PLASMA LEVELS OF ASYMMETRIC DI METHYL ARGININE IN DIABETIC PATIENTS WITH NEUROPATHIC FOOT ULCERATION

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ABSTRACT

Background. Increased cardiovascular mortality had been reported in diabetic subjects with neuropathic foot ulceration (NFU). Endothelial dysfunction (ED) was found to be more remarkable in diabetic subjects with NFU than those without peripheral nerve dysfunction (PND) which could explain the excess cardiovascular morbidity and mortality. By inhibiting nitric oxide (NO) formation, Asymmetric dimethylarginine (ADMA) was found to cause ED.

Aim of the work. To evaluate plasma ADMA level as a marker of ED in diabetic subjects with NFU, and to study the possible relations of plasma ADMA level with ED and atherosclerosis in diabetic subjects.

Subjects and methods. The study included forty diabetic subjects with NFU (Gr.1), twenty diabetic subjects with PND (Gr.2) and twenty without PND (Gr.3), and ten age and sex matched healthy subjects (Gr.4). Subjects with renal impairment, peripheral arterial disease, ischemic heart disease, smoking or using lipid lowering drugs were excluded. ED was evaluated by measuring the flow mediated dilatation (FMD) of brachial artery using high-resolution ultrasound (Toshiba Power vision 6000 with a 7.5 MHz transducer). Carotid intima media thickness (CIMT) was also used to evaluate atherosclerosis. Fasting blood samples were obtained from all subjects to measure plasma ADMA levels by ELISA kit supplied by EAGLE BIOSCIENCES, INC (Germany).

Results. Gr.1 and Gr.2 were having a significantly lower FMD and higher CIMT in comparison to Gr.3 and Gr.4 ***(both P< 0.001)***. However, there was no significant difference between Gr.1 and other study groups as regard plasma ADMA levels ***(P= 0.126).*** FMD was inversely and strongly related to CIMT ***(r= -0.520, P< 0.001)***. Plasma ADMA was found to be positively correlated with CIMT ***(r= 0.330, P= 0.003)*** with no significant correlation with FMD ***(r = -0.176, P =0.118)***.

Conclusion. Plasma ADMA levels are not increased in diabetic subjects with NFU, and are not associated with the remarkable ED in these patients. In diabetic subjects, plasma ADMA levels are positively correlated with atherosclerosis.

INTRODUCTION

There is strong epidemiological evidence of excess mortality among patients with diabetic foot syndrome ***(1).*** A greater than two-fold increase in mortality rate was reported in diabetic foot ulcer subjects compared with diabetic subjects with no ulcers ***(2, 3).*** Coronary artery disease (CAD) was reported as the major cause of death among diabetic foot ulcer subjects, accounting for 50.4% of causes ***(2).*** The excess risk of cardiovascular morbidity may seem logic for patients with ischemic ulcers; however, the risk was also high in patients with neuropathic foot ulceration (NFU) without clear explanation ***(4).*** A more remarkable ED was found in diabetic subjects with NFU in comparison to a matched group without peripheral nerve dysfunction (PND) ***(5).*** ADMA is an amino acid produced by degradation of arginine-methylated proteins ***(6)*** which acts as an endogenous inhibitor of NO synthase, and causes ED ***(7, 8).*** Elevated plasma ADMA level was identified as an independent risk factor for the progression of atherosclerosis, and cardiovascular death ***(9, 10).***

AIM OF THE WORK

To evaluate plasma ADMA level as a marker of ED in diabetic subjects with NFU, and to study the possible relations of plasma ADMA level with ED and atherosclerosis in diabetic subjects.

SUBJECTS AND METHODS

The study included ninety subjects. Eighty diabetic subjects (cases) were recruited from Mansoura Specialized Medical Hospital (diabetic foot clinic, diabetes outpatient clinics, and inpatient wards), from September 2013 to May 2014, and were subdivided into 3 groups; Forty patients with NFU (Gr.1), twenty patients with PND but without foot ulceration (Gr.2), and twenty patients without PND or foot ulcer (Gr.3). Ten participants were age and sex matched healthy subjects (controls) (Gr.4). Diabetic state was confirmed or excluded according to the American Diabetes Association criteria ***(11).*** Subjects with renal impairment, hepatic impairment, peripheral arterial disease, ischemic heart disease, smoking or using lipid lowering drugs were excluded. A written consent was obtained from all participants and the study was approved by the local ethical committee.

All participants were subjected to thorough medical history and clinical general and foot examination. Body weight, height, waist circumferences, and blood pressure were measured in all subjects with standard protocols. Body mass index (BMI) was calculated. Vibration

perception threshold (VPT) was used as a quantitative method for detection of neuropathy and (10-g) nylon monofilament test was used to diagnose loss of protective sensation (LOPS). Evaluation of pedal arteries was done by Doppler on pedal arteries, calculation of ankle brachial index (ABI), and toe brachial index (TBI). ED was evaluated by measuring flow mediated dilatation (FMD) of brachial artery using high-resolution ultrasound (Toshiba Power vision 6000 with a 7.5 MHz transducer). Carotid intima media thickness (CIMT) was also used to evaluate atherosclerosis. All ultrasound scans were performed by a single experienced radiologist blinded to the subjects' data. Ulcer surface area was calculated by multiplying the length by the width of ulcer.

Laboratory assay

Sample collection:

Six ml of venous blood sample was withdrawn from each subject after over night fasting (12h) via proper venipuncture technique under complete aseptic condition.

- One ml was delivered into EDTA tube for CBC analysis using automated counter, Sysmex KX-21 (USA), and HbA1c testing using fast ion exchange resin supplied by Human (Germany).
- Two ml were delivered into EDTA tube then centrifuged and the clear plasma was kept frozen at – 20 °c till analysis of ADMA.
- The rest of the sample was left to clot then centrifuged and the clear none hemolyzed sera were used for traditional laboratory investigation (fasting blood glucose (FBG), total cholesterol, triglycerides (TG), liver enzymes, and creatinine) using commercially available kits supplied by Spinreact (Spain).

Plasma ADMA level was assayed by ELISA kits ***(12)*** supplied by EAGLE BIOSCIENCES, INC (Germany).

Statistical analysis:

Data of continuous variables were expressed as mean ± standard deviation (SD) or median (range) when distribution was normal or skewed, respectively. Statistical comparison between groups was evaluated by ANOVA and Kruskal Wallis test for parametric and nonparametric variables, respectively. Spearman correlation coefficient ® test was used correlating different parameters. Statistical analysis was done by SPSS 20. *P* value was considered statistically significant if less than 0.05.

RESULTS

I. Demographic and clinical data in studied groups (Table 1)

There was no significant difference between study groups as regard age, sex, body mass index, waist circumference, hypertension treatment, ABI and TBI. Systolic and diastolic blood pressures were significantly higher ***(All P < 0.001)*** in Gr1, Gr.2, and Gr.3 in comparison to control group (Gr.4). Duration of diabetes was significantly higher in the Gr.1 and Gr.2 than in Gr.3 ***(P < 0.001)***.

Most of patient in Gr.1 and Gr.2 were on insulin, while in Gr.3 most patients were on oral hypoglycemic drugs with a statistical significant difference ***(P < 0.001)***. All patients in Gr.1 and Gr.2 were having LOPS by monofilament examination with a statistically significant difference from others in Gr.3 and Gr.4 who were having intact sensation ***(P< 0.001)***. VPT was statistically significantly higher among Gr.1 and Gr.2 in comparison to Gr.3 and Gr.4 ***(P< 0.001)***. Range of ulcers surface area in Gr.1 patients was (0.8-10.7) cm^2 with a duration ranged from (2-30) months.

II. Laboratory and radiological data of study groups (Table 2)

There was no significant difference between study groups as regard serum creatinine, TG, Doppler on pedal arteries or plasma ADMA levels. FBG and HbA1c were not significantly different between the patient groups (Gr.1, Gr.2, and Gr.3). Total cholesterol was significantly higher among patient groups than Gr.4 ***(P< 0.001).*** Gr.1 and Gr.2 were having a significantly lower FMD and higher CIMT in comparison to Gr.3 and Gr.4 ***(All P< 0.001).***

III. Correlation of plasma ADMA levels in diabetic subjects (Gr.1, Gr.2, and Gr. 3) with FMD and CIMT (Table 3).

Plasma ADMA levels were found to be positively correlated with CIMT ***(r= 0.330, P= 0.003)*** with no significant correlation with FMD ***(r= -0.176, P= 0.118).*** An inverse and strong correlation was found between FMD and CIMT ***(r=-0.520, P < 0.001).***

Table (1): Demographic and clinical data of study groups

Data		Groups				P
		Gr.1	Gr.2	Gr.3	Gr.4	
Age (years)	Mean	53.10	54.75	48.90	49.90	0.07 A
	±SD	8.55	6.93	6.32	7.95	
Gender	Male	25 (62.5%)	12 (60 %)	7 (35 %)	6 (60 %)	0.2 C
	Female	15 (37.5 %)	8 (40 %)	13 (65 %)	4 (40 %)	
BMI (kg/m2)	Mean	33.56	33.27	36.38	31.99	0.1 A
	±SD	5.08	5.91	5.39	2.95	
Waist (centimeter)	Mean	107.18	105.70	109.55	100.60	0.46 A
	±SD	15.43	18.73	7.99	13.31	
Systolic BP (mmHg)	Median	160	160	140 [ab]	130 [abc]	<0.001B
	Range	100-215	130-190	130-160	110-130	
Diastolic BP (mmHg)	Median	90	90	90	80 [abc]	<0.001B
	Range	70-110	70-100	80-100	70-8	
HTN	Yes	30 (75 %)	18(90 %)	14(70 %)	0 (0 %) [abc]	<0.001C
	No	10 (25 %)	2 (10 %)	6 (30 %)	10 (100 %)	
HTN treatment	ACEI	13 (32.5%)	11 (55%)	8 (40%)		0.7 C
	Others	8 (20%)	3 (15%)	2 (10%)		
	No drugs	9 (22.5%)	4 (20%)	4 (20%)		
DM duration (years)	Median	17.0	19.5	3.0ab		<0.001B
	Range	2.0-40.0	1.0-35.0	1.0-11.0		
DM treatment	Oral	1 (2.5 %)	3 (15 %)	15(75%) [ab]		<0.001C
	Insulin	39(97.5 %)	17(85 %)	5(25 %)		
ABI	Mean	1.24	1.17	1.16	1.14	0.06 A
	±SD	.19	.12	.06	.08	
TBI	Mean	1.30	1.26	1.29	1.13	0.1 A
	±SD	.21	.18	.19	.09	
Monofilament Intact	No.	0	0	20[ab]	10[ab]	<0.001C
	%	0.0%	0.0%	100.0%	100.0%	
Monofilament LOPS	No.	40	20	0	0	
	%	100.0%	100.0%	0.0%	0.0%	
VPT (Volts)	Median	40.00	40.00	15.00 [ab]	13.50 [ab]	<0.001B
	Range	32.00-50.00	31.00-50.00	10.00-20.00	10.00-20.00	

Data are presented as means ± SD for parametric data, and median (range) for non parametric data. P: probability. A: ANOVA test, followed by post-hoc tukey for multiple comparisons if statistically significant. B: Kruskal-Wallis test, followed by Mann-Whitney for multiple comparisons if statistically significant. C: Qui-square test (X2). a : significant relative to group 1. b: significant relative to group 2. c: significant relative to group 3. BMI, body mass index. HTN, hypertension. ABI, ankle-brachial index. TBI, toe-brachial index. LOPS, loss of protective sensation. VPT, vibration perception threshold.

Table (2): Laboratory and radiological data of study groups

Data			Groups				P
			gp1	gp2	gp3	gp4	
FBG (mg/dl)		Median	181	151	147.5	81 [abc]	<0.001B
		Range	104-300	92-366	82-268	75-89	
HbA1c (%)		Mean	9.58	9.43	9.41	4.74[abc]	<0.001A
		±SD	1.69	2.10	1.93	.47	
Serum creatinine (mg/dl)		Mean	.89	.88	.76	.83	0.12A
		±SD	.25	.18	.06	.08	
Total cholesterol (mg/dl)		Median	186	189.5	185.5	140 [abc]	<0.001B
		Range	142-262	80-257	80-221	80-155	
Triglycerides (mg/dl)		Median	139	183	117	115	0.78B
		Range	80-380	78-360	80-346	80-155	
ADMA (ng/L)		Median	704.5	687	678	642	0.126B
		Range	508-3611	286-2863	506-874	383-797	
Doppler on Pedal arteries	Monophasic	No	0	0	0	0	0.30 C
		%	0.0%	0.0%	0.0%	0.0%	
	Biphasic	No	14	7	4	1	
		%	35.0%	35.0%	20.0%	10.0%	
	Triphasic	No	26	13	16	9	
		%	65.5%	65.0%	80.0%	90.0%	
FMD (%)	Median		-5.09-	4.67	15.74 [ab]	20.10 [ab]	<0.001 B
	Range		-22.5-22.92	-15-23.91	8.33-36.59	10-46.15	
CIMT (centimeter)	Median		0.9	0.9	0.6 [ab]	0.7 [ab]	<0.001 B
	Range		0.6-1.5	0.6-1.3	0.5-.8	0.5-.9	

Data are presented as means ± SD for parametric data, and median (range) for non parametric data. P: probability. A: ANOVA test, followed by post-hoc tukey for multiple comparisons if statistically significant. B: Kruskal-Wallis test, followed by Mann-Whitney for multiple comparisons if statistically significant. C: Qui-square test (X2). a : significant relative to group 1. b: significant relative to group 2. c: significant relative to group 3. FBG, fasting blood glucose. HbA1c, glycosylated hemoglobin. ADMA, Asymmetric Dimethyl Arginine. FMD, flow mediated dilatation. CIMT, carotid intima media thickness.

DISCUSSION

CAD was reported as the major cause of death among diabetic foot ulcer subjects, accounting for 50.4% of causes ***(2, 4).*** Elevated plasma ADMA level was identified as an independent risk factor for the progression of atherosclerosis, CAD and cardiovascular death ***(10, 13).*** The excess risk of cardiovascular morbidity may seem logical for subjects with ischemic ulcers; however increased CVD had been reported in diabetic subjects with neuropathic foot ulceration (NFU) without clear explanation ***(4).***

Data of this current study demonstrate a more remarkable ED in diabetic subjects with (PND) either with NFU (Gr.1) or without foot ulcers in comparison to diabetic subjects without PND (Gr.3) and control group (Gr.4) ***(P< 0.001).*** This can be explained by diabetes induced ED. ED was reported as one of the pathogenetic mechanisms of PND ***(14, 15),*** by causing endoneurial hypoxia and impaired blood flow, that play a principal role in causing diabetic neuropathy ***(16, 17).*** This explanation is supported by the endothelial NO reduced expression and gene polymorphism found in diabetic neuropathic subjects regardless of the presence or absences of macrovascular disease ***(18, 19).*** Moreover, serum vascular endothelial growth factor (VEGF), one of

ED markers, was increased in patients with diabetic peripheral neuropathy, particularly in the neurologically symptomatic stage ***(20).***

Similar results were obtained by another study that demonstrated more ED in diabetic subjects with NFU in comparison to a matched group without PND ***(5).***

CIMT was significantly higher in Gr.1 and Gr.2 in comparison to Gr.3 and Gr.4 ***(P< 0.001)***. This denotes more risk of atherosclerosis in diabetic subjects with NFU and DPN. This finding seems logic as these patients had a more remarkable ED. ED was considered as a transducer of atherosclerosis risk factors, and thought to play a principal role in the initiation and the progression of atherosclerosis ***(21)*** both for the loss of endothelial protective capability and for the induction of proatherothrombotic mechanisms ***(22).*** Two studies demonstrated this relation; one reported that FMD was correlated with the progression of preclinical carotid artery disease over a 6-year follow-up, showing a closer relationship with progression of atherosclerosis ***(23).*** The other study also found a negative correlation between CIMT and FMD ***(24).*** These results go in hand with our study which demonstrates an inverse and strong correlation between CIMT and FMD ***(P < 0.001).***

Although the remarkable ED and atherosclerosis found with Gr.1, plasma ADMA levels were comparable in all study groups ***(P= 0.126)***. Studies of ADMA in diabetic subjects reported conflicting results ***(25, 26, 27).*** In literature, there are few data about plasma ADMA levels with DPN with no studies about ADMA levels in diabetic foot ulcers. In agreement with us, one study found no difference between ADMA levels of diabetic subjects with and without DPN ***(28).*** In contrast, one study found lower ADMA levels ***(29)***, while another found higher levels ***(28),*** among diabetic neuropathic subjects in comparison to control healthy group.

Comparable ADMA levels in studied groups have many explanations.

First, ED in these individuals may be not necessarily due to decrease NO production by ADMA. Second, the elevation of ADMA in diabetes, in some studies, may actually had been overestimated because of technical difficulty of separation of ADMA and its biologically inactive isomer, symmetric dimethyl arginine, by chromatography ***(30)***. Chromatography was reported as a time consuming and costly technique that deliver quite unstable results ***(13).*** In contrast, ELIZA was used for measuring plasma ADMA in this current study. Third, about half of the hypertensive subjects in Gr.1 & Gr.2 were using angiotensine converting enzyme (ACE) inhibitors and angiotensine receptor blockers (ARBs), and many studies reported that ACE inhibitors and ARBs reduce ADMA levels in people with diabetes ***(31, 32).***Fourth, there was no significant difference as regard total cholesterol among the studied patients groups or TG among all studied groups, and many other studies demonstrated a link between ADMA and dyslipedemia ***(33, 34, 35)***. Fifth, insulin was found to decrease plasma levels of most amino acids by decreasing release from muscle and activating cellular uptake ***(36, 37).*** It was found that insulin resulted in reduced plasma levels of ADMA ***(38).*** In our study, 97% of Gr1 and 85% of Gr2 versus 25% of Gr3 were using insulin, the finding that may explain disappearance of the effect of neuropathy on ADMA levels. Last explanation, at least 80% of plasma ADMA is metabolized by dimethylarginine dimethyl aminohydrolase (DDAH**)** ***(39).*** So, increased activity of ADMA-metabolizing enzyme DDAH due to increased liver blood flow by chronic hyperglycaemia ***(27)***, may explain our finding.

In this study, plasma ADMA levels were not correlated to FMD ***(r= -0.176, P= 0.118)***, that means no association between ED and ADMA. This suggests that ED in these individuals is not necessarily due to decrease NO production because of NO synthetase inhibition by ADMA. Our study was consistent with a previous study that showed no correlation between plasma ADMA and FMD ***(40)***, while contrasting with results of another study reporting negative correlation between ADMA and FMD ***(41).***

Plasma ADMA levels were found to be positively correlated with CIMT ***(r= 0.330, P= 0.003),*** a result that was found also by other researchers ***(42)***. This correlation suggests that elevated plasma ADMA may be a marker of atherosclerosis. It can be explained by the positive associations between plasma ADMA and atherosclerotic risk factors in many cross-sectional studies ***(43, 44).***

In conclusion, plasma ADMA levels are not increased in diabetic subjects with NFU, and are not associated with the remarkable ED in these patients. This suggests that the impaired endothelial function in these patients may not be a result of decrease NO production by ADMA. DDAH enzyme activity among diabetic neuropathy subjects should be examined in further clinical studies. Plasma ADMA levels are positively correlated with atherosclerosis in diabetic subjects.

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