Original Article

Comparison of Blood Homocysteine Levels between Women with Recurrent Pregnancy Loss and Women with Normal Fertility

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Abstract

Background: *Recurrent early pregnancy loss is a common but frequently unexplained obstetric problem. As it happens in early weeks of pregnancy, abnormal vasculogenesis and disordered cell multiplication are possible causes. Hyperhomocysteinemia is associated with both these pathological processes.* ***Objective:*** *To assess the association between hyperhomocysteinemia and recurrent pregnancy loss.* ***Materials and Methods:*** *This case-control observational study was conducted in the Department of Gynaecology and Obstetrics of Bangabandhu Sheikh Mujib Medical University, Dhaka during January to December 2015. Sixty patients were divided into two groups: 30 with recurrent pregnancy loss (RPL) as cases and 30 normal healthy mothers as controls.* ***Results:*** *All the subjects were matched in terms of age (p = 0.504). All were within normal BMI range (19.5 to 25 kg/square meter). Subjects in both groups were comparable in height, weight and BMI. 33.3% women of control group were in the low income stratum (monthly income 20000 taka or less) compared to50% of the RPL group. Control group had an equal mix of educational level from below Secondary School to Post-Graduates whereas there were fewer Post Graduate patients (3.3%) in the RPL group compared to control group (23.3%). The frequency of hyperhomocysteinemia (>15 micromoles/L) was significantly higher in cases compared to controls (46.7 vs.16.7%, p= 0.012). Mean homocysteine level was also significantly higher in cases compared to controls (13.67+/-4.80 vs. 9.87+/-4.84 micromole/L; p= 0.003).* ***Conclusion:*** *This study shows that blood homocysteine level in recurrent pregnancy loss patients is significantly higher compared to normal fertile mothers. There is a strong association between hyperhomocysteinemia and RPL.*

Key words: *Recurrent early pregnancy loss; Hyperhomocysteinemia; Fertility*

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Introduction

Hyperhomocysteinemia has been identified as an emerging risk factor for several diseases of diverse systems such as vascular thrombosis, adverse pregnancy outcome, congenital malformations, vascular dementia; its role in unexplained infertility and recurrent early pregnancy loss is currently a focal point of research, owing to its association with IVF failures.

Homocysteine (Hcy) is a sulfhydryl containing amino acid which does not occur in natural proteins, but

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rather is a de-methylated derivative of methionine and also occurs as an intermediary product in the metabolism of other amino acids such as glycine, cysteine, cystathionine, serine, glutathione etc. However, normally the homocysteine thus formed is only a transient product, going to produce cysteine (an important amino acid required for protein synthesis) or glutathione (an important anti-oxidant in cells) or being re-methylated into methionine, effectively recycling the homocysteine. Impaired function of these metabolic pathways leads to accumulation of homocysteine, either by insufficient trans-sulfuration or by an impairment of re-methylation.[1] Elevation in intracellular homocysteine concentration with a corresponding increase in blood levels can result from either genetic defects in the enzymes involved in its metabolism or nutritional deficiency of vitamin co-factors.[2]

In atherosclerotic vascular disease models, homocysteine has been shown to a) induce vascular inflammation by enhanced expression of pro-inflammatory cytokines;[3] b) reduce endothelium-dependent vasodilatation by accelerated inactivation of NO (nitric oxide)/eNO (endothelial NO) or by increased serum di-methyl-arginine formation;[4] c) increase endothelial oxidative stress by auto-oxidation by the highly reactive thiol group, formation of intracellular superoxides and peroxyl radicals, and reduced cellular anti-oxidants (superoxide dismutase and glutathione peroxidase)[3] and d) alter cellular redox potential and interfere with disulfide bond formation in endoplasmic reticulum (ER), causing unfolded or misfolded proteins to accumulate in ER, which then activate the so called Unfolded Protein Reaction (UPR) leading to subsequent growth arrest and apoptosis.[5]

NO is involved in almost every step of female reproduction, i.e., ovulation, early embryonic cleavage, implantation, regulation of circulatory dynamics, uterine quiescence, etc. Physiological NO concentration has a very narrow range and either an excess or a lack of NO adversely affects reproductive outcomes. Similarly, oxidative stress and apoptosis play a role in events such as follicular development and cyclical endometrial change.[6]

Traditionally recurrent early pregnancy loss (RPL) has been defined as three or more spontaneous pregnancy losses before 20 weeks of gestation (age of viability). The practice committee of the American Society of Reproductive Medicine (ASRM) defines RPL as " a disease distinct from infertility, defined by two or more failed pregnancies." In young women, 15 to 20% of clinically recognized and more than 50% of all pregnancies undergoes spontaneous loss. These figures increase substantially with maternal age to as high as 40% and 85% respectively in women 40 years and older. The chance of having two consecutive losses is 5% with 1% of couples experiencing three consecutive miscarriages.[7] In efforts to minimize otherwise preventable pregnancy loss, most clinicians favor diagnosing RPL after two consecutive losses, but it is important to note that many of the potential etiologies of RPL are not absolute, so occurrence of interval live births does not preclude a diagnosis of RPL.[8] A large amount of scientific work has been done to investigate the role of hyperhomocysteinemia in malformations and pathogenesis of ongoing pregnancy, while data and observations are only emerging now regarding a possible involvement of this biochemical phenomenon in the early stages of reproduction and related diseases. There is a significant negative correlation between follicular fluid homocysteine concentration and the degree of maturity of retrieved oocytes as well as embryo quality on day 3 in patients undergoing IVF.[9] IVF outcomes in terms of pregnancy rate (defined as USG detected fetus at 7 weeks of IVF), implantation rate (number of gestational sacs per 100 embryo-transfers) were reduced and abortion rate (between USG detected pregnancy and 20 completed weeks of gestational age) were significantly increased in women with hyperhomocysteinemia compared to those in whom interventions were instituted to reduce blood homocysteine levels.[10] Studies also found that folate, cobalamin and tHcy levels in mono-follicular fluid are related to embryo quality in women undergoing IVF/ICSI.[11]

Review of the evidence on the effects of hyperhomocysteinemia on reproductive outcome show that while there are evidence that maternal hyperhomocysteinemia is a risk factor for recurrent

embryo loss and even a first early pregnancy loss, evidence also indicate that genetic polymorphism due to mutation in MTHFR enzyme causing high homocysteine concentration from abnormal folate metabolism increased risk of REPL.[12] Possible mechanisms of the deleterious effects of hyperhomocysteinemia on female reproduction include reduced cell division (e.g., of oogonia or of granulose cells), increased oxidative stress, apoptosis, reduced extra-embryonic vasculogenesis, etc.[13]

There has been a growing awareness about reproductive failure with the advent of reproductive techniques and services in Bangladesh recently. The investigation of recurrent pregnancy loss includes laboratory, imaging, invasive and genetic analysis. Despite all these work-up, the cause of a significant portion of cases of reproductive failures such as recurrent early pregnancy loss remains unknown. Such unexplained cases merit exploration for other uncommon causes. There are reports of 2 to 3-fold higher concentration of homocysteine among such patients, both in western as well as sub continental countries.[14,15] Hyperhomocysteinemia merits consideration in such cases. Because hyperhomocysteinemia is easily detected and is amenable to easy intervention by vitamin supplementation in a majority (i.e., two-thirds) of cases, exploring its relation to and contribution towards recurrent early pregnancy loss are important.

The present study explores the blood levels of homocysteine in women with recurrent early pregnancy loss without an obvious cause compared to women with normal fertility.

Materials and Methods

This was a case-control observational study, conducted from January 2015 to December 2015. The study population consisted of women of reproductive age attending the outdoor and indoor department of Obstetrics and Gynecology at Bangabandhu Sheikh Mujib Medical University with women having normal fertility (as controls), unexplained recurrent pregnancy loss (as cases). The approval of the Local Ethical Committee was taken. The aims, objectives, procedures of collecting samples, risks (if any) and benefits were explained to study subjects in very easily understandable local language. Participation of subjects was strictly on voluntary basis, on the basis of informed consent. Subjects were assured that all records would be kept confidential. It was explained to them that the study would help both physicians and patients by finding out scientific facts that will help in making rational treatment choices regarding the management of patients. A purposive sampling method was used. All subjects were selected by history, examination as well as appropriate diagnostic workup.

Inclusion criteria:

a. Married women aged 20 to 40 years, and BMI between 19 and 25.

b. Control group: Healthy women with one or more successful pregnancy without any obstetrical complications (e.g., intrauterine growth restriction, preeclampsia, abortion and MR) and never required assisted reproduction,

c. Cases: Women who, despite conception, have had two or more pregnancy losses before 20 weeks of gestation and diagnosed as unexplained after i) anatomic evaluation of by TAS, TVS and HSG and/or hysteroscopy with laparoscopy reveal normal uterine cavity and absence of polycystic ovaries, ii) endocrine assessment by basal FSH, LH, estradiol, TSH, prolactin and fasting blood glucose were normal, iii) evaluation of immunological factors antinuclear antibody (ANA), anti-DNA antibody, antiphospholipid antibody, anticardiolipin antibody, iv) karyotyping of both partners were normal and v) normal semen analysis of husband.

Exclusion criteria:

a. Women aged <20 or >40 years, BMI <19 or >25 kg/m^2

b. Women with recurrent pregnancy loss due to identifiable causes

c. Women having other significant medical disorder (chronic cardiac, renal, hepatic or pulmonary disorders requiring chronic medications).

d. Women who have received folate, vitamin B6 and B12 supplementation within last three months.

Operational definitions for the purpose of this study:

1. Recurrent Early Pregnancy Loss: two consecutive or more involuntary termination of pregnancy before twenty weeks of gestation (dated from the last menstrual period).
2. Hyperhomocysteinemia: Defined as a fasting serum homocysteine concentration of more than 15 micromole/L.
3. Reproductive age group: 15−49 years

Samples were identified and data were collected on a structured data collection sheet and divided into controls and cases according to the inclusion and exclusion criteria. All data were collected by interview, physical and laboratory examination of blood samples and recorded in the data collection sheet.

After editing and coding, coded data was entered into computer database of the Statistical Package for the Social Sciences (SPSS) software (SPSS Inc., Chicago, Illinois, USA) Version 22 and data organizing and final analysis were performed on the above software. Categorical data was presented as frequency and percentage, and continuous variables were expressed as mean and standard deviation. The Chi-squared test was used to analyze and compare discrete variable and Students t test where appropriate. The statistical significance (p) threshold was set to ≤0.05 (two-tailed).

Results

Results of comparison of sixty women, 30 in control group (women with normal fertility) and 30 in the unexplained recurrent early pregnancy loss group are presented in the following tables.

All the subjects matched in terms of age group categories. The mean ages of all the groups were comparable, with no statistically significant difference. The subjects in the groups were well-matched in terms of height, weight and BMI. The women included in the study were within the normal BMI range (from 19.5 to 25.0 kg/m^2) with no statistical difference between group means among the pre-specified groups. This excluded any role of height or low body weight contributing to the incidence of cases among these subjects.

Analysis of the socio-economic status in terms of monthly income showed that there were more patients in the lowest stratum of monthly income among those with recurrent early pregnancy loss; however, overall there was no statistically significant difference between the groups in terms of monthly income level. Since the study was conducted in a hospital setting accessible to patients of even low income status, subjects included in the study represented mostly low and middle income family background. The subjects in the control group had an equal mix of educational levels from below secondary school certificate to post-graduate levels whereas there were very low post-graduate patients in the recurrent early pregnancy loss group commensurate with their socio-economic status in terms of monthly income (Table II); however, difference in the frequency of various educational achievements between the groups did not reach statistical significance.

The distribution of various occupations between the study groups did not show any significant difference

Table I: Comparison of biological variables between controls (women with normal fertility) and cases (women with recurrent early pregnancy loss) (N=60)

Variables	Controls (n=30)	Cases (n=30)	p values
Age (years)	28.90±5.09	28.0±5.27	0.504^{ns}
Height (cm)	156.17±6.92	153.20±7.14	0.108^{ns}
Weight (kg)	56.73±6.78	56.37±5.77	0.822 ^{ns}
BMI (kg/m^2)	23.26±1.55	23.75±1.38	0.204 ^{ns}

Data are presented as mean ± SD. Student's t test was used to compare the variables between the groups. N=Number of study population; n=Number in each group; s= Significant; ns=Not significant; SD=Standard deviation

Table II: Comparison of socioeconomic status by monthly income of the subjects between two groups (N=60)

Variables	Controls (n=30)	Cases (n=30)	p values
Monthly income			
Taka < 1000	1 (3.3%)	7 (23.3%)	0.072[ns]
Taka 10000−20000	9 (30.0%)	8 (26.7%)	
Taka >20000	20 (66.7%)	15 (50.0%)	
Educational status			
Below SSC	7 (23.3%)	9 (30.0%)	0.096[ns]
SSC	6 (20.0%)	4 (13.3%)	
HSC	4 (13.3%)	3 (10.0%)	
Graduates	6 (20.0%)	13 43.3%)	
Postgraduates	7 (23.3%)	1 (3.3%)	
Occupations			
Housewives	14 (46.7%)	14 (46.7%)	0.356[ns]
Students	0 (0.0%)	4 (13.3%)	
Services	12 (40.0%)	10 (33.3%)	
Doctors	1 (3.3%)	1 (3.3%)	
Nurses	2 (6.7%)	1 (3.3%)	
Day Labors	1 (3.3%)	0 (0.0%)	

Chi-square test was used to compare between two groups. N=Number of study population; n=Number in each group; s= Significant; ns=Not significant

Table III: Comparison of marital and obstetric history between two groups (N=60)

Variables	Controls (n=30)	Cases (n=30)	p values
Marital history			
Marital period (years)	7.88±3.54	7.94±4.40	0.960[ns]
Coital frequency per week	3.63±0.76	3.40±0.81	0.257[ns]
Obstetric history			
Abortion	0 (0%)	30 (100.0%)	<0.001[s]
Ectopic pregnancy	0 (0%)	1 (3.3%)	0.313[ns]
MR	0 (0%)	3 (10.0%)	0.076[ns]
Parity (mean±SD)	1.50±0.63	0.47±0.73	<0.001[s]

Continuous variables are presented as mean±SD and categorical variables are presented as number and percentage. Student's t test was used to compare continuous variables and Chi-square test for frequency data. N=Number of study population; n=Number in each group; s= Significant; ns=Not significant; SD=Standard deviation

Table IV: Comparison of the frequency of hyperhomocysteinemia between two groups (N=60)

Homocysteine level	Controls (n=30)	Cases (n=30)	p values
≤ 15 µmol/L	25 (83.3%)	16 (53.3%)	0.012[s]
> 15 µmol/L	5 (16.7%)	14 (46.7%)	
Mean, SD µmol/L	9.87±4.84	13.67±4.80	0.003[s]

Frequency data are presented as number and percentage while continuous variables were presented as mean and standard deviation. Statistical analysis was done by Chi-square test. N=Number of study population; n=Number in each group; s= Significant; ns=Not significant; SD=Standard deviation

statistically. Subjects from all social strata were included in the study, owing to the fact that the place of study is an Institution accessible to a wide section of the population of the country.

All the subjects included in both the study groups, had similar duration of marriage and had a healthy marital life in terms of their coital frequencies. This pattern reflects regular marital habits. History of previous Menstrual Regulation procedures were seen in 10% of the subjects with recurrent early pregnancy loss and none in the control group, giving rise to a statistically significant difference among the groups in terms of obstetric history.

Based on the institutional cut-off value of 15 micromoles/L for serum fasting homocysteine level, the frequency of hyperhomocysteinemia was found in significantly higher number of patients in the recurrent early pregnancy loss group compared to the control group. Comparison of serum fasting homocysteine levels among the two study groups showed that patients and the recurrent early pregnancy loss group had significantly higher levels compared to the control group of women.

Discussion

The results of the present study showed that the frequency of hyperhomocysteinemia defined as fasting serum homocysteine levels >15micromole/L (Table IV) are significantly higher in the REPL group (46.7% p = 0.012 for difference from controls) compared to control group (16.7%). Furthermore, the fasting serum homocysteine levels (Table IV) in study women with unexplained recurrent early pregnancy loss (mean 13.67 ± SD 4.80 micromoles/L) were significantly higher compared to those in study control women with normal fertility (mean 9.87 ± SD 4.84 micromoles/L). The findings of this study are consistent with results from previous studies in Europe as well as the subcontinent. The association of hyperhomocysteinemia with adverse pregnancy outcomes, including recurrent pregnancy loss has been in evidence in several previous studies. Furthermore, while considerable bodies of evidence are available from studies in the assisted reproductive background, epidemiological evidence is rare. The findings of comparable levels of serum homocysteine in the recurrent (early) pregnancy loss groups in all these studies – upheld by the findings of the present study – also strengthens the association of hyperhomocysteinemia with unexplained adverse pregnancy outcomes.

As has been already mentioned, a link between hyperhomocysteinemia and adverse pregnancy outcomes have been demonstrated in various previous studies, most notably the large epidemiological Hordaland Homocysteine study.[16] Data available from 5883 women with hyperhomocysteinemia in the age group 40−42 years with over 14,000 pregnancies showed increased incidence of stillbirths and placental abruptions among other adverse pregnancy outcomes. In China, a pilot study exploring the nutritional background of 30 women with an embryonic gestations with absent fetal poles showed higher homocysteine levels compared to 30 controls with a normal healthy delivery.[17] A retrospective case-control study of 200 women in each group found that while MTHF 677 T/T (homozygous genotype) was associated with increased chances of recurrent early and late pregnancy losses as well as similar elevations of serum homocysteine among cases and controls, regression analysis did not reveal any association between hyperhomocysteinemia (defined as serum homocysteine level >15micr-moles/L) and recurrent pregnancy loses.[18] It may be argued that in both studies the presence of other stronger adverse modulators of fertility and pregnancy out- comes have predominated and confounded the effects of hyperhomocysteinemia. For example, it is postulated that the association of MTHFR 677 T/T homozygous genotype and Vitamin B6 status with recurrent early pregnancy loss, independently of hyperhomocysteinemia, was due to interference with red blood cell folate metabolism.

In the present study confounding variables were avoided by matching the cases for age and BMI. However, the study included far smaller sample size than the number suggested by the sample size calculation. Furthermore, since all women with history of recent intake of vitamin folic acid, B6 and B12 supplementation were excluded and no attempts were made to assess their vitamin status, the effects of

such confounders could not be excluded. This study is the first of its kind on the association of high serum homocysteine level on fertility and pregnancy outcomes in Bangladesh.

The levels of homocysteine in normal healthy populations can be found from the control group of various studies relating the effects of homocysteine on conditions such as pre-eclampsia and eclampsia and coronary artery disease. In a study on the effects of serum homocysteine on pre-eclampsia and eclampsia, the healthy control population of 136 women at late middle to late pregnancy (gestational age of mean 30.80 ±SD 4.03 weeks) had a fasting homocysteine level of 6.86±2.47 micromoles/L, which is considerably low compared to the control group in the present study (9.87±4.84 micromoles/L). Likewise, the 84 pre-eclampsia patients (with homocysteine level of mean 9.54±SD 3.21 micromole/L) and 120 eclampsia patients (homocysteine level 10.57±3.39 micromole/L) had considerably lower levels of homocysteine compared to the cases in the current study (Table IV).

However, their difference can be explained by the fact that serum homocysteine level decreases during pregnancy, especially in the later parts.[19]

Das et al[20] found a serum homocysteine level in a control group without coronary artery disease was mean 9.66±SD 3.54 micromole/L, which was comparable to finding in the present study (9.87±4.84 micro-mole/L).

In a large population based cohort, Gamble et al[21] on prevalence of folate and cobalamin deficiency and incidence of hyperhomocysteinemia in Bangladesh, in 973 women, the plasma homocysteine was found to be mean 9.5±4.7 micromole/L, also comparable to the level found in controls in the current study, showing that the subjects were representatives of the women in Bangladesh.

In a limited sample of women with Recurrent Early Pregnancy Loss mean serum fasting homocysteine levels and hyperhomocysteinemia were increased compared to age and BMI matched women with normal fertility and healthy pregnancy outcomes. However, a larger study with adequately powered sample size and assessment of other confounding variables are required to further corroborate the effect of hyperhomocysteinemia on fertility and pregnancy outcomes.

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