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MAGNESIUM SULFATE (MG) PREVENTS MATERNAL INFLAMMATION INDUCED OFFSPRING CEREBRAL INJURY EVIDENT ON MRI BUT NOT VIA IL-1β

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Abstract—*Objective:* As maternal treatment with magnesium sulfate (MG) may protect the fetal brain, we sought to assess the inflammation associated neuroprotective potential of MG and its association to interleukin 1β (IL-1β). *Methods:* Pregnant Sprague–Dawley rats at 18-day gestation received i.p. lipopolysaccharide (LPS) or saline. Dams were randomized to treatment with s.c. saline (control), or MG prior to or following the i.p. injection, resulting in three groups. At the end of the treatment, fetal brain IL-1β was quantified for 18 pregnant rats (six of each group). Another 18 pregnant rats delivered spontaneously and pups were allowed to mature. At postnatal day 25, female offspring were examined by magnetic resonance imaging (MRI) and analyzed using voxel based analysis. Apparent diffusion coefficient (ADC) and T2 relaxation protocols were performed to assess white and gray matter injury. *Results:* Offspring of LPS-treated dams exhibited (1) significantly increased T2 levels, and (2) increased ADC levels in white and gray matter, consistent with diffuse cerebral injury. Offspring of MG-treated LPS dams demonstrated similar T2 and ADC levels as control dams. Fetal brain IL-1β was significantly increased following maternal LPS compared to control (0.125 ± 0.01 vs 0.100 ± 0.01u, $p < 0.05$). No significant decrease in IL-1β level was observed in response to maternal MG. *Conclusions:* Maternal LPS-induced neonatal brain injury can be prevented by maternal MG. Maternal MG therapy may be effective in human deliveries associated with maternal/fetal inflammation. The absence of a decrease in fetus brain levels of IL-1β following MG treatment implies that the mechanism of MG is not through inhibition of IL-1β production.

***Significance statement:* Intrauterine fetal exposure to maternal inflammation and pro-inflammatory cytokines is associated with adverse offspring neurological outcomes. Although its precise mechanism is not elucidated, magnesium sulfate (MG) is commonly used as neuroprotection for white matter brain injuries in preterm fetuses. A proposed mechanism involves the ability of MG to reduce pro-inflammatory cytokine levels. In the current study, we used a rat model of LPS-induced maternal inflammation to investigate the short-term effect of MG on fetal brain IL-1β levels, and its long-term neuroprotective effect on the offspring brain by using MRI. *Significance statement:* We demonstrated that maternal administration of MG can prevent long-term neonatal brain injury but, since no decrease was observed in fetal brain IL-1β levels, the neuro-protective mechanism of MG is not mediated by inhibition of IL-1β production.**

Key words: brain injury, maternal inflammation, magnesium sulfate, interleukin 1β.

INTRODUCTION

Understanding the pathogenesis of perinatal brain injury represents one of the greatest challenges in perinatal medicine today. Whereas brain damage as cerebral palsy (CP) was previously attributed mostly to perinatal hypoxia, recent studies imply that asphyxia accounts for only a small proportion of CP cases (Wu and Colford, 2000; Bax et al., 2006; Thorngren-Jerneck and Herbst, 2006; Ahlin et al., 2013; Himmelmann and Uvebrant, 2014). In addition to fetal infection, an association has been demonstrated of maternal infection/inflammation with fetal brain injury and neuro-developmental disorders. Chorioamionitis induces short- and long-term changes in fetal brain morphology, central nervous system activity and behavior (Grether and Nelson, 1997; Wu and Colford, 2000; Urakubo et al., 2001; Wu, 2002; Brown et al., 2004; Fatemi et al., 2005; Meyer et al., 2009; Elovitz et al., 2011; Brown, 2012; Lee et al., 2015; Jiang et al., 2016). Immunostaining of the fetal and postnatal offspring brain has shown that prenatal lipopolysaccharide (LPS)-induced inflammation in rats and mice leads to white matter damage, microglial activation, decreased hippocampal neurogenesis, dendritic alterations and decreased myelin basic proteins (Rousset et al., 2008;

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Abbreviations: ADC, apparent diffusion coefficient; CP, cerebral palsy; DTI, diffusion tensor imaging; IL-1β, interleukin 1β; LPS, lipopolysaccharide; MG, magnesium sulfate; MRI, magnetic resonance imaging; NS, normal saline; RD, radial diffusivity; SC, subcutaneous; VBA, voxel based analysis.

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Cui et al., 2009). As previously published by our group (Beloosesky et al., 2013), similar cerebral injuries in offspring of LPS-treated dams were exhibited by magnetic resonance imaging (MRI).

Maternal inflammation increases cytokine levels, including interleukin-6 (IL-6), interleukin-1β (IL-1β) and TNFα in maternal circulation, in the placenta and amniotic fluid, and eventually in fetal systemic circulation and the brain (Gayle et al., 2004). Neuropathological studies and experimental animal models reveal that pro-inflammatory cytokines, especially IL-1β, are implicated in the cascade leading to brain damage at varying developmental stages (Urakubo et al., 2001; Allan et al., 2005; Ashdown et al., 2006; Thornton et al., 2006; Rousset et al., 2008; Cui et al., 2009; Knox et al., 2009; Elovitz et al., 2011; Denes et al., 2011; Arrode-Brusés and Brusés, 2012; Beloosesky et al., 2013; Savard et al., 2013; Rosenzweig et al., 2014). Moreover, Leitner et al. (2014) demonstrated that maternal treatment with an IL-1β antagonist might improve the cortical neuronal injury associated with exposure to intrauterine inflammation.

During the last decade, several prospective studies in pregnant patients have demonstrated the neuroprotective effect of MG in preventing preterm white matter brain injury (Nelson, 1996; Crowther et al., 2003; Anon, 2006; Marret et al., 2006; Nguyen et al., 2013; Cho et al., 2015). The putative neuroprotective mechanism of MG has not been elucidated, though one proposed explanation is through its anti-inflammatory properties (Marret et al., 2006; Dowling et al., 2012; Cho et al., 2015). In a study published by Tam Tam et al. (2011), maternal MG was found to significantly reduce pro-inflammatory mediator levels in both maternal and fetal compartments, including the fetal brain. In contrast, Burd et al. (2010) demonstrated increased levels of fetal brain mRNA of pro-inflammatory cytokines following maternal MG administration.

In our current study, we used a rat model of LPS-induced maternal systemic inflammation. We sought to determine the long-term neuroprotective effect of MG on offspring at age 25 days, as evidenced by MRI, and the short-term effect of MG on fetal brain IL-1β levels.

EXPERIMENTAL PROCEDURES

Animals and treatments

All protocols and procedures were approved by the Institutional Animal Care Committee at the Rappaport Research and Education Institute, Haifa, Israel. Sprague–Dawley pregnant rats (n = 36, Harlan, Inc) were obtained at gestational day 11 (term = 21 days) and allowed to acclimate for 7 days prior to initiation of experiments. Animals were maintained at a controlled temperature (37 °C) and lighting (0600-lights on; 1800-lights off); as well as access to food (LabDiet 5001 Rodent Diet, PMI Nutrition International, LLC) and water *ad libitum* throughout the study. Saline and MG were administered subcutaneously and LPS was administered intraperitoneally. LPS (E. coli., serotype 0111:B4, Calbiochem Inc.) was reconstituted in physiological saline and administered at 500 μg/kg birth weight.

We studied the short- and long-term effects of LPS and MG on the brain of the fetus and offspring respectively. At gestational day 18, rats received injections of i.p. LPS (500 μg/kg) or saline of equivalent volume at time 0. Dams were randomized to treatment with s.c. normal saline (NS) or MG (270 mg/kg loading followed by 27 mg/kg q20 min) for 2 h prior to and following the i.p. LPS; or saline and another injection of MG 270 mg/kg at 2 h following the LPS or saline injection. This resulted in three study groups (control: NS-NS-NS; LPS: NS-LPS-NS; LPS + MG: MG-LPS-MG).

Sample collection

Pregnant rats (18 rats – six of each group) were anesthetized 3–4 h after LPS injections, since we previously demonstrated a cytokine peak (IL-1β) 3–4 h following maternal LPS (Gayle et al., 2004) . The peritoneal cavities were exposed via midline incision. The uterus was removed and placed in a chilled Petri dish. Each of the fetal brains was collected and frozen in liquid nitrogen. All samples were subsequently stored at −80 °C for further processing and analyses. All samples were analyzed individually.

IL-1β isolation and western blotting

The fetal brains were homogenized in lysis buffer containing 2% SDS, 10% glycerol, 2% 2-mercaptoethanol and 0.002% bromophenol blue in 75 mm Tris–HCl. The samples were heated at 95 °C for 10 min before separating on 10% Tris/Glycine/SDS acrylamide gels. The proteins were subsequently trans-blotted to polyvinylidene difluoride membranes and blocked in 5% dry milk for 2 h at room temperature. The membrane was incubated with rat anti-IL-1β (Santa Cruz Company, USA) for 2 h at 37 °C. After three washes with TBS/0.05%Tween-20, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rat antibody (Santa Cruz) for 1 h at 37 °C. Protein signal was visualized using the Super Signal West Pico Chemiluminescent Substrate (PIERCE Company, Waltham, Massachusetts, USA) and detected with Imaging System (Syngene Company, Frederick, Maryland, USA). β-actin protein was visualized and detected as above. The ratio between IL-1β and actin density for each sample was determined using densitometer.

MRI protocol

The remaining dams (18 pregnant rats – 6 of each group) delivered spontaneously at term (e21). The male and female pups remained with the dams during lactation. In the current study, we examined only young female rats before sexual maturity. At age 25 days, six female offspring from each group (one from each dam, selected randomly) were examined by brain MRI. The rats were anesthetized with 1–2% isoflurane and oxygen and maintained at 37 °C; breathing was monitored with a breathing sensor.

MRI scans of the rats' brains were performed with a 7 T scanner (BRUKER, Karlsruhe, Germany), with a 30-cm bore and a gradient strength of up to 400 mT/m. For acquisition we used quadrature head coil. Both diffusion tensor imaging (DTI) and T2 relaxation protocols were performed (BioImage, Haifa). DTI is based on measurements of water diffusion in several directions to extract the 3D diffusion profile. The orientation of axon bundles determines the direction of water flow: i.e. parallel bundles of axons and their associated myelin sheaths make diffusion of H_2O molecules easier along the main direction. This property can be imaged and measured with DTI. In our current analysis, we analyzed the apparent diffusion coefficient (ADC), which measures the magnitude of water molecule diffusion. T2 distribution can serve as a highly specific method in the imaging of structures (MacKay et al., 2006). DTI was performed by standard protocols. DTI parameters included the following: spin echo sequence, repetition time (TR) = 4000 ms; echo time (TE) = 25 ms; slice thickness = 1 mm; δ = 4.5 ms; Δ = 10 ms, 15 gradient directions; B value = 1000 meters; matrix: 128 × 96. ADC maps were calculated for each rat from the MRI images. All maps were coregistered and normalized to a template (based on one subject) and smoothed (0.2 mm FWHM) using the SPM software version 2 (SPM Technologies Inc, Morganville, NJ, USA).

Quantitative T2: T2 map was extracted for each rat from the T2 relaxation measurements. T2 parameters: MSME sequence, TR = 3000 ms, 16 different TE (ms):10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 and 160; FOV = 1.920 cm; matrix: 256 × 128; slice thickness = 1 mm.

Data analyses

Voxel-based analysis (VBA) is a whole-brain statistical technique that enables the detection of regionally specific differences in brain tissue composition on a voxel-by-voxel basis. VBA involves transforming the images into a standard space (spatial normalization), using an automated non-rigid registration of the images to an anatomical template. After normalization, an analysis of variance (ANOVA) was performed voxel by voxel, by SPM2 software (Wellcome Trust Center for Neuroimaging, London, UK), to compare with the LPS group, which served as a reference; and to assess white and gray matter injury or any brain tissue changes.

Only significant regions that passed the threshold of $p < 0.001$ are presented. Fetal brain IL-1β levels were compared between fetuses from the different groups (six brains from each group, 1 per dam). All results are expressed as mean ± SD using 1-way analysis of variance followed by post hoc tests for pairwise comparisons (Holm-Sidak method). Differences were considered to be significant at $p < 0.05$.

RESULTS

Fetal brain IL-1β levels

Maternal LPS significantly increased the fetal brain IL-1β protein level compared to the saline group (0.125 ± 0.01 and 0.100 ± 0.01 u, respectively ($p < 0.05$). Maternal MG did not attenuate fetal brain IL-1β protein levels following maternal LPS (0.118 ± 0.007 u) (Fig. 1).

Neonatal brain MRI

Eighteen female rats, six from each group were evaluated by MRI at age 25 days. Measurements of DTI (focusing on the ADC) and T2 relaxation are presented with correlations to the specific brain regions in Table 1. Differences in both gray and white matter areas were well observed among the groups.

Maternal exposure to LPS (NS-LPS-NS) offspring. ADC was significantly higher (associated with injury) in white and gray matter areas in the LPS than the control group in the following regions: entorhinal cortex (Enc), superior colliculus (SupCol), cingulate cortex (CingCor), corpus callosum (CC), external capsule (EC), auditory cortex (AudC), hypothalamus (Hypth), thalamus (Th) and the CA1 of the hippocampus. No area was found with higher ADC levels in the control than the LPS group (Fig. 2A, D).

The T2 levels in white and gray matter areas were higher (higher T2 relaxation values are associated with injured areas (Woo et al., 2009)) in the periventricular fiber system, corpus callosum, sub thalamic radiation, external capsule, forceps major, cortex, thalamus and other white and gray brain areas than in the control group. No area was found with higher T2 levels in the control than the LPS group (Fig. 3A, B).

Maternal exposure to LPS after treatment with MG (MG-LPS-MG) offspring. Offspring of MG-treated LPS dams demonstrated: similar T2 and ADC levels as the controls in both white and gray matter; and an apparently decreased ADC in all the affected white and gray matter areas compared to the offspring of the LPS treated dams (Figs. 2 + 3).

Decreased T2 levels (associated with normal tissue) were observed in all brain areas (Fig. 3A, B) including the periventricular fiber system, the corpus callosum, the external capsule, and the thalamus. In contrast to the LPS group, between the MG treated-LPS exposed dams and the control, no significant difference was observed in most brain regions.

DISCUSSION

Maternal LPS at E18 increases fetal brain IL-1β protein levels in the short term and induces brain injury in offspring at age 25 days, evident by magnetic resonance imaging (MRI). Injury was demonstrated in both gray and white matter areas. MG treatment for 2 h, prior to and following i.p. LPS, prevented brain injury in offspring, as demonstrated by MRI in most brain areas. However, in contrast to the MRI findings, MG did not attenuate fetal brain IL1β protein levels.

LPS, the major structural component of gram-negative bacteria, is a well characterized and widely accepted model of inflammation. LPS activates the immune system, and increases the inflammatory response and

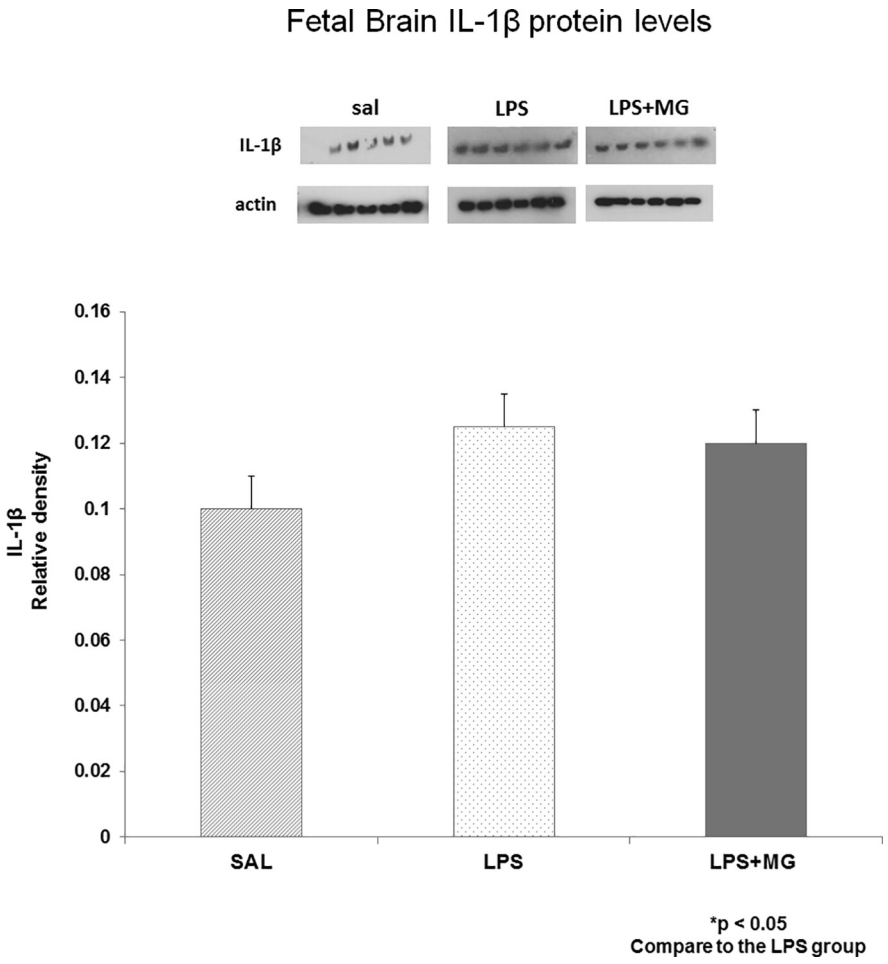

Fig. 1. Fetal Brain IL-1B protein levels (independent-samples Kruskal–Wallis Test). Fetal brain IL-1B protein levels, at embryonic day 18, 4 h following lipopolysaccharide (LPS) injection in three treatment groups: saline (SAL): NS-NS-NS; LPS: NS-LPS-NS; and LPS/MG: MG-LPS-MG. $^{*}p < 0.05$ compared to the LPS group. IL-1B is expressed as IL-1B/actin protein ratio.

cytokine production. As previously demonstrated (Beloosesky et al., 2010), i.p injections of LPS to pregnant rats increase the production of cytokines and stress markers in both the maternal and fetal compartments and may trigger lifelong neuronal damage. White matter damage and decreased myelin basic proteins were demonstrated in immuno-staining brains of fetuses post maternal LPS exposure, while pro-inflammatory cytokines have been implicated in the development of progressive neurologic changes (Urakubo et al., 2001; Rousset et al., 2008; Cui et al., 2009; Meyer et al., 2009). Elevated cerebrospinal fluid and plasma cytokine levels have been reported in infants with hypoxic-ischemic encephalopathy (Oygür et al., 1998) and in infants who later developed poor neurological outcomes (Aly et al., 2006).

In our current study, we demonstrate an increase in the level of interleukin-1β in the fetal brain following maternal LPS administration. IL-1β is a key mediator of inflammatory responses and a well-characterized early response pro-inflammatory cytokine. As demonstrated by Cai et al. (2000) and Pantoni et al. (1998), elevated levels of IL-1β in the brain can accentuate injury and result in adverse neurological outcomes. IL-1β expression

Table 1. Region-function attribution

Fetal brain region-function affected by maternal inflammation

Motor function	Learning & Memory system	Sensory system	Emotional system	General
CPu – Dorsal striatum EC – External capsule	Cpu – Dorsal striatum MS – Medial septal nucleus EnC – Entorhinal cortex DG – Molecular dentate gyrus CingCor – Cingulate cortex CA1 – CA1 of the hippocampus Hip – Hippocampus	S1/S2 - The somato-sensory system PirC - Piriform cortex SupCol - Superior colliculus (visual) AudC – Auditory cortex	InsularC – Insular capsule	CC – corpus callosum Th - Thalamic nucleus: Po – Posterior Vpm – Ventral posterior Va – Ventral anterior Rt - Reticular Hypth - Hypothalamus MI – Medullary cortex Vis C – Visual cortex DoMe – Deep mesencephalic nucleus GenB – Geniculate body (visual)

Function areas of the brain included the LPS group, which had white and gray matter injuries, compared with sal/sal. MG treatment (LPS-MG group) prevented those brain injuries; *LPS,* lipopolysaccharide; *MG, magnesium sulfate.*

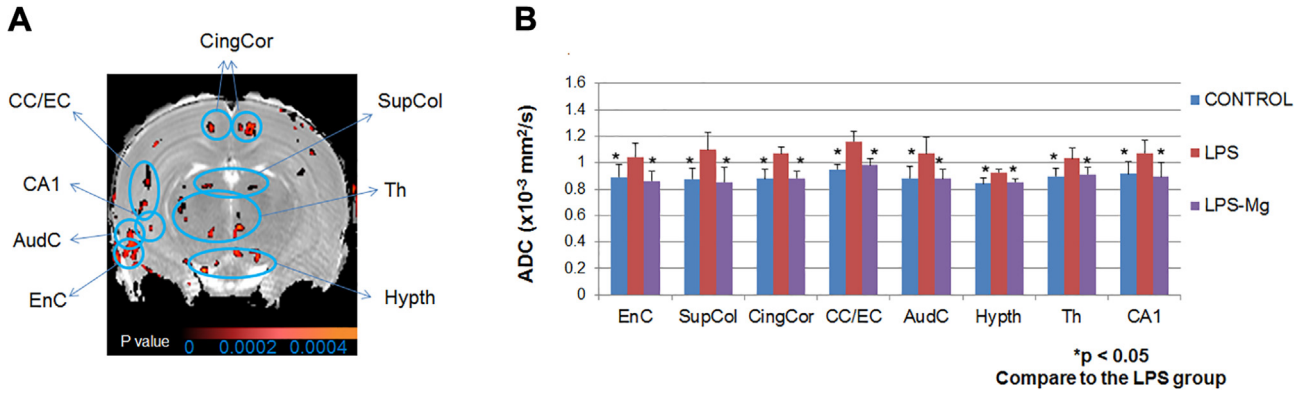

Fig. 2. Neonatal brain MRI at one month after delivery. Neonatal brain MRI at one month after delivery. (A) Images of ADC MRI comparing the LPS groups and LPS-MG group (higher ADC implies brain damage). The *colored areas* indicate areas of significant differences in ADC between the LPS group and the LPS-MG group. The significant regions are superimposed on ADC relaxation maps ($p < 0.001$). (B) In the graphs, averaged ADC values at significant brain regions, in the LPS group, the LPS/MG group, the Sal/MG group and the Sal/Sal group. Asterisk indicates $p < 0.05$. LPS, lipopolysaccharide; MRI, magnetic resonance imaging; MG, magnesium sulfate MG, magnesium sulfate; Sal, saline.

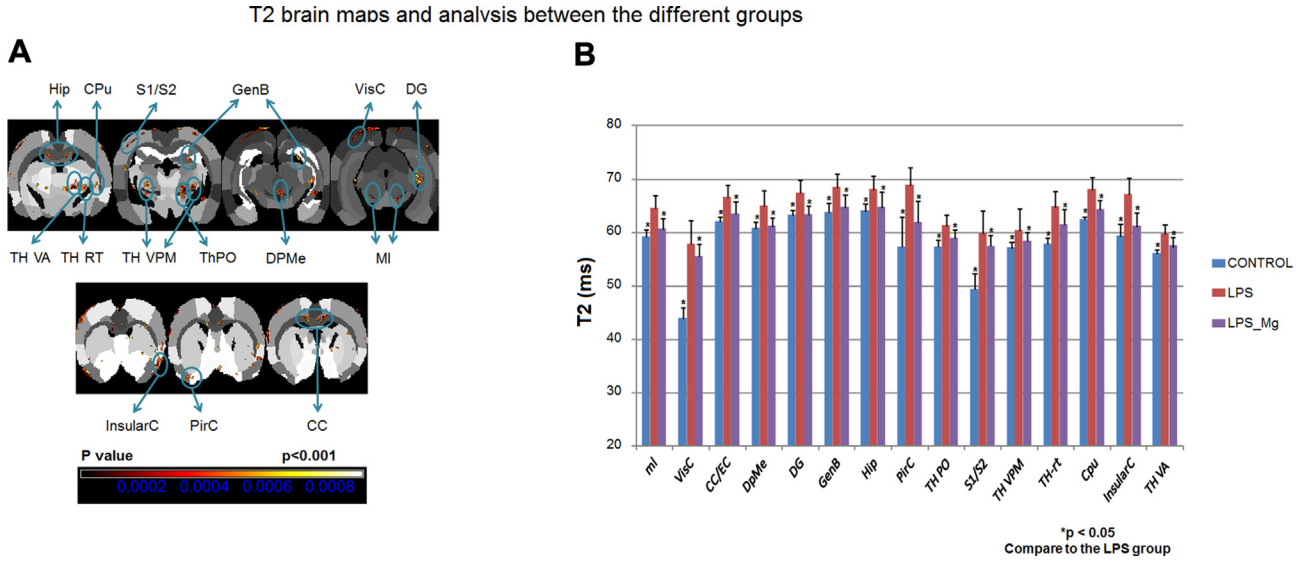

Fig. 3. T2 – ANOVA: Interaction between control LPS and MG treatment. Neonatal brain MRI at day 25 after delivery. (A) Images of T2 MRI comparing the LPS groups and MG treatment (LPS-MG group) group (higher T2 implies brain damage). The colored areas indicate significant differences in T2 between the LPS group and the LPS-MG group. The significant regions are superimposed on T2 relaxation maps ($p < 0.001$). (B) The graphs show averaged T2 values at significant brain regions, in the LPS group, the LPS-MG group and the control group. The asterisk indicates $p < 0.05$. The LPS group included Sal-LPS-Sal; the LPS-MG group included MG-LPS-MG; the control included Sal-Sal-Sal. LPS, lipopolysaccharide; MRI, magnetic resonance imaging; *MG*, magnesium sulfate; Sal, saline.

is increased in the first 48 h following brain injury (Silveira and Procianoy, 2003), and has been linked to hypoxic-ischemic encephalopathy in perinatal animal models. The exact mechanism by which IL-1β damages the developing neuron has not been identified; however, both Girard et al. (2010)) and Leitner et al. (2014)) demonstrated that maternal IL-1 receptor antagonist treatment in a rodent model of systemic inflammation may improve neuronal performance in offspring.

Following maternal LPS, offspring brain MRI demonstrated a significant increase in T2 and ADC levels in both white and gray matter, consistent with diffuse cerebral injury. In this study, we used both DTI (ADC) and T2 for the evaluation of offspring brain injury. DTI appears to be superior to standard MRI techniques for demonstrating white matter brain injury; this promotes further insights into the integrity or disruption of white matter structures. The DTI-ADC measures the magnitude of diffusion (of water molecules) within cerebral tissue. When the tissue is damaged, as demonstrated in the offspring of the LPS-treated dams, the brain tissue is less dense; this facilitates water diffusion in more directions. Such change in water diffusion yields higher ADC. In our experiment we also utilized the T2 map, which provides an estimate of the myelin water fraction, a measurement that is believed to be related to myelin content (MacKay et al., 2006). The offspring of the LPS group demonstrated higher T2 levels (less dense tissue) than the control group in the brain areas associated with motor function, learning and mem-

ory system, sensory system and emotional system (Table 1). Concurring with these findings we recently demonstrated that maternal LPS was associated with impairment of learning ability and memory in rat offspring, though maternal MG prevented this impairment (Lamhot et al., 2015). Contrasting findings were reported by Binette et al. (2016). Although they used a different protocol at different gestational ages, they demonstrated motor deficits in offspring exposed to MG, which may be attributed to a lower level of circulating IL-6.

Skranes et al. (2007) demonstrated in humans that abnormal MRI patterns, similar to the findings in our study (e.g., corpus callosum, internal and external capsule, and other areas corresponding to long association tracts), were related to perceptual, cognitive, motor and mental health impairments found among children born with very low birthweight. This correlation between MRI findings and mental impairment suggests that maternal inflammation-induced offspring brain injury might have long-term offspring sequelae.

Based on several prospective studies, MG may promote fetal neuroprotection. Following a Cochrane meta-analysis review (Nguyen et al., 2013), MG became "the drug of choice" to prevent brain injuries and CP in "at-risk" fetuses, though the mechanism of action is not completely understood. In 2011 Tam Tam et al. (2011) suggested that the key mechanism of MG is anti-inflammatory. By using a LPS rat model, they demonstrated that maternal MG treatment significantly attenuated pro-inflammatory mediator levels in fetal compartments, including the brain. Similar findings were demonstrated by both Suzuki-Kakisaka et al. (2013) and Sugimoto et al. (2012). Additionally, Gao et al. (2013)) recently demonstrated that MG treatment significantly inhibited the release of IL-1β in LPS-activated incubated microglia cells.

According to our results, the significant reduction in offspring brain injury, as demonstrated by MRI, was not accompanied by a similar decrease in the fetal brain IL-1β following maternal MG treatment. Our results imply that the demonstrated neuroprotection modality of MG is probably not mediated via blocking IL-1β production. Support for our data can be found in the study of Burd et al. (2010), which demonstrated morphologic changes in neuronal cultures in an LPS group; these were prevented by MG treatment. Fetal brain IL-1β mRNA levels were increased in both LPS and the LPS + MG, with no statistically significant difference between the groups. We emphasize that we assessed protein levels and not mRNA expression.

Additional results that might correlate with our evidence are those published by Varner et al. (2015) and Blackwell et al. (2001). In the former, using a case-control analysis of 615 participants no correlation was demonstrated between umbilical cord cytokines (including IL-1β) and childhood brain injury. In the latter study, intrapartum MG administration did not affect the concentration of inflammatory cytokines in fetal blood at delivery. We recently demonstrated that MG and the N-methyl-D-aspartate-receptor (NMDA-R) antagonist may have the same protective effect on the fetal brain. This supports involvement of the MG mechanism, at least in part, via blocking the NMDA-R in the fetal brain (Beloosesky et al., n.d.).

In summary, we demonstrated that maternal LPS-induced neonatal brain injury, evident by MRI, can be prevented by maternal MG administration. These findings may have important implications for prevention of newborn neurologic injury.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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