Biochemical analysis of oxidative stress and purinergic system in pregnant women with gestational *diabetes mellitus*

Análise bioquímica do estresse oxidativo e do sistema purinérgico em gestantes com diabetes mellitus gestacional

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ABSTRACT

Background: Pregnancy is characterized as a physiological period with greater sensitivity to insulin resistance and changes in oxidative stress. Purinergic signaling is directly related to diabetes, as this condition modifies the concentration of extracellular ATP and the level of degradation of ATP to adenosine. **Objective:** Analyze oxidative stress and the purinergic system in pregnant women with Gestational Diabetes Mellitus (GDM) and compare them with low-risk pregnant women (LR). **Materials and Methods:** The research was of a quantitative approach of an experimental nature. The study was carried out at the Clínica da Mulher, which serves high-risk pregnant women, and at the Family Health Centers, which serves low-risk pregnant women, both located in Chapecó, Santa Catarina, Brazil. **Results:** From the analysis, it was observed that oxidative stress was increased in pregnant women in LR compared to pregnant women with GDM by increasing the concentration of TBARS and reducing the concentration of Carbonyl Protein in pregnant women with LR. Regarding the purinergic system, there was a significant decrease in the hydrolysis of the nucleotides ATP, ADP, and AMP in pregnant women with GDM, and a significant increase in the hydrolysis of ADA, also in pregnant women with GDM. **Conclusion:** Therefore, pregnant women with GDM have less oxidative stress compared to pregnant women in LR concerning TBARS and Carbonyl Protein markers, thus allowing a greater antioxidant defense mechanism. Furthermore, concerning the purinergic system, there is an increase in the activity of ADA, which is directly related to the immunosuppression process, a necessary condition for the protection of the fetus during the gestational period.

Keywords: Pregnancy complications, Gestational diabetes, Oxidative stress, Antioxidants, Purines.

RESUMO

Introdução: A gravidez é caracterizada como um período fisiológico em que há uma maior sensibilidade a resistência à insulina e alterações no estresse oxidativo. A sinalização purinérgica está diretamente relacionada ao diabetes, pois esta condição modifica a concentração de ATP extracelular e o nível de degradação de ATP em adenosina. **Objetivo:** Analisar o estresse oxidativo e o sistema purinérgico em gestantes com Diabetes Mellitus Gestacional (DMG) e compará-los com gestantes de baixo risco (BR). **Materiais e Métodos:** A pesquisa foi de abordagem quantitativa, de caráter experimental. O estudo foi realizado na Clínica da Mulher, que atende gestantes de alto risco, e nas Unidades de Saúde da Família, que atendem gestantes de baixo risco, ambas localizadas no município de Chapecó, Santa Catarina, Brasil. **Resultados:** A partir das análises, observou-se que o estresse oxidativo apresentou-se aumentado em gestantes de BR quando comparado a gestantes com DMG. No que tange ao sistema purinérgico, houve uma diminuição significativa na hidrólise dos nucleotídeos ATP, ADP e AMP em gestantes com DMG, bem como um aumento significativo na hidrólise de ADA, também em gestantes com DMG. **Conclusão:** Portanto, gestantes com DMG possuem menor estresse oxidativo quando comparado a gestantes de BR, permitindo assim, um maior mecanismo de defesa antioxidante. Para mais, no que se refere ao sistema purinérgico, verifica-se o aumento da concentração de ADA está diretamente relacionada ao processo de imunossupressão, condição necessária à proteção do feto durante o período gestacional.

Palavras-chave: Complicações na gravidez, Diabetes gestacional, Estresse oxidativo, Antioxidantes, Purinas.

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INTRODUCTION

Pregnancy is a period characterized as one of the most important in the life of many women and corresponds to complex transformations such as physiological, emotional, interpersonal and social, being an adaptive phase in the organism, which is expected to not occur in this period; however, some women may experience some complications during pregnancy. Among the most severe complications, Gestational Diabetes Mellitus (GDM) stands out as one of the most common causes, which impairs an adequate evolution of pregnancy and exacerbates the rates of maternal and perinatal morbidity and mortality[1].

The predominance of hyperglycemia in pregnancy varies according to the diagnostic criteria applied and the population evaluated. Studies carried out in recent years show a predominance that varies from 1 to 37.7% and a world average of 16.2%. Currently, it is estimated that one in six births occurs in women with some condition of hyperglycemia during pregnancy since 84% of cases are due to GDM[2].

When changes in the mechanisms of cell physiology occur in both production and metabolism, which leads to an increase in concentration, it can be said that the biological system is faced with a situation of oxidative stress; such factor has been associated with the physiological changes analyzed in the syndrome metabolic, such as insulin resistance in Diabetes[3]. Regarding the purinergic system, there is growing evidence that it is involved in the malfunction of β cells and the inflammatory reactions involved in diabetes[4].

In view of the above, in order to contribute to the scope of the research and in the future assistance provided to pregnant women with GDM, and due to the scarce number of research related to this theme, it was proposed to develop the present study, whose objective was to analyze oxidative stress and the purinergic system in pregnant women with GDM and compare them with LR pregnant women.

METHODS

The research was of a quantitative approach, of an experimental character. The study was carried out at the Women's Clinic, which serves high-risk pregnant women, and at the Family Health Centers, which serves low-risk pregnant women, both located in the city of Chapecó, Santa Catarina, Brazil. Participated in the research, 13 pregnant women with GDM, over the age of 18 years, as well as 13 pregnant women of LR who underwent follow-up at the Family Health Centers in that city. All pregnant women were with gestational age between 37 and 41 weeks. Pregnant women under 18 and pregnant women with Chronic Diabetes Mellitus, chronic hypertensive syndrome, and other comorbidities were excluded from the research.

Data collection procedure

Data collection was carried out from June to December 2018. Twenty-six pregnant women participated in the research, 13 pregnant women with GDM and the other 13 pregnant women of LR. First, the list of pregnant women who were carrying out prenatal care was analyzed, both at the clinic and the Family Health Centers. Afterward, each pregnant woman was approached individually to talk, making an invitation and then explaining the purpose of the research and the importance of their participation. Data was collected after the participants signed the Free and Informed Consent Form (ICF).

Ethical aspects

This study was approved by the Research Ethics Committee of the Federal University of Fronteira Sul (UFFS), Chapecó-SC, Brazil, under the Certificate of Presentation for Ethical Appreciation: 67328417.3.0000.5564, respecting resolution No. 466 of December 12, 2012, of the National Health Council, which considers respect for human dignity and protection of life for participants in scientific research involving human beings[5]. Afterward, the project was presented to the heads of the health service in the municipality of Chapecó, who signed the authorization request for the implementation of the research.

Data analysis

The sequence of the location of the blood samples was coded with the letter P (pregnant

woman) and followed by a number according to the order of collection. The data obtained were tabulated on a spreadsheet in Libre Office and then subjected to appropriate statistical tests for each sample. The statistical analysis was performed as follows: the differences between the groups concerning the study variables, were assessed by ANOVA analysis of variance. Thus, when the samples did not show normal distribution, they were subjected to ANOVA analysis of variance by the Kruskal-Wallis test. And, when there was a difference, the groups were compared with Student-Newman-Keuls correction for parametric variables and Dunn's correction for non-parametric variables. The results were presented with mean ± standard deviation for parametric variables and in the form of median and range of variation for non-parametric variables. Differences were considered statistically significant when the probability of rejection of the null hypothesis was less than 5% ($p<0.05$).

Blood sample separation

Platelet-rich plasma was prepared by the method of Pilla et al. modified by Lunkes et al. [6,7]. Whole blood was collected with sodium citrate with anticoagulant and centrifuged at 1500 rpm for 10 minutes. Then, the platelet-rich plasma was centrifuged at 5000 rpm for 30 minutes and washed with 3.5 mM HEPES buffer, pH 7.0, at least twice. The platelet pellets were suspended in HEPES buffer, and the protein was adjusted to 0.4-0.6 mg/mL.

Analysis of oxidative stress

The determination of substances reactive to thiobarbituric acid (TBARS) was made according to Ohkawa et al[8]. The formation of malondialdehyde, due to the breakdown of polyunsaturated fatty acids, was the method adopted to determine the degree of lipid peroxidation. The activity of glutathione peroxidase (GPx) was according to Paglia and Valentine[9]. The nitric oxide test, which detects the presence of organic nitrite in the sample, was carried out according to the protocol of Choi et al[10]. Protein carbonylation analyses were according to protocols described by Levine et al[11]. The activity of the myeloperoxidase enzyme (MPO) was made according to Suzuki et al[12]. Plasma levels of vitamin C were determined according to Galley et al[13]. This method aims to generate an orange chromogen produced by reacting vitamin C with dinitrophenylhydrazine at 37 ºC, which can be measured spectrophotometrically at 520 nm. Finally, the quantification of thiol groups, classic biomarkers of oxidative stress, was carried out according to Ellman[14].

Analysis of the purinergic system

The analysis of the enzymatic activity of the purinergic system occurred in a similar way to the analysis of oxidative stress, however, with some modifications: E-NTPDase, determined according to Pilla et al., modified by Lunkes et al., and the activity of the ADA enzyme according to the method described by Giusti[6,7,15]. The data obtained were analyzed using the GraphPad Prism 6.0 software using the Student's t-test.

RESULTS

Oxidative stress

Regarding TBARS lipid peroxidation, at the 5% significance level, with $p=0.0087$, it can be seen that the mean TBARS is significantly higher in LR pregnant women compared to pregnant women with GDM (Figure 1a). Regarding the levels of carbonyl protein, at the significance level of 5%, with $p=0.0107$, the mean is significantly higher in pregnant women in LR (Figure 1b). Regarding the levels of glutathione (GSH), there was no significant difference between the groups (Figure 1c). Regarding vitamin C levels, it was also not possible to observe a significant difference between the studied groups (Figure 1d). Regarding the levels of protein thiols (PSH) (Figure 1e) and non-protein thiols (NPSH) (Figure 1f), there was no significant difference between the groups analyzed. Finally, concerning the levels of myeloperoxidase (Figure 1g), there was no significant difference between the groups analyzed.

Purinergic system

Laboratory analyses of the purinergic system indicated a significant decrease in ATP hydrolysis in

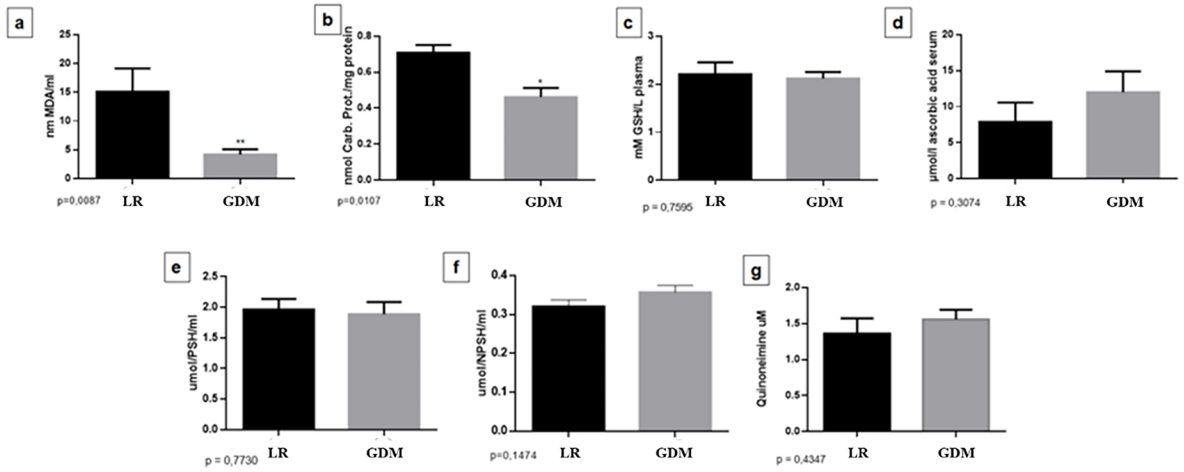

Figure 1: Analysis of oxidative stress indicators. (a) TBARS, (b) Carbonyl Proteins, (c) GSH, (d) Vitamin C, (e) PSH, (f) NPSH, (g) Myeloperoxidase, n = 13.

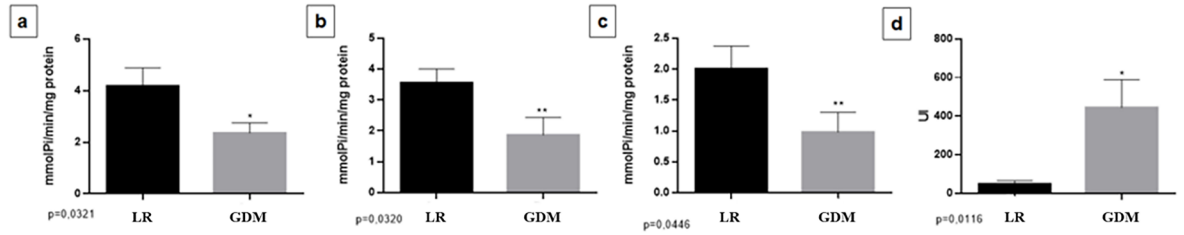

Figure 2: Analysis of purinergic system indicators. (a) Platelet ATP hydrolysis, (b) Platelet ADP hydrolysis, (c) Platelet AMP hydrolysis, (d) Platelet adenosine hydrolysis, n = 13.

platelets of pregnant women with GDM, p=0.0321 (Figure 2a). Regarding the hydrolysis of ADP, also in platelets, with p=0.0320, there is a significant decrease in hydrolysis in the group of pregnant women with GDM (Figure 2b). There was also a significant reduction in AMP hydrolysis in platelets in pregnant women with GDM, with p = 0.0446 (Figure 2c). Furthermore, regarding adenosine hydrolysis levels, there was a significant increase in hydrolysis in pregnant women with GDM, with p=0.0116 (Figure 2d).

DISCUSSION

Pregnancy is characterized as a physiological period in which there is a greater sensitivity to insulin resistance and changes in oxidative stress since it is in the placenta that the production of diabetogenic hormones takes place, which participates in the formation of Reactive Oxygen Species (ROS). In LR pregnancies, this production is repaired by increasing antioxidants. However, in cases of diabetes, increased production of ROS occurs, which exceeds the antioxidant defenses, altering the levels of oxidative stress[16].

In our study, the concentration of TBARS was significantly lower in pregnant women with GDM compared to LR pregnant women; this result was similar to that reported by other authors[17]. In another study with rats, it was observed that TBARS levels were found to be elevated in diabetic rats when compared to the control group. This result proves what was described in a study in which pregnant women with Diabetes Mellitus have high concentrations of TBARS, superoxide dismutase, and malondialdehyde, suggesting an increase in oxidative stress[18].

Carbonyl Protein (CP) is a marker that assesses the damage to proteins through oxidants. Studies show a slight correlation between this marker and glycemic control, observing an impairment in type 2 diabetes mellitus. In our study, the concentration of

CP was significantly higher in pregnant women in LR compared to GDM. Studies have shown an increase in oxidative stress between 16 and 20 weeks prior to the diagnosis of GDM. Such an increase can occur even before the onset of GDM and increase during pregnancy. Therefore, it is suggested that there is a reduction in CP activity during pregnancy due to inadequate redox control resulting from the clinical picture of GDM. Thus, the increase in oxidative stress contributes to the development and maintenance of the pathology in question[19].

Glutathione (GSH) acts in the protection of injuries caused by metals such as iron ion, and in the elimination of residues of lipid peroxidation. Its decrease impairs the functioning of other antioxidant enzymes, resulting in cell damage, subsidizing the pathology of Diabetes Mellitus[20]. There was no significant difference in GSH between these two groups in this study.

Vitamin C in GDM indicates that the changes in its concentration may be related to the fact that oxygen toxicity and the sensitivity of the vascular endothelium. Through the concentration of Vitamin C, it is possible to evaluate the predisposition of antioxidants in the chain break and, in this way, inhibit the formation of lipid peroxidation. In our study, the concentration of Vitamin C, despite not presenting statistically significant values than the two groups under study, presented higher values in the group of pregnant women with GDM, in order to present different results concerning other studies[21]. The increase in free radicals, resulting from oxidative stress, can impair glucose uptake and insulin secretion. However, Vitamin C, by acting as an antioxidant, acts in the reduction of oxidative stress. Thus, studies showed that the increase in Vitamin C levels contributes to the reduction of oxidative stress and, therefore, improves glucose levels in diabetic patients, an essential mechanism in patients with GDM[22].

Thiols are antioxidants that act to protect against reactive oxygen species. Its verification provides an indirect observation of antioxidant defenses. Recent studies have shown that abnormal thiol homeostasis is related to several diseases, including type I diabetes and GDM[23]. In the concentrations of the thiols groups, no significant changes were observed; this fact was similar to other studies[18].

Purinergic signaling is directly related to diabetes, as this condition changes the concentration of extracellular ATP and the level of degradation of ATP and adenosine[4]. In our study, there was a significant difference in ATP hydrolysis between the two groups analyzed. In another study, ATP hydrolysis was evaluated among non-pregnant women and pregnant women without comorbidities, pregnant women with hypertension, and pregnant women with diabetes, and it identified that this increase in ATP possibly occurred due to the physiological mechanisms of pregnancy[24].

Platelet aggregation is triggered by platelet activation, and this activation promotes the release of ATP. In this way, the participation of an enzymatic mechanism responsible for the hydrolysis of ADP in circulation is significant, as it helps in delimiting platelet aggregation, thus preventing the formation of thrombi. ADP-induced platelet aggregation is the result of signaling two types of nucleotide receptors that are linked to G-protein (P2Y1 and P2YAC). Thus, some studies have shown that extracellular nucleotides may be acting as messengers in the hemostatic and vascular mechanisms, inflammatory cells, and exocrine and endocrine systems[24].

Many mediators are involved in the platelet aggregation process, namely: thrombin, epinephrine, thromboxanes, collagen, and ADP. When platelet adhesion occurs in endothelial cells, the repair process begins by releasing mediators, among them, ADP, responsible for the amplification and propagation of the platelet response in relation to platelet aggregation[25].

In our study, there was a significant difference in ADP hydrolysis between the groups analyzed. In another study performed with rats treated with the hyperglycemic agent streptozotocin, there was an increase in ADP in platelet aggregation[26]. It is known that insulin is a signaling molecule and also an inhibitor of platelet activation, a process that occurs through the P2Y12 receptor pathway in the formation of cAMP[27]. When in low quantity or when cells are resistant to it, insulin is unable to perform its functions in inhibiting platelet aggregation. Thus, diabetic patients tend to have a higher platelet aggregation. However, in our study, there was a significant decrease in AMP hydrolysis in pregnant women with GDM, a similar fact mentioned in other studies[26].

In a study with diabetic rats, which were induced by alloxan (a toxic glucose analog, which triggers type 1 diabetes), it was found that the increase in glucose levels may be related to the increase in the hydrolysis of nucleotides with higher adenosine production[7].

Adenosine (ADA) in blood vessels comes from nucleotides released from platelets[28]. Regarding the purinergic system, it appears that the reduction of adenosine transport leads to an increase in the extracellular levels of this nucleoside in the endothelium of the umbilical veins. The endothelium of such umbilical veins exhibits elevated A1a and A2a receptor activities depending on insulin levels, which means that adenosine may be acting as an adjunct to the biological effects of insulin on GDM[29]. In our study, the hydrolysis of ADA into platelets was greater in pregnant women with GDM when compared to pregnant women with LR. In one study, an increase in ADA activity was observed in both normal and GDM pregnant women compared to a group of non-pregnant women and suggested that this increase was due to an imbalance of cytokines. Furthermore, the ADA acts in the immune development in humans, being responsible for the differentiation of monocytes and epithelial cells, as well as acting in neurotransmission and maintenance of pregnancy[30].

Studies suggest that the increase in the hydrolysis of ATP, ADP, and AMP in hypertensive pregnant women, pregnant women with GDM, and pregnant women without comorbidities compared to non-pregnant women, occurs with the purpose of maintaining hemostasis at the time of childbirth or in the puerperium[24].

CONCLUSION

Therefore, from the variables studied, it was possible to verify a lower concentration of TBARS and Carbonyl Protein in pregnant women with gestational diabetes mellitus compared to the physiological stress of the gestational period in pregnant women between 37 and 41 weeks of gestational age. Thus, it can be considered that oxidative stress markers are reduced in the group of pregnant women with GDM. The increase in the antioxidant defense system in pregnant women with GDM may be related to the fact that the lower the oxidative damage, the greater the ability to stimulate insulin secretion and, therefore, the lower the blood glucose level. Furthermore, the increase in the concentration of adenosine suggests an increase in immunosuppression, a factor necessary for the protection of the fetus against the maternal immune system, especially in pregnant women who have comorbidities, such as gestational diabetes mellitus, since it consists of a greater risk to mother-child binomial, among them, traumatic delivery, neonatal hypoglycemia, and fetal macrosomia.

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Conflicts of interest/Competing interests
Bruna Laís Hardt declares that she has no conflict of interest.
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Margarete Dulce Bagatini declares that she has no conflict of interest.
Débora Tavares de Resende e Silva declares that she has no conflict of interest.

Availability of data and material
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability
Not applicable.

Authors' contributions
Conceptualization: [BLH, BDO, EBP and DTRS]; Methodology: [MVGR, MRB, AM and MDB]; Formal analysis and investigation: [BLH, BDO, MVGR, AM and MRB]; Writing - preparation of the original sketch: [BLH, MRB and DTRS]; Writing - revision and editing: [MRB and DTRS].

Ethics approval
The Human Ethics Committee of the Federal University of Fronteira Sul, Santa Catarina, Brazil, approved the protocol under number 2.673.384.

Consent to participate
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