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Determination of serum nitrite and endothelial dysfunction in angiographycally proven coronary artery disease

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Abstract

Endothelial dysfunction can be determined by observing the reduction of the endothelium-derived vasodilators and by local rise in antagonists to these substances and/or by an union of these two factors. Nitric oxide (NO) is a free radical and a bioactive molecule that plays important roles in the regulation of vascular tone, immune system function neurotransmission. NO is biosynthesized from the amino acid L-arginine by nitric oxide synthase (NOS) in vascular endothelial cells. The study was carried out with the aim to assess endothelial dysfunction in angiographically proven cases by using serum nitrite (Nitric oxide) in coronary artery disease (CAD). Present study was conducted on 231 patients with angiographically proven CAD. The control consisted of 40 healthy subjects (26 males and 25 females). A total of 231 CAD patients of both sexes aged 18 to 75 years were included. In this study, a simple, cost-effective, and accurate HPLC method for the determination of serum nitrite was done on ULTIMATE 3000 (Dionex-USA). There was a significant decrease in Serum Nitrite level (CAD: 8.98 ± 4.45 µmol/L; Control: 18.86 ± 4.04 µmol/L) in CAD patients ($p < 0.0001$ S). A biological link between the endothelial damage and atherosclerosis in presumably is related to the decreasing arterial bioavailability of Nitric oxide vide increased in the leukocyte, platelet adhesion, vasoconstriction and smooth muscle cell proliferation.

Keyword: Serum nitrite, Endothelial dysfunction, HPLC, Coronary Artery Disease

Introduction

Coronary artery disease (CAD) is the prominent cause of morbidity and mortality in developed countries. Over many years, atherosclerosis causes coronary artery disease which presents clinically as stable angina or acute coronary syndrome. Traditional risk factors for atherosclerosis are growing age, hypertension, diabetes mellitus, hyperlipidemia, smoking, and family history of cardiovascular disease (CVD). In addition to these

traditional risk factors, identification of novel markers of increased cardiovascular (CV) events such as acute coronary syndrome may help in the finding and risk stratification of patients with CAD.

The fundamental feature of this condition is the hammered NO bioavailability. This can be the consequence of either a decreased production by the endothelial Nitric Oxide Synthase (eNOS) or, more frequently, of an excessive breakdown by Reactive Oxygen Species (ROS)[1]. In the presence of impaired NO bioavailability, the endothelium implements various physiological pathways in the attempt to balance for NO deficiency.

Endothelial dysfunction is defined as a change towards injurious processes and it contributes to vasospasm, vasoconstriction, excessive thrombosis, and abnormal vascular proliferation[2]. Endothelial dysfunction is intended to be the key event in the pathogenesis of conditions like atherosclerosis and hypertension. It may be present in asymptomatic individuals who would develop hypertension, diabetes, and cardiac associated problems in future.

Endothelial dysfunction is also seen in patients with a family history of early CVD and no other risk factors [3], hypertriglyceridemias [4], elevated LDL and reduced HDL cholesterol [5], nicotine use [6], obese patients with minimal coronary artery disease [7].

In Endothelial dysfunction, several factors may damage endothelial cells, which include integrity by inhibiting the platelet aggregation, leukocyte endothelium adhesion, and vascular smooth muscle proliferation. NO produced in cardiac smooth muscle cells monitors cardiac contractility [8, 9]. For NO to manage normal vascular physiology, its suitable levels have to be formed. NO is produced from amino acid L-arginine and molecular oxygen by one of three nitric oxide synthases (NOS): neuronal NOS (nNOS, NOS1), endothelial NOS (eNOS, NOS3), and inducible NOS (iNOS, NOS2).

Decreased nitric oxide bioactivity may cause the narrowing of coronary arteries during exercise or mental stress and provides stimulation of myocardial ischemia in patients with coronary artery disease. Additionally, reduced nitric oxide bioactivity may accelerate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, the precursor of the atherosclerotic plaque.

We think that the different data on the link between NO and CAD are because serum NO levels might be affected by endothelial dysfunction. Hence we plan this study with the aim to assess endothelial status by determining the level of serum nitrite in angiographically proven patients with CAD.

Aim and Objectives

Aim: To determine quantitatively Serum nitrite in CAD patients.

Objectives: To estimate the level of Serum nitrite in Serum nitric oxide (NO) to assess endothelial dysfunction in angiographically proven depicting severity of atherogenesis.

Materials and methods

In a present cross-sectional descriptive study, a total of 231 angiographically proven CAD patients with the age from 18 to 80 years including men and women admitted at MGM Medical College and Hospital, Aurangabad were taken. A healthy 40 volunteers of the similar age were also recruited as normal control. The patients with a history of primary cardiac disease, rheumatic diseases, intestinal diseases, parenchymal liver and sepsis were excluded from this study.

Collection of Blood: Blood samples from each participant were collected into 3.5 mL serum separator tubes in a fasting state. Serum Nitric oxide levels are

estimated by HPLC on ULTIMATE 3000 (Dionex-USA).

Method: There are several *methods for* quantifying *nitrite* in *various* biological samples. The methods include Griess reaction, Chemiluminescence, flu electrochemical probes, Spectrophotometry, electron paramagnetic resonance, electrophoresis high-performance liquid chromatography, gas chromatography and ion chromatography. Among these reported methods, the HPLC methodology with the advantages of high sensitivity was widely applied.

The present method adopted High-performance liquid chromatography (HPLC) for the estimation of quantitative nitrites in biological fluid like serum in study.

Purpose of Examination: was to study the quantity of nitrite, by HPLC technique in serum of angiographically proven CAD patients. Nitric oxide production is essential for overall health because it allows blood, nutrients and oxygen to travel to every part of your body effectively and efficiently. In fact, a limited capacity to produce nitric oxide is associated with heart disease.

Principle of Nitric oxide by HPLC: The purpose of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase.

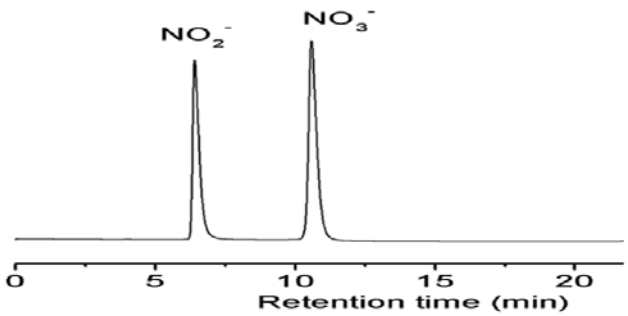
Instrumentation and Reagents: Sample analysis was performed on Ultimate 1200 Dionex (USA) by HPLC method and potassium nitrate and sodium nitrite of analytical grade (HPLC grade) were purchased, potassium dihydrogen phosphate, Disodium hydrogen phosphate, Sulfuric acid, methanol, and distilled water of analytical HPLC grade, tetrabutylammonium perchlorate (TBAP) and syringe filter.

Ultimate 1200 Dionex (USA) systems equipped with a vacuum degasser, an auto sampler, a quaternary pump. The chromatographic separation was performed for 15 minutes on a reversed phase C18 column. The mobile phase used in HPLC method consisted of methanol and water in a ratio of 2:98 by volume, while the water used in mobile phase contained TBAP of 2.5 mM concentration and 0.60 mM phosphate salt (potassium dihydrogen and disodium hydrogen phosphate). Flow rate of mobile phase was 1 ml/minute.

The serums were centrifuged at 5,000 rpm for 10 min after standing at −20 °C for 30 min. The clean supernatants were concentrated under vacuum; then the dried samples were redissolved in 250 µL deionized water, the supernatant was filtered by syringe filter to prevent unwanted spike in graph and immediately analyzed by HPLC.

Nitrite is demanding to be examined for a larger number of interferences. Nitrite is obtained by the following strategy: Each sample (10 ul) was injected twice for HPLC analysis, the amount of nitrite can be calculated as the difference between injections 2 and 1. The UV wavelength was set at 210 nm.

Graph 1: Distribution of Peak Area and Retention Time of Standard



Where spiked with serum samples of known concentrations were estimated. In the Chromatogram

(Graph no 1) the standard peak were observed with 7 minutes as a retention time. These results shown that high reproducibility, accuracy and reliability of our method to estimate serum nitrite.

Calibration curves for nitrite were generated by plotting the peak areas against the concentrations of the standards injected. Each data point was an average of duplicate injections.

Statistical Analysis: This data was compiled in Microsoft excel sheet and the master sheet was prepared. For analysis of this data SPSS (Statistical Software for Social Sciences) software version 25th was used. Data was presented by visual impressions like bar-diagram, pie-diagram, etc. Qualitative was represented in form values. Quantitative data was represented in the form of mean and standard deviation.

Results

Results are presented in Tables no.1-7. The present study was designed to evaluate the vascular endothelial function by studying the metabolic products of nitric oxide. The present study showed significantly low concentrations of serum nitrite (8.97 ± 4.45, $p < 0.0001$) in CAD patients as compared to normal healthy subjects (18.86 ± 4.04).

Table 1: Baseline clinical and demographic characteristics of CAD & Non-CAD

Continuous Variables	CAD Patients (n=231)	Control (n=40)	p-value
Age (years)[a]	56.27 ± 9.93	50.77 ± 8.78	P =0.0004 S
BMI[a]	26.33 ± 4.41	22.3 ± 1.44	P =0.0001 S
Systolic BP (mmHg) [a]	119.7 ± 15.05	120 ± 10.8	P=0.879 NS
Diastolic BP (mmHg) [a]	78.08 ± 7.48	80 ± 8.5	P=0.261 NS
Serum Nitrite (NO) (µmol/L) [a]	8.97 ± 4.45	18.86 ± 4.04	p < 0.0001 S
Categorical Variables			
Male[b]	189 (81.8%)	26 (52%)	--
Female [b]	42 (18.2%)	24 (48%)	
Smokers [b]	91 (39.4%)	--	--
Alcoholic[b]	66 (55%)	--	--
Tobacco Chewers [b]	73 (31.6%)	--	--
Chest pain[b]	217 (93.9%)	--	--
Obesity[b]	133 (57.6%)	--	
Clinical History of			
Diabetes[b]	57 (24.7%)	--	--
Hypertension[b]	91 (39.4%)	--	--

S = significant; NS = non-significant. Significance was calculated using Independent samples t-test at 95% Confidence Interval. A P-value of less than 0.001 was considered significant.

[a] Data are presented as Mean ± SD; [b] Data are presented as number of subjects (Percentage)

Table 1 - shows the clinical characteristics of control and CAD patients. There was a significant decrease in serum nitrite (NO) levels (CAD: 8.97 ± 4.45 µmol/L; Control: 18.86 ± 4.04 µmol/L) in CAD patients ($p < 0.0001$ S). The body mass index (BMI) in CAD subjects (26.33 ± 4.41) was significantly higher ($p<0.0001$) than that of controls (22.3 ± 1.44). The total number of obese cases in categorical variables was 133 (57.6%) and the number of complaints of chest pain was 217 (93.9%) in 231 CAD cases. No significant difference was seen between the systolic and diastolic BP of CAD patients and controls.

Table 2: The concentration and Recovery% of Nitrite in human serum by HPLC Method

No.	Sample	Retention Time	Area	Rel. Std. Deviation (RSD)	Recovery	Height	Relative Area
Unit		Minutes	mAU* min	%	%	mAU	%
37	Serum	7.133	0.185	0	100	0.207	96.53

Table No 2 shows, in the chromatograms Nitrite peaks were observed with 7.133 minutes as retention time with 100 % recovery.

Table 3 : Distribution of patient according to coronary vessel disease for serum nitrite

Serum nitrite (umol/L)		
Single Vessel Disease (n = 120)	Double Vessel Disease (n =52)	Triple Vessel Disease (n =59)
No of CAD Cases (%)	No Of CAD Cases (%)	No of CAD Cases (%)
93 (85.83 %)	48 (75%)	50 (86.44 %)
***p*-value = 0.11**		

Values are presented as number of CAD patients (%).

Table – 3 Shows the distribution of serum nitrite as per vessel disease. They are grouped single, double, and triple vessel disease. The maximum patients were single vessel disease 93 (85.83 %) and the minimum is with double vessels 48 (75%).

Table 4: Pearson's Correlation between serum nitrite levels in CAD and Controls

Variables	r-value	p-value
Control Serum Nitrite (NO) v/s CAD NO	-0.008	0.96 NS
Serum Nitrite CAD V/S Age CAD	0.172	0.009 S

S = significant; NS = non-significant. P value less than 0.01 was considered as ssignificant

Table- 4 shows there is a significant positive correlation between serum nitrite (NO) and the age of CAD patients. We did not find any significant correlation between the serum NO levels in CAD patients and healthy controls.

Table 5: ANOVA test for serum levels of NO in CAD patients with different levels of vessel disease

Parameters	Vessel Disease			ANOVA test	
	Single(n=120)	Double (n=52)	Triple (n=59)	F	p-value
Serum nitrite	9.301 ± 4.22	7.84 ± 3.91	9.32 ± 5.2	2.224	0.11

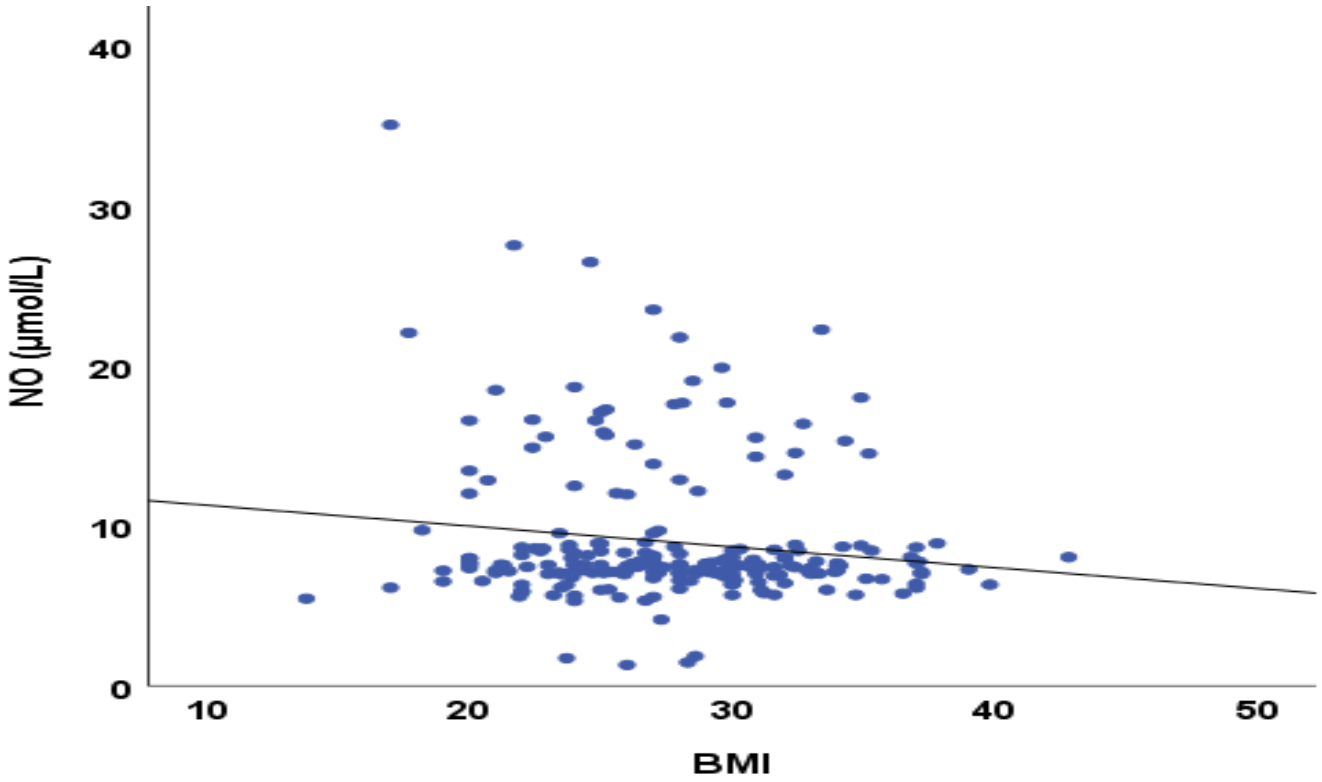
Table-5 shows the analysis of variance (ANOVA) test indicated that no significant variance in Serum nitrite level was found between the three categories of vessel diseases. The serum NO levels were seen to be comparatively low in double vessel disease as shown in graph no. 1, but it was not statistically significant.

Table 6: Chi-Square test between vessel disease and addiction categories

Addiction to CAD cases	Coronary vessel disease				χ^2(Chi-Square)	df	p-value (2-sided)
	Single	Double	Triple	Total			
Smoker	43	22	26	91	1.362	2	0.506
Non-smoker	77	30	33	139			
Total	120	52	59	231			
Alcoholic	37	14	15	66	0.657	2	0.720
Non-Alcoholic	83	38	44	165			
Total	120	52	58	231			
Tobacco	41	17	15	73	1.436	2	0.488
Non-tobacco	79	35	44	158			
Total	120	52	59	231			

Table- 6 shows there was no association between the endothelial dysfunction vessel disease categories and addictions in the CAD patients. Thus addictions were not found to affect the levels of endothelia dysfunction in CAD patients. The above table also shows that the smokers were more than that of tobacco chewers and alcoholic in 231 CAD patients.

Graph 2: Linear regression graph between serum NO and BMI values in CAD patients.



Graph 2 shows negative correlation of NO and BMI is diagrammatically shown by a linear regression graph no. 2

Discussion

The present study done to assess the vascular endothelial dysfunction diagnosed angiographically proven CAD. Analysis of variance test indicated that no significant variance in Serum nitrite level was found between the three categories of vessel diseases (Table no.5). The patients were further categorized according to their addictions of smoking, alcohol, and tobacco which were compared using unpaired t-test. Among the three categories, reduction in the level of serum nitrite was observed in smokers as compared to the Non-smokers. There was no significant difference in the alcohol and tobacco category groups. Thus, we can say that although not statistically significant, smoking habits might have an impact on serum nitrite levels in CAD patient

Endothelial dysfunction in atherosclerotic coronary arteries was illustrated for the first time by Ludmer [10] et al.(1986) and its association with the bioavailability of nitric oxide was later described as well. Diminished bioavailability of NO is the most important mechanism in the multifactorial process of endothelial dysfunction and is involved in the most important cardiovascular dysfunctions.

Reduced NO may accelerate the synthesis and release of the endothelin and proinflammatory cytokines, the release of growth factors, hyperplasia, and migration of the smooth muscle cells and thrombocyte adhesion to the endothelium. All these outcomes of endothelial dysfunction are significant in the initiation, progression, and clinical manifestation of atherosclerosis. The study postulated that the reduced Nitric oxide levels could be found in response to raised oxidative stress in CAD patients. The result showed that Nitric oxide levels were similar to the control group. There are studies, which showed that the serum

NO levels whether decreased or not, changed in coronary artery patients[11, 12].In study which could identify the exact association of serum NO levels with coronary artery disease.

Another study was performed by Thomas Jax [13] (2006), where in375 individuals demonstrated the distribution of the baseline levels of plasma nitrite and their range in individuals with a cardiovascular risk profile (n-351) and a vascular study to assess the effect of endothelial dysfunction (n = 24) on plasma nitrite levels. In this study, they used the flow-injection analysis in combination with the Griess reagent, which is sensitive down to the nanomolar concentration. In this study, they demonstrated that plasma nitrite can be determined with sufficient reproducibility in humans, that plasma nitrite levels progressively decrease with enhanced cardiovascular risk load, and that the presence of endothelial dysfunction and its degree is reflected by the relative difference in plasma nitrite. These results of our study are in line with the results of Thomas Jax (2006) et al. and Stefan Kerber et al. who have found decreased nitrite levels in the obese group as compared to their control counterparts.

Anguo Wu [14] (2013) developed a new simple and beneficial HPLC method for determining nitrite and nitrate in most biological samples. Based on the reaction that nitrite is oxidized rapidly to nitrate with the addition of acidic potassium permanganate. Estimation of nitrite and nitrate was found by the following strategy: Each sample was injected twice for HPLC analysis, i.e. the first injection was to measure nitrate, and the second to determine total nitrate including initial nitrate and the nitrate from the conversion of nitrite with the addition of acid potassium permanganate in the sample. The amount of nitrite can be calculated as the difference between injections 2 and 1.

Sainia V [15] (2020), study was conducted in a tertiary care hospital of northern India and included 110 subjects (60 cases and 50 controls), after being approved by the ethical committee of the institution. Informed consent was taken from all the participants. Patients with a documented history of coronary artery disease (based on ECG and coronary angiography) were selected from the Out-Patient Department of Medicine, Lady Hardinge Medical College and associated hospitals, New Delhi. Age and sex-matched controls comprised of healthy volunteers with no clinical or ECG evidence of CAD and negative history of major CAD risk factors. Estimation of NO determination in plasma was performed indirectly by the measurement of stable decomposition product nitrite (NO_2), employing the Griess reaction.

The mean age of the patients in the study group was 59.46 ± 11.334 years. The study population consisted of 58% females and 42% males. Hypertension and Diabetes mellitus was present in 70% and 36% of the study group, respectively, followed by hypertriglyceridemia (34%) and smoking (26%). The NO level was found out to be significantly lower in the CAD group than in the control group ($p < 0.001$).

Conclusion

In our study, the serum levels of nitrite (NO) were markedly decreased. Therefore, we can conclude that serum nitrite levels play a role as an independent biochemical marker in CAD. This reduction can cause a reduction in endothelium-dependent vasodilatation, which can increase the risk of cardiovascular diseases.

This HPLC method was validated in terms of high recovery efficiency, repeatability short extraction time, and linearity in the range of pathological and

physiological concentrations. HPLC method adopted in the studyfor serum nitrite analysis serum nitrite analysis may greatly facilitate research in the ever-expanding field.

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Ethical Approvals

The required Ethical approval was obtained from Ethical Committee, MGM Medical College, Aurangabad.

References

1. Taddei S, Ghiadoni L, Virdis A, Versari D, SalvettiA. Mechanisms of endothelial dysfunction:Clinical significance and preventive non-pharmacological therapeutic strategies. Curr Pharm Des 2003; 9:2385-402. doi: 10.2174/1381612033453866
2. Celermajer DS. Endothelial dysfunction: does it matter? Is it reversible? J Am CollCardiol1997; 30:325–33.doi: 10.1016/s0735-1097(97)00189-7.
3. Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, Deanfield JE, et al. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. Circulation 1997; 96(10):3378–83. doi: 10.1161/01.cir.96.10.3378
4. Lundman P, Eriksson MJ, Stühlinger M, Cooke JP, Hamsten A, Tornvall P,et al. Mild-to-moderate hypertriglyceridemia in young men is associated with endothelial dysfunction and increased plasma concentrations of asymmetric dimethylarginine. J Am Coll Cardiol 2001; 38(1):111–16. doi: 10.1016/s0735-1097(01)01318-3.
5. Ford M, McConnell JP, Lavi S, Rihal CS, Prasad A, Sandhu GS, Hartman SJ, Lerman LO, Lerman A, et al. Coronary artery endothelial dysfunction is positively correlated with low density lipoprotein and inversely correlated with high density lipoprotein subclass particles measured by nuclear magnetic resonance spectroscopy. Atherosclerosis2009; 207(1):111–15.
6. Lavi S, Prasad A, Yang EH, Mathew V, Simari RD, Rihal CS, Lerman LO, Lerman A, et al. Smoking is associated with epicardial coronary endothelial dysfunction and elevated white blood cell count in patients with chest pain and early coronary artery disease. Circulation2007;115(20):2621–27.
7. Al Suwaidi J, Higano ST, Holmes DR, Jr, Lennon R, Lerman A. Obesity is independently associated with coronary endothelial dysfunction in patients with normal or mildly diseased coronary arteries. J Am Coll Cardiol 2001; 37(6):1523–28.
8. Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Ann Rev Biochem 1994; 63:175–95.
9. Liu VWT, Huang PL. Cardiovascular roles of nitric oxide: a review of insights from nitric oxide synthase gene disrupted mice. Cardiovas Res 2008; 77:19–29.
10. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986; 315:1046–51.

11. Napoli C, Ignore LJ. Nitric oxide and atherosclerosis. Nitric Oxide 2001; 5(2):88-97.
12. Soysal D, Savas S, Susam I, Cetin C, Esin G, Eser S, Sema Get al. The association of plasma homocysteine, coronary risk factors and serum nitrite in patients with coronary artery disease, vascular syndrome X and healthy subjects. Anatol J Cardiol 2003; 3(1):26-34.
13. Thomas Jax, Stefan Kerber. Plasma nitrite concentrations reflect the degree of endothelial dysfunction in human. Free radical Biology and Medicine2006; 40:295-302.
14. Wu A, Duan T, Tang D, Xu Y, Feng L, Zheng Z, Zhu J, Wang R, Zhu Q, et al. Determination of Nitric Oxide-Derived Nitrite and Nitrate in Biological Samples by HPLC Coupled to Nitrite Oxidation. Biomed Chromatogr. 2013; 76(23-24):1649-55
15. Sainia V, Bhatnagarb MK, Bhattacharjeeb J. Association of endothelial dysfunction with endothelin, nitric oxide and eNOS Glu298Asp gene polymorphism in coronary artery disease. Disease Markers 2011; 31:215-22.