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Oxidative stress and immunological alteration in women with preeclampsia

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Objectives: To determine plasma concentrations of malondialdehyde (MDA) and of inflammatory markers in women with preeclampsia. *Methods*: A case–control study was conducted on 50 preeclamptic and 50 healthy pregnant women. The concentrations of MDA were determined by the method of thiobarbituric acid reactive substances. Markers of inflammation were determined by the multiplex method. *Results*: The concentrations of MDA did not differ between groups ($p > 0.05$) and the preeclampsia group had significantly higher IL-6, IL-10, TNF-α and IL-6/IL-10 ratio, compared to those with normal pregnancy. *Conclusions*: The MDA is a nonspecific marker for oxidative stress in preeclampsia, and the gestantes with preeclampsia have immune dysfunction.

Keywords Biomarker, Cytokines, Malondialdehyde, Preeclampsia.

INTRODUCTION

Preeclampsia is a pregnancy-specific syndrome, characterized by hypertension and proteinuria after the 20th week of gestation. This disease affects 3–14% of pregnancies worldwide, and there is a 7.5 to 65% risk of recurrence. It is one of the main causes of maternal and perinatal morbidity and mortality. In Brazil it is responsible for 37% of direct obstetric causes of death (1,2). Its clinical diagnosis is defined as blood pressure higher than 140 mmHg associated with urinary protein excretion higher than 300 mg/day (3,4).

Two factors are important in the pathophysiological mechanism of the disease: lipid peroxidation and exacerbated systemic inflammatory response (5,6). These processes are metabolically interrelated because lipid peroxidation triggers a strong inflammatory response which, in turn, promotes a high release of free radicals (7).

In preeclampsia the ischemic placenta may be a potential source for the increase in products of lipid peroxidation (8). Plasmatic malondialdehyde (MDA) is an important biomarker for cellular oxidative

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damage and lipid peroxidation of cell membranes (9). Previous studies have shown a high concentration of this marker in the plasma, serum and placenta of pregnant women with preeclampsia when compared to healthy subjects (10–12).

Preeclampsia has been associated not only with oxidative stress, but also with the presence of altered levels of seric inflammatory cytokines and with leukocyte activation. These inflammatory response components are responsible for the increased adherence of leukocytes to the endothelium, which causes endothelial dysfunction in the maternal vascular system: the primary cause of the disease's pathophysiology (13,14).

The factors that lead to the disruption of the maternal immune system remain unknown. In preeclampsia there is a predominance of T1 helper cells (Th1), IL-1, IL-2, IL-6, IL-18, interferon-γ (IFN-γ), α tumor necrosis factor (TNF-α) and TNF-β, which induce cellular immunity, compared to Th2 cells, IL-4, IL-5, IL-10 and IL-13, which induce the production of antibodies (15). There is evidence that type 1 cytokines are dominant compared with type 2 due to abnormal activation of the immune system. This activation compromises uteroplacental perfusion and further aggravates vascular damage. In addition, high placental Th1/Th2 ratios have been related to the disease (16,17).

Even though the participation of the systemic inflammatory response and the exacerbated oxidative stress in triggering preeclampsia have been reported in the literature, little is known about the interaction between these two factors. The objective of this study was to determine plasmatic concentrations of MDA and the cytokines IL-2, IL-6, IL-10 and TNF-α in women with preeclampsia and to correlate these markers in order to better understand the involvement of these two processes in the pathophysiology of the disease and how such information can be used for early diagnosis.

MATERIALS AND METHODS

Study Design

This case–control study included 50 adult pregnant women with preeclampsia, which 17 with preeclampsia mild and 33 with preeclampsia severe, and 50 with normal pregnancy in the third trimester of pregnancy who were being treated at the Dona Evangelina Rosa Maternity Hospital, Piauí, Brazil. The project was carried out after approval by the Ethics in Research Committee of both the Federal University of Piauí and the maternity hospital. All of the women taking part signed an informed consent form.

Patients who had systolic blood pressure (SBP) $\geq$140 mmHg and/or diastolic blood pressure (DBP) $\geq$90 mmHg and proteinuria (1–2 +) labstix were classified as having mild preeclampsia and those with SBP $\geq$160 and/or PAD $\geq$110 mmHg and proteinuria (3–4 +) labstix were classified as severe preeclampsia (4).

The study included pregnant women with preeclampsia (exclusively) or with an uncomplicated pregnancy; were non-smokers and had no systemic diseases and/or associated genital tract diseases (diabetes mellitus, essential hypertension, chronic renal failure, thyroid disorders, lupus erythematosus, urinary infection and cervical or vaginal inflammation), since these conditions appear to be related to oxidative stress and inflammation.

Collection and Separation of Blood Components

A sample of 5 mL of peripheral venous blood was collected from the pregnant women who were fasting at least 10 h in tubes with ethylenediaminetetraacetic acid as anticoagulant. The blood was centrifuged at 4 °C with a relative centrifugal force of 1831 *g* for 15 min and the plasma was transferred to *eppendorf* tubes, stored at −80 °C until the time of analysis.

Assays

Malondialdehyde concentrations were determined in all pregnant women by the production of thiobarbituric acid reactive substances, following the method described by Ohkawa et al. (18), with adaptations. The following were added to 200 μL of plasma; 350 μL of acetic acid (pH 3.5) and 600 μL of thiobarbituric acid 0.5%, diluted in acetic acid. The samples were incubated in a water bath with agitation at 85 °C for 1 h and then placed in an ice bath for 15 min. After cooling, 50 μL of 8.1% sodium dodecyl sulfate was added to the tubes which were centrifuged for 15 min at 12 000 rpm at 25 °C. The supernatant was extracted and the absorbance read at 532 nm using a BEL SP 1102 spectrophotometer (Milan, Italy), with 1,1,3,3-tetraetoxipropano as standard. The results were expressed as nmol of MDA/mL.

The cytokines were measured in the plasma of 40 pregnant women with preeclampsia (14 with preeclampsia mild and 26 with preeclampsia severe) and 36 with normal pregnancy, using the multiplex method, with the commercial Human Cytokine Milliplex Kit (Cat. #MPXHCYTO-60 K-04, LINCO Research, Inc., Saint Charles, MO). The difference of the total initial sample resulted from the exclusion by gross hemolysis or lipemia, as recommended by the manufacturer. The assays were carried out *overnight,* according to the manufacturer's guidelines. The test sensitivities for cytokines IL-2, IL-6, IL-10 and TNF-α, were 0.11, 0.30, 0.30 and 0.10 pg/mL, respectively.

Statistical Analysis

The Kolmogorov–Smirnov test was used to verify the normality of the data. The groups were compared using the Student's *t*-test, for variables with normal distribution and the Mann–Whitney test for those with a non-normal distribution. The Pearson linear correlation coefficient was used for parametric correlations and the Spearman's correlation coefficient for the non-parametric correlations. Statistical significance was considered for p value <0.05. The statistical analysis was carried out using SPSS software (version 15.0.1.1, SPSS Inc., Chicago, IL).

RESULTS

The clinical characteristics of the study participants are described in Table 1. There was no significant difference for the maternal and gestational ages between the groups. On the other hand, the SBP and DBP were significantly higher in pregnant women with preeclampsia.

MDA concentrations did not differ between the preeclampsia and ($p > 0.05$) groups; the highest values were in pregnant women with mild preeclampsia ($p < 0.05$) (Table 2).

Table 1. Clinical characteristics of preeclamptic pregnant and healthy pregnant controls.

	Preeclampsia ($n = 50$) Mean ± SD	**Control ($n = 50$) Mean ± SD**
Age (years)	26.02 ± 6.98	24.28 ± 4.97
Gestacional age (weeks)	38.02 ± 3.14	39.22 ± 1.36
Systolic blood pressure (mmHg)	161.10 ± 20.10*	119.10 ± 11.32
Diastolic blood pressure (mmHg)	107.80 ± 16.07*	79.96 ± 10.30

*$p < 0.05$ preeclamptic versus control.

Table 2. Levels plasma of MDA (nmol/mL), pro-inflammatory and anti-inflammatory cytokines (pg/mL) in all study groups.

	Preeclampsia (PE)		
	PE mild	**PE severe**	**Control**
MDA	1.23 (0.27–3.13)##	1.09 (0.41–3.27)	1.50 (0.68–4.63)
IL-2*	0 (0–0.38)	0 (0–0.75)	0 (0–0.68)
IL-6*	20.36 (0.56–85.19)	23.29 (0–144.34)#	4.61 (0–48.35)
IL-10*	10.45 (3.89–65.25)	9.22 (2.99–206.62)#	4.19 (1.19–79.63)
TNF-α**	11.57 ± 5.74	10.59 ± 5.03#	6.62 ± 2.85

*Median (range); Mann–Whitney test; **Mean ± SD; Student's *t*-test; #$p < 0.05$ preeclampsia versus control; ##$p < 0.05$ preeclampsia mild versus preeclampsia severe.

Table 3. Th1/Th2 cytokines ratios between preeclampsia and control groups.

	Preeclampsia ($n = 40$)	**Control ($n = 36$)**	***p* Value**
Th1/Th2*	3.64 ± 2.81	2.55 ± 1.97	0.057
IL-2/IL-10**	0 (0–0.8)	0 (0–0.8)	0.811
IL-6/IL10*	2.35 ± 2.31	0.85 ± 0.80	0.001
TNF-α/IL10*	1.28 ± 0.92	1.69 ± 1.74	0.210

*Mean ± SD; Student's *t*-test; **Median (range); Mann–Whitney test.

The concentrations of IL-6, IL-10 and TNF-α were higher in the preeclampsia group compared to the control, whereas IL-2 did not differ between the groups. There was no significant difference regarding the behavior of the cytokines studied, according to disease severity (Table 2).

The ratios of Th1 to Th2 cytokines are shown in Table 3. The IL-6/IL-10 ratio was significantly higher in pregnant women with preeclampsia compared to controls; the others were similar between groups.

There was no correlation between the concentrations of MDA and IL-2 ($r = -0.145$, $p = 0.374$); MDA and IL-6 ($r = -0.054$, $p = 0.739$); MDA and IL-10 ($r = 0.218$, $p = 0.177$) or MDA and TNF-α ($r = -0.145$, $p = 0.372$).

DISCUSSION

There have been extensive reports on the involvement of lipid peroxidation in the pathophysiology of preeclampsia; in this study this process was evaluate by

determining MDA, its secondary product. The results demonstrated no significant difference in the concentrations of MDA in pregnant women with preeclampsia, compared with those with normal pregnancy, and no increase in its concentration with the severity of the disease.

Our results are consistent with those of Llurba et al. (19), who found that the concentrations of plasmatic and erythrocytary MDA did not differ between women with preeclampsia and those with normal pregnancy and that there was no correlation between MDA and disease severity. The authors dispute that there is widespread oxidative stress in preeclampsia; they believe that lipid peroxidation is immediately interrupted by the physiological activation of antioxidant enzymes.

Women with normal pregnancy have higher seric and plasmatic levels of MDA, compared to healthy non-pregnant women (20). Longitudinal studies conducted with healthy pregnant women have shown a progressive increase in plasmatic and urinary concentrations of markers of oxidative stress with gestational age, especially in the third quarter, and after birth these markers tend to return to baseline levels (21,22). These findings confirm the hypothesis that mild oxidative stress is involved in normal human pregnancy and the placenta seems to be responsible for the increased oxidative stress in this period (21).

Conversely, several authors have found a significant increase in the concentrations of MDA in the serum, plasma (23,24) and placenta (12) of pregnant women with preeclampsia, as well as those with more serious stage of the disease (20,10). Prior study demonstrated positively associated of the plasma concentrations of MDA in early pregnancy with the risk of developing preeclampsia (25). It is worth stressing that all the women involved in the research were in the third trimester of pregnancy and measures of oxidative stress lose their predictive ability for hypertension/preeclampsia with advancing gestational age (26).

As for markers of inflammation, apart from IL-2, the cytokines IL-6, IL-10 and TNF-α differed significantly between the control and preeclampsia groups. These results are in line with previous studies that found higher concentrations of IL-6 (14), TNF-α, circulating chemokines and adhesion molecules in women with preeclampsia, compared to controls with normal pregnancy. In preeclampsia, there is inadequate leukocyte activation and endothelial dysfunction with consequent increased release of IL-6 and pro-inflammatory systemic response (27).

Several studies have shown an association between preeclampsia and high plasma and serum concentrations of IL-6 and TNF-α (17,23,28,29). TNF-α has cytotoxic action and appears to inhibit trophoblastic invasion (30) and promote endothelial activation (31), contributing to an altered production of prostaglandin and an imbalance between vascular regulatory factors (32).

Despite to the large number of reports of the involvement of IL-6 and TNF-α in preeclampsia, previous studies demonstrated conflicting regarding the use of these cytokines as prognostic markers of endothelial damage and inflammation in preeclampsia or for the screening, monitoring or diagnosis of the disease (28,29,33,34).

Reports in the literature differ regarding the concentrations of IL-2 in women with preeclampsia. We found similar concentrations of IL-2 in women

with preeclampsia and normal pregnancy. This finding was consistent with previous work (29,34) and disagree with those found by Sharma et al. (35) and Singh et al. (17). It is worth stressing that about 80% of plasma samples of pregnant women who participated in this study had IL-2 concentrations below the detection limit for the test that was used.

Some authors argue that preeclampsia is associated with reduced concentrations of this cytokine in the maternal circulation (28,29), while others have found no differences in IL-10 in women with preeclampsia and those with normal pregnancy (23,34).

Our results had significantly higher concentrations of IL-10 in women with preeclampsia and suggest that there is a compensatory maternal immune response to the pro-inflammatory medium present in the disease (27,36). In addition to this compensatory increase in IL-10 the difference between the groups can be explained by the physiological reduction in the release of IL-10 by the placenta in the third trimester (37). This reduction enables the expression of the metalloproteinase matrix family of enzymes, which are required for adequate trophoblastic invasion (36,38).

In preeclampsia, the inflammatory process alters the balance between Th1 and Th2 cytokines, resulting in the predominance of Th1. Thus, the cascade of the production of pro-inflammatory cytokines involved in the disease is initiated or intensified (15). In this study, the analysis of the Th1/Th2 cytokines ratios showed that there was only a significant difference between the groups for IL-6/IL-10. The increased expression of Th1 cells and the imbalance of Th1/Th2 cytokines led to hypertensive disorders in pregnancy (39).

For decades the literature has pointed out the role of oxidative stress and inflammation in the etiology of preeclampsia; however, hardly any studies have directly studied the correlation of these two processes in the disease. Therefore, we assessed the concentrations of MDA and the studied cytokines and found that there was no correlation between these markers. This study is in line with that of Bernardi et al. (23) and one of the limitations was the evaluation of these parameters at only one moment of time, just after diagnosis of the disease.

The lack of correlation between the markers investigated is acceptable, since, unlike what was observed for the cytokines, the concentrations of MDA did not differ between the preeclampsia and control groups.

Our results suggest caution in using MDA as a marker of oxidative stress, since it is nonspecific for preeclampsia in the third trimester of pregnancy. The study reinforces the hypothesis that there is immune dysfunction in preeclampsia, with an increase in the production of pro-inflammatory cytokines IL-6 and TNF-α, and a compensatory increase in IL-10.

We only analyzed one marker of oxidative stress and only measured MDA and cytokines in the third trimester of pregnancy; it also did not study the soluble receptors of the cytokines. Prospective and longitudinal studies could contribute to the understanding of the interactions between oxidative stress and inflammation, so that pregnant women at risk can be identified and care directed toward these women.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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