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Research report

Oxidative stress markers in cognitively intact patients with diabetic neuropathy

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ABSTRACT

Various forms of vascular injury are frequently associated with type-2 diabetes mellitus (DM2). Macro-angiopathy has alarming signs and symptoms such as those seen with stroke or heart attack, however the presentation of small vessel disease is generally more subtle and therefore usually unnoticed for a long period of time. While it may affect any organ, complications involving the nervous system such as diabetic poly-neuropathy (DPN) are especially debilitating, and it may also be a risk factor for other brain disorders such as dementia. The underlying mechanisms are likely to be multi-faceted, but piling evidence indicates oxidative stress as one of the crucial factors. Here we evaluate the oxidative profile of patients with DM2. The total anti-oxidant capacity appears to be reduced in DM2 with or without complications. Of the specific bio-markers studied, the levels of tissue-damage indicator malon-dialdehyde (MDA) were significantly lower in the DM2 + DPN population only. These results suggest that diabetic patients present with wavering oxidative status, and the low MDA concentrations in patients with complications such as DPN may represent either an exhausted anti-oxidative defense system or a response to anti-inflammatory medications. The findings may also support the use of anti-oxidants such as vitamins A and E.

1. Introduction

Micro-vascular disease is considered to be one of the most common complications of type-2 diabetes mellitus (DM2). The subtle injuries involving the small blood vessels that supply nerves lead to diabetic poly-neuropathy (DPN) and are the risk factors for other neuro-psychiatric disorders such as cognitive impairment (Iqbal et al., 2018; Strom et al., 2017). DPN affects about one-third of the diabetic population. It causes a great impact on the patients' quality of life and increases the rate of morbidity and mortality (Iqbal et al., 2018; Papanas and Ziegler, 2015a). According to the literature, oxidative stress is considered as one of the main factors in the development of DM2 and its complications (Almogbel and Rasheed, 2017).

Oxidative stress is usually defined as the process of imbalance between free radicals and antioxidants that favors the increase of free radicals (Asmat et al., 2016). The main free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS includes radicals such as superoxide, hydroxyl, peroxyl and hydroperoxyl, whereas RNS comprises nitrite oxide (Mrakic-sposta et al., 2018). Due to its great participation in the development of DM2 and its likely role in the pathogenesis of DPN, the oxidative stress profile must be investigated (Oyenihi et al., 2015).

Both DM2 and its complications are multifactorial, and so the pathogenesis of DPN is rather complex. It has not been well defined yet, but some studies point to several pathways that may lead a diabetic individual to develop PD, including hyperglycemia, dyslipidemia and impaired insulin signaling (Pop-busui et al., 2017). Currently, hyperglycemia is considered as one of the most described mechanisms. There is evidence that chronic hyperglycemia can cause cell damage through several pathways. First, excess glycolysis leads to mitochondrial electron transport chain overload and increased ROS and RNS (Pop-busui et al., 2017). Through the polyol pathway, hyperglycemia increases cellular osmolarity by reducing the nicotinamide adenine phosphate dinucleotide NADPH, which, consequently, leads to oxidative stress (Almogbel and Rasheed, 2017). Through the hexosamine pathway, hyperglycemia can cause inflammatory injury (Pop-busui

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et al., 2017). In addition, increased blood glucose may generate advanced late glycation end products (AGEs). In general, the latter causes the oxidation of biomolecules and irreversible tissue damage, including peripheral nerve damage (Schreiber et al., 2015). Recent studies have been conducted to verify the association between oxidative stress and the development of DM2 or DPN (Strom et al., 2017). Many of these studies evidence a relationship between oxidative stress and DM2.

In light of such evidence on the role of oxidative stress in the development of DM2 and its complications, the present study aims to evaluate the profile of oxidative stress in patients with DM2 and DPN and relate them to factors associated with the development of DPN.

2. Materials and methods

2.1. Study design

This is a cross-sectional study, with descriptive and inferential statistical analysis.

2.2. Patients and control subjects

The study protocol was approved by the Committee for Ethics in Research of the Federal University of Viçosa (CAAE 59855516.0.0000.5153). All subjects signed legal consent forms. Information on demographic characteristics, lifestyle habits and perceived stress level was also assessed.

We assessed a total of 42 male and female subjects at the ages ranging from 40 to 72 years. DM2 patients were selected in the Centro Estadual de Atenção Especializada in Viçosa, Minas Gerais, Brazil. DM2 + DPN patients were selected at the neurology clinic in Viçosa. The diagnosis was confirmed by nerve conduction studies and the presence of peripheral neurological symptoms. The control group was composed of healthy subjects with no previous history of DM2 or DPN. Healthy individuals were recruited via the on-line news and mailing system of the Federal University of Viçosa. On the basis of clinical criteria, the subjects were distributed into three groups: control (n = 13), DM2 (n = 15) and DM2 + DPN (n = 14).

Subjects with renal, hepatic, autoimmune, and myelo-proliferative diseases, coagulation disorders, cancer, in a post-operative period or immobilized during a long time, and those with metabolic syndrome were excluded from the study. All participants with Type 1 diabetes mellitus were also excluded. The individuals were classified as having DM2 if plasma glucose was equal to over 126 mg/dL, under fasting conditions, or if they were receiving oral anti-diabetics or insulin. The mini-mental state examination (MMSE) was performed according to Folstein et al. (1975).

2.3. Sample collection

Venous blood samples were taken from all subjects after fasting for 12 h. Five milliliters of venous blood without anticoagulant were collected from each participant using Vacutainer® System tubes (Becton-Dickinson). The samples were centrifuged at 1.100 *g* for 15 min, for rapid serum separation. The serum aliquots were stored at −70 °C.

2.4. Laboratory measurements

The tissue damage marker Malondialdehyde (MDA), nitric oxide (NO), antioxidant mediators Glutathione S-transferase (GST) and total antioxidant capacity were measured, to evaluate the oxidative stress.

The lipid peroxidation (MDA) was measured by reactive substances with thiobarbituric acid (TBARS), according to the methodology proposed by Buege and Aust (1978). Thus, 200 µL of each sample were added to 400 µL of TBARS solution (15% TCA, 0.375% TBA and 0.25 M HCl), vortexed for 10 s and placed in water bath, at 90 °C, for 40 min. After cooling, the thiobarbituric acid reactive substances were extracted with the addition of 600 µL of n-butanol, followed by centrifugation at 3500 rpm, for 5 min. Finally, after centrifugation, 200 µL of the supernatants were removed and subjected to reading at 535 nm. TBARS values were expressed as malondialdehyde (MDA) ηmols per mg protein.

Nitric oxide was evaluated by the modified Griess method (Romitelli et al., 2007). In this method, nitric is used as an indicator of nitric oxide synthesis and is detected by the Griess Reactive, composed of 1% sulfanilamide and 0.1% naphthylethylene diamine in 2.5% H3PO4. Based on this method, 50 µL of serum was added to 96-well microplates containing 100 µL of the Griess Reagent. This mixture was incubated in the dark for 10 min, at room temperature. After incubation, the Thermo Scientific Multiskan GO spectrophotometer, 570 nm wave, was used for analysis. The concentration of nitric oxide was measured using the standard curve with known concentrations (µM) of sodium nitrite.

The analysis of the enzymatic activity of GST was performed by the method described by Habig et al. (1974). In a Quartz cuvette, 682 µL of Phosphate buffer (0.1 M and pH 7.0) was pipetted along with 6 µL of CDNB 1-chloro-2,4-dinitrobenzene (0.1 M), 6 µL of sample and 6 µl of GSH solution (0.1 M).The reaction rate of the enzyme present in the samples was monitored at 340 nm in a spectrophotometer for 90 s to monitor the enzymatic kinetics. A blank was also made for the experiment, which had no addition of the sample and was used to verify the rate of non-enzymatic reactions. The enzyme activity was expressed in µmol min-1 g -1, where one unit of activity refers to the amount of enzyme that catalyzes the formation of 1 µmol of product per minute and per gram of sample, under the conditions of the assay.

The total antioxidant capacity (FRAP) was determined by the method described by Benzie and Strain (1996). The reaction was performed by adding10 µL of sample or standard, and 220 µL of the FRAP solution were added to polystyrene microplates. The microplates were incubated in the dark for 30 min.

2.5. Statistical analysis

The data were analyzed by the Graph Pad Prism version 7.0 software system, using one-way analysis of variance (ANOVA), followed by the Tukey test. The categorical variables (risk factors) were analyzed by the Chi-square contingency test. The Pearson's correlation coefficient was used to measure the linear association between two continuous variables.

The minimum size of the sample was defined using the coefficient of variation previously obtained for FRAP (21.6%) and GSH (20.2%) in the literature (Gawlik et al., 2016), considering fifteen and twelve percent of variation around the average, respectively, with a minimum number of nine individuals per group. It was possible to verify statistical differences with a 5% level of significance (Hulley and Cummings, 1988).

3. Results & discussion

There were no significant correlations between the variables studied. Table 1 presents the characteristics of age, sex and body mass index of the three study groups, as well as the evaluation parameters of the oxidative stress profile and the result of the stress perceived by the participants. Table 2 displays data about medications and supplements (vitamins A, C, E) used by the participants in the DM2 and DM2 + DPN groups.

Figs. 1 and 2 delineate the significant differences observed for the FRAP and MDA variables, respectively.

When uncontrolled, chronic hyperglycemia can lead to macrovascular and microvascular complications (Oyenihi et al., 2015). Polyneuropathy is considered to be one of the most severe and common microvascular complication (Giriraja et al., 2017). One of the causes associated with the development of polyneuropathy in diabetic individuals is oxidative stress, which is defined as an imbalance process

Table 1
Characterization of sample and evaluated parameters.

	Control (n = 13)	DM2 (n = 15)	DM2 + DPN (n = 14)	*p*
Age (years)	61 ± 6	60 ± 6	61 ± 5	0.655
Male/Female	7/6	7/8	7/7	—
BMI (Kg/m^2)	27 ± 7	33 ± 4*	31 ± 5	0.0187
MMSE	27 ± 6	25 ± 3	25 ± 5	0.325
Perceived stress	—	12 ± 9	14 ± 8	0.619
Nitric Oxide (μM)	19.6 ± 5.7	19.5 ± 8.4	19.8 ± 6.1	0.992
MDA (nmol/mg)	1.04 ± 0.15	1.06 ± 0.16	0.30 ± 0.08*	< 0.0001
FRAP (μM)	332 ± 87**	278 ± 59	256 ± 36	0.023
GST (nmol g -1)	0.59 ± 0.27	0.74 ± 0.38	0.51 ± 0.23	0.191

n = sample size, DM2 = Diabetes mellitus type 2, DPN = diabetic poly-neuropathy, BMI = Body Mass Index, MMSE = mini-mental state examination, p = probability for the hypothesis tests, MDA = malon-dialdehyde, FRAP = ferric reduction capacity, GST = glutathione-S-transferase. ANOVA followed by Tukey's test, data presented as mean ± standard deviation. (*) Significant difference in relation to the control and DM2 groups. (**) Significant difference in relation to the DM2 and DM2 + DPN groups.

Table 2
Distribution of frequencies of use of drugs and supplements per group.

	Control (n = 13)	DM2 (n = 15)	DM2 + DPN (n = 14)	*p*
Simvastatin	0 (0%)	0 (0%)	6 (42.8%)	—
Metformin	0 (0%)	1 (6.66%)	9 (64.28.8%)	0.0011*
Glimepiride	0 (0%)	1 (6.66%)	2 (14.3%)	0.5008
Insulin	0 (0%)	0 (0%)	8 (57.1%)	—
Vitamin A	0 (0%)	0 (0%)	4 (28.5%)	—
Vitamin C	0 (0%)	0 (0%)	4 (28.5%)	—
Vitamin E	0 (0%)	0 (0%)	5 (35.7%)	—

n = sample size; DM2 = Diabetes mellitus type 2, DPN = diabetic poly-neuropathy, p = probability for hypothesis testing, (*). significant difference between DM2 and DM2 + DPN groups (chi-square test).

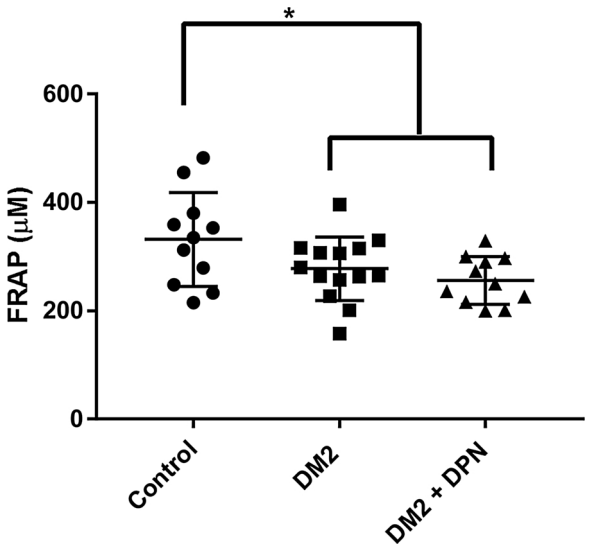

Fig. 1. Distribution of Ferric reduction capacity (FRAP) values in the three groups studied. FRAP is expressed in micromoles μM. The control group showed significantly higher plasma levels of FRAP than the DM2 and DM2 + DPN groups (p = 0.0236).

between ROS and antioxidants, with the occurrence of increased radical production and decreased rate of antioxidants (Asmat et al., 2016; Giriraja et al., 2017).

In this study, we investigated the parameters related to oxidative

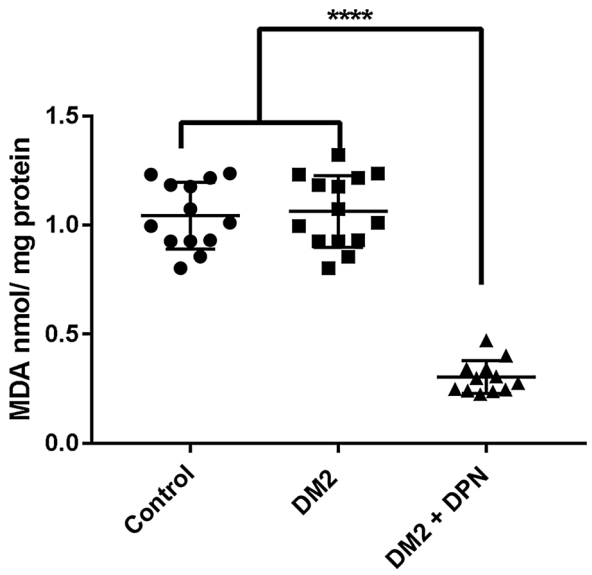

Fig. 2. Distribution of malon-dialdehyde (MDA) values in the three groups studied. MDA concentrations were expressed in nano-mol / mg proteins. The DM2 + DPN group presented significantly lower MDA values than the control and DM2 groups (p < 0.0001).

stress in patients with DM2 and DPN. A decreased total antioxidant capacity was observed in the serum of patients with DM2 and DPN, when compared with participants considered normal in the control group. These findings suggest that patients with DM2 with or without DPN present alterations in the antioxidant defense system in relation to controls. Contrary to other studies, such as de Palem and Abraham (2015) and Ganjifrockwala et al. (2017), a significantly reduced MDA level was observed in the DM2 + DPN group compared to the DM2 and control groups.

The studied population didn't present a cognitive decline considering the data obtained with the MMSE (Table 1) and also the years of schooling of the participants. The characteristics of the participants that were considered in the present study include age, gender, BMI, perceived stress and MMSE. These factors may directly or indirectly affect the oxidative profile of an individual (Takahashi et al., 2013; Papanas and Ziegler, 2015b; Zamani et al., 2015). Age may alter the markers of oxidative stress, which, in its turn, will increase the risk of developing various diseases, including DPN (Papanas and Ziegler, 2015a; Takahashi et al., 2013; Papanas and Ziegler, 2015b). Regarding gender, there is still controversy, but some authors found a significant difference in the profile of oxidative stress in female and male diabetic patients (Brunelli et al., 2014), while others did not observe this difference (Palekar and Ray, 2016). Despite some controversies, BMI is considered as one of the factors that may also alter the markers of oxidative stress (Zamani et al., 2015). The results of our study show a significant difference between the control group and the DM2 group. But there was no statistical difference between the control group and the DM2 + DPN group and the DM2 and DM2 + DPN groups showed no significant differences either. The difference between the control group and the DM2 group may help explain the relationship between obesity and DM2 development. There is lack of very consistent studies on the processes in which psychological stress participates in the development of DM2 and its complications, but some studies point to an association between perceived stress and DM2 (Hackett and Steptoe, 2017). In most of these studies, a high level of perceived stress was observed in DM2 patients when compared with non-patients. The study by Thangavel et al. (2015) concluded that perceived stress along with BMI can be considered as high risk factors for the development of hyperglycemia. Scientific evidence points the oxidative stress as one of the main factors for the cognitive decline (Baierle et al., 2015)

The reaction between NO and superoxide anion forms the peroxynitrite radical, which may damage macromolecules such as proteins and their functions (Adela et al., 2015). In addition, the literature indicates that high NO production is considered an underlying factor in the pathogenesis of DPN (Agrawal et al., 2014). Studies that evaluated the level of NO in DM2 patients present controversial results (Assmann et al., 2016). In some studies, the level of NO in diabetic groups increased, compared with non-diabetic subjects (Adela et al., 2015). However, other studies have observed a decreased NO between the diabetic and control groups (Mishra and Mishra, 2017). The present study was not able to establish a significant difference between the three groups in relation to NO.

MDA is involved in lipid peroxidation (Agrawal et al., 2014) and is one of the mostly used biomarkers in the evaluation of oxidative stress in patients with DM2 (Hackett and Steptoe, 2017; Ayala et al., 2014). In chronic diseases, such as DM2 and its complications, there is usually excessive formation of MDA (Rehman et al., 2017). However, the present study presented decreased MDA in the DM2 + PDN group in relation to the DM2 group and in the control group. No significant difference was observed between the DM2 group and the control group. This reduced MDA in patients with diabetes with neuropathy can be explained by some hypotheses that remain unclear. Among them, we can point out the daily use of alpha lipoic acid, vitamin A, C and E supplements (Schreiber et al., 2015).

The daily use of vitamins A, C and E supplements is one of the strategies to treat or control DM2 + DPN patients. So far, there is a dearth of information on the role of vitamin A and vitamin C supplements, but studies have already shown some benefits of vitamin E for diabetic patients with peripheral neuropathy (Oyenihi et al., 2015; Balbi et al., 2018), including decreased final products of lipid peroxidation, such as MDA (Oyenihi et al., 2015; Balbi et al., 2018). In addition to vitamin supplements, the study by Villegas-Rivera et al (2015) shows the effects of the drug simvastatin, which significantly reduces the MDA marker in patients with DM2 + DPN (Villegas-Rivera et al., 2015). Therefore, we can relate the results of our study with the vitamins A, C and E supplements ingested by some participants of the DM2 + DPN group, as well as the significantly higher use of simvastatin, oral hypoglycemic agents and insulin (Román-Pintos et al., 2016).

A significant FRAP decrease was observed in the DM2 and DM2 + DPN groups compared to the control group. This marker is widely used for measuring total antioxidant capacity in DM2 patients (Patel et al., 2015). Unlike other methods, FRAP provides us information not only on a specific antioxidant or enzyme, but on the overall profile of all antioxidants in the body (Patel et al., 2015). A study conducted by Dasegowda and collaborators in a diabetic population presented results to those of the present study (Dasegowda et al., 2015). Another study conducted by Padel and colleagues also obtained reduced FRAP values in patients with DM2 compared to controls (Patel et al., 2015). The FRAP decrease evidenced in the present study can corroborate the information about the changes in the antioxidant defense system in patients with DM2. However, no difference was observed for this marker between DM2 carriers with and without diabetic neuropathy. The non-significant difference found in the FRAP results for the DM2 and DM2 + DPN groups may be associated with the daily use of significantly higher rates of vitamin A, C and E supplements in the DM2 + DPN group (Oyenihi et al., 2015).

Glutathione is an enzyme present in almost all cells that plays a crucial role in the metabolism of several elements (Asmat et al., 2016; Ayala et al., 2014), mainly in the detoxification, by acting in the defense and protection of cells against free radicals (Stoian et al., 2015). In this study, no significant differences were found between patients with DM2 or DPN, compared with healthy individuals. Giriraja et al. found significantly lower levels of GST among individuals with DM2 with neuropathy, compared to the control group (Rehman et al., 2017). In addition, most studies evaluating GST in patients with DM2, with or without DPN, found a significantly reduced GD (Strom et al., 2017; Rehman et al., 2017). Decreased GST in patients with DPN seems to be associated with nerve damage and neural apoptosis (Aouacheri et al., 2015). The present study did not present a significant difference between the three groups for GST, which can be explained, in part, by the supplementation of vitamins C and E ingested by patients with DM2 + DPN, as well as the increased use of lipid-lowering and antidiabetic agents (Balbi et al., 2018; Román-Pintos et al., 2016).

This study has a few limitations. First, the results of this study may not be completely generalizable because the sample was restricted. Second, data collection has some shortcomings that limited some interesting correlations of results, such as data on non-pharmacological interventions of participants with MD2. Third, lack of resource to evaluate other markers of oxidative stress.

The results obtained lead to the conclusion that DM2 patients with or without diabetic poly-neuropathy presented reduction of total antioxidant capacity in relation to healthy subjects, which may indicate greater oxidative stress in the diabetic patients studied. The decrease in serum MDA levels in the group of diabetic patients with polyneuropathy may be related to the effect of the medications and to the supplementation of vitamins A and E, ingested by the patients.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Academic linkage

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