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ORIGINAL ARTICLE

Somatic DNA Damages in Cardiovascular Autonomic Neuropathy

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Abstract Cardiovascular autonomic neuropathy (CAN) is one of the most clinically significant complications of diabetes mellitus. Even though many ethological factors have been attributed for the pathogenesis of this disease no attempts were made to correlate DNA damage as a causative factor. Hence the present study was undertaken to asses the extent of somatic DNA damages by cytokinesis-block micronuclei assay (CBMN). An attempt is also being made to correlate the habits and/or risk factors and socio-economic status with CAN. The CBMN frequency of 46 patients suffering from autonomic neuropathy was compared with that of 25 healthy age and sex matched controls. All the subjects were suffering from type 2 diabetes for at least 8 years and have varying degrees of coronary artery diseases. The mean CBMN frequency of the patients was statistically higher than that of the healthy control subjects ($P < 0.05$). The CBMN frequency was found to be significantly altered in CAN patients who where physical inactivity and smoking. A significant correlation could also be observed between CAN and smoking, diabetes mellitus, hypertension, dyslipidemia, abdominal obesity, and physical activity.

Keywords Autonomic neuropathy · Cardiovascular autonomic neuropathy (CAN) · DNA damage · Cytokinesis-block micronuclei (CBMN) assay · Coronary artery diseases (CAD)

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Introduction

Cardiovascular autonomic neuropathy (CAN) is one of the most overlooked of all serious complications of diabetes. This encompasses damage to the autonomic nerve fibers that innervate the heart and blood vessels, resulting in abnormalities in heart rate control and vascular dynamics [1]. Cardiovascular autonomic neuropathy (CAN) may carry an increased risk of morbidity and mortality [2]. CAN impair the ability to conduct activities of daily living, lowers quality of life, and increases the risk of death. It also accounts for a large portion of the cost of care [3]. Cardiovascular autonomic neuropathy occurs in about 17% of patients with type 1 diabetes and 22% of those with type 2. An additional 9% of type 1 patients and 12% of type 2 patients have borderline dysfunction [4].

The pathophysiology of the autonomic neuropathy depends on the etiology of each particular type like hyperglycemia, increased oxidative stress, autoimmune factors etc. These may range from genetic disorders with specific gene defects to metabolic disorders with accumulation of toxins and to autoimmune disorders with identifiable autoantibodies.

DNA damage, as evidenced by DNA adducts and oxidative DNA damage has been observed in vascular tissues. Higher levels of DNA adducts in vascular tissues than in

Table 1 Anthropometric and socio-economic data of the patients and control subjects

Age group (years)	Sex	Body mass index				Abdominal circumference (Cm)			
		Patients		Control		Patients		Control	
		LIG/MIG	HIG	LIG/MIG	HIG	LIG/MIG	HIG	LIG/MIG	HIG
31–40	Male	27.48 ± 2.98 (n = 2)	Nil	24.16 ± 3.13 (n = 5)	Nil	98 ± 5.29 (n = 2)	Nil	88.2 ± 5.45 (n = 5)	Nil
	Female	Nil	Nil	25.32 ± 3.05 (n = 3)	Nil	Nil	Nil	80.67 ± 6.55 (n = 3)	Nil
41–50	Male	25.24 ± 4.33 (n = 8)	29.64 ± 3.55 (n = 3)	23.42 ± 2.96 (n = 6)	24.07 ± 2.31 (n = 2)	95.62 ± 8.07 (n = 8)	100 ± 5.99 (n = 3)	85.5 ± 5.88 (n = 6)	90 ± 2.83 (n = 2)
	Female	22.55 ± 4.39 (n = 2)	Nil	22.04 ± 4.97 (n = 2)	Nil	83 ± 8.88 (n = 2)	Nil	81.5 ± 6.68 (n = 2)	Nil
51–60	Male	27.38 ± 4.25 (n = 8)	23.48 ± 4.29 (n = 2)	25.53 ± 3.04 (n = 3)	26.67 ± 3.07 (n = 2)	96.5 ± 6.79 (n = 8)	97 ± 8.81 (n = 2)	89.33 ± 6.03 (n = 3)	90 ± 6.03 (n = 2)
	Female	25.35 ± 4.42 (n = 6)	32.51 ± 0 (n = 1)	Nil	Nil	96.17 ± 7.99 (n = 6)	102 ± 0 (n = 1)	Nil	Nil
>60	Male	25.32 ± 4.21 (n = 6)	26.48 ± 4.78 (n = 5)	31.39 ± 3.58 (n = 2)	Nil	91.17 ± 7.99 (n = 6)	94.85 ± 10.5 (n = 5)	89 ± 5.98 (n = 2)	Nil
	Female	30.24 ± 4.38 (n = 3)	Nil	Nil	Nil	102 ± 9.23 (n = 3)	Nil	Nil	Nil

All values are mean ± SD. *n* Number of subjects; *LIG/MIG* low income/middle income group; *HIG* high income group

Since there is no clear demarcation of urban/rural area in Kerala, there is no relevance in classifying the subjects on this basis

other tissues have been reported [5]. An increased level of micronuclei has been shown to be marker of chromosome damage. The cytokinesis-block micronuclei assay is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells and include (a) micronuclei (MNi)—a biomarker of chromosome breakage and/or whole chromosome loss, (b) nucleoplasmic bridges (NPBs)—a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs)—a biomarker of elimination of amplified DNA and/or DNA repair complexes [6].

No serious attempts were made earlier to correlate somatic DNA damage with CAN. Hence present study was undertaken to quantify the extent of somatic DNA damages by cytokinesis block micronuclei (CBMN) assay in subjects suffering with Diabetic autonomic neuropathy. An attempt is also being made to asses the extend of DNA damage in patients with risk factors associated with cardio vascular diseases.

Materials and Methods

Forty-six subjects suffering from autonomic neuropathy formed the study groups. All the subjects were suffering from type 2 diabetes for at least 8 years and have varying degrees of coronary artery diseases. Twenty-five healthy age and sex matched control subjects were selected. Detailed anthropometric, socio-economic, demographic and other relevant clinical information were recorded using proforma. These subjects were referred from Hridaylaya Institute of Preventive Cardiology, Tiruvananthapuram and General Hospital, Tiruvananthapuram to Genetika, centre for Advanced Genetic Studies.

Three ml blood was collected aseptically in heparinized vacuutainers and used for lymphocyte separation and CBMN assay. Two ml of lymphoprep (pharmacia) added to a 10 ml centrifuged tube and overlaid 3 ml of blood sample to the tube and centrifuged at 1,000 rpm for 10 min. Drawn off the lymphocyte layer and transferred to a 10 ml tube. Suspended the cell pellet in RPMI 1640 medium and centrifuged for 10 min. Removed the supernatant and repeated the above step. Peripheral lymphocyte culture was performed as described by Moorhead et al. [7]. The CBMN test was done using the cytochalasin B technique described by Fenech [6]. The lymphocytes were cultured in sterile bottles using RPMI 1640 medium containing 15% fetal calf serum. Lymphocyte cultures were prepared for each subject. Each culture contained 2.0×10^6 cells in 5 ml RPMI 1640 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum and 1% phytohemagglutinin. At 44 h after initiation,

Table 2 Distribution of CBMN frequency with age

Subjects	Age range				All ages	t	P
	31–40	41–50	51–60	>60			
CBMN frequency of study subjects	13.5 ± 2.08 (n = 2)	14.82 ± 2.34 (n = 13)	15.43 ± 2.32 (n = 17)	15.77 ± 2.32 (n = 14)	15.21 ± 2.3 (n = 46)	9.328	0.001
CBMN frequency of controls	9.86 ± 1.2 (n = 6)	10.8 ± 1.23 (n = 10)	11.0 ± 1.26 (n = 7)	11.0 ± 1.16 (n = 2)	10.6 ± 1.22 (n = 25)		

Values are mean ± SD. Compared to the control group the CBMN frequency was higher in the patients at all age groups ($P < 0.05$)

cells were blocked in cytokinesis by adding cytochalasin B (Sigma, St. Louis, MO; final concentration, 4 μg/ml). The total incubation time for all cultures was 72 h. After incubation, the cells were fixed in 3:1 methanol/glacial acetic acid, dropped onto clean microscopic slides, air-dried, and stained with Giemsa stain. For each sample, 1,000 binucleated cells were scored at 100× magnification. The numbers of micronuclei per 1,000 binucleated cells were recorded.

Statistical Analysis

'*t*' Test was performed using SPSS for comparing the CBMN frequencies of the study subjects and the control subjects. Analysis of variance (ANOVA) was done to compare the CBMN values with and without various risk/life-style factors. Association between various risk/lifestyle factors and socio-economic variables and CAN is analyzed using chi-square test. The contribution of various risk/life-style factors for cardiovascular autonomic neuropathy was studied by Logistic regression analysis.

Results

Anthropometric and Socio-economic data of the patients and control subjects is given in Table 1. Somatic DNA damage in CAN patients at different age groups compared to that of normal control subjects are given in Table 2. The

Table 3 Distribution of CBMN frequencies with risk/life-style factors in patients and control subjects

Risk/life style factors	Control (n = 25)		Study subjects (n = 46)			
	Number	CBMN frequency	Number	CBMN frequency	*t*	*P*
Alcoholism						
Yes	3	12.66 ± 1.3	6	16.83 ± 2.27	1.899	0.064
No	22	10.31 ± 1.22	40	14.97 ± 2.29		
Smoking						
Yes	3	12.66 ± 1.31	27	15.74 ± 2.30	1.867	0.049
No	22	10.31 ± 1.22	19	14.47 ± 2.31		
Diabetes						
Yes	0	0	46	15.21 ± 2.9	–	–
No	25	10.6 ± 1.22	0	0		
Hypertension						
Yes	2	11.0 ± 1.16	36	16.1 ± 2.29	−1.386	0.173
No	23	10.56 ± 1.22	10	14.97 ± 2.29		
Dyslipidemia						
Yes	0	0	39	15.48 ± 2.29	1.935	0.048
No	25	10.6 ± 1.2	7	13.71 ± 2.39		
Abdominal obesity						
High	2	12.0 ± 1.2	22	15.54 ± 2.29	−0.999	0.323
Normal	23	10.47 ± 1.22	24	14.86 ± 2.23		
Physical activity						
Sedentary	16	10.5 ± 1.22	31	16.2 ± 2.30	−2.091	0.042
Non-sedentary	9	10.46 ± 1.21	15	14.47 ± 2.29		
Diet						
Vegetarian	0	0	1	13.0 ± 0	0.343	0.335
Non-vegetarian	25	10.6 ± 1.22	45	15.26 ± 2.3		
Socio-economic status						
High	4	9.5 ± 1.26	11	15.72 ± 2.34	2.399	0.184
Middle/low	21	10.8 ± 1.2	35	15.05 ± 2.32		
Area of residence						
Urban	17	10.5 ± 1.2	28	15.46 ± 2.3	−1.121	0.086
Rural	8	10.46 ± 1.22	18	14.83 ± 2.33		
Family h/o CAD						
Yes	0	0	4	16.00 ± 2.646	1.143	0.259
No	25	10.6 ± 1.22	42	15.03 ± 2.205		

Values are mean ± SD. Comparison of the values is made only in patients with and without risk factors

age of the study subjects ranged from 32 to 72 years with mean age of 55.2 years whereas the age of the control subjects ranged from 25 to 60 years with a mean age of 50.5 years. The mean CBMN frequency of the patients was statistically higher than that of the healthy control subjects ($P < 0.05$). 73.9% of study subjects were males. The mean CBMN frequency for female patients was 13.5 whereas in males it was 15.82. The difference was statistically significant ($P < 0.05$).

The CBMN frequency of patients and control subjects with various risk factors and life style pattern are given in Table 3. Of the major risk factors like diabetes, hypertension, dyslipidemia, abdominal obesity, smoking and alcoholism only smoking showed significant contribution for the increase in CBMN frequency in patients. Other life style factors such as physical activity, diet, socioeconomic status and area of residence were found to influence the CBMN frequency among patients. But significant alteration could be observed only in patients who are physically inactive. This study also revealed increased level of somatic damages in subjects having more than one risk factor.

Contribution of life style/risk factors for cardiovascular autonomic neuropathy is given in Table 4. Subjects above the age of 50 have an increased chance of developing diabetic autonomic neuropathy than subjects below the age of 50 (Odd's ratio = 3.674; Confidence interval = 1.321–10.222). Smoking is another risk factor for developing CAN (Odd's ratio = 10.035 (Confidence interval = 2.618–38.459). Subjects with family history of CAD have 100% occurence of CAN whereas subjects without family history of CAD have 59.7%. This observed difference is statistically significant ($P < 0.05$). Subjects with increased abdominal obesity have 10.542 times higher risk than subjects without abdominal obesity for developing CAN. Subjects with hypertension have 41.4 times more risk for developing CAN. Logistic regression analysis was performed for the following risk factors vis hypertension, smoking, abdominal obesity and age > 50 showed

Table 4 Contribution of life style/risk factors for cardiovascular autonomic neuropathy

Life style/risk factors	Case N (%)	Control N (%)	Total N (%)	χ^2	P
Occupation					
Sedentary	31 (55.4%)	25 (44.6%)	56 (100.0%)	10.336	$P < 0.001$
Non-sedentary	15 (100.0%)	0 (0%)	15 (100.0%)		
Hypertension					
Yes	36 (94.7%)	2 (5.3%)	38 (100.0%)	32.143	$P = .000$
No	10 (30.3%)	23 (69.7%)	33 (100.0%)		Odd's ratio = 41.4
Diabetes					
Yes	46 (100.0%)	0 (0%)	46 (100.0%)	71.000	$P = .000$
No	0 (0%)	25 (100.0%)	25 (100.0%)		
Dyslipidemia					
Yes	39 (100.0%)	0 (0%)	39 (100.0%)	47.028	$P = .000$
No	7 (21.9%)	25 (78.1%)	32 (100.0%)		
Abdominal obesity					
Yes	22 (91.7%)	2 (8.3%)	24 (100.0%)	11.481	$P = .001$
No	24 (51.1%)	23 (48.9%)	47 (100.0%)		Odd's ratio = 10.542
Socio-economic status					
Low	19 (100.0%)	0 (0%)	19 (100.0%)	18.335	$P = .000$
Medium	16 (43.2%)	21 (56.8%)	37 (100.0%)		
High	11 (73.3%)	4 (26.7%)	15 (100.0%)		
Family h/o CAD					
Yes	9 (100.0%)	0 (0%)	9 (100.0%)	5.601	$P = .018$
No	37 (59.7%)	25 (40.3%)	62 (100.0%)		
Smoking					
Yes	26 (89.7%)	3 (10.3%)	29 (100.0%)	13.880	$P = .000$
No	20 (46.3%)	22 (53.7%)	41 (100.0%)		Odd's ratio = 10.035
Age					
Above 50	31 (77.5%)	9 (22.5%)	40 (100.0%)	6.489	$P = .011$
Up to 50	15 (48.4%)	16 (51.6%)	31 (100.0%)		Odd's ratio = 3.674

Table 5 Logistic regression analysis showing correlation between risk factors and cardiovascular autonomic neuropathy

Risk factors	B	SE	Wald	df	Sig.	Exp (B)
Hypertension	3.786	1.047	13.074	1	.000	44.077
Smoking	2.752	.996	7.636	1	.006	15.667
Abdominal obesity	2.747	1.116	6.062	1	.014	15.590
Age > 50	.198	.908	.047	1	.828	1.219
Constant	−16.397	4.022	16.621	1	.000	.000

Variable(s) entered on step 1: hypertension, smoking, abdominal obesity, age > 50

significant correlation with cardiovascular autonomic neuropathy and the results were given in the Table 5.

Discussion

Diabetic neuropathies are the common in cause of incapacitation, morbidity, devastating complications and premature death [8]. These neuropathies comprise of clinical changes in peripheral nerves, autonomic nerves and central nervous system with diffuse or focal damage/regeneration. Previous population-based studies have reported prevalence rates for polyneuropathy ranging from 8 to 54% in type 1 diabetic patients and from 13 to 46% in type 2 diabetic patients [9]. In an earlier study by Fedele et al. [10] the male to female ratio was almost 1:1 whereas in the present study 73.9% were males. These differences may be due the high prevalence alcoholism, smoking among males compared to females in this area. The prevalence of CAN in the present study was found to be increasing with progression of age. Vinik et al. [11] reported a direct correlation between CAN with age and duration of diabetes mellitus. We observed an increased chance of developing cardio autonomic neuropathy in subjects above the age of 50.

Tesfaye et al. [12] reported that, the incidence of neuropathy is associated with potentially modifiable cardiovascular risk factors including a raised triglyceride level, body-mass index, smoking, and hypertension. Higher levels of triglyceride, blood pressure, insulin, weight, and lower HDL cholesterol are associated with both diabetes and macrovascular disease, the latter showing evidence of beginning prior to the onset of the former [13]. Forrest et al. [14] observed hypertension was a strong risk factor for neuropathy in young patients with type 1 diabetes. Dyslipidemia leads to high levels of oxidized LDLs that may injure dorsal root ganglia neurons and contribute to the development of diabetic neuropathy [15]. The present study is in well agreement with the previous reports, as we also found a strong correlation between CAN and various risk factors like diabetes, hypertension, dyslipidemia, abdominal obesity and smoking.

Autonomic dysfunction is very common in patients with diabetes. It has been suggested that autonomic dysfunction can be detected in at least 40% of the patients by formal autonomic nervous system testing [16]. The prevalence of neuropathy was 50% in those with diabetes for 25–29 years and 72% in those with diabetes for >30 years [17]. The development of cardiovascular autonomic dysfunction was independently associated with microvascular complications and glycemic control status in patients with type 2 diabetes [18]. In the present study all the patients with CAN were diabetic for a minimum period of 8 years prior to the study. This prolonged illness may be the reason for the onset of CAN in these patients.

DNA damage is a form of cell stress and injury that has been implicated in the pathogenesis of many neurological disorders [19]. DNA damage is caused by multiple endogenous and exogenous factors such as oxidative stress, age, smoking, hypertension, hyperlipidemia and diabetes mellitus [20]. Andreassi [21] reported that diabetes is a major determinant of somatic DNA instability in patients with CAD. DNA damage might represent an additional pathogenetic dimension and a possible therapeutic target in the still challenging management of coronary artery disease concerning diabetics. Diabetes accelerates the accumulation of the somatic mutation in mitochondrial DNA, which could possibly be a new marker for estimating the duration of diabetes [22]. Somatic damages are detected via chromatin loss from the nucleus leading to micronuclei in the cytoplasm of the cell. MN is scored by Cytokinesis Block Micronuclei Assay developed by Fenech [6]. The purpose of present study was to evaluate the extent of somatic damage by using Cytokinesis Block Micronuclei Assay in patients with cardio autonomic diabetic neuropathy. The CBMN frequency in patients was found to be altered with life style and risk factors like smoking, alcoholism, diabetes mellitus, hypertension, dyslipidemia, abdominal obesity, physical activity, diet, socioeconomic status and area of residence.

A strong correlation between CBMN frequencies and cardio autonomic neuropathy was observed in this study. The higher the number of risk factors higher will be the chance of developing CAN. This may be attributed to the increased DNA damage with risk factors mainly smoking which may cause an increase in blood pressure. Subjects with risk factors like family history of CAD, increased abdominal obesity, hypertension and age above 50 years, have profound influence in developing cardiac autonomic neuropathy. Life style modification with diet and exercise, maintaining blood pressure at normal level, lowering serum lipids and blood sugar and avoiding tobacco and alcohol will reduce the risk of CAD and autonomic neuropathy.

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