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ORIGINAL ARTICLE

Alterations in antioxidant capacity, lipid peroxidation, and nitrite levels in type 2 diabetes

Alterações na capacidade antioxidante, níveis de peroxidação lipídica e nitritos em pacientes com Diabetes tipo 2

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Abstract

Background: this case-control study delves into oxidative and antioxidant markers, including Total Antioxidant Capacity (TAC), lipid peroxidation, and nitrites, alongside glucose levels in individuals with hyperglycemia due to type 2 diabetes mellitus (T2DM) in comparison to their healthy counterparts. Furthermore, the study explores the impact of glucose on these variables.

Methods: twenty patients diagnosed with T2DM and hyperglycemia were included, along with a control group of 20 healthy subjects (Control Group – CG). Glucose levels, as well as oxidative markers (malondialdehyde and nitrites) and antioxidants, were assessed in the blood plasma of each participant. To ascertain statistically significant differences between the studied variables across groups, Student t-test or Mann–Whitney tests were employed. Pearson's Linear Correlation test was utilized to evaluate associations between the variables.

Results: glucose and TAC levels exhibited significant elevation in the T2DM group compared to the CG. In T2DM patients, malondialdehyde and nitrites demonstrated reduced levels (1.83±0.84 μM/L and 29.5±16.6 μmol/L, respectively) in contrast to the CG (4.00±1.11 μM/L and 82.9±29.2 μmol/L, respectively), with both parameters showing a robust positive correlation ($p = 0.0002$).

Conclusions: individuals with diabetes manifest alterations in the redox state, characterized by diminished lipid peroxidation and nitrite levels, suggesting disruptions in the constitutive pathway for nitric oxide production. This underscores the intricate interplay between oxidative stress, antioxidants, and glucose metabolism.

Keywords: diabetes mellitus, hyperglycemia, oxidative stress, nitrites.

Resumo

Objetivos: este estudo de caso-controle busca investigar marcadores oxidativos, capacidade antioxidante e níveis de glicose em pacientes com Diabetes mellitus tipo 2 (T2DM), em comparação com participantes saudáveis de perfil semelhante. Adicionalmente, analisa-se o impacto da glicose nessas variáveis.

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Metodologia: foram incluídos 20 pacientes com T2DM e hiperglicemia, bem como 20 voluntários saudáveis no grupo controle (CG). Nos participantes, os níveis de glicose, malondialdeído, nitritos e capacidade antioxidante total (TAC) foram determinados no plasma. Para analisar diferenças significativas entre as variáveis nos grupos, aplicaram-se o teste t de Student ou Mann–Whitney. O teste de correlação linear de Pearson foi empregado para avaliar associações entre as variáveis.

Resultados: os níveis de glicose e TAC mostraram elevação significativa no grupo T2DM em comparação com o CG. Participantes do grupo T2DM apresentaram redução nos níveis de malondialdeído e nitritos (1,83±0,84 μM/L e 29,5 ± 16,6 μmol/L, respectivamente) em relação ao CG (4,00±1,11 μM/L e 82,9±29,2 μmol/L, respectivamente), ambos demonstrando forte correlação positiva (p = 0,0002).

Conclusão: pacientes com Diabetes mellitus tipo 2 exibem alterações no estado redox, evidenciadas pela diminuição da peroxidação lipídica e dos níveis de nitritos, indicando comprometimento na via constitutiva de produção de óxido nítrico. Esses achados ressaltam a complexa relação entre estresse oxidativo, antioxidantes e metabolismo da glicose.

Palavras-chave: diabetes mellitus, hiperglicemia, estresse oxidativo, nitritos.

Introduction

Diabetes Mellitus (DM) is a chronic metabolic syndrome characterized by persistent hyperglycemia due to changes in insulin secretion and/or resistance to the hormone, hindering glucose absorption in most tissues and promoting the emergence of cardiovascular, renal, and ocular complications, among others (1, 2).

Approximately 537 million people worldwide are living with DM, and it is projected that the number of cases will reach 783 million by the year 2045. Moreover, it is estimated that 6.7 million deaths are directly related to the disease each year around the world. In Brazil, the number of diagnoses reached 15.7 million in 2021, occupying the sixth position with the highest prevalence in the world. In addition, among the known diabetes types, type 2 diabetes mellitus (T2DM) has the highest incident among the population, corresponding to about 90% of cases (3). The high prevalence of the disease in Brazilian adolescents has drawn attention (4, 5).

Despite intensive efforts aimed at unraveling the pathophysiology of DM, there is still a need for a better understanding of this complex condition. Nevertheless, it is known that a combination of factors contributes to its development, which may be genetic, metabolic and/or behavioral. This pathology has multiple etiologies with diverse pathogenic process involved, having in common elevated blood glucose levels. However, the underlying abnormalities of this increase vary widely (6, 7).

Hyperglycemia is the main laboratory alteration initially identified in DM and the main factor for the development of complications associated with the disease, including the presence of oxidative stress, characterized by the presence of alterations in antioxidant activity, lipid peroxidation, nitric oxide (NO) levels and its metabolites (nitrites - NO_2^-, and nitrates - NO_3^-) (8-10).

The mechanisms linking hyperglycemia and antioxidant activity have not been fully elucidated; however, it is believed that the increased production of reactive oxygen species (ROS) is further stimulated by the presence of an inflammatory process. Therefore, limiting the production of radicals may offer therapeutic benefits. In this context, some researchers reported that increased antioxidant levels can fight ROS in DM, while others suggest the need for the increase of specific antioxidant enzymes (8).

Besides these alterations, chronic hyperglycemia can promote lipid peroxidation, which frequently happens in the fatty acid chains of cell membranes, impairing their selectivity and producing lipid peroxides that can trigger oxidative chain reactions that ultimately result in cell damage. Case-control studies have demonstrated changes in oxidized lipid levels in patients with T2DM (9, 11).

Regarding NO, it is a free radical that has both beneficial and harmful roles in the organism, once it is involved in mechanisms ranging from relaxation of the vascular endothelium and neuronal signaling, to toxic effects in stressful situations, which generate highly reactive oxygen intermediates, impairing the antioxidant system (12) In addition, to date, the relationship between NO and hyperglycemia is controversial, as some studies affirm that NO concentrations are higher in diabetic patients, while others observed reduced expression of nitric oxide synthase (NOS) and consequent impairment of the conversion of arginine into NO and L-citrulline, as well as other metabolites of reactive nitrogen species (9, 10, 13 – 15).

Therefore, the present work aimed to verify the glucose levels, the antioxidant activity, lipid peroxidation and nitrites in hyperglycemic T2DM patients, in comparison to healthy subjects, as well as the influence of hyperglycemia on the variables analyzed.

Methods

This is a case-control study, developed with the participation of 20 patients diagnosed with T2DM and hyperglycemia (T2DM group), as well as 20 healthy subjects (control group – CG), both with a similar profile, and ages between 35 and 60 years old. All patients were treated at Socorrão Hospital and the CG was recruited in the Dom Afonso community, both from September 2021 to November 2022 in Imperatriz, State of Maranhão-Brazil. Patients who had T2DM were included, regardless of gender or age. The exclusion criteria were applied to patients with type 1 diabetes mellitus, normoglycemic T2DM patients, with any kind of infection, smokers, alcoholics, drug addicts, those being treated with antidepressants or anxiolytics, pregnant women and those taking antioxidant supplements, such as ascorbic acid, tocopherol, among others. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (16).

Personal data was obtained from all study participants and then 5 mL of blood was collected in EDTA tubes after an 8-hour fast. Samples were centrifugated (1000 rpm for 15 minutes) to obtain plasma for the laboratory assessment of glucose, total antioxidant capacity (TAC), thiobarbituric acid reactive substances (TBARS), and nitrites. Furthermore, all participants were previously informed about the study and signed the Free and Informed Consent Form. The study was carried out respecting ethical standards of the Declaration of Helsinki (17) and was approved by Human Research Ethics Committee of the Federal University of Maranhão, as stated on Brazil Platform (ethical approval document number 2.935.105).

In the determination of the glycemic index blood glucose was quantified using the Liquiform glucose kit (Ref. 133 Labtest®, Brazil). Hyperglycemic patients were considered as having glucose levels ≥ 100 mg/dL (2).

The determination of TAC was developed based on the reduction of the ABTS radical (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), which generates a change in absorbance read at 734 nm in the presence of antioxidants. Analyzes were carried out in duplicates, using the average as the result. The TAC plasma concentration was determined based on the linear regression curve ($y = 0.3653x + 0.0407$, R2 = 0.9683) calculated with the use of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard (18).

Lipid peroxidation analysis was determined by the quantification of thiobarbituric acid reactive substances based on malondialdehyde (MDA), without the use of trichloroacetic acid, as the concentration determined in the plasma was below the detection limit (0.5 μM/L) in the presence of this deproteinizing agent. Plasma MDA and thiobarbituric acid (TBA at 0.38%) prepared in acidic environment (pH 2.5) were heated at 94°C for 30 minutes, giving rise to the formation of the TBA-MDA-TBA complex, with peak absorption at 532 nm. Plasma TBARS concentration was determined based on the linear regression curve ($y= 0.1608x +0.0474$, R2 = 0.9621), calculated with the use of 1,1,3,3-tetramethoxypropane, MDA standard (19).

The determination of nitrites (NO2-) was carried out using the Griess reagent, which, at low pH, reacts with nitrites in the presence of sulfanilamide ($C_6H_8O_2N_2S$) and N-(1-Naphthyl) Ethylenediamine (NED). It was then read spectrophotometrically at 540 nm. As nitrite is a more stable metabolite of NO, researchers suggest that NO2- determination is a better way to assess NO concentration. It was determined based on the linear regression curve ($y=335.11x+0.4467$, R2 = 0.9998), calculated with the use of sodium nitrite ($NaNO_2$) as standard (20).

In the statistical analysis the data obtained were analyzed using the BioEstat 5.3 (Mamirauá Institute, Brazil). Aiming at investigating the existence of statistically significant differences between the variables studied between groups, we applied Shapiro-Wilk test, when the assumption of normality and homoscedasticity was met, while Student t-test or Mann–Whitney test were used when the assumption of normality was not met. Furthermore, Pearson's Linear Correlation test was used to assess the association between the variables studied, assuming up to 0.30 ($r < 0.30$) as a weak correlation, between 0.31 and 0.70 ($0.31 < r < 0.70$) as moderate correlation and between 0.71 and 1.00 ($0.71 < r < 1.00$) as strong correlation. The statistical significance was set at $p < 0.05$.

Results

The variables TAC, TBARS and nitrites showed normal distribution in the Shapiro-Wilk test in both groups studied, while glucose for the T2DM group showed non-normal distribution and normal distribution for the CG (**Table 1**).

TABLE 1 – Distribution of Glucose, TAC, TBARS and Nitrites in normoglycemic and hyperglycemic groups.

Variables	Normoglycemic p*	Hyperglycemic p*
Glucose	0.2476	0.0076
TAC	0.2970	0.2968
TBARS	0.3824	0.3679
Nitrites	0.2796	0.1333

TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances. **Shapiro-Wilk test.*

For glucose, as expected, CG showed significantly lower levels (77.9±10.1 mg/dL) than T2DM (184.9±84.074 mg/dL). Likewise, for TAC, CG showed a significantly low antioxidant capacity when compared to T2DM (**Figure 1**).

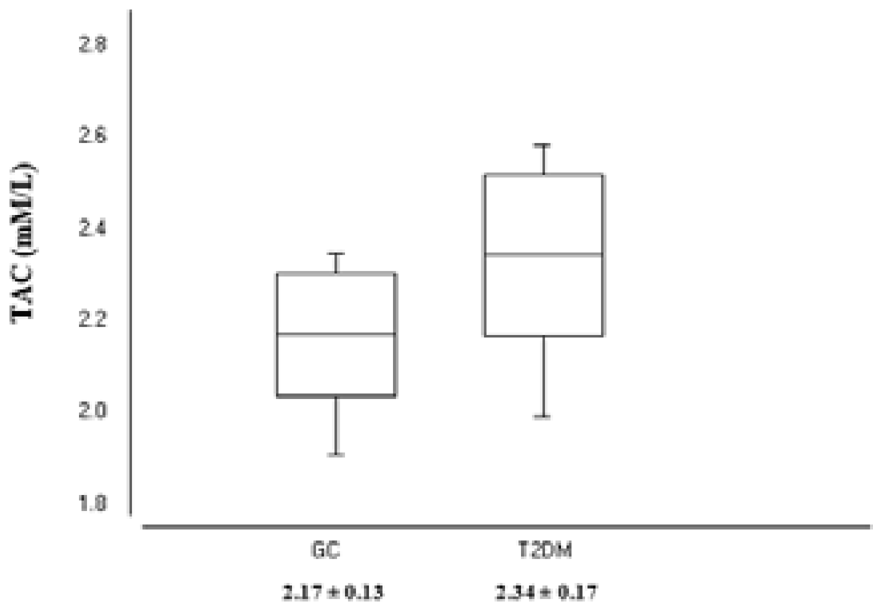

Figure 1. Comparison of total antioxidant activity (TAC) between control group (CG) and type 2 diabetes mellitus and hyperglycemia group (T2DM). Data are presented as mean ± standard deviation (mM/L). *Student's t-test*: p<0.0001.

In the lipid peroxidation assessment, TBARS levels in hyperglycemic patients were significantly lower than CG (**Figure 2**). Likewise, nitrite levels were significantly lower for T2DM compared to CG (**Figure 3**).

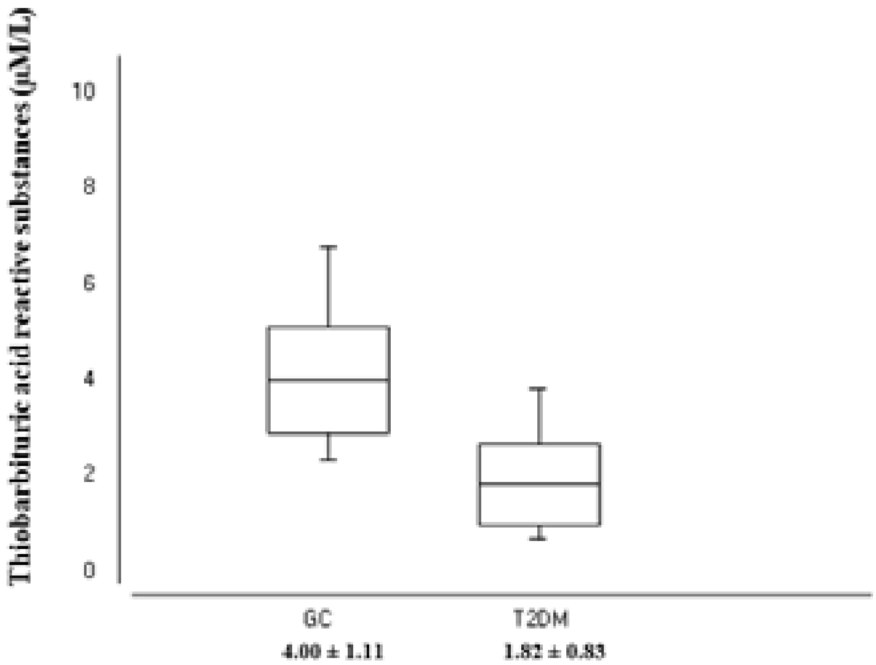

Figure 2. Comparison of thiobarbituric acid reactive substances (TBARS) levels between control group (CG) and type 2 diabetes mellitus and hyperglycemia group (T2DM). Data are presented as mean ± standard deviation (µM/mL). *Student's t-test*: p<0.0001.

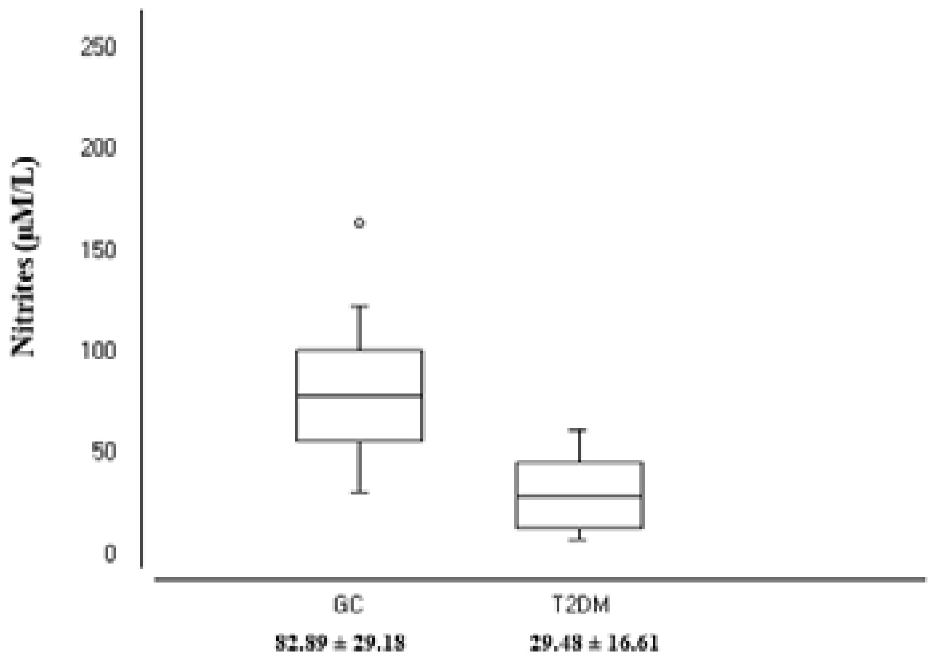

Figure 3. Comparison of nitrite levels between control group (CG) and type 2 diabetes mellitus and hyperglycemia group (T2DM). Data are presented as mean ± standard deviation (μM/mL). *Student's t-test*: p<0.0001.

Regarding the correlation studies, a moderate positive correlation between glucose and TBARS was observed only in the CG group (p = 0.0196 and r = 0.5168). Additionally, a strong positive correlation was identified between TBARS and nitrites in patients with T2DM (**Figure 4**). No other significant correlations were observed.

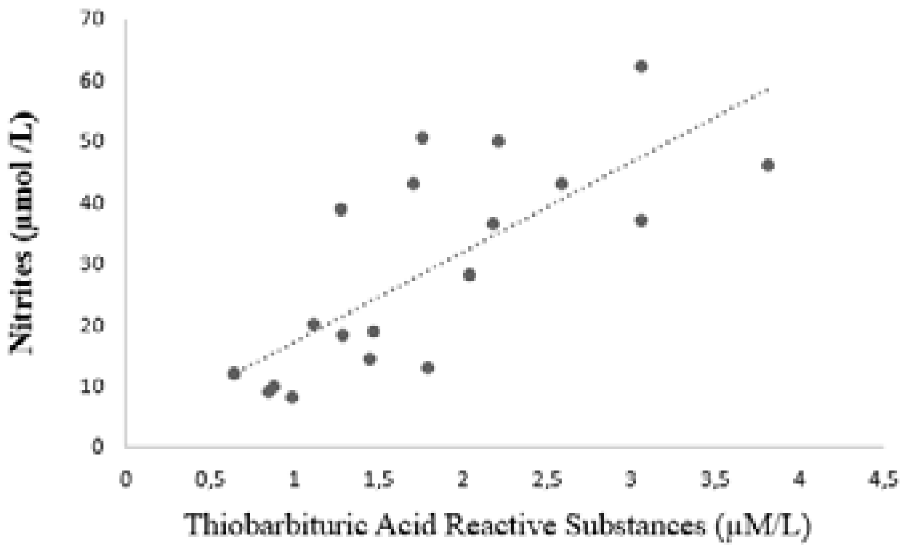

Figure 4. Correlation between Nitrites and Thiobarbituric Acid Reactive Substances in type 2 diabetes mellitus and hyperglycemia group. Pearson test was used to analyze the relationship between both variables. Correlation coefficient (r = 0.7462, p < 0.0002) indicating a strong correlation.

Discussion

In the present study, we choose to use glucose, level together with the analysis of redox variables, allowing us to access the direct correlation of carbohydrate levels with oxidative stress, in order to unravel its involvement in the pathophysiology of the disease, where all parameters should be in the same condition at the time of sample collection.

Glucose did not exhibit normal distribution in the T2DM group, as patients with DM often manifest an organic response and widely varying glycemic levels. Some studies suggest that these fluctuations may contribute to the development of diabetes-related complications (23, 24). Furthermore, despite undergoing glucose reduction treatment, patients with T2DM did not achieve glucose levels considered normal, that is, below 100 mg/dL. These results are in line with the findings of Sá et al. (25), where patients had an average glucose level of 122 mg/dL. Such studies highlight the challenges associated with restoring normal insulin function in patients with T2DM.

Interestingly, we found that elevated TAC values were found in T2DM patients, when compared to CG. By assessing TAC through the method based on the ABTS radical, we confirmed the existence of a general state of oxidative stress in these participants, as some antioxidant defenses may increase in response to an unbalanced redox state, since such antioxidant molecules can be synthesized by the patients, corresponding to what is classified as mobilized antioxidants (26). Therefore, an increase in TAC, as seen in T2DM, indicate an active state of oxidative stress. This result may occur due to the need for a higher antioxidant level to neutralize the production of oxygen reactive species in the disease condition. This finding differs from the one found by Rosa et al. (27), as they identified lower levels of TAC, through the iron reduction method (FRAP), in the serum of T2DM patients, and low glucose levels (median glucose = 103 mg/dL) to show in this study (average glucose = 189.6 mg/dL). Notwithstanding, the role of antioxidants must be further explored. T2DM and hyperglycemia conditions may lead to a better understanding of the underlying reactions of this disease. Moreover, it can lead to better treatments or to the development of wiser prevention strategies.

In addition, another possibility for the TAC values shown by T2DM is due to the use of metformin, a commonly used drug for the treatment of the disease in Brazil, since it, besides increasing the sensibility of the tissues to insulin, has showed its effect on the control of the oxidative stress through the increase in antioxidants defenses, in addition to having inhibitory actions on the levels of *advanced glycation products* (AGEs – advanced glycation end-products) (28, 29).

Notwithstanding, we believe that the increase in TAC values by T2DM may occur as a response to the increasing presence of ROS, which affect the functioning of the machinery involved in glucose absorption by the tissues, thus increasing the peripheral insulin resistance, which increases inflammatory response, generating even more ROS, establishing a vicious cycle that contributes to the pathogenesis of DM and several other diseases (30-32).

Another parameter analyzed was TBARS, an indicator of lipid peroxidation, which was found at lower levels in the T2DM group, suggesting low oxidative action on lipids or that the increased TAC values have effectively reversed the oxidative stress condition. This finding is similar to the one by González and Rodriguez (9), who used the same method and type of study in a sample composed of healthy and hyperglycemic patients. However, different results were identified by Kesavulu et al. (33), since they reported higher levels of TBARS in patients diagnosed with T2DM and microvascular complications. Even so, the patients in this study did not have a diagnosis of vessel impairment. Therefore, it is suggested that different and cumulative clinical conditions of the patients, aggravated by the metabolic syndrome, may have different effects on lipid peroxidation, as oxidative stress arises from different forms of molecular oxidation that go beyond lipid peroxidation, such as the oxidation of DNA proteins and carbohydrates themselves.

Regarding nitrite, a recent study, which dosed separately NO2- and nitrates (NO3-), observed lower values of the first metabolite in diabetics when compared to controls, confirming the findings of the present study about nitrites in the T2DM, which showed lower levels than control.10 However, Konokoglu et al. (13), when dosing total nitrites in a study with the participation of diabetic and control, identified high levels of total nitrites in hyperglycemic diabetics. The elevated production of nitrites seems to be present in diabetics who had complications and were alcoholics (34). Given these findings, we highlight the need for more specific studies regarding the role of nitrites and NO according to the condition and complication of the patients affected by DM. The role of nitrite as a hallmark of NO production, as a signaling molecule or as an antioxidant, should be further explored in the T2DM.

However, concerning NO was observed in this study that its production through the constitutive way in diabetic patients does not seem to be adequate, with reported reduced nitrite levels in diabetics when compared to healthy volunteers. By virtue of their condition, healthy subjects are expected to have basal production of nitrites involved in homeostasis. A recent experimental study reinforces this theory, since the reduced expression of the enzyme involved in the constitutive production, the endothelial nitric oxide synthase (e-NOS), was shown in type 2 diabetic patients (15). With low activity of e-NOS, the arginine metabolism is impaired, harming the production of NO and L-citrulline; the first one has an important vasodilating role, and the former seems to contribute to insulin secretion, capturing glucose from the skeletal muscle, lipolysis, -oxidation, and reduction of gluconeogenesis in adipose tissue (35).

A possible cause for the decrease of e-NOS is its monomerization, that is, the change in conformation and uncoupling from the endothelial tissue. These alterations are promoted by the excess of mitochondrial ROS produced in diabetes, which contribute to the occurrence of these effects and vascular dysfunctions, among them, blockage of vasodilation (36), increase in vascular permeability, platelet aggregation, atherogenesis, and macrovascular problems (37, 38). In cases of complications, such as diabetic retinopathy clinical study, the i-NOS enzyme is a high-risk for developing retinopathy (39). Therefore, the effect of NOS and the consequent production of NO seem to act in different concentrations and effects on the course of the disease.

Concerning the reducing action of nitrite due to its ability to donate electrons, the low concentration evidenced may be involved in the underlying mechanisms of the disease, as studies have demonstrated that this molecule induces insulin secretion due, partially, to its action in fighting oxidative stress, as well as stimulating the production of antioxidants (40).

Among the results of the present study, it was seen a moderate positive correlation between glucose and lipid peroxidation levels, only for healthy subjects of the CG, suggesting a moderate stimulation of lipid oxidation by glucose, a relationship not found in the diabetic patients studied. In that case, the presence of glucose at normal levels in the body may favor the action of pathways that involve lipid oxidation to maintain homeostasis. Moreover, as there is a physiological association between growth hormone (GH) and insulin, when both are at physiological levels, beta-oxidation of lipids occurs, which are prioritized as a source of energy in GH metabolism. However, the insulin resistance in T2DM promotes a negative relationship with the referred hormone, preventing the oxidative effect on fats (41).

The TBARS and NO2- levels in this study showed reduced concentrations for both compounds, suggesting an environment of low oxidative action of lipids by reactive nitrogen species in the patients studied. On the other hand, high levels of NO in the face of oxidative stress react with the superoxide radical (O2-) and form peroxynitrite (ONOO-), a powerful inductor of lipid peroxidation (42). Therefore, given the low production of NO due to changes in the constitutive pathway, there is no formation of ONOO- and lipid peroxidation, effects evidenced in the present study.

Possible alterations involved in reduced production of nitrites are: (I) Endothelial damage promoted by excessive glucose; (II) Low synthesis of e-NOS by exhaustion and/or degeneration of its synthesis pathway by a situational need for hyperproduction of NO with vasodilator action; (III) Changes in e-NOS conformation by oxidative action. However, intrinsic characteristics of diabetic complications may cause increase of nitrogen reactive species and its metabolites, nitrites and nitrates.

In this way, increasing antioxidant activity is crucial as an intrinsic response to the oxidative environment associated with diabetic hyperglycemia, and that NO availability was decreased in T2DM. Lipid peroxidation was found at lower levels in the same individual. Moderate positive correlation was found between glucose and lipid peroxidation in the healthy subjects. Based on these findings, the diabetic condition can affect the inherent pathway of NO production. However, we emphasize the need for additional research to explore the connection between NO and its constitutive pathway in DM.

Notes

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Conflicts of interest disclosure

The authors declare no competing interests relevant to the content of this study.

Authors' contributions

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content; and to approve the version to be published.

Availability of data and responsibility for the results

All the authors declare to have had full access to the available data and they assume full responsibility for the integrity of these results.

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