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Abstract

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Keyword: biomarkers; δ-ALA-D; diabetes mellitus in pregnancy; oxidative markers; pregnant women.

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Abstract

The aim of this study was to perform a comparative evaluation between pregnant women: healthy, with pre-gestational type 2 diabetes (T2DM) and gestational diabetes mellitus (GDM), to determine if diabetes mellitus developed at different times interferes with the oxidative impact on the pregnant woman maternal body. A total of 90 pregnant women were recruited in the third trimester of pregnancy, subdivided into the three groups, and evaluated through their clinical characteristics, oxidative stress markers and delta-aminolevulinate dehydratase (δ-ALA- D) activity. Pregnant women with diabetes mellitus (DM) showed an increase in: age, pre-gestational and gestational body mass index (BMI), blood pressure, blood glucose and platelet count; those with T2DM had higher pre-gestational BMI and glycated hemoglobin A1c (HbA1c). The levels of thiobarbituric acid reactive substances were higher and the levels of non-protein thiols and catalase activity were lower in the DM groups compared to the control. Vitamin C was decreased in the T2DM group. The δ-ALA-D activity was decreased in pregnant women with GDM and the rate of enzymatic reactivation was higher in the DM groups. DM presented in gestation, regardless of the moment of its development, generates increase of the oxidative stress and decrease of the antioxidant defences, representing the largest difference with the control group. It is suggested that the insulin used in the treatment of T2DM acts in a beneficial way in the δ-ALA-D activity.

Keywords: biomarkers; δ-ALA-D; diabetes mellitus in pregnancy; oxidative markers; pregnant women.

1. Introduction

Diabetes mellitus (DM) consists of a systemic disorder characterized by consistently high glycemia and defects in the release and/or action of insulin produced by the body (Sociedade Brasileira de Diabetes, 2017). It is considered the most frequent metabolic problem that affects women in the gestational period (Araújo, Keating, Martel, 2015). It may be present before conception, such as type 1 or type 2 DM (T2DM), increasing incidence, or it may develop during pregnancy, called gestational diabetes mellitus (GDM), which affects about 3-25% of pregnancies, varying according to with the population, ethnicity and diagnostic criteria used (Sociedade Brasileira de Diabetes, 2017; Witczak et al., 2017). This pathology can lead to long-term complications for both mother and fetus (Toljic et al., 2017) being associated with an increase in the rate of spontaneous abortion, congenital abnormalities and macrosomia, which are largely related to the hyperglycemic conditions to which the organism is exposed (Lambert, Holt, 2013).

Closely involved in the pathophysiology of many diseases, including diabetes and complications related to pregnancy, is oxidative stress (Burton, Jauniaux, 2011; Chikezie, Ojiako, Ogbuji, 2015; Pescosolido, Campagna, Barbato, 2014). This state is described when oxidizing substances, especially reactive oxygen species (ROS), overlap with the neutralizing capacity of antioxidants in the body (Cuffe, Xu, Perkins, 2017; Zanini et al., 2014).

Physiological gestation is a period in which oxidative stress is elevated by increased oxygen demand for the placenta, rich in mitochondria, as well as by a decrease in antioxidant power (Hastie, Lappas, 2014). Complementing this physiological structure, in the presence of a hyperglycemic environment, advanced glycation end products are generated, which are involved in the production of free radicals, causing the oxidation state to be exacerbated (Souza et al., 2007; Witczak et al., 2017). These species can react with biomolecules, such as membrane lipids, proteins and DNA, generating irreversible damage through their ability to induce biochemical changes (Cuffe et al., 2017; Zanini et al., 2014).

Research has revealed increased free radical production and depletion of antioxidant levels in pregnant women with DM, leading to an increased risk of complications during these pregnancies (Murthy, et al., 2018; Rueangdetnarong et al., 2018; Toljic et al., 2017). Thus, markers of oxidative stress are one of the main targets of research related to DM, since they are the viable option for the prognosis and management of this disorder (Bonfanti et al., 2011; Cuffe et al., 2017).

The δ-aminolevulinate dehydratase (δ-ALA-D), an essential enzyme in aerobic organisms, participates in the formation of the heme group and other tetrapyrrol molecules. This enzyme has sulfhydryl groups that are sensitive to oxidizing agents and can be reduced in situations of oxidative stress (Bonfanti et al., 2011; Souza et al., 2007). The decrease of the enzymatic activity ends up affecting the biosynthesis of compounds and also, leads to the accumulation of its substrate 5-aminolevulinic acid, contributing to the overproduction of ROS (De Lucca et al., 2016). In the DM scenario, this enzymatic analysis may provide additional information to determine the dysregulation of glucose metabolism (Souza et al., 2007), functioning as an indirect marker of oxidative stress (De Lucca, Gallarreta, Gonçalves, 2015).

The objective of this study was to perform a comparative analysis between pregnant women: healthy, with pre-gestational T2DM and GDM, evaluating clinical characteristics, oxidative and antioxidant markers, as well as δ-ALA-D enzyme activity, not yet evaluated in this relation.

2. Materials and Methods

2.1 Study population

The study population consisted of 90 pregnant women, subdivided into three experimental groups. The control group (C), composed of healthy pregnant women, undergoing prenatal at the Wilson Paul Noal Basic Health Unit in the city of Santa Maria, Brazil. Pregnant women with T2DM and those developed GDM, from the University Hospital of Santa Maria (HUSM), which were distributed in groups according to the parameters established in the Clinical Protocol - Diabetes Mellitus and gestation (Hospital Universitário de Santa Maria, 2015). According to this protocol, pregnant women with GDM had altered and confirmed fasting glucose results (92-125 mg/dL) (up to 23 weeks gestation) or the Oral Glucose Tolerance Test (OGTT) (between 24 - 28 weeks). Samples were collected in the third trimester of gestation and the data obtained by consulting the medical records. Pregnant women with multiple gestation, smokers, alcoholics, hypertensives or any other condition than pre-gestational T2DM and GDM, including other types of diabetes, were excluded from the study. Pregnant with T2DM controlled diabetes with insulin administration and did not have major complications caused by the disease, whereas those in the GDM group only controlled with diet. This work was approved by the Research Ethics Committee with Human Beings of the Federal University of Santa Maria (UFSM, 33665314.4.0000.5346), and the participants agreed to the study by signing the Free and Informed Consent Term.

2.2 Sample Collection

Blood samples in EDTA (hemogram and glycated haemoglobin (HbA1c)), sodium fluoride (plasma glucose) and heparin (oxidative stress) were collected after 8h of fasting. From heparin, plasma was obtained by centrifugation, and after washing with 0.9% sodium chloride, it was obtained the red blood cells. The analyzes were performed after obtaining the material.

2.3 Clinical and laboratory parameters

Body mass index (BMI) was obtained by dividing weight by squared height (kg/m²). Blood pressure was measured with calibrated aneroid sphygmomanometer (mmHg). Biochemical parameters, such as glucose, determined by hexokinase/glucose-6-phosphate dehydrogenase (Siemens, USA) and glycated hemoglobin A1c by high performance liquid chromatography with cation exchange Analyzer D-10 (Bio-Rad, USA). Hematological analysis was performed using Sysmex® XE-5000 (Sysmex Corporation, Kobe, Japan).

2.3.1 Pro-oxidant

To evaluate lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were determined in plasma and erythrocytes, as described by Lapenna et al., 2001. Malondialdehyde (MDA) was used as

standard, and reaction product measured spectrophotometrically at 532 nm and expressed in nmol MDA/mL plasma and nmol MDA/mL erythrocytes.

2.3.2 Antioxidants

Plasma quantification of protein (P-SH) and non-protein thiol groups (NP-SH) in erythrocytes was verified as proposed by Boyne and Ellman, 1972, changed by Jacques-Silva et al., 2001. The reaction product was read at 412 nm, and the results were expressed as nmol P-SH/mL plasma and nmol NP-SH/mL erythrocytes.

Plasma vitamin C dosage was performed as described by Galley et al., 1996, modified by Jacques-Silva et al., 2001. Ascorbic acid was used as standard and the orange reaction product was measured at 520 nm and expressed in µg VIT C/mL plasma.

2.3.3 Enzymatic activity

The activity of the catalase enzyme was determined according to Aebi, 1984. This was measured by the rate of decomposition of hydrogen peroxide, which was verified in a spectrophotometer at 240 nm and expressed in K/mg Hb.

The δ-ALAD activity was determined as described by Berlin and Schaller, 1974. The porphobilinogen formed, is read at 555 nm and expressed as U/L (nmol PBG/h/mgHb). The reactivation index was determined by the concomitant test of tubes containing the same incubation medium, except for the addition of 2 mM dithiothreitol (DTT), a reducing agent. Being estimated by equation: A-B/A*100, where A = δ-ALA-D absorbance with DTT and B = δ-ALA-D absorbance without DTT.

2.3.4 Statistical analysis

GraphPad Prism software version 6.01 (GraphPad Software, San Diego, CA, USA) was used. The Shapiro-Wilk normality test was applied to evaluate the sample distribution. When analyzed three groups, analysis of variance (ANOVA) was used, for data with normal distribution, followed by the Tukey test for comparisons between groups, the results expressed as mean ± standard deviation (SD), and the Kruskal-Wallis test for non-parametric data, with the results presented as median (interquartile range). However, when two groups were evaluated, the Student t test was used for normally distributed data and the results expressed mean ± SD, whereas the Mann-Whitney test was used for non-parametric data and the results presented as median (interquartile range). The analysis of gestational parameters was performed using the chi-square test. Correlations among all participants were assessed by the Spearman correlation coefficient. $p < 0.05$ were considered significant.

3. Results

The clinical and laboratorial parameters evaluated, presented in Table 1, show significant differences in the increase in maternal age, pre-gestational and gestational BMI, systolic and diastolic blood pressure, fasting plasma glucose levels and platelet count in pregnant women with DM, when compared to the control group. Only pre-gestational BMI and HbA1c (measured in patients with the pathology) revealed

a considerable difference between the types of diabetes, showing an increase in the T2DM group compared to GDM. The gestational characteristic cited as a victim of previous fetal death was considered when the pregnant woman had any loss in pregnancy or stillbirth.

The levels of oxidative stress markers were shown in Table 1, indicating that TBARS levels (erythrocytes and plasma) were considerably higher in pregnant women with DM compared to the control group. Significantly lower levels of NP-SH and catalase activity in DM groups, while vitamin C levels decreased significantly only in the T2DM.

Table 1. Characteristics and parameters analyzed.

Parameter	C (n = 30)	T2DM (n = 30)	GDM (n = 30)	p
Clinical characteristics				
Maternal age (years)	23.0 (20.0–28.0)	33.0 (30.0–35.5)[a]	29.5 (25.7–35.2)[a]	
Gestational age of collection (weeks)	33.0 (31.0-37.0)	32.0 (28.0-35.0)	33.0 (30.0-36.0)	
Pre-gestational BMI (kg/m^2)	24.9±3.8	33.8±5.7[a,b]	29.8±5.8[a]	
Gestational BMI (kg/m^2)	29.4 ± 4.3	35.3 ± 5.1[a]	32.6 ± 5.3[a]	
Systolic pressure (mmHg)	110.0 (100.0–110.0)	112.5 (108.0–120.3)[a]	115.0 (110.0–120.0)[a]	
Diastolic pressure (mmHg)	70.0 (60.0–70.0)	70.0 (62.7–80.0)[a]	70.0 (70.0–80.0)[a]	
Laboratory parameters				
Glucose fasting plasma (mg/dL)	78.0 (68.7–80.2)	119.0 (98.5–154.5)[a]	95.0 (85.0–105.0)[a]	
HbA1c (%)		6.4 ± 1.1[b]	5.4 ± 0.5	
Erythrocytes ($10^6/mm^3$)	4.0 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	
Hematocrit (%)	35.6 ± 2.6	36.1 ± 2.3	35.9 ± 2.8	
Hemoglobin (g/dL)	11.6 (10.8–13.2)	12.0 (11.7–12.5)	12.1 (11.4–12.6)	
Platelets ($\times 10^3/mm^3$)	161.5 ± 6.6	222.4 ± 38.1[a]	222.1 ± 50.0[a]	
Gestational characteristics				
Suffered previous fetal death (%)		40.0	26.6	0.273
Macrosomia (fetal weight > 4Kg) (%)		10.0	3.3	0.301
Oxidative stress parameters				
TBARS erythrocytes (nmol/mL)	15.7 (10.4–18.5)	25.2 (20.4–32.2)[a]	24.9 (22.3–27.9)[a]	
TBARS plasma (nmol/mL)	3.9 ± 1.6	5.2 ± 2.0[a]	5.4 ± 1.6[a]	
NP-SH (nmol NP-SH/mL)	824.7 ± 152.4	705.0 ± 133.0[a]	691.9 ± 118.0[a]	

P-SH (nmol P-SH/mL)	153.4 (127.2–170.9)	137.3 (114.5–155.8)	140.1 (122.1–151.8)	
Vitamin C (µg vit C/mL)	14.0 ± 5.3	10.5 ± 3.2[a]	11.7 ± 3.2	
Catalase (K/mg Hb)	53.5 (40.6-59.5)	45.2 (35.5-50.0)[a]	45.0 (36.8-49.0)[a]	
Correlations			**Spearman r**	
δ-ALA-D vs. TBARS, erythrocytes			-0.233	0.048*
δ-ALA-D vs. Glucose			-0.267	0.035*

The parametric results were analyzed by ANOVA, followed by the Tukey test and expressed in mean ± SD, and the non-parametric values determined by the Kruskal-Wallis test and represented as median (interquartile range). Analysis of the levels of HbA1c was used Student's t-test, the data being expressed as mean ± SD. [a]$p < 0.05$ when compared to the control group. [b]$p < 0.05$ when compared to the group of pregnant women with GDM. Gestational characteristics were expressed as percentage (%) and the p value obtained by the chi-square test. Correlation of the data using the Spearman correlation coefficient. *$p < 0.05$ was considered statistically significant. BMI, body mass index; C, control group; δ-ALA-D, delta-aminolevulinate-dehydratase; GDM, gestational diabetes mellitus; HbA1c, glycated hemoglobin; NP-SH, non-protein thiol groups; P-SH, protein thiol groups; TBARS, barbituric acid reactive substances; T2DM, pre-gestational type 2 diabetes mellitus.

Figure 1A depicts the activity of δ-ALA-D in the presence and absence of the reducing agent DTT. Considerable difference was found in relation to control and GDM. However, the rate of enzymatic reactivation was significant in both DM groups compared to the control (Figure 1B).

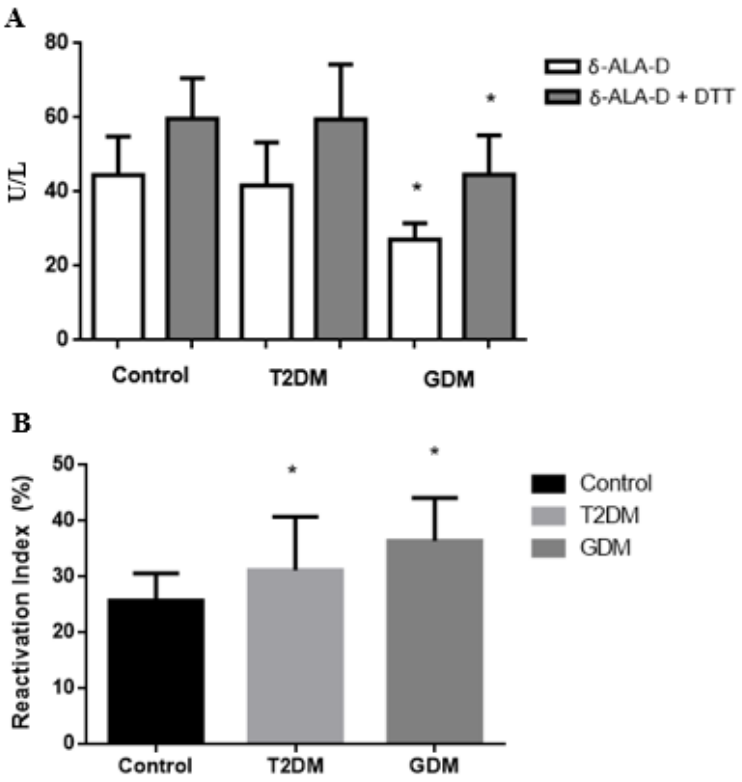

Figure 1. (A) Enzymatic activity of δ-ALA-D in the groups of pregnant women analyzed, results expressed as U/L. (B) Reaction index of δ-ALA-D in the different groups, data expressed as percentage (%). The results analyzed by ANOVA, followed by the Tukey test and expressed in mean ± SD. * $p < 0.05$ when compared to the control group. δ-ALA-D, δ-aminolevulinate-dehydratase; DTT, dithiothreitol; GDM, gestational diabetes mellitus; T2DM, pre-gestational type 2 diabetes mellitus.

Correlations between δ-ALA-D activity and oxidative stress markers (Table 1), show a negative correlation between enzymatic activity and TBARS erythrocytes, and glucose.

4. Discussion

This study aims to evaluate the clinical and oxidative differences between pregnancies in which DM is present. It also evaluates the activity of the δ-ALA-D enzyme, which, to our knowledge, has not yet been verified in this comparison.

In general, risk factors for the development of DM are similar for their subtypes and include advanced maternal age and obesity (Dirar, Doupis, 2017) and therefore this pathology develops more easily in these individuals. This issue is confirmed by the significant difference between the ages of the mothers and gestational BMI, higher in the presence of the condition, and the significant pre-gestational BMI in the T2DM group. Maternal obesity or higher weight has been associated with adverse outcomes in pregnancy such as pre-eclampsia and cesarean section (Sato et al., 2014) and pre-gestational BMI as a factor influencing fetal growth (Alberico et al., 2014).

Possible explanations for the increase in blood pressure may be the effects of hyperinsulinemia, weight gain and renal sodium retention (Dirar, Doupis, 2017), however, in both groups blood pressure values do not exceed the reference limits to be classified as hypertensive.

In pregnant women with DM, fasting glycemic levels were higher than those in the control group, as expected, for their diagnoses. The results of HbA1c were higher in pregnant women with T2DM, probably because the condition was present before pregnancy. These data together may suggest that the pre-gestational group with T2DM would have a higher degree of carbohydrate intolerance compared to the GDM group (Sugiyama et al., 2014).

Higher platelet count was obtained for pregnant women with DM, consistent with the results of Sahbaz et al., 2016, for the GDM. It is known that platelets play an important role in homeostasis and blood coagulation, currently in conjunction with other platelet parameters, is associated with inflammatory diseases. Obesity, as an important risk factor for this disorder, is considered a chronic inflammatory state. And inflammation in turn is said as one of the main causes to increase insulin resistance. Therefore, inflammation is implicated in the pathogenesis of diabetes (Sahbaz et al., 2016).

One of the most well known products of lipid peroxidation is MDA, which is used as a measure of damage and changes in the structure and function of cell membranes (Shang et al., 2015). In the present study, an increase in lipid damage in plasma and erythrocyte levels was observed in pregnant women with DM. Shang et al., 2015, also revealed an increase in MDA in women with GDM, suggesting a relationship between lipid peroxidation and changes in prostaglandin synthesis, which could also be related to fetal

malformations attributed to DM (Arribas et al., 2016). The negative correlation between erythrocyte TBARS and glucose dosages, and δ-ALA-D activity suggest a high ROS production (De Lucca et al., 2016) in pregnant women with DM.

It is also relevant to note that there was a decrease in the levels of thiol groups, which are molecules containing a sulfhydryl functional group attached to a carbon atom and have an important action against free radicals (De Lucca et al., 2016). This result is consistent with that of Rajdl et al., 2005.

Vitamin C plays an important role in non-enzymatic antioxidant defense (Gonçalves et al., 2005). In this study, vitamin C levels were significantly decreased in the groups of pregnant women with T2DM. As these play an important role in the protection of thiol groups, their decreased levels may reduce their antioxidant activity.

The enzyme catalase is an important participant in the enzymatic antioxidant defense system (Polachini et al., 2016). Our results showed that the decrease of the enzymatic activity was significant in the groups with the pathology, data consistent with those of López-Tinoco et al., 2013, for the GDM.

In general, these results show that there is a decrease in the antioxidant defense, which may have occurred due to the inactivation caused by the excess free radicals (Palma et al., 2014; Rajdl et al., 2005) leading to a greater consumption of the same.

A decrease in δ-ALA-D activity was observed in pregnant women with GDM compared to the other groups (Figure 1A), which is consistent with the results of Rodrigues et al., 2018. Pregnant women with T2DM exhibited similar levels of δ-ALA-D activity with the control group, which may be explained by the fact that pregnant women with pre-existing T2DM use insulin. Palma et al., 2014, showed that the use of insulin prevented the reduction of δ-ALA-D activity in mice with induced type 1 DM, and its decrease occurred when it was not used. The enzymatic reactivation index (Figure 1B) shows significant results in both DM groups in relation to the control group, which shows the involvement of thiol groups in the reestablishment of δ-ALA-D activity in the presence of DTT in vitro (De Lucca et al., 2016).

5. Conclusion

The parameters evaluated confirmed an increase in oxidative stress and were associated with the reduction of the antioxidant response in DM in both T2DM and GDM, which may be an adaptive and protective response. Although both disorders are generated by carbohydrate intolerance and are differentiated events for the organism, they present many similarities between the evaluated markers, representing the largest difference with the control group. Considering the data, we identified some differences between the groups, such as the high pre-gestational BMI observed in pregnant women with T2DM, representing an important risk factor for complications. For the knowledge, this is the first time that this comparative evaluation of δ-ALA-D enzyme activity was performed, and shows that the use of insulin in pregnant women with T2DM may have been beneficial in preventing the reduction of δ-ALA-D enzyme activity, preventing an even greater increase of oxidative stress and, consequently, the possibility of damages to both the fetus and the mother.

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