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*Original Research*

# Nitric oxide levels in response to the patients with different stage of diabetes

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**Abstract:** The association between hyperglycaemia and serum nitric oxide (NO) levels has not yet been fully clarified. Thus, it was aimed to evaluate the concentration of NO metabolites (nitrate, nitrite) in diabetic patients and to find the relationship between hyperglycaemia and serum NO levels in diabetic and prediabetic patients, and control groups. In this study, 100 subjects were divided into 3 groups: healthy control (n=20), prediabetic (n=40) and diabetic (n=40). Glycemic control was assessed using glycated hemoglobin (HbA1c). Nitrate and nitrite levels were measured using the Griess reagent. NO was obtained from the sum of nitrates and nitrites. The serum NO level was higher in diabetic (53.4±2.0 µM) and prediabetic patients (51.6±1.6 µM) as compared to that in the control (45.6±1.2 µM), ($p<0.05$). The NO level was not significantly different in diabetic and prediabetic patients. Higher levels of serum glucose, insulin and HOMA-IR may be responsible for the activation of endothelial cells to increase NO levels. This study supports the role of insulin resistance in increased NO levels in both diabetic and prediabetic patients.

***Key words:*** Nitric oxide; Diabetes; Prediabetes; Hyperglycaemia; Endothelial dysfunction.

## Introduction

Type 2 diabetes mellitus (Type 2 DM) leads to endothelial dysfunction and arterial hypertension (1, 2). The incidence of DM has been expected at 382 million and going to increase to 592 million till 2035 (3). Metabolic disorders, such as Type 2 DM, are generally related to an altered glucose metabolism, insulin resistance, abnormal fasting glucose levels and impaired glucose tolerance. Moreover, prediabetes (impaired glucose regulation), which the World Health Organisation describes as impaired fasting glycemia (IFG) and impaired glucose tolerance (IGT), also seriously impairs glucose metabolism (4). The DM involved in cellular injury, and the inflammation and failure of various organs (5). Endothelial dysfunction seems to be a widespread problem in all diabetic and prediabetic patients. Endothelial cells secrete vasodilators, such as nitric oxide (NO). The impairment of NO production may occur due to hyperglycaemia and other metabolic changes (6). NO is a gaseous molecule secreted by the endothelium. It is a major modulator of endothelial function (7) and is known to have various biological effects on systems in the body (8). NO is a key regulatory molecule that has extensive metabolic, vascular and cellular effects (9, 10), and induces vasodilatation (11, 12). On the contrary, low levels of NO are useful for various physiological and cellular functions, and high levels of NO may lead to harmful effects in the cells (13, 14). Thus, NO is presumed to play an important role under physiological and pathological conditions (12). Previous studies conducted by different investigators showed altered serum NO levels in Type 2 DM (9, 15, 16). In the literature, serum NO data in Type 2 DM patients are controversial. Some studies reported increased NO levels in diabetic patients (16) whereas others reported decreased levels (9). In the present study, we have considered diabetic patients as a separate group compared to prediabetic patients and control groups. Therefore, it may be able to understand whether NO can be utilised as an assistive tool in the diagnosis and treatment of glucose metabolic impairments. This study was designed to understand the alteration in the NO levels of diabetic and prediabetic patients and to discover if hyperglycaemia can induce NO production in endothelial cells.

## Materials and Methods

### Subjects

A total of 100 subjects participated: 80 patients (39 females; 41 males) with different impaired glucose tolerances (who attended the Diabetes, Endocrine and Metabolism Unit at Fırat University Hospital) and 20 (11 females; 9 males) healthy subjects. The subjects included 3 different groups: 20 healthy subjects as the control group (age 40.5±1.6 years, height 167±2 cm, weight 70±2.0 kg), 40 prediabetic patients (age 42.2±1.0 years, height 165.6±1.4 cm, weight 81.1±2.6 kg) and 40 diabetic patients (age 39.9±0.8 years, height 165.4±1.5 cm, weight 81.1±1.8 kg).

Before participating in the study, diabetic and prediabetic patients and control subjects underwent medical screening to rule out abnormalities; this screening involved taking medical histories, physical examinations, cardiovascular risk assessments, laboratory evaluations (including lipid parameters), thyroid function tests and

serum cortisol measurements for probable endocrine pathology.

**Clinical included and excluded criteria**

Control groups had no prior history of Type 2 DM (a normal glucose tolerance), endocrine disorders, hy-pertension and any other cardiovascular diseases, and were not taking medication for any chronic medical condition. Control groups had normal fasting blood glucose, HbA1c and blood chemistry.

The diabetic patients group included diabetic patients who were chosen according to the American Diabetes Association criteria and these participating subjects has HbA1c levels as high as 6.5%.

The prediabetic patients group was defined by the fasting glucose level ≥100 and <126 mg/dl (17) or by an elevated 2-hour glucose level ≥140 and <200 mg/dl after a 75-gr glucose load on the oral glucose tolerance test in the presence of a fasting glucose level <126 mg/dl (17).

The criteria for both (diabetic and prediabetic) groups excluded Type 1 DM, gestational DM, pregnancy, clinical or laboratory evidence of liver failure, renal failure (plasma creatinine levels > 1.5 mg/dl), hormone repla-cement therapy, major depressive disorders and cancer, a history of drug use (systemic glucocorticoids and antipsychotics), thyroid and other endocrine disorders, severe acute or chronic infectious disease and systemic diseases (heart failure, liver or kidney disease, or lung disease).

All procedures were managed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study protocol was approved by the Fırat

University local ethics committee, and written informed consent was obtained from each subject at the start of the study.

**Biochemical parameters analyses**

Blood samples (5 ml) were taken from a forearm vein in the morning after overnight fasting. All samples were placed into aprotinin-containing tubes. The samples were separated by centrifugation at 4,500 rpm for 5 min at 4 °C. The separated serum was stored at -80 °C and analysed immediately at the end of the study (Hettich, Zentrifugen Universal 32 R, Germany). Samples from each subject were analysed in the same assay. Direct measurement of NO was difficult, but the serum concentration of its more stable end products (nitrite + nitrate = NO) could be considered to be a marker of the total NO synthesis (18). The total serum nitrate and nitrite were measured using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company Item No. 780001). Serum nitrate was initially converted into nitrite by nitrate reductase, followed by colorimetric detection of nitrite using a Griess reaction. NO was obtained from the sum of nitrate and nitrite.

The plasma insulin level was determined according to the electrochemiluminescence immunoassay method using an automated immunoassay analyser (Immulite 2500 Insulin, Diagnostic Products Corporation, Los Angeles, CA, USA). Serum glucose, total cholesterol, very low-density lipoprotein (VLDL), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels were determined using commercial kits in an Olympus AU600 autoanalyser. The glycemic control of the diabetic and prediabetic patients was evaluated by glycated hemoglobin (HbA1c) (i.e. the mean plasma glucose level over the preceding 2 to 3 months). HbA1c levels were measured by an autoanalyser from EDTA containing blood samples. The homeostasis model assessment of insulin resistance (HOMA-IR) was detected using computer analyses (i.e. fasting blood glucose and insulin levels).

**Statistical analyses**

Number of subjects in each group was determined by power analysis. Values were expressed by mean ± standard error. The Kolmogorov-Smirnov Z test showed that the data were normally distributed and, thus, using the parameters test was justified. The unpaired t test was used to analyse the between-group differences, and $p<0.05$ was accepted as significant statistically.

**Results**

The biochemical results for diabetic and predia-betic patients with control groups are given in Table 1.

Serum NO levels (μM) were found to be significantly higher in diabetic (53.4±2.0 μM) and prediabetic patients (51.6±1.6 μM) patients compared to those in the control (45.6±1.2 μM), $p<0.05$) subjects (Fig 1). Serum NO levels in diabetic patients were not significantly different compared to those in prediabetic patients ($p>0.05$) (Fig. 1).

The insulin (ulU/ml) levels were significantly higher in diabetic (11.2±0.8 ulU/ml ) and prediabetic patients (7.1±0.6 ulU/ml ) compared with those in the control subjects (2.9±0.3 ulU/ml) ($p<0.001$). In addition, insulin levels were significantly different in diabetic patients compared to prediabetic patients' ($p<0.001$) (Fig. 2).

The HOMA-IR (units) (Fig. 3) levels were significantly higher in prediabetic (1.9±0.1 units) and diabetic (4.9±0.5 units) patients compared to those in the control subjects (0.9±0.1 units) ($p<0.0001$).

The HbA1c (%) (Fig. 4) levels were significantly

**Table 1.** The clinical characteristics of the study groups. Fasting blood glucose ($BG_F$), postprandial blood glucose ($BG_{PB}$), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL).

| | $BG_F$ (mg/dl) | $BG_{PB}$ (mg/dl) | LDL (mg/dl) | VLDL (mg/dl) | HDL (mg/dl) | Triglyceride (mg/dl) |
|---|---|---|---|---|---|---|
| Control | 90.2±0.8 | 124.2±2 | 74.5±4.5 | 17.6±1.0 | 63.5±2.0 | 135.0±2.0 |
| Prediabetic | 113.2±1.5* | 162.4±5.9* | 121.3±6.0* | 33.6±3.5* | 45.3±1.5* | 172.8±20.0* |
| Diabetic | 174.2±11.5* | 244.0±17.8* | 125.0±5.1* | 41.7±3.7* | 40.1±1.2* | 215.5±17.6* |

Values are presented as mean (±SE).

* indicates significant differences compared to the control ($p<0.05$).

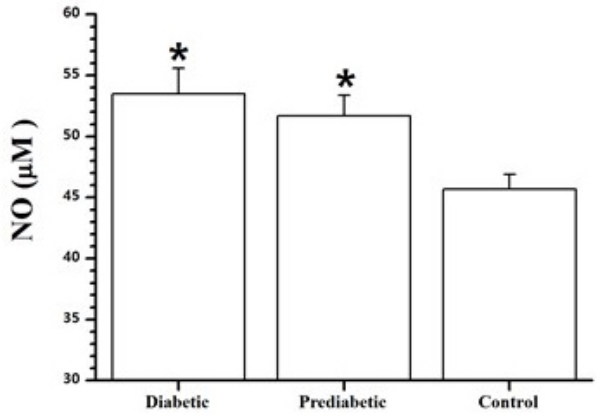


**Figure 1.** Nitric oxide levels in response to the control (C), pre-diabetic, diabetic groups. Values are presented as mean (±SE).
* indicates significant differences compared to the control levels.

higher in prediabetic (6.0±0.1 %), and diabetic patients (7.2±0.3 %) compared to those in the control subjects (4.7±0.08 %) ($p<0.0001$).

## Discussion

In this study, we examined the variation of serum NO levels in diabetic and prediabetic patients, and found higher NO levels. In the literature, increased glucose levels may stimulate NO production through the elevated expression of eNOS and iNOS protein levels (19-21). In our study, we compared NO levels in prediabetic and diabetic patients, as determined by HOMA-IR.

HOMA-IR was measured as being significantly higher in diabetic groups compared to what was measured in control groups. Importantly, we also found significant differences ($p<0.05$) in insulin resistance between diabetic and prediabetic groups, as determined by HOMA-IR. Thus, insulin resistance and impaired insulin secretion could possibly explain the significantly higher NO levels in the diabetic group (22). Despite extensive research during the last few decades, the main mechanism of insulin resistance, which is complicated, is still not fully understood (23). In the previous literature, NO levels were elevated in diabetic patients but not in non-diabetic patients who had insulin resistance (24). The NO levels were associated with the results of a previous study (24). However, other studies showed decreased NO levels (25).

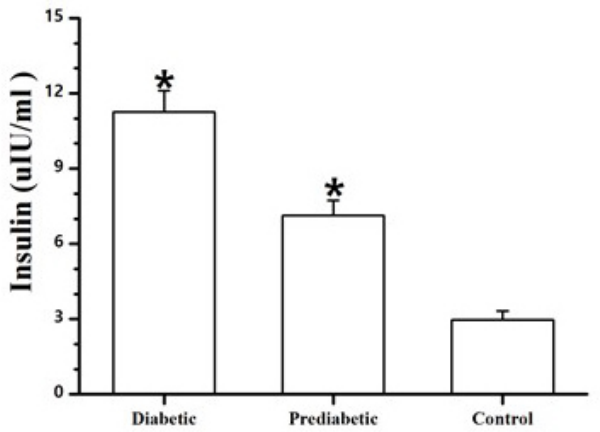


**Figure 2.** Insulin in response to the control, prediabetic, diabetic groups. Values are presented as mean (±SE).
* indicates significant differences compared to the control levels.

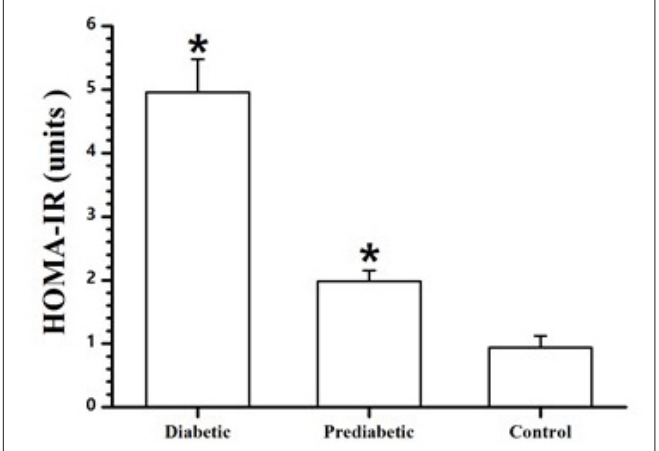


**Figure 3.** HOMA-IR in response to the control , prediabetic, diabetic groups. Values are presented as mean (±SE).
* indicates significant differences compared to the control levels.

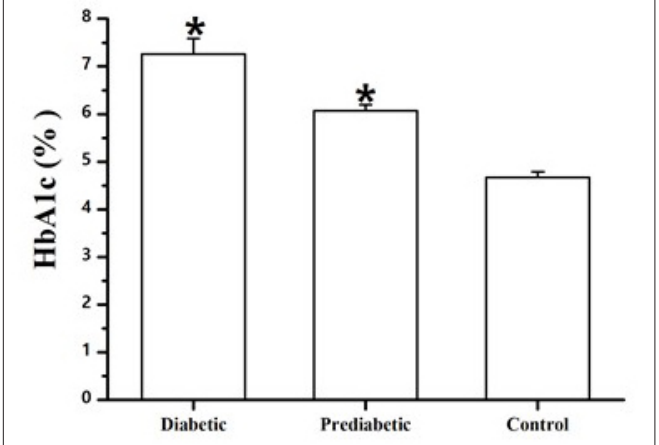


**Figure 4.** HbA1c in response to the control, prediabetic, diabetic groups. Values are presented as mean (±SE).
* indicates significant differences compared to the control levels.

Elevated NO levels in vivo might have both useful as well as deleterious effects, depending on the NO concentration. NO in physiological levels can lead to the relaxation of blood vessels and reduce hypertension, and may interact with the superoxide radical-caused inactivation of NO (26, 27). This leads to the formation of a potent oxidant radical, peroxynitrite. This may be conducive to disturbed endothelial function (26, 27). It is suggested that enhanced glucose levels in blood and hyperglycaemia may increase the NO levels in blood. The endothelium is the source of nitric oxide in blood. Endothelial function may change with hyperglycaemia in diabetic patients (28). Enhanced oxidative stress in diabetic patients is recently explained endothelial dysfunction because of the impaired release and/or action of nitric oxide via several mechanisms initiated by hyperglycaemia (16). Hyperglycaemia other than HbA1c may contribute to the increase in the NO level.

The higher NO level is related to the higher glucose level in diabetic and prediabetic patients. To confirm whether higher NO levels in diabetic patients are useful or deleterious during the disease's progression needs further study.

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