

## Serum nitric oxide and homocysteine as biomarkers of ectopic pregnancy

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### ABSTRACT

**Background:** Aim of current study was to evaluate the role of serum homocysteine and nitric oxide in the diagnosis of ectopic pregnancy.

**Methods:** The study included 32 patients with ruptured ectopic, 29 miscarriage patients and 30 normal pregnant women as controls. Fasting plasma homocysteine, serum folate, vitamin B<sub>12</sub> levels and nitric oxide levels were estimated at the time of admission.

**Results:** Plasma homocysteine levels were significantly lower in patients with ectopic pregnancy than normal pregnancy. Nitric oxide levels were significantly lower in patients with abortion.

**Conclusions:** Patients with abortion have decreased circulating nitric oxide levels in serum while those with ectopic pregnancies have decreased homocysteine levels.

**Keywords:** Ectopic, Homocysteine, Nitric, Abortion, Fallopian

### INTRODUCTION

Ectopic pregnancy and abortion are two common complications seen in early pregnancy.<sup>1</sup> Ectopic pregnancy denotes the implantation of the gestational sac outside the uterus and is the most common life threatening emergency in early pregnancy.<sup>2</sup> Most of the cases of ectopic pregnancy are tubal. This could be because of the retention of the embryo within the fallopian tube due to impaired embryo-tubal transport.<sup>3</sup>

Abortion is the most common complication of pregnancy. Early spontaneous abortion is seen in 15-20% of the couples desiring pregnancy. In spite of various efforts to find an etiological factor for early pregnancy loss, more than 50% of the cases remain unexplained.<sup>4</sup>

Further, both abortion and ectopic pregnancy present with similar features of abdominal pain and vaginal bleeding in the first trimester of pregnancy. Tubal ectopic pregnancy must be diagnosed as early as possible since it is one of the leading causes of pregnancy related death in the first trimester. Currently, it is diagnosed by serial measurement of  $\beta$ -hCG and transvaginal ultrasonography.<sup>5</sup> However, the sensitivity of these tests is poor demanding new serum biomarkers for diagnosis of ectopic pregnancy and to differentiate it from abortions and early ongoing intrauterine pregnancy.

Recent studies have demonstrated an increasing role of nitric oxide on the outcome of pregnancy. It has been known to regulate myometrial contractions and placental blood flow.<sup>6,7</sup> Nitric oxide levels have been implicated in both ectopic pregnancy and abortion. The levels in

recurrent abortions are found to be decreased, leading to increased myometrial contraction, whereas in ectopic pregnancy, the levels are found to higher, leading to decreased motility of fallopian tube and ectopic implantation.<sup>8-12</sup> One of the factors affecting nitric oxide concentration, is the levels of plasma homocysteine in circulation. Homocysteine decreases nitric oxide level by increasing the oxidative degradation of nitric oxide through the formation of disulfides and the generation of hydrogen peroxide and superoxide anion.<sup>13,14</sup> Homocysteine levels are in-turn dependent upon the Vitamin B<sub>12</sub> and folate status of an individual.

Therefore, in the present study, we observed the circulating levels of homocysteine, nitric oxide, vitamin B<sub>12</sub> and folate in patients with ectopic pregnancy & abortion and compared it to normal pregnancy, in an attempt to use homocysteine and nitric oxide to differentiate ectopic pregnancy from abortion.

We hypothesize that circulating nitric oxide levels are raised in ectopic pregnancy and decreased in abortion, whereas, homocysteine levels should be raised in abortion and decreased in ectopic pregnancy, leading to a decrease in the circulating levels of nitric oxide in abortion and an increase in abortion.

## METHODS

This was a prospective case control study conducted in the department of biochemistry in collaboration with the department of obstetrics & gynecology, JIPMER, Puducherry, India. The protocol and informed consent were approved by the institutional review board and research ethical committee.

### Study subjects

Tubal ectopic pregnancy and intrauterine abortion cases were included based on both clinical assessment and transvaginal ultrasonography. These cases were treated surgically and diagnoses were confirmed by

histopathological examination. The study subjects with clinical conditions that alter the study parameters were excluded from the study.

Blood sample was drawn at the time of presentation prior to surgical intervention and were centrifuged at 3000 rpm and serum samples were stored at -70 degree Celsius until use.

### Biochemical parameters

Fasting plasma homocysteine, serum folate and vitamin B<sub>12</sub> were measured using chemiluminescent immunoassay in automated Advia Centaur CP, USA. Nitric oxide levels were assessed in serum spectrophotometrically using Griess reaction (Enzo USA). Assays were done in duplicate whenever necessary. Quality control samples were monitored.

### Statistical analysis

It was performed using the SPSS 42 package for Windows, version 16 (SPSS, Chicago, IL, USA). Results were compared using Kruskal Wallis tests and one-way ANOVA followed by post hoc test Tukey HSD for non-parametric and parametric data respectively.

## RESULTS

We included 32 patients with ruptured ectopic pregnancy matched for gestational age with 29 patients of intrauterine abortion with no antecedent history of abortion and 30 women with normal intrauterine pregnancies (controls). The mean age of study subjects was 25.8 ± 5 years, 24.2 ± 4 years and 24.3 ± 3.2 years for ectopic pregnancy, intrauterine abortion and normal pregnancy respectively. Gestational age of study subjects are depicted in Table 1 and were matched among the study groups. Serum levels of β-hCG, as shown in Table 1 were found to be low in both ectopic pregnancy and abortion cases when compared to controls.

**Table 1: General characteristics of the study subjects.**

Parameter	Normal pregnancy	Intrauterine abortion	Ectopic pregnancy
Number	30	29	32
Age (years)	25 ± 5	24.2 ± 4	24.3 ± 3.2
Gestational age (weeks)	5.4 ± 2.1	5.2 ± 1.2	4.6±1.5
B-hCG (IU/L)	54613 ± 62350	19515 ± 38224 <sup>a</sup>	10845 ± 18918 <sup>a</sup>

a: P <0.05 when compared with normal pregnancy

The levels of homocysteine, folate, vitamin B<sub>12</sub> and nitrate in serum of the study subjects are shown in Table 2. Homocysteine levels are significantly lower in patients

with ectopic pregnancy and nitrate levels are significantly lower in patients with abortion when compared to normal pregnancy. The levels of serum folate and vitamin B<sub>12</sub> are not significantly different in the three groups.

**Table 2: Levels of homocysteine, folate, vitamin B<sub>12</sub> and nitric oxide among the study subjects.**

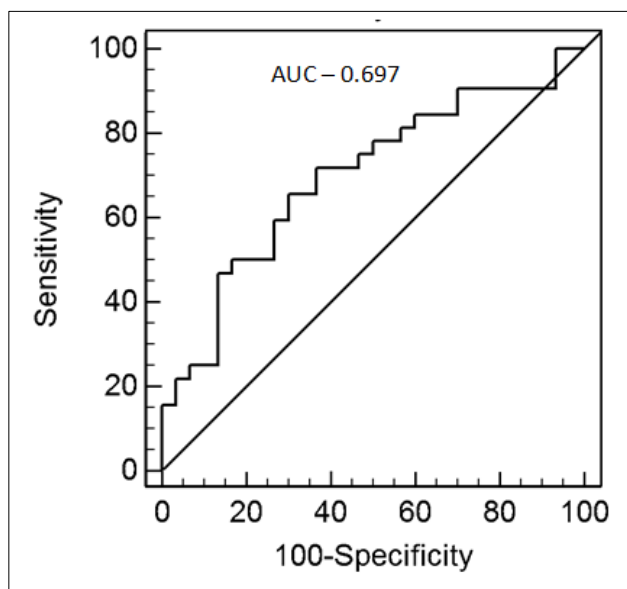
Parameter	Normal pregnancy (n=30)	Intrauterine abortion (n=29)	Ectopic pregnancy (n=32)
Homocysteine (μmol/L)	12.99 ± 4.98	10.9 ± 5.23	9.5 ± 5.01 <sup>a</sup>
Folate (ng/ml)	9.47 ± 17.73	7.22 ± 6.05	7.67 ± 6.28
Vitamin B <sub>12</sub> ( pg/ml)	306.6 ± 180.82	414.62 ± 401.04	350.5 ± 468.95
Nitrate (μM)	172.94 ± 38.59	134.34 ± 47.91 <sup>a</sup>	167.61 ± 75.85

a: P <0.05 when compared with normal pregnancy

**Table 3: Variables from ROC curve of homocysteine (Figure 1).**

Variables	AUC ± SE	95% CI (Lower limit - Upper limit)	Cut-off value	Sensitivity (%)	Specificity (%)	P value	Youden index	+LR	-LR
Homocysteine	0.697 ± 0.0677	0.567-0.807	≤ 10.9	65.62	70.00	0.003	0.3563	2.19	0.49

AUC: Area under curve; SE: Standard error; CI: Confidence interval; LR: Likelihood ratio



**Figure 1: Receiver-operating characteristic (ROC) curve of Homocysteine.**

**DISCUSSION**

In the present study, we explored the potential of circulating levels of homocysteine and nitric oxide to differentiate between ectopic pregnancy and abortion from early ongoing intrauterine pregnancy.

The study group was matched for their gestational age. Serum levels of β-hCG were found to be low in both ectopic pregnancy and abortion cases when compared to controls. This is in accordance with the fall of HCG seen with adverse outcomes of pregnancy.

We found that the serum nitric oxide levels were significantly decreased in abortion cases when compared

to the controls, whereas, patients with ectopic pregnancy had circulating nitric oxide levels similar to the control group. This is in favor of study conducted by Baban RS et al. in which they have found a significant decrease in serum nitric oxide levels in women with recurrent pregnancy loss.<sup>9</sup> The significant decrease in the nitric oxide levels in cases of abortion may be due to an increase in the production of free radicals such as superoxide which inactivates nitric oxide in the chemical reactions. Due to the decreased NO levels, the potential functions of NO during pregnancy, i.e. regulation of placental blood flow and suppression of myometrial contractions are disrupted resulting in increased risk of abortion. Paradisi et al. observed that patients with missed abortion showed decreased serum NO levels compared with non-pregnant patients and patients with regular pregnancy and threatened abortion, supporting a direct functional role of the NO mediator in early embryonic development.<sup>10</sup> Similarly, Maronini et al. found trophoblasts staining for iNOS positivity at the maternal-decidual interface to be significantly lower in spontaneous abortions than in controls.<sup>11</sup>

For the ectopic pregnancy, we expected higher circulating levels of nitric oxide, leading to myometrial relaxation and tubal implantation, but this was not observed in the present study. Animal studies by Dixon et al show that there is up-regulation of inducible nitric oxide synthase in the oviducts of Chlamydia infected mice.<sup>12</sup> This disrupts the pacemaker activity in the oviduct and slows the transport of the embryo through it. Similarly, Refaat et al. found iNOS (inducible nitric oxide synthase) expression to be significantly increased in patients with tubal pregnancy.<sup>15,16</sup> But the levels of nitric oxide in our study were similar to the control group of normal pregnancy. This can probably be attributed to the discrepancy between local enhanced expression of nitric oxide and circulating levels of nitric oxide. Also, serial

measurements of nitric oxide would probably give a clearer assessment of the circulating levels.

We had hypothesized that as homocysteine levels decrease the level of nitric oxide, its level should be increased in abortion and decreased in ectopic pregnancy. Homocysteine decreases the level of nitric oxide by multiple ways. It directly inhibits the enzyme dimethylarginine dimethylaminohydrolase (DDAH) leading to the accumulation of ADMA. Homocysteine also increases oxidative degradation of NO by generating free radicals.<sup>13</sup> Zang et al. demonstrated that concentrations of homocysteine more than 20  $\mu$ M produce a significant indirect suppression of eNOS activity without any discernible effects on its expression. It was also observed that folates, superoxide ions, and peroxynitrite scavengers restore the NO-generating activity to eNOS, suggesting that cellular redox state plays an important role in homocysteine suppressed NO-generating function of this enzyme.<sup>14</sup> Nelen et al. found elevated homocysteine levels and low folate levels to be associated with recurrent spontaneous early pregnancy losses.<sup>17</sup> A study in Italian population by D'Uva et al. also found increased plasma homocysteine concentration in patients with recurrent pregnancy loss compared to the control group. 17 out of the 20 patients of recurrent pregnancy loss showed methyl tetrahydro folate reductase C677T homozygosity, without any significant differences in the levels of vitamin B<sub>12</sub>.<sup>18</sup> Kumar et al. found the highest values of homocysteine concentration in women experiencing recurrent pregnancy loss with both the methyl tetrahydro folate reductase CT genotype and folate deficiency.<sup>19</sup> From the results observed, we found the levels of plasma homocysteine to be significantly decreased in both the patients with ectopic pregnancy and abortions as compared to controls in this study. These results were in contrast to the expected outcome of increased homocysteine levels, leading to increased oxidative stress in abortion. On the other hand, in patients with ectopic pregnancy, we found the plasma homocysteine levels to be significantly decreased as compared to the control group. We hypothesize that, the lower homocysteine levels causes an increase in the activity of nitric oxide resulting in tubal relaxation and retention of embryo in the fallopian tube.

The serum levels of vitamin B<sub>12</sub> and folate were not significantly altered in cases of abortion and ectopic pregnancies when compared to normal pregnancy. Studies in this regard have varied results. According to Sutterlin et al, the serum concentrations of folic acid and vitamin B<sub>12</sub> are not significantly altered in women with recurrent abortions.<sup>20</sup> Study by Willianne et al. show that serum folate levels are significantly decreased while vitamin B<sub>12</sub> levels are unaltered in patients with recurrent pregnancy loss.<sup>21</sup> Another study done in 43 Syrian women with recurrent abortion demonstrated significantly decreased vitamin B<sub>12</sub> levels in these patients compared to controls, along with elevated

homocysteine. Folate and methylmalonic acid did not differ significantly between the study groups.<sup>22</sup> Similarly, Hoffman et al. did not find any significantly difference in the homocysteine and red blood cell folate levels in the women with recurrent abortions compared to the control group.<sup>23</sup> Our results indicate that folic acid and vitamin B<sub>12</sub> do not play a crucial role in the etiology of abortion or ectopic pregnancy.

There is emerging evidence on the effect of homocysteine on nitric oxide concentration and vice versa. Woo et al. observed that homocysteine at concentrations of 0.05-0.1 mM significantly stimulated NO production and iNOS activity in macrophages via increased expression of iNOS mRNA and protein.<sup>24</sup> On the other hand, Lee et al. demonstrated that nitric oxide protects the cells from the effects of homocysteine by S-nitrosylation of homocysteine.<sup>25</sup> These results predict that a dynamic redox balance is maintained in the tubular cells, by the factors that influence concentration of homocysteine and nitric oxide, which further dictates the pregnancy outcome. It is a promising area in the pathogenesis and biomarker discovery for ectopic pregnancy and abortions.

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## REFERENCES

1. Khaskeli M, Baloch S, Baloch AS. Risk factors in early pregnancy complications. *J Coll Physicians Surg Pak.* 2010;20(11):744-7.
2. Murray H, Baakdah H, Bardell T, Tulandi T. Diagnosis and treatment of ectopic pregnancy. *Can Med Assoc J.* 2005;173(8):905-12.
3. Shaw JLV, Dey SK, Critchley HOD, Horne AW. Current knowledge of the aetiology of human tubal ectopic pregnancy. *Hum Reprod Update.* 2010;16(4):432-44.
4. Lauzikiene D, Drasutiene GS, Mecejus G, Zakareviciene J. Serum folate & homocysteine concentrations in women with the first early spontaneous pregnancy loss. *Acta Medica Lituanica.* 2003;10(4):207-12.
5. Cartwright J, Duncan WC, Critchley HOD, Horne AW. Serum biomarkers of tubal ectopic pregnancy: current candidates and future possibilities. *Reproduction.* 2009;138:9-22.
6. Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. *Am J Physiol.* 1997 Feb;272(2 Pt 2):R441-63.
7. Norman JE, Cameron IT. Nitric oxide in human uterus. *Rev Reprod.* 1996;1:61-8.
8. Sydow K, Schwedhelm E, Arakawan N, Bode-Böger SM, Tsikas D, Hornig B, et al. ADMA and oxidative stress are responsible for endothelial dysfunction in hyperhomocysteinemia: effects of L-arginine and B vitamins. *Cardiovasc Res.* 2003;57:244-52.

9. Baban RS. Oxidative stress in recurrent pregnancy loss women. *Saudi Med J.* 2010;31(7):759-63.
10. Paradisi R, Fabbri R, Battaglia C, Facchinetti F, Venturoli S. Nitric oxide levels in women with missed and threatened abortion: results of a pilot study. *Fertil Steril.* 2007 Sep;88(3):744-8.
11. Marinoni E, Di Iorio R, Lucchini C, Di Netta T, Letizia C, Cosmi EV. Adrenomedullin and nitric oxide synthase at the maternal-decidual interface in early spontaneous abortion. *J Reprod Med.* 2004 Mar;49(3):153-61.
12. Dixon RE, Hwang SJ, Hennig GW, Ramsey KH, Schripsema JH, Sanders KM, et al. Chlamydia infection causes loss of pacemaker cells and inhibits oocyte transport in the mouse oviduct. *Biol Reprod.* 2009;80:665-73.
13. Stuhlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP. Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. *Circulation.* 2001;104:2569-75.
14. Zhang X, Li H, Jin H, Ebin Z, Brodsky S, Goligorsky MS. Effects of homocysteine on endothelial nitric oxide production. *Am J Physiol Renal Physiol.* 2000 Oct;279(4):F671-8.
15. Refaat B, Simpson H, Britton E, Biswas J, Wells M, Aplin JD, et al. Why does the fallopian tube fail in ectopic pregnancy? The role of activins, inducible nitric oxide synthase, and MUC1 in ectopic implantation. *Fertil Steril.* 2012 May;97(5):1115-23.
16. Al-Azemi M, Refaat B, Amer S, Ola B, Chapman N, Ledger W. The expression of inducible nitric oxide synthase in the human fallopian tube during the menstrual cycle and in ectopic pregnancy. *Fertil Steril.* 2010 Aug;94(3):833-40.
17. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. *Obstet Gynecol.* 2000;95(4):519-24.
18. D'Uva M, Di Micco P, Strina I, Alviggi C, Iannuzzo M, Ranieri A, et al. Hyperhomocysteinemia in women with unexplained sterility or recurrent early pregnancy loss from Southern Italy: a preliminary report. *Thromb J.* 2007 Jul;5:10.
19. Kumar KS, Govindaiah V, Naushad SE, Devi RR, Jyothy A. Plasma homocysteine levels correlated to interactions between folate status and methylene tetrahydrofolate reductase gene mutation in women with unexplained recurrent pregnancy loss. *J Obstet Gynecol.* 2003 Jan;23(1):55-8.
20. Sütterlin M, Bussen S, Ruppert D, Steck T. Serum levels of folate and cobalamin in women with recurrent spontaneous abortion. *Hum Reprod.* 1997 Oct;12(10):2292-6.
21. Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. *Obstet Gynecol.* 2000;95(4):519-24.
22. Hübner U, Alwan A, Jouma M, Tabbaa M, Schorr H, Herrmann W. Low serum vitamin B<sub>12</sub> is associated with recurrent pregnancy loss in Syrian women. *Clin Chem Lab Med.* 2008;46(9):1265-9.
23. Hoffman ML, Scoccia B, Kurczynski TW, Shulman LP, Gao W. Abnormal folate metabolism as a risk factor for first-trimester spontaneous abortion. *J Reprod Med.* 2008 Mar;53(3):207-12.
24. Woo CW, Cheung F, Chan VW, Siow YL. Homocysteine stimulates inducible nitric oxide synthase expression in macrophages: antagonizing effect of ginkgolides and bilobalide. *Mol Cell Biochem.* 2003 Jan;243(1-2):37-47.
25. Lee SJ, Kim KM, Namkoong S, Kim CK, Kang YC, Lee H, et al. Nitric oxide inhibition of homocysteine-induced human endothelial cell apoptosis by down-regulation of p53-dependent noxa expression through the formation of S-nitrosohomocysteine. *J Biol Chem.* 2005;280:5781-8.

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