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Profile of angiotensin-converting enzyme activity on nitric oxide levels in type 2 diabetes mellitus patients

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ABSTRACT

Nitric oxide and renin angiotensin system are involved in the pathophysiology and progression of diabetes mellitus chronic complications. To evaluate angiotensin-converting enzyme activity on nitric oxide levels in patients with type 2 diabetes mellitus. Were recruited 20 patients and 20 health volunteers. Blood and urine samples were collected to measure: fasting blood glucose, glycated hemoglobin, plasmatic Na+, K+, urea, creatinine, total cholesterol, triglycerides, thiobarbituric acid reactive substances, nitric oxide levels, angiotensin-converting enzyme activity and microalbuminuria.The mean arterial pressure and body mass index were obtained from the medical records. The results were considered significant when $p<0.05$. Fasting blood glucose, glycated hemoglobin, body mass index, nitric oxide, urinary thiobarbituric acid reactive substances, angiotensin-converting enzyme activity and microalbuminuria were increased and total cholesterol was reduced in diabetic vs. controls; meanwhile MAP, Na+, triglycerides, urea and creatinine were similar between these two groups. Our study showed that although the angiotensin-converting enzyme was elevated, favored by the high oxidative stress level, demonstrating that there was protection on the cardio-renal axis. Our data suggest that maybe angiotensin-converting enzyme inhibitors acted on AT2, demonstrated by increased nitric oxide and stable blood pressure, revealing how dynamic the renin angiotensin system is and reacts to treatment.

Keywords: Diabetes mellitus, Microalbuminuria, Nitric oxide, Renin angiotensin system.

1 INTRODUCTION

In the World we had 424.9 million people with diabetes in 2017, and the prevalence will be 628.6 million in 2045. In Brazil, the number of patients with diabetes in 2017 was 12.5 million and might reach 20.3 million in 2045 (Cho et al. 2018). Diabetes mellitus (DM) is a disease characterized by metabolic and hemodynamic alterations, which could result from the chronic hyperglycemia. Type 2 Diabetes mellitus (T2DM), the most common type of diabetes mellitus, it occurs due to failure in insulin secretion or to insulin resistance, affecting some specific tissues, such as blood vessels, heart and kidney. In addition, T2DM is a metabolic disorder that can be triggered by several factors such as genetics, epigenetics, environment and lifestyle (diet and exercise). There is evidence that oxidative stress induced by reactive oxygen species (ROS) contributes to DM pathogenesis and the primary activators of these pathways appear to be hyperglycemia and hyperlipidemia (Tanhauserova et al. 2014).

The diabetic nephropathy (DN) is the main cause of morbidity and mortality related to DM, as it is the most common cause of end stage renal disease (ESRD). About 15% to 20% of T2DM patients present nephropathy after 20 years of disease progression. Besides, being the first manifestation of nephropathy, the microalbuminuria is also a marker of endothelial dysfunction and a risk factor to the increase of cardiovascular morbidity and mortality in DM patients (Tanhauserova et al. 2014, Satirapoj 2012).

Endothelial dysfunction is responsible by cardiovascular changes occurring in DM and it could be involved with the impairment of nitric oxide (NO) synthesis. The NO or endothelium derived relaxing factor (EDRF), is a very unstable gas produced from the substrate L-arginine (L-Arg) (Palmer, Ashton and Moncada 1988) under the action of the enzyme nitric oxide synthase (NOS), with consequent formation of a co-product, the L-citrulline (Iyengar, Stuehr and Marletta 1987). NO is involved not only with the DM but with another diseases, such as atherosclerosis and hypertension (Napoli and Ignarro 2001, Taddei et al. 2001).

The DM patients also present an alteration in angiotensin II (Ang II), such as elevated intraglomerular pressure, microalbuminuria and high risk of DN. ACE inhibitors or the antagonists of Ang II receptors minimize the renal alterations (intraglomerular hemodynamics and renal structural changes in the glomerulus and tubules) induced by DM, suggesting a potential role of renin-angiotensin system (RAS) in the progression of DN. It is know that any imbalance in the RAS could contribute to the pathophysiology of hypertension, DM and ischemic heart disease, having an important role in the cardiorenal axis (Boudoulas et al. 2017, Kohzuki et al. 1995, Leehey et al. 2000).

Due to the high prevalence, morbidity, mortality and high cost of the DM treatment, studies are necessary to identify the profile of patients and understand the mechanisms involved in its chronic complications. The aim of this study was to evaluate the ACE activity on NO levels in patients with T2DM.

2 METHODS

2.1 STUDY POPULATION

Diabetic group (T2DM) n=20 was recruited from out-patient clinic of Diabetes and Endocrinology Center of Universidade Federal de Sao Paulo (UNIFESP) and diagnosed according to the American Diabetes Association criteria (2021). The control group (CTL) n=20 was selected by a specialized team from volunteers at the Division of Health and Medicine (SESMT) of UNIFESP and these ones did not have diabetes nor hypertension, nor did they use any medications. All participants signed an informed consent form in accordance with the Research Ethics Committee 1406/11 (ANNEX 1).

The patients and volunteers from both groups had samples of blood and urine (the samples were collected and stored at -80 before being analyzed) collected for the routine screening after 8 to 12 hours overnight fasting. Glycosylated hemoglobin (HbA1c) was analyzed by HPLC - high performance liquid chromatography in Tosoh equipment (normal value: 3.5 to 5.6%). For the other analyzes, we used the Cobas c501 equipment from Roche.

For fasting plasma glucose (FBG) was used an enzymatic reference method with hexokinase, hexokinase catalyzes glucose phosphorylation into glucose-6-phosphate by ATP.

Sodium and potassium were analyzed by an ion selective electrode (Ion Selective Electrode - ISE), which uses the unique properties of some membranous materials to develop an electrical potential (electromotive force, EMF) for the measurement of ions in solution.

Urea kinetic test was analyzed with urease and glutamate dehydrogenase, urea was hydrolyzed by urease, giving rise to ammonia and carbonate. Creatinine kinetic colorimetric assay was based on the Jaffé method, in an alkaline solution, creatinine forms a reddish-yellow complex with picrate. The rate of dye formation is proportional to the concentration of creatinine in the sample.

Cholesterol was measured by enzymatic colorimetric method, cholesterol esters are cleaved through the action of cholesterol esterase and produce free cholesterol and fatty acids. Cholesterol oxidase catalyzes the oxidation of cholesterol to colest- 4 -en- 3 - one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide formed affects the oxidative coupling of phenol and 4-aminoantipyrine, forming a red quinone-imine dye.

Triglycerides was measured by enzymatic colorimetric assay and microalbuminuria was analyzed by immunoturbidimetric assay, anti-albumin antibodies react with the antigen in the sample and form antigen / antibody complexes. These, after agglutination, were determined by turbidimetry.

The laboratory exams were performed at the Central Laboratory of the Hospital Sao Paulo (UNIFESP), and the results were available in their medical records along with the identification of patients, such as sex, age, duration of diabetes, hypertension, BMI (kg/m^2) and, clinical data.

At the time of collection, 10 mL of blood and a urine sample were obtained for the determination of NO and ACE, which were performed in the Laboratory Nitric Oxide & Oxidative Stress, at the Nephrology Division, UNIFESP.

2.2 STUDY DESIGN

This was an observational, analytical and prospective study of patients diagnosed with T2DM and updated medical records. A total of 40 T2DM individuals were selected, but by convenience only 20 were included because of the age (mean 50 years old) of the volunteers in the control group (n = 20). Besides that, since getting a control group is more difficult, we consider that removing this age bias would help to become the information more reliable and either demonstrate the importance of this parameter in the pathogenesis of this disease.

The inclusion criteria for CTL group were FBG < 100mg/dL, blood pressure < 120/90 mmHg, age < 60 years old and no medication; for T2DM group were FBG > 100mg/dL, blood pressure > 120/90 mmHg, age < 60 years old, HbA1c < 8%, with or without the use of drugs such as oral hypoglycemic agents, statins and, ACE inhibitor.

Patients with highly uncontroled diabetes (HbA1c > 10%) and serious illnesses such as liver cirrhosis and cancer were excluded. Thus, follow a flowchart for further understanding (Figure 1).

2.3 NITRIC OXIDE (NO)

NO was determined by a chemiluminescence method of highsensitivity for detection of this molecule; serum and urine samples were deproteinated and read on Nitric Oxide Analyzer – NOA™ 280, Sievers Instruments, Inc. Boulder, CO, USA.

Nitrite (NO_2^-) and nitrate (NO_3^-), the stable metabolites of NO were reduced to NO by reaction with vanadium. NO, now in the form of gas, is captured on specific compartment of the apparatus, and reacts with ozone, resulting in light emission, represented by the reaction below:

$$NO + O_3 \rightarrow NO_2^- + O_2$$

$$NO_2^- \rightarrow NO_2 + h\nu$$

The light emission from the electron nitrogen dioxide in the region of the infrared spetrum is detected by a photomultiplier tube. This reading is processed through a "software" (NOAnalysis™ Software) and results are provided in µM of NO. The sensitivity of NOA for measurement of NO in the gas phase is about 1 picomol.

2.4 ESTIMATION OF OXIDATIVE ESTRESS

The lipid peroxidation characterized by malondialdehyde (MDA) levels was analyzed using TBARS method. The MDA concentration was calculated using a molar extinction coefficient ($1.56\times10^{5}M^{-1}cm^{-1}$), in plasma and urine samples (Bernheim, Bernheim and Wilbur 1948).

2.5 DETERMINATION OF ACE ACTIVITY

ACE activity was determined fluorometrically, using Z-Phe-Leu-His (Z-Phe-HL) as a substrate. The standard buffer used for the tests was 100 mM potassium phosphate, pH 8.3, containing 300 mM NaCl and 0.1 mM ZnSO4. Serum aliquots (10µL) were incubated at 37 °C with 200 µL of the substrate Z-Phe-H (1 mM), for a period of 15 minutes, and the enzymatic reactions were stopped with 1.5 mL of 0.28 NaOH N (mol / L). The released His-Leu dipeptide was coupled to the orthophthalaldialdehyde fluorescent marker (20 mg / mL, in methanol), and the fluorometric reaction was interrupted after 10 minutes by adding 200 µL HCl 3 N. Then, the fluorescence reading (excitation: 360 nm, emission: 500 nm) was performed by the spectrofluorometer. All samples were tested in duplicate, and the intrinsic fluorescence of the sample was corrected using blank (negative control). The standard curve was obtained using different concentrations of -HL (His-Leu, Sigma-Aldrich, USA) used as positive control, in the blank reaction mixture and it showed a linear relation between relative fluorescence and -HL concentration.

2.6 STATISTICAL ANALYSIS

Categorical variables were presented by absolute numbers and percentages and, were analyzed by Fisher's exact test.For the comparisons between the groups, the Student's t-test unpaired or the Mann-Whitney test were used, depending on the normality tested by the Kolmogorov-Smirnov test. Continuous variables with normal distribution were described as mean and standard deviation (SD) and those without normal distribution were described as median and interquartile range (IQR). The results were considered significant for $p<0.05$. Statistical analysis was performed using Statistical Package for Social Science (SPSS), version 19.0 (IBM, Chicago, IL, USA).

3 RESULTS

Most of the population studied was white and male (Table 1) without statistical significance, however, it was observed that there were more brown patients in the DM vs. CTL ($p<0.05$). As for the drugs of continuous use, only the T2DM group used the following drugs: oral hypoglycemic agents, ACE inhibitors, still a portion of them, associated insulin with these medications and only a single patient used an ACE inhibitor simultaneously with the angiotensin receptor blocker (ARB), as seen in Table 1. In addition, these patients used statins to control cholesterol (data not shown).

Our results showed that the mean age of the patients was 50.7 ± 7.6 years. We observed that MAP was slightly increased in T2DM group *vs.*CTL, although within normal levels.The T2DM patients presented higher BMI than the CTL group ($p<0.01$), revealing that T2DM group had overweight in the mean terms . Fasting glycemia and HbA1c were significantly increased ($p <0.0001$) in T2DM *vs*. CTL. The Na^+ and K^+ electrolytes had different behaviors in plasma, Na^+ had no difference between groups, whereas K^+ was decreased in T2DM group when compared to CTL, with a statistical difference of $p <0.005$ (Table2).

Renal parameters, urea and creatinine, did not differ between groups. However, the microalbuminuria was increased in T2DM group when compared to CTL, $p<0.05$ (Table 2).

Cholesterol was decreased in T2DM group in relation to the CTL group, with statistical significance $p<0.009$, but the triglycerides did not present any difference between them (Table 2).

Plasma and urinary NO were increased in T2DM group when compared to CTL, $p<0.005$; concerning oxidative stress markers urinary TBARS were significantly increased in T2DM; plasma TBARS showed no statistical difference. Serum ACE activity was elevated in T2DM in contrast to control patients considering the reference value for this analysis (13.3 to 63.9 U/L; $p<0.0006$). When we classified those who received ACE inhibitor, we observed that their serum ACE activity was not different from those who were not taking this medication (Table 1), assessed by blood pressure (main pharmacologic effect), revealing that patients who did not use controlled medication with a diet, to maintain blood pressure at normal levels, since these patients in specific out patients follow-up always have nutritional guidance, thus maintaining average blood pressure levels (MAP), as seen in Table 2.

Table 1. Ethnicity, gender and medicines of control and Type 2 diabetes individuals studied

	Groups				Total		
	CLT		T2DM				p-value
	N	%	N	%	N	%	
White	16	80.0	9	45.0	25	62.5	
Black	3	15.0	3	15.0	6	15.0	
Brown	1	5.0	8	40.0	9	22.5	0.0360
Total	20	100.0	20	100.0	40	100.0	
Gender	N	%	N	%	N	%	

Female	9	45.0	10	50.0	19	47.5	1.0000
Male	11	55.0	10	50.0	21	52.5	
Total	20	100.0	20	100.0	40	100.0	
T2DM							
Oral Hypoglycemic	-	-	N	%			
no	-	-	2	10.0			
yes	-	-	18	90.0			
Total	-	-	20	100.0			
ACE Inhibitor	-	-	N	%			
no	-	-	9	45.0			
yes	-	-	11	55.0			
Total	-	-	20	100.0			

CTL: Control; T2DM: Type 2 diabetes mellitus; ACE: Angiotensin converting enzyme.CTL: Control; T2DM: Type 2 diabetes mellitus. N = 40. p<0.05: Fisher's exact test.

Table 2. Demographic and clinical characteristics of control and Type 2 diabetes individuals studied

Variables	CTL	T2DM	P-value
Age years; mean ± SD	50.35 ± 7.09	50.75 ± 7.63	0.8645[2]
MAP mm HG; median (IQR)	100.83 (93.33 – 106.66)	100 (98.33 – 113.33)	0.3949[1]
BMI kg/m^2; median (IQR)	**24.07 (22.33 - 28)**	**29.1 (27.1 – 32.6)**	**0.0104[1]**
FPG mg/dL; mean ± SD	**85.2 ± 9.3**	**163.7 ± 53.5**	**0.0002[2]**
HbA1c %; mean ± SD	**5.4 ± 0.5**	**7.9 ± 1.1**	**0.0001[2]**
Na^+ mEq/L; median (IQR)	143 (139 - 143)	138.5 (138 - 140)	0.3229[1]
K mEq/L; median (IQR)	**4.9 (4.7 – 5.1)**	**4.1 (4.1 – 4.4)**	**0.0024[1]**
Urea mg/dL; median (IQR)	32 (26 - 38)	36 (23 - 43)	0.9563[1]
Creatinine mg/dL; median (IQR)	1 (0.8 - 1)	1 (0.9 - 1)	0.9159[1]
Total Cholesterol mg/dL; median (IQR)	220 (203 - 243)	165 (149 - 214)	0.0094[1]
Triglycerides mg/dL; median (IQR)	117.5 (73 - 149)	204 (77 - 287)	0.2962[1]
Serum NO uM; median (IQR)	**32 (27.5 - 44)**	**55 (36.75 – 72.75)**	**0.0032[1]**
Urinay NO uM; median (IQR)	**252.5 (195 - 325)**	**369.65 (263.75 - 787)**	**0.0304[1]**
Serum ACE nmol/mL.min; median (IQR)	**52.96 (43.67 – 63.66)**	**73.4 (63.75 – 91.6)**	**0.0006[1]**
Serum TBARS nmol/dL; median (IQR)	6.56 (5.66 – 7.64)	5.64 (5.34 – 6.19)	0.1038[1]
Urinary TBARS nmol/dL; median (IQR)	**2.58 (2.29 – 3.55)**	**3.43 (3.11 – 4.23)**	**0.0308[1]**
Microalbuminuria mg/L; median (IQR)	2.25 (1.1 – 3.75)	5.35 (1.9 – 39.05)	0.0582[1]

CTL: Control; T2DM: Type 2 diabetes mellitus; MAP: Mean arterial pressure; BMI: Body mass index; FPG: Fasting plasma glucose; HbA1c: Glycosylated hemoglobin; NO: Nitric Oxide; ACE: Angiotensin converting enzyme; TBARS: Thiobarbituric acid reactive substances; SD: Standard deviation; IQR: Interquartile range. N = 40. p< 0.05: [1]Mann-Whitney test or [2]Student's t test.

4 DISCUSSION

In the present study, we found high levels of serum ACE, NO (serum and urinary) and TBARS (urinary) in a group of long-duration T2DM patients with glycemic uncontrolled and microalbuminuria.

Diabetes induces microvascular complications due to chronic hyperglycemia, advanced glycation end products (AGE), increased production of ROS and abnormal stimulation of the rennin angiotensin- aldosterone system (RAAS)(Cade 2008). Chronic hyperglycemia (high HbA1c) is one of the cardiovascular risk factors (Pieme et al. 2017). In our study, the T2DM patients were with FBG and HbA1c out off control. A study showed that control of these glycemic parameters in Type 2 dibatetes improved renal and endothelial complications (Wei et al. 2016).

In the last decade, studies have been shown that some hypoglycemic agents may have also renoprotective effects independently of their glucose-lowering effects. The combination of sodium glucose cotransporter-2 (SGLT-2) inhibitors with a RAAS inhibitor might slow the progression of DN (Gnudi and Karalliedde 2016). Different patterns in the use of certain drugs, such as Ang II receptor antagonists or ACE inhibitors, were likely associated with treatment recommendations for patients with nephropathy (Brugnara et al. 2018).

T2DM is frequently associated to obesity and conditions related as dyslipidemia, hypertension and “chronic inflammation”. However the patern of dyslipidemia may be heterogenous and some patients with poorly controlled glycemic levels are found to have high BMI and cholesterol, with no difference in triglycerides (Dizdarevic-Bostandzic et al. 2018) .Our data also shown that there was no statistical difference in serum triglycerides, but there was a difference in BMI and serum total cholesterol between T2DM patients and controls. Nevertheless, we need to consider that some of our T2DM patients could be on treatment of your dyslipidemia and, therefore consuming statins.

In relation to electrolytes, like sodium and potassium evaluated. Our patients have decreased potassium, and this can occur because some patients use insulin, which leads to a decrease in plasma glucose and potassium. As for sodium, there was no difference, is similar very to others studies (Kang 2015). In this last study, the authors evaluated electrolytes in T2DM and they showed hypokalemia and hypoglycemia, situations could be one of the risk factors to precipitate cardiovascular complications in these patients.

In a study comparing newly diagnosed T2DM patients, 5-10 years of diabetes and patients with more than 10 years with disease, it is shown that markers of renal function such as microalbuminuria, urea and creatinine increased progressively over the years (Idowu et al. 2017). In our study, we observed only a significant increase in microalbuminuria levels, which it may indicate the presence of renal lesion. We hypothesized that other plasma markers analyzed did not change yet, due to the treatment, which may be acting as renal protectors.

Chronic hyperglycemia is one of the factor to modulate the blood pressure and could collaborate to increase this condition in T2DM patients (Vidotti et al. 2004). In our study, the MAP in T2DM was maintained at normal levels, however the NO was higher in this group, suggesting that this vasodilator may have contributed to the normalization of blood pressure.

TBARS are formed from a byproduct of lipid peroxidation (fat breakdown products) that can be detected by the assay using thiobarbituric acid as a reagent.The ROS have short half-life time and they are difficult to measure. Thus, we can only measure the various products of the damage produced by oxidative stress, such as TBARS (Vidotti et al. 2004).

Our study showed that plasma TBARS levels were decreased in the T2DM group as Piemeet *al* (Pieme et al. 2017) showed that patients with T2DM either with or without complications (nephropathy, retinopathy or hypertension). On the other hand, our patients presented increased levels of urinary TBARS as we think, based in previous studies of our laboratory (Rodrigues et al. 2014, Punaro et al. 2014), since the kidney it is one oth the targets organs in diabetes (Pieme et al. 2017). We hypothesized that there is a renal local higher production of ROS, thus increasing urinary TBARS in the T2DM group.

It is known that NO reduces the renal vascular resistance .Therefore, a decreased synthesis of NO increases the renal vascular resistance, and could explain the reducing of glomerular filtration rate (GFR) (Baylis, Mitruka and Deng 1992, Raij and Baylis 1995). Our data showed that serum and urinary NO concentrations of T2DM individuals were significantly higher than CTL groups. In T2DM group an alteration in the NO concentration was probably caused due to the function of chronic hyperglycemia. Maejima *et al* (Maejima et al. 2001), observed that in T2DM plasma, the expression of NOS is increased and consequently enhanced NO production. According to the authors, the increase of NOS expression in individuals submitted to hyperglycemia for long periods of time was associated to increased shear stress present in high blood pressure and it could contribute to increasing plasma NO in T2DM patients. However, another study showed no-change (Francesconi et al. 2001) or decreased (Honing et al. 1998) serum NO in T2DM patients. Some authors believe that exposition of endothelial cells to high glucose concentrations leads the production of ROS, which are capable to inhibit NO (Honing et al. 1998); others believe that increased glucose itself caused cellular injury and decreased the NO production (Giugliano, Ceriello and Paolisso 1996, Bohlen 2004).

Another study (Apakkan Aksun et al. 2003) showed that the urinary excretion of NO is increased in uncontrolled T2DM patients, being probably associated to an increase in NO synthesis (Honing et al. 1998). However, Hoshiyama *et al* (Hoshiyama et al. 2003) demonstrated that hyperglycemia lead to increase of eNOS expression but decreased the NO production in human glomerular endothelial cells. The diminished NO bioavailability seems to be associated with increased superoxide production and L-arginine deficiency. Maybe, these mechanisms are responsible for the imbalance between renal NO synthesis and NO urinary concentration (Hoshiyama et al. 2003). Therefore, there is not yet a consensus in the literature about the NO imbalance synthesis in diabetes.

Familial studies showed a strong relation between the onset of DN and the presence of determined genetics factors (Seaquist et al. 1989). The polymorphisms insertion/deletion of ACE gene

are one of the most studied and contribute with more than 40% for the individual variability of serum activity or tissue levels of ACE (Tiret et al. 1992). The ACE activity is important because it determines the level of intrarenal angiotensin which controls the intra glomerular pressure and the development of renal injuries caused by Ang II concentration increase (Hall et al. 1977, Soubrier et al. 1993). In the present study, 55% of patients were treated with ACE inhibitor and presented mild microalbuminuria, without hypertension or elevated serum urea or creatinine. A review study has shown that there are no differences in the use of ACE inhibitors and angiotensin II receptor blockers (ARB) in relation to renal protection in patients with albuminuria and other risk factors (Maione et al. 2011).

When we analyzed the serum ACE activity, it was increased in our T2DM patients compared to CTL individuals; this activity was basically the same in the patients who received ACE inhibitor compared to the ones who were not taking it; we thought in the possibility that those patients were not taking the medication properly. This scenario is common to occurn when the patients have to take it daily during your lifetime. We then hypothesized that the increased ACE activity in T2DM group, stimulated Ang II, which bound preferentially to AT2, instead to AT1. This could explain the increased serum and urinary NO, caused by AT2 stimuli explaining how the kidneys of these patients were protected agonist the deleterious effects of DM, once it is know that AT2 has an antiproliferative, vasodilatory and apoptosis effect. It also activates the production of NO. Likewise, the production of Ang 1-7 by ECA2 translates into beneficial effects such as antiproliferative and antihypertensive. Study reported that increased Na+ concentration contributed to disrupted glycocalyx with a reduced NO production and ativaction of Na+ channel in the vascular endothelium leading to endothelium stiffness, revealing that Na+ can also modulate the efficiency of the RAS blockers (Luft 2014), which it was observed in our study, since, both, serum and urinary NO levels were augmented and Na+ unchanged.

In DM, generally, the vasodilatory response by NO is lost. This may occured by the inactivation or unavailability of NO due to an increase of ROS. Mechanisms have been reported to explain reduced NO availability induced by elevated oxidative stress (Vanhoutte et al. 2017). Previous studies in our Laboratory showed that diabetic rats had reduced NO levels in urine, however when these animals received treatment with antioxidants or exercises presented increase in this parameter, revealing proctetive effect of this vasodilatador in diabetes (Punaro et al. 2014, Rodrigues et al. 2014). Another study suggested that reduced plasma NO contributed to greater endothelial dysfunction in cardiac patients (Chirinos and Zamani 2016). In the present study, the T2DM patients showed elevated urinary NO levels with stable blood pressure, which may have occurred due to the use of ACE inhibitors and better control of the lipid profile through medication.

The relevance of this study was the fact that we found high levels of NO, since this molecule is reduced in diabetics (Punaro et al. 2014, Rodrigues et al. 2014) and that this increase may have been

caused by the ACE inhibitor via AT2, which is already known to provide cardiovascular protection in this population.

The major limitation of our study was having performed the laboratory measurements in an isolated urine sample, instead of collecting a 24h urine, which could give us more accurate data, not only for renal function, but also for NO and TBARS measurements; in addition, we cannot carry out an ACE2 activity and nor about AT2 receptor due to lack of material, since the use of ACE inhibitors can contribute to the increase of ACE2, which has been related to cardiovascular protection, delaying heart failure; since it is known that ACE2 is cardio- and renoprotective, being counter-regulated by ACE (Anguiano et al. 2016). Other limitation was the use of RAS modulators in part of the diabetic group.

5 CONCLUSION

In summary, our study showed that although most patients used ACE inhibitor, presented augmented ACE activity, which was favored by the increase of oxidative stress; however, we believe this elevation in the ACE activity occurred via angiotensin AT2 receptor, causing increase in systemic and urinary NO levels and stable blood pressure, showing cardiovascular protection in this population.

In addition, the T2DM group showed some protection on the cardio-renal axis and a potential delay in the diabetic complications, revealing how dynamic the RAS system is and how it reacts to treatment.

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AUTHORS CONTRIBUTION

Margaret conducted the research, analyzed the data, and wrote the manuscript. Ana helped with data collection and laboratory techniques. Giovana helped with the interpretation of the results and revised the manuscript. Deyse, Marcos and Sergio assisted in revising the manuscript. Fernanda, Adelson and Dulce assisted in carrying out the biochemical techniques. Elisa supervised the study and reviewed the manuscript.

CONFLICT OF INTEREST

All the authors declared that they have no conflicts of interests.

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ANNEX 1

UNIFESP

Universidade Federal de São Paulo
Escola Paulista de Medicina

Comitê de Ética em Pesquisa
Hospital São Paulo

São Paulo, 25 de novembro de 2011
CEP Nº **1406/11**

Ilmo(a) Sr(a)
Pesquisador(a): MARGARET GORI MOURO
Disciplina/Departamento: Medicina Translacional
Pesquisadores associados: Elisa Mieko Suemitsu Higa (orientadora)

Parecer Consubstanciado do Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo

TÍTULO DO ESTUDO: Avaliação dos níveis do oxido nítrico e da enzima conversora de angiotensina em pacientes com diabetes mellitus tipo 2 :

CARACTERÍSTICA PRINCIPAL DO ESTUDO: Intervenção diagnóstica

RISCOS ADICIONAIS PARA O PACIENTE: Risco mínimo, envolvendo coleta de sangue

OBJETIVO DO ESTUDO: Avaliar os níveis de NO e enzima conversora de angiotensina em pacientes com DM tipo 2.

RESUMO: Os voluntários serão divididos em dois grupos, o grupo diabético (DM), que será recrutado no Ambulatório de Diabetes da Disciplina de Endocrinologia da Universidade Federal de São Paulo (UNIFESP), com um mínimo de 120 participantes e um grupo controle que será selecionado pela divisão do Serviço Especializado em Saúde e Medicina do Trabalho (SESMT), recomenda-se que os pacientes selecionados não tenham diabetes e nem hipertensão, com um mínimo de 50 participantes. Os indivíduos, tanto do Ambulatório de Endocrinologia quanto do SESMT, terão coletadas amostras de sangue e urina para a determinação de exames de rotina, tais como: glicemia de jejum, hemoglobina glicosilada, uréia, creatinina, microalbuminuria. Os exames serão feitos no Laboratório Central da UNIFESP, cujos resultados estarão à disposição em seus prontuários junto com a identificação dos pacientes, ou seja, sexo, idade, tempo de diabetes, hipertensão arterial, IMC e história clínica em geral. Na hora da coleta, 10ml. de sangue e uma amostra de urina isolada serão obtidas para a determinação do (NO) e da (ECA), que será realizada no Laboratório da Disciplina de Nefrologia da UNIFESP.

MATERIAL E MÉTODO: Estão descritos os procedimentos do estudo

TCLE: Adequado, contemplando a resolução 196/96

DETALHAMENTO FINANCEIRO: CAPES - R$ 113268,50

CRONOGRAMA DO ESTUDO: 24 meses

PRIMEIROS RELATÓRIOS PARCIAIS PREVISTOS PARA : 19/11/2012 e 14/11/2013

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo ANALISOU e APROVOU o projeto de pesquisa referenciado.
1. Comunicar toda e qualquer alteração do projeto e termo de consentimento livre e esclarecido. Nestas circunstâncias a inclusão de pacientes deve ser temporariamente interrompida até a resposta do Comitê, após análise das mudanças propostas.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/Hospital São Paulo