

## SERUM NITRATE LEVELS AS AN INDEX OF ENDOTHELIAL FUNCTION IN PRE-ECLAMPSIA AND NORMAL PREGNANCY

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**Abstract :** The study was conducted in St. John's Medical College Hospital and Department of Physiology, with the aim of studying the serum nitrate levels in pre-eclampsia and normal pregnancy. The total number of subjects studied in various groups were 159, control (n = 55), first trimester (n = 13), second trimester normal (n = 42), second trimester pre-eclampsia/PET (n = 5), third trimester normal (n = 32), third trimester pre-eclampsia/PET (n = 12). The serum nitrate was measured by one step enzymatic assay using Nitrate reductase from *Aspergillus* species. The nitrate levels in the third trimester pre-eclamptic group was found to be significant lower (P = 0.02), as compared to normal subjects, however the renal functions were normal in all the subjects.

**Key words :** pregnancy  
pre-eclampsia

serum nitrate  
pre-eclamptic toxemia

### INTRODUCTION

Nitric oxide is synthesised ubiquitously in the body from amino acid L arinine, and is produced predominantly from vascular endothelium. It rapidly degrades to nitrates and nitrites, which are its stable end products and are solely excreted through kidney (1). The serum nitrates are often considered as an index endothelial function.

Nitric oxide is a powerful vasodilator and is an important modulator of vasomotor tone (2). The normal pregnancy is

characterised by widespread vasodilation (3), there is fall of systemic vascular resistance, which is associated with increased serum nitrate levels (4, 5). Endothelial dysfunction due to any reason will result in lower production of nitric oxide and hence lower serum nitrates. Rise of systemic arterial pressure and increased vasoconstriction (6) could result due to lower production of vasodilators (7, 8). Pre-eclamptic toxemia (PET) is multi factorial disorder characterised by oedema, albuminuria, and hypertension. It is also associated with defective endothelial function (9, 10).

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Studies of serum nitrates in preeclampsia have yielded conflicting results. The studies showing increased levels of nitrates in PET attribute it to compensatory mechanisms, for associated hyper coagulable states (11, 12). On the other hand some studies have shown lower levels, due to lower production of nitric oxide by endothelium (7, 8, 13) while in some reports no change was observed (14).

The aim of the present study was to test the hypothesis that serum levels are lower in patients with pre-eclampsia as compared to normal pregnancy, provided renal functions are normal.

## METHODS

**Subjects:** The study is prospective, conducted at SJMCH by the Departments of Physiology and Obstetrics & Gynaecology. The patients were recruited from those attending OPD and admitted to SJMC Hospital. The inclusion criteria was pregnant women belonging to age group of 20–35 yrs. They were divided into groups, one group of normal pregnancy and the other PET group, having evidence of any two of the following; oedema, systolic blood pressure >140 mm Hg and diastolic >90 mm Hg, Albuminuria, and of pregnancy more than 20 weeks duration. Exclusion criteria were fever, infections, intake of any medications, diarrhoea or allergic disorders in last 2 weeks, and history of threatened abortion, molar or multiple pregnancy.

The control subjects were not pregnant women in same age 20–35 yrs, parity nil, nondiabetic, normotensive and no history of recent infection. All the subjects were on

vegetarian diet for previous 2 days and the samples were collected during 1st–6th day of menstrual cycle. This was because diet rich in nitrate would effect the serum nitrate levels. The subject characteristics are shown in Table I. The written consent was taken from all the subjects and the project was approved by ethical committee of the institution.

**Methods:** The samples were collected in the Central lab after the subjects were recruited in the out patient department. They came to laboratory in fasted state in the morning, they were asked to rest for half an hour, prior to collection of blood sample. This was to avoid the effect of physical exercise on serum nitrate (20). In the patients who were admitted, the samples were collected at the time of admission. Out of total 340 subjects selected, only 159 could be included in the study. The distributions of the subjects was : control (n = 55), first trimester (n = 13), second trimester (normal, n = 42; PET n = 5), third trimester (normal n = 32; PET n = 12).

**Procedure:** The serum was separated and stored at  $-20^{\circ}\text{C}$  till further analysis. The samples were analysed by one step enzymatic assay using nitrate reductase (15). Briefly serum was processed as follows: Blank (reagent), serum blank, sample and standard.

**Principal of the assay:** Change in the absorbance of B-NADPH at 340 nm is measured as it gets oxidised in the process of the chemical reaction.

**Source of materials:** Nitrate reductase enzyme (from *Aspergillus* species) and FAD were

obtained from Sigma-Aldrich, B-NADPH from Spectrochem, and Sodium nitrate, Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate were obtained from Nice chemicals.

**Reagent blank:** 250  $\mu\text{L}$  of potassium phosphate buffer (pH 7.5) was taken and to this 50  $\mu\text{L}$  of 0.2  $\mu\text{mol/L}$  FAD and 10  $\mu\text{L}$  of 12 mM of B-NADPH and 100  $\mu\text{L}$  of distilled water (instead of nitrate calibrator for standard or serum for sample), and 50  $\mu\text{L}$  of distilled water in presence of 40  $\mu\text{L}$  of 500 units/L of nitrate reductase, incubated in dark for 45 min. and absorbance value noted as reagent blank by U-V spectrophotometer (Pye-unicam-SP8-400).

**Serum blank:** Same procedure as above except for 100  $\mu\text{L}$  of serum instead of distilled water and 40  $\mu\text{L}$  of distilled water to replace nitrate reductase. It was incubated in dark for 45 min and absorbance value was noted as serum blank.

**Sample:** Same as serum blank except that 40  $\mu\text{L}$  of nitrate reductase was added instead of distilled water, incubated for 45 min and absorbance value was noted.

Absorbance values thus recorded were used to calculate the levels of Nitrate in the sample.

Standardisation of the assay was done using pooled serum samples, having mean nitrate level 9.33  $\mu\text{mol/L}$ . The intra assay co-efficient of variation was 4.39% and the inter assay co-efficient of variation was 4.67%. Linearity of the assay was also checked up to 200  $\mu\text{mol/L}$  using the sodium nitrate solution as calibrator (16).

## RESULTS

The Table I shows the values of mean  $\pm$  SD body weight, serum creatinine, fasting blood sugar (FBS), haemoglobin, total platelet count and resting blood pressure of the subjects (n = 159), grouped

Table I: Subject characteristics (Mean  $\pm$  SD).

| Groups                        | Control         | 1st Tri.       | 2nd Tri.<br>(N) | 2nd Tri.<br>(PET) | 3rd Tri.<br>(N) | 3rd Tri.<br>(PET) |
|-------------------------------|-----------------|----------------|-----------------|-------------------|-----------------|-------------------|
| Body wt. in K.G.              | 48 $\pm$ 5      | 53.4 $\pm$ 11  | 49.5 $\pm$ 7.4  | 50.3 $\pm$ 3      | 57.1 $\pm$ 8.3  | 55.6 $\pm$ 6.5    |
| Serum creatinine mgs%         | 0.57 $\pm$ 0.1  | 0.6 $\pm$ 1    | 0.53 $\pm$ .01  | 0.58 $\pm$ 0.1    | 0.51 $\pm$ .01  | 0.69 $\pm$ 0.1    |
| FBS mgs%                      | 80.3 $\pm$ 11.0 | 82 $\pm$ 10.6  | 77.8 $\pm$ 11.2 | 86.6 $\pm$ 24.0   | 78 $\pm$ 9.2    | 82.2 $\pm$ 4.0    |
| Hb Gm%                        | 11.9 $\pm$ 6.0  | 11.7 $\pm$ 1.7 | 10.9 $\pm$ 1.2  | 11.2 $\pm$ 5.0    | 10.9 $\pm$ 1.0  | 11.2 $\pm$ 2.0    |
| Platelet count lakhs/c.mm     | 2.3 $\pm$ 0.6   | 2.3 $\pm$ 0.7  | 2.1 $\pm$ 0.5   | 2.3 $\pm$ 0.5     | 2.1 $\pm$ 0.3   | 2.1 $\pm$ 0.7     |
| <b>Blood pressure (mm Hg)</b> |                 |                |                 |                   |                 |                   |
| Systolic                      | 110 $\pm$ 6     | 113 $\pm$ 8    | 110 $\pm$ 5     | 142 $\pm$ 16      | 115 $\pm$ 8     | 149 $\pm$ 18      |
| Diastolic                     | 76 $\pm$ 4      | 72 $\pm$ 4     | 72 $\pm$ 3      | 90 $\pm$ 2        | 74 $\pm$ 6      | 100 $\pm$ 1       |
| <b>Parity</b>                 |                 |                |                 |                   |                 |                   |
| Primi                         | Nil             | 4              | 24              | 2                 | 13              | 9                 |
| 1                             | Nil             | 4              | 12              | 1                 | 16              | 3                 |
| 2                             | Nil             | 5              | 6               | 2                 | 3               | 0                 |

Table II: Serum nitrate levels in  $\mu\text{mol/L}$  in various trimesters of pregnancy, Normal (N) and Pre-eclampsia (PET).

| Groups        | Control         | 1st Tri.        | 2nd Tri. (N)    | 2nd Tri. (PET)  | 3rd Tri. (N)    | 3rd Tri. (PET) |
|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| Numbers       | 55              | 13              | 42              | 5               | 32              | 12             |
| Mean $\pm$ SD | 27.7 $\pm$ 14.7 | 15.7 $\pm$ 12.7 | 15.5 $\pm$ 15.5 | 21.7 $\pm$ 15.7 | 22.2 $\pm$ 14.4 | 11 $\pm$ 7.5   |
| Median        | 28.0            | 16.0            | 8.0             | 31.2            | 24.4            | 8.8            |
| IQR           | 20.8            | 22.45           | 20.4            | 29.2            | 25              | 7.2            |
| (Values)      | (16.8–37.6)     | (2.8–25.6)      | (4.0–24.0)      | (4.8–34.0)      | (7.0–32.0)      | (6.4–13.6)     |

as control (n = 55), first trimester (n = 13), second trimester normal (n = 42), second trimester PET (n = 5), third trimester normal (n = 32) and third trimester PET (n = 12).

The Table II gives the mean values of serum nitrate level in  $\mu\text{mol/L}$ , median and IQR (with values), of all the groups.

The standardisation of the assay is described in Methods. The sensitivity of assay was 0.8  $\mu\text{mol/L}$ .

**Control (n = 55):** The mean serum nitrate level in the control was 27.7  $\pm$  14.7  $\mu\text{mol/L}$ , IQR 20.8(16.8–37.6). The mean body weight was 48  $\pm$  5 kg, the serum creatinine was 0.57  $\pm$  0.1 mg%, fasting blood sugar 80.3  $\pm$  11 mg%, haemoglobin 11.9  $\pm$  1.6 g/100 ml of blood. Total platelet count 2.3  $\pm$  0.6 lakhs/cu.mm. of blood, and the mean blood pressure, systolic was 110  $\pm$  6 mm Hg and diastolic was 76  $\pm$  4 mm of Hg. Urine albumin was absent.

**The first trimester (n = 13):** The mean nitrate level was 15.7  $\pm$  12.7  $\mu\text{mol/L}$  median 16  $\mu\text{mol/L}$ , IQR (2.8–25.2). The mean body weight was 53.4  $\pm$  11.0 kg, serum creatinine 0.6  $\pm$  0.1 mg%, fasting blood glucose level 82  $\pm$  10.6 mg% haemoglobin 11.7  $\pm$  1.7 g/

100 ml, total platelet count 2.3  $\pm$  0.7 lakhs/cu.mm, and blood pressure systolic 113  $\pm$  8 mm Hg, diastolic 72  $\pm$  4 mm Hg. Urine albumin was absent.

**The second trimester:** There were 42 subjects in the normotensive group and the mean nitrate was 15.5  $\pm$  15.5  $\mu\text{mol/L}$ , median 8  $\mu\text{mol/L}$  IQR 20 (4–24). The mean body weight was 49.5  $\pm$  7.4 kg., serum creatinine 0.53  $\pm$  0.01 mg%, fasting blood sugar 77.8  $\pm$  11.2 mg%, haemoglobin 10.9  $\pm$  1.2 g%. Total platelet count 2.1  $\pm$  0.5 lakhs/cu.mm, and systolic blood pressure 110  $\pm$  5 and diastolic 72.3 mm Hg respectively.

The PET group in second trimester had only n = 5 subject. This included all the subjects who were more than 20 weeks of gestation. The mean serum nitrate was 21.7  $\pm$  15.7  $\mu\text{mol/L}$ , the median being 31.2  $\mu\text{mol/L}$ , IQR 29.2 (4.8–34). The mean body weight was 50.3  $\pm$  3.0 kg., serum creatinine was 0.58  $\pm$  0.1 mg%, fasting blood sugar ws 86.6  $\pm$  24 mg%, the mean haemoglobin 11.2  $\pm$  0.5 g%. Total platelet count 2.3  $\pm$  0.5 lakhs/cu.mm. The mean systolic blood pressure 142  $\pm$  16 mm Hg, and diastolic 90  $\pm$  2 mm Hg.

There was no difference in these parameters between normotensive and PET groups, with exception of blood pressure

which was higher in PET group, oedema was present in 3 subjects and 5 had albuminuria. The serum nitrate was higher in hypertensive group.

**The third trimester:** This group included 32 normotensive subjects. The mean serum nitrate was  $22.2 \pm 14.4$   $\mu\text{mol/L}$ , the median being  $22.4$   $\mu\text{mol/L}$ , IQR 25 (7–32). The mean body weight was  $57.1 \pm 8.3$  kg, serum creatinine  $0.51 \pm 0.01$  mg%, fasting blood sugar  $78 \pm 9.2$  mg%, haemoglobin  $10.9 \pm 1.0$  g%. Total platelet count  $2.1 \pm 0.3$  lakh/cu.mm of blood. The systolic blood pressure of  $115 \pm 8$  and diastolic  $74 \pm 6$  mm Hg respectively. The mean nitrate levels in PET group was  $11 \pm 7.5$   $\mu\text{mol/L}$  and  $22.2 \pm 14.4$   $\mu\text{mol/L}$  in normotensive group. In PET group median  $8.8$   $\mu\text{mol/L}$  and  $24.4$   $\mu\text{mol/L}$  in normotensive. The difference was statistically significant ( $P = 0.02$ ). The creatinine levels were slightly higher in PET group, though all the values were within normal range, indicating normal renal function.

The levels of serum nitrate were lower in PET group in third trimester, and the inter quartile ranges were narrower.

## DISCUSSION

The nitrate levels in non pregnant women are in range from  $27.7 \pm 14.7$   $\mu\text{mol/L}$ . It is generally believed that during pregnancy there is rise in nitrate level (4, 5). Our results however do not confirm this. We find that there is actually a fall in nitrate levels in first trimester as compared to non-pregnant control. The serum nitrates start rising as the pregnancy progresses and by third trimester it almost reaches the pre pregnancy levels. The vasodilator metabolites,

including nitric oxide are produced in increasing amounts as pregnancy advances (17). In patients who have PET we found that rise in nitrate levels in the third trimester were lower. This probably is due to the failure of 2nd wave of trophoblastic invasion (21). Though the numbers in our study are small we feel that estimation of nitrate levels longitudinally in normal and PET pregnancy and their correlation with doppler flow velocitometry may be helpful in detecting early onset of PET.

The nitrate levels in the second trimester in PET group were higher than second trimester normal pregnant group. This is against our initial hypothesis. Review of literature of such studies have explained it as an effect of compensation to increased peripheral resistance and tone due to hypercoagulable states (11, 12).

The nitrate levels in third trimester are significantly lower in PET group. One of the characteristic features of PET is defective endothelial function (9). This could be due to injury to the endothelium, as shown by some of the studies showing higher circulating antagonists (18), or antibodies to the endothelium (19), and low levels of nitrate in the serum (10).

The low levels in the PET group and their lower dispersion, IQR 7.2 (6.4–13.6) it might have been due to patients being hospitalised and having limited physical activity. It is well known that physical activity can alter the nitrate levels in subjects (20).

The lower nitrate levels and the normal renal functions as assessed by serum

creatinine levels in our subjects indicate lower production of nitric oxide in the third trimester PET group. The present study design however gives the over all levels of nitrate in all the groups and does not identify the source of nitric oxide. This is a limitation of our study.

Based on the results of our study we conclude that in the third trimester patients in PET group have significantly lower level of serum nitrates as compared to normal pregnant group, and that nitrate levels increase during normal pregnancy from first to third trimester.

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