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The Evaluation of Plasma Vitamin E and Plasma Nitrite/Nitrate Anion Levels in Newly Diagnosed Type 2 Diabetes Mellitus Patients

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ABSTRACT

Aim: Oxidative stress induces the onset of type 2 Diabetes mellitus (T2DM). Both plasma vitamin E (VE) and nitrite/nitrate anion (NOx) levels might be crucial factors to determine diabetes induced endothelial damage through different mechanisms. Therefore, in this study, we aimed to evaluate plasma VE and NOx concentrations of newly diagnosed T2DM patients and to understand whether any correlation exists with the patients' lipid profiles.

Material and Methods: Blood samples were taken when the patients (n=15) and the controls (n=15) first came to our university's diabetes policlinic for investigation and were used to detect plasma VE and NOx levels. Patients' files were evaluated to record both the demographic data and biochemical parameters. The data was distributed normally according to Kolmogorov-Smirnov test. Therefore, Student t test was chosen to compare patient and control data and Pearson test was applied for correlation analysis.

Results: In newly diagnosed T2DM patients, total cholesterol (TC) and triglyceride levels were found to be significantly elevated (p=0.036, and p<0.001, respectively), whereas, HDL-cholesterol levels were significantly reduced (p<0.001). Plasma NOx levels increased in T2DM patients and this was in positive correlation with TC levels (r=0.392, p=0.032). Plasma VE levels reduced in the diabetic group. A significant negative correlation was found between plasma VE and TC (r=-0.415, p=0.022) as well as triglyceride (r=-0.380, p=0.039) levels.

Conclusion: This study demonstrates that the protective effect of VE on cell membrane decreases in T2DM. The elevation of plasma NOx levels is due to increased nitric oxide synthase levels in hyperglycemia. Although no correlation was found between plasma VE and NOx levels, they can be used as specific biomarkers in understanding T2DM induced vascular damage especially in patients with high TC levels.

Keywords: *Vitamin E, Nitrites, Nitrates, Cholesterol, Diabetes mellitus;type 2*

Yeni Tanı Almış Tip 2 Diabetes Mellitus Hastalarında Plazma E Vitamini ve Plazma Nitrit/Nitrat Anyon Düzeylerinin Değerlendirilmesi

ÖZ

Amaç: Oksidatif stres, tip 2 Diabetes mellitus (T2DM) başlangıcını indükler. Hem plazma E vitamini (EV) hem de nitrit/nitrat anyon (NOx) seviyeleri, farklı mekanizmalar yoluyla diyabetin neden olduğu endotelyal hasarı belirlemek için çok önemli faktörler olabilir. Bu nedenle bu çalışmada yeni tanı almış T2DM hastalarının plazma EV ve NOx konsantrasyonlarını değerlendirmeyi ve hastaların lipid profilleri ile herhangi bir korelasyon olup olmadığını anlamayı amaçladık.

Gereç ve Yöntemler: Hastalar (n=15) ve kontroller (n=15) üniversitemiz diyabet polikliniğine sağlık muayenesi için ilk geldiklerinde kan örnekleri alındı ve plazma EV ve NOx düzeylerinin saptanması için kullanıldı. Hasta dosyaları hem demografik verileri hem de

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biyokimyasal parametreleri kaydetmek için değerlendirildi. Veriler Kolmogorov-Smirnov testine göre normal dağıldı. Bu nedenle hasta ve kontrol verilerinin karşılaştırılmasında Student t testi ve korelasyon analizi için Pearson testi uygulandı.

Bulgular: Yeni tanı konmuş T2DM hastalarında total kolesterol (TK) ve trigliserit düzeyleri anlamlı olarak yükselirken (sırasıyla p=0,036 ve p<0,001), HDL-kolesterol düzeyleri ise anlamlı düzeyde azaldı (p<0,001). T2DM hastalarında plazma NOx seviyelerindeki artış TK seviyeleri ile pozitif korelasyon gösterdi (r=0,392, p=0,032). Diyabet grubunda plazma EV seviyeleri azaldı. Plazma EV ve TK (r=-0,415, p=0,022) ve ayrıca trigliserit (r=-0,380, p=0,039) düzeyleri arasında anlamlı bir negatif korelasyon bulundu.

Sonuç: Bu çalışma, EV'nin hücre zarı üzerindeki koruyucu etkisinin T2DM'de azaldığını göstermektedir. Plazma NOx seviyelerinin yükselmesi, hiperglisemide artan nitrik oksit sentaz seviyelerine bağlıdır. Plazma EV ve NOx seviyeleri arasında bir ilişki bulunmamakla birlikte, özellikle yüksek TK seviyeleri olan hastalarda T2DM'nin neden olduğu vasküler hasarı anlamada spesifik biyobelirteçler olarak kullanılabilirler.

Anahtar Sözcükler: *E vitamini, Nitritler, Nitratlar, Kolesterol, Diabetes mellitus;Tip 2*

INTRODUCTION

Oxidative stress is formed by an enhancement in reactive oxygen species as a result of an enhancement in free radical production, together with a decay in the antioxidant defense mechanism. Lipid peroxidation occurs by the exposure of the lipid layer of the cell membrane to free radicals. It is known that Vitamin E (VE) is localized in the cell membrane and demonstrates an antioxidant effect by inhibiting lipid peroxidation (1). Hypertriglyceridemia and the reduction in high density lipoprotein-cholesterol (HDL-C) levels are the characteristics of dyslipidemia and it is generally found in type 2 diabetics (2). The deterioration of lipid profiles causes endothelial damage mediated vascular diseases.

Under the conditions of type 2 Diabetes mellitus (T2DM), pancreatic beta cells cannot produce insulin properly or insulin resistance may occur in the target tissues of the body. International Diabetes Federation data indicate that in 2015, almost 415 million people (aged 20-79 years) worldwide were diagnosed as diabetic and this number is estimated to be 642 million by 2040 (3).

Nitric oxide synthase reacts with L-arginine to produce nitric oxide (NO). L-citrulline is formed as a byproduct. NO has a very short half-life (< 4 seconds). Before being excreted into the urine, first nitrite and then, with the effect of oxygenated haemoglobin, nitrate occurs. Detection of plasma nitrite/nitrate (NOx) levels of the patients reflect both the amount of NO formed from NO synthesis and the amount of NO stored and can be converted back to NO in case of need (4). It has been reported that the endothelial dysfunction occurs during insulin resistance, before the formation of T2DM (5). During impaired endothelial dependent vasodilatation, NO activity declines and this usually happens at the early stages of hypercholesterolemia, before the structural changes occur in the vascular wall (6). In T2DM, due to insufficient effects of plasma VE, lipid peroxidation gets triggered and the integrity of the cell membrane deteriorates. This might lead to an increase in plasma NOx levels and endothelial dysfunction occurs.

Even though hyperglycemia induced microvascular complications in T2DM has been clearly established, the mechanisms between insulin action, endothelial dysfunction, and NO production has not been cleared. In newly diagnosed T2DM patients, vascular endothelial function may be affected by the dual action of plasma VE and NOx levels. Therefore in this study, we aimed to detect plasma VE and NOx levels and to investigate whether a correlation exists between these parameters as well as the lipid profiles.

MATERIAL and METHODS

Study Population

Individuals who applied to our university's Health Practice and Research Center Endocrinology Polyclinic between September 2015 and March 2016 to be searched for Type 2 diabetes and found healthy formed our control group and the ones who were diagnosed with T2DM for the first time [fasting blood glucose level ≥126 mg/dl, 75-gm oral glucose tolerance test (GTT, 2-hour value) ≥200 mg/dl, and the glycosylated haemoglobin (HbA1C) ≥6.5%)] formed our patient group (7). Both patients (n=15) and controls (n=15) had no other disease related to organ damage, were all above 18 years of age, were not pregnant or breastfeeding, were non-alcoholic and non-smokers. In addition, patients were randomly selected among people who are not using any medications or vitamins

Biochemical Data Collection and Laboratory Analysis

On the individuals' first visit to the hospital to be diagnosed as T2DM, an additional 10 ml venous blood samples were taken into gel containing tubes to measure plasma VE and NOx levels spectrophotometrically. Plasma samples were collected into the eppendorf tubes after centrifugation the whole blood at 3500 rpm for 10 minutes. Eppendorf tubes were stored at -20 C firstly and then transferred to -80 C

deep freeze within 2 months period. The demographical data and the lipid profiles; HDL-C, low density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and triglycerides(TG) of the patients were all collected from the patients' files.

The study was approved by our University's Ethics Committee adhering to the Declaration of Helsinki (approval number 2014-157-30/09) and informed consent form was signed by all the patients and healthy controls.

Measurement of Plasma Vitamin E (VE)

Plasma VE levels were measured as described by Martinek, in 1964 (8). The principle of this method is based on the reduction of ferrous iron to ferric iron with the effect of VE and the spectrophotometrical determination of the specific color formed by 2,4,6, tripridyl-s-triazine.

Measurement of Plasma Nitrite/Nitrate (NOx) levels

Plasma NOx levels were determined according to the method of Green (9). Plasma samples were mixed with Griess reagent and incubated for 45 minutes at 37°C. The principle of the assay depends on the formation of diazonium ion with the interaction of nitrite and sulfanilamide which then formed a chromophoric azo product with the addition of N-[1-Naphthyl]ethylenediamine (9, 10).

Statistical Analysis

Statistical analysis were performed by using SPSS 19.0 software (SPSS Inc., Chicago, Ill., USA), and the distribution of data was determined by the Kolmogorov-Smirnov test. According to the results, plasma nitrite, plasma VE, TG, TC, LDL-C and HDL-C test results distributed normally. Student t test was chosen to compare patient and control data. Pearson test was applied for correlation analysis. Variables were expressed as mean ±SD. A value of $p<0.05$ was considered as statistically significant for all tests.

RESULTS

A total of 15 newly diagnosed T2DM patients and 15 controls were included in this study. There were less men than women in both groups (33.33% in T2DM patients and 40.00% in controls). Creatinine, plasma NOx and LDL-C levels were all found to be increased in the T2DM patients but the differences were not significant ($p=0.204$, $p=0.603$ and $p=0.236$, respectively). We found statistically significant differences in plasma VE levels ($p<0.005$) as well as TC ($p=0.05$), TG ($p<0.001$) and HDL-C ($p<0.001$) levels (Table 1).

According to Pearson correlation analysis, TC was found to be positively correlated with plasma NOx ($r=0.392$; $p=0.032$) and negatively correlated with plasma VE levels ($r=-0.380$; $p=0.039$), which were all significant at the 0.05 level. TG levels were found to be negatively correlated with both plasma VE levels ($r=-0.415$; $p=0.022$) (significant at the 0.05 level) and HDL-C levels ($r=-0.547$; $p=0.002$) (significant at the 0.01 level) (Table 2).

DISCUSSION

In the present study , it was aimed to determine plasma VE and NOx levels in T2DM patients and to investigate wheth-

Table 1: Differences between HbA1c groups in demographic data, creatinine, plasma vitamin E and plasma nitrite/nitrate levels, together with lipid profiles.

	HbA1C>6.5% (n=15)	HbA1C≤6.5% (n=15)	*p* value
	Mean ± SD	Mean ± SD	
Age (years)	47.72 ± 11.02	44.40 ± 10.05	NS
Sex ratio (% male)	33.33	40.00	NS
BMI (kg/m^2)	31.21 ± 3.28	28.42 ± 2.12	NS
Creatinine (mg/dl)	0.77 ± 0.13	0.72 ± 0.09	0.204
Nitrite/nitrate (umol/L)	7.89 ± 3.60	7.13 ± 4.38	0.603
Vitamin E (umol/L)	9.26 ± 1.23*	12.18 ± 3.08*	0.002
Lipid Profiles			
Total cholesterol (mg/dl)	210.93 ± 38.00*	186.87 ± 18.45*	0.036
Triglycerides (mg/dl)	196.93 ± 40.82*	115.33 ± 35.96*	0.000
LDL cholesterol (mg/dl)	138.80 ± 32.90	126.93 ± 18.97	0.236
HDL cholesterol (mg/dl)	43.47 ± 5.53*	61.53 ± 12.69*	0.000

HbA1c: Hemoglobin A1c, **SD:** Standard deviation, **BMI:** Body mass index, **LDL:** Low density lipoprotein, **HDL:** High density lipoprotein, **NS:** Non significant.

Table 2: Pearson correlation analysis of plasma vitamin E, plasma nitrite/nitrate, creatinine and lipid profiles.

Variables		Vitamin E	Nitrite/nitrate	Triglyceride	Total cholesterol	LDL-C	HDL-C	Creatinine
Vitamin E	r	1						
	p							
Nitrite/nitrate	r	-0.105	1					
	p	0.579						
Triglyceride	r	-0.415*	0.081	1				
	p	0.022	0.672					
Total cholesterol	r	-0.380*	0.392*	0.408*	1			
	p	0.039	0.032	0.025				
LDL-C	r	-0.177	-0.219	0.184	-0.116	1		
	p	0.348	0.246	0.330	0.543			
HDL-C	r	0.201	0.083	-0.547**	-0.080	-0.090	1	
	p	0.287	0.661	0.002	0.675	0.636		
Creatinine	r	-0.114	0.262	0.023	0.342	0.096	-0.212	1
	p	0.549	0.161	0.903	0.064	0.613	0.260	

er there is a correlation between these parameters and lipid profiles. Our study showed that total cholesterol levels are negatively correlated with plasma VE levels and positively correlated with plasma NOx levels which may suggest that the protective role of VE on the cell membrane is reduced in T2DM.

Insufficient insulin secretion causes hyperglycemia and this could lead to changes in plasma lipoproteins in T2DM patients. Especially in non-insulin dependent (type 2) diabetes, the lipoprotein abnormalities include hypertriglyceridemia and reduced plasma HDL-C (11-13). Similar to these findings, we also found a significant increase in TC ($p<0.05$) and TG ($p<0.001$) levels of newly diagnosed T2DM patients and a significant decrease in HDL-C ($p<0.001$) levels. LDL-C levels of our patients also increased but this increase was not significant. As explained by Goldberg (2001), the deficiency of lipoprotein lipase enzyme which converts very low density lipoprotein (VLDL) to LDL may supress LDL synthesis in untreated T2DM patients (14).

Due to its very short half-life, the quantification of NO is difficult. Therefore, in this study, stable degradation products of NO, nitrite (NO2–) and nitrate (NO3–), are measured by using Griess reagent which is defined as a reliable method (9). Plasma NOx content is assessed by several studies. According to our results, plasma NOx levels were also found to be higher than the non-diabetics, but this was not significant ($p=0.603$). We found "a positive correlation between TC levels and plasma NOx levels ($r=0.392$; $p=0.032$). In another study, basal plasma nitrate levels were found to be significantly higher in T2DM patients compared to non-diabetic subjects ($p<0.01$). This was in correlation with the increasing intima ± media thickness (IMT) levels of the carotid artery (15). In a study, it was reported that in human aortic endothelial cells, NO synthase (NOS) levels elevate with high blood glucose levels. An elevation of NOS cause an increase in blood flow and leads to reduced relaxation of blood vessels, especially, in the further stages of Diabetes mellitus (16). On the contrary, in pulmonary arterial hypertension, it was reported that the circulating NOx levels reduced almost three times in patients (17). Another study reported a reverse correlation between plasma NOx and plasma LDL-C as well as TC levels in patients with coronary artery disease. Similar to our study, the difference between patients and healthy individuals was not found significant (18).

The present study reports a significant reduction of plasma VE levels ($p<0.005$) in newly diagnosed T2DM patients. There are also other studies reporting reduced VE levels in T2DM patients (19-21). This finding might be related with the depletion of antioxidants due to an increase in oxidative stress levels. According to the results of a study conducted in Sweden, high serum VE concentration has a protective role in the development of diabetes mellitus (22). The present finding of decreased plasma VE and increased plasma NOx levels in patients with type 2 diabetes mellitus can be ameliorated by VE supplementation. Study results suggesting that type 2 diabetes patients should take VE supplements are conflicting. Mayer-Davis et al. could not establish a relationship between VE intake and diabetes development (23).

Similarly, in a study conducted in type 2 diabetes patients, 600 IU VE was supplied for ten years and it was found that the risk of type 2 diabetes development did not decrease (24). Also in another study no significant changes in plasma NOx levels and lipid profiles were found in T2DM patients given 400 IU VE for 8 weeks (25). However, many studies have reported that the risk of developing type 2 diabetes is reduced by VE supplementation. Engelen et al. reported no change in glycated haemoglobin, lipid profiles and blood biochemistry values, but a reduction in TBARS production and lipoprotein (LDL and VLDL) peroxidizability in Type 1 diabetes mellitus patients after 3 months. These effects were not found as a result of VE supplementation for an additional 3-9 months (26). As a result, it has been concluded that long-term VE supplements are not effective on diabetes.

The main limitation of our study was its small sample size. The relationship between plasma VE, plasma NOx and lipid profiles has not been previously investigated in newly diagnosed T2DM patients. Total cholesterol levels which is being detected as part of a routine laboratory analysis has negative correlation with plasma VE levels (r=-0.380; p=0.039) and positive correlation with plasma NOx levels (r=0.392; p= 0.032). This finding may help to develop new diagnostic and therapeutic approaches for T2DM patients.

To our knowledge, this is the first study that investigates plasma VE and NOx levels to understand the factors related with oxidative stress induced endothelium derived vascular changes in T2DM patients. In the light of our findings, we believe that larger studies could be designed to better understand the underlying mechanisms of T2DM induced vascular damage.

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Author Contributions

Ayse Ceylan Hamamcioglu, Zehra Safi Oz and Taner Bayraktaroglu together designed the work. **Ayse Ceylan Hamamcıoglu** wrote the draft after interpreting the data. The authors all revised the draft critically for important intellectual content. **Ayse Ceylan Hamamcioglu** prepared the manuscript for submission to the journal. They both finally approved the version to be published. The authors both agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors report no conflicts of interest.

Financial Disclosure

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Ethical Approval

The authors state that they have obtained appropriate institutional review board approval from Zonguldak Bulent Ecevit University Local Ethics Committee decision no: 2014-157-30/09) and have followed the principles outlined in the Declaration of Helsinki for human investigations. In addition, informed consent has been obtained from the participants involved in this study.

Peer Review Process

Extremely peer-reviewed and accepted.

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