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Original Article

Prenatal Exposure to L-Citrulline Has Positive Effects on Reflexive Motor Behavior in Newborn Mice

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Abstract

L-citrulline (L-cit) is a nonessential amino acid that interacts with the veracity of neurotransmitters in the brain and might have a positive effect on fetal growth. However, there is no information about the possible effect of L-cit on reflexive motor behavior. Thus, this study aimed to determine the effects of prenatal exposure to L-cit on reflexive motor behavior in mice offspring. Forty pregnant female mice were allocated into four groups. In the control group, mice received water, while in groups 2-4, female mice orally gavage with L-cit (25, 50, and 100 mg/kg) at 5, 8, 11, 14, and 17 days of gestation (GD). Following delivery, pups were selected, and reflexive motor behaviors were determined using ambulation, hind-limb foot angle, surface righting, hind-limb strength, grip strength, front limb suspension, and negative geotaxis tests. Also, serum Nitric oxide (NO), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined. Based on the findings, maternal exposure to L-cit improved ambulation score, hind-limb suspension score, grip strength, and front-limb suspension in offspring (P<0.05). Prenatal exposure to L-cit decreased surface righting, hind-limb foot angle, and negative geotaxis in offspring (P<0.05). L-cit decreased immobility time in forced swimming test (FST), tail suspension test (TST), and increased number of squares crossed in the open field test (OFT) and spending time on rotarod on postpartum mice (P<0.05). L-cit increased serum NO levels (P<0.05). L-cit decreased blood MDA and increased SOD and GPx levels in offspring (P<0.05). Findings revealed that L-cit improves postpartum behaviors in mice and reflexive motor in their pups.

Keywords: L-Citrulline, Maternal exposure, Motor reflex, Mice

1. Introduction

L-citrulline (L-cit) is a nonessential amino acid and a major watermelon component produced by the liver (1). Because L-cit is not only a precursor of L-arginine but also a by-product of nitric oxide (NO), several beneficial properties are reported for it (1). Maternal exposure to the L-cit by enhancing NO production decreases hypertension in rat offspring (2). L-cit improves cerebral blood flow in migraine model rats and prevents the excessive and uncontrolled generation of the NO (2). Supplementation of the L-cit enhances performance during high-intensity anaerobic exercise (3). Reactive oxygen species (ROS) have been proposed to contribute to and/or maintain chronic pain conditions (4). Free radicals derived from ROS such as hydrogen peroxide, superoxide, and NO enhanced nociceptive response (5). Interestingly, L-cit acts as a potent antioxidant by reducing ROS production, but scarce information exists on this effect on oxidative stress (6).

Gestational exposure to chemicals might have positive and negative effects which influence infant

growth, development, and long-term mental health (7). Animal studies revealed maternal exposure to drugs or chemicals leads to behavioral despair such as decreased play behavior, novel object exploration, conspecific interactions even suppressed pain response in rats (8). However, it is important to note the differences between prenatal development in humans and rodents. Rodent brains are less developed following delivery than human babies, so this concern must be noticed in findings from animal model studies (9).

It is assumed that L-cit plays a vital role in several physiological functions. Decreased NO bioavailability and increased reactive oxygen species (ROS) production were seen in the hypertension pathophysiology. Maternal supplementation of the L-cit decreases hypertension by enhancing NO production and decreasing ROS generation (10). NO plays a crucial role in angiogenesis and the regulation of vascular tone, particularly during intrauterine fetal growth restriction (11). L-cit increases fetal growth by increasing NO production and muscle protein synthesis (11). Even though the beneficial effect of the L-cit during pregnancy is reported, there is no information about the possible effect of the L-cit in pregnancy on subsequent reflexive motor development in neonatal. Thus, the current study aimed to determine the effect of prenatal exposure to L-cit on reflexive motor behavior in neonatal mice.

2. Materials and Methods

2.1. Animals and Procedure

The 16 male and 40 virgin female NMRI mice (28–30 g weight and 8–10 weeks old) were supplied from Pasture Institute (Tehran, Iran). The mice acclimatized for a week before the beginning of the study at laboratory conditions (temperature of 22±2°C and 12/h light/dark cycle) with *ad libitum* access to standard chow pellet and fresh water. Then, the female mice were caged with fertile male mice. Each morning, the female mice were examined for the presence of the sperm or vaginal plug to determine pregnancy. The pregnant mice were randomly assigned into four groups. In control, pregnant mice received water as a placebo, while in groups 2-4, pregnant female mice orally gavage with L-cit (25, 50, and 100 mg/kg) at 5, 8, 11, 14, and 17 days of gestation (GD), respectively. Following parturition, 20 male pups from each litter were selected based on anus-vaginal distance and used to determine postnatal (PD) reflexive and behavior neonatal motor tests. Mice offspring are removed from the dam for no more than 15 minutes to prevent body heat and hunger/ separation issues. Also, postpartum depressive tests were done in dams. The dosage of the L-cit was determined based on previous reports (1, 12, 13).

2.2. Ambulation

Crawling behavior appears in mice offspring up to PD 5 and transitions to walking starting when they are 5-10 days old (14). To take advantage of transitional time, the ambulation test was performed in 8 days old offspring. Mice were placed in a transparent enclosure, visible from the top to all 4 sides. To motivate pups to walk, we used a gentle tail prod. The ambulation score was: 0= no movement, 1= crawling with asymmetric limb movement, 2= slow crawling but symmetric limb movement, and 3= fast crawling/ walking. To eliminate alterations in the test due to learning, the test was performed in triplicate within 3 min (15).

2.3. Hind-Limb Foot Angle

This test was carried out on 8 days old pups. By maturing the pups from crawling to walking, hind limb posture changes wherein walking; the hind limbs are positioned under the body. Therefore, the angle among the hind limbs in the walking position was less than in the crawling position (15). A plain open field box with a camera above was used to record the pup's movements around the box. The mice were forced to walk by touching the pup's tail. Recorded videos determined the foot angle. In recorded videos, a line was drawn from the end of the heel/shin to the tip of the toe. The measurements were recorded only in the pups that were performing a full stride in a straight line and their feet were flat on the ground. To minimize experimental errors, the average of the 3–5 sets of foot

angles was calculated for each pup. No repeat-related learning was observed in this test (15).

2.4. Front‑Limb Suspension

This test was conducted on PD 12–15 days, in which pups were permitted to hang onto the wire with both forepaws. After grasping the wire, pups were released, and the time needed to fall was recorded in seconds. To minimize alterations in testing due to learning, the test was performed in triplicate within 3 min (15).

2.5. Hind-Limb Suspension

The hind-limb suspension test was performed to determine pups' strength and neuromuscular function in pups younger than 10 days old and beginning on day 3. Pups were placed face down into the standard 50 mL conical laboratory tube. The mice's hind legs hung over the wire and were released, and the hind-limb posture score was recorded as score 0: constant clasping of the hind limb by holding onto the tube; score 1: weakness was apparent, and the hind-limb were almost in a clasped position with the tail raised; score 2: hind-limb were close to each other and often touching; score 3: weakness was apparent, closer together and rarely touched each other; score: 4 normal hind limb separation with tail raised (15). To minimize alterations in testing due to learning, the test was carried out in triplicate within 3 min (15).

2.6. Surface Righting

The surface righting reflex is a motor ability of the offspring to flip onto their feet from their supine position (16). This can be measured by the surface righting Test. This test was performed on 5–7 days old pups, placed on their backs on a cotton sheet, and kept in position for 5 seconds. Then, they were released, and the time needed to return to their prone position was recorded. As no-repeat-related learning was reported in this test, triplicate within 3 min was done (15).

2.7. Grip Strength

The test was conducted on 7–8 days old offspring to determine the grip strength in which animals can grab onto a screen and generate a reading of the grip force. In summary, a 16 × 18 fiberglass screen was used in which the surface was rotated slowly from a horizontal to a vertical position to challenge the grasping of all four limbs (17). The hanging impulse which indicated the force needed to resist gravity was calculated per the below formula:

[weight (g) × latency to fall (s)] (18).

2.8. Negative Geotaxis

This test was performed on PD 7 pups. Mice were placed down on a 45° wooden surface which is used as a slope. Then, they were released, and the time needed by pups to face the slope upward due to vestibular cues of gravity was documented (15).

2.9. Forced Swimming Test (FST)

The FST was done according to a previously reported method (19). On day 2 postpartum, dams were individually plunged in ht: 25 cm; diameter: 15 cm) containing 10 cm of water to a cylindrical glass container at 25 °C. Each Mouse was left in the cylinder for 6 min. When dams ceased struggling and remained motionless in the water, the total duration of immobility during the last 4 min of the 6 min testing period was measured.

2.10. Tail Suspension Test (TST)

TST is a common technique for assessing mice's antidepressant-like activity (20). The TST was performed based on the method described by Steru, Chermat (21) and Alimohammadi, Hosseini (22). Briefly, on day 2 postpartum, dams were away from nearest objects and were both acoustically and visually isolated from observing or interacting with each other. Then, dams were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the extremity of the tail. Immobility time was monitored during a 6 min period.

2.11. Open Field Test (OFT)

The OFT was used to determine the possible effects of L-cit on the locomotor and exploratory activities in mice. The test was done using a 45×45×30 cm^3 wooden box. The floor of the open field box was divided by masking tape markers into 9 squares. On day 2 postpartum, each dam was placed individually at the center of the apparatus. Then, the number of segments crossed with the four paws was recorded for a period of 6 min (23).

2.12. Rotarod Test

The accelerated rotarod test is a standard sensory-motor test to investigate animals' motor coordination and learning skills by measuring the ability of the dams to stay and run on the accelerated rod. The test was done for 8 min with an acceleration of 0–20 rpm. The time was recorded when dams fell off the rod or started to rotate with the rotarod without running. After an initial training trial, dams were tested for 5 trials over two days. The recovery phase between trials was 10 min (24).

2.13. Biochemical Assay

At the end of the tests, blood samples were collected via cardiac puncture, and serum MDA, SOD, and GPx were determined using Zell Bio GmbH (Germany) assay kits. Also, the Greiss colorimetric method determined NO concentration in the blood serum. An ELISA reader measured samples' optical density (OD) at the wavelength of 540 nm (25).

2.14. Statistical Analysis

Obtained data were analyzed by one-way analysis of variance (ANOVA) and presented as the mean±standard error (SE). For treatments having differences, mean values were compared with the Tukey HSD test. $P<0.05$ were considered to indicate significant differences among the treatments.

3. Results

Based on figure 1, L-cit (50 and 100 mg/kg) increased gestational weight in mice offspring compared to the control mice ($P<0.05$).

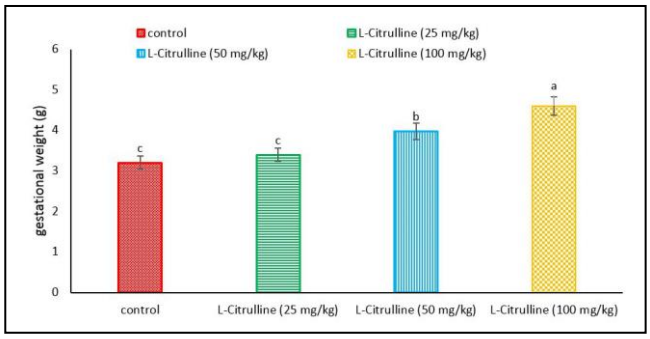

Figure 1. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on gestational weight in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments ($P<0.05$)

According to figure 2, maternal exposure to L-cit (50 and 100 mg/kg) increased ambulation scores in newborns compared to the control mice ($P<0.05$) (Figure 2).

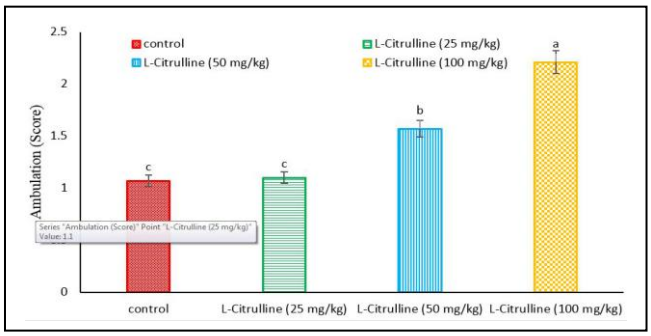

Figure 2. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on Ambulation score in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments ($P<0.05$)

Prenatal exposure to L-cit (50 and 100 mg/kg) decreased hind-limb foot angle in pups compared to the control mice ($P<0.05$) (Figure 3).

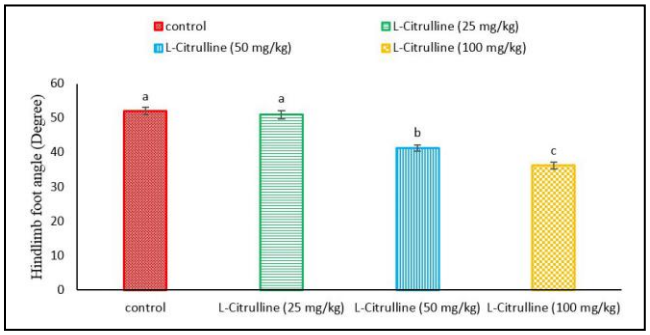

Figure 3. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on Hindlimb foot angle in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments ($P<0.05$)

L-cit (50 and 100 mg/kg) decreased surface righting in offspring than in the control mice ($P<0.05$) (Figure 4).

Prenatal exposure to L-cit (50 and 100 mg/kg) enhanced grip strength in mice offspring in comparison to control mice ($P<0.05$) (Figure 5).

As seen in figure 6, maternal exposure to the L-cit (50 and 100 mg/kg) improved front limb suspension than the control mice offspring ($P<0.05$).

In experiment 7, L-cit (50 and 100 mg/kg) increased the hind-limb suspension score compared to the control pups ($P<0.05$) (Figure 7).

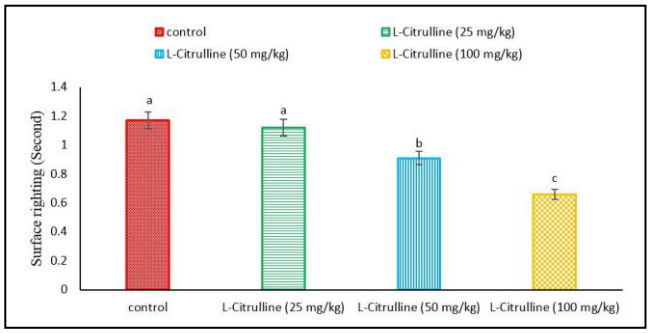

Figure 4. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on surface righting (s) in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

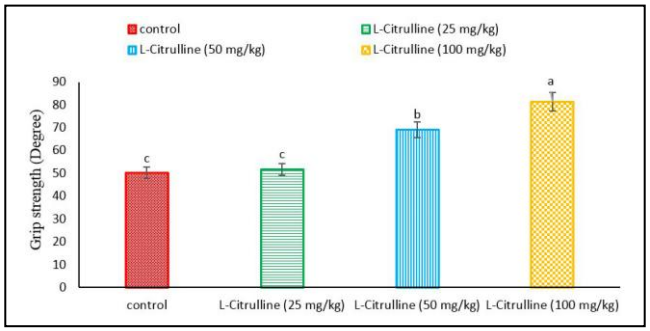

Figure 5. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on grip strength in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

Maternal exposure to L-cit (50 and 100 mg/kg) significantly diminished negative geotaxis in mice offspring (P<0.05) (figure 8).

According to figure 9, L-cit (50 and 100 mg/kg) increased stay on the rotarod in postpartum mice compared to the control pups (P<0.05).

In this study, L-cit (50 and 100 mg/kg) increased the number of the cross in the OFT following postpartum than in the control group (P<0.05; figure 10).

Also, exposure to L-cit (50 and 100 mg/kg) decreased immobility time in FST in postpartum mice compared to the control mother (P<0.05) (Figure 11).

Additionally, exposure to L-cit (50 and 100 mg/kg) significantly decreased immobility time in TST in postpartum mice (P<0.05) (Figure 12).

The results of the biochemical assay are presented in table 1. L-cit (25, 50, and 100 mg/kg) in a dose-dependent manner increased serum NO level

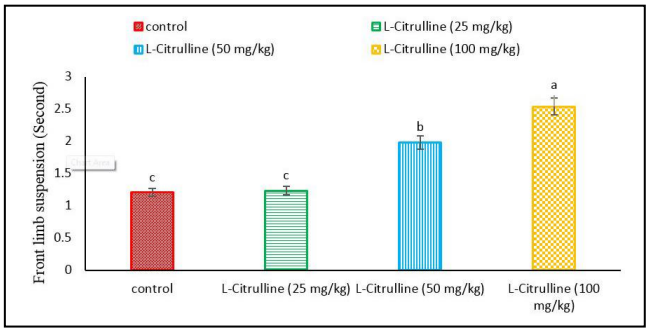

Figure 6. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on front limb suspension (s) in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

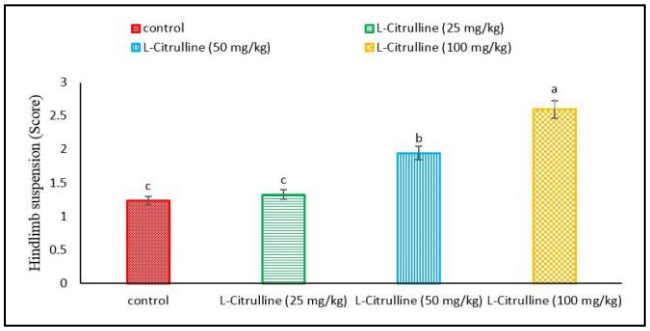

Figure 7. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on hindlimb suspension score in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

compared to the control group (P<0.05). L-cit (50 and 100 mg/kg) significantly reduced the MDA levels in offspring compared to the control group (P<0.05). Also, L-cit (50 and 100 mg/kg) significantly amplified the SOD and GPx levels compared to the control group offspring (P<0.05).

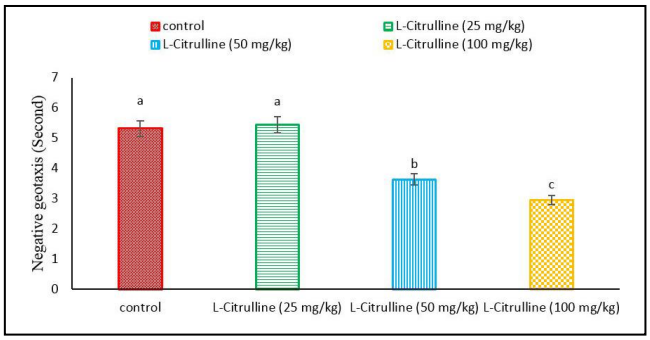

Figure 8. The effect of prenatal exposure to L-Citrulline (25, 50 and 100 mg/kg) on negative geotaxis (s) in mice offspring. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

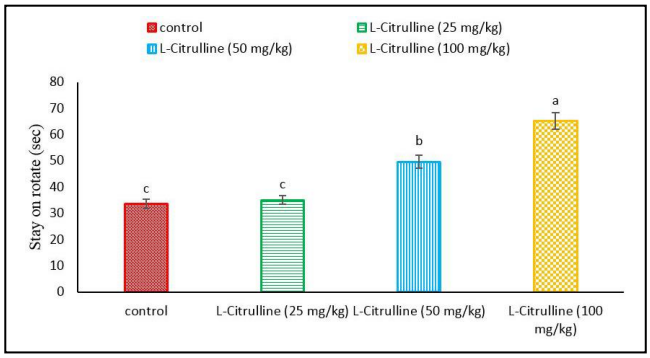

Figure 9. Effect of exposure to different levels of L-Citrulline (25, 50 and 100 mg/kg) during pregnancy on stay on the rotarod (s) in postpartum mice. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

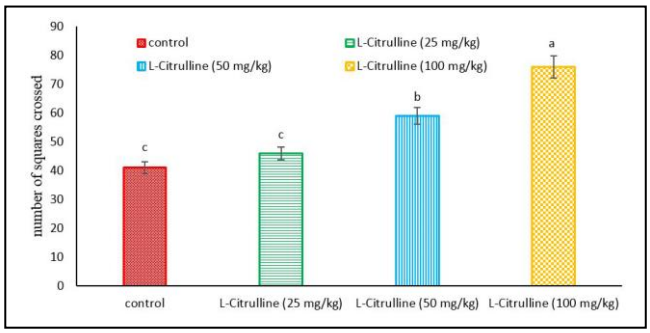

Figure 10. Effect of exposure to different levels of L-Citrulline (25, 50 and 100 mg/kg) during pregnancy on number of squares crossed in open field test (OFT) in postpartum mice. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

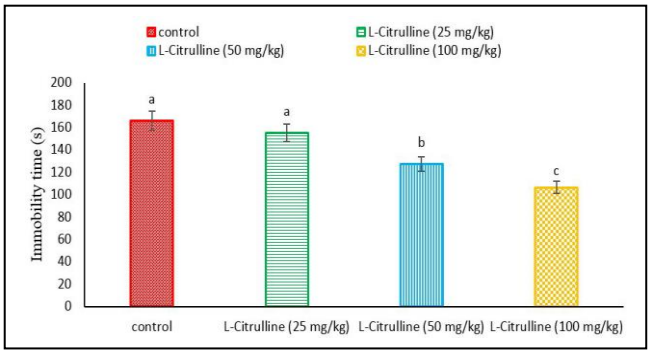

Figure 11. Effect of exposure to different levels of L-Citrulline (25, 50 and 100 mg/kg) on immobility time (sec) in forced swimming test (FST) on postpartum mice. Data are expressed as mean±SE. Different letters (a–c) indicate significant differences between treatments (P<0.05)

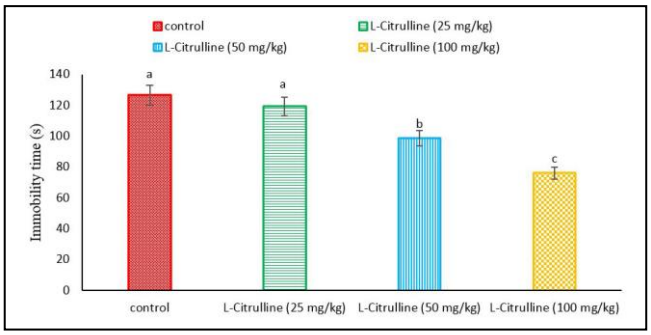

Figure 12. Effect of exposure to different levels of L-Citrulline (25, 50 and 100 mg/kg) during pregnancy on tail suspension test (TST) in postpartum mice. There are significant differences between groups with different superscripts (a-c; P<0.05)

Table 1. Effect of different levels L-citrulline on serum values of Malondialdehyde, Superoxide dismutase and Glutathione peroxidase in mice offspring (n=20)

Group	NO (OD%)	MDA (nmol/ml)	SOD (IU/ml)	GPx (IU/ml)
Control	24.2±2.33^{d}	7.84±0.54^{a}	11.99±0.84^{c}	5.35±0.67^{c}
L-citrulline (25 mg/kg)	29.65±2.62^{c}	7.49±0.64^{a}	11.46±0.61^{c}	5.99±0.39^{c}
L-citrulline (50 mg/kg)	45.95±3.62^{b}	5.99±0.55^{b}	15.58±0.61^{b}	6.99±0.95^{b}
L-citrulline (100 mg/kg)	50.64±3.86^{a}	4.56±0.53^{c}	19.77±0.67^{a}	7.54±0.55^{a}

NO: nitric oxide, MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase. Different letters (a-c) indicate significant differences between treatments (P<0.05)

4. Discussion

This is the first report on prenatal exposure to the L-cit on reflexive motor behaviors, antioxidant activity in mice offspring, and anti-depressive behaviors in postpartum dams. Based on the findings, maternal exposure to L-cit improved ambulation score, hind-limb suspension score, grip strength, and front-limb suspension in pups. Prenatal exposure to L-cit decreased surface righting, hind-limb foot angle, and negative geotaxis in pups. Maternal exposure to L-cit dose-dependently increased pups' serum NO levels. Several beneficial effects have been reported to exhibit on physiological functions for L-cit. Orally administration of the L-cit increases NOS expression in the hippocampal CA_1 region following brain ischemia (1). Also, NO has neuroprotective effects and increases arteriogenesis during cerebral ischemia (26). Oral L-cit supplementation improved alveolar growth and pulmonary hypertension in bronchopulmonary dysplasia (27).

NO has a deniable role in physio-pathological conditions in the brain, from cell signaling to cytotoxic host defense mechanisms. In the neonatal hypoxia-ischemia model, vast NO production with superoxide radicals leads to oxidative stress and mitochondrial dysfunction. Thus, adequate NO levels are needed for proper brain function (28). Both L-arginine and L-cit increase NO production. Although the direct mechanism for How L-cit acts is not fully elicited, it is assumed that L-cit enhances fetal growth by different mechanisms. Maternal exposure to L-cit improves maternal nutritional status and placental function, which can directly influence fetal growth (11). Also, L-cit-induced NO production enhances placental angiogenesis and blood supply. This is proved in an animal model in which treatment with L-NAME, the NO inhibitor, decreases NO production and induces a preeclampsia-like syndrome (29).

Abnormal reflexes lead to delays in acquisition, absence, or reappearance in life and are predictive of developmental disabilities. Reflexes provide a quick and easy method of assessing neurological development at very young ages where more complex behavioral testing is not feasible (30). Thus, experimental models of neurodevelopmental disabilities can use to determine reflex delays and rodents are the ideal animal model to mimic these motor skills (30). Chemicals or medications might have influenced the brain's prenatal development during gestation, which has positive and adverse effects on cognitive functions during adulthood (31). Neurodevelopmental reflexes are a valuable technique for assessing neurodevelopmental in neonates. Abnormal synaptogenesis, functioning, and myelination lead to delayed neurodevelopmental reflexes or absences (30).

Interestingly, NO-induced by L-cit can increase brain-derived neurotrophic factor (BDNF) in the differentiation of neural stem cells (32). BDNF signaling is associated with neurogenesis, and there is an interaction between BDNF and NO, which can act as trans-synaptic signaling molecules in the brain (32). However, we could not determine BDNF levels following L-cit administration in pups based on limitations. Perhaps, L-cit enhanced reflexive motor behaviors mediated by BDNF in mice offspring.

As observed, L-cit decreased immobility time in FST and TST, increased the number of squares crossed in OFT, and spent time on rotarod on postpartum mice. Postpartum depression is a kind of depression that happens following parturition. Its main signs are sadness and anxiety in mothers. Even though numerous animal and human-based studies have been done, the physiological mechanisms and role of the underlying brain areas are not yet well understood (33). Commonly applied antidepressants act by the monoaminergic system and selective serotonin reuptake inhibitors and tricyclic antidepressants are routine remedies. However, side effects and low effectiveness lead to an interest in natural bioactive components or herbal remedies with fewer side effects (34). The FST and TST do not reproduce the

pathophysiology of depression, but they are useful in that they induce changes that are sensitive to therapeutic agents in a manner predictive of their effects in humans. The FST and TST have been used extensively for this purpose, but the selectivity of these tests for monoamine-based mechanisms may limit their ability to detect novel mechanisms (35).

L-cit decreased blood MDA and increased SOD and GPx levels in offspring. Hassan-Danboyi, Jimoh (13) reported orally administration of the L-cit (200, 400, and 800 mg/kg/day) decreased MDA levels and improved suppressed catalase, SOD, and GPx levels in high‐fat diet‐ and dexamethasone‐induced Type‐2 Diabetes Mellitus in rats. Oseni, Odesanmi (36) reported that administration of the watermelon crude extract for 1 week increases GPx and SOD levels in diabetic rats. L-cit increases SOD and GSH activity in acute renal failure in rats (37). It is suggested that an increase in SOD activity scavenges a large amount of H_2O_2, which triggered the increase in the CAT activity. Also, L-cit-induced NO production can augment by endogenous GPx. Thus, L-cit acts by preventing excessive and uncontrolled ROS production and the production and metabolism of NO (13). Interestingly, a relation was observed between NO and the antioxidant system. It is observed elevated mRNA expression of iNOS with increased SOD and CAT expression can increase the antioxidant defense system (38).

The first limitation of our study was not to measure the NO levels in the brain—the second limitation was not to measure the concentration of the antioxidant enzymes in the brain during training. In conclusion, findings revealed that L-cit improves postpartum behaviors in mice and reflexive motor in their pups.

Authors' Contribution

Design, Proofing the paper: Sh. H.

Graduate student, Experimental procedure, Draft of paper: A. R.

Graduate student, Experimental procedure, Draft of paper: P. H.

Ethics

All the procedures were approved by the ethics committee of the Islamic Azad University, Tehran, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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