Original article

Estimation of salivary nitrite in patients having hypertension

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Abstract

Introduction: Hypertension is the most common cardiovascular disease and one of the most important public health concerns all over the world. Nitric oxide (NO) is the key vasorelaxing agent produced by vascular endothelium. Nitrate and Nitrite (NO_3^- and NO_2^-) is stable end product of NO oxidation and considered as markers of endothelial dysfunction associated with hypertension.

Aim: This study was aimed to estimate NO_2^- levels in patients with essential hypertension (EH) and its correlation with Systolic and diastolic blood pressure of hypertensive patients.

Methods: Saliva samples of 50 hypertensive patients and 50 healthy subjects aged 40 years and above were taken from OPD and IPD of Department of Medicine, MMIMSR, Mullana, Ambala. Griess Reagent Kit for NO_2^- quantitation, Molecular Probes, Catalog No. G-7921 was used and the readings were obtained on a Spectrophotometric Microplate Reader.

Results/Finding: We observed relatively low salivary nitrite levels (4.56±1.87µM/ml) in patients with essential hypertension compared to healthy controls (7.78±2.29µM/ml) and the difference was statistically highly significant ($p=<0.001$). The mean level of salivary nitrite in the patients was found to be 38% less than that of the controls. We also observed a highly significant negative correlation between both SBP ($r=-0.6$, $p<0.001$) and DBP ($r=-0.5$, $p<0.001$).

Conclusion: Salivary nitrite level was found to be lower in essential hypertensive (EH) patients which was significant statistically compared to that of controls. This may be used as a diagnostic tool in the patients with EH.

Keywords: Essential hypertension, Nitric oxide, Nitrite

Introduction

High blood pressure (BP) or Hypertension (HTN) is a chronic medical state of concern due to its role in the causation of coronary heart disease (CHD), stroke and other vascular complications. It is the commonest cardiovascular disorder, posing the major public health challenge worldwide in various studies.[1] It is one of the major risk factors for cardiovascular mortality[2] and leading risk factors for coronary heart disease (CHD), congestive heart failure (CHF), stroke and end-stage renal disease (ESRD), affecting the health of millions of the people worldwide.[3] Cardiovascular diseases account for a large proportion of deaths and disability worldwide.[4] High blood pressure is a multi-factorial disease, developed by the grouping of genetic, environmental, and living factors.[5] HTN is defined as a systolic blood pressure (SBP) 140 mm Hg or greater and diastolic blood pressure (DBP) of more than 90 mm Hg. When SBP and DBP falls into two categories, for the selection of individual's blood pressure the higher categories should be selected.[2]

HTN is categorized as primary (essential) or secondary hypertension. Essential hypertension (EH) is the main form of hypertension. Essential, primary or idiopathic hypertension is characterized as high BP without any identifiable cause in which different secondary causes such as renovascular disease, renal failure, pheochromocytoma, aldosteronism or other causes of secondary hypertension are not present. This the most

common form of hypertension accounting for about 80-95% of all hypertensive patients. The underlying cause of the remaining 5-15% hypertensive patients has been identified.[6] cardiovascular risk factors such as aging, abdominal obesity, dyslipidemia, glucose intolerance; insulin resistance, diabetes, and hyperuricemia are usually related with essential hypertension.[7] Essential hypertension was proposed to be a familial disease and probably occurs due to the interaction between environmental and genetic factors. It is predominantly identified by endothelial dysfunction and increased vascular resistance due to a mismatch between endothelial-derived relaxing factor (EDRF) and contracting factors.[8] Epidemiological studies have shown that hypertension is present in 25% of urban and 10% of rural subjects in India. According to the 2001 census, there are 600 million adults in India, of whom 420 million are in rural and 180 million in urban areas and the absolute number of hypertensives in India was 31.5 million rural and 34 million urban subjects, a total 65.5 million.[9] According to the World Health Organization (WHO) and the international society of Hypertension (ISH), 600 million people globally have hypertension and nearly 3 million die every year as a direct result. HTN is responsible for 57% of all stroke deaths and 24% of all coronary heart disease deaths in India. This fact is important because hypertension is a controllable disease and a 2 mmHg population-wide reduce in BP can stop 151,000 strokes and 153,000 coronary heart disease deaths in India.[10] Blood pressure (BP) is directly associated with risks of several types of cardiovascular disease, and the associations of BP with disease risk are continuous, indicating that large proportions of most populations have non optimal BP values.[11]

Inorganic anions Nitrate (NO_3^-) and Nitrites (NO_2^-) were considered inert end products of nitric oxide (NO) metabolism. However, a new view is evolving with the accumulating evidence that nitrate and nitrite metabolism occurs in blood and tissues to form NO and other bioactive nitrogen oxides.[12] NO is synthesized by L- Arginine in the presence of enzyme Nitric oxide synthase (NOS). It converts Guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) by the activation of soluble guanylate cyclase.[6] NO, as a second messenger is of immense importance in the maintenance of blood pressure. It is a vasodilator, hence reduces peripheral resistance.[13]

NO_2^- is considered to be a major water pollutant. High concentration of NO_2^- in drinking water is hazardous to health, especially for infants and pregnant females.[14, 15] Nitrosamines are produced in the human body through reaction with amines or amides of NO_2^- and are causative agents of cancer.[16] Nitrite react with Nitrosamine formation is inhibited by antioxidants such as vitamin C and vitamin E both of which are abundant in the diet.[17] NO_2^- has various applications. It is used as a food preservative, an antibacterial agent in food industry, and also used in fertilizers, detergent, wood pulp, dye and synthetic fiber industries.[16] Increased levels of circulating NO_2^- causes vasodilatation and reduce blood pressure in healthy subjects.[18] Nitrite administration causes vasodilatation at low concentrations, and this is ultimately mediated by activation of cGMP(cyclic guanosine monophosphate)in the vascular smooth muscle.[12]

Aims and Objectives

The study was conducted in the Department of Biochemistry in collaboration of Department of Medicine, Maharishi Markandeshwar Institute of Medical Sciences and Research, Mullana, Ambala with the following aims and objectives.

1. To estimate Nitrite in saliva in patients diagnosed with essential hypertension
2. To find out correlation of Nitrite with both systolic and diastolic blood pressure of hypertensive patients.

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The selection criteria for the controls were patients with Essential Hypertension (ii) Either sex (male or female) of age 40 years and above (iii) B.P. < 140/80 mm Hg. Patients with salivary gland lesion in mouth, patients of Ischemic Heart Disease which were on nitrate medication and patients with diabetes mellitus, endocrine illness and renal failure were excluded from the study. Clearance from the ethics committee and informed consents from study subjects were taken

Material and Methods

Our hospital based study was undertaken with 50 hypertensive patients aged 40 years and above. 50 age and sex matched apparently healthy subjects were taken as control. The hypertensive patients were taken from the OPD and Wards of Department of Medicine, Mullana, Ambala. The controls participating in the study were volunteers amongst staff.

Sampling

About 5 ml of human saliva samples were collected from the patients and controls in a sterile glass container. To prevent further reduction of nitrate and nitrite after saliva sampling, the following preservation procedure was used:-

The 5ml saliva sample was mixed with 0.5ml of 1 N sodium hydroxide (NaOH) as stabilizer and added to a glass container. 0.2 ml of 0.5M Zinc Sulfate (ZnSO4) was added to an aliquot of 3 mL of the above prepared saliva sample, followed by mixing. The mixture was subsequently centrifuged at 3000 rpm for 10 minutes and only the supernatant was used. Treatment with ZnSO4 removes proteins and other substances which could inhibit chromogen formation from nitrite. Nitrite estimation in the saliva was measured by the Griess reaction method.[19]

Estimation of salivary nitrite level

Salivary Nitrite (NO_2^-) Estimation-

Griess Reagent Kit for Nitrite quantitation, Molecular Probes, Catalog No. G-7921[20] was used and the readings were obtained on a Spectrophotometric Microplate Reader.

Preparation of the Griess Reagent

Depending on the number of samples to be tested, the corresponding amount of Griess reagent was prepared by mixing together equal amount of N-(1-naphthyl)ethylenediamine and sulfanilic acid. In acidic form Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite. The diazonium salt is then united with N-(1-naphthyl) ethylenediamine, forming a deep purple azo dye compound that can be spectrophotometrically measured based on its absorbance at 540 nm.

Calibration Curve

The curve was prepared by accomplishing the following steps: - 1ml of the provided 1mM/ml stock $NaNO_2$ solution was diluted in 9ml distilled water (D/W) to make a 10 ml working solution of 0.1 mM/ml (100µM/ml) concentration. (1mM= 1000µM)

Serial Dilution: - 7 calibrators of concentrations 100, 50, 25, 12.5, 6.25, 3.12 and 0 µM/ml were prepared by serial dilution of the above prepared working nitrite solution in such a way that final volume of calibrator in each well was 150 µL. As shown in the following table-1

Nitrite Standard reference curve was plotted for accurate determination of NO_2^- levels in samples using the previously prepared calibrators. The absorbance of the nitrite-containing samples was calculated relative to the

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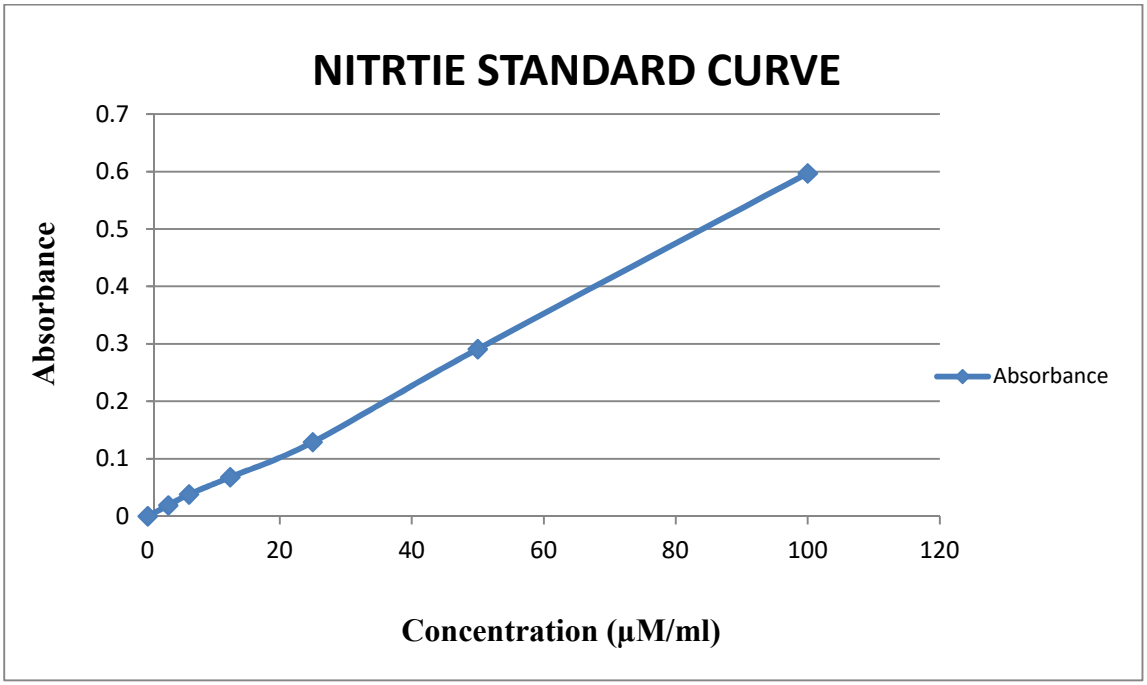
reference sample in a spectrophotometric microplate reader. The optical density was measured at wavelength of 540 nm.

Results

50 hypertensive and 50 healthy subjects age 40 years and above were selected for the study. All the statistical tests (e.g. - Mean, standard deviation, Sig. (2-tailed) t test and Pearson's coefficient) were done using SPSS software. P value of less than 0.05 was considered significant. The mean age of cases (55.46±7.53) and control (53.92±7.53) was non-significant statistically (p value = 0.31). Our study showed relatively low salivary nitrite levels (4.56±1.87µM/ml) in patients with essential hypertension compared to healthy controls (7.78±2.29µM/ml) and the difference was statistically highly significant (p=<0.001) as shown in Table-2.

Table 3 shows age distribution of the both groups. The male subjects were more in both the groups however the distribution appeared similar. The mean level of salivary nitrite in the patients was found to be 38% less than that of the controls. We also observed a highly significant negative correlation of nitrite with both SBP (r=−0.6, p<0.001) and DBP (r=−0.5, p<0.001) of hypertensive patients as shown in figure 3, 4.

Figure 1: Standard curve for nitrite level



Parameter	Controls	Cases	P value
Age	53.92±7.53	55.46±7.53	0.31
NO_2^- (µM/ml)	7.78±2.29	4.56±1.87	<0.001**

Table 1:- Comparison of mean age and salivary nitrite levels between both in cases and controls

** Statistically Significant, NO_2^- : Nitrite

Age group	Cases (%)	Controls (%)
40-49	15	17
50-59	23	20
60-69	8	11
70 & above	4	2

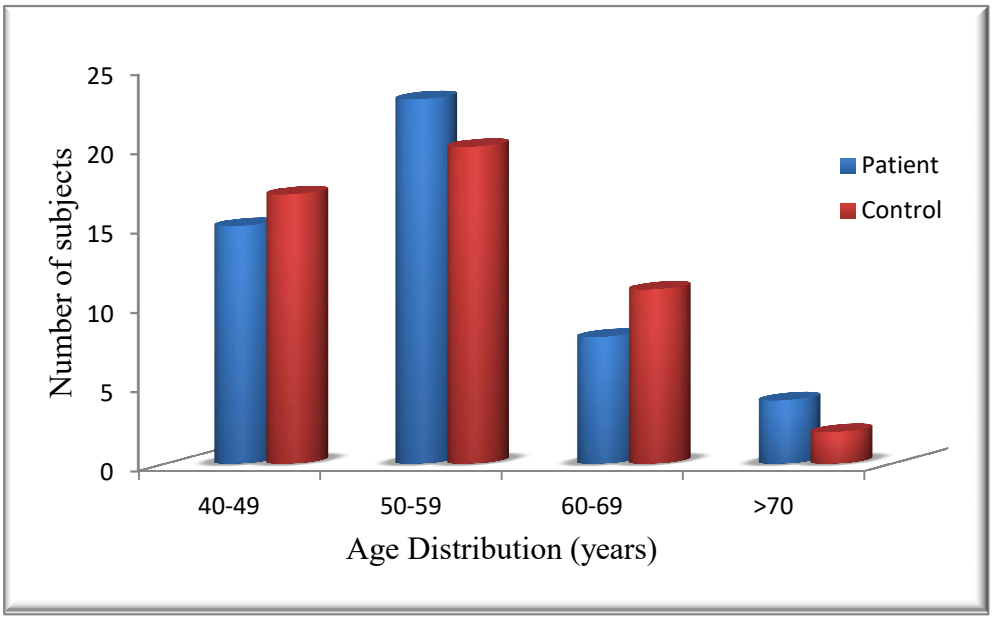


Figure 2:- Demographic age distribution of patients and controls

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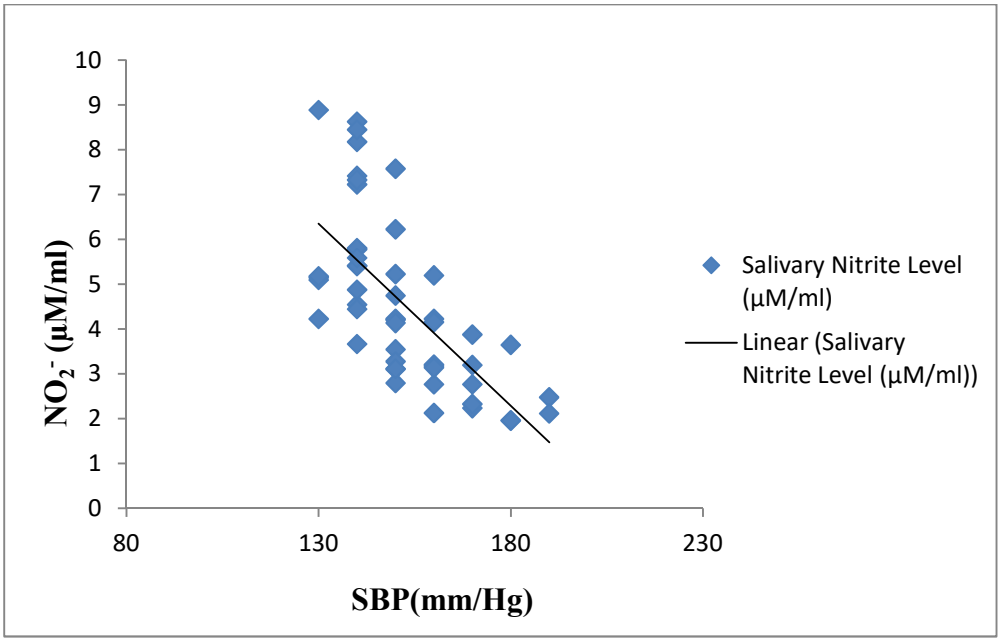

Figure 3:- Scatter diagram shows correlation between Systolic blood pressure and salivary nitrite level in cases (r = -0.673)

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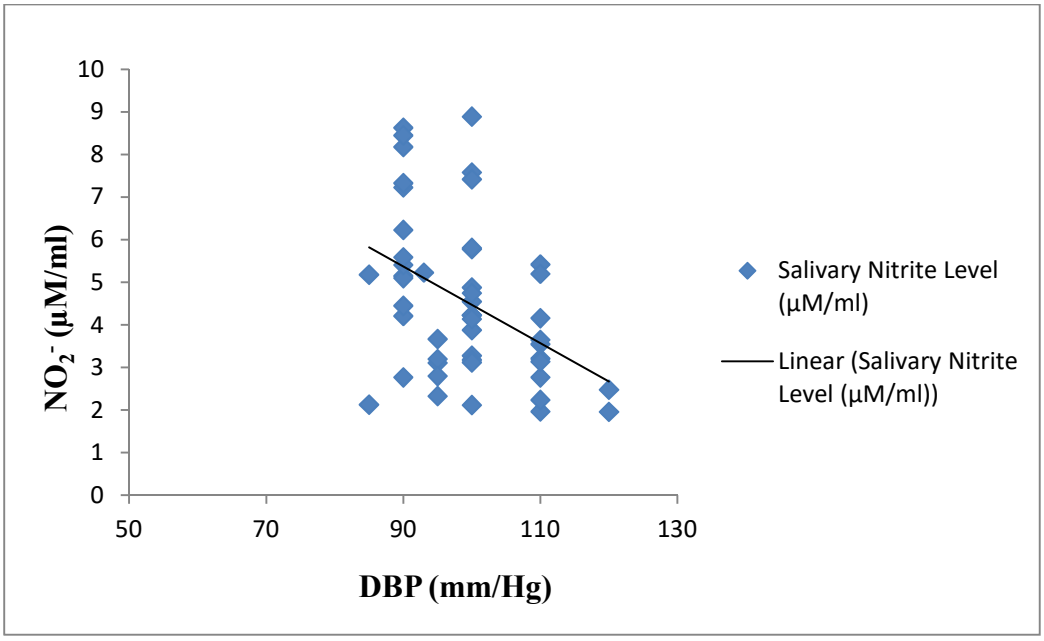

Figure 4:- Scatter diagram shows correlation between diastolic blood pressure and salivary nitrite levels in cases (r = -0.533)

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Study	Year	NO or its metabolite level in cases	NO or its metabolite in level in controls
Node et al	1997	15.7+1.1 mmol/l	22.8+1.4 mmol/l
SÖZMEN, et al.	1998	4.7±2.4 µM /L	8.1±3 µM /L
Chandra, et al.	2003	18.59±7.46 mmol/l	33.04±2.8 mmol/l
Arora, et al.	2009	4.0±1.7µM	6.7±3.2µM
Zaheera Sultana S, et al.	2014	13.06 ± 0.005 µM /L	18.001 ± 0.306 µM L
Nayak et.al.	2016	8.14±0.33 µM /L	13.53±0.38 µM /L
Our study	2016	4.56µM±1.87 µM/ml	7.78µM±2.29 µM/ml

Table 2:- Nitrite Levels in cases and controls in different studies.

Discussion

NO is continuously synthesized from L-arginine by different isoforms of NOS enzyme. NO induces vasodilatation of vascular smooth muscle cells, via stimulation of cGMP and inhibits platelets adhesion and aggregation. Further NO gets sequentially oxidized to nitrate and nitrite in the plasma. The endothelium plays an important role in the maintenance of vascular homeostasis, achieved through release of a variety of local vascular mediators. Endothelium-derived NO has been reported to control vascular tone, smooth muscle cell proliferation and growth, Thus, alterations in endothelium-derived NO bioavailability can result in endothelial dysfunction, failure of the endothelium leading to impaired vascular homeostasis and thus to the pathogenesis and clinical expression of a variety of cardiovascular diseases [21]

Collecting saliva is noninvasive, safe, and easy. It is also easier to handle during diagnostic procedures than blood as it does not clot, so it was preferred over serum for the estimation of NO_2^- in our study. Our study is well supported by the study of Arora et al (2009), [13] who found statistically significant (p=<.001) lower salivary nitrite levels in patients compared to controls. NO levels were 42% less than controls. Studies done in serum have also found lower NO level in hypertension patients. SÖZMEN, et al (1998) [22] found lower levels of nitrate and nitrite in plasma with hypertensive patients which were significantly lower compared with controls ($p<0.05$). These studies have been summarized in Table- 4

Some studies have also tried to correlate the nitrite level with blood pressure. Node [23] et al. who found a significant inverse correlation of NO with the SBP ($r=-0.68$, $p<0.005$) and DBP ($r=-0.61$, $p<0.05$) Similarly, the study of Nayak [6] et al. established that NO showed a highly significant negative correlation to both systolic ($r=-0.89$, $p<0.001$) as well as diastolic ($r=-0.64$, $p<0.001$) BP

Conclusion

Hypertension is a global health problem which affects millions of people worldwide. Arterial hypertension represents one of the most common conditions associated with endothelial dysfunction. Impaired production of

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NO from endothelium causes endothelial dysfunction which is usually related with reduced NO bioavailability. From various studies it has been confirmed that NO is a vasodilator and plays various important physiological roles in the human body. Salivary nitrite (NO_2^-) level was found to be statistically significantly lower in essential hypertensive (EH) patients compared to healthy controls. There was statistically significant inverse correlation of salivary nitrite with both systolic and Diastolic blood pressure in patients with EH. Salivary nitrite estimation is a non invasive, simple and economic method which can be useful in clinical setting. A large sample size study is needed to establish the reference value for salivary NO_2^- and the assertion of the clinical utility of this test.

Reference

1. Kokiwar PR, Gupta SS, Durge PM. Prevalence of hypertension in a rural community of central India. Int J Biol Med Res. 2011;2(4):950-3.
2. Park K, Park's Textbook of Preventive and Social Medicine. 18th ed. Jabalpur: M/s Banarasidas Bhanot Publishers; 2005. 293-8 p.
3. GARG R. What Health Problems are Associated with Hypertension? Indian J. of Clin. Pract. 2014;25(7):683-4.
4. Kreatsoulas C, Anand SS. The impact of social determinants on cardiovascular disease. Can J Cardiol. 2010;26(Suppl C):8C-13C.
5. Kantanchuvessiri A, Hypertension: A social impact disease. East J Med 2003;8(2):22-6.
6. Ranjan Nayak S, Jena I, Mishra PK, Behera S, Ray S. Evaluation of serum nitric oxide in essential hypertension and its correlation with severity of disease. Asian J Pharm Clin Res. 2016;9(2):179-82.
7. Victor RG. Arterial hypertension. In: Goldman L, Schaferai, editors. Goldman-Cecil medicine. 25th ed. Philadelphia, pa: Elsvier Publication; 2016. P. 381-96.
8. Staessen JA, Wang J, Bianchi G, Birkenhäger WH. Essential hypertension. Lancet 2003;361(9369):1629-41.
9. Gupta R. Trends in hypertension epidemiology in India. J Hum Hypertens. 2004;18(2):73-8.
10. Gupta R, Gupta VP. Hypertension epidemiology in India: Lessons from Jaipur heart watch. Curr Sci. 2009;97(3):349-55.
11. Deedwania P, Gupta R. Hypertension in South Asians. In: Izzo, Black (eds). Primer on Hypertension. American Heart Association, Dallas, USA, 2002.
12. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, et al. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol. 2009;5(12):865-9.
13. Arora S, Das N, Srivastava K. Nitric Oxide and eNOS Gene in Essential Hypertension. Int J Collab Res Intern Med Public Health. 2009;1(2):56-71.
14. Ensafi AA, Rezaei B, Nouroozi S. Simultaneous spectrophotometric determination of nitrite and nitrate by flow injection analysis Anal. Sci. 2004;1749:20.
15. Kazemzadeh A, Ensafi AA, Anal, Chim, Acta 2001;442:319.
16. Nagaraja P, Al-Tayar NGS, Shivakumar A, Shrestha AK, Gowda AK A simple and sensitive spectrophotometric method for the determination of trace amounts of nitrite in environmental and biological samples using 4-amino-5-hydroxynaphthalene-2,7-disulphonic acid monosodium salt. SAA. 2010;75:1411-6
17. Nitrate and Nitrite in drinking water. National Research Council, National Academy of Sciences. National Academy Press. 1995

18. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008 Feb;7(2):156-67
19. Diaconu R, Vasilov M, Mancaş G. Colorimetric assay of salivary nitrite content. J Med Prev. 2001;9(1):74-8.
20. Information P. Griess Reagent Kit for Nitrite Determination (G-7921). 2003;1–3.
21. Sandoo A, van Zanten JJC. V, Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. The Open Cardiovascular Medicine Journal. 2010;4:302-312.
22. Sözmen B, Kazaz C, Taskiran D, Aslan L, Akyol A, Soezmen EY. Plasma antioxidant status and nitrate levels in patients with hypertension and coronary heart disease. Turkish Journal of Medical Sciences. 1998;28:525-32.
23. Node K, Kitakaze M, Yoshikawa H, Kosaka H, Hori M. Reduced plasma concentrations of nitrogen oxide in individuals with essential hypertension. Hypertension. 1997;30(3):405-8.