Physiology and Pharmacology 28 (2024) 295-303
Experimental Research Article

Effects of dietary nitrate or nitrite supplementation on inhibitory avoidance task and pentylenetetrazole-induced clonic seizure threshold in mice

Shima Davoudi[1], Ablofazl Ardjmand[2,3], Azhdar Heydari[2,3*]

1. Student Research Center, Kashan University of Medical Sciences, Kashan, Iran
2. Physiology Research Center, Institute for Basic Sciences, Kashan University of Medical Sciences, Kashan, Iran
3. Department of Physiology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

ABSTRACT

Introduction: The nitrate-nitrite-nitric oxide (NO) is considered a possible alternative pathway for NO production. Consequently, this research aimed to assess how adding dietary nitrate or nitrite affects the inhibitory avoidance task, the threshold for clonic seizures induced by pentylenetetrazole (PTZ), and levels of nitric oxide metabolites (NOx) in mice.

Methods: In this research, 40 male NMRI mice were used, with 8 mice in each of the five groups including control and four experimental groups (given 50 or 100 mg/l nitrate or nitrite in drinking water for 21 days). The mice's memory retention was assessed through the step-down passive avoidance test, while their locomotor activity was measured using the open-field apparatus. The seizure threshold was determined by administering PTZ through intravenous infusion. Additionally, the levels of NOx in the brain tissue were quantified using the Griess method.

Results: Supplementation with either nitrate or nitrite at a concentration of 100 mg/L resulted in a significant increase in the step-down passive avoidance latency compared to the control group ($P<0.01$). Only nitrate at a concentration of 100 mg/L significantly increased the threshold for PTZ-induced clonic seizures ($P<0.001$). The levels of NOx were significantly elevated in all groups that received nitrate or nitrite at concentrations of 50 and 100 mg/L ($P<0.05$).

Conclusion: We conclude that the nitrate-nitrite-NO pathway is partly involved in the memory-improving effects of nitrate or nitrite and the increase of PTZ-induced clonic seizure threshold following nitrate supplementation.

Keywords:
Nitric oxide
Nitrite
Pentylenetetrazole
Memory
Step-down passive avoidance

Introduction

Nitric oxide (NO) is a gas-phase molecule that plays diverse roles in various physiological processes. NO is produced through the conversion of L-arginine by an enzyme known as nitric oxide synthase (NOS), which comes in different isoforms including neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms (Andrabi et al., 2023). The half-life of NO is short (about

* Corresponding author: Azhdar Heydari, heydariazh@kaums.ac.ir
Received 2 September 2023; Revised from 22 March 2024; Accepted 6 April 2024

Citation: Davoudi S, Ardjmand A, Heydari A. Effects of dietary nitrate or nitrite supplementation on inhibitory avoidance task and pentylenetetrazole- induced clonic seizure threshold in mice. Physiology and Pharmacology 2024; 28: 295-303. http://dx.doi.org/10.61186/phypha.28.3.295

1 second) and is quickly converted to NO_3^- (nitrate) and NO_2^- (nitrite) or reacts with amines or thiols (Bryan et al., 2004). The production of NO from its metabolites, including nitrite and nitrate, has been of significant interest (Tiso and Schechter, 2015). Dietary nitrate is reduced to nitrite by oral commensal bacteria. Furthermore, approximately a quarter of the nitrate present in circulation is actively absorbed by the salivary glands. Subsequently, the same bacteria in the oral cavity work to convert it into nitrite (Bedale et al., 2016). Subsequently, within the stomach's acidic conditions, nitrite undergoes reduction to form NO. Once formed, NO is metabolized to nitrite and nitrate, and these metabolites are converted to NO again (Bryan and Ivy, 2015).

NO plays a significant role in shaping the processes of learning and memory formation, as well as in triggering the occurrence of long-term potentiation (LTP) within the central nervous system (CNS). It has been reported that nitrite levels increased following learning of spatial tasks in the hippocampus (Harooni et al., 2009). Also, learning of foot-shock avoidance tasks and induction of LTP was accompanied by an increase in NO formation in the hippocampus (Bernabeu et al., 1995). NO precursors such as L-arginine or NO donors such as sodium nitroprusside (SNP) facilitated (Paul et al., 2005), while NG-nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, impaired learning and memory (Reddy et al., 2002). In contrast, the results of some studies imply that NO impairs learning and memory processes. For example, excess formation of NO by NO donors or L-arginine (as a NO precursor) at high doses impaired learning tasks (dos Reis et al., 2002; Pigott and Garthwaite, 2016). Meanwhile, selective nNOS inhibitor, 7-nitroindazole (7-NI), and L-NAME prevented the memory-impairing effect of high doses of L-arginine (dos Reis et al., 2002).

For many years, nitrate and nitrite have been considered harmful to human health due to their potential carcinogenic effects. Based on some contradictory reports, nitrite and nitrate have no or limited carcinogenic effect (Grosse et al., 2006). The results of animal and human studies imply that dietary nitrate supplementation has potential beneficial effects on blood pressure, exercise capacity, and metabolic syndrome (Siervo et al., 2013). However, less information is available on the effect of nitrate and nitrite in the CNS. Some studies have reported improvement of cognitive function by nitrate in humans (Lefferts et al., 2016; Wightman et al., 2015) or impairment of learning and memory in animals (Hu et al., 2015; Chen et al., 2016). To our knowledge, there is no data about the effects of inorganic nitrate or nitrite on seizure susceptibility. On the other hand, the role of NO in seizures is not exactly clear, and anticonvulsant (Xu et al., 2023) or proconvulsant (Zandieh et al., 2010) properties of NO have been reported in different seizure models. Overall, it seems that the nitrate-nitrite-NO pathway plays a role in both learning and memory functions, as well as seizure susceptibility. Hence, the objective of this research was to assess how nitrate or nitrite supplementation impacts tasks involving inhibitory avoidance, locomotor activity, thresholds for clonic seizures induced by pentylenetetrazole (PTZ), and the concentrations of total nitric oxide metabolites (NOx) in mice.

Material and methods

Animals

Male NMRI mice weighing between 25 and 30 grams were acquired from the animal facility at Kashan University of Medical Sciences. The animals were kept in typical polypropylene cages at a standard temperature of 25±2 ºC and a relative humidity of 50-60%. They experienced a controlled 12-hour light and 12-hour dark cycle, with the lights being turned on at 7:00 AM. The animals were fed with regular food and had unrestricted access to water. Randomly, animals were divided into five groups (with 8 animals in every group), consisting of a control group and four experimental groups. The experimental groups were exposed to sodium nitrate (at levels of 50 and 100 mg/l) or sodium nitrite (at levels of 50 and 100 mg/l) through their drinking water for 21 days. The animals in the control group were given regular tap water. Water consumption (ml/day), food intake (g/day), and body weights (g) were determined before and after nitrite or nitrate supplementation. Experiments were carried out during the light phase between 9.00 a.m. and 4.00 p.m. All experimental and animal care procedures accepted by the Research and Ethics Committee of "details omitted for double-blind reviewing" Kashan University of Medical Sciences (ethics code: I.R KAUMS.MEDNT.REC.1396.23) and conducted in accordance with the guidelines on laboratory animal care published by the National Institutes of Health (NIH Publication 8023, 1996).

Chemicals and Drugs

The provided substances were PTZ, sodium nitrate, and sodium nitrite (Sigma, US). All drugs were freshly prepared daily to the desired concentration.

Inhibitory avoidance task

The apparatus consisted of a plexiglass box (Height: 30 cm, Length: 40 cm, Width: 30 cm) resting on a stainless-steel floor with parallel bars 0.3 cm in diameter, spaced 1 cm apart. A plexiglass plate (4 cm×4 cm×4 cm) was positioned at the center of the grid floor. An electric shock (with a frequency of 1 Hz, lasting 0.5 seconds, and an intensity of 45 V) was administered to the grid floor via an isolated stimulator (from Borj Sanat Co., Iran). During the training phase, mice were gently positioned on the plate and given a foot shock lasting 15 seconds once they fully descended to the grid floor. This process was duplicated immediately and then repeated after a 1-hour interval from the initial training. Mice that remained on the plate for more than 60 seconds were deemed to have acquired the task. After 24 hours, the mice were once again placed on the plate, and the time it took for them to step down was measured using a stopwatch as a quantifiable measure of inhibitory avoidance behavior. The maximum time allowed for this test was 300 seconds (Nasehi et al., 2016).

Locomotor activity

The spontaneous locomotor activity of each animal was assessed using a designated apparatus called an open field (from Maze Router, Iran) for 5 minutes. This open field apparatus consisted of a square arena (length:50 cm, width: 50 cm, height: 40 cm) with black flooring and walls, and it was illuminated by overhead fluorescent lights (120 lx). During 5 minutes, mice receiving nitrate or nitrite, as well as those in the control group, were allowed to explore this open field. Before testing a new mouse, the arena was properly cleaned. The extent of locomotor activity was gauged by the horizontal distance (in centimeters) traveled by each mouse during the session, and this measurement was facilitated by a camera (Nasehi et al., 2016).

Seizure induction

The tail vein of each mouse was catheterized by connecting a needle (30-gauge) to a polyethylene (PE) tube (No. 10). The needle was secured in place using adhesive tape. A heparinized solution of PTZ (5 mg/ml) was infused into each mouse's tail vein. This was achieved using a syringe pump (from Top, Japan) operating at a rate of 0.5 ml/min. The time it took from the start of the infusion until the onset of clonic seizure was noted in seconds. Using the concentration of PTZ, the volume of infused PTZ in milliliters, and the body weight of the mouse, the recorded timings were converted into a seizure threshold value in mg/kg (Esmaili and Heydari, 2019).

Measurement of NOx

Brain tissue NOx levels were measured by the Griess reaction. In the first step, nitrite standard curves were prepared. To homogenize the brain tissue, a 50 mM Tris HCl solution containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) was used. The Griess reagent was then added to 100 µl of tissue suspension. This reagent comprises vanadium (III) chloride (VCl_3) (100 µl), sulfanilamide (50 µl), and N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) (50 µl). In order to convert nitrate into nitrite, VCl_3 was used. After centrifugation, the supernatants were transferred to flat-bottomed 96-well microplates. Based on standard nitrite curves, absorbance values were calculated using a microplate reader at 540 nm (Heydari and Davoudi, 2017).

Statistical analysis

The results were reported as mean ± SEM. The analysis of variance (ANOVA) was used to analyze the seizure thresholds and locomotor activity followed by Tukey's post-hoc for multiple comparisons. The significance level was the P value < 0.05.

Due to the skewed distribution of step-down latencies and NOx levels, we examined the data using the nonparametric Kruskal-Wallis ANOVA, followed by a two-sided Mann-Whitney U-test. In cases where suitable, we applied the Holmes Sequential Bonferroni Correction test for making paired comparisons.

Results

Effect of supplementation with nitrate or nitrite on the avoidance learning task or locomotor activity

The results depicted in Figures 1A and 1B illustrate the effects of supplementation with nitrate (50 and 100 mg/l) or nitrite (50 and 100 mg/l) for 21 days on the step-down latency in passive avoidance learning tasks

Fig.1A.

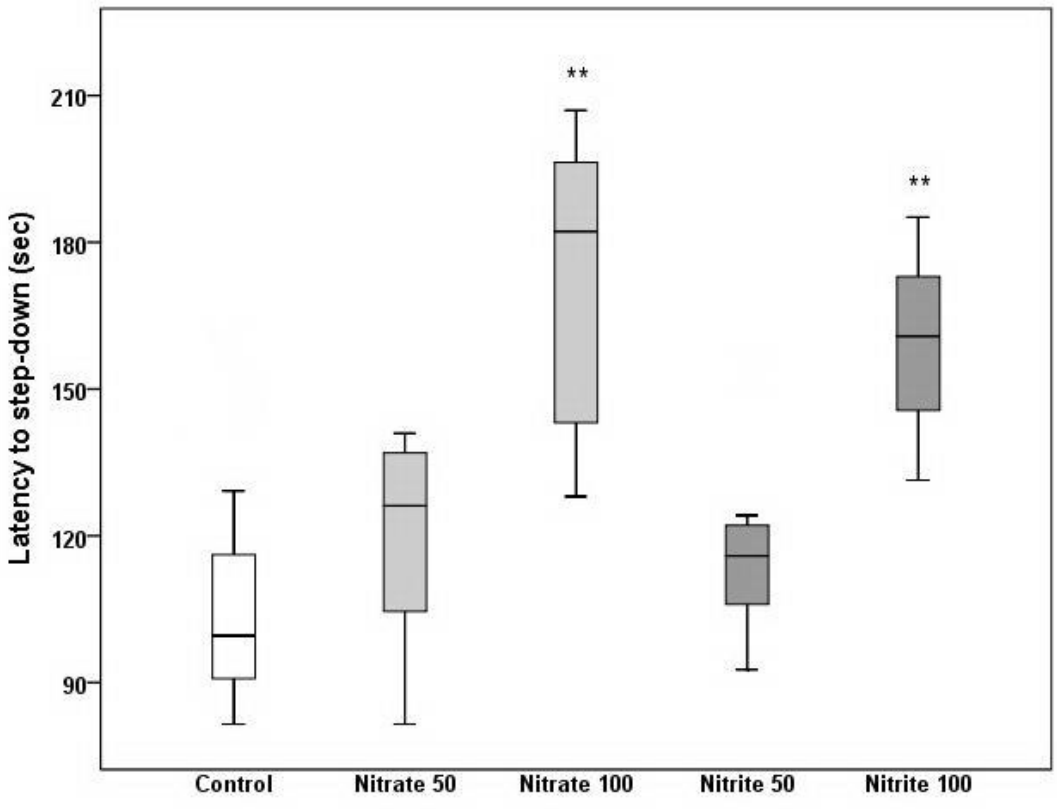

Fig.1B.

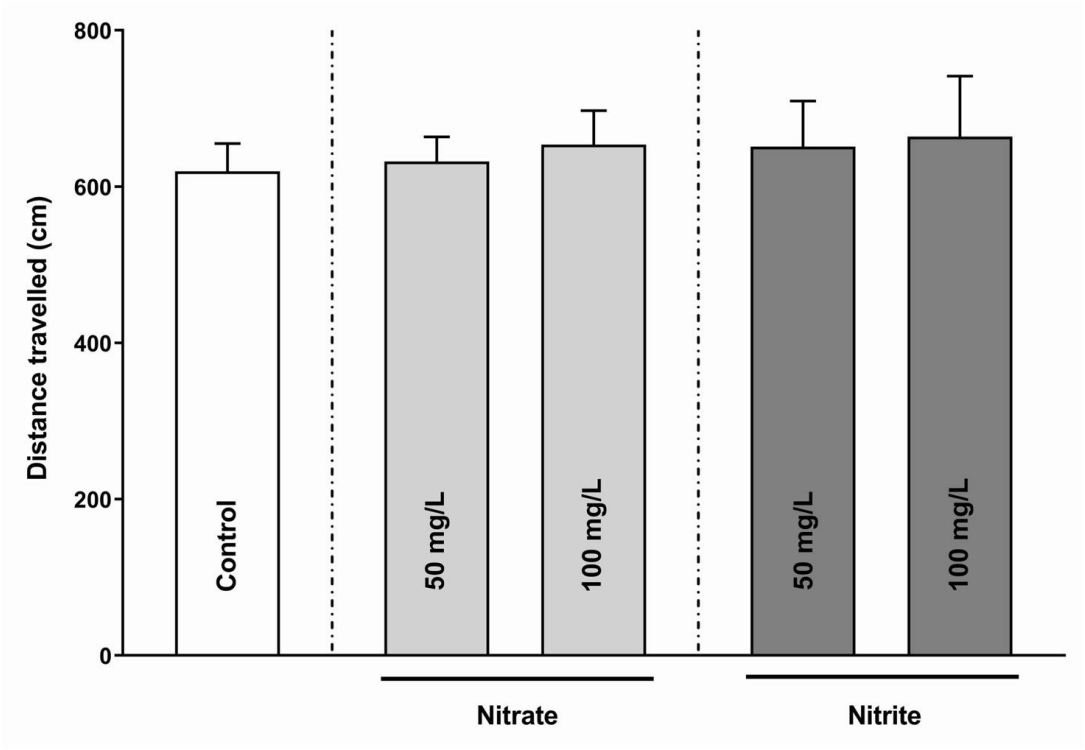

FIGURE 1. Effect of nitrate or nitrite supplementation at doses of 50 and 100 mg/l for 21 days on the step-down latency in passive avoidance learning task (1A) or locomotor activity as distance traveled in 5 min (1B). $^{**}P<0.01$ compared to the control group. Step-down latencies are expressed as median and quartile (n=8).

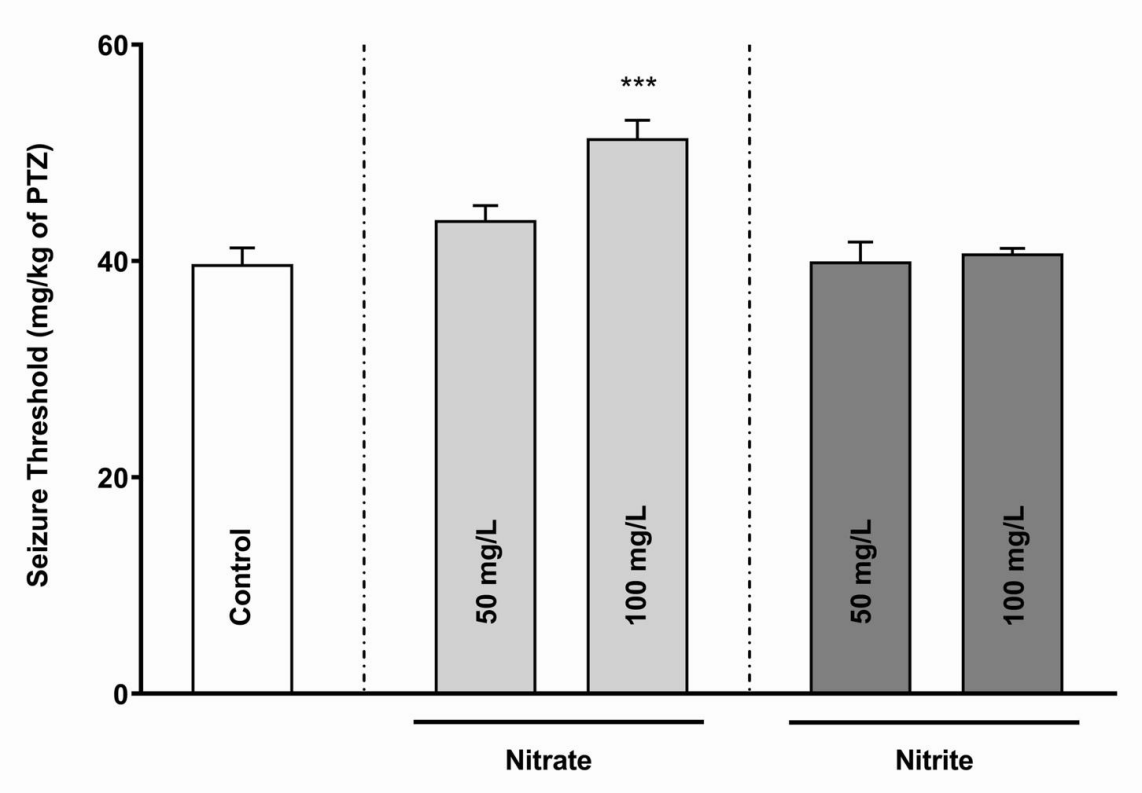

FIGURE 2. Effect of nitrate or nitrite supplementation at doses of 50 and 100 mg/l for 21 days on the PTZ-induced clonic seizure threshold. ***P<0.001 compared to control group (mean±SEM, n=8).

or locomotor activity, respectively. Kruskal–Wallis nonparametric ANOVA indicated that groups that received nitrate or nitrite experienced an enhancement in step-down latencies during the passive avoidance task [H (5) =134.99, P<0.001], which shows the memory-improving effects of nitrate or nitrite supplementation (Fig. 1A). Post-hoc analysis showed that nitrate or nitrate at a dose of 100 mg/l significantly improved the latency in passive avoidance learning task (P<0.01).

The locomotor activity was measured as the distance (cm) traveled in 5 min (Fig. 1B). The one-way ANOVA revealed that none of the doses of nitrate or nitrate significantly changed locomotor activity compared to the control group.

Effect of supplementation with nitrate or nitrite on the threshold for clonic seizures induced by PTZ

The results depicted in Figure 2 illustrate the effects of supplementation with nitrate (50 and 100 mg/l) or nitrite (50 and 100 mg/l) over 21 days on the PTZ-induced clonic seizure threshold. The results from conducting a one-way ANOVA and subsequent post-hoc examinations of the data indicated that solely the application of 100 mg/l of nitrate led to a significant enhancement in seizure threshold when compared with the control group. (F2, 27 = 16.89, P<0.001).

Effect of supplementing with nitrate or nitrite on the NOx levels

The results depicted in Figure 3 illustrate the effects of supplementation with nitrate (50 and 100 mg/l) or nitrite (50 and 100 mg/l) over 21 days on the NOx levels in brain tissue. Kruskal–Wallis nonparametric ANOVA indicated that the NOx levels increased in the brain tissue of all nitrate or nitrite receiving groups [H (5) =64.61, P<0.001]. The highest rise in NOx levels was achieved by using nitrite at a concentration of 100 mg/l. (P<0.001).

Discussion

The purpose of this research was to investigate the impact of supplementing the mice with nitrate or nitrite on the step-down passive avoidance test, seizure threshold, locomotor activity, and the NOx level. Although some studies have examined the effect of nitrite supplementation on learning and memory (Hu et al., 2015; Chen

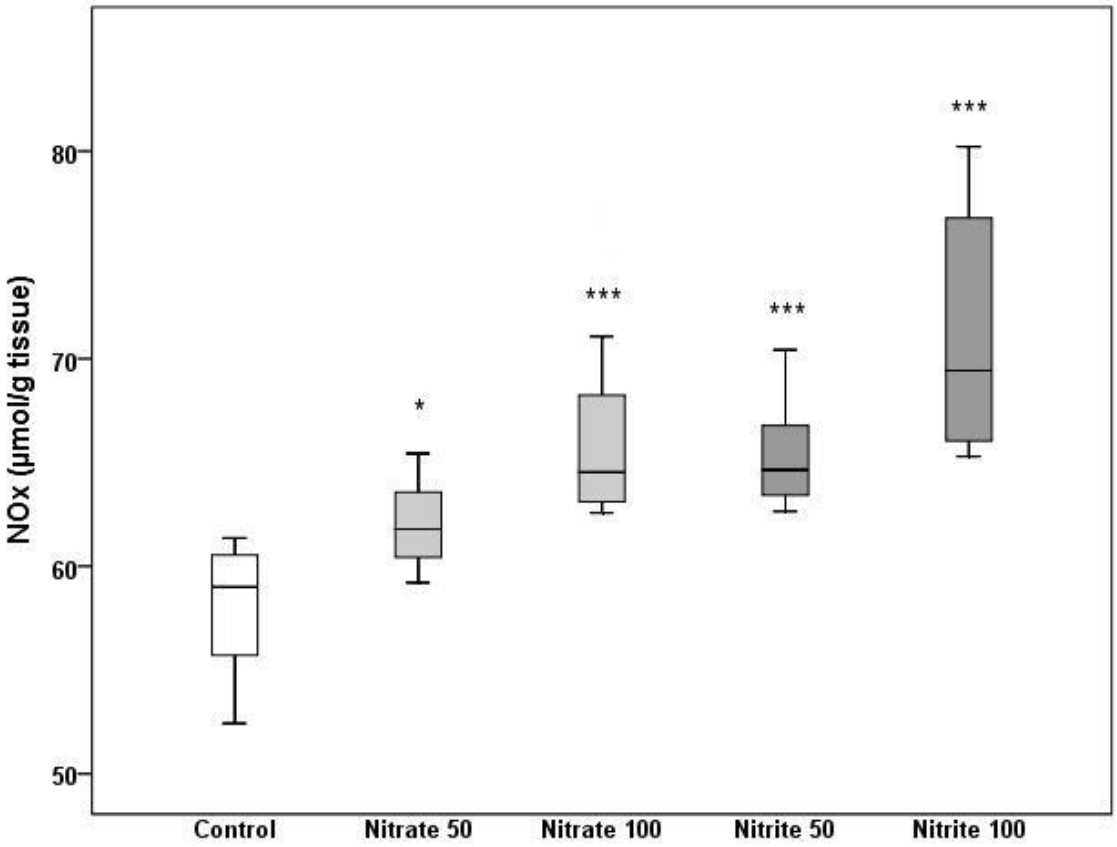

FIGURE 3. Effect of nitrate or nitrite supplementation at doses of 50 and 100 mg/l for 21 days on the NOx levels in the brain tissues. $^{*}P<0.05$ and $^{***}P<0.001$ compared to control group. The NOx levels are expressed as median and quartile (n=8).

et al., 2016), to our knowledge, this study is the first of its kind to investigate how nitrate or nitrite supplementation affects memory retention and seizure threshold in mice. The uniqueness of our study lies in its novelty. Our findings show that both nitrate and nitrite at a dose of 100 mg/l had a positive impact on memory enhancement. Contrarily, only nitrate at a dose of 100 mg/l had a protective effect against our seizure model. The NOx levels were increased in the groups supplemented with nitrate or nitrite at doses of 50 and 100 mg/l for 21 days.

The results of NOx measurement in our study confirm those studies showing that nitrate and nitrite supplementation increases NOx levels (Larsen et al., 2011; Lundberg et al., 2009; Piknova et al., 2011). Nitrite can be reduced to NO, which produces cyclic guanosine monophosphate (cGMP) through activation of soluble guanylate cyclase (sGC) (Bailey et al., 2018; DeMartino et al., 2019). Therefore, the formation of NO from nitrate and nitrite via the nitrate-nitrite-NO reduction pathway is likely to be a protective mechanism in our study. In most studies, the advantageous effects of inorganic nitrate or nitrite were observed without toxicity in the concentration range of 50 to 100 mg/L in drinking water (Bryan and Ivy, 2015). Excessive production of NO in high amounts is toxic and can lead to neurotoxicity (Dawson and Dawson, 2018). However, it seems unlikely that the supplemented doses of nitrate and nitrite in our study caused neurotoxicity.

NO production is likely the underlying mechanism by which nitrite or nitrate supplementation at 100 mg/kg significantly increased passive avoidance latency in our study. NO promotes the LTP of synaptic transmission by stimulating sGC and the subsequent increase of the cyclic adenosine monophosphate (cAMP) signaling pathway (Fukaya et al., 2023). The cAMP pathway plays an important role in the formation of memory (Glebov-McCloud et al., 2024). Therefore, the cAMP pathway, as a downstream target of the NO-cGMP pathway, to some extent can be involved in the memory-improving effects of nitrate or nitrite supplementation.

Moreover, it seems that some effects of nitrite and nitrate supplementation on learning depend on the dosage and are not directly mediated by NO production. For instance, nitrite increases cAMP levels by inhibiting cAMP-degrading phosphodiesterase (PDE) isoforms, namely PDE2A and PDE4A (Guimaraes et al., 2017). Nitrite and nitrate have a direct antioxidant effect with or without relation to NO production (Guimaraes et al., 2018). Another possible mechanism for the direct action of inorganic nitrite or nitrate may be through improvements in neuronal metabolism and cerebral blood flow (CBF). Some studies have reported that nitrite supplementation in healthy humans increases CBF and has an advantageous effect on cognitive performance (Lefferts et al., 2015) or neurovascular coupling (NVC) during increased cognitive demand (Wightman et al., 2015). Contrary to the above-mentioned mechanisms, chronic nitrite administration for 60 or 90 days resulted in the impairment of spatial learning and memory in rats (Hu et al., 2015; Chen et al., 2016). Different types of memory tests or different protocols for the timing of nitrate or nitrite supplementation may explain these discrepancies.

Nitrate or nitrite at a dose of 50 mg/l did not increase the passive avoidance latency. Likely, an adequate amount of NO is required for its direct or indirect positive impact on memory function. It has been reported in this regard that a basal tone of NO is required for its memory-improving effect in synapses (Paul and Ekambaram, 2011).

Only nitrate supplementation at a dose of 100 mg/l for 21 days had a protective effect against PTZ-induced clonic seizure. Since the production of NO needs the conversion of nitrate to nitrite, it is unlikely that the ability of nitrate to protect against seizures is solely due to NO production. Instead, it appears that pathways other than NO-cGMP may participate in the protective effect of nitrate against seizures induced by PTZ. Gamma-aminobutyric acid (GABA) as the principal inhibitory neurotransmitter in the CNS has an important role in the prevention and treatment of epilepsy (Richardson et al., 2024). Supplementation with sodium nitrate (60 mg/l) for 18 days significantly increased the plasma level of GABA with a concomitant decrease in glutamine as a GABA metabolic precursor (Roberts et al., 2017). Thus, an increase in the GABA level by nitrate may be an important possible protective mechanism against seizures. Dietary nitrate reduces oxidative stress and improves mitochondrial efficiency (Carlstrom, et al., 2011). Since free radicals are involved in seizure development (Devi et al., 2008), it is possible that the protective effect of nitrate is in part through the attenuation of oxidative stress. Meanwhile, sialin expression by nitrate in the CNS may contribute to its protective effect on seizures. Sialin is a mammalian membrane nitrate transporter that delivers nitrate and other anions, including glutamate and aspartate (Qin and Wang, 2022). Overexpression of sialin in the presence of high amounts of nitrate but not nitrite may partially explain the different effects of nitrate and nitrite on seizures, although this possibility needs further investigation.

Conclusion

This study is the first to demonstrate the advantageous impacts of supplementation with both nitrate or nitrite on the inhibitory avoidance task and nitrate alone on the seizure threshold. Increased production of NO and NO-like species or potentiating of NO-cGMP pathway are likely the main mechanisms by which nitrate or nitrite increased the latency in the step-down passive avoidance test. Contrarily, pathways independent of NO may also be responsible for the advantageous impact of nitrate to prevent the seizures induced by PTZ. Further research is necessary to fully understand the effects of nitrate or nitrite supplementation on the CNS.

Acknowledgments

This work was supported by the Deputy of Research, Kashan University of Medical Sciences, Kashan, Iran [grant number 94166]. The authors report no disclosures, nor conflicts of interest.

Conflict of interest

The authors declare no conflicts of interest.

Ethics approval

All experimental and animal care procedures accepted by the Research and Ethics Committee of "Kashan University of Medical Sciences (ethics code: I.R KAUMS.MEDNT.REC.1396.23) and carried out in accordance with the National Institutes of Health guidelines (NIH Publication 8023, 1996) on laboratory animal care standards.

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