Original article

Plasma total homocysteine is not associated with peripheral neuropathy in a groups Bangladeshi type 2 diabetic subjects

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Abstract

Aims: The present study was undertaken to explore the relationship of plasma homocysteine in the pathogenesis of neuropathy in diabetic patients. ***Subjects and Methods:*** Forty two type 2 diabetic patients [22 with neuropathy (DN group) and 20 without neuropathy (DNN group)], age range between 35-70 years had relatively controlled glycemia and duration of diabetes 7-15 years, were studied. Motor and sensory nerve conduction velocities and action potential amplitudes of peripheral nerves were determined by following standard protocol. HbA1c was estimated by modified HPLC (BIO-RAD Variant, USA). Serum C-peptide was measured by enzyme linked immunosorbentassay (ELISA), plasma total homocysteine by Fluorescent Polarization Immunoassay (FPIA). ***Results:*** Age, BMI and blood pressure of the study subjects were. Duration of diabetes between DN and DNN groups was comparable. DN group had significantly higher fasting glucose levels (9.8±3.8, mmol/l) compared to the DNN group (6.9±1.8, p=0.004). This trend was also reflected in the HbA1c level: 8.7± 2.1 vs 7.2±1.6 in DN group and DNN group respectively (p=0.009). The two diabetic groups had relatively higher absolute C-peptide level compared to the controls (p=ns). DN and DNN groups had significantly higher plasma homocysteine level compared to the Controls. But between the two diabetic groups no significant difference was observed. Ulnar and peroneal motor nerve conduction velocities and compound muscle action potentials in the diabetic neuropathy group significantly lower compared to diabetic counterpart and the controls. Ulnar and sural sensory nerve conduction velocities and action potentials were significantly lower in the diabetic neuropathy group compared to the diabetic counterpart and the controls. Plasma homocysteine did not show any correlation with nerve conduction velocities and action potential amplitudes. ***Conclusions:*** The data concluded that (i) Diabetic neuropathy may not be related to hyperhomocysteinemia in type 2 diabetic patients of Bangladeshi origin; (ii) Hyperglycemia, even at milder level, is related to neuronal dysfunction in these subjects; and (ii) Hyperinsulinemia don't seem to be prerequisite for neuropathy in these subjects.

Key words: type 2 diabetes, nerve conduction and neuropathy.

Introduction

Diabetes mellitus is an increasingly prevalent disorder all over the world [1]. Of the two major diabetic classes type 2 diabetes mellitus is linked with more aggressive complications. Most common chronic complications of T2DM include retinopathy, nephropathy, neuropathy, acute myocardial infarction and peripheral vascular disease. Diabetic peripheral neuropathy (DPN) found to occur in up to 50% of diabetic patients and causes sensory-motor and/or autonomic dysfunction [2]. Several pathogenic mechanisms have been suggested to contribute the pathogenesis and severity of diabetic peripheral neuropathy which include microangiopathy, oxidative stress, polyol flux, insulin deficiency, glycemic control and advanced glycation end products [3-5]. The course and severity of DPN found to be further affected by a wide range of comorbid conditions.

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Most importantly accumulation of homocystine (Hcy), an intermediary metabolite of amino acid methionine and methylmalonicacid (MMA) [6,7] in the blood. Homocysteinemia have been implicated in the pathogenesis of peripheral sensory-motor and autonomic neuropathy. Hyperhomocysteneinemia was shown to be associated with diabetic autonomic neuropathy but not peripheral neuropathy[8]. However, others have demonstrated that total plasma homocystein is linked to peripheral neuropathy [9]. On the other hand hyperhomocysteinemia was presumed to be a risk factor for diabetic autoneuropathy but not peripheral neuropathy[10]. These observations strongly suggest that role of blood homocysteine level in the pathogenesis of peripheral neuropathy still be clearly understood.

T2DM, like other countries of the world, constitutes the major bulk of diabetic patients in Bangladesh. Recent estimates suggest the prevalence of T2DM in this population is around 7% [11,12]. It is assumed that both micro- and macrovscular complications are more frequently in T2DM and ethno-geographic factors exerts influences in its development. Frequency of diabetes related complications was explored. Highest proportion of study subjects had 69.2% nephropathy, followed by 52.9% retinopathy, 50.9% hypertension, 28.5% peripheral neuropathy, 20.2% autonomic neuropathy, 26.5% ischemic heart disease and 5.3% cerebrovascular diseases [13]. However, data are lacking regarding the pathophyiological basis of diabetic complications. As a part of series of experiments to explore the pathological basis of diabetes and its complications of Bangladeshi population we have measured plasma total homocysteine levels and evaluated sensory motor nerve functional status in a group of T2DM patients with or without neuropathy to investigate their relationship.

Materials and Methods
Subjects
Forty two type 2 diabetes subjects [with neuropathy (DNP), n=22 and without neuropathy (DNNP), n=20) were purposively recruited from the Outpatient department of BIRDEM. Inclusion criteria for of diabetic patients were: age between 35-50 years, duration of disease 7-15 years, relatively controlled blood glucose and exclusion criteria: clinically manifested neuropathy, patients treated with insulin, serum creatinine level above the cut-off (>1.2 mg/dl), features of microvascular complications (evidenced by ophthalmoscopic examination), recent (<3 months) medication with vitamins (particularly B6 and B12, and folic acid) gastrointestinal disorders and pregnancy. Smokers were also excluded.

Methods
Informed consent was obtained form the all the respondents. Overnight (8-10 hours) fasting patients reported at Biomedical Research (BMRG) Laboratory, BIRDEM between 8-10 am. Blood samples were allowed to clot, centrifuged at 3000 rpm for 10 minutes. Separated serum was preserved at -40oC for future biochemical analyses. Nerve conduction velocities were estimated on the same day.

Laboratory methods
Glucose was measured by glucose-oxidase (GOD-PAP) method, HbA1c estimated by modified HPLC method using BIO-RAD Variant Analyzer. C-peptide was measure by chemiluminessence based ELISA using IMMULITE®2000. Plasma total homocysteine level was measured by Fluorescent Polarization Immunoassay (FPIA) using AxSYM Analyzer.

Nerve conduction study
Nerve conductions were performed in NCV-EMG Lab, BIRDEM. The study was performed following the protocol of San Antonio Conference (1988) of diabetic neuropathy 14. Unilateral studies of motor and sensory conduction of ulnar nerve including F wave latency were measured for upper limb and unilateral study of peroneal nerve for motor conduction including F wave and unilateral study sural nerve for sensory conduction were measured for lower limb. All measurements were performed with surface electrodes. Nerves were stimulated using 1 millisecond (ms) electrical pulses at a repetition rate of 1 per sec with intensity sufficient to elicit maximum amplitude of compound muscle action potential (CMAP) and sensory nerve action potential (SNAP). Conduction and action potentials above two standard deviations of the controls were considered as abnormal value in the study groups.

Statistical methods
The data were managed using Statistical Package for Social Science (SPSS) for Windows version 10. All the values were expressed as mean SD. Statistical comparison between groups was performed using unpaired Student's-'t' test. P value <0.05 was considered as level of significant. This study was approved

by 'Research and ethics committee of BIRDEM.'

Results

Age, height BMI and blood pressure of the study subjects were matched. Duration of diabetes of the two groups did not show statistical difference between two diabetes groups (Table 1). Glycemic and insulin secretory status

Fasting glucose (mean SD, mmol/l) was significantly higher in the DN group compared to the DNN group (9.7 3.8 vs 7.1 1.8, p=0.006). This level of glycemic control was reflected in their HbA1c levels. Diabetic neuropathy group had significantly higher HbA1c level compared to the nondiabetic counterpart (p=0.009) (Table).

Absolute C-peptide level in the DNN and DN groups did not show significant difference compared to the Controls and between themselves. But fasting C-peptide glucose ratio in both the DNN and DN groups was significantly higher (p=0.001). No significant difference was observed for the ratio between two diabetic groups (p=0.519) (Table I).

Homocystein status

Plasma total homocysteine (mean SD, mol/l) in the diabetic nonneuropathy and neuropathy group was 12.93 2.78 and 13.47 3.76 respectively which was significantly higher compared to the Controls (10.83 2.25) (p=0.012 and 0.009). Homocysteine level between two diabetic groups did not show statistical difference (p=0.600) (Table I).

Peripheral nerve functional status

Ulnar motor nerve conduction velocity (UMNCV) (mean±SD, m/s) in the DN group (50.2±5.1) was significantly lower compared to DNN group (59.9±6.4) and Controls (63.2±5.4) (p<0.001 for both). Ulanr compound muscle action potential (UCMAP) (mean±SD, mV) in the DN group (5.7±2.4) was significantly lower compared to the DNN (7.1±1.6; p=0.022) and Controls (8.4±2.8, p=0.002) (Table II).]

Peroneal motor nerve conduction velocity (PMNCV) (mean±SD, m/s) in the DN group (40.1±6.2) was significantly lower from controls (55.3±5.7, p<0.001) and the DNN group (51.8±5.4, p<0.001). P MNCV between DNN group and the controls no statistical difference was observed (p=0.054). Peroneal compound muscle action poten-

tial (PCMAP) (mean±SD, mV) in the two diabetic groups did not show statistical difference with the Controls and between themselves (Table 2).

Ulnar sensory nerve conduction velocity (USNCV) (mean±SD, m/s) in the DN group (47.95±4.6) was significantly lower compared to controls (54.7±4.8, p<0.001) and DNN group (52.0±3.2, p=0.002). USNCV in DNN groups was significantly lower compared to controls (p=0.023) (Table 2). Ulnar sensory nerve action potential (USNCV) (mean±SD, mV) in the DN groups (15.3±4.1) was significantly lower compared to the controls (22.0±6.9, p=0.001) and DNN group (20.3±5.1, p=0.003).

Sural sensory nerve conduction velocities (SSNCV) and sural sensory nerve action potential (SSNAP) could be measured in 6 (27.3%) individuals out of 22 in the DN group. SSNCV (mean±SD, m/s) in the DN group (31.9±11.3) was significantly lower compared to the controls (52.3±4.3, p=0.006) and DNN group (52.8±5.2, p=0.005). USNAP (mean±SD, μV) level was almost similar in the groups (Table 2).

Correlation analyses

Correlation analyses were performed for total homocysteine and HbA1c with nerve conduction parameters. tHcy and HbA1c either in diabetic neuropathy (r= -0.192, p=0.392) or in nonneuropathy group (r= -0.363, p=0.116) did not show significant association between itself. The trend was similar when two diabetic groups were polled together (r= -0.205, p=0.208).

In the control group tHcy showed positive significant correlation with circulating C-peptide level (6=0.691, p=0.001). No significant association was observed between tHcy and nerve functional status in DN and DNN groups (Table III).

In DN group HbA1c was found to be significantly negatively associated with USNCV (r= -0.565, p=0.006). In DNN group HbA1c was also significantly negatively associated with PMNCV (r= -0.450, p=0.047). When the DN and DNN groups were considered together HbA1c was significantly negatively associated with USNCV (r= -0.433, p=0.004) and PMNCV (r= -0.421, p=0.008).

Discussion

Racial differences presumed to influence nutritional and biochemical variations in a population.

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Heterogeneity in etiopathogenesis, basic defects in pancreatic B cell secretory defect and/ or insulin resistance of diabetes and its complications, including nephropathy has also been attributed to racial differences [15,16]. T2DM constitutes the major bulk of diabetics, like other countries of the world, in Bangladesh as well. Both secretory defects and insulin sensitivity were found to be predominantly involved in the pathogenesis of T2DM [17,18].

In the present study peripheral sensory-motor nerve conductions parameters were determined and explored its relationship with the insulin secretory status and homocysteine level in a group of diabetic patients with and without of neuropathy of almost similar mean duration of diabetes (6.7 3.8 and 8.0 4.9 years respectively, p=ns).

Glycemic status and duration of diabetes implicated as independent risk factors in the pathogenesis of peripheral neuropathy [19,20]. In the present study diabetic neuropathy group was found to have relatively uncontrolled glycemic status as evidenced by significantly higher fasting blood glucose and well as HbA1c levels. This has reconfirmed the importance of need for strict control of blood glucose to avert or at least delay diabetic complications.

Motor and sensory nerve conduction parameters in diabetic neuropathy groups were significantly lower. It was interesting to note that compound muscle action potential of ulnar nerve was significantly lower in DN group but not that of peroneal nerve. Correlation analysis demonstrated that HbA1c was significantly negatively correlated with USNCV in DN group and PMNCV in DNN group. This has possibly highlighted the fact that deterioration of USNCV and PMNCV might not be related to blood glucose related parameter but attributed to other yet unidentified factor(s).

C-peptide has long been thought to be the connecting peptide, as the name coined and devoid of any biological significance. However, recent volume of articles on the C-peptide suggests renewed interest on researchers of its possible biological functions 21-25. We have estimated serum C-peptide levels of the study subjects. The two diabetic groups had relatively higher level of circulating C-peptide level. However, calculation of C-peptide glucose ratio unmasked the compromised secretory status in the DN and DNN groups which had significantly lower fasting C-peptide glucose ratio compared to the controls. But between the DN and DNN groups no difference was observed which precludes reduced basal B cell secretion as judged by C-peptide level as a risk of developing peripheral neuropathy in diabetic patients.

Homocysteine is a sulfur-containing small compound not present in the food rather generated from methionine metabolism [26]. Elevated level of total homocysteine has been considered to be an independent risk factor for atherescerotic disease in both nondibetic and diabetic subjects [27-29]. Homocysteinemia has been attributed to neuropathy. However, controversies exist regarding the role of homocysteine in the pathogenesis of neuropathy and types of nerve involved. When some investigators suggested that hyperhomocystenemia was probably not related to the risk of distal somatic neuropathy other had stressed its link with poly neuropathy [30-35]. Recently it was demonstrated that total homocysteine level was associated with occurrence of neuropathy in a Chinese T2DM patients [9]. However, others have argued that hyperhomocysteineimia might be a risk for diabetic autonomic neuropathy [10].

In the present study significantly higher plasma homocysteine was observed in both DN and DNN groups compared to the controls. However, between the two groups no significant difference was observed which suggests that the higher level of plasma homocysteine in these two groups might be resulted due to metabolic derangements and not linked to development of neuropathy in DN group. This plausibility has been substantiated by absence of correlation between plasma homocysteine level and nerve conduction parameters. The major weakness of the study is failure to measure vitamin B12 and folic acid which are the main determinant of the homocysteine metabolism. The weakness was tried to circumvent during designing the study by excluding the patients taken any form of vitamin B and folic acid in the preceding three months. However, measurement of the two variable would have given additional strength of data and conclusively comment on the issue.

It may be concluded that (i) hyperhomocysteinmia may not be related to peripheral neuropathy type 2 diabetes subjects of Bangladeshi origin; (ii) hyperglycemia, even at low level, is related to neuronal dysfunction in type 2 diabetes mellitus; and (iii)

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hyperinsulinemia don't seem to be prerequisite for neuropathy- rather hyperglycemia resulting from secretory defects of B cells is likely to be the major pathophysiologic factor in the development of diabetic neuropathy

Table I: Clino-biochemical variables of the study subjects

Parameters	Controls (n=20)	DNN Group (n=20)	DN Group (n=22)
Age (yrs)	50.4±8.1	51.3±8.0	54.3±7.8
Height (m)	1.61±0.08	1.57±0.09	1.62±0.08
BMI (Kg/m^2)	23.2±3.0	24.5±2.6	23.4±3.2
SBP (mmHg)	128±9	123±12	129±14
DBP (mmHg)	79±8	79±8	80±7
DiabDur (yrs)	-	7.96±4.92	6.66±3.84
Fasting glucose (mmol/l)	4.9±0.6[a]	7.1±1.8[b]	9.7±3.8[c]
HbA$_{1c}$ (%)	5.6±0.3[a]	7.2±1.7[b]	8.6±2.1[c]
FC-peptide (nmol/l)	0.66±0.26	0.71±0.17	0.78±0.56
C-peptide:Gl(nmol/mmol)	0.141±0.047[a]	0.106±0.041[b]	0.083±0.044[b,c]
tHcy(µmol/l)	10.83±2.25[a]	12.93±2.78[b]	13.47±3.76c[b,c]

Results are expressed as mean±SD. Statistical difference between groups was calculated by Student's unpaired 't'-test. A p<0.05 was taken as level of significance. Different superscript in each column indicates significant difference between groups. DNN group, diabetes nonneuropathy group; DN group, diabetes neuropathy group; BMI, body mass index; SBP, systolic blood pressure; DBP diastolic blood pressure; DiabDur, diabetes duration; C-peptide:Gl, fasting C-peptide glucose ratio; tHcy, total homocysteine,

Table II: Nerve conduction functional status of major nerves of the study subjects

Parameters	Controls (n=20)	DNN group (n=20)	DN group (n=22)
U MNCV (m/s)	63.2±5.4[a]	59.9±6.4[a,b]	50.2±5.1[c]
U CMAP (mV)	8.4±2.8[a]	7.1±1.6[a,b]	5.7±2.4[c]
P MNCV (m/s)	55.3±5.7[a]	51.8±5.4[b]	40.1±6.2[c]
P CMAP (mV)	5.6±2.7	5.8±3.4	5.0±3.6
U SNCV (m/s)	54.7±4.7[a]	52.0±3.1[a,b]	48.0±4.7[c]
U SNAP (µV)	22.1±7.0[a]	20.3±5.1[a,b]	15.3±4.0[c]
S SNCV (m/s)*	52.3±4.3[a]	52.8±5.2[a,b]	31.9±11.3[c]
S SNAP (µV)*	14.8±8.0	12.5±4.8	9.4±3.6

Results are expressed as mean±SD. Statistical difference between groups was calculated by Student's unpaired 't'-test. A p<0.05 was taken as level of significance. Different superscript in each column indicates significant difference between groups. DNN group, diabetes nonneuropathy group; DN group, diabetes neuropathy group; m/s, meter per second; mV; milli volt; µV, micro volt.
*UMNCV, ulnar motor nerve conductive velocity; UCMAP, ulnar compound muscle action potential;; PMNCV, peroneal, motor nerve conductive velocity; PCMAP, peroneal compound muscle action potential; USVCV, ulnar sensory nerve conductive velocity; USNAP, ulnar seneory nerve action potential; SSNCV, sural sensory nerve conductive velocity; SSNAP, suralseneory nerve action potential. *nerve conduction could be revealed only in 5 cases out of 21 in the DN group.*

Table III: Correlation analysis for total homocysteine and HbA$_{1c}$ with C-peptide glucose ratio and nerve conduction parameter

	C-pep:Gl	UMNCV	UCMAP	USNCV	USNAP	PMNCV	PCMAP
tHcyvs				*r/p values*			
DN Group (n=22)	0.367/ 0.093	0.142/ 0.527	0.093/ 0.679	0.170/ 0.450	-0.043/ 0.848-	0.192/ 0.431	0.002/ 0.993
DNN Group (n=20)	-0.131/ 0.583	-0.283/ 0.227	-0.081/ 0.735	-0.185/ 0.435	0.297/ 0.203	0.216/ 0.361	0.065/ 0.784
HbA$_{1c}$vs				*r/p values*			
DN Group (n=22)	-0.128/ 0.570	-0.239/ 0.284	-0.144/ 0.523	-0.565/ 0.006	-0.037/ 0.872	0.031/ 0.901	-0.180/ 0.476
DNN Group (n=20)	-0.121/ 0.611	0.376/ 0.102	0.331/ 0.154	0.190/ 0.422	-0.022/ 0.926	-0.450/ 0.047	-0.179/ 0.451
C-peptide Glucose Ratio vs				*r/p values*			
DN Group (n=22)	-	-0.086/ 0.703	-0.010/ 0.963	-0.013/ 0.954	-0.286/ 0.198	-0.304/ 0.205	-0.460/ 0.055
DNN Group (n=20)	-	0.547/ 0.013	0.435/ 0.055	-0.025/ 0.916	-0.127/ 0.594	0.118/ 0.620	-0.026/ 0.912

C-pep:Gl, fasting C-peptide glucose ration; UMNCV, ulnar motor nerve conductive velocity; UCMAP, ulnar compound muscle action potential;; PMNCV, peroneal, motor nerve conductive velocity; PCMAP, peroneal compound muscle action potential; USVCV, ulnar sensory nerve conductive velocity; USNAP, ulnar seneory nerve action potential; Since nerve conduction could be revealed only in 6 cases out of 22 in the DN group sural sensory nerve conductive velocity; and suralseneory nerve action potential were not considered for correlation analyses.

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