Title: Prenatal Zinc Supplementation Ameliorates Hippocampal Astrocytes Activation and Inflammatory Cytokines Expression Induced by Lipopolysaccharide in a Rat Model of Maternal Immune Activation

Running title: Zinc Inhibits Hippocampal Inflammation

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Abstract

Objective: There is evidence that gestational exposure to lipopolysaccharide (LPS) results in fetal zinc deficiency, and eventually neurodevelopmental abnormalities. In this study, we utilized a rat model of maternal immune activation (MIA) to investigate the possible neuroprotective effect of zinc supplementation throughout pregnancy on hippocampal astrocytes activation as well as inflammatory cytokines expression in adult offspring.

Methods: Pregnant rats received intraperitoneal injections of either LPS (0.5 mg/kg) or saline at gestational day (GD) 15 and 16 and orally gavaged with zinc sulfate (30 mg/kg) throughout pregnancy. Astrocyte density and histological assessment were evaluated in the hippocampus of adult offspring at postnatal day (PND) 60-62. Also, the mRNA levels of IL-6, TNF-α, IL-1β, NF-κB, and glial fibrillary acidic protein (GFAP) were measured using qPCR analysis.

Results: Prenatal exposure to LPS resulted in up-regulated expression levels of IL-6, TNF-α, NF-κB, and GFAP in the hippocampus of adult pups. Moreover, offspring from LPS group showed an increased astrocyte density in CA1 region with no histological alterations in CA1 and CA3 areas. Conversely, maternal zinc supplementation ameliorated these inflammatory alterations induced by LPS.

Discussion: This study provides support for the premise that zinc supplementation during pregnancy might be an early treatment option to inhibit hippocampal inflammation induced by the maternal immune response to infectious agents.

Keywords: Maternal immune activation, Maternal zinc supplementation, Schizophrenia, Lipopolysaccharide, Hippocampus, Inflammatory markers
Introduction

Schizophrenia is a psychiatric disorder with neurodevelopmental origin, for which an interaction between genetic and environmental risk factors is known to induce the development of this mental disease. Based on the neurodevelopmental theory, a widely considered theory of schizophrenia, stressful events during specific stages of embryogenesis lead to disturbance in normal brain development with deleterious and long-lasting impact on the brain function and structure later in life (Lang, Puls, Müller, Strutz-Seebohm, & Gallinat, 2007). Of particular interest is gestational infection, a prenatal risk factor which is known to increase the incidence of schizophrenia in the progeny, supported by the numerous epidemiological and experimental studies (Boksa, 2010; Khandaker, Zimbron, Lewis, & Jones, 2013; Markham & Koenig, 2011; Meyer, Feldon, & Yee, 2009; Meyer, Yee, & Feldon, 2007). In this regard, maternal immune response to pathogenic agents is shown to result in cytokine imbalance in the fetal brain and eventually the manifestation of mental disorders such as schizophrenia (Solek, Farooqi, Verly, Lim, & Ruthazer, 2018). Although the exact mechanism of these detrimental alterations in the brain is unclear, animal models of maternal immune activation (MIA) has been established based on the administration of infectious agents such as lipopolysaccharide (LPS) to pregnant rodents at specific neurodevelopmental time points. Indeed, several studies detailing the use of MIA model with various protocols have supported the face validity of this model to represent several phenotypes related to schizophrenia (Alizadeh, Davoodian, Kazemi, Ghasemi-Kasman, & Shaerzadeh, 2020; de Souza et al., 2015; Hao, Hao, Li, & Li, 2010; Mattei et al., 2014;
A number of human studies have provided evidence for the involvement of neuroinflammation as well as abnormal morphology and functionality of glial cells in the etiology of schizophrenia (Fillman et al., 2013; Müller, 2018; Potvin et al., 2008). More recently, a postmortem study has highlighted a significant upregulation in cellular pathways associated with inflammation in dorsolateral prefrontal cortex, striatum, and particularly hippocampus of subjects with schizophrenia (Lanz et al., 2019). Consistently, the association between schizophrenia and immune dysregulation has been further supported by the numerous MIA animal model studies which dominantly evaluated density or activity of microglia and astrocytes in different brain regions (Solek et al., 2018). Although microglia are the principal innate immunity component in the brain, astrocytes, as most numerous cells in the central nervous system (CNS), are also pivotal players in brain immune activity (Farina, Aloisi, & Meinl, 2007). As such, remarkable astrogliosis along with neuroinflammation has been detected in the hippocampus of pups with prenatal exposure to LPS or IL-6 (Hao et al., 2010; Samuelsson, Jennische, Hansson, & Holmang, 2006). This is further evidenced by a recent study demonstrating the significant astrogliosis in both prefrontal cortex and hippocampus of adult pups born to Polyriboinosinic–polyribocytidylic acid (poly[I:C])-treated dams (Ding et al., 2019). These results indicate that maternal infection leads to long-lasting alterations in the brain of offspring. However, there is controversy surrounding the changes in density and activity of astrocytes in both postmortem brains of schizophrenic patients (Trepanier, Hopperton, Mizrahi, Mechawar, & Bazinet, 2016) and MIA animal models (de Souza et al., 2015), which might be due to the difference in experimental design.

Epidemiological studies have well documented that the response to MIA and not specific kind of pathogen is involved in the etiology of neurodevelopmental disorders, including schizophrenia (Brown et al., 2004; Solek et al., 2018; Yolken, Dickerson, & Fuller Torrey, 2009). The main obvious response to maternal immune challenge is the increased production of pro-inflammatory cytokines, however, the secondary consequences of systemic immune activation need to be considered as well (Reisinger et al., 2015). Accordingly, gestational
exposure to LPS acts as a potent inducer of maternal metallothionein (MT) which results in maternal hypozincemia and ultimately fetal-zinc deficiency (Carey, Berbée, Coyle, Philcox, & Rofe, 2003; Coyle, Tran, Fung, Summers, & Rofe, 2009). Zinc is an essential and abundant trace element required for the correct functioning of numerous proteins and exerts a major influence on the broad spectrum of the cellular process including immunity, wound healing, and normal brain functions (Chasapis, Loutsidou, Spiliopoulou, & Stefanidou, 2012). Interestingly, zinc dyshomeostasis has been identified as being involved in a range of psychiatric diseases, such as depression and schizophrenia (Portbury & Adlard, 2017). This is further supported by recent human studies demonstrating a significant reduction in the serum concentration of zinc in the subjects with schizophrenia (Cai et al., 2015; Cao et al., 2019). Added to this, zinc supplementation during pregnancy is confirmed to reduce neurobehavioral alterations and teratogenicity as well as fetal death induced by LPS in MIA animal model (Alizadeh et al., 2020; Carey et al., 2003; Coyle et al., 2009; Kirsten et al., 2015). However, the cellular mechanism underlying the protective effect of zinc supplementation against LPS-induced impairments is yet to be fully elucidated.

Due to the putative role of hippocampus in neurogenesis and learning as well as its involvement in neuropsychological impairments associated with schizophrenia (Ewing & Winter, 2013), in the present study, we utilized MIA animal model to evaluate the possible neuroprotective effect of zinc supplementation during pregnancy on astrocyte activation as well as several inflammatory mediators in the hippocampus of adult pups.

**Materials and methods**

**Animal**

Female and male Wistar rats (female: 200-230 g, male: 250-300 g) were obtained from animal house of Hormozgan University of Medical Sciences (HUMS). Animals were kept under standard environmental conditions (temperature 22 ºC, humidity 60-70%, 12h light-dark cycle), with unlimited access to food and tap water. In the present study, eight-week-old male offspring were used, which were selected based on the results of our previous study (Alizadeh et al., 2020). All experimental procedures were based on the National Institutes of Health guide for the care and use of laboratory animals and approved by the ethical committee of HUMS (IR.HUMS.REC.1397.276). To minimize the number and suffering of
animals based on the Three Rs principle, we shared the treated animals in two other published articles (Alizadeh et al., 2020; Mousaviyan et al., 2021).

**Treatment**

Adult male and female rats were housed overnight, and the first day of pregnancy confirmed by the presence of spermatozoa in vaginal smears, which was designated as gestational day 1 (GD1). Pregnant dams were randomly assigned into four treatment conditions with 6 litters per group. Control: pregnant dams were received intraperitoneal (i.p.) injections of saline at GD15 and 16; LPS: pregnant dams were received LPS injections (0.5 mg/kg, i.p., *Escherichia coli* L2630) (Waterhouse et al., 2016; Wischhof et al., 2015) at GD15 and 16; LPS+Zinc: pregnant dams were received LPS injections (0.5 mg/kg, i.p.) at GD15 and 16 and orally gavaged with zinc sulfate (30 mg/kg) (Moazedi, Ghotbeddin, & Parham, 2007) throughout pregnancy; Zinc: pregnant dams were received i.p. injections of saline at GD15 and 16 and orally gavaged with zinc sulfate (30 mg/kg) throughout pregnancy. The control and LPS groups were administered with an equal volume of water during pregnancy by gavage. The experimental timeline is provided in Fig.1.

**Fig.1.** Schematic diagram describing the experimental timeline. On gestation day (GD) 15 and 16, pregnant dams were intraperitoneally administered with either LPS (500 µg/kg) or saline and gavaged with zinc sulfate (30 mg/kg)/vehicle throughout pregnancy by gavage. The resulting offspring were submitted to qPCR, immunostaining and morphological analysis at PND60.
Based on sex and treatment, the resulting offspring were weaned on postnatal day 21 (PND21) and maintained undisturbed until PND60. For sample size calculation, we conducted Resource equation method (Charan & Kantharia, 2013). To minimize litter effects, one male offspring from each litter was randomly selected for future analysis. The remaining pups were used for other experiments that were not reported here.

**Tissue collection, RNA isolation, and qPCR analysis**

At PND 60, six male pups from each group (n=6, one pup per litter and 6 litters per group) were sacrificed using CO2 euthanasia. The whole brains were rapidly removed, placed on the ice, followed by the microdissection of hippocampus (Chiu, Lau, Lau, So, & Chang, 2007). The tissues were immediately snap-frozen and kept at -80 ºC.

All the experimental procedures described below were carried out based on the MIQE guidelines (Bustin et al., 2009). Total RNA was extracted using Trizol reagent (Sigma-Aldrich, USA), based on the manufacturer’s instructions. After assessment of RNA integrity using agarose gel electrophoresis, RNA (1 μg) of each sample was reverse-transcribed using the cDNA synthesis kit (Thermo Ficher, USA), as described by the manufacturer’s protocol. qPCR reactions were performed using a Mic qPCR system (Australia) with the primer sets for *IL-6*, *TNF-α*, *NF-κB*, *GFAP*, and *IL-1 β* as the target genes and *GAPDH* as a reference gene (Table 1). The reactions were conducted in SYBR Premix Ex Taq II (Takara, Japan) with a three-step protocol, as described previously (Alizadeh et al., 2020). After normalization with *GAPDH*, relative gene expression analysis was calculated using the \(2^{-\Delta\Delta Ct}\) method.
Table 1. The primer sets applied for qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>il-6</em></td>
<td>NM_012589.2</td>
<td>5′-TGATGGGATGCTTCCAAACTG-3′ 5′-GAGCATTGGAAGTTGGGTA-3′</td>
</tr>
<tr>
<td><em>il-1β</em></td>
<td>NM_031512.2</td>
<td>5′-GCTGTGGGCAGCTACCTATGTCTTG-3′ 5′-AGGTCGTCATCATCCCACGAG-3′</td>
</tr>
<tr>
<td><em>tnf-α</em></td>
<td>NM_012675.3</td>
<td>5′-AAATGGGCTCCCTCTCATCAGTTC-3′ 5′-TCTGCTTGGTGTTTGCTACGAC-3′</td>
</tr>
<tr>
<td><em>nf-κB</em></td>
<td>XM_006233360.3</td>
<td>5′-TGCAAGAAGAAGACATTGAGGTG-3′ 5′-AGGCTAGGGTCAGCTATGG-3′</td>
</tr>
<tr>
<td><em>gfap</em></td>
<td>NM_017009.2</td>
<td>5′-TGGCCACCAGTAACATGCAA-3′ 5′-CAGTTGGCGGCGGATAGTCAT-3′</td>
</tr>
<tr>
<td><em>gapdh</em></td>
<td>XM_017593963.1</td>
<td>5′-ACGGCAAGTCAACAGGCACAG-3′ 5′-GACATACTCAGCACCAGCCTACC-3′</td>
</tr>
</tbody>
</table>

**Immunostaining**

Immunofluorescence staining was carried out, as described previously (Mousaviyan et al., 2021). After anesthetization with ketamine/xylazine (100 mg/kg- 10 mg/kg), animals (n=4/group) were transcardially perfused with phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA). The brain tissues were harvested, post-fixed in PFA overnight, and finally immersed in sucrose solution (30%) for 48h. After freezing in O.C.T compound, coronal sections of the hippocampus (6-μm) were obtained by a cryostat apparatus (MICROM HM 525, Germany) and mounted on charged slides.
For immunostaining, tissue sections were washed with PBS for three 5-min, followed by blocking in 10% normal goat serum and 0.3% Triton X-100 for 1h. The slides were then incubated overnight with rabbit anti-glial fibrillary acidic protein (GFAP) (1:400, Z0334, Dako) at 4 °C. Afterwards, the slides were washed with PBS for three 10-min and incubated with Goat anti-rabbit Alexa Fluor®594 (1:1000 dilution, ab150080) secondary antibody for 1h, followed by staining with 4,6-diamidino-2-phenylindole (DAPI) for 10 min. Subsequently, the images were obtained by fluorescence microscopy (Nikon, Japan). The fluorescence images were manually quantified as the number of GFAP positive cells soma/total cells using Image J 1.42 software (NIH, USA).

Histopathological examination

Like immunofluorescence staining procedure, animals (n=4/group) were perfused by PBS and 4% PFA. Then, the cerebral samples were rapidly harvested and stored in 4% PFA overnight. After dehydration with a series of alcohol and paraffin embedding, the tissues were cut into 6-μm-thick coronal sections. Finally, H&E staining was performed, and the slides were evaluated under a microscope.

Statistical analysis

Data analysis was conducted by GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA). The data from qPCR and immunostaining were analyzed by two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Prenatal treatment (saline vs. LPS) and maternal supplementation (vehicle vs. zinc) was considered, as between-group variables. The data are presented as mean±SD, and statistical=p<0.05.
Results

Prenatal zinc supplementation suppressed the upregulation of pro-inflammatory markers induced by LPS

To evaluate the LPS induced inflammatory reaction in the hippocampus of the offspring as well as the possible protective effect of prenatal zinc supplementation, the expression levels of $IL-6$, $TNF-\alpha$, $NF-\kappa B$, $GFAP$, and $IL-1 \beta$ were measured by qPCR technique. As illustrated in Fig.2a, the expression level of $IL-6$ was differentially affected by LPS, with a moderate increase in the hippocampus of offspring prenatally exposed to LPS compared to the corresponding control (main effect of prenatal treatment, $F (1, 20) = 8.829$, $P = 0.0075$; LPS group vs control group Tukey’s multiple comparison, $P < 0.01$), and this effect was prevented by zinc supplementation in LPS+Zinc group (interaction of main effects, $F (1, 20) = 9.403$, $P = 0.0061$; LPS+Zinc group vs LPS group Tukey's multiple comparison, $P < 0.05$) (Fig.2a).
Fig. 2. Prenatal LPS treatment enhanced the expression of pro-inflammatory mediators in the hippocampus of offspring at PND60, which were suppressed by maternal zinc supplementation. Prenatal LPS exposure significantly induced the expression of IL-6 (a), TNF-α (c), NF-κB (d), and GFAP (e) in the hippocampus of pups, which was mitigated by maternal zinc supplementation. The data are presented as mean±SD, n=6 per group, *p < 0.05, **p < 0.01, and ***p < 0.001 compared to control group. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared to LPS group.

Furthermore, a significant effect of prenatal treatment was also detected for tumor necrosis factor-alpha (TNF-α) mRNA level, reflected by the slight increase in the hippocampus of LPS-exposed pups related to the control group (main effect of prenatal treatment, F (1, 16) = 8.577, P = 0.0083; LPS group vs control group Tukey's multiple comparison, P < 0.01), that
returned to the control level upon maternal zinc supplementation in LPS+Zinc group (interaction of main effects, F (1, 16) = 8.401, P = 0.0089; main effect of maternal supplementation, F (1, 16) = 4.754, P = 0.0413; LPS+Zinc group vs LPS group Tukey's multiple comparison, P < 0.01) (Fig.2c). Gene expression analysis, however, revealed no significant effects of prenatal treatment (F (1, 17) = 0.7645, P = 0.3923), maternal supplementation (F (1, 17) = 0.1752, P = 0.6800), and their interaction (F (1, 17) = 1.507, P = 0.2339) for IL-1β in the experimental groups (Fig.2b). Conversely, LPS treatment had a moderate but significant effect on the expression level of nuclear factor kappa B (NF-κB), depending on prenatal supplementation. NF-κB expression level was significantly upregulated in the offspring from LPS-treated mothers compared to the control pups (main effect of prenatal treatment, F (1, 17) = 6.305, P = 0.0207; LPS group vs control group Tukey's multiple comparison, P < 0.01), whereas this increment was suppressed in the LPS+Zinc group (interaction of main effects, F (1, 17) = 14.12, P = 0.0012; main effect of maternal supplementation, F (1, 17) = 5.549, P = 0.0288; LPS+Zinc group vs LPS group Tukey's multiple comparison, P < 0.01) (Fig.2d). Similarly, there was a significant increase in the GFAP mRNA level in the pups of LPS-exposure mother related to the control group (main effect of prenatal treatment, F (1, 20) = 4.822, P = 0.0401; LPS group vs control group Tukey's multiple comparison, P < 0.05), while in the offspring of LPS+Zinc group, GFAP expression level was approximately back to the control level (LPS+Zinc group vs LPS group Tukey's multiple comparison, P < 0.05). Although, no significant interaction or main effect of maternal supplementation were found for GFAP expression level (interaction of main effects, F (1, 20) = 4.157, P = 0.0549; main effect of maternal supplementation, F (1, 20) = 3.139, P = 0.0917) (Fig.2e).

**Prenatal zinc supplementation alleviated the increased density of astrocytes induced by LPS in the CA1 hippocampus**

In order to examine whether the alterations in the mRNA levels of GFAP as well as pro-inflammatory markers were associated with changes in astrocyte density, we carried out immunostaining in the both CA1 and CA3 areas (Fig.3, 4).
Fig. 3. Immunostaining for GFAP in the CA1 hippocampus of MIA offspring. Immunofluorescence images (a) and the quantified graph (b) of GFAP in the CA1 area of pups at PND60. The data are presented as mean±SD, n=4 per group. Scale bar: 50 μm, **p <0.01 compared to control group and #p < 0.05 compared to LPS group.
Fig. 4. Immunostaining for GFAP in the CA3 hippocampus of MIA offspring. Immunofluorescence images (a) and the quantified graph (b) of GFAP in the CA3 area of pups at PND60. The data are presented as mean±SD, n=4 per group. Scale bar: 50 μm.
In CA1 region of hippocampus, a significant effect of prenatal treatment was revealed (main effect of prenatal treatment, F (1, 12) = 13.86, P = 0.0029), with pups born to LPS-exposure dams exhibited a marked increment in GFAP+ cells compared to the control group (LPS group vs control group Tukey's multiple comparison, P < 0.01). This effect was restrained by maternal zinc supplementation in LPS+Zinc group (interaction of main effects, F (1, 12) = 11.32, P = 0.0056; LPS+Zinc group vs LPS group Tukey's multiple comparison, P < 0.05), with no main effect of maternal supplementation (main effect of maternal supplementation, F (1, 12) = 3.382, P = 0.0908) (Fig.3b). In CA3 area of hippocampus, however, no statistically significant effect for prenatal treatment, maternal supplementation as well as their interaction was detected on astrocyte density (interaction of main effects, F (1, 12) = 0.0, P > 0.9999; main effect of prenatal treatment, F (1, 12) = 0.2297, P = 0.6404; main effect of maternal supplementation, F (1, 12) = 0.2297, P = 0.6404) (Fig.4b).

**No histological changes were observed in CA1 and CA3 hippocampus of offspring**

H&E staining was performed to examine the possible histological alterations including changes in arrangement and morphology structure of cells in both CA1 and CA3 hippocampus of offspring. As depicted in Figure 5a, the CA1 area of the hippocampus from all groups exhibited arranged neurons with normally rounded nuclei (Fig.5a). Similarly, we detected no evidence of histological changes in the CA3 hippocampus of pups in all experimental groups (Fig.5b).
**Fig.5.** Histological assessment of CA1 and CA3 hippocampus. No histological alterations were observed in the both CA1 (a) and CA3 (b) areas for the offspring in all experimental groups. Black arrows represent the normal (N) neurocyte. Scale bar: 50μm, (n=4).

**Discussion**

Maternal infection is known as a prenatal risk factor associated with deleterious effect on the brain function and structure in the progeny (Garay, Hsiao, Patterson, & McAllister, 2013). In our previous study, we demonstrated that prenatal exposed to LPS on GD15 and 16 results in significant behavioral impairments in adult offspring being a phenotype associated with schizophrenia (Alizadeh et al., 2020). Of note, these behavioral deficits were observed only among male pups, which were selected for further investigation. In the study described here, our findings revealed the long-lasting alterations in the hippocampus of adult offspring prenatally exposed to LPS, characterized by the enhanced expression levels of IL-6, TNF-α, NF-κB, and GFAP as well as increased astrocyte density in CA1 region of hippocampus. Furthermore, the mentioned changes were alleviated by maternal zinc supplementation.

Hippocampus is a region in the brain with a prominent role in memory, learning, and neurogenesis. Accordingly, both human and animal studies have repeatedly highlighted the importance of the hippocampus as one of the main areas involved in the pathophysiology of...
schizophrenia (Antoniades et al., 2018; de Souza et al., 2015; Hao et al., 2010; Lieberman et al., 2018). This is further evidenced by the well-described reduction in hippocampal volume in schizophrenic patients as well as MIA offspring (Adriano, Caltagirone, & Spalletta, 2012; van Erp et al., 2016; Zhou, 2015). Furthermore, a recent postmortem study has detected a robust upregulation of inflammatory pathways, especially IL-6 pathway, in the hippocampus of subjects with schizophrenia. This finding provides support for the persistent neuroinflammation in the brain of patients with schizophrenia (Lanz et al., 2019). In MIA model studies, however, contradictory findings have been reported regarding the alterations in hippocampal pro-inflammatory markers of resulting offspring. Herein, we detected the increased mRNA levels of NF-κB, TNF-α, and IL-6 with unchanged expression level of IL-1β in the hippocampus of the offspring born to LPS-treated mothers. In this regard, Pups exposed in utero to Poly I:C (4 mg/kg, i.v.) exhibited the upregulation in expression of both TNF-α and IL-1β in the hippocampus at PND120 (Mattei et al., 2014). In the same region, another study showed the elevated mRNA level of IL-6 in adult offspring prenatally exposed to IL-6 (9 μg/kg, i.p.) (Samuelsson et al., 2006). However, protein levels of IL-6, IL-1β, and TNF-α were reported unchanged in the hippocampus of pups prenatally exposed to Poly I:C (5 mg/kg, i.v.) (Giovanoli et al., 2015). Similar results were demonstrated by a recent study using a different dose of Poly I:C (10 mg/kg, i.v.) (Ding et al., 2019). In addition, in the only MIA study, to our knowledge, examining the expression levels of different inflammatory genes, the mRNA level of NF-κB was found to be unchanged in the hippocampus of offspring at PND21 (Zhou, 2015). Methodological differences, including difference in dose, immunogenic substances, and the gestational stage during that MIA is induced might be responsible for the discrepancies in reported results. Notably, our study revealed the upregulation in GFAP expression level as well as the increased GFAP positive cells in the CA1 hippocampus of offspring prenatally exposed to LPS with no alteration in the CA3 region. This discrepancy observed in hippocampal CA1 and CA3 is probably due to the difference in astrocyte sensitivity and vulnerability of these regions, which is reported to be responsible for the selective neural death in CA1 after forebrain ischemia (Sun, Fukushi, Wang, & Yamamoto, 2018). Using a similar protocol, de Souza et al. noted the increased level of GFAP protein (measured by ELISA) and GFAP immunocontent in the CA1 hippocampus of adult rats born to LPS-challenged mothers (0.5 mg/kg, i.p.) (de Souza et al.,
Consistently, adult offspring of dams treated with LPS (0.79 mg/kg, i.p.) exhibited higher numbers of GFAP immunoreactive cells related to the control (Hao et al., 2010). In contrast, mice exposed prenatally to Poly I:C (5 mg/kg, i.v.) showed no alterations in density of GFAP positive astrocytes in CA1, CA3 and dentate gyrus areas at PND28 and 140 (Giovanoli et al., 2015). The difference in the type of infectious agent and its concentration might explain these conflicting results. Importantly, human studies have also highlighted the pivotal role of astrocytes in the etiology and pathophysiology of schizophrenia (Catts, Wong, Fillman, Fung, & Shannon Weickert, 2014; Tarasov et al., 2019). Therefore, dysregulation of astrocytes function, which are morphologically and functionally associated with neurons, might be a new point of view to illustrate metabolic and transmitter alterations in the brain of schizophrenic patients (Tarasov et al., 2019). Moreover, we performed histological evaluation in CA1 and CA2 hippocampus of adult offspring. In contrast to the previous study in which disordered and reduced number of nuclei were reported in CA1 hippocampus of pups born to LPS challenged mothers (0.79 mg/kg, i.p.) (Hao et al., 2010), we detected no significant histological changes in both areas. Similarly, the number of neurons has been found to be unchanged in the hippocampus of schizophrenic patients (Heckers & Konradi, 2002) as well as prenatally immune challenged offspring in MIA model of schizophrenia (de Souza et al., 2015). This finding reinforces the hypothesis that disruption in neural connectivity (Ruiz, Birbaumer, & Sitaram, 2013), instead of reduced neuron number, might be involved in the development of this mental disease.

As a complex mental health disease, schizophrenia characterized by three major manifestations, including positive, negative, and cognitive symptoms. Currently, the available pharmacological treatment options mainly provide relief for positive symptoms with a lack of effective treatment for negative and cognitive symptoms (Patel, Cherian, Gohil, & Atkinson, 2014). With this background, the discovery of satisfactory pharmacological options as well as early therapeutic interventions is of interest. Using MIA animal model with well-established face and predictive validity (Reisinger et al., 2015), we found that maternal zinc supplementation alleviated LPS-induced increased expression of IL-6, TNF-α, NF-κB, GFAP, and also GFAP positive cells in the hippocampus of male pups. Interestingly, it is reported that gestational infection with LPS is correlated to maternal and fetal hypozincemia mainly due to the stimulating of metallothionein synthesis in the maternal liver (Carey et al., 2015).
In addition to being involved in innate and adaptive immunity, zinc is strongly required for the normal brain functions and fetal hypozincemia can eventually lead to neurodevelopmental damage in the offspring (Chua, Cowley, Manavis, Rofe, & Coyle, 2012; Coyle et al., 2009). In support of this possibility are findings of several studies demonstrating the beneficial effect of zinc supplementation during pregnancy to protect against fetal death, neurobehavioral impairments, autistic-like behaviors, and preterm delivery induced by LPS with unclear molecular mechanism (Alizadeh et al., 2020; Chen et al., 2012; Chua et al., 2012; Kirsten et al., 2015). One possible mechanism is that zinc supplementation during pregnancy might counteract the LPS-induced reduction of zinc availability to the fetus and ultimately prevent the abnormal neurodevelopment in the progeny. Another mechanism can be explained by the antioxidant and anti-inflammatory properties of this element. In support of this, numerous studies have consistently documented the link between zinc deficiency and increased production of oxidative stress and inflammatory markers (Jarosz, Olbert, Wyszogrodzka, Młyniec, & Librowski, 2017). More interestingly is the literature that demonstrates the negative regulatory effect of zinc on NF-κB, which is one of the main inflammatory pathways (Jarosz et al., 2017). This signaling cascade positively regulates the expression of pro-inflammatory genes IL-6, TNF-α, IL-1β, etc (Lawrence, 2009). In this context, it has been suggested that zinc exerts an inhibitory effect on LPS-induce NF-κB and eventually suppresses the expression of inflammatory mediators which further supports our findings (Jarosz et al., 2017). As mentioned earlier, we also found that maternal zinc supplementation alleviated LPS-induced increment in GFAP mRNA level and astrocyte density in the CA1 hippocampus of pups. To our knowledge, there is no MIA model study evaluating the effect of maternal zinc supplementation on the astrocyte density of adult offspring. In the fetal hippocampus at GD18, however, the previous study reporting the protective influence of prenatal zinc treatment on astrogliosis induced by LPS (Chua et al., 2012).

Finally, this study has several potential limitations as followed. MIA animal model is profoundly considered as a suitable tool to evaluate pathomechanism as well as the development of new therapeutic agents for some of the most complex mental diseases, including schizophrenia (Reisinger et al., 2015). However, there is a stigma associated with this model, as this approach is not able to represent all behavioral and pathological features
of a particular neurodevelopmental disease. Added to this, the main focus of this study was to investigate hippocampal astrocyte changes in adult offspring. It should be taken into consideration that another glial cells including microglia cells have a contributing role in producing inflammatory markers. Despite the strong data for the cytokine disturbance in the brain of MIA offspring, literature has provided conflicting results about microglial activation in different brain regions. While some studies have documented an increase in microglial activation, other groups have demonstrated no significant alterations (Bergdolt & Dunaevsky, 2019; Solek et al., 2018). Therefore, the investigation of possible changes in both astrocyte and microglia density in different hippocampal regions of MIA offspring is of utmost importance.

**Conclusion**

Collectively, in the present study, we took advantage of MIA animal model to examine the beneficial effect of maternal zinc supplementation to protect against the LPS-induced hippocampal inflammation in adult offspring. Our findings showed that prenatal LPS exposure induced long-lasting alterations in the hippocampus of the resulting offspring, evidenced by the increased expression of NF-κB, TNF-α, IL-6, GFAP as well as astrocyte density in CA1 area. In addition, our findings showed that zinc supplementation given throughout pregnancy mitigated the mentioned LPS-induced impairments. Therefore, due to the lack of a comprehensive therapeutic strategy for schizophrenia, zinc supplementation during pregnancy might be an early treatment option to inhibit neurodevelopmental abnormalities induced by the maternal immune response to infectious agents.

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**Conflicts of interest**

For the present study, the authors declare no conflict of interest.
Data availability statement

The analyzed data are available from the corresponding author upon reasonable request.

Author contributions

Study design: ND

Data collection: ES, RM, ND

Data analysis: ND, MGK, EE, AA

Manuscript preparation: ND, MGK
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