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LPS-Induced Inflammation Alters Cytokines in Amniotic Fluid and Serum of Pregnant Wistar Rats and their Offspring

Ana Claudia Januario[1]*, Alexandre Ademar Hoeller[1], Joana Margarida Gaspar[2,3], Roberta de Paula Martins[2,4], Tuany Eichwald[2], Monique Michels[5], Felipe Dal-Pizzol[5], João Carlos Xikota[1,6,7], Maria Marlene de Souza Pires[1,6,7]

[1]Programa de Pós Graduação em Ciências Médicas, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

[2]Laboratório de Bioenergética e Estresse Oxidativo (LABOX), Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

[3]Programa de Pós Graduação em Bioquímica, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

[4]Departamento de Ciências da Saúde, Universidade Federal de Santa Catarina (UFSC), Araranguá, SC, Brazil

[5]Laboratório de Fisiopatologia Experimental, Universidade do Extremo Sul Catarinense (UNESC), Criciúma, Brazil

[6]Laboratório de Nutrologia e Metabologia (MENULAB), Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

[7]Departamento de Pediatria, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil

***Corresponding Author**: Ana Claudia Januario, Programa de Pós Graduação em Ciências Médicas, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brazil.

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Abstract

Intrauterine inflammation is a major cause of early pregnancy loss and impaired fetal growth, besides associated with the biogenesis of neuropsychiatric disorders such as autism spectrum and schizophrenia. Animal models of maternal immunity activation aim to elucidate the inflammatory mechanisms and their relationship with neurodevelopmental disorders. Thus, we investigated the effects of LPS-induced inflammation at different doses in female rats and their relation with the duration of the inflammatory event and maternal changes. Pregnant Wistar rats were grouped and treated once (on E17) or 3 times (on E15-E17) with LPS (15 or 150 µg/kg, IP). LPS at a concentration of 150 µg/kg, both in single and repeated doses, impaired gestational weight gain, in addition to cause death in 23.07% of the rats and significantly decreasing the number of live-born pups. LPS injection increased the level of TNF-α in the amniotic fluid which decreased 72 hours after injection, with no changes in these cytokines in the maternal serum. IL1-β was increased in amniotic fluid after a single dose of LPS (150 µg/kg) but decreased after the application of 3 doses, with a difference between the duration of treatments at this dose. Also, was increased in the offspring serum following 3 repeated doses of LPS 150 µg/kg. IL-6 was increased in amniotic fluid at a single dose of 150 µg/kg and decreased after three days of daily LPS injections. There was an increase in maternal serum IL-6 levels at doses of 15 µg/kg applied once and 150 µg/kg after 3 consecutive injections. IL-10 was increased in amniotic fluid after both doses of LPS applied once but decreased 72 h after application of the highest dose. In the maternal serum, only the highest dose increased IL-10 after 72 hours of LPS. These findings demonstrate that even a single dose of low LPS concentration is sufficient to activate the maternal immune system and may have a direct implication on gestational progress that could affect fetal development.

Keywords: *Gestation; Inflammation; LPS; Cytokines; Autism*

Introduction

Prenatal inflammation harms the mother and also the fetus, causing a restriction in fetal development and weight gain, increasing the risk of miscarriage, death of the pregnant woman and premature birth [1,2]. Intra-amniotic inflammation affects one in four premature births, showing the relevance of the inflammatory process in the early pregnancy outcome [3]. It is estimated that 15 million children are born prematurely (i.e. before the thirty-seventh week of pregnancy) worldwide each year. As evidenced in clinical and experimental research in the last decade, about 40% of these children [4] have some kind of alteration in neurodevelopment [5-9], resulting in psychiatric disorders such as schizophrenia and autism spectrum [10-13].

In the experimental field, systemic administration of LPS in pregnant animals is widely used for maternal immune activation (MIA) [9]. The relationship between the augmentation of cytokines in the fetal milieu and the increase of cytokines in maternal serum levels is still unclear [1]. Experimental MIA is used to clarify the mechanisms involving premature births, interruption of spontaneous pregnancy and chorioamnionitis [15-19]. The cytokines TNF-α, IL-1, IL-6, and IL-10, besides participating in the maternal and fetal immune system, have an important role in embryonic and fetal development, participating in the normal maturation of the fetus. Specifically, the concentration of IL-1 in the amniotic fluid is usually elevated during pregnancy, especially in the third trimester [20,21].

Cytokines orchestrate innate or adaptive immune responses by stimulating the expression of each other, triggering a cascade of molecular events or exerting autocrine, paracrine and endocrine activities. These mediators are categorized into two main groups that exert pro- and anti-inflammatory activities [22]. A very conflicting issue is the fact that the elevation of cytokines in the fetal milieu can lead to embryonic and fetal development, but also cause serious damage to fetal nervous system, even leading to death [1,20]. The participation of cytokines involved in the maternal-fetal immune system is well established in the model of prenatal inflammation, with the main mediation of IL1-β, IL-6 and IL-10 and TNF-α [23,25].

Substantial evidence reinforces that prenatal inflammation is associated with adverse effects on maternal and fetal health and may even persist after birth until adulthood when affects the fetus. About forty percent of pregnant women have premature births caused by intra-amniotic inflammation [27]. Despite the evidence, the mechanisms involved in the inflammation-maternal-fetal change interface are poorly elucidated [25].

Importantly, the involvement of intra-amniotic inflammation can occur in different gestational periods and manifest in different ways - including a short-term or long-lasting acute inflammation, which can trigger diversified inflammatory cascades. Thus, we aim to investigate the relationship between the LPS-induced inflammation and the outcome on cytokines measured in pregnant rats and their offspring. We also aimed to observe whereas this response can be influenced by the duration of the inflammatory process.

Materials and Methods

Animals and experimental design

All experiments were conducted in accordance with international standards of animal welfare as outlined by the Brazilian Law (number 11.794 - 10/08/2008) and approved by the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (# 687170217). The number of animals was defined considering the power of the study and the size of the expected effect, ensuring the minimum number of animals required without compromising the results.

We used 95 nulliparous female rats and 31 male Wistar rats (between 12 - 14 weeks of age, 200 - 250g body weight) housed in groups and kept under standard laboratory conditions (controlled room temperature 22 ± 2ºC, 12-h light/dark cycle and access to food and water *ad libitum*).

Rats were allocated in the animal´s facilities, where the female cages were placed next to the male cages for 5 days, in order to synchronize the estrous cycle of the females. After this period, the body weight of the females and the estrous cycle were verified through vaginal washing. Then, 3 females were placed in a cage with 1 male. The vaginal lavage was performed using a Pasteur pipette filled with 100 µl of 0.9% NaCl, where the tip was lightly and carefully introduced into the rat´s vagina. Immediately after, a drop of 0.9% NaCl was injected, aspirated and placed on a glass slide to view the estrous cycle in an optical microscope. Mating started the next day. The vaginal wash was carried out to identify the copulatory plug, or the presence of estrous cells and sperm. After confirmation of the pregnancies, the female rats were placed in cages with supposedly pregnant females.

At E15, rats were randomly divided into six groups: control groups, which were single (E17) or daily (E15-17) injected IP with 0.9% NaCl; groups treated with 15 µg/kg of LPS at E17 or daily at E15-17; groups treated with 150 µg/kg of LPS at E17 or daily at E15-17.

Drugs and treatment

Pregnant rats were randomly divided into groups, weighed for the correct determination of the volume to be administered, followed by the measurement of body temperature with an infrared thermometer after LPS injection. LPS, a liposaccharide derived from E. coli serotype 0111: B4 (number L-2630; Sigma, St. Louis, MO) and saline were injected intraperitoneally (IP) with a volume of 1 µl/g of 0.9% NaCl saline and LPS at doses of 15 or 150 µg/kg.

Experimental procedures

Female rats from each group, and posteriorly their offspring at age of 60 days, were anesthetized (ketamine 150 mg/kg/IP; xylazine 20 mg/kg/IP) and submitted to *in vivo* thoracotomy to extract 1 mL of blood by cardiac puncture (the mothers) or by decapitation (the offspring), which was centrifuged at 3000 rpm for 5 minutes to obtain the serum. Approximately 1 mL of amniotic fluid was collected from the gestational sacs by suction and centrifuged. The samples were frozen in a freezer -80ºC until analysis. The rats that were not used to collect biological samples remained with the gestation until the offspring births, where body temperature, gestational weight gain and the LPS effects on pups were observed. For inflammatory markers analysis, a pool of serum and amniotic fluid was performed by ELISA kits (R&D SYTEMS).

Statistical analysis

The results are expressed as mean ± S.E.M., and were analyzed by two-way ANOVA, with "treatment duration" and "treatment" as factors, followed by the Student Newman-Keuls' post hoc test for multiple comparisons when appropriate. Differences were considered significant when $p < 0.05$. All tests were performed using the software Statistica® (StaSoft Inc., Tulsa, USA), version 8.0 and graphs were drawn with the GraphPad Prism@ software, version 5.0.

Results

The effects of the LPS-induced inflammatory process in pregnant rats were observed through the following parameters: body temperature, gestational maternal weight gain and number of live births. As observed in table 1, regarding the body temperature, only the "treatment" factor was significant according to two-way ANOVA [$F(2,87) = 6.04$, $p < 0.005$], with no differences in the multiple comparison between groups, despite a trend in the hypothermic effect caused by a single injection of LPS (150 µg/kg) compared to the control group ($p = 0.06$). In relation to maternal weight gain, the "treatment" factor is altered [$F(2,77) = 21.81$, $p < 0.00001$], with a significant decrease of maternal weight gain caused by single ($p < 0.005$) or multiple injections ($p = 0.001$) of the highest dose of LPS (150 µg/kg) in relation to the respective control groups. Similarly, the higher dose of LPS used once ($p < 0.005$) or three times ($p = 0.001$) significantly decreased the number of pups born alive compared to rats treated with saline [Treatment: $F(2,87) = 17.74$, $p < 0.00001$). Also, death was observed

in 23.07% of pregnant rats that received three doses of LPS 150 µg/kg and 11.11% of rats that received one dose of LPS 150 µg/kg, with no influence of lower doses on mortality. Gestational weight gain was compromised in pregnant rats submitted to LPS injections in the highest dose.

Treatment duration	Treatment (µg/kg)	Gestational parameters (mean ± S.E.M.)						
		Body temperature (ºC)	N	Maternal weight gain (g)	N	Puppies born alive	N	Maternal death (%)
1 day	Control	35.61 (0.13)	17	111.82 (5.01)	17	9.75 (1.01)	16	0
	LPS 15	35.68 (0.24)	6	99.45 (5.34)	11	7.3 (0.83)	18	5.55
	LPS 150	35.14 (0.15)†	19	82.18 (5.08)*	17	5 (1.23)*	17	11.11
3 days	Control	35.26 (0.07)	19	114.19 (4.71)	16	9.87 (1.13)	15	0
	LPS 15	35.65 (0.11)	15	108.67 (7.30)	6	7.29 (1.30)	7	0
	LPS 150	35.25 (0.08)	17	81.56 (4.69)*	16	2.65 (0.87)*	20	23.07

Table 1: *Effects of the LPS-induced inflammatory process in pregnant rats on physiological and demographic parameters. Values are represented as mean ± S.E.M. and assessed by two-way ANOVA ("treatment duration" and "treatment" as factors) followed by the Newman Keuls post-hoc test when necessary. *p < 0.05 in relation to the respective control group (treated with saline). †p = 0.06 in relation to the respective control group (treated with saline). "N", number of rats per group according to the evaluated parameter.*

The LPS-induced inflammatory process were also investigated through the quantification of cytokines present in maternal serum and amniotic fluid of pregnant rats. As seen in figure 1A, two-way ANOVA revealed significant differences in the factors "days of treatment" [$F(1,42) = 30.38$, $p < 0.00001$) and interaction between this factor and "treatment" [$F(2,42) = 17.68$, $p < 0.00001$) in the measurement of IL-1β in amniotic fluid. According to post-hoc comparison, LPS (150 µg/kg) single injection increased the expression of IL-1β comparing to the saline group ($p < 0.05$). Nevertheless, LPS (150 µg kg) administered over 3 consecutive days significantly decreased the expression of this cytokine in relation to control animals ($p = 0.0001$) and also when compared with the same dose administered only once ($p = 0.0001$). The measurement of IL-1β in maternal serum (Figure 1B) was significantly different in the interaction between the factors "treatment" and "treatment days" [$F(2,39) = 4.26$, $p < 0.05$], with no differences in comparison between groups. When measured in the serum of adult offspring, IL-1β changed the factors "treatment" [$F(2,45) = 29.38$, $p < 0.00001$], "duration of treatment" [$F(1,45) = 12,01$, $p < 0.01$] and interaction between them [$F(2,45) = 16.03$, $p = 0.00001$]. In the post-hoc comparison, the single injection of LPS (150 µg/kg) increased the levels of IL-1β compared to the saline group ($p < 0.0001$). The same dose administered three times did not cause the same effect, being significantly lower in relation to the single application ($p = 0.0001$, Figure 1C).

The expression of IL-6 in the amniotic fluid (Figure 1C) showed a significant difference according to two-way ANOVA in the factors "treatment days" [$F(1,45) = 6.90$, $p < 0.05$] and interaction between this factor and "Treatment" [$F(2,45) = 12.04$, $p < 0.0001$]. The post-hoc analysis showed an increase in IL-6 expression after a single administration of LPS (150 µg/kg) compared to the control group ($p < 0.05$). Consecutive treatment with LPS (150 µg/kg) decreased the levels of IL-6 in the amniotic fluid compared to the control group ($p < 0.01$) and when compared to the same dose administered once ($p < 0.001$). Two-way ANOVA showed a difference in the "treatment" factor when measuring IL-6 maternal serum [$F(2,37) = 10.99$, $p = 0.0001$). Comparison between groups showed an increase in IL-6 after a single administration of LPS (150 µg/kg) compared to rats treated with saline ($p < 0.05$). Multiple LPS administrations (15 µg/kg) also increased levels of IL-6 in relation to the control group ($p < 0.01$, Figure 1D). When measured in the serum of offspring, IL-6 was not modified, despite the significant change in the "treatment" factor after two-way ANOVA [$F2,46) = 4.09$, $p < 0.05$, Figure 1F).

Two-way ANOVA showed significant changes in the factors "treatment" [$F(2,50) = 4.52$, $p < 0.05$] and interaction between this factor and "treatment days" [$F(2,50) = 20.19$, $p < 0.00001$] when measuring IL-10 in amniotic fluid. In the comparison between groups, when administered only once, LPS (15 µg/kg) decreased ($p < 0.05$), while the dose of 150 µg/kg increased the levels of IL-10 in relation to saline treatment ($p = 0.01$). When administered for three consecutive days, LPS 150 µg/kg decreased IL-10 in the amniotic fluid in relation to the respective control group ($p < 0.001$) and when compared to the same dose treated only once ($p < 0.001$, Figure 1G). When IL-10 was measured in maternal serum, two-way ANOVA showed differences in the "treatment" factor [$F(2,44) = 8.51$, $p < 0.001$). In the comparison between groups, the multiple administration of LPS 150 µg/kg increased the levels of IL-10 in the maternal serum in relation to the control group ($p < 0.01$) and to the same dose used only once ($p < 0.05$, Figure 1H). In the serum of offspring, ANOVA revealed statistical differences in the "treatment" factor [$F(2,46) = 6.50$, $p < 0.01$], showing an increase in IL-10 levels after consecutive administration of LPS 150 µg/kg in relation to the respective control group and the same dose applied only once (Figure 1I).

TNF-α was measured in the amniotic fluid. According to two-way ANOVA, factor "days of treatment" [$F(1,38) = 5.62$, $p < 0.05$] and interaction between this factor and "treatment" [$F(2,38) = 9.05$, $p < 0.001$] showed a significant difference. Post-hoc analysis showed an increase in the levels of TNF-α after a single injection of LPS 150 µg/kg compared to the control group ($p < 0.05$). Multiple administration with LPS 150 µg/kg significantly decreased TNF-α levels comparing to the same dose administered once (Figure 1J). No changes were observed in maternal or adult offspring serum levels of TNF- α (Figure 1K-1L).

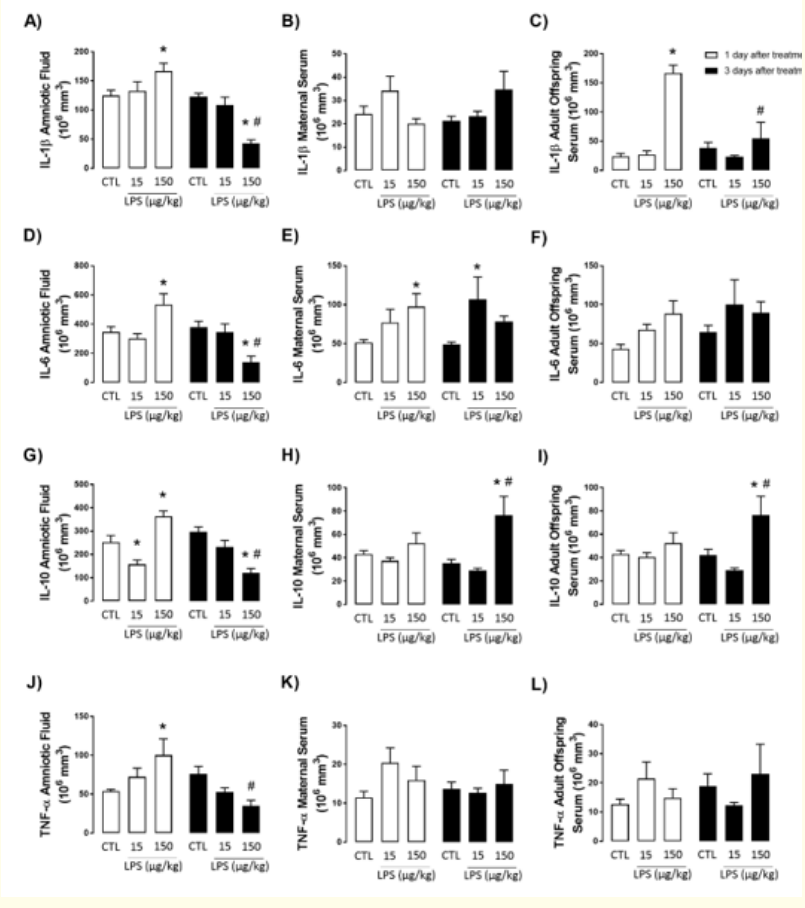

Figure 1: *Effects of the LPS-induced inflammatory cytokines in the amniotic fluid and serum from pregnant Wistar rats and serum from their adult offspring. Values are represented as mean ± S.E.M. and assessed by two-way ANOVA ("treatment duration" and "treatment" as factors) followed by the Newman Keuls post-hoc test when necessary. *p < 0.05 in relation to the respective control group (treated with saline). #p < 0.05 in relation to the respective dose administered only once (n = 4 - 14/group).*

Discussion

A growing body of evidence implies the inflammatory process during the prenatal period as harmful to pregnant women and associated with abortion and impaired fetuses development [1,2]. Here we show that treatment with LPS slightly alters body temperature besides decrease maternal weight gain and the number of pups born alive. In addition, it has been shown that intrauterine inflammation in pregnant rats changes the levels of cytokines measured in the amniotic fluid differently, with an increase in inflammatory markers after a short exposure to LPS and decreasing these after successive injections. Interestingly, only interleukins 6 and 10 were sensitive to inflammation measured in maternal serum, increasing after single or consecutive injections of LPS (except IL-10 levels after single injection), and interleukins 1β and 10, increased after the administration of LPS 150 μg/kg (IL-10 only augmented following 3 consecutive injections) in the adult offspring serum.

In this study, LPS did not alter the body temperature of pregnant rats at E17, despite a slight tendency to hypothermia caused by a single injection of LPS 150 μg/kg. Bison., *et al.* [28] observed in rodents the influence of different doses of LPS on physiological responses, showing that low doses of LPS did not interfere in the body temperature, despite the hypothermic effect observed at concentrations above 125 μg/kg. Previous research by our group also revealed that a single injection of LPS (at a concentration of 150 μg/kg) caused hypothermia in pregnant rats [2], indicating the activation of the immune system and association with severe or fatal inflammation. Gomez-Lopez and collaborators [29] observed that LPS at a dose of 15 μg/kg (i.p. injection) caused hypothermia and premature birth of the offspring. When administered directly into the intrauterine cavity, LPS did not reduce body temperature in mice. Corroborating these findings, Tsang, Fewell and Moore [30] found a similar hypothermic effect in pregnant rats after the systemic injection of LPS.

Hypothermia indicates the activation of the immune system and, although fever is considered a fundamental characteristic in the process of sepsis, the reduction in body temperature is associated with severe or fatal inflammation. The binding of LPS to its receptor, Toll-like Receptor 4 (TRL4), activates the production of pro-inflammatory cytokines, including TNF, which may have a protective function and is linked to thermal modulation, although these mechanisms are not clear [31]. Also, considerable death was observed in pregnant rats receiving LPS 150 μg/kg, with no influence of lower doses on mortality. Gestational weight gain was compromised in pregnant rats submitted to LPS injections in the highest dose, an effect also demonstrated by Lins., *et al*. [32], Peiris., *et al*. [3] and Toyama., *et al* [2].

As observed here, IL-1β was increased in amniotic fluid after a single injection of LPS 150 μg/kg and decreased after the application of 3 doses. Importantly, Reznikov., *et al.* report that even in pregnancies without complications, there is a significant increase of IL-1β in amniotic fluid [17], especially in the third gestational trimester [20,21]. Thus, our results are justified by showing a considerably greater amount of cytokines in the amniotic fluid in relation to the maternal environment, reinforcing the questioning about the immune system activation, being the maternal or fetal environments capable of activating immune responses without maternal interference, or the existence of an interface between the two. In addition, several studies reported increased levels of IL-1β in maternal serum but not in amniotic fluid [2,5,18,19], highlighting the needed of additional studies to clarify this issue.

TNF-α and IL-1β, the first cytokines to be activated in amniotic fluid [1,2,5,14-17,21,27], have pro-inflammatory characteristics and activate anti-inflammatory cascades in response to the entry of infectious agents. Similar to our findings, maternal-fetal immune activation activates IL-6 and IL-10 [6,14,33], corroborating our results, where the administration of LPS 150 μg/kg increased the concentrations of IL-6 and IL-10 in maternal serum and amniotic fluid, although in amniotic fluid this increase occurred after 3 hours and decreased after 72 hours hours. In maternal serum, IL-6 increased by 3 hours (150 μg/kg) and remained elevated only at the dose of 15 μg/kg after 72 hours of LPS-induced inflammation. IL-10 was also activated in the amniotic fluid in 3 hours, normalizing after 72 hours, while in the maternal serum it remained activated after 72 hours.

IL-6 can be considered a pro-inflammatory cytokine that also participates in the molecular anti-inflammatory cascades. In turn, IL-10 plays a crucial role in anti-inflammatory mechanisms [21,22]. In accordance, we shown that TNF-α and IL-1 were initially activated for

three hours in the fetal environment, subsequently triggering the anti-inflammatory cytokines that remained elevated for 72 hours. Importantly, the relationship between maternal and fetal activation is still unclear, since we did not observe an increase in pro-inflammatory cytokines in the maternal environment. Perhaps this can happen soon after the induction of the inflammatory process by LPS. These results indicate that both the maternal and fetal environments respond to LPS, not evidencing the maternal or fetal environment participation in triggering the autoimmune response or whether they participate in a dependent way or not.

We observed that LPS (150 μg/kg) increased the levels of TNF-α present in the amniotic fluid three hours after the injection, in accordance with studies that observed a decrease in this cytokine 72h after administration of LPS [2,5,17,18]. Interestingly, LPS elevated TNF-α in the placenta of rats [14]. TNF-α signaling in the placenta can reach the fetus playing a critical role in hypoxia, decreased fetal neurogenesis [6], placental hemorrhage [16], involved in fetal death mediation [1]. Paradoxically, high levels of TNF-α in the fetal environment, in addition to causing problems, can play a protective function since its role in thermal regulation and in the identification of the infecting target, activating anti-inflammatory cascades.

Although not fully understood, amniotic cells produce a variety of anti-inflammatory factors such as IL-10 [6,13,14], which increases with the presence of TNF-α, IL-1 and LPS [26]. Activation of TNF-α occurs immediately after infection, being produced by amnion and released directly into the amniotic fluid [22]. These findings could justify the non-elevation of TNF-α in the maternal serum level, although this has been observed in some studies [6,18,19,22]. On the other hand, similar to our findings, Carleigh., *et al*. [16] reported an increase in TNF-α in amniotic fluid after LPS injection, but not in maternal serum, although this study was carried out in pregnant mares and not in rodents. These results raise doubts about the relationship of the maternal-fetal immune system and who would be responsible for triggering the anti-inflammatory cascades, the mother or the fetal environment, a question also previously carried out [1]. The fact that TNF-α maternal serum levels were not elevated highlights that the peak of maternal activation may occur before serum collection, which occurred three hours after LPS administration.

Importantly, even at low doses, LPS activated anti-inflammatory molecules. This animal model of activation of the maternal immune system is frequently used in studies on changes in neurodevelopment, mainly in autism spectrum disorder. It is a validated and well accepted model, although previous studies have reported doses of LPS ranging from 100 to 200 μg/kg injected in pregnant rats [1,2,10,13]. Therefore, the alterations caused by a low dose of LPS presented here can bring contributions to future studies on MIA, encouraging new researchers to use protocols with lower doses of LPS and, with this, reducing the financial investment with experiments, besides reinforcing the attention that must be given to maternal-fetal inflammation, since there is robust evidence that this event can lead to negative outcomes for the fetus, such as changes in neurodevelopment [25,27,33].

The present study also shows that the inflammatory responses activated by MIA can remain altered in the offspring until adulthood. This was observed through the quantification of inflammatory and anti-inflammatory cytokines in the serum of adult offspring, where the rats that were exposed to inflammation in the intrauterine period with LPS 150 μg/kg maintained high serum levels of IL-1β and IL-10. These results corroborate with two reviews, Depino [34] and Bergdolt and Dunaevsky [5], which refer to alterations in the immune response and increase in peripheral cytokines in the offspring exposed to MIA, which may last throughout life. Other studies have shown changes in the quantification of cytokines in brain regions, such as prefrontal cortex, hippocampus, and hypothalamus in adult animals submitted to MIA [6,10].

Conclusion

Nevertheless, our results reveal the negative outcome caused by the induced-inflammation in pregnant rats, affecting the offspring and the mother herself. Importantly, the activation of inflammatory markers in the maternal and offspring serum, and mainly in the amniotic fluid after treatment with low doses of LPS, stands out showing that a mild harmful stimulus may trigger inflammatory markers and activate the maternal immune system during the gestational period.

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