MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society for Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ERYTHROCYTE ARGINASE ACTIVITY AND SERUM NITRIC OXIDE IN DIABETES MELLITUS

Harika Vemugadda[1], Prajna P Shetty[1], Monalisa Biswas[1], Revathi P Shenoy[1], Nalini K[*1]

[1]*Department of Biochemistry, Kasturba Medical College, Manipal Academy of Higher Education, Manipal-576104*

*Corresponding Author: nalini.kbhat@manipal.edu

History
Received: 16th July 2019
Accepted: 22nd December 2019

Keywords:

Arginase; Nitric oxide; Prediabetes; Type 2 Diabetes Mellitus; Vascular Complications

Abstract

The study is to estimate and correlate the erythrocyte arginase activity and serum nitric oxide levels in normal, prediabetes and type 2 diabetes mellitus. This is a case control study with total 124 samples which were grouped as normal, pre-diabetes and diabetes based on HbA1C values. Blood samples were collected from Clinical Biochemistry laboratory, Kasturba Hospital, Manipal, after the completion of HbA1C analysis. Erythrocyte Arginase activity is estimated by measuring the ornithine formed by Chinard reaction and Arginase activity is expressed as ornithine released per minute per gram hemoglobin under assay conditions. Nitric oxide is estimated by reducing the serum nitrate to nitrite by using Griess reaction method. The increase in arginase activity was seen in both prediabetes and diabetes compared to normals. Compared to normal group, there was significant decrease in nitric oxide level in pre diabetes (P=0.013) however decrease is not significant in type 2 diabetes. Significant positive correlation between the arginase and nitric oxide levels is seen in normals and type 2 diabetes whereas negative correlation in prediabetes. Increase in arginase activity is indirectly affecting the nitric oxide levels and causing the macrovascular (atherosclerosis, hypertension, gangrene of foot, diabetic neuropathy) and microvascular (diabetic nephropathy, diabetic retinopathy) complications in type 2 diabetic patients.

INTRODUCTION

Diabetes is characterized by hyperglycemia and insulin resistance or deficiency and is a top 10 cause of death worldwide [1, 2]. Type 2 diabetes is the most common health problem across the world when compared to type 1 [3, 4]. Increase in population, ageing, obesity, unhealthy diet and sedentary lifestyles are some of the main reasons to develop type 2 diabetes [5]. Diabetes is the fastest growing disease in India with more than 85% to 95% that is seen in developed countries [6]. 80% of type 2 diabetic patients are more vulnerable to macrovascular complications (cerebrovascular, coronary artery, and peripheral arterial diseases) and 65% deaths are seen in this group [7-9]. The type 2 diabetes morbidity is also substantial in contribution to microvascular complications (retinopathy, nephropathy and neuropathy) [10, 11].

The three most important gasotransmitters namely, Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) are endogenously produced by various cellular enzymes and play an important role in physiology and disease. The reduced bioavailability of gasotransmitters is seen in type 2 diabetes when compared to the healthy individuals [12]. The first endothelial-derived relaxing factor (EDRF) recognized in the body is nitric oxide [13] and this is endogenously formed from its substrate L-arginine by three different nitric oxide synthase (NOS) enzymes; eNOS, iNOS, and bNOS and plays vital role in maintaining vascular tone, promoting inflammation, and neurotransmission respectively [14].

Arginase is a urea cycle enzyme that catalyzes the reaction, L-arginine to L-ornithine and urea [15]. Arginine is the common substrate for the arginase and nitric oxide synthase therefore the availability of arginine is one of the rate-limiting factors in cellular nitric oxide production [16]. Thus, arginase may reciprocally regulate the production of nitric oxide and thereby induce endothelial dysfunction [17-19]. In the body, nitric oxide is relatively unstable product and undergoes oxidation process; finally presenting in the form of nitrate in plasma [20].

Hence the current study is taken up to find out the relationship between erythrocyte arginase activity and nitric oxide levels in type 2 diabetes.

MATERIALS AND METHODS

In this case control study, total 124 residual blood sample collected with EDTA for HbA1C estimation to Clinical Biochemistry laboratory, Kasturba Medical College, Manipal, after annonymization. The study was approved by institutional ethics committee (IEC No: 14/2019). As per the American Diabetes Association Diabetes Care 2013, the samples were grouped based on HbA1C values [21] into normals, pre diabetic and diabetics with the age group of 40 to 65yrs without cardiovascular diseases. The normal group with HbA1C values 4 to 5.6 (n=35), group 2, pre-diabetes with HbA1C 5.7 to 6.4 (n=49), and group 3, diabetes with HbA1C ≥6.5 (n=40).

Materials

The chemicals zinc sulphate, sodium hydroxide GR, glycine GR, cadmium granules were purchased from MERCK, copper sulphate was purchased from RANBAXY, sulphuric acid (98% AR) was purchased from SDFCL, sodium nitrate was purchased from Sarabhai M Chemicals, griess reagent and tris(hydroxymethyl) aminomethane were purchased from SRL, manganese chloride tetrahydrate, L-Ornithine hydrochloride, sodium bicarbonate, sodium carbonate, L-Arginine monohydrochloride, potassium ferricyanide, potassium cyanide were purchased from Sigma Aldrich Inc., orthophosphoric acid (88% GR), glacial acetic acid (100% GR, aldehyde free), ninhydrin GR, potassium dihydrogen phosphate, potassium hydroxide, sodium hydroxide, sodium chloride, hydrochloric acid were purchased from MERCK

Methods

The whole blood was centrifuged at 2000g (15 mins) to separate RBCs and plasma. The RBCs are used for the estimation of arginase activity, whereas plasma for nitric oxide estimation. The catalytic activity of arginase is determined colorimetrically by measuring the increase in the concentrations of ornithine by Chinard reaction and the absorbance is read at 515nm. Blood was centrifuged at 2000g to extract red cells. The separated red cells were washed 3 times and centrifuged 2000g (15 min) with 5 volumes of normal saline. Supernatant was discarded after the last wash and, diluted with 5mmol/L Tris buffer, pH 7.5. The suspension obtained was used for estimation of arginase activity and hemoglobin concentration. Hemoglobin was estimated using Drabkin's method. Arginase activity is expressed as the amount in millimoles (mmol) of ornithine released per minute per gram hemoglobin under the assay conditions (units/g hemoglobin) [22].
The plasma is used for nitric oxide estimation by the griess reaction that involves the indirect assay of stable decomposition products in plasma nitrite and nitrate levels as an index of nitric oxide generation. Nitric oxide is estimated by the amount of nitrite formed by reducing nitrate present in the plasma of the sample. The absorbance is read at 540nm in ELISA reader [20, 23]. Statistical methods used are ANOVA test with Dunnett Post Hoc comparison and Pearson's correlation analysis using SPSS 15.0

RESULTS

The increase in arginase activity is seen in both prediabetes and diabetes compared to normals. There was statistical significant decrease in Nitric oxide level in pre diabetes and their decrease in type 2 diabetes statistically not significant compared to the normals (**Table 1**). Statistical significant mean difference is seen with Nitric oxide levels in prediabetes at 95% CI (**Table 2**). Significant positive correlation between the arginase and nitric oxide levels is seen in normals and type 2 diabetes whereas negative correlation in prediabetes (**Table 3**).

DISCUSSION

In diabetes type 2 about 80% are developing macrovascular complications (cerebrovascular, coronary artery, and peripheral arterial diseases) causing 65% deaths. The contribution of microvascular complications (retinopathy, nephropathy and neuropathy) to type 2 diabetes morbidity is also substantial. It is hypothesized that derangement in nitric oxide and arginase levels leads to these macro and microvascular complications.

Our study found the mean of Arginase level increased in the prediabetes (μ=693.4, ±362.1) and diabetes (μ=733.8, ±416.3) when compared to the normals (μ=581.6, ±361.9), while the mean of nitric oxide level is decreased in both prediabetes (μ=20.4, ±8.5) and diabetes (μ=23.4, ±11.95) than normals (μ=27.1, ±9.43). The significant decrease of nitric oxide levels is seen in between the groups (p=0.013). This is the generally expected pattern as per literature because the increase in arginase activity will reduces the availability of L-arginine for nitric oxide synthase and finally decreases the nitric oxide production [24]

The nitric oxide levels in prediabetes shows the significant difference when compared to normal group (p=0.006). However, the difference was not significant with diabetes group, which might be due to the increased levels of insulin in these patients (either due to insulin treatment or short-term hyperinsulinemia condition) [25]

In normals, the high positive correlation (r=0.718) is seen between arginase activity and nitric oxide levels and this shows that arginase activity and nitric oxide levels are may be independent to each other (unaffected by endogenous insulin) in normoglycemic physiological state [26-29]. The negative correlation in prediabetes (r=-0.206) might be because of the action of insulin in hyperglycemic condition that increases the arginase activity and reduces the nitric oxide levels [30]. The high positive correlation of arginase and nitric oxide levels in diabetes (r=0.574) is may be due to short term physiologic hyperinsulinemic condition seen during the early stages of diabetes, arginase activity might also be increased due to insufficient insulin levels either during treatment or endogenously. In advanced stages of the disease, overproduction of insulin in response to prolonged hyperglycemia, damages the beta cells of pancreas and reduce its function that ultimately lowers the insulin levels in diabetic patients. This may be the reason of developing vascular complications caused in diabetes at late stages [31-34]. According to previous literature, elevated levels of glucose may enhance NO production through increased expression of eNOS and iNOS gene and protein levels [35-38]. Elevated levels of NO in *in-vivo* might have both beneficial as well as adverse effect based upon the amount of NO concentration that is present. On one hand, NO can cause relaxation of blood vessels and reduce hypertension, and on the other hand, NO may interact with superoxide radical (O_2^-) leading to inactivation of NO. The interaction of O_2^- with NO is rapid and leads to the formation of potent oxidant radical, peroxynitrite. This stimulates the metabolism of arachidonic acid, lipid peroxidation, and prostanoid generation in the body and finally contributes to endothelial dysfunction [39, 40].

Table 1. Comparison between erythrocyte arginase activity and plasma NO levels in controls and patients

Variables	Group 1		Group 2		Group 3		P value
	MEAN	SD	MEAN	SD	MEAN	SD	
HbA1C	5.300000	.3547990	6.012245	.2297033	8.445000	1.9252639	.000
Arginase	581.5809	361.82408	693.3796	362.01708	733.7750	416.32375	.208
Nitric oxide	27.01429	9.430803	20.39796	8.490025	23.37500	11.945959	.013

Table 2. Post Hoc comparison

Dunnett t (2-sided)

Dependent Variable	(I) gp	(J) gp	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower	Upper
HbA1C	2	1	.7122449*	.009	.160072	1.264418
	3	1	3.1450000*	.000	2.567523	3.722477
Arginase	2	1	111.79873	.306	-75.9563	299.5538
	3	1	152.19414	.149	-44.1648	348.5531
NO	2	1	-6.616327*	.006	-11.54279	-1.68986
	3	1	-3.639286	.200	-8.79150	1.51293

*The mean difference is significant at the 0.05 level.
Dunnett t-tests treats group 1-control, group 2-prediabetes and group 3-type 2 diabetes

Table 3. Pearson correlation between Nitric oxide and Arginase

Parameters	r values in the groups		
	Group 1	Group 2	Group 3
Nitric oxide and Arginase	.718**	-.206	.574**

Our results clearly indicate that there is decrease in the nitric oxide levels in T2DM contradictory with the previous literature that the plasma NO levels are increased in T2DM and not in non-diabetic insulin resistance [41]. In prediabetic patients (prone to diabetes), plasma NO level might be increases, but decreased in nitric oxide levels are also reported in some literature [42].

The study is limited with the smaller sample size. The study needs to be replicated with a larger sample size in a prospective or cohort model to observe the expected statistical significance and validate the findings of this study. Further studies should be carried out accounting for the insulin levels, insulin resistance status of subjects along with history of duration of diabetes and presence of other comorbidities.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Vasudeva Guddattu, Assistant Professor, Department of statistics, Manipal Academy of Higher Education, Manipal, for his guidance in the statistical analysis of the research study and also Kasturba Medical College, Manipal Academy of Higher Education for their financial support by a postgraduate research grant.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

REFERENCES

1 Geneva, Switzerland, (2014) Global Status Report on Noncommunicable Diseases. World Health Organization

2 Wild S, Roglic G, Green A, Sicree R, King H. (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care.* **27***:*1047–1053

3 Bagust A, Hopkinson PK, Maslove L, Currie CJ. (2002) The projected health care burden of Type 2 diabetes in the UK from 2000 to 2060. Diabet Med. **19**: 1-5.

4 Motala AA, Pirie FJ, Gouws E, Amod A, Omar MK. (2003) High incidence of Type 2 diabetes mellitus in South African Indians: a 10-year follow-up study. Diabet Med. **20**: 23-30.

5 Harris MI, Flegal KM, Cowie CC, et al. (1998) Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988-1994. Diabetes Care. **21**: 518-524.

6 International Diabetes Federation [IDF]. Diabetes e-Atlas. Retrieved June 20, 2005, from http://www.-eatlas.idf.org.

7 Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, et al. (2007) Primary prevention of cardiovascular diseases in people with diabetes mellitus: a scientific statement from the American heart association and the American diabetes association. Circulation.;**115**:114–126. doi: 10.1161/CIRCULATIONAHA.106.179294.

8 Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, et al. (1999) Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. Circulation.**100**:1134–1146.

9 Kirkman MS, McCarren M, Shah J, Duckworth W, Abraira C. (2006) The association between metabolic control and prevalent macrovascular disease in Type 2 diabetes: the VA Cooperative Study in diabetes. J Diabetes Complicat. **20**:75–80.

10 Fowler MJ. (2011) Microvascular and macrovascular complications of diabetes. Clin Diabetes.**3**:116–122.

11 Mohammedi K, Woodward M, Marre M, Colagiuri S, Cooper M, Harrap S, et al. (2017) Comparative effects of microvascular and macrovascular disease on the risk of major outcomes in patients with type 2 diabetes. Cardiovasc Diabetol. 16:95.

12 Yamagishi S, Matsui T (2011) Nitric oxide, a janus-faced therapeutic target for diabetic microangiopathy-friend or foe? Pharmacol Res. 64:187–194

13 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A.* 84:9265–9269

14 Alderton WK, Cooper CE, Knowles RG *(*2001)Nitric oxide synthases: structure, function and inhibition. *Biochem J.* **357***:*593–615

15 Shetty Sukanya, Rao Ashalatha V, and Bhandary Roopa. (2011) Role of maternal erythrocyte arginase activity in pregnancy – A pilot study. NHUJS **1**: 1-3.

16 Mathew G, Glende, Jack L. (1996) Quantitation of nitrite and nitrate in extra-cellular fluids. Met Enzymol. **268**:237-46

17 Pernow, J, Jung, C. (2013) Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? Cardiovasc Res. **98**: 334–343.

18 Durante, W, Johnson, FK, Johnson, RA. (2007) Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol. **34**: 906–911.

19 Caldwell, RB, Toque, HA, Narayanan, SP. (2015) Arginase: an old enzyme with new tricks. Trends Pharmacol Sc. **36**: 395–405.

20 Cortas N, et al. (1990) Determination of inorganic nitratein serum and urine by a kinetic cadmium-reduction method. Clin. Chem., **36**: 1440

21 Diagnosis and Classification of Diabetes Mellitus. American Diabetes Association Diabetes Care 2013; 36

22 Bascur L, Cabello M, Veilz M, Gonzalez A. (1966) Molecular forms of human-liver arginase. Biochem. Biophys. Acta;**128**: 149-154

23 Relationship between Arginase activity and nitric oxide production (Chapter 12). Availablefrom: https://www.sciencedirect.com/science/article/pii/B9780123704207500137

24 Cederbaum SD, Yu H, Grody WW, Kern RM, Yoo P, Iyer RK. (2004) Arginases I and II: do their function overlap? Mol Genet Metab.81

25 Sangeeta R. Kashyap, Abigail Lara, Renliang Zhang, Young Mi Park, MD, and Ralph A. DeFronzo, Insulin Reduces Plasma Arginase Activity in Type 2 Diabetes Patients

26 Zhang C, Hein TW, Chang C, Kuo L. (2001)Constitutive expression of arginase in microvascular endothelial cells counteracts nitric oxide-mediated vasodilatory function. FASEB.**15**:164–1266

27 Loscalzo J. (2000) What we know and don't know about L-arginine and NO. Circulation. **101**:2126–2129.

28 Loscalzo J. (2003) Adverse effects of supplemental L-arginine in atherosclerosis. Arterioscler Thromb Basc Biol.**23**:3–5.

29 Chen J, Kuhlencordt P, Urano F, Ichinose H, Astern J, Huang PL. (2003) Effects of chronic treatment with L-arginine on atherosclerosis in apoE knockout and apoE/inducible NO synthase double-knout mice. Arterioscler Thromb Vasc Biol.**23**:97–103.

30 Du XL, Edelstein D, Dimmeler S, Ju Q, Sui C, Brownless N. (2001) Hyperglycemia inhibits endothelial nitric oxide synthase activity by post translational modification at the Akt site. J Clin Invest.**108**:1341–1348.

31 Owen K, Hattersley AT. (2001) Maturityonset diabetes of the young: from clinical description to molecular genetic characterization. Best Pract Res Clin Endocrinol Metab. **15**: 309-323.

32 Kahn CR, Vicent D, Doria A. (1996) Genetic of noninsulin-dependant (Type-II) diabetes mellitus. Ann Rev Med. **47**: 509-531.

33 Tuomi T, Carlsson AL, Li H, et al. (1999) Clinical and genetic characteristics of type 2 diabetes with and without GAD antibodies. Diabetes. **48**: 150-157.

34 Pozzilli P, Di Mario U. (2001) Autoimmune diabetes not requiring insulin at diagnosis (latent autoimmune diabetes of the adult): definition, characterization, and potential prevention. Diabetes Care. **24**: 1460-1467.

35 Cosentino F, Hishikawa K, Katusic ZS, Lusher TF (1997) High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation. **96**(1): 25–28

36 Cai S, Khoo J, Channon KM (2005) Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. Cardiovascular Research **65**: 823–831.

37 Yang P, Cao Y, Hua LI (2010) Hyperglycemia induces iNOS gene expression and consequent nitrosative stress via JNK activation. Am J Obstet Gynecol. **203**(2): 185–185.

38 Zhang X, Fu Y, Xu X, Li M, Du L, Han Y, Ge Y (2014) PERK pathway are involved in NO-induced apoptosis in endothelial cells cocultured with RPE under high glucose conditions. Nitric Oxide. **40**C:10–16.

39 Francesco C, Keiichi H, Zvonimir SK (1997) High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation **96**:25–28

40 Naohito I, Kaushik PP, Pascale HL, Traci T, Ka B, et al. (2001) Nitric oxide synthesis and oxidative stress in the renal cortex of rats with diabetes mellitus. J Am Soc Nephrol. **12**:1630–1639.

41 Chien WY, Yang KD, Eng HL, Hu YH, Lee PY, et al. (2005) Increased plasma concentration of nitric oxide in type 2 diabetes but not in non diabetic individuals with insulin resistance. Diabetes Metab **31**: 63–68.

42 Amrita G, Mingma LS, Yazum B, Ranabir P, Sanjay D (2011) Serum nitric oxide status in patients with type 2 diabetes in Sikkim. Int J Appl Basic Med Res **1**(1): 31–35.