ANKARA MEDICAL JOURNAL

Research Article

Ankara Med J, 2022;(4):533-541 // 10.5505/amj.2022.65707

EVALUATION OF LARGE UNSTAINED CELLS (LUC) AND NITRIC OXIDE IN DIABETES MELLITUS

Funda Eren[1], Arzu Kösem[2], Esra Fırat Oğuz[1]
Salim Neselioglu[3], İhsan Ateş[4], Ozcan Erel[3]

[1]Ankara City Hospital, Department of Medical Biochemistry Laboratory, Ankara, Turkey
[2]Ankara Dışkapı Research and Training Hospital, Department of Medical Biochemistry Laboratory Ankara, Turkey
[3]Yıldırım Beyazıt University, Department of Medical Biochemistry, Ankara, Turkey
[4]Ankara City Hospital, Clinic of Internal Medicine, Ankara, Turkey

Correspondence:
Funda Eren (e-mail: fundakarakoyunlu@gmail.com)

Submitted: 27.09.2022 // Accepted: 05.12.2022

Ankara Yıldırım Beyazıt University Faculty of Medicine
Department of Family Medicine

ANKARA MEDICAL JOURNAL

Abstract

Objectives: The large unstained cells (LUC) is a differential count parameter measured by routine hematology analyzers and reflects activated lymphocytes and peroxidase-negative cells in leukocytes. Nitric oxide (NO) is produced in all tissues in enzymatic and non-enzymatic ways. This study aimed to determine the levels of LUC and NO products (nitrite and nitrate) and to evaluate the LUC/NO ratio in patients with Diabetes Mellitus (DM).

Materials and Methods: The study included 103 DM patients and 84 healthy controls. HbA1c, LUC/%LUC and total NO levels were measured. All the statistical calculations were performed using the Statistical Package for Social Sciences (SPSS) software program.

Results: Nitric oxide levels of the patients were statistically significantly lower compared with the control group (p=0.004). LUC levels, LUC% values, LUC/NO, and LUC%/NO ratios were significantly higher in the DM group (p=0.002, p=0.009, p= <0.001, and p= <0.001, respectively). Statistically significant correlations were observed between HbA1c and nitrite, nitrate, NO, LUC/NO ratio, and LUC % /NO ratio.

Conclusion: In this study, we determined the LUC/NO percent ratio and LUC%/NO percent ratio for the first time, according to our knowledge. We predict that these two parameters may be useful markers in the diagnosis and the follow-up of the disease and may provide target pathways for further studies that may contribute to the etiopathogenesis of the disease.

Keywords: Diabetes Mellitus, inflammation, LUC, NO.

Introduction

Diabetes mellitus (DM) is a carbohydrate metabolism disorder that is increasing in prevalence worldwide and manifests itself with hyperglycemia due to the underuse of glucose. Acute complications of DM are often serious, but long-term micro- and macrovascular complications of the disease are responsible for most of the morbidity and mortality in patients with DM.[1,2] Hemoglobin A1c (HbA1c) is a parameter used in the evaluation of glycemic status for both diagnosis and follow-up of DM and is a marker that shows glucose tolerance and glucose regulation in DM, formed by slow and non-enzymatic glycosylation of hemoglobin. HbA1c reflects the risk of developing diabetic complications and the quality of diabetic care, together with demonstrating glycemic control in DM.[3] While HbA1c reflects the mean plasma glucose of erythrocytes for 120 days. It is also the parameter that best correlates with the mean plasma glucose over the previous 8-12 weeks.[4] In 2009, the American Diabetes Association (ADA) approved the use of HbA1c level of ≥ 6.5% for the diagnosis of DM.[5] Also in our country, according to the guideline published by the Turkish Endocrinology and Metabolism Society in 2020, HbA1c levels of ≥6.5% (≥48 mmol/mol) make the diagnosis of overt DM. In the same guideline, people with HbA1c levels of 5.7-6.4% (39-47 mmol/mol) were considered to be a high-risk group for DM.[6]

Large unstained cells (LUC) are large peroxidase-negative cells that could not be determined as large lymphocytes, virocytes, blasts, or stem cells on the automatic cell counters. The percentage of LUC (%LUCs) is a count test measured by hematology analyzers automatically and shows activated lymphocytes and peroxidase-negative cells in leukocytes.[7,8] Increased levels of LUCs may be related to some viral and fungal infections, inflammation, or leukemia.[8]

Nitric oxide (NO) is produced in all tissues in enzymatic and non-enzymatic ways. NO is mostly produced from L-arginine by nitric oxide synthase (NOS) enzymes. NO plays an important role in the regulation of metabolism, energy balance, food intake, and insulin sensitivity. High morbidity and mortality rates of DM may be due to the early development of atherosclerosis in these patients.[9] NO affects endothelial permeability for macromolecules and also the proliferation and migration of vascular smooth muscle cells.[10,11] The concept of impaired NO activity for increased cardiovascular complications in DM is gaining more and more support.[12] Traditionally, nitrate and nitrite (NOx) were assumed to be inert derivatives of NO production.[13] The last stage in the NO pathway is an electron transfer from nitrite to NO. This nitrite reduction is catalyzed by deoxyhemoglobin, deoxymyoglobin NOS, cytochrome P-450, xanthine oxidase, and the mitochondrial electron transfer complexes. This reaction occurred in hypoxic conditions. The alternative NO generation pathway limits the production of NO from NOS under hypoxia and oxidative stress conditions.[14]

DM leads to endothelial dysfunction and accelerates the progression of atherosclerosis. Inflammation is known to play a key role in atherosclerosis.[9] This study aimed to determine the levels of LUC indicating inflammation

and nitric oxide products nitrite and nitrate (NOx), which are markers of endothelial dysfunction, and to evaluate the LUC/NO ratio in patients with DM.

Materials and Methods

In the study, the subjects were divided into two groups according to their HbA1c levels patients with DM (HbA1c≥6.5%) and control (HbA1c<5.7%). Our study included 103 patients with DM over the age of 18 who applied to the internal medicine outpatient clinic of our hospital and 84 healthy volunteers as the control group. Blood samples were collected from the patients and healthy volunteers into two separate tubes, EDTA (ethylenediaminetetraacetic acid) containing tube and a serum separator tube used for routine biochemical tests. HbA1c values were measured by Atellica CH 930 Analyzer (Siemens Healthineers, Erlangen, Germany) with the principle of latex agglutination inhibition assay in our laboratory. The %LUC values were calculated automatically with the Advia2120 (Siemens Healthcare Diagnostics, Forchheim, Germany) fully automatic blood count analyzer. Nitrite, nitrate, and NO levels were measured using the spectrophotometric method. Briefly, samples were initially deproteinized. Then, nitrite levels were measured with the Griess reaction. Total nitric oxide (nitrite and nitrate) was measured after the conversion of nitrate to nitrite by cadmium granules by a spectrophotometer at 545 nm.[15] A standard curve was established with sodium nitrite, and this curve was used to calculate the unknown sample concentrations. The results were expressed as micromoles per liter plasma (μmol/L).

Our study was approved by Ankara City Hospital Ethics Committee number E-19-022.

The conformity of continuous variables to normal distribution was examined using Kolmogorov–Smirnov test). Variables were evaluated considering their distribution using statistical tests. Since continuous variables showed normal distribution, the results were presented as mean and standard deviation. Categorical variables are given as numbers and percentages (%). Categorical variables were evaluated by the Chi-square test. Group comparisons (control group vs. patient group) were made using Student's t-test. Correlation analyses were performed using Pearson's correlation. The analysis of the cut-off value, sensitivity, and specificity of tests was done by the receiver operating characteristic (ROC). SPSS software program (v22 IBM, Armonk, NY, USA) was used for the statistical analysis, and a *P* value less than 0.05 was set as statistically significant for all analyses.

Results

Our study included 103 patients with the diagnosis of DM (52 females (50.48%), 51 males (49.52%)) and 84 healthy controls (41 females (48.80 %), and 43 males (51.20%)). The mean age of the patients and the control group were similar. Nitric oxide levels of the patients were statistically significantly lower than healthy controls

(p=0.004). LUC levels, LUC% values, LUC/NO and LUC%/NO ratios were significantly higher in the DM patient group (p=0.002, p=0.009, p= <0.001, and p= <0.001, respectively) (Table 1, Figure 1).

Table 1. Laboratory findings of individuals in study groups.

Parameters	**Group**	**Mean ± SD**	***p*-value**
HBA1c (%)	Control	5.05 ± 0.33	<0.001
	DM	8.58 ± 1.93	
FG (mg/dL)	Control	78.17 ± 9.11	<0.001
	DM	176.74 ± 80.41	
PG (mg/dL)	Control	89.33 ± 21.55	<0.001
	DM	286.79 ± 125.03	
LUC (x10^9/L)	Control	0.11 ± 0.03	0.002
	DM	0.15 ± 0.07	
LUC (%)	Control	1.5 ± 0.43	0.009
	DM	1.82 ± 0.65	
Nitrite (µmol/L)	Control	3.23 ± 0.68	0.011
	DM	2.81 ± 0.51	
Nitrate (µmol/L)	Control	8.06 ± 0.76	0.039
	DM	7.66 ± 0.7	
NO (µmol/L)	Control	11.29 ± 1.02	0.004
	DM	10.51 ± 1.06	
LUC/NO ratio	Control	0.97 ± 0.28	<0.001
	DM	1.4 ± 0.47	
LUC %/NO ratio	Control	13.16 ± 3.9	<0.001
	DM	17.39 ± 6.11	

(FG: fasting glucose; PF: postprandial glucose; LUC: large unstained cells; SD: standard deviation.)

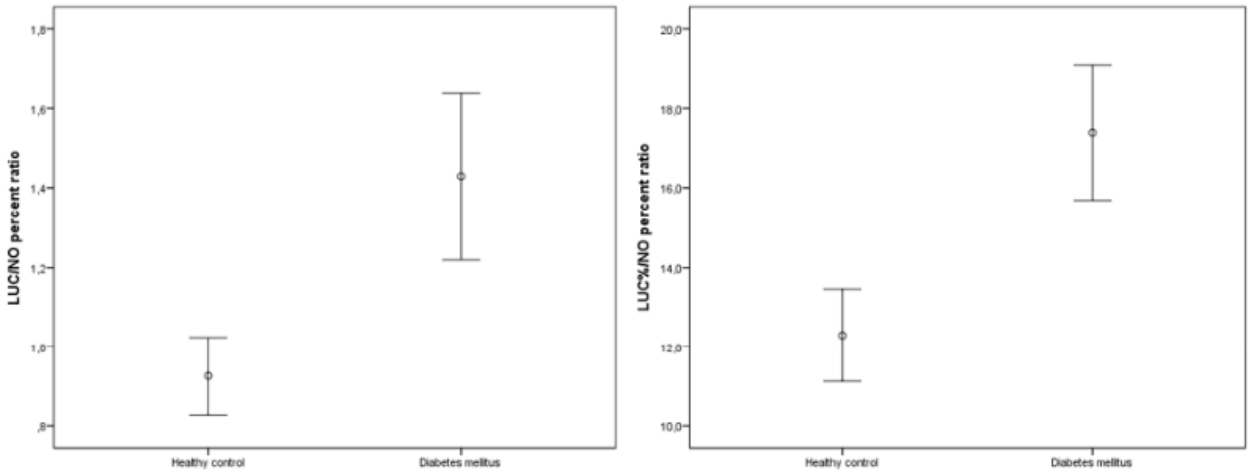

Figure 1. The alteration of LUC/NO and LUC%/NO percent ratios in Diabetes Mellitus and healthy control groups.

According to ROC analysis performed to evaluate the possible contribution of the tests to prediction, LUC/NO ratio and LUC%/NO ratio parameters provided the highest contribution (Table 2, Figure 2.). The cut-off level for LUC/NO ratio was 1.01, the sensitivity was 73.10%, and the specificity was 60.90%. The optimal cut-off level for the LUC%/NO ratio was 13.05, the sensitivity was 75.00 %, and the specificity was 65.20 %.

Table 2. The value of the tests in predicting cases of Diabetes Mellitus

Parameters	AUC %	Std. Error	95% CI	*p*-value
LUC	66.9	0.062	0.547-0.792	0.014*
%LUC	64.1	0.065	0.515-0.768	0.065
NO	69.8	0.063	0.574-0.822	0.006*
LUC/NO Ratio	78.2	0.052	0.679-0.884	<0.001*
%LUC/NO Ratio	78.4	0.052	0.683-0.885	<0.001*

(AUC: area under the curve; CI: confidence interval; LUC: large unstained cells)

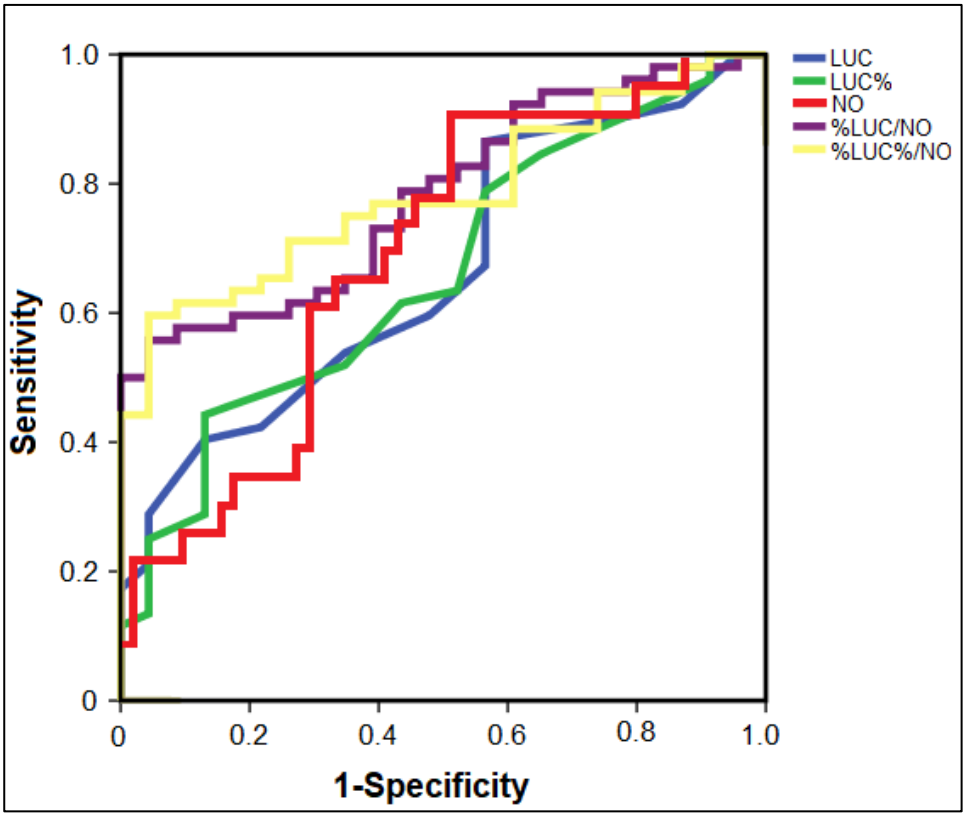

Figure 2. ROC curve of tests predicting Diabetes Mellitus

While statistically significant correlations were observed between HbA1c and NO_2, NO_3, NO, LUC/NO ratio, and LUC % /NO ratio, no correlation was found with LUC and LUC % levels (Table 3).

Table 3. The relationship between the HbA1c levels and other laboratory parameters.

		LUC	%LUC	NO_2	NO_3	NO	LUC/NO Ratio	%LUC/NO Ratio
HbA1c	*r*	0.115	-0.005	-0.507	-0.396	-0.547	0.261	0.239
	p	0.281	0.961	<0.001*	<0.001*	<0.001*	0.024*	0.038*

Discussion

DM is a chronic metabolic disorder that the metabolism of carbohydrates, fats, and proteins is disrupted due to insulin deficiency or defects in the effect of insulin on the target organs. Oxidative stress is thought to be associated not only with DM complications but also with the progression of insulin resistance. [6,16] DM leads to endothelial dysfunction and accelerated progression of atherosclerosis.[17] Inflammation is known to be a component of DM.[18] Another parameter that has recently been shown to be related to inflammatory markers is LUC. It has been emphasized in previous studies that LUC levels are significantly positively correlated with inflammatory biomarkers.[19]

LUCs are larger than lymphocytes and may be abnormal lymphocytes, peroxidase-negative blasts, or myeloperoxidase-deficient cells. Aberrations in the number of LUCs may be an indicator of some viral infections or leukemia.[7,20] In a study, it was determined that leukocyte and neutrophil counts were higher and lymphocyte counts were lower in patients with DM.[21] According to our knowledge, there is not enough information about LUC levels in patients with DM. Therefore, it is important to evaluate the relationship between DM and LUC levels. In our study, we found increased LUC levels in DM patients when compared to healthy controls. DM is an inflammatory disease.[22] The increase may be associated with the inflammation status in DM patients. The results of the study of Vozarova B et al. [23] support our findings, and it can be said that elevated leukocyte levels and elevated LUC levels can be indicators of inflammation in DM.

Another parameter associated with the development of DM is nitric oxide, which is known to be important in the maintenance of vascular endothelial functions. NO plays an important role in the regulation of systemic metabolism, energy balance, and insulin sensitivity. Endothelial dysfunction and increased oxidative stress in DM are suggested as common mechanisms in the development of the metabolic syndrome and insulin resistance.[24,25] An imbalance of oxidant-antioxidants in the body results in oxidative stress, which contributes to impaired bioavailability of nitric oxide and vascular dysfunction. It has been shown that endothelial cells cannot produce enough NO in patients with DM and do not relax in response to some vasodilators, such as

endothelium-dependent acetylcholine and bradykinin.[26,27] With these findings, it can be said that oxidative and nitrosative stress has a potential contribution to the pathogenesis of complications related to DM, where glycemic control cannot be achieved.[28] In the study of Paolo Tessari et al., it was shown that whole-body NOx synthesis was decreased in patients with type 2 DM and was not stimulated appropriately by hyperinsulinemia. In this study, it has also been shown that there is a decrease in whole-body NOx synthesis and the conversion of arginine to NOx in DM in response to insulin.[29] Similarly, in our study, we found lower NO levels in DM patients indicating impaired NOS activity and decreased NO production. Lower NO levels in our study indicated endothelial dysfunction in DM patients in accordance with the literature. The stimulation of NOS activity downregulates the effect of insulin; and, insulin resistance may be associated with decreased nitric oxide production in DM.[30]

When the healthy controls and DM patients were compared, LUC, LUC%, NO, and the oxidation products of NO (nitrite and nitrate) were statistically significant. In this study, we determined the LUC/NO percent ratio and LUC%/NO percent ratio for the first time, according to our knowledge. LUC/NO percent ratio and LUC%/NO percent ratio were also statistically significant. Various visual and statistical analyzes were performed to evaluate the effectiveness of these parameters for the diagnosis of DM. According to the analyzes performed, LUC/NO percent ratio and LUC%/NO percent ratio parameters provided the highest contribution to the diagnosis of DM patients (Table 2 and figure 2). In addition, significant correlations were observed between HbA1c, which is an important test in the diagnosis of DM, and NO, the oxidation products of NO, LUC/NO percent ratio, and LUC%/NO percent ratio parameters (Table 3).

In line with these findings, the LUC/NO percent ratio and LUC%/NO percent ratio parameters are remarkable, as they are found to be significantly higher in DM patients, making the highest contribution to the diagnosis of DM. We predict that these two parameters may be useful markers in the diagnosis and the follow-up of the disease and may provide target pathways for further studies that may contribute to the etiopathogenesis of the disease.

Ethical Considerations: The study was approved by Ankara City Hospital Ethics Committee (Date: 05.09.2019, Approval number: E-19-022).

Conflict of Interest: Authors declare that there is no conflict of interest

References

1. Diabetes Control Complications Trial Research Group. Clustering of long-term complications in families with Diabetes in the diabetes control and complications trial. Diabetes. 1997;46(11):1829-39.
2. UK Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). The lancet. 1998;352(9131):837-53.
3. İsmail K. Gycated Hemoglobin (HbA1c) determination and its utilization for monitoring long-term glycemic control of diabetes mellitus. Gülhane Tip Dergisi. 2003;45(4):387-95.
4. Rohlfing CL, Wiedmeyer H-M, Little RR, England JD, Tennill A, Goldstein DE. Defining the relationship between plasma glucose and HbA1c: analysis of glucose profiles and HbA1c in the Diabetes Control and Complications Trial. Diabetes care. 2002;25(2):275-8.
5. Yun JH, Lee H-S, Yu H-Y, et al. Metabolomics profiles associated with HbA1c levels in patients with type 2 diabetes. PLoS One. 2019;14(11):e0224274.
6. TEMD Diabetes Mellitus Çalışma ve Eğitim Grubu. Diabetes Mellitus ve komplikasyonlarının tanı, tedavi ve izlem kılavuzu. Ankara Endokrinoloji ve Metabolizma Derneği. 2022:1-324.
7. Vanker N, Ipp H. Large unstained cells: a potentially valuable parameter in the assessment of immune activation levels in HIV infection. Acta haematologica. 2014;131(4):208-12.
8. Bononi A, Lanza F, Dabusti M, et al. Increased myeloperoxidase index and large unstained cell values can predict the neutropenia phase of cancer patients treated with standard dose chemotherapy. Cytometry: The Journal of the International Society for Analytical Cytology. 2001;46(2):92-7.
9. Jensen T, Feldt-Rasmussen B, Bjerre-Knudsen J, Deckert T. Features of endothelial dysfunction in early diabetic nephropathy. The lancet. 1989;333(8636):461-3.
10. Honing ML, Morrison PJ, Banga JD, Stroes ES, Rabelink TJ. Nitric oxide availability in diabetes mellitus. Diabetes/metabolism reviews. 1998;14(3):241-9.
11. Loscalzo J. Nitric oxide and vascular disease. Vol 333: Mass Medical Soc; 1995:251-3.
12. Joost H-G, Tschöp MH. NO to obesity: does nitric oxide regulate fat oxidation and insulin sensitivity? Endocrinology. 2007;148(10):4545-7.
13. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. New England journal of medicine. 1993;329(27):2002-12.
14. Bailey SJ, Vanhatalo A, Winyard PG, Jones AM. The nitrate-nitrite-nitric oxide pathway: Its role in human exercise physiology. European Journal of Sport Science. 2012;12(4):309-20.
15. Cortas NK, Wakid NW. Determination of inorganic nitrate in serum and urine by a kinetic cadmium-reduction method. Clinical chemistry. 1990;36(8):1440-3.

16. Gorudko I, Kostevich V, Sokolov A, et al. Functional activity of neutrophils in diabetes mellitus and coronary heart disease: role of myeloperoxidase in the development of oxidative stress. Bulletin of Experimental Biology and Medicine. 2012;154(1):23-6.
17. Maiese K, Daniela Morhan S, Zhong Chong Z. Oxidative stress biology and cell injury during type 1 and type 2 diabetes mellitus. Current neurovascular research. 2007;4(1):63-71.
18. Pickup J, Mattock M, Chusney G, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia. 1997;40(11):1286-92.
19. Cakir I, Cakir N, Atalay MA, Koc AN. Large unstained cells are correlated with inflammatory biomarkers in patients with invasive aspergillosis. Turkish Journal of Biochemistry. 2018;43(3):306-11.
20. Thirup P. LUC, What is that? Clinical chemistry. 1999;45(7):1100.
21. Yan Y, Yang Y, Wang F, et al. Clinical characteristics and outcomes of patients with severe covid-19 with Diabetes. BMJ open diabetes research and care. 2020;8(1):e001343.
22. Özdemir İ, Hocaoğlu Ç. Tip 2 diabetes mellitus ve yaşam kalitesi: Bir gözden geçirme. Göztepe Tıp Dergisi. 2009;24(2):73-8.
23. Vozarova B, Weyer C, Lindsay RS, Pratley RE, Bogardus C, Tataranni PA. High white blood cell count is associated with a worsening of insulin sensitivity and predicts the development of type 2 diabetes. Diabetes. 2002;51(2):455-61.
24. Ghasemi A, Jeddi S. Anti-obesity and anti-diabetic effects of nitrate and nitrite. Nitric oxide. 2017;70:9-24.
25. Maas R, Xanthakis V, Göen T, et al. Plasma nitrate and incidence of cardiovascular disease and all-cause mortality in the community: the Framingham Offspring Study. Journal of the American Heart Association. 2017;6(11):e006224.
26. Pitocco D, Zaccardi F, Di Stasio E, et al. Oxidative stress, nitric oxide, and Diabetes. The review of diabetic studies: RDS. 2010;7(1):15.
27. Pitocco D, Zaccardi F, Di Stasio E, et al. Role of asymmetric-dimethyl-L-arginine (ADMA) and nitrite/nitrate (NOx) in the pathogenesis of oxidative stress in female subjects with uncomplicated type 1 diabetes mellitus. Diabetes research and clinical practice. 2009;86(3):173-6.
28. Ceriello A, Esposito K, Piconi L, et al. Oscillating glucose is more deleterious to endothelial function and oxidative stress than mean glucose in normal and type 2 diabetic patients. Diabetes. 2008;57(5):1349-54.
29. Tessari P, Cecchet D, Cosma A, et al. Nitric oxide synthesis is reduced in subjects with type 2 diabetes and nephropathy. Diabetes. 2010;59(9):2152-9.
30. Kashyap SR, Roman LJ, Lamont J, et al. Insulin resistance is associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. The Journal of Clinical Endocrinology & Metabolism. 2005;90(2):1100-5.