An Acad Bras Cienc (2020) 92(4): e20190925 DOI 10.1590/0001-3765202020190925
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Ammonia exposition during gestation induces neonatal oxidative damage in the brain and long-term cognitive alteration in rats

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Abstract: Ammonia is involved in the pathogenesis of neurological conditions associated with hyperammonemia, including hepatic encephalopathy. Few is known about the effects of gestational exposition to ammonia in the developing brain, and the possible long-term consequences of such exposure. We aimed to evaluate the effects of ammonia exposure during the gestation and the possible long-term cognitive alterations on pups. Eight female rats were divided into two groups: (1) control (saline solution); (2) ammonia (ammonium acetate, 2,5mmol/Kg). Each rat received a single subcutaneous injection during all gestational period. The brains from 1-day-old rats were obtained to the determination of thiobarbituric acid reactive species (TBARS), protein carbonyl and nitrite/nitrate levels. Some animals were followed 30 days after delivery and were subjected to the step-down inhibitory avoidance task. It was observed a significant increase in protein carbonyl, but not TBARS or nitrite/nitrate levels, in pups exposed to ammonia. Rats exposed to ammonia presented long-term cognitive impairment. Gestational exposition to ammonia induces protein oxidative damage in the neonatal rat brain, and long-term cognitive impairment.

Key words: hyperammonemia, gestational period, offspring, oxidative stress, cognitive dysfunction.

INTRODUCTION

Ammonia is a cytotoxic metabolite that is removed primarily by hepatic ureagenesis in humans (Davuluri et al. 2016). It is involved in the pathogenesis of neurological alterations associated with hyperammonemia, including hepatic encephalopathy (HE) (Skowronska & Albrecht 2012). In excess can be neurotoxic caused impairs glutamatergic and GABAergic neurotransmission by altering membrane expression of glutamate and GABA receptors, resulting in impaired spatial learning (Felipo & Butterworth 2002, Hernandez-Rabaza et al. 2016). In addition, ammonia can induce oxidative stress and disturbances of nitric oxide (NO) production, besides, induces astrocytes and microglia activation in the hippocampus, increasing pro-inflammatory cytokines IL-1β and IL-6 (Cagnon & Braissant 2007, Haussinger & Schliess 2008, Hernandez-Rabaza et al. 2016).

Brain is more susceptible to the deleterious effects of ammonium in childhood when compared to adulthood (Braissant et al. 2013). Several general mechanisms are associated with brain injury in the neonatal period and these include increased levels of intracellular calcium, oxidative stress, inflammation, decreased levels of trophic factors, and mitochondrial

dysfunction (DiMauro & Schon 2008, Morato et al. 2014). At later times, this increase may cause irreversible damage to developing CNS, such as cognitive impairment and cerebral palsy (Braissant et al. 2013). In fact, Gropman et al. (2007), Enns (2008) and Tuchman et al. (2008) demonstrated that neonates and infants present hyperammonemia develop cortical atrophy, ventricular enlargement, and demyelination. In addition, all these effects of ammonia on CNS may lead to energy deficit, oxidative stress and cell death, mainly in neuronal cells (Braissant et al. 2013, Hernandez-Rabaza et al. 2016).

Cell culture studies and animal experiments suggest that the induction of oxidative/nitrosative stress is closely related to the pathogenesis of cerebral ammonia toxicity (Reinehr et al. 2007, Schliess et al. 2002). In addition, the toxic effects of ammonia could be related to alterations in cerebral microcirculation, zinc homeostasis and gene transcription (Kruczek et al. 2009). Even though the effects of hyperammonemia in the brain are recognized, few is known about the effects of gestational exposition to ammonia, and the possible long-term consequences of such exposure to pups. In this context, we aimed to evaluate the effects of ammonia exposure during gestation and the possible long-term cognitive alterations on pups.

MATERIALS AND METHODS

Animals

Female Wistar albino rats (60-90 days old, weighting mean 300±50g, n = 8), and their pups, were used in this study. All experimental procedures were performed with the approval of the Ethics Committee from Universidade do Extremo Sul Catarinense (protocol number: 61/2012) and conformed to international regulations. Animals had water and food *ad libitum* and were maintained on a 12 h light–dark cycle (lights on at 7:00 a.m.), at a temperature of 23 °C ± 1 °C. These conditions were maintained constant throughout the experiments.

Experimental procedure

Animals were randomly divided in two groups: (1) control group (phosphate buffered saline solution – PBS, n=4); (2) ammonia group (ammonium acetate, 2.5mmol/Kg, n=4). Each rat received a daily, subcutaneous injection during all gestational period, as previously described (Yonden et al. 2010). The dose was selected on the basis of earlier reports that indicated increased ammonia levels in the brain after systemic injections (Hermenegildo et al. 2000). Twenty-four hours after delivery, ten pups of each group were killed and their brains were removed to the determination of thiobarbituric acid reactive species (TBARS), protein carbonyl, nitrite/nitrate and ammonia levels. Thirty days after the delivery, a total of 15 animals each group were subjected to the step-down inhibitory avoidance task. The experimental design is shown in Figure 1.

Ammonia levels

Ammonia levels were determined using a colorimetric kit (Sigma-Aldrich). Briefly, ammonia reacts with α-ketoglutaric acid and reduced nicotinamide adenine dinucleotide phosphate in the presence of L-glutamate dehydrogenase to form L-glutamate and oxidized nicotinamide adenine dinucleotide phosphate. The decrease in absorbance at 340 nm is proportional to the ammonia concentration (SpectraMax Plus 384). The Ammonia Assay Kit may be used to determine ammonia concentrations in the range of 0.2-15 µg/ml.

Protein carbonyl levels

The oxidative damage to proteins was assessed by the determination of carbonyl groups content

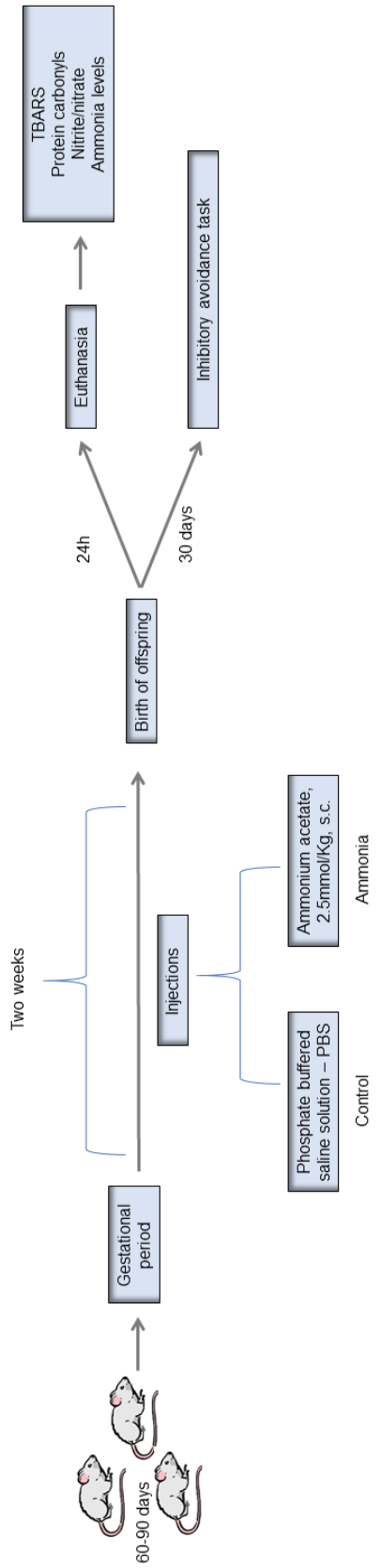

Figure 1. Experimental design.

based on the reaction with dinitrophenylhidrazine (DNPH), as previously described (Levine et al. 1990). Proteins were precipitated by the addition of 20% trichloroacetic acid (Sigma-Aldrich) and were re-dissolved in DNPH (Sigma-Aldrich). The absorbance was monitored spectrophotometrically at 370 nm (SpectraMax Plus 384).

Thiobarturic acid reactive species (TBARS) measurement

The substances reactive to thiobarbituric acid (TBARS) are formed as a by-product of lipid peroxidation, and their quantification is analyzed as a primary cytotoxic event capable of triggering a sequence of cell injuries, caused by reactive oxygen species. The test consists of evaluating the levels of malondialdehyde (MDA) present in the sample, as well as generated from lipid hydroperoxides through a substance reactive to the heating of thiobarbituric acid, formed during peroxidation in membrane systems (Draper & Hadley 1990).

First, the samples were weighed and homogenized with a specific buffer for this technique (Na2PO4 - KCl (Sigma-Aldrich)) in a 10x potter in 1ml of TBARS buffer. Soon after, the standard curve was performed with different concentrations of TBA (0; 0.02; 0.1; 0.5; 1 nmol of TBA) diluted in distilled water. 500 µL of each sample was homogenized and mixed with 10% trichloroacetic acid (TCA) (1: 2 portion) (Sigma-Aldrich). The contents were centrifuged (10 min at 1000 rpm). Right after, 500ul of the supernatant was removed from the centrifuged samples and placed in a test tube. 500ul of 0.67% thiobarbituric acid (TBA) (Sigma-Aldrich) diluted in distilled water was added to each sample. The samples were heated in a water bath at 100 ° C for 30 min, and the product generated from the reaction was read spectrophotometrically at a wavelength of 532 nm (SpectraMax Plus 384).

TBARS concentrations were expressed as MDA equivalents (nmol / mg protein).

Nitrite and nitrate levels (NOx)

The principle of the assay used is reduction of nitrate by vanadium (III) (Sigma-Aldrich) combined with detection by the acidic Griess reaction, as previously described by (Miranda et al. 2001). The absorbance at 540 nm (SpectraMax Plus 384) was measured using a plate reader following incubation for 30 minutes. NOx levels were expressed as nmol/mg protein.

Protein measurement

Total protein measurements were determined by the Lowry et al. method (1951) and bovine serum albumin was used as a standard. first, to perform the standard curve 0.0005g of bovine albumin (BSA) was used for 1ml of distilled water (concentration of 0.10; 20; 50; 80; 100 ug of BSA). To perform the technique, 10 µL of the homogenized tissue were used, and mixed with 190ul of distilled water.

Reactive C was prepared which consists of reactive A (0.1N NaOH - 4g + 2% Na2CO3 - 20g; diluted in 1000ml of distilled water) + reactive B (cupric sulfate (1% in distilled water) and sodium / potassium tartrate (2% in distilled water)) (Sigma-Aldrich). 1000ul of reactive C was added to each sample and to the curve and waited for 10 min. Then Folin 1N (Sigma-Aldrich) was added, stirred, and waited for 30 min, in order for the reaction to occur. Right after the readings were performed on a spectrophotometer with a wavelength of 700nm (SpectraMax Plus 384).

Step-down inhibitory avoidance task

The animals were subjected to inhibitory avoidance procedure as previously described by Roesler et al. (1999). The apparatus was an acrylic box (50 × 25 × 25 cm) whose floor consisted of parallel-caliber stainless-steel bars (1 mm diameter) spaced 1 cm apart, and a platform that was 7 cm wide and 2.5 cm high (EP 104MC – insight ltda). Animals were placed on the platform and their latency to step down on the grid with all four paws was measured with an automatic device. Training session was performed 10 days after surgery. Immediately after stepping down on the grid, animals received a foot shock of 0.4 mA and 2 s. In test session carried out 24 h after training, no foot shock was given and the step-down latency (maximum of 180 s) was used as a measure of retention. This behavioral test was performed by a same blinded person.

Statistical analysis

All data from biochemical analyses were fitted in a standard distribution curve and were therefore, subjected to parametric analyses. Data from biochemical were presented as mean $\pm$ SEM, and to compare the levels of TBARS, carbonyl protein, nitrite and nitrate levels between control and ammonia exposed animals it was performed a Student´s t test (n=10, each group). Data from the inhibitory avoidance task were reported as median and interquartile ranges and comparisons among groups were performed using Mann–Whitney *U* tests. The within individual groups were analyzed by Wilcoxon tests. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed using the SPSS 20.0 software for Windows (SPSS, Chicago, IL).

RESULTS

In this study, there were no perceptible alterations in the behavior of pregnant rats exposed to ammonia. In addition, there was no increase in neonatal mortality, birth weight or gross malformations in ammonia-exposed

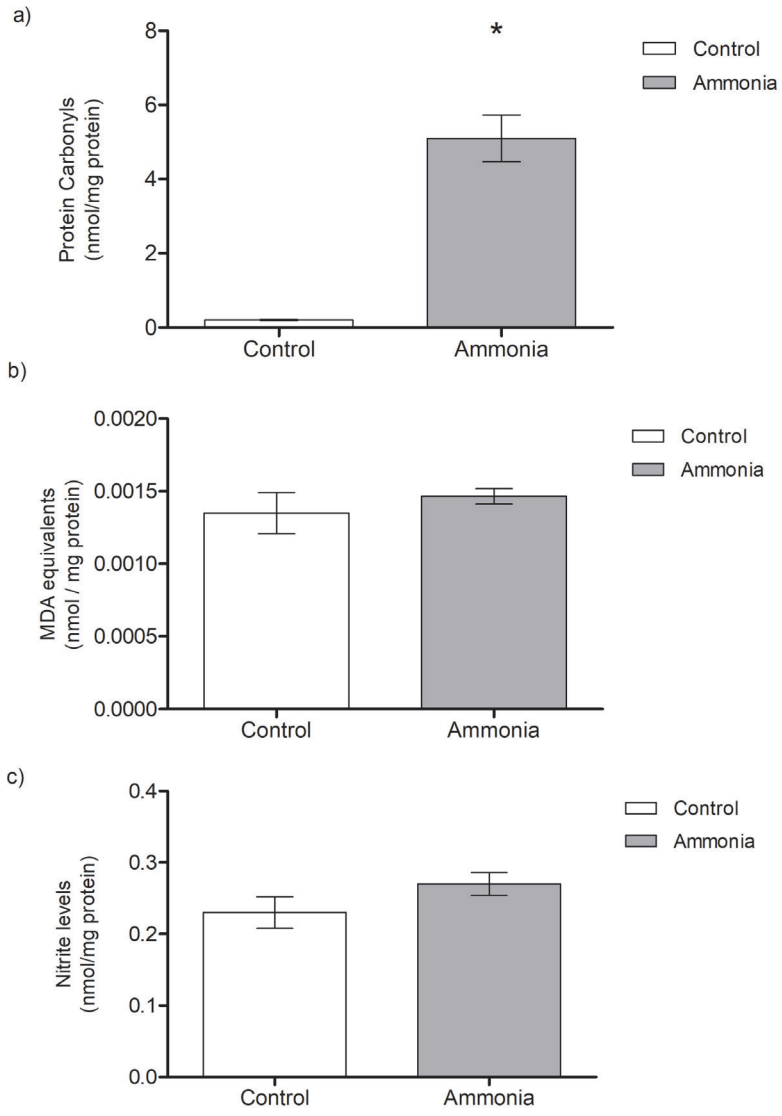

Figure 2. (a) Protein oxidative damage in the brain of pups after gestational ammonia exposure. (b) Thiobarbituric acid reactive species (TBARS) levels in the brain of pups after gestational ammonia exposure. (c) Nitrite / nitrate (nox) levels in the brain of pups after gestational ammonia exposure. Pregnant rats were daily treated with a single injection of ammonium acetate (2.5mmol/Kg) or saline during all gestational period. Twenty-four hours after delivery pups were killed and their brains were removed to the determination of nox. Data are presented as mean ± SD, (n=10, each group). *Different from control, (*P*<0.05, Student T test).

pups when compared to control. Plasma ammonia concentration in pregnant rats was not significantly different between groups (data not shown). In contrast, brain ammonia levels were significantly higher in pups of exposed pregnant rats (0.034 + 0.039 vs 0.018 + 0.0057 µg/mg protein, p = 0.03).

Early after delivery, gestational exposure to ammonia increased brain carbonyl groups content when compared to offspring of females that received the treatment with PBS solution (p<0.05) (Figure 2a). However, there was no significant increase in TBARS and nitrite/nitrate levels in ammonia-exposed animals when compared to control group (Figures 2b and 2c). Since oxidative damage in proteins can be associated with cognitive dysfunction it was determined the performance on the inhibitory avoidance task 30 days after delivery.

As shown in Figure 3, in the inhibitory avoidance test, the animals of both groups presented significant difference between the training and test sessions. However, animals exposed to ammonia presented a decreased latency time in the test section when compared to control group, suggesting memory impairment.

DISCUSSION

We here demonstrated that gestational exposition to ammonia induced protein oxidative damage in brain during neonatal period as well as long-term cognitive impairment. This seems to be more relevant since there was no increase in neonatal mortality, birth weight or gross malformations in ammonia-exposed pups, as well as in plasma ammonia concentration in pregnant rats. Hyperammonemia in the adult brain does not provoke significant neuronal loss or structural damage to neurons, in contrast to the observed in developing CNS (Walker 2009). In general, perinatal brain injury is predominantly caused by inflammation/infection and hypoxic-ischemic events that cause metabolic dysfunction and cell death (Thornton et al. 2012). In addition, the heperammonemia during brain development is associated with neuronal cell loss and cerebral atrophy leading to mental retardation and cerebral palsy in pediatric patients (Braissant et al. 2013).

The exact mechanism of ammonia neurotoxicity is not completely understood. In pediatric patients, the pathogenic mechanisms of NH_4^+ toxicity to the brain involve alterations in amino acids pathways, neurotransmission systems, cerebral energy, NO synthesis, axonal and dendritic growth or signal transduction pathways (Braissant et al. 2013). Furthermore, it is commonly accepted that astrocytes are the primarily affected cells in HE (Aschner & Allen 2000, Northrop et al. 2016). In pathological circumstances, astrocytes can induce neuronal dysfunction, but they can also play an essential role in the protection of neurons against excitotoxicity (Mates et al. 2002, McKenna 2007, McKenna & Ferreira 2016). Ammonia can lead to impairment of astrocyte function (Bai et al. 2001) and oxidative stress appears to be a major mechanism related to this effect (Alvarez et al. 2011, McKenna & Ferreira 2016).

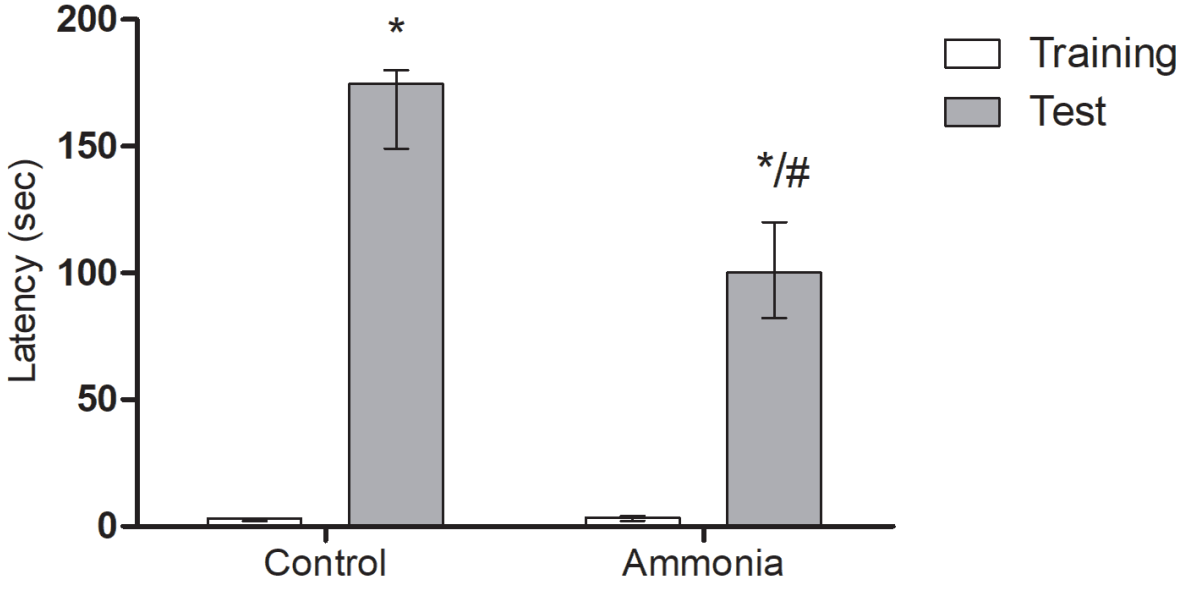

Figure 3. Long-term inhibitory avoidance performance pups after gestational ammonia exposure. Pregnant rats were daily treated with a single injection of ammonium acetate (2.5mmol/Kg) or saline during all gestational period. Thirty days after delivery animals were subjected to the step-down inhibitory avoidance task. Data are presented as median and interquartile ranges, (n=15, each/group). *Different from training section in the same group (*P*<0.05, Wilcoxon test). #Different from test section compared to control group (*P*<0.05, Mann-Whitney test).

The role of reactive oxygen species (ROS) in the pathogenesis of brain injury has been investigated. Probably an immature brain is more susceptible when compared to an adult one and this can be secondary to lower levels of endogenous antioxidants (Khan & Black 2003). Furthermore, excitotoxic mechanisms appear to be involved in brain oxidative stress being a major death pathway in different models of brain injury (Davuluri et al. 2016, Deng et al. 2006, Gorg et al. 2010). There is a close relationship between astrocyte dysfunction and oxidative stress involving a self-amplifying cycle: astrocyte dysfunction induces oxidative stress through a NMDA receptor-dependent mechanism. In addition, NMDA receptor activation and oxidative stress triggers astrocyte dysfunction (Haussinger & Schliess 2008, Hermenegildo et al. 2000, Walker 2009). In this context, ammonia can trigger release of glutamate from astrocytes (Weaver 2007) leading to NMDA receptor activation (McKenna & Ferreira 2016, Schliess et al. 2002, Shen et al. 2008).

During the perinatal period, toxic factors do not lead to major brain injury, but they can cause long-lasting cognitive, motor, and/or behavioral impairments (Volpe 2009, 2011). In addition, neonatal exposure to ammonium impairs memory and conditioned learning, but do no produce such effects in adults (Aguilar et al. 2000). Long-term potentiation is significantly decreased in hippocampal slices from rats exposed to ammonia during the neonatal period (Munoz et al. 2000), and these findings corroborate with our results.

Some limitations must be noted when interpreting our results. Firstly, it was not measured intrauterine or pups' brain levels of ammonia. Thus, we could not ascertain that gestational ammonia administration really increased ammonia levels in pups' brain. On the other hand, since the only intervention that these animals were submitted was ammonia injection during pregnancy it is plausible to suppose that the observed alterations in pups were secondary to ammonia exposure. Secondly, from three measured oxidative damage parameters only protein carbonyl was increased in pups' brain. Oxidative damage is a dynamic process, and repair mechanisms, as well as turnover of molecules involved could interfere in its identification. To ascertain that oxidative damage is involved in the observed long-term cognitive damage interventions aimed to decrease oxidative damage should be a target for further studies.

In conclusion, our results suggest that gestational exposition to ammonia induces protein oxidative damage in the neonatal rat brain. Moreover, this exposition induces long-term cognitive impairment that could be relevant to human exposition.

Acknowledgments

This research was supported by Universidade do Extremo Sul Catarinense (UNESC) and Instituto Nacional de Ciências e Tecnologia – Medicina Translacional (INCT).

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How to cite

DOMINGUINI D, DALL'IGNA DM, NOGUEIRA L, STECKERT AV, GONÇALVES RC, MICHELS M, QUEVEDO J, RITTER C, BARICHELLO T & DAL-PIZZOL F. 2020. Ammonia exposition during gestation induces neonatal oxidative damage in the brain and long-term cognitive alteration in rats. An Acad Bras Cienc 92: e20190925. DOI 10.1590/0001-3765202020190925.

Manuscript received on August 29, 2019;
accepted for publication on October 7, 2020

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Dhébora Mozena Dall'Igna, Lauro Nogueira designed the study. Diogo Dominguini and Dhébora Mozena prepared the manuscript. Felipe Dal-Pizzol edited the manuscript and supervised the study. Lauro Nogueira, Amanda Valnier Steckert, Renata Casagrande Gonçalves, Dhébora Mozena Dall'Igna, Diogo Dominguini and Monique Michels, were responsible behavioral tests and biochemical analysis. João Quevedo, Cristiane Ritter and Tatiana Barichello supervised the study. All authors have approved the final version of the manuscript.

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