International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

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International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
Pages 56-71

ISSN 1840-4529

http://www.iomcworld.com/IJCRIMPH

Paper review summary:
Paper submission date: March 2009
Paper acceptance date: April 20, 2009
Paper publication date: April 2009

International Journal of Collaborative Research on Internal Medicine & Public Health
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pp. 56-71

Nitric Oxide and eNOS Gene in Essential Hypertension

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Abstract

Background: Currently hypertension grips around 25% of the entire world population. More than 90% of the hypertensive patients suffer from essential hypertension. In Asian Indians hypertension is the predominant risk factor for Coronary Artery Disease among others. Nitric Oxide (NO) is synonymous with endothelial derived relaxation factor. Acting via cGMP (cyclic guanosine monophosphate) it causes smooth muscle relaxation, prevents platelet aggregation and acts as an anti-inflammatory agent. iNOS (inducible Nitric oxide synthase), nNOS(neuronal nitric oxide synthase) and eNOS (endothelial nitric oxide synthase) are the three enzymes producing the gas nitric oxide in the human body. eNOS is the main source of NO under physiological conditions. It is known to have a number of polymorphisms. The most well known ones being the G to T polymorphism in exon 7, the T to C polymorphism in the promoter region and the a/b polymorphism in the intron 4. While the G to T polymorphism has been associated with hypertension in many races including the north Indian population, the association of other polymorphisms has been more of a controversy. Not much study has been done on the Asians especially those in India regarding these polymorphisms.

Aims: To elucidate the association between the intron4a/b polymorphism in the eNOS gene and nitric oxide levels and essential hypertension.

Objectives:

1. To determine the genotype frequencies of the above mentioned polymorphism in patients and controls
2. To study the levels of NO in the plasma of the patients and controls
3. To find out correlation if any between this polymorphism and plasma NO levels
4. To find a correlation if any between this polymorphism and essential hypertension

Materials and Methods: The study design was a case control study. 10 ml of venous blood was taken from 45 patients (selected from the department of Cardiology All India Institute of Medical Sciences, ages between 25 to 55 yrs and not on any antihypertensive medications) and controls (healthy volunteers with normal blood pressure, ages between 25 to 55 yrs, not on antihypertensive treatment, not having any diabetes mellitus or endocrine disorder or any acute illness and responding to a publicized call. It was separated into plasma and packed cells. The alleles were identified by method of Wang et al with slight modification using PCR and Agarose Gel electrophoresis with 2.7% agarose. The nitric oxide was measured using levels of the surrogate marker, nitrite by the simple and economical method of Ding et al using Griess reagent. The Data was analyzed using SPSS software. The level of significance was fixed at $p<0.05$.

Results/Findings: The patient and control populations were found to be in Hardy Weinberg equilibrium [p value (patient)= 0.05, p value(control) >0.05] . The mean level of nitric oxide in the patients was found to be 42% less than that of the controls. The majority of patients (76%) had plasma nitrite levels below 5uM. On the other hand a majority of controls (64%) had plasma nitrite levels more than or equal to 5uM. The fraction of patients with nitrite levels below 5uM (76%) was more than double that of the controls (34%). The patients were equally distributed between the lower two groups. The controls however showed two peaks the higher one being that of greater than 7uM.
There was no significant difference (p value= 0.79) in the genotype distribution between patients and controls. There was also no significant difference between in the levels of nitrite between ab and bb genotypes in both patients and controls.

Conclusion: The levels of nitrite were found to differ significantly between patients and controls. Although no statistically significant correlation could be found in the present study a trend towards higher frequency of a allele could be envisaged among the patients of essential hypertension. A detailed study with a larger sample size is needed to establish or refute the role of this polymorphism in essential hypertension in the Indian population.

Keywords: Essential Hypertension, Nitric Oxide, eNOS gene intron 4a/b polymorphism

Introduction

Essential hypertension is an increase in the systemic arterial blood pressure without any apparent cause. It places the patient at an increased risk for target organ damage. Hypertension affects about 25% of the world population. According to a study conducted in urban areas, the prevalence of systolic and diastolic hypertension in India is 40.9% and 29.3%, respectively (Das, Sanyal, & Basu, 2005). In Asian Indians, hypertension is the predominant risk factor for coronary artery disease (CAD) of all ethnic groups. Therefore, understanding the pathophysiology of hypertension is important. More than 90% of hypertensive individuals suffer from essential hypertension. It shows an earlier onset in men than in women. The factors linked to essential hypertension are age, obesity, smoking, and stress. A strong genetic predisposition is also suggested. Blood pressure is mainly under the control of blood volume and peripheral resistance determined predominantly by the arterioles. It is influenced by hormones as well as by the local factors. Nitric oxide (NO) as a second messenger is of immense importance in the maintenance of blood pressure. It is a

vasodilator, hence reduces peripheral resistance.

Nitric Oxide (NO) is synonymous with Endothelial Derived Relaxing Factor (Katzung, 2004). It acts via the heme moiety of guanylyl cyclase, which produces cyclic guanosine monophosphate (cGMP). This reduces the levels of cytosolic Ca^{++} and also phosphorylates myosin light chain kinase. NO also decreases the activity of platelets and neutralizes free radicals. In this way it helps in preventing atherosclerosis, an important factor contributing to hypertension. It also prevents binding of leucocytes to the endothelium and decreases inflammation. Because of all these reasons, it is considered an important factor in preventing hypertension. It is thus logical to envisage a close link between the level of available NO and blood pressure. It is pertinent to hypothesize that factors influencing the levels of NO will have an important role in the pathophysiology of and susceptibility to hypertension.

The main source of circulating NO is the endothelium, where it is produced from the amino acid L-arginine by the action of endothelial nitric oxide synthase (eNOS). This is a constitutive enzyme with its gene (eNOS/*NOS3*) on chromosome 7. NOS is a heme protein that exists in its inactive form as a monomer, but dimerizes before action. NO is also produced by the neurons where neuronal nitric oxide synthase (nNOS/ *NOS1*), another constitutive enzyme, is responsible for its production. Inducible nitric oxide synthase (*iNOS/NOS2*) on chromosome 17 is induced mostly during inflammation. It is responsible for the harmful effects of the gas. It is expressed in macrophages, smooth muscle cells and hepatocytes and is responsible for pathological vasorelaxation. The eNOS is membrane bound while the other two are present in soluble form. NO is removed from circulation mostly by reaction with free radicals such as superoxide. The balance between the production and removal of NO is very important with regard to hypertension (Fig. 1).

Because eNOS is the major enzyme responsible for nitric oxide production, variation in its expression and activity can be linked to hypertension. It has been found that a G to T polymorphism in the exon 7 region leading to a change from Glu at 298 position to an Asp decreases the expression of the enzyme but has no effect on the activity (Kato et al., 1999). Another study has suggested that such a change causes the enzyme to undergo selective proteolysis (Hingorani, 2003).

In intron 4 of *NOS3* there can be four 27 bp repeats (allele a) or five (allele b). Presence of allele a, rather than the wild-type allele b, has been shown to increase the expression of the enzyme but to reduce its activity (Kato et al. 1999). The promoter region T to C polymorphism has also been shown to reduce the expression of the enzyme. The association of these polymorphisms to hypertension has been controversial. Whereas the G to T polymorphism has been associated with hypertension in many ethnic groups (Miyamoto et al., 1998), including the north Indian population (Srivastava K, Narang R, Sreenivas V, Das S and Das N ,2008), the association of the other two polymorphisms has been more controversial (Hingorani, 2003; Kato et al., 1999). Little study has been conducted on Asians, especially those living in India, regarding these polymorphisms.

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

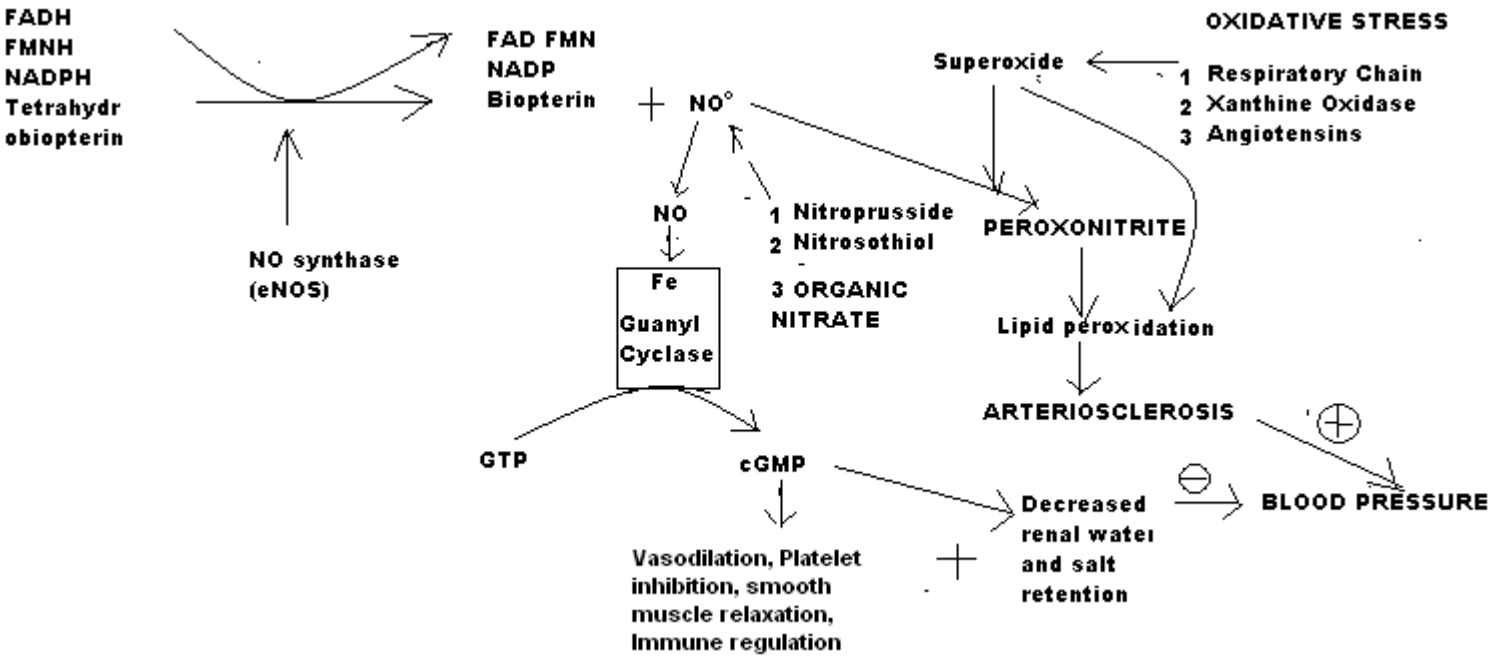

Figure 1: Nitric oxide, generation and functions

In this study, we aimed to elucidate the association between the intron 4a/b polymorphism in *NOS3* with NO levels and essential hypertension. The specific objectives were as follows:

1. To study the levels of NO in the plasma of patients with hypertension and controls
2. To determine the genotype frequencies of the above-mentioned polymorphism in patients with hypertension and controls.
3. To determine any correlation between these polymorphisms and essential hypertension
4. To determine any correlation between this polymorphism and plasma NO levels.

Materials and Methods

Materials

1. Chemicals and reagents

Ethylene diamine tetra acetate (EDTA), Sodium dodecyl sulphate (SDS) and agarose were purchased from the Sigma chemical co. St Louis , USA. Orthophosphoric acid was purchased from S.D. fine-chemical Ltd, New Delhi. All other chemicals were of analytical regent grade and purchased from Sisco Research Laboratories, Central drug House, Mark and Qualigens, India.

2. Biological reagents

The sense and antisense primers for intron 4, deoxynucleotides, Taq DNA polymerase with buffers and DNA markers were purchased from Biobasics, Canada, distributed here by Life technology (India) Pvt Ltd, Pitampura, New Delhi. Proteinase K was obtained from Bangalore Genei, India.

3. Buffers
a) Buffers for DNA isolation
i) RCL (Red Cell Lysis) Buffer, pH 7.6
10mM Tris (1.21g) 5mM $MgCl_2$ (1.016g) and 10mM NaCl (0.584) were dissolved in one liter of distilled water and autoclaved.
ii) SE (sodium chloride EDTA buffer pH 8.0
75 mM NaCl (4.39g) and 25 mM Na_2EDTA (8.41g) were dissolved in one liter of distilled water and autoclaved.
iii) TE (Tris EDTA) buffer, pH 7.6
10mM Tris (1.2 g) and 1mM EDTA (0.29g) were dissolved in one liter of distilled water and autoclaved.
b) Buffers for Gel Electrophoresis
TBE (Tris Boric acid EDTA) Buffer pH 8.0
For 10X TBE buffer 108g Tris base, 55g Boric acid and 7.4 g of EDTA were dissolved in one litre of EDTA. 1X TBE buffer was used as working solution.
c) Buffer for Nitrite assay
PBS (phosphate buffer saline) pH 7.2 to 7.4.
NaCl (5.84g), Na_2HPO_4 (4.72g) and $NaH_2(PO_4)_2.2H_2O$ (2.64g) were dissolved in 1liter of distilled water and autoclaved.

Methods

1. Leucocytes were isolated from the peripheral blood and DNA was extracted from the leucocytes.
2. The DNA was subjected to polymerase chain reaction (PCR) to amplify the region with the polymorphism.
3. It was then analyzed by agarose gel electrophoresis.
4. The NO levels were measured in the plasma using the Griess reagent.
5. Statistical methods were used to test any correlations between hypertension and the polymorphism.

Subjects and Study Design

Blood samples were taken from 45 patients and 45 controls. Selection criteria of hypertensive patients were – (i) Subject age, 25-55 years, (ii) B.P.> 140/90 mmHg visiting the hospital for the first time with no treatment started .Hypertensive patients with (i) history of drugs and alcohol abuse, (ii) women on oral contraceptives (iii) oedema, (iv) secondary forms of hypertension (v) subjects with a history of diabetes mellitus, endocrine illness and renal failure were excluded from the study.The selection criteria for the controls were- (i) subject age, 25-55 years (ii) B.P. < 140/90 mm Hg, (iii) absence of any antihypertensive therapy, (iv)Not suffering from any diabetes mellitus endocrine or any acute illness . They were the healthy volunteers who had responded to a publicized call. Clearance from the ethics committee of All India Institute of medical sciences and informed consents from study subjects were taken.

Sampling

Samples of 8 mL of venous blood were drawn from every subject into a Falcon tube containing an anticoagulant (EDTA). Plasma was separated from packed cells for nitrite estimation.

DNA extraction

The method of Miller Dykes & Polesky (1988) was used .Packed cells were lysed with Red Cell Lysis buffer. The solution was centrifuged at 4000rpm for 15min at 4°C to pellet out the nucleated cells i.e. WBCs. Nucleated cells were subjected to detergent (10% SDS) and protease (Proteinase K) treatment in Sodium Chloride-EDTA buffer and left at 37°C overnight on a shaker. Subsequently proteins were salted out with 5M NaCl. Proteins were pelleted out by centrifugation at 4000 rpm at room temperature for 15 min. DNA was precipitated by ethanol addition to the supernatant. DNA isolated is stored in TE buffer and stored at 4°C for further use.

Concentration and Purity Check

DNA is diluted in double distilled water and OD (Optical Densities) is taken at 260nm. Concentration of DNA is estimated by assuming that 50µg of DNA corresponds to an OD of 1 at 260 nm. Purity of DNA is checked by taking ratios of ODs at 260nm to 280nm (a ratio of 1.6-1.8 indicates nearly pure DNA). Protein impurities absorb prominently at 280 nm hence altering the ratio. The buffers were prepared in the laboratory itself.

Analysis of Variable Number Tandem Repeat (VNTR) Polymorphism in Intron 4 of the eNOS Gene

The method of Wang, Sim, Badenhop, McCredie, and Wilcken (1996) was used with slight modification. For PCR amplification of the DNA, two oligonucleotide primers flanking the 27 bp repeats in the intron 4 were used. The forward primer was 5′–AGGCCCTATGGTAGTGCCTTT–3′ and the reverse primer was 5′–TCTCTTAGTGCTGTGGTCAC–3′. Genomic DNA was amplified in final reaction volume of 25 µL containing 10 mM Tris chloride pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each of the four dNTPs, 1 µM each of the primers, and 2U Taq DNA polymerase. It was subjected to the following steps. The PCR machine used was PTC 100 (MJ research co.)

Step 1: 94 °C for 10 min (initial denaturation).
Step 2: 94 °C for 45 sec (denaturation).
Step 3: 56 °C for 1 min (annealing).
Step 4: 72 °C for 1 min 30 sec (extension).
Step 5: repeat steps 2–4 for 34 cycles.
Step 6: 72 °C for 10 min (final extension).
Step 7: hold at 4 °C.

The PCR products were analyzed by electrophoresis in 2.7% agarose gel and stained with ethidium bromide (EtBr). Molecular weight marker DNA was also run along with the test samples. The 420 bp wild-type product contained five 27 bp repeats (the b allele) and the 393 bp mutant type contained the four 27 bp repeats (the allele).

Estimation of NO Levels

Plasma levels of NO were estimated in the controls and patients with essential hypertension by the method of Ding, Nathan, and Stuehr (1998). NO is difficult to measure because it is unstable. Nitrite, a stable end product produced in the circulating plasma was estimated as an

index of NO using the Griess reagent (GR). It was prepared fresh by mixing equal volumes of 1% sulphanilamide and 0.1% naphthylene diamine dihydrochloride in 2.5% orthophosphoric acid. To measure nitrite, 50 µL of plasma was incubated with an equal volume of GR at 37 °C for 30 min. The absorbance at 550 nm was measured against plasma with phosphate-buffered saline (PBS) as a control, distilled water with GR as blank, and sodium nitrite as a standard.

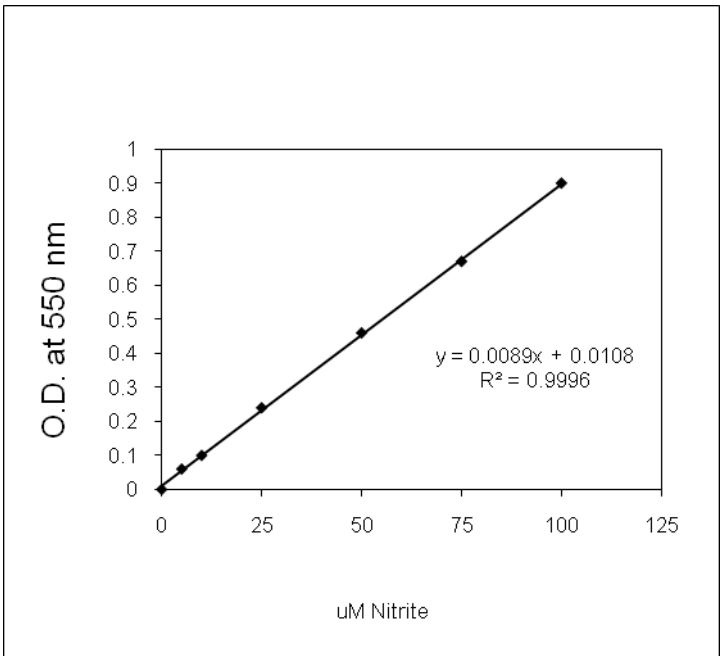

Figure 2: The standard curve for nitrite level

Analysis of Data

Data were analyzed using SPSS 7.5 software (SPSS Inc., Chicago, IL, USA). Chi square goodness of fit was used to verify the agreement of the observed genotype frequencies with those of the expected. Differences between the means of the groups were analyzed by the Students t-test. Genotype frequencies were compared by the Chi square test. Odds ratio [95%confidence interval (CI)] was calculated as an index of association of the eNOS genotypes (4b/b, 4a/b, 4a/a) with disease. Statistical significance was defined as $p<0.05$.

Results

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

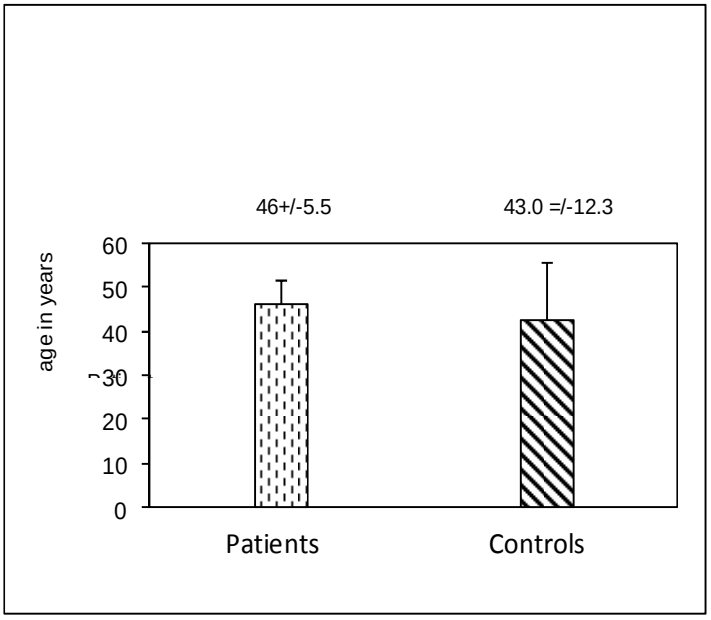

Figure 3: The mean ages of patients and controls

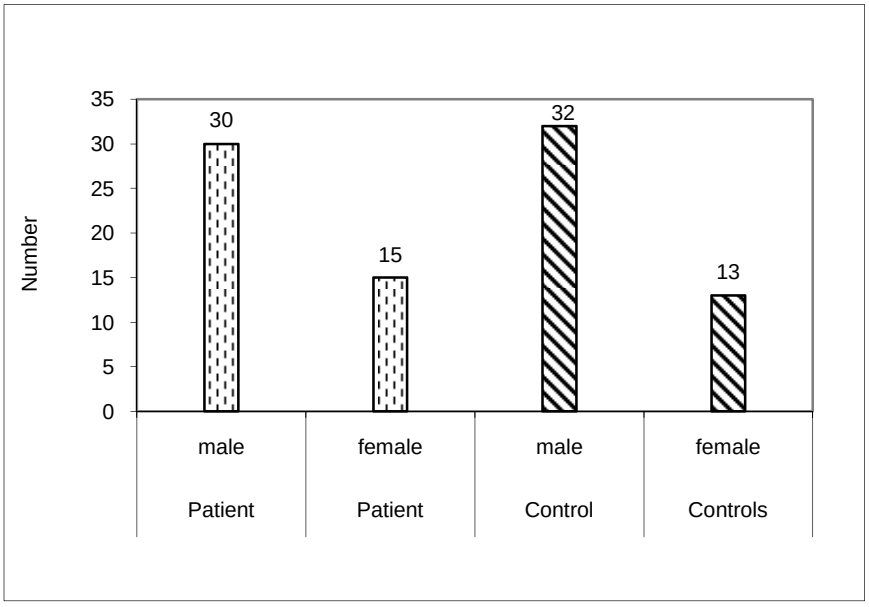

Figure 4: The gender distribution in patients and controls

As is clear from figures 3 the average age of patients and controls was comparable (p=0.14). The number of male volunteers was greater in both the groups however the distribution appeared similar.

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

Table 1: Comparison of various parameters of controls and patients

Parameter	*Patient*	*Control*	*P*
Age (years)	46.15 ± 5.5	43.0 ± 12.8	0.14
Nitrite (μM)	4.0 ± 1.7	6.7 ± 3.2	<.001
Nitrite in a/b genotype (μM)	4.2 ± 1.6	7.4 ± 1.5	<.01
Plasma nitrite <3uM	17(38%)	3(6%)	
Plasma nitrite >/=3to <5uM	17(38%)	12(27%)	
Plasma nitrite>/=5to<7uM	6(13%)	8(18%)	
Plasma nitrite>/=7uM	5(11%)	22(49%)	
Nitrite in b/b (μM)	3.9 ± 1.8	6.8 ± 3.4	<.001
Genotype b/b	36 (80%)	37 (82%)	0.79
Genotype a/b	7 (16%)	8 (17%)	
Genotype a/a	2 (4%)	0	
Genotype a/a +a/b	9 (20%)	8 (17%)	
Allele frequency of a	0.12	0.09	
Allele frequency of b	0.88	0.91	

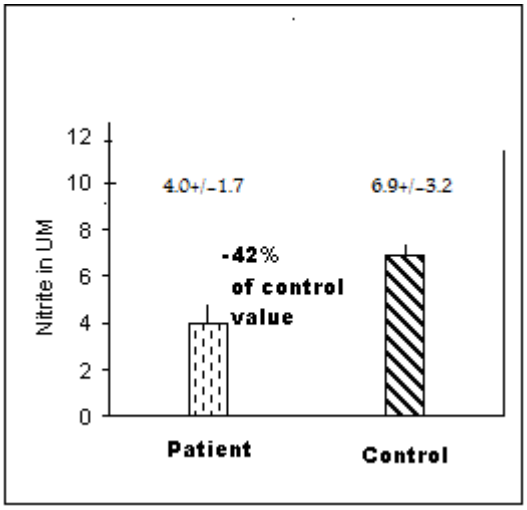

Figure 5: Nitrite levels in patients of hypertension and controls

The mean level of NO in the patients with hypertension (4.0 +/-1.7) (Fig. 5) was 42% less than that of the controls (6.9 +/-3.2). The difference is highly significant ($p<0.001$).

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

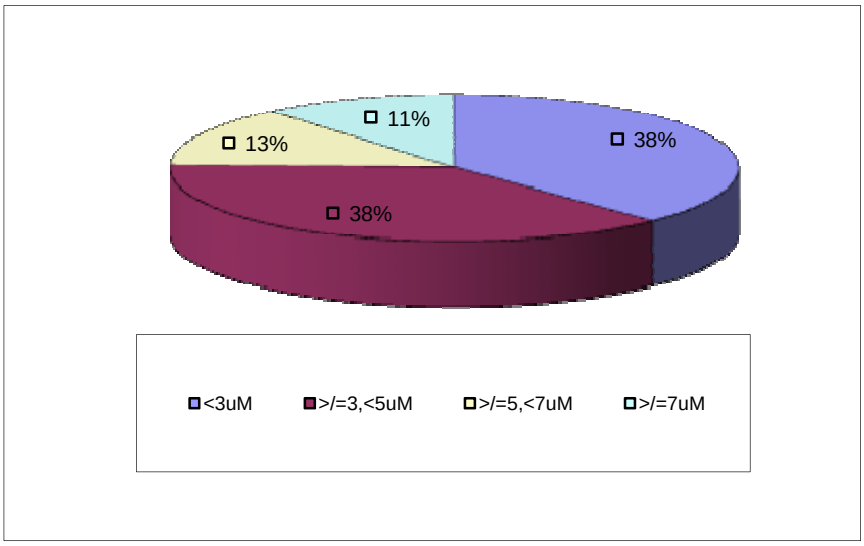

Figure 6: Distribution of nitrite in patients

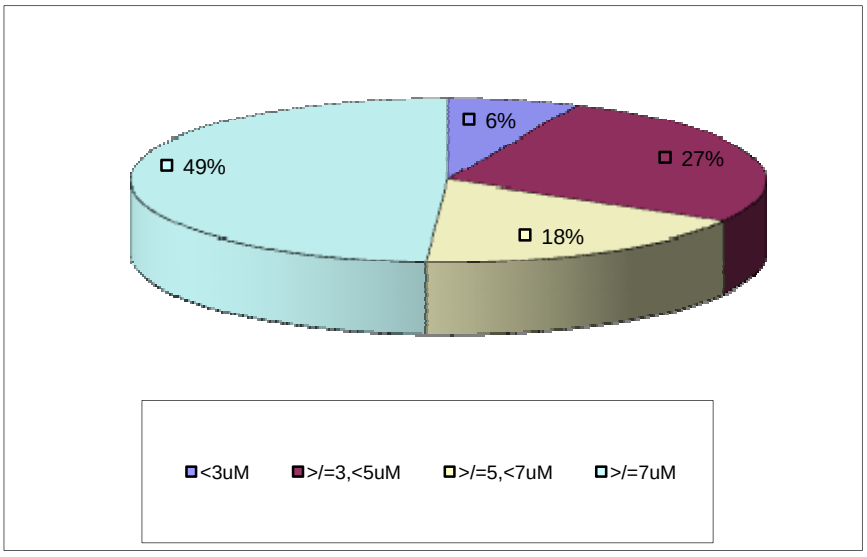

Figure 7: Distribution of nitrite in controls

It is clear from Figs 6 and 7 that most patients with hypertension (76%) had plasma nitrite levels below 5 μM. On the other hand, most controls (64%) had plasma nitrite levels of 5 μM or greater. The fraction of patients with nitrite levels below 5 μM (76%) was thus more than double that of the controls (34%). The patients were equally distributed between the two lower groups. However, the controls showed two peaks with the higher one being greater than 7 μM.

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

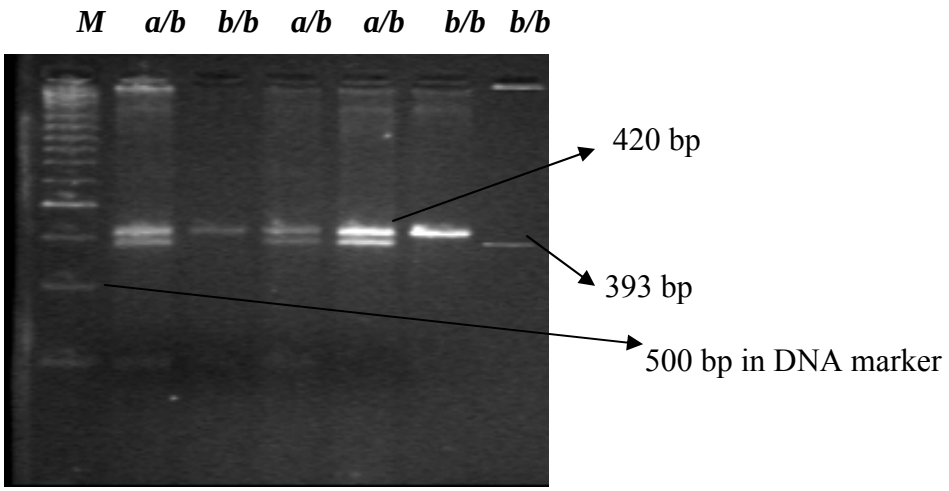

Figure 8

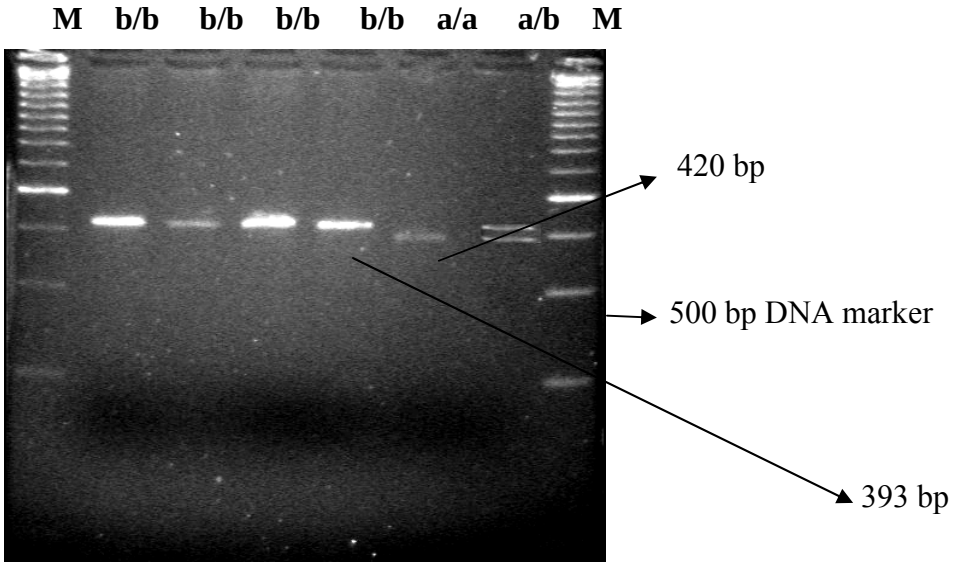

Figure 9

Figures 8 and 9. In these figures M- marker; a/a- a/a genotype of the eNOS gene; a/b- a/b genotype of the eNOS ; b/b- b/b genotype of eNOS gene The 420 bp band is of the b allele (five repeats) and the 393 bp band is of the a allele (four repeats).

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

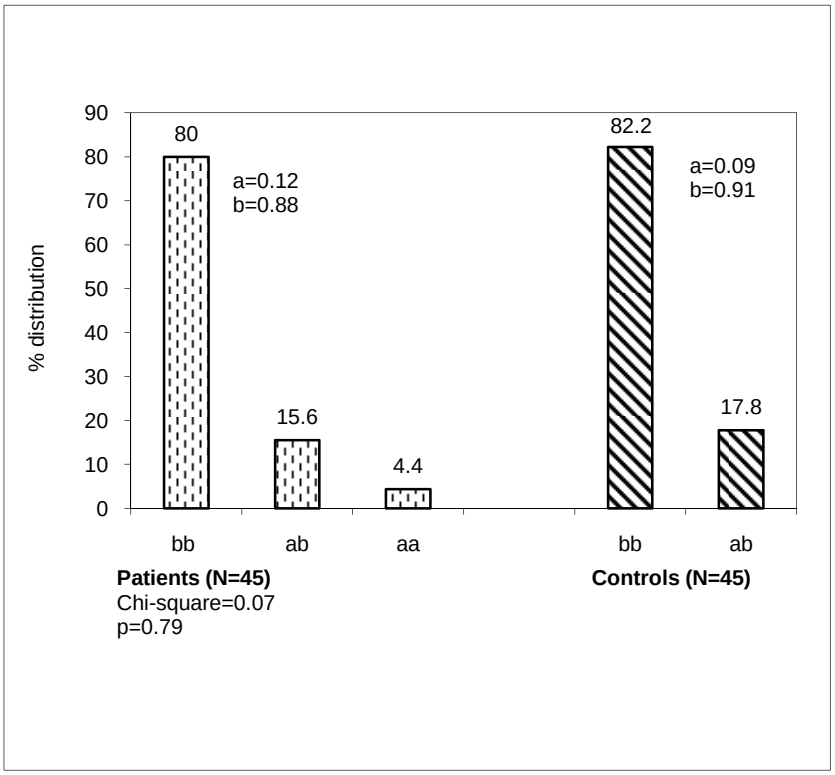

Figure 10: Distribution of genotypes and allele frequencies in study subjects

The patient and control populations did not differ significantly from that expected under Hardy Weinberg equilibrium [X^2=3.49, p=0.05(patient) X^2=0.06, p value>0.05 (control)]. The genotype frequencies in both the patients and controls were in the order of 4b/b > 4a/b> 4a/a. There was no significant difference [X^2=0.07, p = .79, Odds ratio=0.869(0.27-2.8) at 95% confidence interval adjusted for age and sex] in the genotype distribution between patients and controls. The a/a genotype could only be detected in two of the patients with hypertension.

International Journal of Collaborative Research on Internal Medicine & Public Health
Vol. 1 No. 2 (April 2009)
pp. 56-71

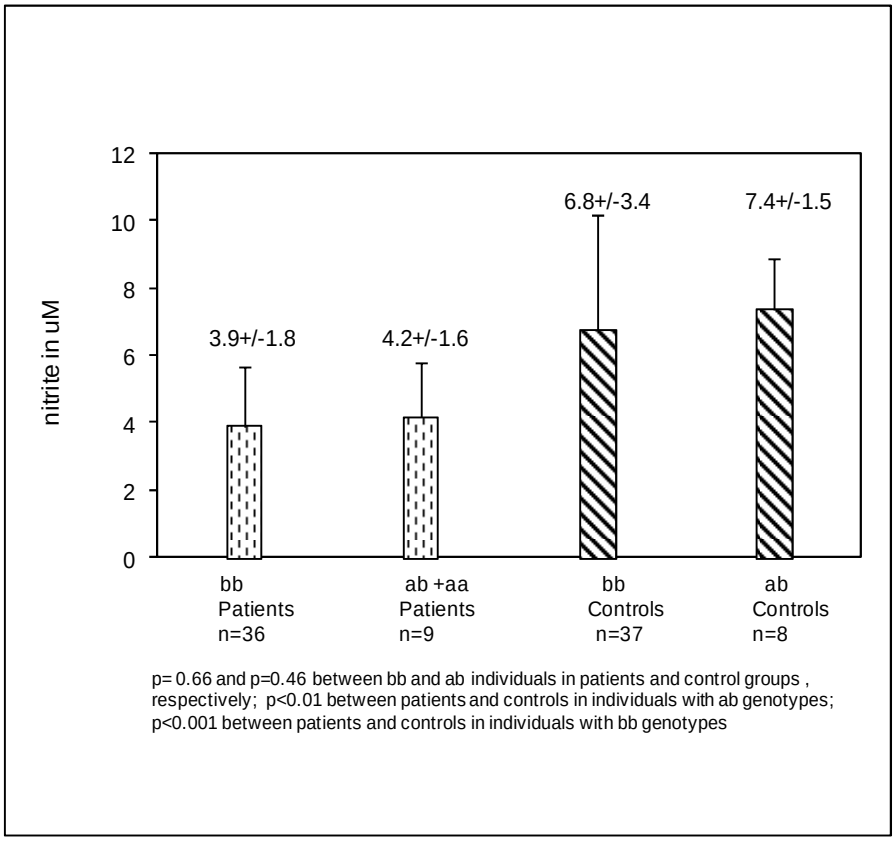

Figure11: Intergenotypic variations in the levels of nitrite in the study subjects

Figure 11, shows that there was no significant difference in the levels of nitrite between ab and bb genotypes in both patients (p=0.66) and controls(p=0.46). However, in both the genotypes, levels of nitrite were significantly lower in patients than in controls ($p<0.01$ for ab and $p<0.001$ for bb.)

Discussion

The participation of males in this study was nearly twice that of the females. This could be due to the social forces in the society where this study was conducted. Another study done in the same institute, on similar patient and control populations, but a larger sample size showed an even more unequal distribution between male and female subjects. (Srivastava K et al, 2008).

The nitrite levels were significantly lower in patients than in controls. The nitrite levels in the patients were 42% less than that in the controls. Previous studies (Afrasyap & Ozturk, 2004; Kumar & Das, 2000: Node, K., Kitakaze, M., Yoshikawa, H., Kosaka, H., & Hori, M. (1997)) have shown similar results. Node K et al (1997) in fact recorded a very similar (43%) decline in patients as compared to controls. The normal range of NO showed a wide variation in different studies (Ferlito, Gallina, Pitari, & Bianchi, 1998; Klahr, 2001; Kumar & Das, 2000; Jeerooburkhan et al., 2001). No relationship could be found between nitrite levels and gene polymorphism.

This study did not find any correlation between the presence of the eNOS 4 a/b variant and hypertension. This may be because of the small sample size. According to the literature survey, only two studies, one on Caucasians in general (Rodríguez-Esparragón, Rodríguez-

Pérez, Macías-Reyes, & Alamo-Santana, 2003), and another on the Ukrainian population (Dosenko, Zagoriy, Haytovich, Gordok, & Moibenko, 2005), have shown a correlation between this polymorphism and essential hypertension. Other studies have found no such correlation (Gouni-Berthold et al., 2005; Miyamoto et al., 1998; Zhao, Su, Chen, Li, & Gu, 2006). Although no statistically significant correlation could be found in the present study, a trend toward a higher frequency of the a allele was seen among the patients with essential hypertension. A detailed study with a larger sample size is needed to establish or refute the role of this polymorphism in essential hypertension in the Indian population. It is interesting to note that the a/a genotype was found only among two of the patients.

In the present study, though intron 4a/b polymorphism of NOS3 had no association with essential hypertension, a trend toward a higher frequency of the a allele was apparent among the patients' group. However, this suggests a need to conduct a large cohort study so that the nature of any association between essential hypertension and this polymorphism can be tested. If the a allele is found to be a disease-associated allele, screening of the population for individuals at risk might help save lives. If not, we can rule out an association of essential hypertension with this polymorphism.

NO levels showed a significant difference between patients and controls. This suggests that an estimation of NO levels could be included as a routine lab investigation to screen people at risk and to devise appropriate individualized therapeutic strategies. However, we stress that the reference value for NO in normal Indian subjects remains to be established. Estimating total NO is rather cumbersome, as it involves converting nitrate back to nitrite using the enzyme nitrate reductase. However, estimation of nitrite alone using an economic and simple method is a workable alternative.

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