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Chronic administration of methylmalonate on young rats alters neuroinflammatory markers and spatial memory

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ABSTRACT

The methylmalonic acidemia is an inborn error of metabolism (IEM) characterized by methylmalonic acid (MMA) accumulation in body fluids and tissues, causing neurological dysfunction, mitochondrial failure and oxidative stress. Although neurological evidence demonstrate that infection and/or inflammation mediators facilitate metabolic crises in patients, the involvement of neuroinflammatory processes in the neuropathology of this organic acidemia is not yet established. In this experimental study, we used newborn Wistar rats to induce a model of chronic acidemia via subcutaneous injections of methylmalonate (MMA, from 5th to 28th day of life, twice a day, ranged from 0.72 to 1.67 μmol/g as a function of animal age). In the following days (29th–31st) animal behavior was assessed in the object exploration test and elevated plus maze. It was performed differential cell and the number of neutrophils counting and interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) levels in the blood, as well as levels of IL-1β, TNF-α, inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT) in the cerebral cortex were measured. Behavioral tests showed that animals injected chronically with MMA have a reduction in the recognition index (R.I.) when the objects were arranged in a new configuration space, but do not exhibit anxiety-like behaviors. The blood of MMA-treated animals showed a decrease in the number of polymorphonuclear and neutrophils, and an increase in mononuclear and other cell types, as well as an increase of IL-1β and TNF-α levels. Concomitantly, MMA increased levels of IL-1β, TNF-α, and expression of iNOS and 3-NT in the cerebral cortex of rats. The overall results indicate that chronic administration of MMA increased pro-inflammatory markers in the cerebral cortex, reduced immune system defenses in blood, and coincide with the behavioral changes found in young rats. This leads to speculate that, through mechanisms not yet elucidated, the neuroinflammatory processes during critical periods of development may contribute to the progression of cognitive impairment in patients with methylmalonic acidemia.

Abbreviations: 3-NT, 3-nitrotyrosine; BBB, blood–brain barrier; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium; BSA, bovine serum albumin; CNS, central nervous system; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus maze; HPLC–UV, high performance liquid chromatography/ultraviolet; IEM, inborn error of metabolism; IL-1β, interleukin-1 beta; iNOS, inducible nitric oxide synthase; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acid, methylmalonate; NMDA, *N*-methyl-D-aspartate; NFκB, nuclear factor kappa B; OET, objects exploration test; PBS, phosphate buffered saline; R.I., recognition index; s.c., subcutaneously; SDS, sodium dodecyl sulfate-polyacrylamide gels; SEM, standard error of the mean; TBS, Tris–borate saline; TBS-T, Tween 20 in Tris–borate saline; TNF-α, tumor necrosis factor-alpha.

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Introduction

Methylmalonyl-CoA mutase (MCM) deficiency or its cofactor, 5-deoxyadenosylcobalamin, leads to accumulation of methylmalonic acid (MMA) (Chandler et al. 2009; Oberholzer et al. 1967). This condition is known as methylmalonic acidemia, an inborn error of metabolism (IEM) with incidence of approximately 1:50,000 births (Shigematsu et al. 2002). Extracellular accumulation of MMA acts as a potent neurotoxic metabolite which causes excitotoxicity (Kolker et al. 2000), breakdown of mitochondrial energetic metabolism (Chandler et al. 2009) and oxidative stress in the central nervous system (CNS) (Fernandes et al. 2011). In line of this view, it has been demonstrated that MMA accumulation plays a role in neurological alterations including failure to thrive and psychomotor delay in patients with this organic acidemia (Manoli and Venditti 1993). Furthermore, the neurological crises evidenced in patients with methylmalonic acidemia are typically precipitated by a high intake of protein or minor infections inducing a catabolic state during critical periods of development (Horster and Hoffmann 2004).

Neuroinflammation is closely related to pathologies of the CNS, which can lead to neuronal death as well as to learning and memory deficits. Neuroinflammatory response is characterized by a breakdown of the blood–brain barrier (BBB), activation of the microglia, infiltration of peripheral immune cells and increases in cytokine release (Lucas et al. 2006), which can lead to neuronal death (Glass et al. 2010) and learning or memory deficit (Hein and O'Banion 2012). For example, Chen et al. (1995) showed that convulsions induced by kainate raise the expression of cyclooxygenase-2 (COX-2) in the brain of rats; this enzyme is expressed in most of tissues during inflammatory response, which can lead to chronic lesions development. Furthermore, systemic or central bacterial lipopolysaccharide (LPS) injections activate microglia, potently block neuronal differentiation and disrupt the integration of neurons into existing hippocampal circuitry (Ekdahl et al. 2003; Monje et al. 2003; Belarbi et al. 2012).

Although the studies have shown that neuroinflammation has a main role in human neurological diseases, such as epilepsy (Aronica and Crino 2011), autism (Vargas et al. 2005), multiple sclerosis (Lu et al. 2010), Alzheimer's (Venneti et al. 2009), Huntington's (Moller 2010) and Parkinson's disease (Chung et al. 2010), there are no studies in clinic and experimental literature about the relation between methylmalonic acidemia and the above-mentioned condition. Therefore, the objective of the present work was to verify if the experimental model of MMA chronic injection in young rats alters the inflammation markers in cerebral cortex and/or in behavioral parameters.

Materials and methods

Animals and reagents

The present study utilized Wistar rats with 5 days of life. Pregnant rats and/or the pups were kept in laboratory-controlled conditions (12:12 h light–dark cycle, lights on at 07 am; 24 ± 1 °C; 55% relative humidity) with free access to water and food (Supra; Santa Maria, RS, Brazil). The utilization of animals reported in this study was conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) policies, revised in 1996, and in according to the Brazilian regulations of National Council of Control of Animal Experimentation (CONCEA). All possible efforts were made in order to reduce the number of animals utilized, as well as to minimize the animal suffering. All reagents were purchased from Sigma (St. Louis, MO, USA), except the antibodies, which were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Experimental design

The pregnant rats were placed in individual cages during the final days of gestation. Forty-eight hours after labor, only a number of eight pups (males preferentially) were selected to continue in cages. On the fifth day, the pups received the treatment (saline solution or MMA). On the twenty-first day, the rats were weaned. The treatment finished on the twenty-eighth day. The behavioral tests occurred at days 29, 30 and 31; after this procedure, the rats were euthanized to biochemistry analysis (Fig. 1).

Drug treatment

Methylmalonic acid was diluted in saline and buffered to pH 7.4 with NaOH 6 M. MMA was subcutaneously (s.c.) applied twice a day (with 8 h of interval between the injections), from the fifth to the twenty-eighth day of life, in order to reproduce a methylmalonic acidemia. MMA doses were based on previous studies by Dutra et al. (1991). Therefore, from the fifth to the twelfth day of life the animals received 0.72 μmol of MMA by gram of body weight, from the thirteenth to the nineteenth day the rats received 0.89 μmol/g, and from the twentieth to the twenty-eighth day 1.67 μmol/g (Fig. 1). According to these protocols, MMA concentration reaches similar levels to the ones found in the plasma (2.0 and 2.5 mM) and in the brain (1 and 2 μmol/g cerebral) of patients with acidemia (Dutra et al. 1991). The control group animals were s.c. injected with saline (0.9%). All solutions were prepared so that the animals received 10 μl of solution by gram of body weight in each injection.

Objects exploration test (OET)

OET was adapted from Cippitelli et al. (2010). Approximately 12 h after the last drug injection, the animals were acclimatized (section 1) during 10 min to the open-field. The field consisted of a square box without ceiling, with 40 cm of width and 20 cm of height, painted in white, divided in 12 areas and visually uniform.

One day after the acclimatization, the animals were once again put in the field (section 2), which contained two identical objects (A = A′) placed in a linear configuration for training. The objects were clean and counterbalanced between each animal.

Section three, named of recognition test with spatial alteration, took place 4 h after the second section, to test spatial learning and memory. On this section, one of the objects (A′) was moved to a diagonal configuration. After the test, once again the objects were clean and counterbalanced between the animals.

According to the protocol, 10 min after section three, section four was initiated, named of object recognition test. On this section, one of the objects was replaced by a new object (A ≠ B) still on a diagonal configuration on the field space.

In order to avoid that the animals were guided by smell, the field and the objects were clean with ethanol solution 30% and dried after each section. Each section lasted 10 min and was recorded to later behavioral analysis. The criteria analyzed in section one was the number of crossing and rearing; on the other sections, it was analyzed the percentage of time each object was explored, which is also measured by the recognition index (R.I.) of memory on the moved (A′) or new (B) object. The mentioned procedure is calculated by the formula: (time of investigation of the moved or new object × 100)/(time of investigation of both objects) (Bevins and Besheer 2006).

Elevated plus maze (EPM)

On the following day of OET, in the morning, the animals were submitted to the elevated plus maze test. The maze consists of a wood structure 50 cm from the floor, with two opposite open

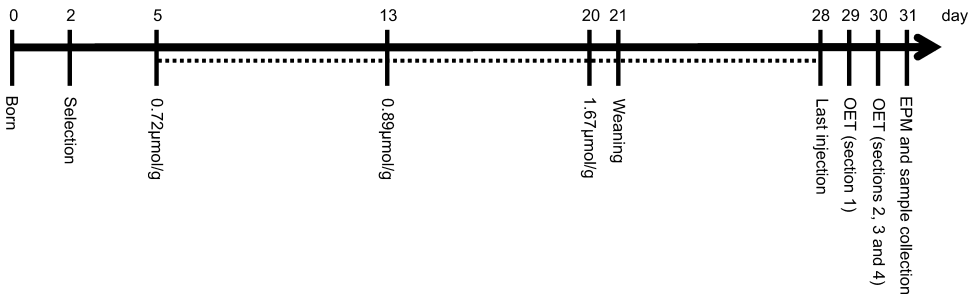

Fig. 1. Experimental design female Wistar rats which gave birth (day 0) were separated into individual cages some days before. On day 2 rats were selected in each litter 8 (preferably male) to remain in the cages. On day 5 the animals began receiving twice a day s.c. injections of MMA ($n = 4$/cage, 0.72 mmol/g) or saline solution ($n = 4$/cage). On day 13 and 20 the dose of MMA was increased (0.89 mmol/g and 1.67 mmol/g, respectively). On day 21 weaning of animals occurred with the removal from their mothers. On day 28 the animals received the last two injections and on days 29–31 were subjected to behavioral tests (OET and EPM). After testing the EPM, in the morning of day 31, the animals were euthanized, blood was collected and the cerebral cortex was removed and frozen for posterior biochemical analysis.

arms (50 cm of length × 10 cm of width each), which cross with two opposite enclosed arms (with the same dimensions of the open arms, but containing walls with 40 cm of height).

Initially, the animals were put on the crossroad of the maze in a similar way (File and Gonzalez 1996). The animals could explore the maze during 5 min. During this period of time, the animals were recorded and, subsequently, it was analyzed the following behaviors: percentage of the total number of entrances in the enclosed and open arms and time (s) of exploration of each arm.

The apparatus was cleaned with ethanol solution 30% and dried with clean cloths after each animal made the test.

Sample collection

Right after EPM test or three days after the last injection, the animals were euthanized. The blood was collected and the cerebral cortex was removed and immediately frozen in liquid nitrogen for posterior biochemical analysis. Then for each biochemical analysis was randomly selected equal numbers of cerebral cortex per group, which were homogenized in specific buffers.

Differential blood cell count

The total number of neutrophils in blood was determined through the differential count by the method of May-Grünwald–Giemsa (Bins et al. 1989). The total number of leukocytes was determined in Neubauer chamber after the dilution (1:20; v/v) in Turk solution (Gencian Violet 0.01%; glacial acetic acid 1% in distilled water).

Determination of cytokines

For the measurement of cytokines in the blood serum, blood samples of a part of these animals were collected by cardiac puncture, centrifuged at 1500 × *g* for 15 min (room temperature) and the serum was collected and stored at −80 °C. Then, on the day of analysis, samples were unfrozen gradually and measurement of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) was assessed using commercial ELISA kits for rat as described by the manufacturer (eBIOSCIENCE®, San Diego, USA).

The content of IL-1β and TNF-α was determined in cerebral cortex of other animals, which were homogenized in phosphate buffered saline (PBS, pH 7.4) containing bovine serum albumin (BSA, 10 mg/mL), EGTA 2 mM, EDTA 2 mM and PMSF 0.2 mM. The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) kit for rodents, commercially provided by R&D Systems® (Minneapolis, MN, USA), in accordance with the manufacturer protocols, and the results are expressed in pg/mg of protein.

Western blot

Western blot technique was conducted with small modifications on the method described by Casu et al. (2007). To perform this technique, a part of the cerebral cortex of animals was homogenized in rate 1:5 (w/v) with ice-cold A buffer (10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, and 2 mM of sodium orthovanadate in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 × *g* for 45 min at 4 °C.

The supernatant (S1), denominated cytosolic fraction, was reserved for posterior processing. The pellet (P1) was resuspended in the same used above volume of ice-cold buffer B (10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, and 1% Triton-X in 10 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 × *g* for 45 min at 4 °C. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 100 μL of ice-cold buffer C (50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 10 μg/mL aprotinin, 10 mM β-glycerolphosphate, 1 mM PMSF, 1 mM DTT, 2 mM sodium orthovanadate, 420 mM NaCl, and 25% glycerol in 20 mM HEPES, pH 7.9), incubated for 15 min on ice, and centrifuged at 16,000 × *g* for 45 min at 4 °C. The supernatant (S3) was considered the nuclear fraction (Medeiros et al. 2007).

The protein concentration in the cytosolic and nuclear fractions was determined using the Bradford method (Bradford 1976). Equivalent amounts of protein (80 or 20 μg for cytosolic or nuclear fractions, respectively) were added to 0.2 volumes of concentrated loading buffer (200 mM Tris, 10% glycerol, 2% SDS, 2.75 mM β-mercaptoethanol, and 0.04% bromophenol blue) and boiled for 10 min. Proteins were separated in 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinilidenedifluoride membranes.

Ponceau staining (data not shown) served as a loading control (Romero-Calvo et al. 2010). Western blot analysis of inducible nitric oxide synthase (iNOS) was carried out in cytosolic fractions. Membranes were processed using a SNAP i.d. system (Millipore, Billerica, MA, USA). First, the membrane was blocked with 1% BSA in Tris–borate saline (TBS), then incubated for 10 min with specific primary antibody diluted 1:300 in TBS. iNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA catalog numbers sc-372 and sc-8310). Blots were washed three times, with 0.05% Tween 20 in Tris–borate saline (TBS-T) followed by incubation with adjusted alkaline phosphatase-coupled secondary antibody (1:3000, anti-rabbit IgG; Santa Cruz Biotechnology, Inc.) for 10 min. Protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and p-nitro blue tetrazolium (BCIP/NBT; Millipore).

Membranes were dried, scanned, and quantified with the ImageJ program.

High performance liquid chromatography/ultraviolet (HPLC–UV) detection

The determination of 3-nitrotyrosine (3-NT) and tyrosine levels was performed by HPLC–UV, based on the method of Erdal et al. (2008). Basically, to perform this analysis, another part of cerebral cortex samples was homogenized in five volumes of Tris–HCl 30 mM (pH 7.4), and a part was hydrolyzed in HCl 12 N (1:1) at 100 °C during 12 h. The samples were then filtered through a membrane (0.45 μm pore size, Millipore®) before the injection in the HPLC-UV equipment (Shimadzu®).

The analytical column was a 5 μm particle and 100 Å pore size Phenomenex® ODS-2 C18 reverse-phase column (4.6 mm × 250 mm, Allcrom, BR). The mobile phase was 50 mM sodium acetate, 50 mM sodium citrate and 8% (v/v) methanol, pH 3.1 (corrected with 12 N HCl). The HPLC analysis was performed under isocratic conditions at a flow rate of 1 ml/min and UV detector set at 274 nm. 3-NT levels were expressed as 3-NT (μM)/total tyrosine (μM).

Protein quantification

The protein content was colorimetrically determined by the method of Bradford (1976). BSA (1 mg/mL) was the pattern used.

Statistical analysis

Data from behavioral and neurochemistry experiments were analyzed by unpaired *t* test or two-way ANOVA test when appropriated, and were expressed as means and standards error of the mean (SEM). Statistical analyses were performed using the SPSS (statistical Package for the Social Sciences) software in a PC-compatible computer. The value of *t* or Fare presented only if $P < 0.05$. The results in all the experiments showed no significant differences between animals of different genders within groups.

Results

Chronic MMA induces spatial memory deficits

During all the period of the chronic injection of drugs, the animals were weighted and, similar to the original protocol (Wajner et al. 1988), there was no weight differences between the groups (Fig. 2), indicating no malnutrition or failure to thrive.

On the following morning after the last drug injection, the animals of both groups were acclimatized to the open field (section 1, Fig. 1) in order to initiate the OET protocol. It was verified no difference between groups in the number of crossing ($t = 0.2775$, Fig. 3A) or rearing ($t = 0.9153$, Fig. 3B).

In the morning of the following day (section 2, Fig. 1) the animals were put in open field with two identical objects disposed in a linear configuration. On this evaluation it was verified that the animals did not show preference by any of the objects (Fig. 3C).

Section 3 was made 4 h after section 2. Section 3 aimed at verifying if there was any type of spatial recognition impairment. When the objects were positioned in a diagonal configuration, the animals treated with MMA present a smaller R.I. of the dislocated objected if compared to the control group ($t = 2.929$, $P < 0.01$; Fig. 3D).

Following the protocol, section 4 was made 10 min after the end of section 3. On section 4, the objected which was moved before was substituted by a new one. However, there was no difference between the groups in this object recognition test ($t = 0.3670$, Fig. 3E).

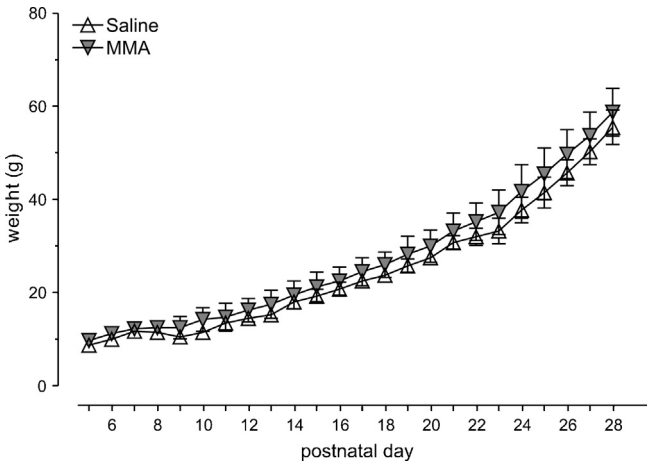

Fig. 2. Treatment effects on the body weight of the animals. From 5th to 28th day, the animals were weighed (g) to daily adjust of doses of saline or MMA to be injected. There was no difference in weight gain between the two groups. Data represent mean ± standard error of the mean (SEM) for $n = 12$ in each group.

On the following day, the animals were tested in the morning in the EPM (Fig. 1).

The EPM test has been used to verify the occurrence of anxiogenic or anxiolytic effects in various experimental models (File et al. 1998; Pinton et al. 2011), which could influence in others cognitive tests, such as OET. Table 1 presents the results on the analyzed criteria in the EPM test. There was no difference between groups regarding the time (in s) at which the animals were in the open or closed arms ($t = 0.9272$), or the percentage of entries into the open or closed arms ($t = 0.6234$). It indicates that there were no alterations in anxiety-like behavior.

Chronic MMA alters the number of leukocytes and cytokines levels in blood

After the EPM, the animals were euthanized and were collecting the samples (Fig. 1).

In the blood there was a count of the total leukocytes, in which was possible to perceive an increase in the mononuclear cells and a reduction in the number of polymorphonuclear cells in the group treated with MMA ($t = 5.505$, $P < 0.0001$; Fig. 4A and B, respectively).

When the differential count was made in blood, it was possible to perceive an increase in the number of other types of blood leukocytes and a reduction in the neutrophils number in MMA-treated group ($t = 2.612$, $P < 0.05$; Fig. 4Cand D, respectively).

To investigate the MMA effects on pro-inflammatory cytokines levels in blood, IL-1β and TNF-α were quantified in serum. Quantification and statistical analysis of ELISA technique showed an increase in IL-1β ($t = 12.20$, $P < 0.0001$; Fig. 5A) and TNF-α ($t = 14.61$, $P < 0.0001$; Fig. 5B) levels in blood serum of MMA-treated group.

Table 1
Assessment of the behavior in the EPM test.

Parameter evaluated in EPM	Saline	MMA
Entries into open arms (%)	23.1 ± 3.3	20.1 ± 3.5
Entries into closed arms (%)	76.9 ± 3.3	79.9 ± 3.5
Time spent into open arms (s)	23.8 ± 4.4	18.3 ± 4.0
Time spent into closed arms (s)	241.5 ± 7.0	247.2 ± 5.4

EPM test was conducted on the 31st day of the experimental protocol. Data represent mean ± SEM for $n = 12$ in each group. According to unpaired *t* test, there were no differences between the groups.

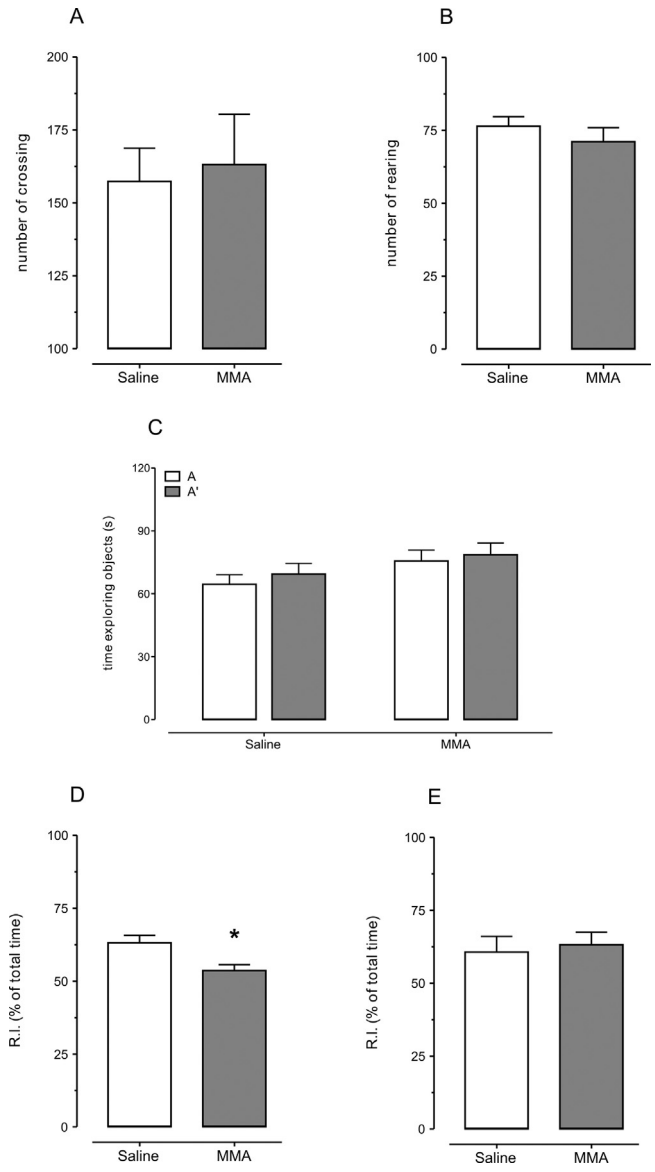

Fig. 3. Treatment effects on OET. Was observed the number of crossing (A) and rearing (B) during the acclimatization of the animals (section 1, 29th day) in the open field. The next day (30th day), the animals returned to the open field and the preference for each one of the similar objects was checked (section 2) by the exploration time (in s) (C). Four hours later, one of the objects in the open field was placed in a diagonal position (section 3) and the R.I. by the percentage of time exploring the objects was assessed (D). Ten minutes later, the animals returned to the open field containing two different objects (section 4) and the R.I. was accessed again (E). Data represent mean ± SEM for $n = 12$ in each group. According to unpaired t test, $^*P < 0.05$ was considered significant.

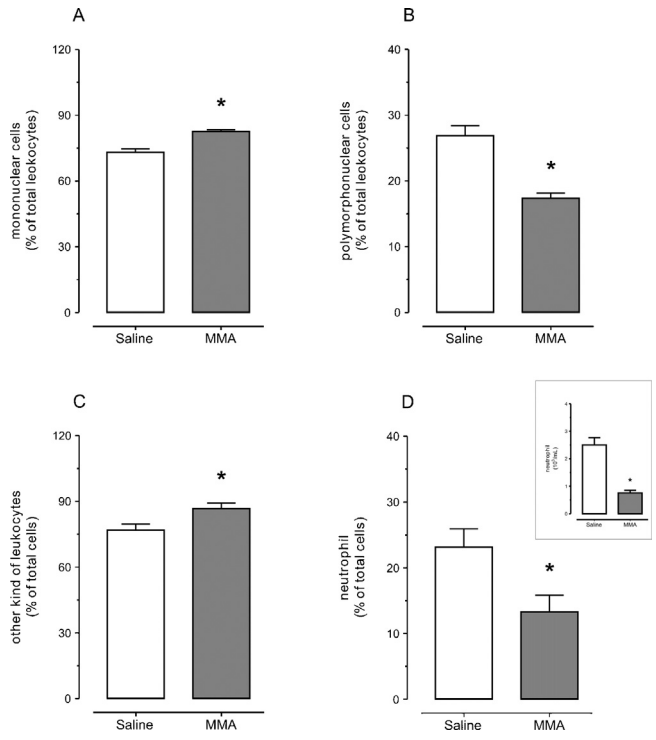

Fig. 4. Treatment effects on differential blood cell count. The levels of mononuclear (A) and polymorphonuclear cells (B) were estimated in a Neubauer chamber, and the result is expressed as a percentage of total leukocytes. The cellular differentiation estimated the number of other leukocytes types (C) and neutrophil granulocytes (D), and is displayed as percentage of total cells. Moreover, in the box (upper right corner of D) are expressed the absolute neutrophil counts ($t = 6.174$, $P < 0.001$). Data represent mean ± SEM for $n = 8$ in each group. According to unpaired t test, $^*P < 0.05$ was considered significant.

Chronic MMA induces an increase on inflammatory markers in the cerebral cortex

The cerebral cortex was frozen in liquid nitrogen and after homogenized in the ideal buffer for each biochemical analysis.

The measurement of the two pro-inflammatory cytokines IL-1β and TNF-α was made by the ELISA technique. As a result, it was observed an increase both in the IL-1β ($t = 3.199$, $P < 0.01$; Fig. 5C) levels as in the TNF-α in cerebral cortex of the animals treated with MMA ($t = 5.357$, $P < 0.0001$; Fig. 5D).

Knowing that the inflammatory cascade also leads to the induction of iNOS (Aktan 2004), it was utilized the western blot method to quantify the immunoreactivity of this enzyme. Once again, the group treated with MMA presented an increase in the iNOS ($t = 2.402$, $P < 0.05$; Fig. 6A) levels in the cerebral cortex.

Besides that, the iNOS causes a quick and excessive production of nitric oxide, which can result in nitrosative stress, another marker associated to inflammatory processes (Schopfer et al. 2003). Therefore, making use of the detection method by HPLC–UV, it was possible to measure the 3-NT levels, in which was verified an increase in the levels of this marker ($t = 2.718$, $P < 0.05$; Fig. 6B) in the cerebral cortex of the group treated with MMA.

Discussion

Patients with methylmalonic acidemia usually present acute clinical features early in life resulting from metabolic decompensation, with recurrent vomiting, dehydration, respiratory distress and neurological symptoms, including psychomotor delay, irritability, lethargy, hypotonia, convulsions and coma. Most children survive to the first acute metabolic crisis, but develop long-term complications including neurological deficits (Baumgarter and Viardot 1995; Leonard 1995; Horster et al. 2007). Although it is believed that these abnormalities occur as result of the primary metabolic impairment, the underlying mechanism of brain damage and neurological deficits in methylmalonic acidemia is poorly understood.

It is known that patients and experimental models of this IEM exhibit neuronal damage and changes in several areas of the central nervous system, as well as in several other neurological diseases (Melo et al. 2011; Radmanesh et al. 2008). Often this is related to oxidative stress and neuronal death, causing cognitive impairment and neuroinflammatory processes (Lee et al. 2009; Cameron and Landreth 2010; Hein and O'Banion 2009). Recent articles in experimental models have elucidated the role of inflammatory mediators

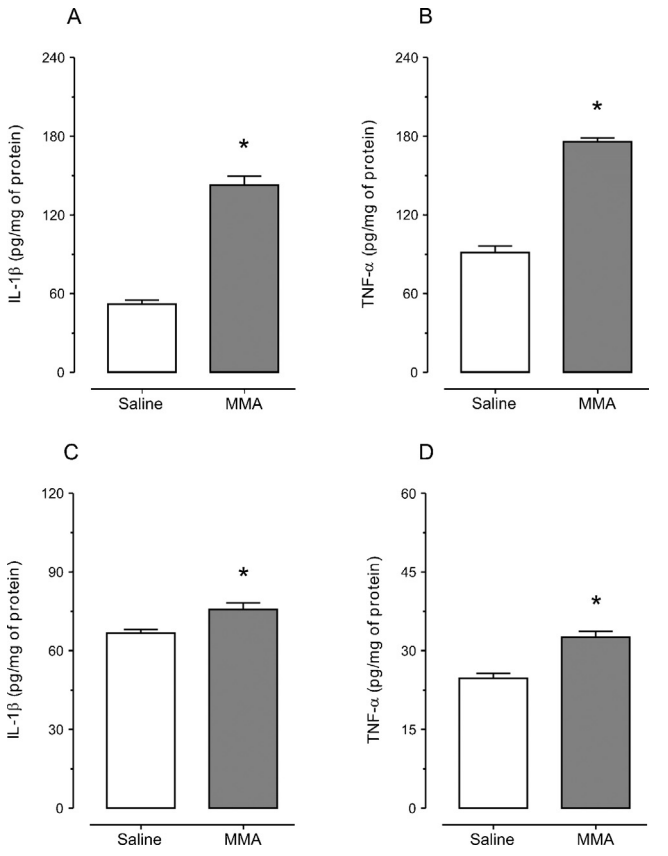

Fig. 5. Treatment effects on the levels of pro-inflammatory cytokines in blood serum and cerebral cortex. Levels of IL-1β and TNF-α in the blood serum (A and B, respectively) and cerebral cortex (C and D, respectively) of rats were estimated by ELISA and are expressed in pg/mg of protein. Data represent mean ± SEM for $n = 8$ (blood serum) and 14 (cerebral cortex) in each group. According to unpaired t test, $^{*}P < 0.05$ was considered significant.

in models of acute seizures by MMA (Salvadori et al. 2012; Ribeiro et al. 2009). However, it is not known if MMA itself might increase these mediators.

We conducted this study with rats until the 31st day of life, a period of development with proven synaptogenesis and cell proliferation in several brain structures involved in learning and memory. We found that the chronic MMA administration (5°–28° day of life) at doses that raise its concentration in the blood and in the brain (Dutra et al. 1991) causes memory deficits in spatial recognition, decreased number of polymorphonuclear cells (neutrophils) while increasing blood mononuclear leukocytes, and induces increased levels of pro-inflammatory cytokines (IL-1β and TNF-α) in blood serum and cerebral cortex, in addition to increasing iNOS immunoreactivity and consequent nitrosative stress in the cerebral cortex.

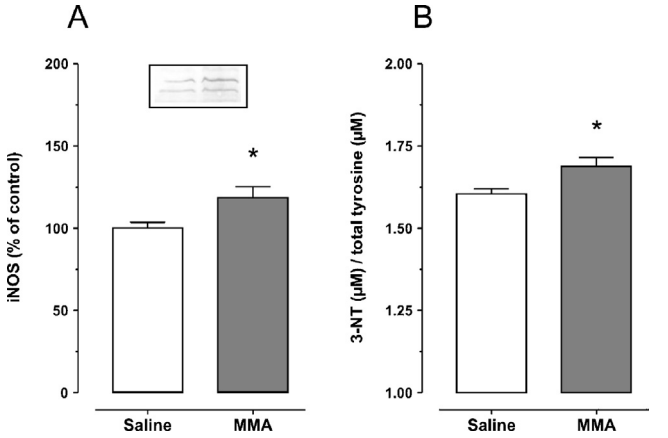

Fig. 6. Treatment effects on induction of iNOS and nitrosative stress. The levels of iNOS (A) in the cerebral cortex of rats were estimated by Western blot (representative bands of groups in the box) and are expressed as percentage of control. The levels of 3-NT (B) in the cerebral cortex of rats were determined by HPLC–UV and are expressed in μmol by the total of tyrosine residues. Data represent mean ± SEM for $n = 8$ in each group. According to unpaired t test, $^{*}P < 0.05$ was considered significant.

This is the first study to investigate the effect of this experimental protocol on learning/memory through the OET in the days succeeding injections of MMA, and showed that the accumulation of the same may cause a decrease in R.I. when the objects are arranged in a spatial configuration different of the original. In previous studies by Pettenuzzo et al. (2003a,b), there was a deficiency in the purchase of a new paradigm of spatial localization in Morris water maze test of learning/memory, performed approximately two weeks after the probe trial, indicating a perseverative behavior. It also demonstrated that the damage MMA-caused on the CNS can be observed even in a long period of time after its last administration (more than one month and half). Thus, despite differences (temporal and techniques) between protocols performed in this and the aforementioned studies, it was found a similarity in the case of the inflexibility behavioral presented by the animals of MMA-injected group, which can result from damage caused by even at the CNS (Pettenuzzo et al. 2003a; Royes et al. 2006). It should be remembered that several subcortical structures are involved in the acquisition, consolidation and evocation of memory (Izquierdo and Medina 1997; Izquierdo et al. 2006; McGaugh 2000), and these could also suffer consequences arising from the accumulation of MMA, as has been observed in experimental models of this acidemia (Wajner et al. 1988; Pettenuzzo et al. 2003b; Vasques et al. 2006; Malfatti et al. 2003). However, this is only a supposition and would require more experiments to verify the possibility of neuroinflammatory or neurodegenerative processes in other brain structures.

Out of CNS, the blood presents as first line of defense of the organism the innate immune system. The neutrophils are polymorphonuclear leukocytes of immune system and have the function of phagocyte microorganisms or particles (Kobayashi and DeLeo 2009). Patients with variants of methylmalonic acidemia show reduction in the number of these cells, a condition called neutropenia (Watkins and Rosenblatt 2011; Guerra-Moreno et al. 2003). In addition, Hutchinson and colleagues elegantly demonstrated that MMA causes a suppression of granulopoietic progenitor cell proliferation in marrow culture, suggesting that it could be one cause of neutropenia in patients (Hutchinson et al. 1985). On the other hand, treatment with MMA also induced an increase in other types of leukocytes, peripheral blood mononuclear cells, such as lymphocytes and monocytes. It should be pointed out that on the total white cell count did not differ between groups (data not shown), and this large number of mononuclear cells may be only a "relative leukocytosis" due to the lower number of neutrophils.

In the CNS, neutrophils are usually not present because they do not cross the blood brain barrier. At this place, the first and foremost immune defense is the microglia cells (Ransohoff and Brown 2012). They are one of the responsible for the production of pro-inflammatory cytokines, such as IL-1β and TNF-α, which are accepted as modulators of neurotransmission within the brain (Merrill 1992). However, in the present study we showed that MMA administration induced the increase these pro-inflammatory cytokines (IL-1β and TNF-α) both in the cerebral cortex and in blood serum. Therefore, since the BBB is still in process of maturation during this period of life (Song et al. 2002), the observed increase of cytokines in the CNS may be a consequence of this inflammatory response in the periphery, and this can act as an insult to

the developing brain triggering behavioral alterations (Stolp et al. 2011). Current evidence indicates that cytokines, particularly IL-1β, increase neuronal excitability by activating IL-1 receptors (Vezzani et al. 1999; Bernardino et al. 2005). The neuronal IL-1R1 stimulation induces Src kinase-mediated tyrosine phosphorylation of the NR2B subunit in *N*-methyl-D-aspartate (NMDA) receptor. As a consequence, IL-1β facilitates NMDA receptor-mediated Ca^{2+} influx into neurons, promoting excitotoxicity (Viviani et al. 2003). Considering that IL-1β can also inhibit glutamate uptake in astrocytes (Hu et al. 2000) and increase its glial release possibly via TNF-α production (Bezzi et al. 2001) it is plausible to propose that increase of pro-inflammatory cytokines result in elevated extracellular glutamate levels and toxicity in this model of organic acidemia. In agreement of this view, a considerable body of evidence has demonstrated that excessive glutamate receptor stimulation, in particular the NMDA receptor, has been implicated as a major pathway that leads to MMA-induced toxicity (de Mello et al. 1996; de Mattos-Dutra et al. 2000).

Furthermore, TNF-α and IL-1βacts in their respective receptors and cause activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a transcription factor that migrates to the cell nucleus and promotes the expression of iNOS (Aktan 2004). The iNOS is one of three isoforms of nitric oxide synthase (EC 1.14.13.39) which, even though it does not present a greater specific activity than the other isoforms, can be highly expressed during the inflammatory process and, for presenting activity even at normal concentrations of intracellular calcium, can cause an increased production of NO (Hemmens and Mayer 1998; Nathan and Xie 1994). NO is a free radical that when reacting with superoxide anion ($O_2^{-\bullet}$, also formed during the inflammatory process by enzymes such as NADPH oxidase) generates the highly reactive peroxynitrite ($ONOO^-$) (Chung 2006). This free radical then can nitrate tyrosine residues to 3-NT in proteins, a condition known as nitrosative stress, and can also induce lipid peroxidation, and DNA damage (Chung 2006; Ischiropoulos and Beckman 2003). In the present study we revealed that chronic MMA administration induced increase in iNOS expression and consequent nitrosative stress in the cerebral cortex of rats.

In the literature, until the present moment, only the study of Goyenechea et al. (2012) investigated the effect of chronic injections of MMA on pro-inflammatory markers but in renal cortex, one of the organs affected by acidemia. Among the results obtained in the study it was found, for example, increased levels of TNF-α and a positive correlation between the levels of mRNA of TGF-β, as well as urinary excretion of MMA, which can be important in inflammation and renal damage. Other studies using the same model of this acidemia observed small amounts of ganglioside related to synaptogenesis in the cerebellum and cerebrum (Wajner et al. 1988; Brusque et al. 2001), and reduced activity of the enzyme Na^+, K^+-ATPase activity in cerebral cortex (Wyse et al. 2000). These effects may be due to inhibitory effects on several metabolic pathways and/or by oxidative stress caused by MMA (Wyse et al. 2000; Mirandola et al. 2008; Royes et al. 2005; Wajner and Coelho 1997). In another model of this acidemia, [U-^{14}C]acetate incorporation into the lipids of cerebral cortex was reduced by MMA, which may explain the hypomyelinization and/or demyelinization characteristic of patients and, together with the findings of this paper, we can hypothesize that is mediated by immune system (de Mello et al. 1997; Mayo et al. 2012). Moreover, experimentally or clinically, cytokines interfere directly or indirectly in the process of memory consolidation, synaptic plasticity and/or neurogenesis, and chronic expression of neuroinflammatory mediators potentially implies in neuronal damage leading to cognitive impairment (Bossu et al. 2012).

Although the results of the present study may suggest an association between memory deficits and increased pro-inflammatory markers in the cerebral cortex of animals treated with MMA, we are only aware that these data do not point out if/how a direct association actually exists. Changes in these pro-inflammatory markers may influence behavior by affecting neurotransmission, endocrine system, neuronal plasticity and brain circuitry (Bossu et al. 2012), mechanisms and functions which evaluation is beyond the scope of this study. Therefore, it is interesting that more experiments are performed to question the assumption that the neuroinflammatory process is linked to pathophysiology of methylmalonic acidemia.

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