Induction of systemic inflammation by hyaluronan and hsp70 in women with pre-eclampsia

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A R T I C L E   I N F O
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A B S T R A C T
Pre-eclampsia (PE) is a human pregnancy syndrome with abnormal activation of the innate immune response. The study evaluated the involvement of molecular structures called damage-associated molecular patterns (DAMPs), such as hyaluronan (HA) and heat shock proteins (Hsp) on NLRP1 and NLRP3 inflammasome activation in peripheral blood monocytes. Twenty pre-eclamptic women, 20 normotensive pregnant women (NT) and 20 non-pregnant women (NP) were studied. Enzyme immunoassay was employed for the determination of HA, Hsp70 and High mobility group Box 1 (HMGB1) in plasma, as well as for the detection of Interleukin-1β (IL-1β), IL-18 and tumor necrosis factor alpha (TNF-α) in the supernatant of monocytes cultured with or without HA and Hsp70. The inflammasome induction was evaluated by the quantification of mRNA for NLRP1, NLRP3, caspase-1, IL-1β, IL-18, HMGB1 and TNF-α by qPCR in monocyte culture. The results showed significantly higher plasma levels of HA, Hsp70 and HMGB1 in pre-eclamptic women than in NT and NP women. Monocytes from women with PE showed endogenous activation of NLRP1 and NLRP3 inflammasomes, and expressed high amounts of IL-1β, IL-18 and TNF-α. The stimulation of monocytes with HA increased the gene expression of NLRP1, NLRP3, caspase-1, TNF-α, IL-1β, HMGB1 and IL-18 and the production of IL-1β in pre-eclamptic women. Monocytes cultured with Hsp70 produced elevated levels of IL-1β and TNF-α through a mechanism independent of inflammasomes activation. These results suggest the participation of these DAMPs in the systemic inflammatory response that is characteristic of PE.

1. Introduction
Maternal immune adaptation is required for pregnancy involving innate and adaptive immunity, to permit normal development and growth of the fetal semi-graft [1]. A specific syndrome called pre-eclampsia (PE) can be present in 2–10% of human pregnancies [2], and is the leading cause of mortality, morbidity and premature labor. This pathology is primarily identified by hypertension and proteinuria from 20 weeks of gestation [3] or by hypertension associated with maternal neurologic or hematologic complications, kidney failure, liver involvement or fetal growth restriction [4,5].

PE shows intense inflammatory response that seems to be related to molecules capable of inducing inflammation, which are called damage-associated molecular patterns (DAMPs) [6]. DAMPs are represented by molecules such as uric acid [7], reactive oxygen species, heat shock proteins (Hsps) [8], proteins released from cells such as HMGB1 [9] and products released from the extracellular matrix such as hyaluronan [10,11]. Plasma concentration of Hsp70 was significantly higher in pregnant women with early-onset PE than in the late-onset PE [12]. The release of Hsp70 by monocytes/macrophages acts as a “danger signal” stimulating TNF-α, IL-6 and IL-8 production by the human monocyte cell line THP-1 [13].

High mobility group box 1 (HMGB1) is considered as a “danger signal”, acting as a pro-inflammatory mediator [14]. The release of HMGB1 in endotoxemia is dependent on activation of the NLRP3 inflammasome [15]. Plasma level of this protein is increased in women associated with pre-eclampsia (PE) [4,5].
with PE compared to normotensive pregnant women [16] and may contribute to the development of the inflammatory response in women with PE [17].

Hyaluronan (HA) is a glycosaminoglycan of the extracellular matrix, which undergoes rapid degradation in inflammatory environments, resulting in the accumulation of low molecular weight fragments [10]. These fragments activate the pro-inflammatory response via Toll-like receptors (TLR) 2 and TLR4, and can also activate the NLRP3 inflammasome acting as a DAMP [18]. Elevated plasma levels of HA are described in women with PE [19,20].

DAMPs exert their inflammatory effect by interacting with the main pattern recognition receptors (PRRs) such as Toll-like (TLRs) and NLR receptors expressed on innate immune cells involved in the inflammatory response [21]. Activation of NLR receptors by DAMPs induces the cleavage of the inactive form of caspase-1 that becomes biologically active [22,23]. Caspase-1 is responsible for cleaving pro-IL-1β and pro-IL-18 to the biologically active forms IL-1β and IL-18, which are subsequently secreted into the extracellular medium [24]. Since NLRP3 inflammasome may be activated by DAMPs in monocytes from women with PE, the present study aimed to evaluate the involvement of hyaluronan (HA) and Hsp70 in NLRP1 and NLRP3 inflammasomes activation in monocytes from women with PE, as well as normotensive pregnant and non-pregnant women.

2. Materials and methods

2.1. Subjects

The study included 40 pregnant women admitted to the Obstetric Unit of Botucatu Medical School, Botucatu, SP, Brazil. Twenty women, without preexistent hypertension or obstetric and medical complications, were diagnosed with PE. A group of 20 pregnant women with an uncomplicated pregnancy matched for gestational age with the pre-eclamptic group were recruited as controls. Gestational age was confirmed by early (<12 weeks gestation) ultrasound examination. Twenty healthy non-pregnant women, volunteer donors of the Blood Bank from the Hemocenter of the Botucatu Medical School were included to compare the immunological parameters between groups. Proteinuria was measured in 24-h urine by the Technicon RAXT autoanalyzer system, a colorimetric method and uric acid was determined by uric acid enzymatic Trinder (Biotrol Diagnostic). Exclusion criteria included prior pre-eclampsia, illicit drug use, multiple gestation and pre-existing medical conditions such as renal disease, diabetes and chronic hypertension. The Ethics Committee of the Botucatu Medical School approved the study (Protocol n° 349.847) and all women signed a written informed consent. Parents or guardians have signed for women with age below 18 years old.

2.2. Blood sampling

Ten milliliters of blood from all women was collected into plastic tubes containing 5% EDTA and centrifuged for 10 min at 1200 g. The plasma was collected and stored in aliquots at −80°C until analyses.

2.3. Monocyte cultures

After plasma separation, density gradient centrifugation method was used to isolate peripheral blood mononuclear cells (PBMCs) on Ficoll-Paque Premium [density (d) = 1.077] (GE Healthcare Bio-Sciences, Uppsala, Sweden) as previously described [25]. Cell viability was >95% in all experiments as determined by 0.2% Trypan Blue dye exclusion. Neutral red dye (0.02%) was used to count monocytes to final concentration of 5 × 10° monocytes/mL in complete medium and distributed (1 mL/well) in 24-well flat-bottomed plates (NalgeNunc, Rochester, NY, USA). After 2 h of incubation at 37°C in 5% CO2 atmosphere, each well was rinsed twice with complete medium to remove non-adherent cells. Monocyte culture routinely contained >90% monocytes as determined by morphology and staining examination [26]. These cells were incubated with or without 100 ng/mL of low molecular weight hyaluronan (HA) (R&D Systems, Minneapolis, USA) or 2.5 ng/mL of Hsp70 (Sigma-Aldrich). The concentrations of HA and Hsp70 were previously standardized employing monocytes from healthy non-pregnant women. To rule out the presence of endotoxin in HA and Hsp70 stimuli preparation, a culture with these stimuli was performed employing polymixin B (PMX-B). The addition of PMX-B to monocyte cultures significantly decreased TNF-α production by LPS-stimulated cells, while there was no effect on the cytokine release after HA and Hsp70 stimulation (data not shown). Culture supernatants were collected and stored at −80°C until cytokines determination.

2.4. DAMPs and cytokines determination

Quantification of DAMPs (Hyaluronan and Hsp70) in plasma and TNF-α and IL-1β cytokines in the supernatants of monocytes cultured in the presence or absence of hyaluronan (HA) or Hsp70 were done by specific commercial kits obtained from R&D Systems. IL-1β was determined in monocytes supernatant using the quantitative ELISA kit (MBL – Medical & Biological Laboratories, Japan). HMGB1 was quantified in plasma using the IBL kit International - Shino Test (Hamburg, Germany). In the tests, the concentrations of monoclonal and polyclonal antibodies, as well as the specific recombinant cytokines used in standard curves were those recommended by the manufacturer. Assay sensitivity limits were 0.4 ng/mL hyaluronan, 125 pg/mL Hsp70, 0.1 ng/mL HMGB1, 15.6 pg/mL TNF-α, 3.9 pg/mL IL-1β and 12.5 pg/mL IL-18.

2.5. Expression of transcripts related to inflammation

Monocytes were incubated with or without 100 ng/mL of HA (R&D Systems) or 2.5 ng/mL of Hsp70 (Sigma-Aldrich) for 4 h and were submitted to gene expression of the genes encoding the proteins NLRP1, NLRP3, caspase-1, HMGB1, IL-1β, IL-18 and TNF-α. Glibenclamide (Sigma-Aldrich) was added to monocytes cultures from 5 non-pregnant women for 30 min at concentration of 50 and 200 μM before incubating HA (100 ng/mL) culture for 4 h this procedure allowed evaluating the inhibitory effect of glibenclamide on HA activation of NLRP3 inflammasome. Total RNA Purification Kit (NorgenBiotek Corp., Thorold, Canada) was used to extract total mRNA from monocytes according to the manufacturer’s protocol. The purity and relative quality of samples, synthesis of complementary DNA (cDNA) and the gene expression of NLRP1, NLRP3, caspase-1, HMGB1, TNF-α, IL-1β and IL-18 by real-time quantitative Polymerase Chain Reaction (RT-qPCR) were performed as described by Matias et al. [7]. The primer sequences are shown in Table 1. Differential expression of genes was performed by the data processing method in relation to a standard curve [27]. To relative expression analysis an mRNA sample was select from each group and received 100 as relative value. All other samples received values based on that sample.

2.6. Statistical analysis

Nonparametric tests (Mann-Whitney U test or Kruskal-Wallis) were employed to analyze the characteristics of the women and concentration of DAMPs. Cytokine production and gene expression data were evaluated by parametric analysis of variance (ANOVA). Results were evaluated using the statistical program PRISM, (Graph Prism, version 6.01, GraphPad, CA, USA) and statistical significance was accepted at p < 0.05.
3. Results

3.1. Clinical characteristics

Analysis of the clinical characteristics of women with PE, normotensive (NT) pregnant women and non-pregnant (NP) women (Table 2) showed no statistical difference in age and race parameters between the groups studied. Similarly, there was no significant difference in gestational age and parity between women with PE and NT pregnant women. Systolic and diastolic blood pressure were significantly higher in the pre-eclamptic group (p < 0.05) than in the NT and NP groups. Additionally, proteinuria concentration was significantly higher (p < 0.05) in women with PE compared to NT pregnant women.

3.2. Concentration of DAMPs in plasma

Plasma concentrations of uric acid, Hyaluronan, Hsp70 and HMGB1 were significantly higher in women with PE compared with NT and NP women (Table 3).

3.3. Basal gene expression and cytokine production by monocytes

The basal gene expression of NLRP1 (Fig. 1A), NLRP3 (Fig. 1B), caspase-1 (Fig. 1C), IL-1β (Fig. 1D) and TNF-α (Fig. 1E) is significantly increased in monocytes from women with PE when compared to NT and NP groups. HMGB1 (Fig. 1F) and IL-18 (Fig. 1G) showed significantly different expression between pre-eclamptic and non-pregnant women. Lower gene expressions of NLRP1 (Fig. 1A), NLRP3 (Fig. 1B), caspase-1 (Fig. 1C), IL-1β (Fig. 1D) and HMGB1 (Fig. 1F) were detected in the NT group compared to the NP group.

Monocytes from the PE group produced higher levels of IL-1β (Fig. 1H) and TNF-α (Fig. 1I) compared to the NT and NP groups, while IL-18 (Fig. 1J) was elevated only in the PE group compared to the NT group. The production of TNF-α (Fig. 1I) and IL-18 (Fig. 1J) by monocytes from NT women was significantly lower compared to NP group.

3.4. Gene expression and cytokine production by monocytes stimulated with hyaluronan

Stimulation of monocytes with Hyaluronan (HA) led to a significant increase in the gene expression of NLRP1 (Fig. 2A), NLRP3 (Fig. 2B), caspase-1 (Fig. 2C), IL-1β (Fig. 2D) and TNF-α (Fig. 2E) in cells from women with PE compared to the NT and NP groups. Moreover, HMGB1 (Fig. 2D) and IL-18 (Fig. 2E) expression in monocytes stimulated with HA was higher in PE women than in the NT group. It is also possible to notice the lower gene expression of all genes in monocytes stimulated with HA in the NT group compared to the NP group (Figs. 2 and 3). The PE group showed a significant difference between non-stimulated (Co) and HA-stimulated (HA) cells, with an increase in the expression of NLRP3, NLRP1, caspase-1, HMGB1, TNF-α, IL-1β and IL-18 by cells stimulated with HA (Figs. 2 and 3).

Figs. 2 and 3 show that treatment of monocytes with 200 μM of glibenclamide, an NLRP3 inflammasome inhibitor, led to a decrease in the expression of caspase-1 (Fig. 2D) and IL-1β (Fig. 3C) by monocytes from NP women, even when these cells were stimulated with HA. These results demonstrated that HA participate in NLRP3 inflammasome activation.

Analysis of cytokine production showed that monocyte stimulation with HA induced higher IL-1β (Fig. 4A) and TNF-α (Fig. 4B) production.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>GenBank</th>
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<tr>
<td>NLRP1</td>
<td>(1728)TCCGCTCcTTACATGCAGA (1747)</td>
<td>(1810)AGACCATTcTGGcTcATcT (1791)</td>
<td>NM_030043.4</td>
</tr>
<tr>
<td>NLRP3</td>
<td>(2826)GAGGAGAAAGGAACCGCACA (2845)</td>
<td>(2917)TGGCTGTTCAcCACCATCA (2897)</td>
<td>NM_004895.4</td>
</tr>
<tr>
<td>CASP1</td>
<td>(1065)AGACATCCCAATGCGGCTC (1084)</td>
<td>(1172)TAAAGATGAGGGAGATCAAG (1151)</td>
<td>NM_032923.3</td>
</tr>
<tr>
<td>HMGB1</td>
<td>(1404)TGAGGAAAGGATATGCGGCTG (1423)</td>
<td>(1505)CTCGCTCTCTCTTCTTTCGT (1484)</td>
<td>NM_00131893.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>(544)GAGCACAAGGATGTTGCTCTG (564)</td>
<td>(652)ACAGCGAGGAGGAGGAGCT (634)</td>
<td>NM_000576.2</td>
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<tr>
<td>IL-1β</td>
<td>(438)ACTGATAGAGATAATGCACCCCG (459)</td>
<td>(517)AGTTACAGCCATACCTCTAGGC (496)</td>
<td>NM_001562.3</td>
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<tr>
<td>TNF</td>
<td>(325)GCTGCACTTTGGAGTGATCG (344)</td>
<td>(462)GGGTTTGCTACAACATGGGC (443)</td>
<td>NM_000594.3</td>
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<tr>
<td>GAPDH</td>
<td>(684)CGTGGAGGAGGTCTGAGCA (703)</td>
<td>(801)GCGAGGATGAGGCAGCAGGA (782)</td>
<td>NM_002464.6</td>
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</table>

Table 2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pregnant women with pre-eclampsia (n = 20)</th>
<th>Normotensive pregnant women (n = 20)</th>
<th>Non-pregnant women (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 (15–40)</td>
<td>26 (14–41)</td>
<td>24 (21–40)</td>
</tr>
<tr>
<td>Race Caucasian (%)</td>
<td>82.3</td>
<td>89.6</td>
<td>84.0</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>34 (23–40)</td>
<td>35 (24–39)</td>
<td>–</td>
</tr>
<tr>
<td>Parity Nulliparous (%)</td>
<td>63</td>
<td>68</td>
<td>–</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>160 (140–200)</td>
<td>110 (90–112)</td>
<td>114 (100–120)</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>110 (90–120)</td>
<td>69 (63–70)</td>
<td>70 (65–80)</td>
</tr>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>1510&lt;sup&gt;a&lt;/sup&gt; (300–18,800)</td>
<td>&lt; 300</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are expressed in percentage or median, with the minimum and maximum values in parentheses. * (p < 0.05) vs. normotensive and non-pregnant women (Kruskal-Wallis test).

<sup>a</sup> (p < 0.05) vs. normotensive pregnant women (Mann-Whitney U test).

Table 3

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pregnant women with pre-eclampsia (PE)</th>
<th>Normotensive pregnant women (NT)</th>
<th>Non-pregnant women (NP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.8 (4.5–10.1)</td>
<td>3.8 (2.3–4.7)</td>
<td>4.1 (2.6–4.8)</td>
</tr>
<tr>
<td>Hyaluronan (ng/mL)</td>
<td>135.5 (20.4–296.8)</td>
<td>62.0 (2.5–199.7)</td>
<td>42.8 (3.3–165.0)</td>
</tr>
<tr>
<td>Hsp70 (pg/mL)</td>
<td>907.1 (404.6–1272.8)</td>
<td>680.2 (7.5–1090.4)</td>
<td>655.4 (12.7–1083.4)</td>
</tr>
<tr>
<td>HMGB1 (ng/mL)</td>
<td>8.11 (1.864–97.809)</td>
<td>2.09 (1.682–4.789)</td>
<td>2.15 (1.848–18.854)</td>
</tr>
</tbody>
</table>

Values are expressed in median, with the minimum and maximum values in parentheses. * (p < 0.05) vs. normotensive pregnant women and non-pregnant women (Kruskal-Wallis test).
by the PE group when compared to the NT group. A significant decrease in IL-1β and TNF-α levels produced by HA-stimulated monocytes was observed in the NT group compared to the NP group. When basal (Co) and HA-stimulated monocytes were compared, there was a significant difference in the production of IL-1β (Fig. 4A) in the three groups studied, and in IL-18 levels only in the pre-eclamptic group. However, there was no significant difference between basal and HA-stimulated levels of TNF-α (Fig. 4B) in monocytes from the PE and NT groups. The
stimulus with HA only induced the higher production of this cytokine in the NP group. No significant production of IL-18 was detected by monocytes stimulated or not with HA in the NT and NP groups (Fig. 4C).

3.5. Gene expression and cytokine production by monocytes stimulated with Hsp70

Gene expression of NLRP1 (Fig. 5A), NLRP3 (Fig. 5B), caspase-1 (Fig. 5C), IL-1β (Fig. 5D) and TNF-α (Fig. 5E) by monocytes stimulated with Hsp70 is higher in the group of women with PE compared to the NT and NP groups, while HMGB1 (Fig. 5F) and IL-18 (Fig. 5G) gene expression stimulated with Hsp70 was higher in the PE group only compared to the NT group. Lower expression of NLRP1 (Fig. 5A), NLRP3 (Fig. 5B), caspase-1 (Fig. 5C), IL-1β (Fig. 5D) and HMGB1 (Fig. 5F) genes was observed in monocytes from NT women, stimulated with Hsp70 and compared to the NP group. In the three groups studied, there were no significant differences between unstimulated (Co) and Hsp70-stimulated monocytes in relation to NLRP1, NLRP3, caspase-1, IL-1β, HMGB1 and IL-18. Higher gene expression of TNF-α (Fig. 5E) was only observed when monocytes from these three groups were stimulated with Hsp70.

The stimulation of monocytes from NT pregnant women with Hsp70 induced lower IL-1β (Fig. 6A) and TNF-α (Fig. 6B) production compared to the PE and NP groups. In addition, significantly higher differences in these cytokine levels were detected between cultures of monocytes stimulated with Hsp70 and the basal (Co) production by these cells in the three groups studied. No significant difference in relation to IL-18 production after Hsp70 monocyte stimulation was observed when the three groups were compared (Fig. 6C).

4. Discussion

The present study evaluated the in vitro involvement of DAMPs, HA and Hsp70 in the inflammatory response detected in PE. The significantly higher plasma concentrations of uric acid, HA, Hsp70 and HMGB1 in women with PE compared with normotensive pregnant women and non-pregnant women confirms studies in the literature showing elevated systemic levels of these DAMPs in pre-eclamptic women and suggest their participation in the pathogenesis of PE [12,17,20,25]. The origin of these DAMPs in plasma is unknown, but there is evidence that they can be released from damaged tissues, stressed or necrotic cells. It is possible that these DAMPs may induce inflammasome hyper activation, resulting in an exaggerated...
inflammatory state in PE [6].

The spontaneous basal activation of inflamasomes in circulating monocytes of pre-eclamptic women, and the higher plasma levels of uric acid, HA, Hsp70 and HMGB1 detected in these women suggest that monocytes can be activated by alarmins or DAMPs present in the plasma. Analysis of the endogenous gene expression of caspase-1, NLRP1 and NLRP3, as well as IL-1β, IL-18, HMGB1 and TNF-α on monocytes of women with PE showed elevated expression, associated with higher protein production of the inflammatory cytokines TNF-α and IL-1β, confirming previous results [7].

Monocytes from women with PE stimulated with HA showed higher gene expression of NLRP3 and caspase-1. On the other hand, differently from non-pregnant and normotensive pregnant women, monocytes from pre-eclamptic women had increased NLRP1 expression when stimulated with HA. To our knowledge this is the first study in the literature showing NLRP1 and NLRP3 activation by HA in monocytes from preeclamptic women. This effect may be explained due to an NLRP1 polymorphism variant rs12150220 (L155H) found in women from pre-eclamptic women detected in the present and in previous studies [30]. Although not directly related to the inflamasomes, a recent study demonstrated that in vitro stimulation with TNF-α induces the early expression of NLRP3 mRNA in a 3T3-L1 adipocyte line and it is detected after one hour of culture suggesting that NLRP3 gene expression is immediately responsive to TNF-α [34].

The stimulated response of monocytes from normotensive pregnant women cultured with HA and Hsp70 were different from the response of monocytes from pre-eclamptic and non-pregnant women, showing diminished expression of the inflammatory cytokines IL-1β, IL-18, TNF-α and HMGB1. This lower expression observed in normotensive pregnant women could be due to the regulation exerted by IL-10 in these cells. The anti-inflammatory cytokine predominance in normal pregnancy could regulate the inflammatory response that occurs during pregnancy by controlling the gene expression of IL-1β and TNF-α [35].

5. Conclusion

The present study demonstrated that monocytes from pre-eclamptic women show endogenous activation of NLRP1 and NLRP3 inflamasomes, and express elevated levels of TNF-α, IL-1β, IL-18 and HMGB1. The activation of these inflamasomes was associated with the higher plasma concentration of HA in these pregnant women. The stimulation of monocytes with HA induced increased gene expression of NLRP1, NLRP3, caspase-1, IL-1β, TNF-α, HMGB1 and IL-18, with the production of IL-1β being more evident in pre-eclamptic and non-pregnant women. Monocytes stimulated with Hsp70 contributed to IL-1β and
TNF-α production by these cells through a mechanism that is independent of inflammasome activation. These results suggest the participation of these DAMPs in the systemic inflammatory response characteristic of PE. The study of mechanisms involved in the activation of monocytes by DAMPs in women with PE, whether or not dependent on inflammasomes, will allow a better understanding of this important inflammatory syndrome of pregnancy.

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Author contributions

MRV, MLM, VRR and PRN performed experiments. VTB and JCP selected pregnant women for the study. MRV and MTSP conceived the ideas, designed experiments, analyzed data and prepared the manuscript.
Disclosure

The authors declare no conflict of interest.

References


