The role of oxidative stress and endothelial injury in diabetic neuropathy and neuropathic pain

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Abstract

OBJECTIVE: The roles of endothelin-1 (ET-1) and oxidative stress causing vascular injury in the pathogenesis of diabetic neuropathy are debatable. The present study was undertaken to clarify the possible effects of oxidative stress and ET-1 in diabetic patients with and without peripheric neuropathy.

METHODS: We studied plasma ET-1, nitric oxide (NO), catalase, glutathione (GSH) levels of fifty (22 females, 28 males) patients with Type 2 diabetes in order to evaluate endothelial dysfunction and oxidative stress. The neuropathy types (motor, sensorial and sensorimotor), comorbid diseases, antidiabetic treatments, smoking, diabetes duration were also considered. Short McGill Pain Questionnaire (SF-MPQ) was also performed for patients with neuropathy.

RESULTS: There were no significant differences between patients with (n=23) and without (n=27) neuropathy with regards to demographic features except diabetic disease duration. The statistical analysis was done considering this difference. Although NO and ET-1 levels were higher, and catalase and GSH levels were decreased in neuropathic patients, no statistical significancy was found. We also couldn't find any correlations between the parameters and SF-MPQ scores.

CONCLUSIONS: Although there were no relationships between neuropathy and the studied parameters, we found lower levels of catalase and GSH as intracelluler antioxidants and higher NO and ET-1 as markers of endothelial injury in patients with neuropathy. Our data suggest that there is a need of further studies with larger study groups in order to clear out the role of endothelial injury and oxidative status in the pathogenesis of diabetic neuropathy.

ORIGINAL ARTICLE

INTRODUCTION

The diabetic vascular complications have become an increasingly important issue because of being the major cause of morbidity and mortality in the diabetic population (Cooper & Johnston 2000). Abnormal hemodynamic changes in blood flow and contractility can be seen in many organs of diabetics, especially in microvessels of peripheral nerves (Koya & King 1998). One of the possible

mechanisms is protein kinase C activation with an increase of endothelin-1 (ET-1) expression. It has been shown that ET-A antagonist can prevent neurovascular dysfunctions in diabetic rats (Cameron & Cotter 1996). Additionally oxidative stress is a major contributor to the diabetic neuropathy. Reactive oxygen species (superoxide radical, hydroxyl radical and hydrogen peroxide) and reactive nitrogen species (peroxynitrite) contribute to pathophysiological changes in diabetic neuropathy (Vincent *et al.* 2004; Pacher *et al.* 2005). Increased oxidative stress within the cell leads to activation of the Poly (ADP-ribose) polymerase (PARP) pathway, which regulates the expression of genes involved in promoting inflammatory reactions and neuronal dysfunction. High levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes in diabetics (Edwards *et al.* 2008). In the present study endothelial dysfunction and oxidative stress parameters were investigated in order to clarify their possible role on pathogenesis of diabetic neuropathy.

METHODS

We included fifty type 2 diabetic patients (22 females, 28 males) who were admitted to our neurology clinic. ADA (American Diabetic Association) diagnostic criteria were used for diagnosis of diabetes mellitus. Those with a history of an endocrinologic, infectious or inflammatory disease, cancer, autoimmune disorder, hematological disorder, cardiac, renal or hepatic disease or use of immune-suppressant, antioxidant, anti-inflammatory drugs in the previous two months were excluded. Each patient had a complete physical and neurological examination, together with complete blood count, blood chemistry, sedimentation rate and electroneuromyography (EMG). Diabetic neuropathy (motor, sensorial, sensorimotor) diagnosis was made according to EMG findings. The neuropathy types (motor, sensorial and sensorimotor), antidiabetic treatments, smoking, diabetes duration and comorbid diseases causing atherosclerosis like hypertension, hyperlypidemia were also considered. The severity of the neuropathic pain was also assessed by Short McGill Pain Questionnaire (SF-MPQ). The blood samples were drawn from patients and centrifuged at 3000 rpm for 10 min to seperate the sera to be kept in –80 °C until analysis of ET-1, nitric oxide (NO), catalase, glutathione (GSH). The patients were grouped into two as with and without neuropathy and the groups were compared according to the studied parameters.

NO (nitrite + nitrate) was assayed by a modification of cadmium-reduction method as mentioned by Navarro-Gonzalves (Navarro-Gonzalves *et al.* 1998). The nitrite produced was determined by diazotization of sulphanilamide and coupling to naphthlethylene diamine. For the measurement of NO (nitrite + nitrate), 400 µl sample was denatured by adding 80 µL 30% $ZnSO_4$ solution, stirring and then centrifuging at 10,000 × g for 20 min at 4 °C. First, we activated Cd granules using CuSO4 solution in glycine-NaOH buffer. Then 100 µL of deproteinized samples and standarts were added. This reaction using pre-treatment of samples to reduce nitrate to nitrite, which can be accomplished by catalytic reactions using enzyme or Cd. The samples were analyzed spectrophotometrically using a microplate reader and quantified automatically against KNO_3 standard curve and the results were expressed as µM/L. In samples ET-1 levels were determined via commercial ELISA kit Human Endothelin, catalog number was BI-20052, lot number was 983C (Biomedica Medizinprodukte GmbH& Co KG, A-1210 Wien, Divischgasse 4, Germany). The results were determined via microplate reader ELX 800 and calculated automatically standart curve and were given fmol/mL. CAT activity measurement in erytrocyte lysate was measured by the method of Aebi (Aebi 1974). The reaction mixture was 50 mM phosphate buffer (pH 7.0), 10 mM H_20_2 and erytrocyte lysate. The reduction rate of H202 was followed at 240 nm for 30 sn at room temperature. Catalase activity was expressed in U/g Hb. Total glutathione content in the samples were measured according to the method of Beutler *et al.* (1963) using metaphosphoric acid to precipitate the protein and 5,5' dithiobis (2-nitrobenzoic acid) for colour development. The standart curve was used to calculate GSH content (Beutler *et al.* 1963).

Statistical analyses:

The Statistical Package for the Social Sciences (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) was used or statistical analysis and statistical significance was defined as $p<0.05$. Results were given as mean ± standard deviation. Comparisons of numeric values of all variables were performed using the Mann–Whitney U-test or Student's t-test. Chi square tests were used for analyzing categorical variables. We tested whether or not the variables which were detected in numeric scales in both groups had normal distribution by using the Kolmogorov–Smirnov test. In the patient group we used Spearman's test for correlation analysis of variables which had no normal distribution. The variables which had normal distribution were examined by Pearson correlation analysis. We also used covariance analysis for statistically standardization of studied two groups.

RESULTS

This study included 50 type II diabetic patients with (n=27) and without neuropathy (n=23). Their ages were between 43–74 (59.5±8.22) years. There were no significant differences between two groups with regard to age, gender, comorbid diseases, antidiabetic treatments and smoking ($p>0.05$) The demographic and clinic parameters of diabetics with (group 1, n=23) and without neuropathy (group 2, n=27) are sum-

marized in Table 1. DM disease duration in group 1 and 2 was 11.95±8.4 and 7.5±4.9 years respectively and this showed statistically significancy (p=0.025). The covariance analysis was done for the standardization according to disease duration. We found higher NO (41.19 ± 5,33 µM/L vs. 34.48 ± 4.9 µM/L; p>0.05) and ET-1 (2.35 ± 0.09 fmol/mL vs 2.23 ± 0.08 fmol/mL; p>0.05) levels in group 1. Additionally intracellular antioxidants catalase (298.26 ± 30.35 U/g Hb vs. 309.38 ± 27.88 U/g Hb; p>0.05) and GSH (376.91 ± 16.00 vs. 390.15 ± 14.70 mU/g Hb; p>0.05) serum levels were found lower in the same group. Although NO and ET-1 levels were higher, and catalase and GSH levels were decreased in patients with neuropathy, the results weren't statistically significant (Table 2). SF-MPQ was also performed for group 1 which had 12 sensorial, 4 motor, 7 sensorimotor neuropathic patients. The SF-MPQ scores were between 22 and 42 (30.2 ± 6.05) and did not differ according to the neuropathy types (p>0.05). SF-MPQ scores for sensorial, motor and sensorimotor neuropathy were detected as followed 30.7 ± 6.2, 27 ± 2.4 and 31.2 ± 7.1. There were no correlations between the studied biochemical parameters and SF-MPQ scores either.

Tab. 1. The demographic and clinical features of patients with (group 1) and without neuropathy (group 2).

		Group 1	Group 2	*p*-value
Age		61.3±6.4	57.96±9.12	>0.05
Gender (female)		10 (43.47%)	12 (44.44%)	>0.05
DM disease duration (year)		11.95±8.4	7.5±4.9	0.025
Hypertension		12 (52.17%)	13 (48.18%)	>0.05
Hyperlipidemia		12 (52.17%)	15 (55.55%)	>0.05
DM treatment	OAD	15 (68.18%)	15 (55.55%)	>0.05
	Insulin	6 (27.27%)	10 (37.03%)	>0.05
	OAD+Insulin	1 (4.5%)	2 (7.4%)	>0.05
Smoking		5 (21.73%)	7 (25.92%)	>0.05

OAD:oral antidiabetic drugs

Tab. 2. The studied endohelial dysfunction and oxidative stress markers for patients with (group 1) and without neuropathy (group 2).

	Group 1 (n=27)	Group 2 (n=23)	*p*-value
NO	41.19±5.33 µM/L	34.48±4.9 µM/L	>0.05
ET-1	2.35±0.09 fmol/mL	2.23±0.08 fmol/mL	>0.05
Catalase	298.26±30.35 U/g Hb	309.38±27.88 U/g Hb	>0.05
GSH	376.91±16.00 mU/g Hb	390.15±14.70 mU/g Hb	>0.05

DISCUSSION

Microvascular complications are the most important causes of increased morbidity and mortality in diabetic patients. Neuropathy is one of these complications affecting up to half of those with this disease. There are metabolic, humoral and haemodynamic factors all contribute to the pathogenesis (Cooper *et al.* 1998). Axons are susceptible to hyperglycemic damage both due to their direct access to nerve blood supply and their large population of mitochondria. The hyperglycemic environment with abnormal hemodynamic changes overloads the metabolic capacity of the mitochondria, producing oxidative stress (Brownlee 2001). Hyperglycemia leads to increased mitochondrial activity, raising ROS production. This causes mitochondrial damage followed by axonal degeneration and death.

Reactive oxygen species are produced under normal conditions through the electron transport chain and are normally removed by cellular detoxification agents such as catalase and glutathione (Leinninger *et al.* 2006). Therefore NO and intracelluler antioxidants such as catalase and GSH can play important roles in pathogenesis of diabetic neuropathy. In spite of the fact that there is no statistically significancy, NO levels were found higher, while catalase and GSH levels were lower in our diabetic neuropathy group. This may be due to our small study group. The other unstudied microvascular complications like nephropathy and retinopathy and different treatment choices with different oral antidiabetics may have an effect on oxidative stress in diabetics as well.

We could not also find statistically significant elevated ET-1 levels in diabetic neuropathy group. There is a controversy on the relation between ET-1 levels and diabetic microvascular complications. Although in some studies patients with diabetic microvascular complications had higher ET-1 levels, some studies reported normal levels. In these studies there is no correlation between ET-1 levels and diabetic microangiopathy and neuropathy similar with our results (Bertello *et al.* 1994; Guvener *et al.* 1997; Veglio *et al.* 1993; Vertello *et al.* 1993). There can be many reasons causing this controversy like using different immunological methods, heterogenous and small study groups.

In our study SF-MPQ which showed no difference between neuropathy types was also performed to assess neuropathic pain severity. There were no relationships between endothelial dysfunction, oxidative stress and pain severity as well. However in literature there is a growing evidence that endothelin axis plays a role in the development of tactile allodynia in diabetic neuropathy. In experimental animal models anti-allodynic effect of the selective blockade of ET_A receptor is an evidence of endogenous ET-1 mediating diabetic neuropathy (Berti-Mattera *et al.* 2006). In sciatic nerves extracted from chronic diabetic rats showed reduced expression of ET_B receptor. These rats also exhibited both mechanical hyperalgesia and tactile allodynia (Berti-Mattera *et al.* 2006). However we could not find any correlations between ET-1 and SF-MPQ scores.

In literature it has been also shown that NO combines with superoxide to form peroxynitrite, which rapidly causes protein nitration or nitrosylation, lipid peroxidation, DNA damage and cell death and has direct toxic effects on the nerve tissue leading to neuropathic pain (Kim *et al.*, 2003). In addition tocotrienol as an antioxidant can be useful in neuropathic pain through modulation of oxidative–nitrosative stress in the diabetic rats (Kuhad *et al.* 2009). However there were no correlations between the studied oxidative stress markers and SF-MPQ scores either.

To our knowledge the relationships between oxidative stress, endothelial dysfunction and neuropathic pain in diabetic patients have not been studied before in literature. Nevertheless we could not find any statistically significant result in our study. In spite of the fact that our data do not state any relationships between oxidative stress, endothelial dysfunction and neuropathy in diabetics, this may be due to our small study group, the effects of unstudied microvascular complications or different oral antidiabetic treatments.

Our findings suggest that the association of oxidative stress and endothelial dysfunction should be studied in larger groups to determine their effects on pathogenesis of diabetic neuropathy and neuropathic pain.

CONCLUSION

Although there were no statistically significancy, we found elevated plasma ET-1 and NO levels while catalase and GSH as intracelluler antioxidants were decreased in presence of diabetic neuropathy. Further studies may help us to obtain a broader view of oxidative stress and endothelial dysfunction in pathophysiology of diabetic neuropathy and neuropathic pain for developing novel treatment strategies.

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