Serum inducible and endothelial nitric oxide synthase in coronary artery disease patients with Type 2 Diabetes mellitus

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Abstract. – OBJECTIVE: Coronary artery disease (CAD) is a well-known cause of morbidity and mortality in type 2 diabetes mellitus (T2DM). The role of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in T2DM patients in relation to CAD is not well understood. We examined serum inducible and endothelial nitric oxide synthase activities in patients with T2DM in relation to the presence of coronary artery disease.

PATIENTS AND METHODS: The present study was conducted in the Department of Physiology, King Saud University, Riyadh, Saudi Arabia. Subjects were grouped into control (Group A, n=87), T2DM without CAD (Group B, n=70), and T2DM patients with CAD (Group C, n=49). The selection of T2DM subjects was according to the American Diabetes Association (ADA). Serum iNOS, eNOS, hsCRP, nitrates and nitrites along with lipid profile were compared between different groups. Spearman's correlation and ROC analysis were also performed.

RESULTS: Serum eNOS levels were significantly high in the control group (112.38±47.16 U/ml) than in DM without CAD (81.43±49.91 U/ml) and DM with CAD (84.80±43.32 U/ml, $p<.001$). Serum iNOS levels were significantly higher in DM with CAD (42.87±28.83 U/ml) compared to both control (22.08±11.77 U/ml) and DM without CAD (16.24±12.30 U/ml, $p<.001$). Additionally, the differences in nitrite and NO were not significant between the three groups (34.06±24.75, 33.02±21.50, 38.83±24.34 uM, $p = .384$), and (56.51±36.78, 49.89±28.83 *vs.* 55.77±30.34 uM, $p=.416$) respectively. ROC curve analysis revealed a sensitivity and specificity of 73.5% and 68.6% of iNOS level at a cutoff point of 21.1 U/ml to predict CAD in T2DM patients. The ROC analysis for iNOS, eNOS, and hs-CRP were .782 ($p<.001$), .574 ($p=.170$), and .726 ($p<.001$), respectively.

CONCLUSIONS: Patients with T2DM have significantly higher levels of serum iNOS and lower levels of eNOS. However, iNOS levels were significantly higher in T2DM patients with concomitant CAD. Moreover, iNOS activity positively correlated with glycemic control and hsCRP. Therefore, iNOS could be an emerging future marker for CAD in T2DM patients and its antagonists could be useful in the management of these patients.

Key Words:
  Endothelial nitric oxide synthase, Inducible nitric oxide synthase, Coronary artery disease, Type 2 diabetes mellitus, HsCRP, HbA1c.

Introduction

Coronary artery disease (CAD) is one of the commonest types of cardiovascular dysfunctions. CAD is the major cause of mortality in most countries and is estimated to rise in the coming decades[1]. World Health Organization (WHO) declared that approximately 32% of deaths in 2019 worldwide were due to cardiovascular disease (CVD)[1]. According to an estimate, CAD might have caused over 11 million deaths globally by 2020 globally[2]. It is assumed that by 2030, CVD alone will surpass death rates from other infectious, maternal, perinatal, and nutritional diseases, especially in developing countries[3,4].

Endothelial dysfunction is a common feature of patients with cardiovascular diseases after hyperlipidemia and hypertension. Under normal physiological conditions, the endothelium protects the vessels against processes that lead to inflammation and thrombosis. Thus, endothelial

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dysfunction may be a common leading pathway in cardiovascular diseases. Since endothelial cell dysfunction causes inflammation and increases polymorphonuclear cells aggregation, these can increase reactive oxygen species[5]. Interestingly, obesity, diabetes, and insulin resistance could lead to endothelial dysfunction and subsequently lead to cardiovascular diseases[6]. Conversely, deficiency of endothelial nitric oxide synthase (eNOS) as a part of endothelial dysfunction can lead to metabolic disorders[7].

When glucose is elevated in the blood after a meal, eNOS will produce more NO, and hence, vasodilatation will occur. This will lead to increase blood flow and subsequent delivery of more insulin and glucose to muscle and adipose tissues[8]. Therefore, endothelial dysfunction leads to impaired glucose and insulin delivery to tissues, causing hyperglycemia and glucotoxicity. Furthermore, insulin resistance and increased levels of free fatty acid usually exaggerate endothelial dysfunction[9].

Three distinct nitric oxide synthase (NOS) isoforms, neuronal (nNOS), inducible (iNOS), and endothelial (eNOS), can produce lipophilic short-lived gas nitric oxide (NO)[10]. The fast binding of NO with oxyhemoglobin and plasma proteins precisely determines its half-life[10,11]. These bound molecules are rapidly broken down to give oxidative end products (nitrite and nitrate), excreted by the kidneys[10-12].

Reduced eNOS expression may lead to endothelial dysfunction through two mechanisms[13]. First, reduced eNOS activity may lead to reduce NO production, and secondly, due to elevated level of superoxide anion, which might lead to the breakdown of endothelial NO. This occurs due to negative feedback inactivation of eNOS by the reaction of eNOS with superoxide anion to produce peroxynitrite (a strong oxidant). Subsequently, lipid peroxidation of LDL and atherosclerosis will be triggered. In patients with cardiovascular disease, superoxide dismutase, a powerful antioxidant enzyme in the blood vessel wall, is downregulated, leading to an increased level of superoxide anion[14]. Endothelial dysfunction might initiate vascular inflammation that leads to the stimulation of inducible NOS (iNOS), which is widely distributed in tissues. Due to the increased amount of NO (produced by iNOS), more free radicals and peroxynitrite are formed, contributing to cytotoxicity and tissue injury[15].

Specifically, overexpression of iNOS might be attributed to cardiovascular dysfunction in diabetes mellitus[16]. The expression of iNOS could be a compensatory mechanism for the decreased level of eNOS and hence loss of endothelial function[17]. Suppression of iNOS activity could lead to improvement of diabetic patients and cardiac performance in cardiovascular disease patients[18]. Assessing the association between iNOS and eNOS expression in cardiovascular diseases associated with diabetes mellitus is of particular importance. Therefore, we aimed to assess serum inducible and endothelial nitric oxide synthase levels in patients with T2DM and its association with coronary artery disease.

Patients and Methods

The study was performed in the Department of Physiology, College of Medicine, King Saud University, Riyadh, Saudi Arabia. Enrollment of patients was from the outpatient clinic of the King Khalid University Hospital. All participants signed consent forms. For each participant, demographic characteristics, personal data, and family history were obtained. The study protocol was approved by the institutional review board of King Saud University Medical City (KSUMC).

American Diabetes Association (ADA) criteria was used to recruit all the subjects. About 119 subjects, including 65 men and 54 women with T2DM, were selected for study from a sample of 149 subjects. Furthermore, 87 subjects without T2DM, matched for gender, age, and weight distributions were recruited as controls. The subjects were divided into Group A (control without T2DM and CAD), Group B (T2DM without CAD, n=70), and Group C (T2DM with CAD, n=49). Patients with nephrotic syndrome, acute or chronic renal failure, thyroid disorders, acute infections, stroke, diabetic ketoacidosis, non-ketotic hyperosmolar, history of oral contraceptives, steroid intake, and familial hypercholesterolemia were excluded.

Laboratory Analysis and Assays

Fasting venous blood samples were analyzed for Triglycerides (TG), Total cholesterol (TC), high-density lipoprotein (HDL-c), low-density lipoprotein (LDL-c), fasting blood glucose (FBG), and glycosylated hemoglobin (HbA1c) levels. Lipid profiles and FBG were analyzed by an enzymatic colorimetric method using an auto-analyzer (Dimension, USA). HbA1c levels were measured

using an HbA1c Clover analyzer by reflectance spectrophotometry.

Serum Nitrate/Nitrite (Nitric Oxide) Colorimetric Assay

Because NO is a very fast degradable molecule, we measured nitrite (NO_2^-) and nitrate (NO_3^-) anions concentrations as end products of NO [Cayman Chemical Company, Ellsworth, MI, USA, (Catalog Number 780001)]. Using the Nitric Oxide Fluorometric Assay Kit of BioVision, one could easily and quite accurately measure the total nitrite/nitrate concentration. It comprises a 2-step process; firstly, with the help of nitrate reductase, nitrate is converted to nitrite. Secondly, the nitrite is then converted into a fluorescent compound by the addition of DAN led by NaOH. The precise and accurate measurement of this fluorescent compound then helps us determine the total production of NO.

Endothelial Nitric Oxide Synthase (eNOS)

These kits were supplied by USCN Life China (Catalog Number: E0868h). These kits are specified for eNOS because they are precoated with eNOS antibodies. We added samples along with a biotin-conjugated polyclonal antibody preparation which was specific for eNOS, to the microtiter plate wells. This was followed by the addition of avidin conjugated to horseradish peroxidase (HRP), to each microplate well and incubated. Next, a TMB substrate solution was added to each well. As a result, there was a color change in the wells with eNOS which were enzyme-conjugated avidin and biotin-conjugated antibody. Then, the reaction was stopped by the addition of sulfuric acid. The color change was measured through a spectrophotometer at a wavelength of 450 nm and the eNOS samples were then compared to the standard curve.

Inducible Nitric Oxide Synthase (iNOS)

The kits were supplied by USCN Life China (Catalog Number: E0837h). In this assay, a monoclonal iNOS specific antibody that is pre-coated onto a microplate was utilized. A polyclonal iNOS specific antibody was added to the wells. Afterward, an unbound antibody-enzyme reagent was removed. A substrate was added to the wells to detect the change in the intensity of the color, and color developed in proportion to the number of iNOS bound in the initial steps.

Statistical Analysis

Statistical Package for Social Sciences (SPSS Version 19, Chicago, IL, USA) was used for data analysis. Mean ± standard deviation (SD) was obtained for descriptive characteristics. Group comparisons were performed using one-way ANOVA followed by post hoc analysis for comparison within different groups. Spearman's correlations were determined between iNOS, HbA1c, and hsCRP levels. ROC curve comparison was performed for iNOS, eNOS, and hsCRP as predictors of CAD in DM. p-value < .05 was considered significant.

Results

The clinical characteristics of all groups are compared in Table I. A comparison of glycemic status, lipid profile, and cardiovascular risk markers is shown in Table II. FBS, HOMA-IR, and HbA1c were significantly increased in both groups B & C compared to group A (p<.001); however, there was no significant difference be-

Table I. Comparison of clinical characteristics between different groups.

Variables	Group A n=87	Group B n=70	Group C n=49	*p*-value
Age (years)	49.625±12.73	52.91±11.44	51.02±10.23	.715
Height (cm)	167.698±8.40	164.44±15.91	164.74±13.81	.987
Weight (Kg)	78.83±14.77	84.19±22.66	84.95±19.12	.753
BMI (Kg/m²)	28.024±4.94	29.40±5.09	29.87±4.96	.598
Pulse (beats/min)	79.24±9.34	81.26±10.64	81.90±10.34	.775
SBP (mmHg)	127.38±19.32	134.36±21.37	126.42±24.97	.100
DBP (mmHg)	76.21±12.32	80.01±15.41	72.39±15.00	.021

Group A (control), group B (T2DM without CAD), group C (T2DM with CAD), body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP). Differences were analyzed by one-way ANOVA.

Table II. Comparison of glycemic status, Lipid control & cardiovascular risk markers between different groups.

Variables	Group A n=87	Group B n=70	Group C n=49	A vs. B (p-value)	A vs. C (p-value)	B vs. C (p-value)
FBS (mmol/dl)	5.04±0.53	8.55±3.11	9.03±3.25	<.001*	<.001*	.780
Insulin (µIU/ml)	24.30±13.25	24.34±9.27	25.08±8.31	.995	.997	.946
HOMA-IR	5.49±3.31	9.43±5.18	9.89±4.55	<.001*	<.001*	.788
HbA1C (%)	5.09±0.53	7.91±1.73	7.62±1.25	<.001*	<.001*	.900
TGs (mmol/L)	1.41±0.63	2.12±1.48	2.22±1.90	.668	.528	.951
TC (mmol/L)	4.70±0.97	4.40±1.10	4.30±1.23	.749	.966	.973
HDL-c (mmol/L)	1.11±0.21	0.98±0.08	1.14±0.53	.013*	.967	.316
LDL-c (mmol/L)	2.86±0.85	2.69±0.90	2.59±1.21	.632	.450	.949

Group A (Control), Group B (T2DM without CAD), Group C (T2DM with CAD), nitric oxide (NO), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS), high sensitivity C-reactive protein (hs-CRP). Differences were studied using ANOVA with post hoc analysis. *Significant.

tween groups B and C (Table II). There were no significant differences in other parameters, as observed in Table II.

The results for NO and its isoforms are summarized in Table III. The iNOS levels were significantly elevated in group C (42.87±28.83 U/ml), followed by group A (22.08±11.77 U/ml) and group B (16.25±12.31 U/ml). Similarly, hsCRP levels were significantly elevated in group C than in groups A and B. Contrarily, eNOS levels were significantly elevated in group A than in the other two groups, and there was no significant difference between groups B and C. Nitrate levels were significantly elevated in group A compared to other groups. Additionally, the differences in nitrite and NO were not significant between the three groups (34.06±24.75, 33.02±21.50, 38.83±24.34 uM with p=.384), and (56.51±36.78, 49.89±28.83 vs. 55.77±30.34 uM, with p=.416) respectively (Table III).

Spearman's correlation showed a significant association among iNOS and glycosylated hemoglobin (HbA1c) (r=.206, p<0.05) and hsCRP (r=.330, p<.001), as shown in Figure 1 and 2, re-

spectively. ROC curve analysis (Figure 3) showed a sensitivity of 73.5% and specificity of 68.6% of iNOS level at a cutoff point of 21.1 IU/ml to predict CVD in DM patients. Table IV showed the ROC analysis of iNOS, eNOS, and hsCRP in patients with CAD (group C) and those without CAD (group B).

It revealed that area under the curve (AUC) values for iNOS, eNOS, and hsCRP were 0.782 (p<.001), 0.574 (p=.170), and 0.726 (p<.001), respectively.

Discussion

The present study highlights the importance of iNOS and eNOS measurements in diabetic patients in relation to CAD. Some studies[19,20] show that suppression of iNOS activity could lead to improvement of diabetic patients with CAD.

Several pathways have been identified that are linked with an increase in oxidative stress of the cells, including derangements in hexosamine biosynthetic pathway, poly-ADP ribose polymerase,

Table III. Comparison of nitric oxide and its isoforms between different groups.

Variables	Group A n=87	Group B n=70	Group C n=49	A vs. B (p-value)	A vs. C (p-value)	B vs. C (p-value)
Nitrate (µM)	23.11±18.76	17.59±9.09	16.94±8.96	.052	.033*	.973
Nitrite (µM)	34.06±24.75	33.03±21.51	38.83±24.34	.990	.625	.456
NO (µM)	56.52±36.79	49.89±28.84	55.78±30.34	.503	.994	.643
hsCRP (mg/L)	3.73±2.50	3.26±1.69	5.18±2.76	.423	.009*	<.001*
eNOS (U/ml)	112.39±47.17	81.43±49.91	93.39±43.22	<.001*	.057	.421
iNOS (U/ml)	22.08±11.77	16.25±12.31	42.87±28.83	.009*	<.001*	<.001*

Group A (Control), Group B (T2DM without CAD), Group C (T2DM with CAD), nitric oxide (NO), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS), high sensitivity C-reactive protein (hs-CRP). Differences were studied using ANOVA with post hoc analysis. *Significant.
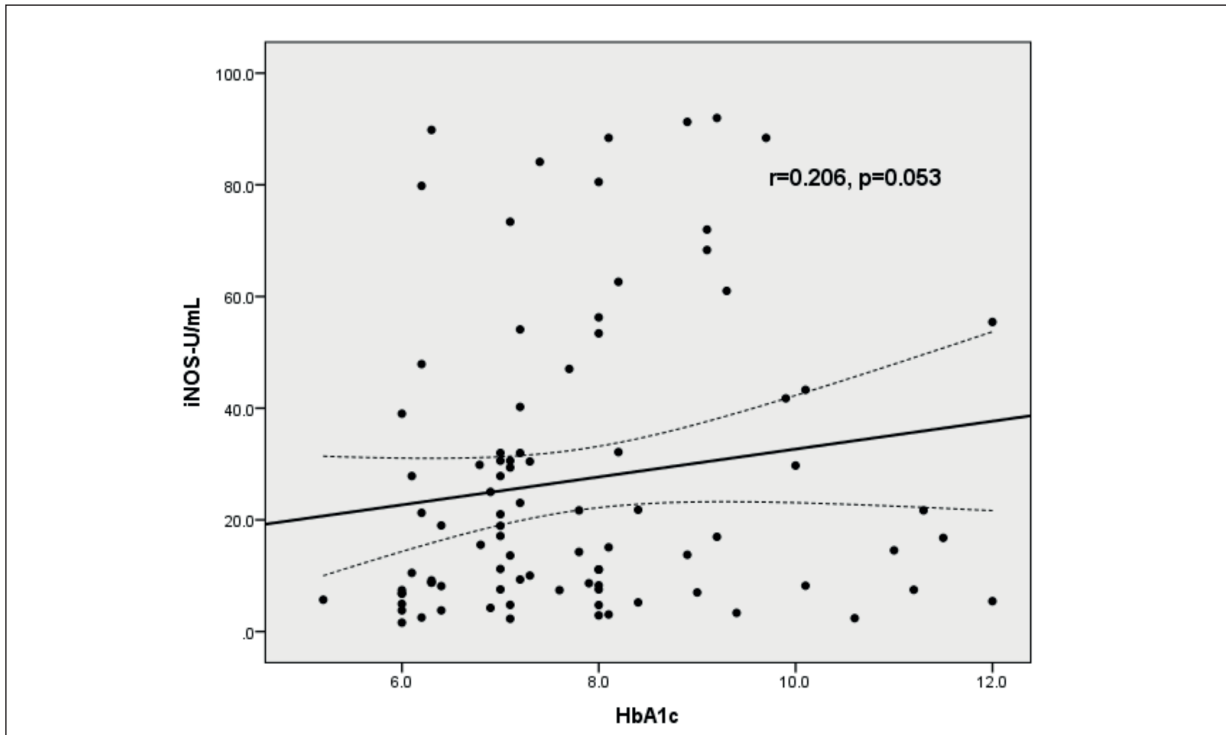
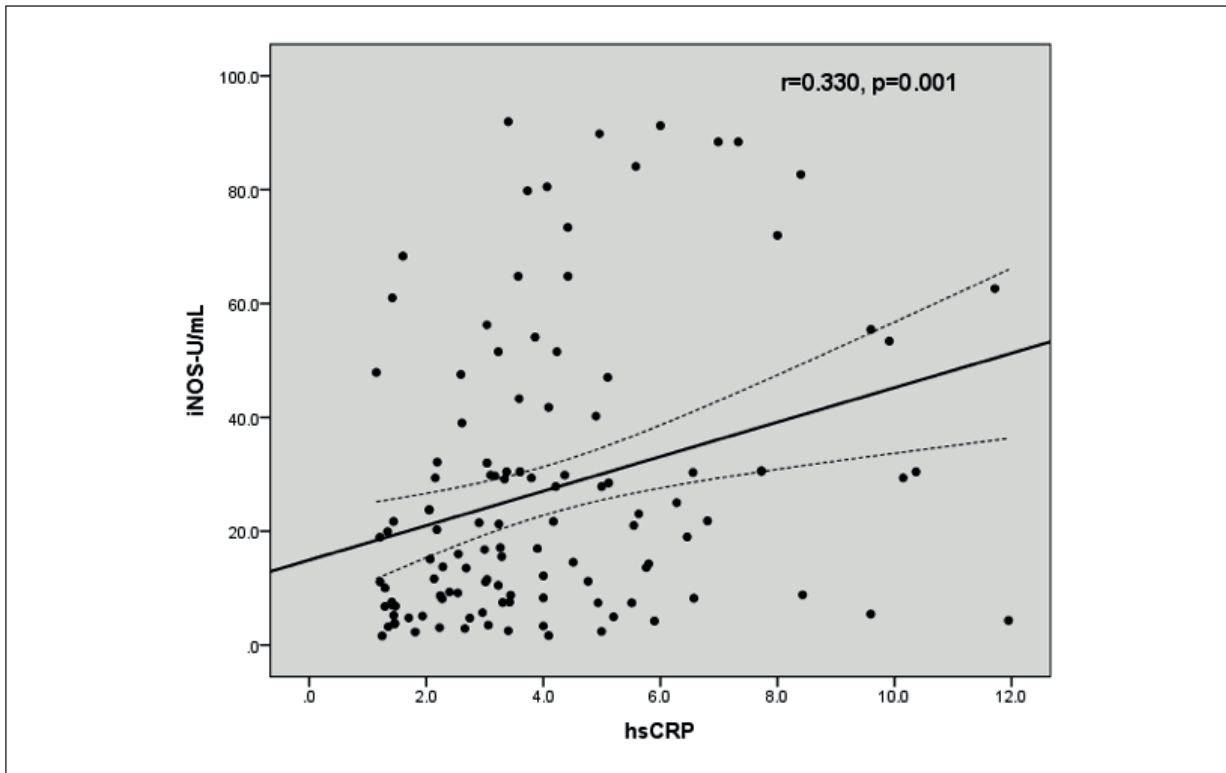**Figure 1.** Spearman's correlations between serum iNOS and HbA1c in all T2DM patients.

Figure 2. Spearman's correlations between serum iNOS and hsCRP in all T2DM patients.
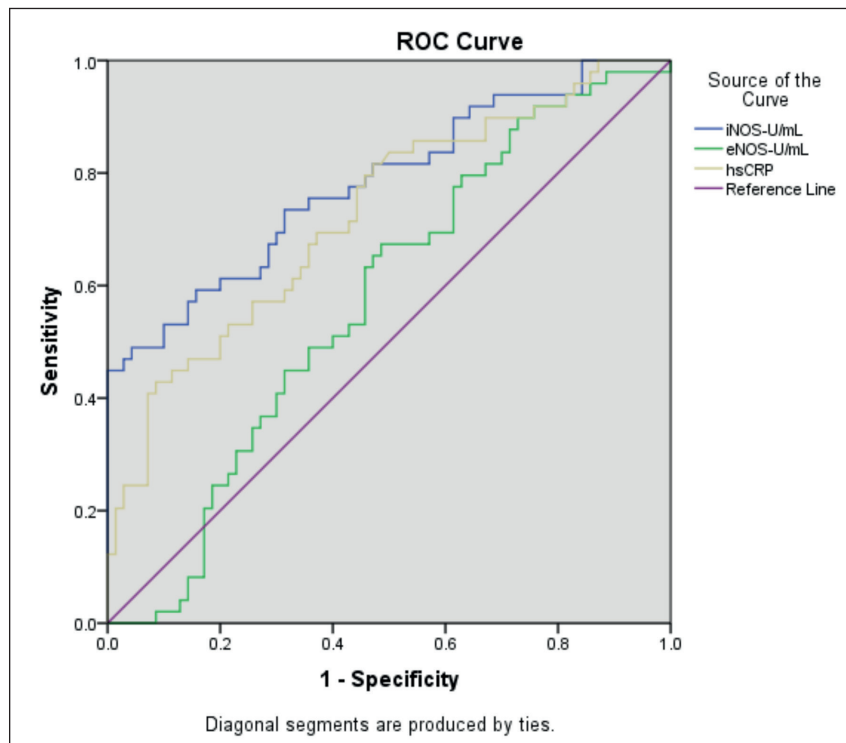
Figure 3. ROC analysis between serum iNOS, eNOS, and hsCRP in T2DM patients with CAD (group C) and without CAD (group B).

and protein kinase C pathways. Subsequently, oxidative stress eventually increases the production of reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, and peroxides. If ROS levels are elevated, they might overcome or exceed the antioxidant defense mechanisms by superoxide dismutase, glutathione, and ascorbic acid. Although the mechanisms of macrovascular complications are not well identified, elevated level of glucose and endothelial dysfunction of vascular smooth muscle is well proven. Due to the pernicious effects of hyperglycemia on endothelial injury, macrophage-monocyte activation, inflammatory cytokines, and growth factors, there is accelerated progression of microvascular and macrovascular complications of diabetes[20].

The major role of increased iNOS expression in various cardiovascular disorders, such as heart failure, cardiomyopathies, and arteriosclerosis has been well documented[15]. Specifically, an increase in iNOS expression and a decrease in eNOS expression is associated with atherosclerosis[21]. However, both eNOS and iNOS produce NO. However, NO produced by eNOS could be a useful tool, while NO produced by iNOS could have harmful effects[5,22,23]. This observation is in line with our study. Endothelial NO can inhibit atherosclerotic plaque formation, such as monocyte adhesion, platelet aggregation, and vascular smooth muscle cell proliferation[5,22,23]. In addition, endothelial NO plays a protective role exerted in the vascular wall against oxidative stress[24]. On the other hand, the rise of NO could be part of many disorders other than cardiovascular diseases, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease[25,26]. Using selective iNOS inhibitors is of value as a treatment approach[25,26].

In this study, we examined the association of iNOS and eNOS in CAD in T2DM and we found that elevated iNOS level was associated with CAD in diabetic patients. Our results were consistent with the results of others who identified the detrimental effect of increased iNOS level in correlation with various cardiovascular[15,21] and other diseases[25,26]. In our results, elevated iNOS level in CAD patients was accompanied by elevated hsCRP level in the same group, which is a well-known predictor of cardiovascular disorders[27-29]. Moreover, a significant correlation between iNOS and hsCRP was observed in all T2DM subjects.

Table IV. ROC analysis of iNOS, eNOS, and hsCRP comparing patient with CAD (group C) and without CAD (group B).

Test Result Variable(s)	AUC	p-value	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
iNOS-U/ml	.782	<.001	.696	.868
eNOS-U/ml	.574	.170	.471	.677
hsCRP mg/L	.726	.001	.634	.819

AUC: Area under the curve.

In addition, Liu et al[30] focused on the association between the eNOS -786C>T polymorphism and CAD, and this polymorphism might be a marker for the risk evaluation of CAD.

We found that there is an increase of NO production (even though it is not statistically significant) in CAD patients when compared to T2DM without CAD, which could be produced by the highly activated iNOS. This suggests that the increase in NO concentration in CAD patients has detrimental effects rather than the protective role of NO.

Weakness and Strengths of the Study

The diagnosis of coronary artery disease was based on hospital records and not based on angiography. The size of the sample and the design of the study (cross-sectional) can be considered the weaknesses of our research. The strength is that no study has previously reported these observations. Evaluation of iNOS and eNOS expression and their association with cardiovascular dysfunctions of diabetic patients is of particular importance and may open a new door for therapeutic interventions.

Conclusions

Patients with T2DM have significantly higher levels of serum iNOS and lower levels of eNOS. However, iNOS levels were significantly higher in T2DM patients with concomitant CAD. However, iNOS activity positively correlated with glycemic control and hsCRP. Therefore, iNOS could be an emerging future marker for CAD in T2DM patients and its antagonists could be useful in the management of these patients.

Conflict of Interest

The authors declare that they have no conflict of interest.

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