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Indian Journal of Obstetrics and Gynecology Research

Journal homepage: www.ijogr.org

Original Research Article

Nutritional deficiency and MTHFR gene polymorphism in obstetrics

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ARTICLE INFO

Article history:
Received 27-02-2023
Accepted 22-03-2023
Available online 18-05-2023

Keywords:
Folic acid
Vitamin B12
Homocysteine
MTHFR gene
NTD
RPL
AP
PTL

ABSTRACT

Aim: This study was carried out to determine the association of folic acid deficiency, vitamin B12 deficiency, hyperhomocysteinemia and methylenetetrahydrofolate reductase (MTHFR) gene polymorphism with obstetrical events linked to folic acid deficiency like neural tube defects (NTD); unexplained abruption placentae (AP), recurrent pregnancy loss (RPL) and preterm labour(PTL).

Materials and Methods: In the present study 87 pregnant females with past or present history of either neural tube defect (NTD), abruptio placentae, recurrent pregnancy loss (RPL) or preterm labour and 100 pregnant females without any such history were enrolled. Serum levels of folic acid, vitamin B12 and homocysteine in these females were estimated using chemiluminescence and Polyacrylamide gel electrophoresis (PAGE) was used to detect MTHFR gene polymorphism.

Results: No significant association was observed between serum folic acid levels and NTD (p = 0.495), RPL (p = 0.832) or preterm labour (p = 0.724). However, folic acid deficiency had significant association with the occurrence of abruptio placenta (p = 0.001). Serum vit B12 deficiency was found to be a significant risk factor only in patients with RPL. Increased homocysteine revealed significant association with RPL (p= 0.024), abruption placentae (p=0.002) and preterm labour (0.015). No polymorphism in MTHFR gene could be revealed in the above pregnancy complications.

Conclusion: In the present study, deficiency of folic acid was uncommon probably due to its routine supplementation throughout the first trimester. However, preconceptional folic acid supplementation still needs to be emphasized to build up adequate folic acid levels required during embryogenesis. The relationship between vit B12 and RPL needs studies on larger number to establish the association before supplementation is suggested. MTHFR677 gene polymorphism may have remained undetected due to small sample size.

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1. Introduction

Folate is an important micronutrient needed for protein and nucleic acid synthesis which are prerequisites for cell growth and division. Inability of the human body to synthesize folate requires it to be provided through dietary intake (folate) or through vitamin supplements (folic acid). But both folate and folic acid are metabolically inactive. They are absorbed from the gastrointestinal tract and sequentially reduced by the enzyme dihydrofolate reductase (DHFR) to tetrahydrofolate(THF).[1] THF is then transformed enzymatically to 5-MethylTHF by methylene tetrahydrofolate reductase(MTHFR).[1] 5-Methyl THF is the biologically active form of folate found in plasma.[2]

This 5-MethylTHF acts as a methyl group donor in one-carbon metabolism required for protein and nucleic

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https://doi.org/10.18231/j.ijogr.2023.040
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acid synthesis.[3] The amino acid homocysteine accepts a methyl group donation from 5-MethylTHF and converts to methionine. A vitamin B12 containing enzyme methionine synthase carries out this step. Methionine also acts as a methyl group donor in one carbon metabolism[4] required for protein synthesis.

Therefore, insufficient supply of either 5-MethylTHF (due to folate deficiency or genetic polymorphism in MTHFR gene) or cofactor vitaminB12 halt this conversion process resulting in hyperhomocysteinemia[5] and low levels of methionine which compromises DNA and protein synthesis.

Folate requirement increases through the antenatal period as it is essential for fetal maturation.[1] The importance of folate was first of all established in the prevention of neural tube defects (NTD)[6] during pregnancy. NTDs are birth defects of the brain and spinal cord where the neural tube is unable to close during embryogenesis. Commonest among these are anencephaly and meningomyelocele. Anencephaly usually leads to stillbirth whereas infants with meningomyelocele survive but with serious longterm disability.[7] The etiology of NTDs has been explained to be multifactorial including nutritional, environmental, and genetic factors.[2] Nutritional causes of NTDs are dietary folate and vitamin B12 deficiency whereas the genetic causes are polymorphisms in various genes present in the folate-B12 pathway discussed above. In our study we have tried to correlate the nutritional deficiency factor with MTHFR C677T gene polymorphism.

We have already discussed that folate deficiency invariably leads to hyperhomocysteinemia. Elevated plasma homocysteine damages the vascular endothelium which causes oxidative stress and promotes generation of free radicals. Endothelial damage favours thrombosis and inadequate placental perfusion resulting in obstetrical problems like preeclampsia, placental abruption, growth restricted fetus, early pregnancy loss and venous thrombosis.[5]

Furthermore, pregnancy is itself a hypercoagulable state with increased risk of thromboembolic disease even in women without thrombophilia.Inherited and acquired thrombophilias, if present, have additive effect and further predispose to thrombosis. Thrombophilias are the underlying cause in around fifty percent of those affected by thromboses.The thermolabile variant of methylene tetrahydrofolate reductase MTHFR C677T gene (replacement of cytosine by thymine at nucleotide 677)is one of the common inherited thrombophilias.[8]

Abruptio placenta which is explained as early separation of placenta from the uterine wall before delivery, is an obstetrical complication with grave maternal and neonatal prognosis. Common risk factors include preeclampsia, smoking, polyhydramnios, premature rupture of membranes, trauma and prior history of abruption. Etiology of some cases still cannot be explained.[9] Low folate levels,[10] hyperhomocysteinemia[11] and MTHFR C677T mutation[12] increase the chances of abruption placentae due to their thrombotic tendency.

Recurrent pregnancy loss (RPL) which is explained as two or more consecutive pregnancy lossesbefore 20 weeks is a considerable issue with common causes being chromosomal anomalies, uterine malformations, thrombophilia, endocrinopathy, and immunological. After evaluation for these causes, around 50% of the cases still remain unexplained.[13] Folic acid is necessary for oocyte quality, maturation, blastocyst implantation and ensuing normal pregnancy. Low folate levels and MTHFR C677T mutation are associated with hyperhomocysteinemia which increases oxidative stress and promotes apoptosis. This interferes with functioning of the placental trophoblast thereby disturbing implantation and pregnancy maintenance.[14]

Preterm birth (PTB) resulting from preterm labour defined as delivery prior to 37 completed weeks of gestation is the most important cause of neonatal morbidity and mortality. It has both short and long term detrimental health sequelae in the form of respiratory problems, cognition and behavioural problems and also puts a significant financial burden on the family and the society as well. Etiology of preterm birth has been shown to be multifactorial with poor socioeconomic strata, previous history of abortions, multiple pregnancy, previous preterm birth and medically indicated preterm delivery being the common causes.[15] Low serum folate levels have been linked to preterm birth with folate supplementation having shown to decrease its incidence.[16,17] Various studies have also tried to establish a genetic association to spontaneous preterm birth.[18–20]

To the best of our knowledge, there is not much of literature among the north Indian population where the role of genetic polymorphism of methylene tetrahydrofolatereductase gene has been studied in relation to vitamin B_{12} and folic acid deficiency along with different clinical outcomes in pregnant females. Therefore, the present study was planned to study the genetic polymorphism of MTHFR C677T gene, hyperhomocysteinemia and clinically correlate it with vitamin B_{12} and folic acid deficiency among pregnant females.

2. Materials and Methods

This study enrolled 187 pregnant females, visiting the Obstetrics and Gynaecology department, Government Medical College and Hospital, Chandigarh for antenatal care from June 2015 to September 2016. The work on genetic polymorphism was done in collaboration with the Parasitology department, Postgraduate Institute of Medical Education and Research (PGIMER Chandigarh). The Institute's Ethics Committee at GMCH Chandigarh

gave ethical clearance. Inclusion criteria for the case group were any of the following: women with pregnancy affected by neural tube defect, recurrent pregnancy loss, spontaneous preterm labor or with placental abruption. The exclusion criteria was subjects with induced abortions, infection, uterine structural abnormalities, those on drugs affecting folate metabolism such as anticonvulsants, antitubercular drugs, alcohol and those with pregestational diabetes mellitus. The control group included 100 antenatal women without a history of NTD, preterm labour, recurrent pregnancy loss or placental abruption. Matching of the test and control groups was done for age, geographical region, social class and ethnic area to have a similar gene pool. Sample size was estimated according to the study by James et al[21] where the odds ratio was 2.6. Using the 95% confidence coefficient (α=5%) and a confidence interval for the odds ratio with an amplitude of d=4.6, the sample size was estimated as a minimum of 80 cases and 100 controls. A detailed history and clinical examination findings were recorded.

Blood sample collection: After written informed consent, six ml of blood was collected. Two ml was transferred into a plain vial for folic acid and vitamin B_{12} levels estimation, two ml into an EDTA vial for homocysteine level estimation and another two ml into an EDTA vial for molecular analysis. After centrifugation the samples were stored at -20^{0}C for biochemical estimation. Serum folic acid, vitamin B_{12} and serum homocysteine levels were estimated via chemiluminiscence method using Advia Centaur XP machine.

For detection of genetic polymorphism, following steps were followed sequentially:

a) DNA extraction –

This was carried out from whole blood using Qaigen kit. The separated DNA was stored at -20^{o}C till further analysis.

b) PCR amplification

The target gene (MTHFR) was amplified by using previously published primers by Kohli et al.[22] The basis of PCR amplification- denaturation, annealing and elongation were standardized and used for amplifying the target region of MTHFR gene. Storage of the PCR products was done at -20 ° C.

c)Restriction fragment length polymorphism (RFLP)

RFLP was performed for analysing mutations in the MTHFR gene by digesting the amplified product with restriction enzyme. The amplified product was analysed in 2% agarose gel using ethidium bromide staining. The C to T substitution at nucleotide 677 creates TaQ1 restriction site. Restriction enzyme Taqα1 was used which cleaves the original 134 base pair PCR fragment into 75 and 59 base pair fragments. In the absence of this mutation, the PCR product remained uncleaved as 134 bp after Taq I digestion. Size fractionation of PCR products was established by electrophoresis. Allelic sizes were compared with known molecular weight markers.[23]

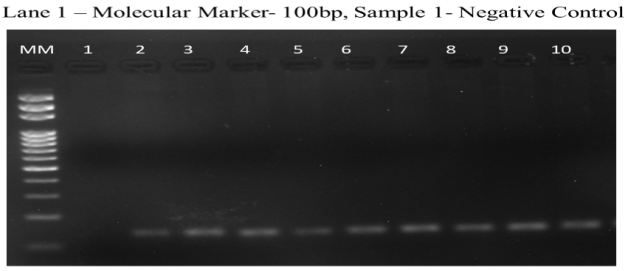

Fig. 1: Photograph of agarose gel electrophoresis for PCR amplification of MTHFR gene fragment

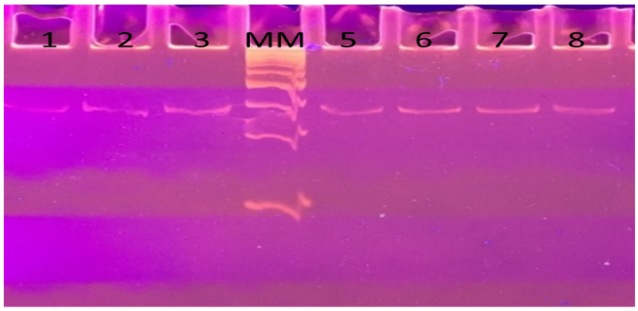

Fig. 2: DNA bands visualizedin UV light after ethidium bromide staining

Subjects having absence of this polymorphism showed one 134 bp fragment. Subjects heterozygous for the polymorphism would show both 134bp and 75bp fragments.

2.1. Statistical analysis

While carrying out statistical analysis, correlation of genetic polymorphism in MTHFR gene with vitamin B_{12} and folate deficiency was done. Quantitative data was presented as mean and range as appropriate. Normality of quantitative data was checked by measures of Kolmogorov Smirnov tests of normality. For normally distributed data, mean were compared using T-test. For skewed data or scores, Mann-Whitney U-test was used. For discrete categorical data, number and percentages were calculated. Chi-Square test or Fisher's Exact test were applied for categorical data. All statistical tests were two sided. A P value of <0.05 was considered statistically significant. Analysis was conducted using SPSS for windows (version 17.0; SPSS Inc., Chicago, IL, USA).

3. Results

Comparing the age of pregnant women in the study group, the mean age in the test group was 26.13 years whereas in the control group was 25.29 years. Both the groups were found to have no significant difference with respect to the

age distribution. (p value of 0.160)

Serum folic acid deficiency was considered at less than 5.38ng/ml. 16.7% of cases with NTDs, 21.4% of cases with RPL, 55.2% cases of placental abruption and 25% cases with spontaneous preterm birth were folic acid deficient compared to 24% of controls. Analysis of this data by Pearson Chi-Square Test revealed insignificant role of folic acid in the causation of NTD (p value 0.495), RPL (p value 0.832) and preterm birth (p value 0.724) but a statistically significant association with abruptio placentae (p value 0.001).

Analysis of vit B12 deficiency was done and a cutoff value of <211 pg/ml was considered. 61.1% of cases with NTDs, 85.7% of cases with RPL, 75.9% cases with placental abruption and 62.5% cases with preterm birth showed vit B12 deficiency as against 58% of controls. Chi-Square Test revealed insignificant association of vit B12 with NTD (p 0.805), abruption placentae (p 0.081) and preterm birth (p 0.462) but a statistically significant association with RPL (p 0.046).

Considering hyperhomocysteinemia above a level of 13.9μmol/L, 27.8% of cases with NTDs, 50% of cases with RPL, 51.7% cases with placental abruption and 46.2% cases with preterm birth showed hyperhomocysteinemia as against 22% of controls. Chi-Square Test revealed significant association of hyperhomocysteinemia with RPL (p 0.024), abruption placentae (p 0.002) and preterm birth (p 0.015) but not with NTD (p 0.591).

In our study we found that serum folic acid deficient pregnant females were at higher risk for occurrence of abruptio placentae (p = 0.0001). Similarly, pregnant females with low serum vit B12 levels showed significant difference in the occurrence of RPL (0.046). But in rest of the clinical conditions no association could be found with low serum folic acid and vit B12. Increased serum homocysteine levels in pregnant females were significantly associated with RPL (p = 0.024), AP (p = 0.002) and PTL (p = 0.015).

No genetic polymorphism in MTHFR gene was detected in the present study as the Taqα1 restriction enzyme did not cleave the 134 base pair fragment in any sample during gel electrophoresis.

4. Discussion

The present study tried to find association of folic acid, vitamin B12, homocysteine and MTHFR gene polymorphism with neural tube defects, recurrent pregnancy loss, placental abruption and preterm birth in North Indian women.

Initial research work has established folic acid deficiency to be a causative factor for neural tube defects. Most of the pregnant women start taking folic acid tablets after pregnancy detection when they first visit the antenatal clinic. But fetal neural tube closure is already completed by 28days of gestation which is well before this first

Table 1: Comparison of serum levels FA, Vit B12 and homocysteine with NTD, RPL, AP and PTL

	Normal FA	Folic acid deficiency	P value	Normal vit B12	B12 deficiency	p value	Normal homocysteine	Hyperhomocysteinemia	p value
Pregnancy with NTD	15(83.3%)	3(16.7%)	0.495	7(38.9%)	11(61.1%)	0.805	13(72.2%)	5(27.8%)	0.591
Pregnancy with RPL	11(78.6%)	3(21.4%)	0.832	2(14.3%)	12(85.7%)	0.046	7(50%)	7(50%)	0.024
Pregnancy with AP	13(44.8%)	16(55.2%)	0.001	7(24.1%)	22(75.9%)	0.081	14(48.3%)	15(51.7%)	0.002
Pregnancy with PTL	20(75%)	6(25%)	0.724	9(37.5%)	17(62.5%)	0.462	14(53.8%)	12(46.2%)	0.015
Pregnancy without NTD, RPL, AP, PTL	76(76%)	24(24%)		42(42%)	58(58%)		78(78%)	22(22%)	

pregnancy detection.[24] Hence the role of periconceptional folic acid supplementation has been emphasized.[25] This includes intake of 400-800μg folic acid since the time of pregnancy planning upto 12 weeks gestation so that folic acid levels are adequate during the period of neurulation. Those having history of NTD in prior pregnancy should raise this folic acid supplementation to 4mg.[26] The role of folic acid supplementation during the periconceptional period has been found to be preventive against NTD in the offspring. This has been supported by two large randomized controlled trials, the MRC trial[27] and the Budapest trial.[25] This has been further established through a large body of evidence[28] and is shown to decrease the probability of NTDs by 50- 70%.[29]

In our study, folic acid deficiency was not significantly associated with neural tube defects. 16.7% of cases with NTDs showed folic acid deficiency compared to 24% controls. This can be explained as in our study blood sampling was done once NTD was detected, which was through a target ultrasound at around 18 to 20 weeks period of gestation. And folic acid tablets given during the first three months of pregnancy could have built up normal folic acid levels upto the second trimester, probably concealing the low folic acid levels present during the period of organogenesis. A caveat in our study was that information was not collected regarding folic acid intake during the periconceptional period which if done could have provided greater insight into the subject.

In our study, vit B12 was also not associated with NTDs with 61.1% cases showing deficiency against 58% controls. Neither was homocysteine associated with 27.8% cases showing hyperhomocysteinemia compared to 22% controls. This is similar to studies by Ceyhan et al[30] and Mcmullin et al[31] who observed no association between vit B12 and homocysteine with NTDs.

When we study the folic acid- homocysteine pathway, vitamin B12 is a cofactor necessary for normal activity of the enzyme methionine synthase which accepts one carbon atom from the methyl donor 5-methyl THF and remethylates homocysteine to methionine. Vitamin B12 deficiency impairs this conversion resulting in hyperhomocysteinemia. Also, decreased methionine synthase activity prevents conversion of 5-methyl THF to 5,10- methylene THF. Absence of this tetrahydrofolate regeneration, the so called "folate trap" leads to impairment of DNA synthesis[32] despite adequate folic acid levels. In our study, this concept very well explains hyperhomocysteinemia despite normal folic acid levels in subjects with recurrent pregnancy loss (RPL) who were vitB12 deficient. Deficiency of folic acid in 21.4% and of vitamin B12 in 85.7% of cases with RPL was found in our study with resultant hyperhomocysteinemia in 50% of these cases.

Similar outcomes are reflected in studies by Katre et al which show that vitamin B12 insufficiency leads to raised plasma homocysteine levels in pregnant Indian women who had adequate folic acid levels.[33] Since majority of the Indian population follow a vegetarian diet with only minimal intake of animal products, they are found to be deficient in vitamin B12.[34] At present there are no national guidelines regarding periconceptional vitamin B12 supplementation. Considering the fact that vitamin B12 has been shown to decrease homocysteine levels which could probably decrease the incidence of various obstetric complications cited above, serious efforts should be made to frame guidelines regarding periconceptional vitamin B12 supplementation.

Also, folic acid absorption can be affected despite adequate dietary intake. This can be explained by defects at the molecular level with genetic polymorphism in various genes interfering with the folate and homocysteine-remethylation pathway.[24] This concept can explain the occurrence of preterm labour despite normal folic acid and vitamin B12 levels in our study subjects. Naushad et al studied genetic polymorphism in four genes for having susceptibility to NTDs in south Indian population. One polymorphism out of these, the MTHFR 677 C→T has been studied by us. We used the method of RFLP (restriction fragment length polymorphism) for analyzing mutations in the MTHFR gene where the amplified product was digested by restriction enzyme and then analyzed on PAGE (polyacrylamide gel electrophoresis). None of our samples were cleaved by the restriction enzyme into two fragments showing absence of mutation in the MTHFR gene at nucleotide 677. This may be attributable to small sample size. Also, genetic sequencing which shows actual nucleotide substitution could not be carried out as it was not cost effective in our setup. Due to the same reason, only one gene polymorphism could be studied by us and there may be mutations of other genes in the folate pathway responsible for these obstetric complications.

In our study, 55.2% of the cases with placental abruption were deficient in folic acid levels, 75.9% had low vit B12 levels and 51.7% had hyperhomocysteinemia. The folate pathway easily explains folic acid deficiency, normal vit B12 and raised homocysteine levels in these cases with abruption placentae. Hibbard and Hibbard first reported the relation of deficient folic acid with abruption placentae.[35] This has been supported by many other studies.[36,37] Hyperhomocysteinemia is also known as an important cause for placental abruption.[38] The relationship of MTHFR 677 C→T polymorphism with placental abruption is supported by some[39,40] as well as refuted by[41,42] other studies. We are of the opinion that women with placental abruption not justifiable by the well known causes like smoking, hypertensive disorders of pregnancy, trauma, multiple pregnancy, premature rupture of membranes, etc need

to be investigated atleast for hyperhomocysteinemia, if thrombophilia screen is not possible for all being not cost effective. Those having hyperhomocysteinemia should take periconceptional folate and vit B12 in subsequent pregnancy. Effective guidelines need to be formulated in this direction.

The well-known relationship of shorter interval between consecutive pregnancies with increased incidence of preterm labour can be clarified by the fact that folate consumption occurs throughout the antenatal as well as postnatal period.[43] This has been supported by the fact that periconceptional folate supplementation decreases the incidence of preterm labour.[44] Preterm labour has been associated with increased serum homocysteine levels.[45] This is also evident in our study where 46.2% of the cases with spontaneous preterm labour showed hyperhomocysteinemia. Endothelial dysfunction and vascular injury due to hyperhomocysteinemia has been known to cause placental vasculopathy and thrombosis which leads to overexpression of proinflammatory cytokines and increase in cellular gap junctions. These iniate the cascade of events eventually causing preterm birth.[45]

It is well known that a prior preterm birth increases the probability of a subsequent preterm birth.[46] Studies showing recurrence in individual women, in families and increased prevalence in particular ethnic groups point towards a genetic etiology of preterm labour.[47,48] Wu et al, in their meta- analysis to analyze the relationship of maternal MTHFR gene mutation and the risk of spontaneous preterm birth, showed a positive conclusive relationship between them, especially in developing countries.[49] Still, studies with a larger sample size need to be done to validate the approach of screening high risk women for gene polymorphism to determine their probability of having a preterm delivery.

5. Conclusion

Efforts should be directed towards periconceptional folic acid supplementation. Along with folate deficiency, vitamin B12 deficiency and hyperhomocysteinemia have also been associated with birth defects and pregnancy complications. Serious thought should be given to vitamin B12 supplementation during pregnancy after appropriate discussion with clinicians, scientists and policy makers. Patients having recurrent placental abruption, recurrent preterm birth, recurrent pregnancy loss or recurrent NTDs, and who have been investigated but the cause still remains unexplained, can be tested for genetic mutations of genes in the folate-B12 pathway.

6. Source of Funding

None.

7. Conflict of Interest

None.

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Cite this article: Kaur N, Sehgal A, Sinha S. Nutritional deficiency and MTHFR gene polymorphism in obstetrics. *Indian J Obstet Gynecol Res* 2023;10(2):189-195.