supplementation also improved vascular function in Wistar rats without affecting their survivability (Carvalho et al., 2021). Thus, even if augmenting NO activity via dietary intervention does not extend the lifespan, it may promote vascular health and reduce the risk for adverse cardiovascular events that can impede the quality of life. Because of the potential toxicity and carcinogenicity concerns of nitrite, efforts to augment NO activity should focus on increasing the intake of nitrate rather than nitrite, which can be safely accomplished by enhancing the consumption of nitrate-rich green leafy and root vegetables

(Lundberg et al., 2006, Hord, Tang, 2009, Gee and Ahluwalia, 2016, Khatri et al., 2017, Mills et al., 2017).

The protective effects of nitrite most likely stem from its ability to enhance NO production, which in turn mitigates hypertension, a primary driver of ICH. It provides additional benefits through its anti-inflammatory and antioxidant properties. Even in the absence of any survival-enhancing advantage, the capacity of nitrite in decreasing the occurrence and extent of hemorrhagic stroke underscores its potential in preserving cerebrovascular integrity and improving health outcomes in the context of hypertension. Given the concerns regarding the potential toxicity of dietary nitrite, these findings advocate for a further exploration of nitrate, derived from safe dietary sources such as green leafy and root vegetables, as a viable alternative for augmenting NO activity.

Future studies should aim to validate these observations across broader experimental models and human cohorts and also incorporate continuous blood pressure monitoring to fully assess the potential of dietary augmentation of NO activity in preventing hemorrhagic stroke and related cardiovascular conditions.

AUTHOR DISCLOSURE STATEMENT

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

351 None.

352

353 **AUTHOR CONTRIBUTIONS.**

354 Concept and design: SW, KS, KK, TWK, SH. Analysis and interpretation: SR, YKo, MM, HN,

355 TF, YKa, HT, NW, IS, SY. Data collection: SR, YKo, KO, JK, HN, TF, YKa, HT. Writing the

356 article: SR, TWK. Critical revision of the article: SR, KS, KK, KO, MM, TWK, YY, NW, SH.

357 Final approval of the article: all authors. Statistical analysis: YKo, HN, TF, YKa, HT, IS, SY.

358 Obtained funding: SW, KK, YY. Overall responsibility: SW.

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516

Figure 1.

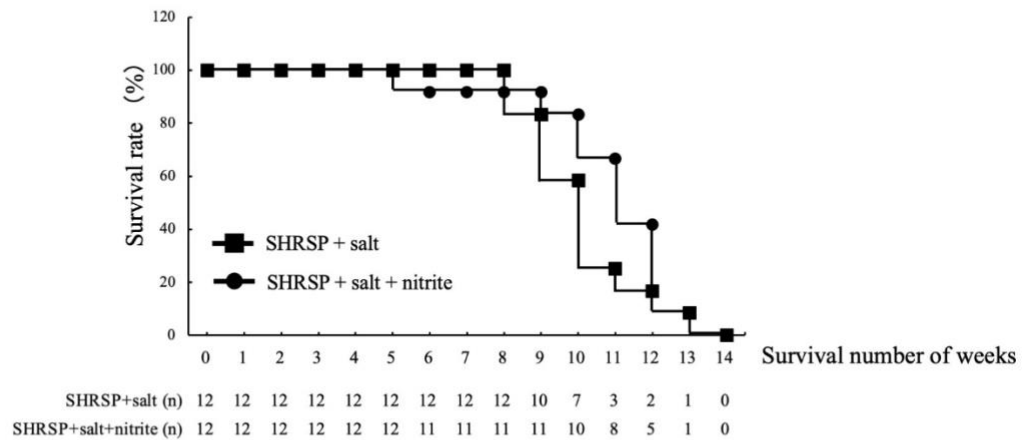


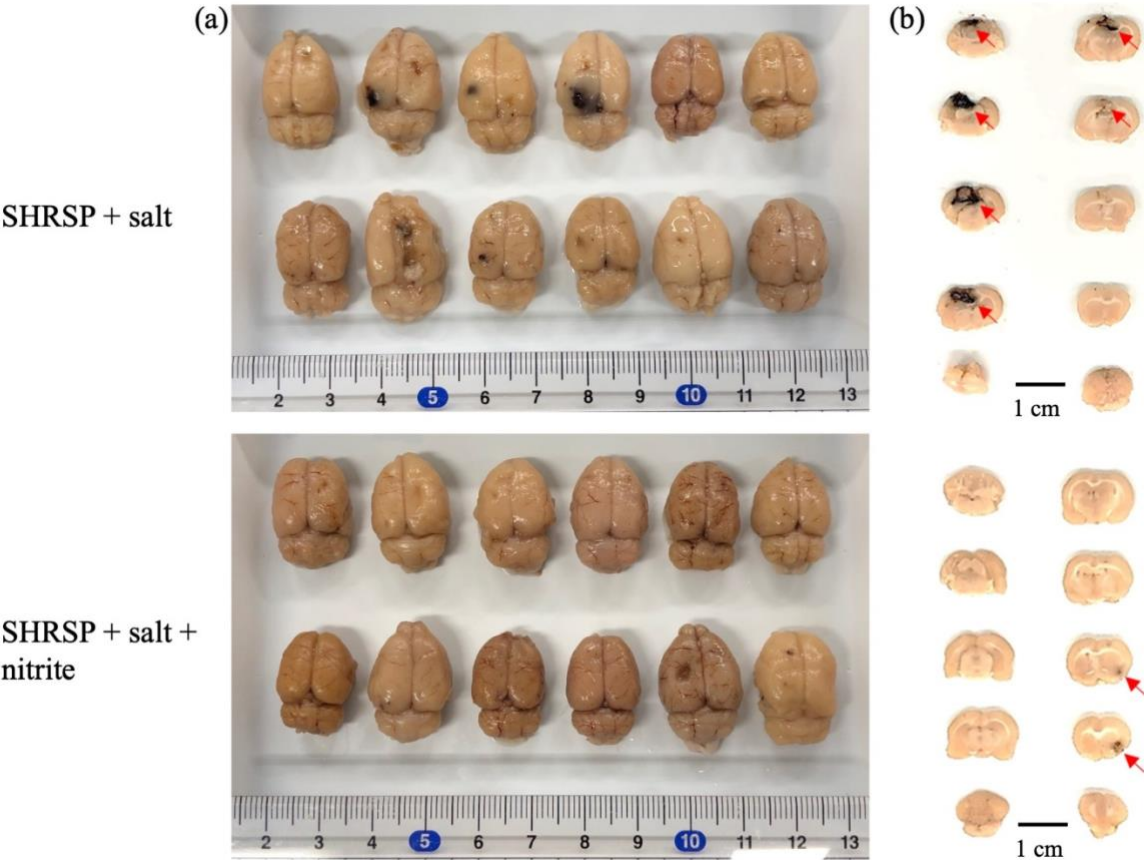
Figure 1. Survival rates of salt-loaded SHRSP rats with and without nitrite supplementation during the experimental study I.

The experiment started with $n = 12$ rats in each of the SHRSP + salt and SHRSP + salt + nitrite groups. At 14 weeks of age, 100% mortality was observed in both groups. The survival curves did not differ significantly, the significance of which was ascertained by log-rank testing ($P = 0.169$).

SHRSP, stroke-prone spontaneously hypertensive rats

527

Figure 2.



528

529 **Figure 2.** Idiopathic intracerebral hemorrhage was visible on the brain surface and in the images
530 of the cross-sections at the time of death during the experimental study I.

531 (a) Appearance of ICH sites in SHRSP + salt (n = 12) and SHRSP + salt + nitrite (n = 12) groups;
532 (b) Representative examples of brains divided into ten sections to confirm the extent of stroke
533 development. The black areas represented by the arrows indicate the ICH sites. The ICH
534 incidence was significantly lower in the nitrite-treated rats (41.7%, 95% CI: 19.3%–68.0%)
535 compared to control rats (91.7%, 95% CI: 64.6%–98.5%; $P = 0.027$, Fisher's exact test).

536 ICH, intracerebral hemorrhage; SHRSP, stroke-prone spontaneously hypertensive rats

Figure 2 consists of two dot plots, (a) and (b), comparing the effects of SHRSP + salt and SHRSP + salt + nitrite on brain cross-sections with ICH lesions and intracerebral hemorrhage area, respectively.

(a) Proportion of brain cross-sections with ICH lesions (%). The y-axis ranges from 0 to 50. The x-axis shows two groups: SHRSP + salt and SHRSP + salt + nitrite. A horizontal bar with two asterisks (**) indicates a significant difference between the groups.

Group	Individual Data Points (%)	Mean ± SEM (%)
SHRSP + salt	0, 10, 10, 10, 10, 20, 20, 20, 30, 40, 50	19.5 ± 1.5
SHRSP + salt + nitrite	0, 0, 0, 0, 0, 0, 10, 10, 10, 10, 20	5.5 ± 1.5

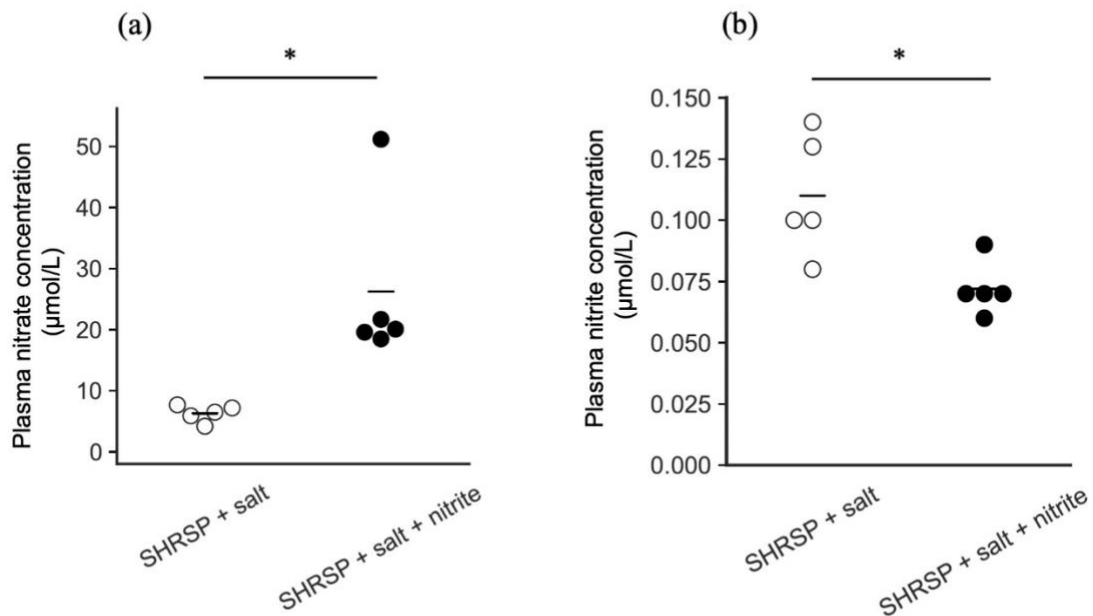
(b) Intracerebral hemorrhage area (%). The y-axis ranges from 0.0 to 2.0. The x-axis shows two groups: SHRSP + salt and SHRSP + salt + nitrite. A horizontal bar with $p=0.35$ indicates no significant difference between the groups.

Group	Individual Data Points (%)	Mean ± SEM (%)
SHRSP + salt	0.1, 0.1, 0.2, 0.2, 0.2, 0.5, 0.5, 0.6, 1.2, 1.6	0.48 ± 0.12
SHRSP + salt + nitrite	0.0, 0.0, 0.0, 0.0, 0.0, 0.0, 0.1, 0.1, 0.2, 0.3, 0.3	0.08 ± 0.05

538 **Figure 3.** Quantitative analysis of idiopathic intracerebral hemorrhage during the experimental
539 study I.

546 ICH, intracerebral hemorrhage; SHRSP, stroke-prone spontaneously hypertensive rats.

Figure 4.



547

548 **Figure 4.** Plasma nitrate (a) and nitrite (b) levels in SHRSP + salt (n = 5) and SHRSP + salt +
549 nitrite (n = 5) groups in the experimental study II.

550 The plasma nitrate levels were significantly greater in the SHRSP rats given nitrite + salt,

551 compared to the control SHRSP given salt alone for 4 weeks (a). In contrast, the plasma nitrite

552 levels were lower in the nitrite-supplemented animals than in the control ones (b). The total

553 plasma NO metabolites (nitrate + nitrite) levels were greater in the nitrite-treated rats than in the

554 controls. * $P < 0.05$; SHRSP + salt vs. SHRSP + salt + nitrite.

555 SHRSP, stroke-prone spontaneously hypertensive rats; NO, nitric oxide.

Figure 5.

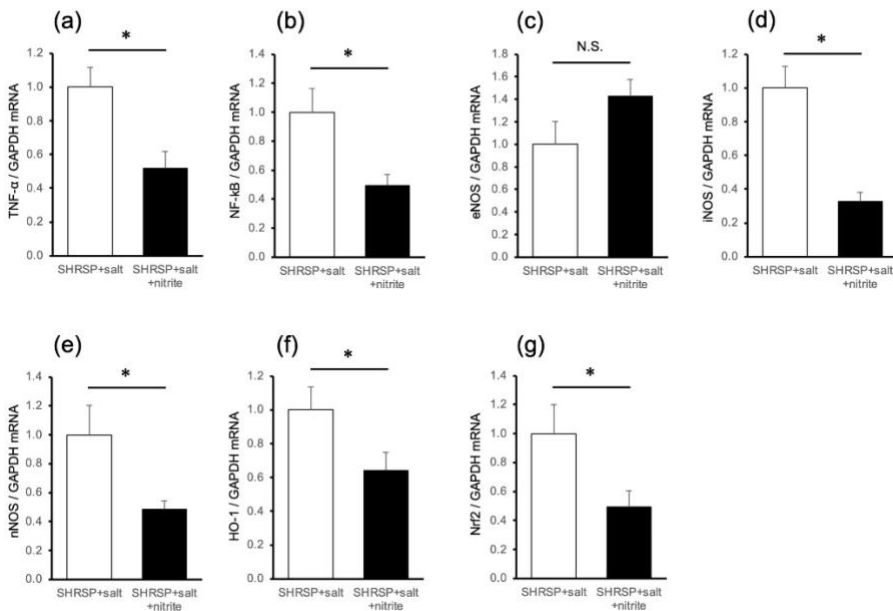


Figure 5. qRT-PCR analysis of inflammation-, NOS-, and oxidative stress-related genes in the brain of rats in the experimental study II.

mRNA levels of (a, b) the inflammatory cytokines, TNF- α and NF- κ B. (c, d, e) three NOS isoforms, eNOS, iNOS, and nNOS. (f, g) oxidative stress markers, HO-1 and Nrf2. *GAPDH* was used as the internal standard. All data are shown as the means $\pm$ SE; $n = 5$ in the two groups. * $P < 0.05$; SHRSP + salt vs. SHRSP + salt + nitrite.

NOS, nitric oxide synthase; TNF- α , tumor necrosis factor- α ; NF- κ B, nuclear factor- kappa B; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid 2-related factor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SE, standard error

Table1. Primer sequences used in mRNA quantitation by reverse transcription-polymerase chain reaction

Gene Name	Primer
Tumor necrosis factor-α (TNF-α)	F: 5'-ACTGAACTTCGGGGTGATTG-3' R: 5'-GCTTGGTGGTTTGCTACGAC-3'
Nuclear Factor kappa B (NF-kB)	F: 5'- GCGCATCCAGACCAACAATA -3' R: 5'- GCACTGTCACCTGGAAGCAG -3'
Endothelial nitric oxide synthase (eNOS)	F: 5'-GAATGCCCACAGCATCAGTT-3' R: 5'-TAGGCAAGCGCTTACCACT-3'
Inducible nitric oxide synthase (iNOS)	F: 5'- CCCTTCCGAAGTTTCTGGCAGCAGC -3' R: 5'- GGCTGTCAGAGCCTCGTGGCTTTGG -3'
Neuronal nitric oxide synthase (nNOS)	F: 5'- GAATACCAGCTGATCCATGGAAC -3' R: 5'- TCCTCCAGGAGGGTGTCCACCGCATG -3'
Heme Oxygenase-1 (HO-1)	F: 5'- CGAAACAAGCAGAACCCA -3' R: 5'- CACCAGCAGCTCAGGATG -3'
Nuclear factor erythroid 2-related factor 2 (Nrf2)	F: 5'- GGGGACAGAATCACCATTG -3' R: 5'- GATGCAGGCTGACATTCTGA -3'
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	F: 5'- TCAAGAAGGTGGTGAAGCAG -3' R: 5'- AGGTGGAAGAATGGGAGTTG -3'

F, forward; R, reverse

Table 2. The transition of body weight during 6-20 weeks of age in SHRSP + salt and SHRSP + salt + nitrite groups.

weeks	SHRSP + salt			SHRSP + salt + nitrite		
	Average	95%CI	n	Average	95%CI	n
6	155	150.8 - 159.2	12	152	147.8 – 156.2	12
7	193	187.2 - 198.8	12	192	188.1 – 195.9	12
8	220	214.1 - 225.9	12	217	213.6 – 220.4	12
9	237	232.1 - 241.9	12	238	235.0 – 241.0	12
10	254	249.0 – 259.0	12	255	251.3 – 258.7	12
11	270	264.6 - 275.4	12	274	268.3 – 279.7	12
12	283	277.4 - 288.6	12	286	279.3 – 292.7	11
13	291	285.2 - 296.8	12	305 *	298.0 – 312.0	11
14	281	263.9 - 298.1	12	313 *	300.8 – 325.8	11
15	255	228.3 - 281.7	10	306 *	286.2 – 325.8	11
16	237	209.4 - 264.6	7	268	240.6 – 295.4	10
17	250	176.9 - 323.1	3	258	230.0 – 286.0	8
18	227	-	2	243	216.7 – 269.3	5
19	212	-	1	265	-	1
20	-	-	0	209	-	1

Data are the means and 95% confidence intervals (CI)

*: $P < 0.05$, vs. SHRSP + salt group

Table 3. Comparison of organ weights removed after four weeks of feeding with nitrite in experimental study (2).

	SHRSP + salt	95%CI	SHRSP + salt + nitrite	95%CI
Body weight (g)	265.2	247.4 - 283.0	274.4	258.6 – 290.2
Brain weight (g)	1.95	1.84 – 2.06	1.77 *	1.71 – 1.83
Heart weight (g)	1.35	1.24 – 1.46	1.16 *	1.13 – 1.19
Liver weight (g)	9.53	8.43 – 10.63	9.90	9.12 – 10.68
Kidney weight (g)	2.18	2.01 – 2.35	2.31	2.12 – 2.50
Brain / body weight (%)	0.74	0.71 – 0.77	0.65 *	0.62 – 0.68
Heart / body weight (%)	0.51	0.48 – 0.54	0.42 *	0.39 – 0.45
Liver / body weight (%)	3.59	3.37 – 3.81	3.61	3.42 – 3.80
Kidney / body weight (%)	0.82	0.79 – 0.85	0.84	0.78 – 0.90

Data are means and 95% confidence intervals (CI). Sample size is n = 5 per group.

*: $P < 0.05$, vs. SHRSP + salt group

Table 4. The transition of systolic blood pressure during 8-12 weeks of age in experimental study (2).

	8 weeks of age	9 weeks of age	10 weeks of age	11 weeks of age	12 weeks of age
SHRSP + salt	191.4	222.8	227.4	228.0	257.2
(95%CI)	(179.7 – 203.1)	(215.0 – 230.6)	(215.2 – 239.6)	(216.9 – 239.1)	(237.0 – 277.4)
SHRSP + salt + nitrite	192.8	205.2 *	221.2	234.0	242.4
(95%CI)	(187.8 – 197.8)	(195.8 – 214.6)	(217.3 – 225.1)	(219.3 – 248.7)	(226.0 – 258.8)

Data are means and 95% confidence intervals (CI). Sample size, n = 5 per group.

*: $P < 0.05$, vs. SHRSP + salt group

Table 5. The biochemical data after 4 weeks of nitrite administration in experimental study (2).

	SHRSP + salt	95%CI	SHRSP + salt + nitrite	95%CI
Glu (mg/dL)	228.8	225.0 – 232.6	220.6	210.2 – 231.0
TG (mg/dL)	68.4	54.6 – 82.2	107.6 *	100.8 – 114.4
FFA (μEQ/L)	359.2	339.6 – 378.8	356.8	327.7 – 385.9
BUN (mg/dL)	16.4	15.5 – 17.3	18.5	15.9 – 21.1
Cre (mg/dL)	0.2	0.19 – 0.21	0.3 *	0.27 – 0.33
AST (IU/L)	84.6	81.0 – 88.2	77.8 *	75.4 – 80.2
ALT (IU/L)	66.6	61.9 – 71.3	63.2	59.3 – 67.1

Data are means and 95% confidence intervals (CI). Sample size, n = 5 per group. Glu, glucose; TG, triglycerides; FFA, free fatty acids; BUN, blood urea nitrogen; Cre, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

*: $P < 0.05$, vs. SHRSP + salt