Relationship between Serum Asymmetric Dimethyl L-arginine and Type1 Diabetes Mellitus

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Abstract:Background: Asymmetric dimethyl-L-arginine (ADMA) acts as an endogenous inhibitor of nitric oxide synthase (NOS). Several studies indicate that ADMA is a critical mediator of the adverse effects on NOS of all cardiovascular risk factors. The present study aimed to examine any associations between ADMA concentration level and Type1 diabetes in children.**Methods:** A total of 84 Saudi school children from both genders (42 healthy and 42 children diagnosed with type 1 diabetes mellitus) with an age ranged from 6 to 18 years were randomly selected. Serum ADMA concentration was measured using competitive ELISA, and 6 other parameters: glucose, Total Cholesterol (TC), Triacylglycerol (TAG), High Density Lipoprotein-Cholesterol (HDL-C), Low Density Lipoprotein-Cholesterol (LDL-C) concentrations were determined by enzymatic analyses, and BMI, were measured in both groups to examine any correlations with ADMA level. **Results:** Serum Asymmetric dimethylarginine (ADMA) concentration in diabetic children was found to be significantly higher than that in healthy children (0.91±0.03 µmol/L, 0.55±0.04 µmol/L $P<0.0001$ respectively). These concentrations remained significantly high even after both diabetic and healthy children were compared to their matched gender. Glucose, TC, HDL-C and LDL-C serum concentrations in diabetic children were also found to be significantly high compared to healthy children. However, only glucose and HDL-C concentrations remained significantly high in both genders of diabetic children compared to their matched group in healthy children. **Conclusion:** The findings of the study suggested an association between the increase of ADMA serum concentration in Type1 diabetes in children with the possibility of developing atherosclerosis or heart disease with aging, particularly in males. Moreover, our study results also suggested a strong possibility of gender differences that may lead to ADMA distribution fluctuation. Further investigation in conjunction with this type of research is therefore required.
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Introduction

Asymmetric dimethylarginine (ADMA) can be found in blood and urine as natural chemical compound. Endothelial cells in human are able to synthesize ADMA by catalyzing a group of enzymes named S-adenosylmethionine protein, N-methyltransferases (protein methylases I and II) (Rawal et al., 1995). ADMA is closely related to the essential amino acid, L-arginine, which plays an important role in nitric oxide (NO) synthesis, a key chemical involved in normal endothelial function and cardiovascular health. ADMA interferes with L-arginine and acts as an endogenous competitive inhibitor of NOS (Boger, 2004) and it has protrude recently as a novel cardiovascular risk factor (Boger, 2003).

An enzyme named dimethylarginine dimethylamino hydrolase (DDAH) is able to metabolize ADMA to L-citrullin and dimethyl amine (Tsikas et al., 2003). The regulation of intercellular ADMA levels achieved by changes in DDAH activity can lead to changes in NO production (Boger, 2003). Accumulation of ADMA in the body is prevented in healthy humans by renal excretion and by metabolic degradation with DDAH (Dayoub et al., 2003, Nijveldt et al., 2003).

High levels of plasma ADMA have been reported in diseases linked with defect in endothelial L-arginine-NO pathway and endothylial dysfunction (Boger, 2003). ADMA concentration level has been associated with many conventional and novel risk factors in the initiating of atherosclerosis. Therefore it represents a well-characterized sign for progression of cardiovascular diseases (Ito et al., 1999, Miyazaki et al., 1999, Abbasi et al., 2001, Valkonen et al., 2001, Achan et al., 2003). An increase in plasma ADMA concentrations have also been related to other various clinical conditions, such as chronic renal disease, liver disease, insulin resistance, diabetes mellitus, hypertension, and dyslipidaemia, (Chan et al., 2000a, Boger, 2006, Mookerjee et al., 2007a, Mookerjee et al., 2007b, Damiati and Khoja, 2009, Damiati and Khoja, 2011, Boger et al., 2003, Lin et al., 2002, Maas, 2005, Mittermayer et al., 2006). However, the concentrations of ADMA association with diabetes mellitus appear to be fluctuated and cause lots of controversy (Anderssohn et al., 2010, Heilman et al., 2009, Marcovecchio et al., 2011). The distribution of ADMA

level found to be varies in different ethnicities (Sydow et al., 2010, Schulze et al., 2005), as well as it found to be sex and age dependent (Damiati and Khoja, 2009, Damiati and Khoja, 2011, Ahmed et al., 2007).

The present study aimed to measure the concentration of ADMA in diabetic children with diabetes mellitus Type1 compared to healthy school children. The study also aimed to find out a possible correlation between sera ADMA concentrations along with various biochemical blood components namely glucose, TAG, cholesterol, HDL-Cholesterol and LDL-cholesterol in diabetic and healthy school children.

Materials and methods

Subjects

A total of 84 school children attending the pediatric clinic at King AbdulAziz University Hospital in Jeddah were recruited for the present study (27 males and 57 females) and divided into two groups:

42 healthy children (11 males and 31 females with age ranged from 6-16 years) were considered as a control group and 42 diabetic children (16 males and 26 females with age ranged from 6-16 years) were diagnosed with type 1 diabetes mellitus. The weight and height of all subjects were recorded in a special sheet in order to calculate the body mass index (BMI) (weight/height^2).

Collection of Samples

Venous blood samples were collected from subjects in plain tubes after overnight fast (8-10 hours). Serum samples were then separated by centrifugation at 3000 g for 10 minutes and stored at -20ºC until assayed.

Determination of ADMA

ADMA was measured in serum samples by competitive ELISA (DLD diagnostika GmbH, Hamgurg, Germany) based on the method described by Damiati and Khoja, 2009 (Damiati and Khoja, 2009).

- Pre-treatment of samples (acylation):

20 µl of every standards were used, control 1 and 2 solutions (20 µl), and 20 µl from each samples were pipetted into the reaction plate (96-wells). Acylation and adjustment buffers (25 µl/ each) were added to each well, respectively. The acylation reagent (25 µl) freshly prepared was added and shaken immediately. The horizontal shaker was use for the reaction plate 30 min in the room temperature. Diluted equalizing reagent (100 µl) was added and incubated at room temperature for 45 min.

- ELISA test procedure:

50 µl of Pre-treated standards were pipetted into the well of of the microtiter plate of both controls and samples, followed by adding 50 µl of antiserum solution to each well. The microtiter plate was then shaked for seconds and covered with adhesive foil. After incubation time for 15–20 h at 2–8 ºC, the plate was cleaned from the solution and the wells were washed with washing buffer (250 µl) four times. Then, to each well enzyme conjugate solution (100 µl) was added and incubated for 1 h at room temperature with shaking. The wells were then washed with the washing buffer for four times again. After washing, a 100 µl of substrate solution was added to each well and incubated for 20–30 min with shaking. Stopping solution was used to stop the reaction then optical density was read at 450 nm (reference wavelength 570–650 nm) within 1 h.

Determination of glucose and lipid profiles

Glucose, TC, TAG, HDL-C, LDL-C concentrations were determined by direct enzymatic analyses using commercial kits (Biosystems, Barcelona-Spain) following the manufacturer's indications.

Serum level of glucose was determined by the glucose oxidase/peroxidase method (Trinder, 1969). Levels of total-cholesterol were measured in serum samples by using a cholesterol assay kit. The enzymatic procedure involves cleavage of the cholesterol esters by cholesterol esterase and oxidation of the free cholesterol by cholesterol oxidase (Natio and Kaplan, 1984, Meiattini et al., 1978). Indeed, enzymatic colorimetric methods were employed for the estimation of serum triglyceride as described by Buccolo, et al. (Bucolo and David, 1973). HDL-C levels were also determined after separation from other lipoproteins using a mixture of phosphotungstic acid and magnesium chloride (Assmann et al., 1983). LDL cholesterol was determined by a homogeneous enzymatic direct LDL assay (Friedewald et al., 1972).

Statistical analysis:

All recorded data were subjected to statistical analysis using statistical package social science (SPSS) program version 12, GraphPad Prism version 5 and Microsoft Excel. The data are expressed as means ± Standard error of mean (SEM) comparison between mean of two groups was performed using independent-sample t test. Statistically significant results were considered at $P<0.05$ .The scatter plot was used to express the correlations.

Results

Biochemical Data

The anthropometric and metabolic characteristics of both diabetic and healthy children are summarized in Table 1. The age mean of the diabetic children participated in this study was lower than control healthy children but not significant (11.8±0.48 year and 12.69 ±0.65 year respectively). Similarly the data showed that the BMI mean was lower but not significant in diabetic children (19.85±0.80 kg/m^2) when compared to control healthy children (20.47±0.73 kg/m^2).

The concentration of serum ADMA in the diabetic children was found to be significantly higher than in healthy children (0.91±0.0.03 µmol/L and 0.55±0.04 µmol/L P<0.0001 respectively). Serum concentration of TAG, glucose, TC, HDL-C and LDL-C in the diabetic children were also determined and compared to healthy children (Table 1). The concentration of TAG was found to be high but not significant in diabetic children (0.87±0.08 mmol/L) compared to healthy children (0.77±0.07mmol/L). Moreover, all other parameters measured were found to be significantly higher in diabetic children compared to healthy control children group, i.e., the concentration of glucose was (11.16±1.10 mmol/L and 4.19±0.17mmol/L, P<0.0001, respectively), total cholesterol concentration was (3.70±0.23 mmol/L and 2.80±0.17 mmol/L, P<0.005, respectively), HDL-C concentration was (1.62±0.10 mmol/L and 1.22±0.07 mmol/L, P<0.005, respectively) and LDL-C (2.50±0.14 mmol/L and 2.06±0.13 mmol/L, P<0.05 respectively).

However, not all the parameters examined above remained significantly high after subjects were divided into two groups and then compared to their matched gender. The anthropometric and metabolic characteristics of the diabetic and healthy children from both genders are summarized in Table 2. As the previous result age, BMI and TAG concentration showed no statistical difference between healthy and diabetic children from both genders. For example no statistical differences were detected in the age, BMI and TAG serum concentrations mean of diabetic boys (11.81±0.87 year, 19.77±1.19 kg/m^2, and 0.93±0.14 mmol/L, respectively) when compared to healthy boys (10.18 ±1.20 year, 18.75±1.14 kg/m^2, and 0.75±0.11 mmol/L, respectively). Similarly in diabetic girls, the age, BMI and TAG serum concentrations mean wear (11.88±0.57year, 19.90±1.08 kg/m^2, and 0.83±0.10 mmol/L, respectively) and in healthy girls were (13.58 ±0.72 year, 21.08±0.89 kg/m^2, and 0.78±0.86 mmol/L, respectively) with no statistical differences.

Only ADMA, glucose and HDL-C concentrations were remained significantly high when each group compared to their matched gender. For instance, ADMA concentration in the diabetic boys remained significantly higher than in healthy boys (0.94±0.05 µmol/L, 0.63±0.09 µmol/L P<0.05, respectively) (Figure 1). Similarly, ADMA concentration was significantly high in the diabetic girls than in healthy girls (0.89±0.04 µmol/L, 0.52±0.04 µmol/L P<0.0001, respectively).

The same pattern were found for glucose and HDL-C levels, the concentrations of both parameters remained significantly high when the diabetic boys compared to healthy boys, i.e., for glucose serum concentration was (13.17±2.10 mmol/L versus 3.90±0.31 mmol/L, P<0.005 respectively) and for HDL-C serum concentration (1.77±0.17 mmol/L, versus 1.24±0.18 mmol/L P<0.05 respectively). Similarly, were found in the diabetic girls compared to healthy girls, the glucose serum concentration was (9.93±1.21 mmol/L versus 4.30±0. 20 mmol/L, P<0.0001 respectively) and the HDL-C serum concentration was (1.53±0.13 mmol/L, versus 1.21±0.10 mmol/L P<0.05 respectively).

Total cholesterol and LDL-C concentrations were only found to be significantly higher in diabetic boys compared to healthy ones. For example, in diabetic boys the concentration of TC was significantly high compared to healthy boys (4.00±0.41 mmol/L and 2.54±0.31 mmol/L, P<0.05, respectively), and high but not significant in diabetic girls compared to their mach group (3.52±0.27 mmol/L and 2.89±0.21 mmol/L respectively). Similarly, LDL-C concentration was significantly high in the diabetic boys compared to healthy ones (2.61±0.25 mmol/L and 1.77±0.18 mmol/L P<0.05, respectively), while it's high but not significant in diabetic girls compared in healthy girls (2.43 ±0.17 mmol/L and 2.16±0.20 mmol/L, respectively).

Discussion

The present study was designed to measure the ADMA concentrations in diabetic school children living in Jeddah area since only few investigations on ADMA were performed in Saudi children. Age, BMI and 5 others parameters including: glucose, TC, TAG, HDL-C, LDL-C concentrations were also measured.

In this study, ADMA concentration differed significantly between the children with type 1 diabetes and the healthy sex-matched controls, with significantly higher levels in the type 1 diabetes group. Elevated ADMA levels have been described previously in patients with type 1 and 2 diabetes mellitus with an assumption that ADMA may contribute to vascular complications (Damiati and Khoja, 2009, Tarnow et al., 2004, Altinova et al., 2007, Marcovecchio et al., 2008). In both types of uncomplicated diabetes, the release and/or bioavailibility of NO are decreased due to increased oxidative stress, high glucose level or elevated ADMA levels as an endogenous nitric oxide synthase (NOS) inhibitor that considered as a key factor in NO biosynthesis (Tarnow et al., 2004, Chan et al., 2000b, Chan et al., 2003). However, these observations show the prominently close correlation between ADMA levels and indices of insulin resistance. Glucose itself may increase ADMA by suppress DDAH activity (Vallance and Leiper, 2004).

ADMA increased levels found in type 1 diabetic patients may cause endothelial damage, even though the hypertension or hyperlipidemia does not exist (Mahfouz et al., 2009). In order to prevent the endothelial damage in these patients, ADMA and the

ratio of L-arginine to ADMA measurements may proceed as biomarkers of any endothelial dysfunction (Altinova et al., 2007). Tarnow et al., in 2004 (Tarnow et al., 2004) has examined the association between ADMA and type 1; and reported that patients with type 1 diabetic and early diabetic nephropathy have higher levels of circulating ADMA, while the correlation between ADMA levels and GRF was negative. However, in renal failure patients, high ADMA level may participate to the high risk of cardiovascular morbidity and mortality (Tarnow et al., 2004, Malecki et al., 2007).

Contrary, other studies showed that ADMA concentration was lower in the children and adolescents with type 1 diabetes mellitus without vasculopathy compared with the healthy group (Huemer et al., 2011). They suggest that ADMA would protect the system from NO overproduction and perpetuation of oxidative stress. This theory is supported by the physiologically higher ADMA concentrations in healthy children. Nonetheless, the finding of lower ADMA concentrations in children with type 1 might not be interpreted as positive considering vasculopathy, but instead may be the hallmark of impaired protection against oxidative stress in type 1 (Pitocco et al., 2009). This assumption is also supported by the correlation between higher hemoglobin A1c (HbA1c) level and low ADMA concentration given the association between poor disease control and enhanced oxidative stress (Kostolanska et al., 2009). Interestingly, Glowinska-Olszewska, et al., 2010 (Glowinska-Olszewska et al., 2010) reported that diabetic children with type 1, without clinically evident vascular complications, have ADMA concentrations similar to healthy children.

This study also represents gender-related differences in ADMA with higher levels in males. Previous studies have reported the effect of gender and age on ADMA levels in blood. In healthy subjects, males have showed a higher levels of ADMA than females and that possibly related to a greater lean body mass a higher protein turnover (Ahmed et al., 2001). In contrast, females who were older than 50 years had significant increase in ADMA levels with the onset of menopause which may reflect hormonal statues (Damiati and Khoja, 2011, Schulze et al., 2005, Sydow et al., 2010). Similarly, diabetic adults, who were younger than 50 years, with type 2 have showed the higher ADMA concentrations comparing to females at the same age (Damiati and Khoja, 2009). Likewise, in this study, circulating ADMA levels were higher in the healthy and diabetic male children compared with the healthy and diabetic female children. Thus, our data are remarkably determine that females younger than 50 years had significantly lower ADMA concentrations than males of any age.

Glucose, TC, TAG, HDL, and LDL serum concentrations were also found to be higher in diabetic children compared to healthy children. However, only glucose and TC concentrations remained significantly higher in both genders of diabetic children compared to their matched group in the healthy children.

Clinical investigations in patients indicate direct relation between ADMA and blood glucose levels (Abbasi et al., 2001, Worthley et al., 2007). These studies showed that ADMA levels are elevated in hyperglycemic patients with type 2 diabetes, and contribute to the accelerated coronary heart disease which is the major cause of morbidity and mortality in type 2 diabetic patients. Thus, strict glycemic control may exert anti-atherogenic effects by reducing ADMA levels in type 2 diabetic patients (Yasuda et al., 2006). However, it was obvious that the diabetic children of the present study did not show a good glycemic control.

Several studies showed that a positive correlation was obtained between lipid profile and ADMA concentrations except HDL-cholesterol (Chan et al., 2000b, Boger et al., 1998, Lundman et al., 2001). Native or Oxidized LDL-cholesterol (OxLDL) and tumor necrosis factor-a (TNF-α) are capable to up-regulate expression and activity of several arginine N-methyltransferases. Thus, offers a putative mechanism for elevated ADMA concentrations that associated with hyperlipidemic patients. However, elevated levels of ADMA can result from increased ADMA synthesis and from reduced degradation by reducing DDAH activity.

Table 1. Clinical characteristic of subject's studies.

Variables	Control (42)	Diabetic (42)
Age (years)	12.69 ±0.65	11.8±0.48
BMI (kg/m^2)	20.47±0.73	19.85±0.80
ADMA (µmol /L)	0.55±0.04	0.91±0.03***
TAG (mmol/L)	0.77±0.07	0.87±0.08
Glucose (mmol/L)	4.19±0.17	11.16±1.10***
TC (mmol/L)	2.80±0.17	3.70±0.23**
HDL-C (mmol/L)	1.22±0.07	1.62±0.10 **
LDL-C (mmol/L)	2.06±0.13	2.50±0.14*

Results are expressed as mean ± SEM, with (n) is the number of subjects. Significant differences between control and diabetic group for females are shown as: * = P<0.05, **= P<0.005, *** = P<0.0001 using Mann-Whitney U test.

One of the clinical findings on ADMA was its elevation in plasma of clinically asymptomatic hypercholesterlemic subjects. Importantly, this elevation was found in the absence of overt cardiovascular disease, suggesting that the increase in ADMA levels occurs early in the pathogenesis of atherosclerosis and may contribute to its progression

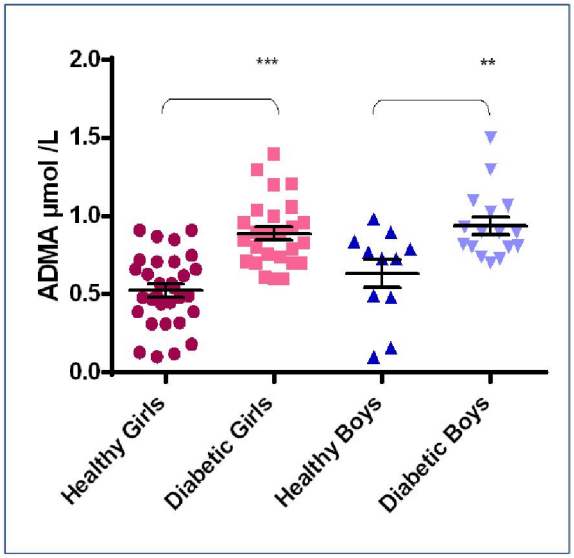
(Boger et al., 1998). Moreover, Lundman et at., 2001 (Lundman et al., 2001) reported that chronic mild-to-moderate hypertriglyceridemia in young men is associated with impaired endothelium-dependent vasodilation and increased concentration of ADMA.

In conclusion, the present study proved many major points that are considered important in clinical trials related to the field of ADMA such as the increase in ADMA serum concentration in children in both sexes was associated with increased TC, LDL-C, and glucose, which suggested that measurement of ADMA may be considered as a causative of the cardiovascular events. According to these results, it is suggested that measurement of ADMA may be considered for cardiovascular marker.

Table 2. The anthropometric and metabolic characteristics of diabetic and healthy children from both genders.

Variables	Male		Female	
	Control (11)	Diabetic (16)	Control (31)	Diabetic (26)
Age (years)	10.18 ±1.20	11.81±0.87	13.58±0.72	11.88±0.57
BMI (kg/m^2)	18.75±1.14	19.77±1.19	21.08±0.89	19.90±1.08
ADMA (µmol /L)	0.63±0.09	0.94±0.05*	0.52±0.04	0.89±0.04 *
Glucose (mmol/L)	3.90±0.31	13.17±2.10**	4.30±0. 20	9.93±1.21***
TC (mmol/L)	2.54±0.31	4.00±0.41*	2.89±0.21	3.52±0.27
TAG (mmol/L)	0.75±0.11	0.93±0.14	0.78±0.86	0.83±0.10
HDL-C (mmol/L)	1.24±0.18	1.77±0.17 *	1.21±0.10	1.53±0.13 *
LDL-C (mmol/L)	1.77±0.18	2.61±0.25*	2.16±0.20	2.43 ±0.17

Results are expressed as mean ± SEM, with (n) is the number of subjects. Significant differences between control and diabetic group for females are shown as: * = P<0.05, **= P<0.005, *** = P<0.0001 using Mann-Whitney U test.



Asymmetric dimethyl-L-arginine (ADMA) results in different gender groups compared to their healthy control groups. Results are expressed as mean ± SEM. Significant differences between healthy controls and diabetic groups for girls and boys are shown as: **= P<0.005 and *** = P<0.0001 using Kruskal-Wallis test.

Figure 1.

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Competing interests

The authors declare that they have no competing interests.

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