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Original Research Article

Correlation of Folic Acid, Homocysteine and Vitamin B_{12} Levels in Neonates with Neural Tube Defects and Their Mothers with the Disease Occurrence

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Abstract

Aims: The study aimed at elucidating relationship between folic acid (FA), homocysteine and vitamin B12 levels in neonates with neural tube defects (NTDs) as well as their mothers with the occurrence of the disease.

Material and methods: The prospective case control study included 26 neonates with NTDs and their mothers in group-I. Neonates with minor illnesses with their mothers formed the control group (Group-II). Both neonates and mothers were subjected to the measurement of folic acid, homocysteine and vitamin B12 levels in their blood.

Results: Neonates were comparable with regard to the age, gender and maturity. Most of the neonates (46%) had defects at lumbosacral region. Group-I neonates had significantly lower FA levels in serum, red blood cells (RBCs) and whole blood when compared to group-II (p-values <0.001, <0.001, 0.005). Whole blood and RBCs FA levels were higher and serum FA levels were lower in group-I mothers in comparison to group-II (p-values <0.001, 0.10, 0.310). Homocysteine levels were found to be significantly higher in both mothers and neonates in group-I (p-values 0.023, 0.030). Vitamin B12 levels were lower in group-I mothers and neonates (p-values 0.135, 0.695).

Conclusion: Both maternal and neonatal hyperhomocysteinemia play an independent role in the development of NTDs whereas vitamin B12 deficiency carries moderate risk. There is dissociation between maternal and neonatal folic acid levels as high maternal FA levels may not reflect in their neonates. Evaluation of defects in placental FA receptors and transporters which prevent FA transfer to developing fetus may help to understand this enigma.

Keywords: Neural Tube Defects, Folic Acid, Homocysteine, Vitamin B12, Placental FA Receptors.

Introduction

Neural tube defects (NTDs) are characterized as congenital defects of the central nervous system resulting from incomplete or incorrect closure of the

neural tube during the early fetal development. [1] A number of nutritional, environmental and genetic or a combination of these factors are known to play a role in the development of NTDs. [2] Maternal nutritional deficiency of folic acid and vitamin B12, which is reflected as their low serum values is considered to be the most important cause in the occurrence of spina bifida. [3, 4] Folic acid along with vitamin B12 acts as a co-factor in the homocysteine metabolism, necessary for its conversion to methionine. The reason for failure of neural tube closure may be a relative shortage of methionine (methylation capacity) and tetrahydrofolate (THF) at a crucial stage of fetal development due to diminished amounts of the nucleic acids, proteins and the lipids. [5] There are certain genetic defects such as polymorphisms in the homocysteine remethylation gene (C677T and A1298C) which render methyl tetrahydrofolate reductase (MTHFR) enzyme thermolabile and thus less active, resulting in increased serum homocysteine levels. [2] Elevated concentration of homocysteine in blood is considered as teratogenic and it has a detrimental effect on NTD-risk even when serum vitamin B12 or RBC folate levels are normal or even high. [6, 7]

Although the etiopathogenesis of this congenital anomaly has been studied in detail, there have been few cases where mothers took FA supplementation as per the standard dosage and also had normal serum FA levels, still their fetuses developed NTD. This disproportion of serum FA levels in the pregnant mothers and the fetuses could be a result of poor trans-placental transport of the folic acid. A defect in the folic acid receptors at the placental level has been demonstrated by the use of isolated perfused-placental cotyledon model in experimental animal studies. However, in-vivo confirmation of the same in the humans is still needed. [8]

The present study measured the levels of folic acid, vitamin B12 and homocysteine in the NTD neonates and their mothers and compared them with the control population. These levels were further correlated with various demographic variables among NTD neonates and their mothers and the severity of the disease.

Materials and methods

This prospective case control study consisted of 26 consecutive neonates with NTDs and their mothers (Group-I), who presented to our out-patient department. The children with multiple anomalies and poor general condition were excluded from the study. Twenty-six age matched neonates with minor illnesses such as congenital inguinal or umbilical hernias, undescended testis, hypospadias along with their mothers formed the control group (Group-II). The institute ethics' committee approved the study.

Enrolled neonates in group-I were examined for numerous demographic factors like- the level of neural tube defects, associated congenital hydrocephalus and neurological deficit in form of bowel-bladder involvement and weakness of lower limbs. Fenton growth charts were referred to for the measurement of weight, length and head circumference percentiles and WHO guidelines were followed to classify the mothers based on their body mass index (BMI). [9]

Blood samples were collected for the measurement of folic acid levels in whole blood, serum and the red blood cells and for the estimation of serum homocysteine and vitamin B12 levels. Folic acid levels were determined with the help of microbiological assay using *Lactobacillus casei*. [10] Commercial ELISA kits and BioTek's Epoch™ ELISA plate reader were used to measure the serum homocysteine and vitamin B12 levels.

The data was analysed using SPSS® version 15. Mann-Whitney *U*-test was

used for statistical analysis of skewed continuous variables. For normally distributed data, t-test was applied for comparison of two groups and one-way ANOVA was used in case of more than two variables. Proportions were compared using Chi square or Fisher's exact test, whichever was applicable. Correlation between different variables was calculated using Spearman or Pearson Correlation Coefficient. The p-values less than 0.05 were considered significant.

Results

The parameters of age at presentation, gender, gestational age, birth order, and maternal age in both the groups were comparable and there was no significant difference. However, 50% of the neonates in NTD group had weight and length percentile of less than 3 as compared to only 12% and 4% respectively in the control group (Table-1).

Table 1: Neonatal factors

Variables	NTD group (n-26)	Control group (n-26)	p value
Mean age at presentation	8.3	10.8	0.223
(Days)			
Gender			
Male	19	14	0.150
Female	7	12	
Maturity			
Term	23	23	0.666
Preterm	3	3	
Birth order			
I	9	13	0.135
II	10	9	
III	7	2	
IV	0	2	
Mean weight (Kilograms)	2.7	3.1	0.027*
Mean length (Centimeters)	47.2	51.9	0.000*

Maternal FA intake, dietary habits and body mass index (BMI) were also compared between the two groups (Table-2). Most of the mothers in the NTD group took FA tablets for periods ranging from 1-2 months only during the pregnancy whereas mothers in the control group took FA supplementation throughout the 1st and 2nd trimesters. However, none of the mothers, in either group, consumed folic acid tablets in the pre-conception period. The intake of FA was erratic in the NTD group as compared to the control group (p value- 0.012). Using linear regression test, it was found that the risk of occurrence of NTD was 4.8 times higher, if the mother was vegetarian and lacking adequate FA resources in her diet. No mother in either group suffered from substance addiction, radiation exposure, and other illnesses during the course of pregnancy. Most of the NTD neonates (77%) were born to fathers, who were in low-income category occupations such as farmer, labourers, sweeper, servant, factory worker and driver as compared to the control group (46%) and majority of the mothers in both the groups were housewives (73% and 69% respectively).

Table 2: Maternal factors

Variables	NTD group (n-26)	Control group (n-26)	p value
Mean maternal age	24.8	25.7	0.523
(Years)			
Folic acid intake			
Yes	8	17	0.012*
No	18	9	
Diet			
Vegetarian	16	8	0.026*
Non-vegetarian	10	18	
Body mass index (BMI)			
< 18.5	7	0	0.002*
18.5-24.99	15	16	
25-29.99	4	10	

Lumbosacral region (46%) was found to be the most affected site followed by the lumbar NTD (11%). Occipital encephalocele, thoracic, thoracolumbar and sacral NTDs; each were seen in 8% of the cases. Spina bifida occulta was present in 11% of the neonates. Nine neonates (35%) had associated congenital hydrocephalus on presentation and 50% of the neonates with NTDs had either partial or complete neurological deficit in form of lower limbs' weakness, congenital talipes equino varus (CTEV), neurogenic bowel and bladder involvement.

On biochemical analysis of the FA; maternal whole blood FA levels were significantly high in NTD group (Group-I) as compared to the controls (Group-II) (p value- <0.001) whereas, there was no significant difference in the serum or RBC FA levels. However, the whole blood, serum and RBC FA levels were significantly high in the control neonates in comparison to the NTD group neonates (p values- <0.001, 0.05 and <0.001 respectively). Maternal and neonatal vitamin B12 levels were also found to be on the lower side in the NTD group though not statistically significant (p values- 0.135 and 0.695 respectively). Homocysteine levels were found to be significantly high in both the mothers and neonates of the NTD group (p values- 0.023 and 0.030 respectively) (Table-3).

Table 3: Biochemical analysis

Variables	NTD group (n-26)	Control group (n-26)	p value
Maternal serum FA	10.40 ± 6.09	11.55 ± 2.98	0.310
Maternal blood FA	303.96 ± 53.13	227.47 ± 74.58	< 0.001*
Maternal RBC FA	791.21 ± 180.59	643.54 ± 216.69	0.10
Neonatal serum FA	10.75 ± 5.81	17.18 ± 5.68	< 0.001*
Neonatal blood FA	349.30 ± 78.92	474.46 ± 203.76	0.005*
Neonatal RBC FA	626.41 ± 172.25	917.99 ± 311.28	< 0.001*
Maternal homocysteine	11.57 ± 3.97	9.06 ± 3.73	0.023*
Neonatal homocysteine	7.90 ± 3.12	6.32 ± 1.79	0.030*
Maternal vitamin B12	165.50 ± 87.28	202.51 ± 88.29	0.135
Neonatal vitamin B12	279.59 ± 125.21	293.09 ± 121.93	0.695

(Folic acid – ng/ml, Vitamin B12 – pg/ml, Homocysteine – µmol/l)

Folic acid, vitamin B12 and homocysteine levels were further compared with the maternal diet and FA intake using the t-test. The values were comparable in either group and no statistical significant correlation was identified. Among the neonates with NTDs, these levels were found to be independent of the gender & birth order of the patient. Level and severity of the neural tube defect too didn't have any causal correlation with the values of these biochemical parameters in the study group.

Discussion

The incidence of NTDs varies widely between 1 and 10 per 1,000 births, depending on the geographic region and ethnical grouping. The prevalence is noted to be 6 per 1000 live births in India and it remains particularly higher in the Northwest India and more so in the Sikh population. [2, 11, 12] Despite intensive epidemiological and experimental research, the exact etiology of NTD remains rather complex and poorly understood. [2, 4] Maternal hyperhomocysteinemia and low serum levels of FA & vitamin B12 have been linked to the etiopathogenesis of NTD in developing fetus. [11] Research in the past 10–20 years has been focused on certain genetic defects in the methyl cycle of homocysteine metabolism. [5]

In the present study, most of the neonates in the NTD group were males (73%) similar to many previous studies; however, in a report by Houcher et al in Algeria, females outnumbered the male patients.[13] Age at the presentation was higher (mean 8.23 days) as compared to previous studies where most of the patients presented early. [11] Inadequate health knowledge of parents, poor socio-economic status and the non-availability of health facilities leads to delay in seeking the medical care. In our study, most of the affected neonates were born at term (88%) though they were found to be small for the gestational age. Half of the neonates had a weight and length percentiles of less than 3. Such analysis was noted to be missing in the literature search for the comparison. Mean age of the mothers in NTD group was 24.8 years and it was 25.7 years in the control group.

Here, none of the patients in either group could receive FA at appropriate dose. NTD risk was found to be 4.8 times more with the vegetarian diet due to its lesser content of vitamin B12 and folic acid. These nutritional deficiencies further lead to altered gene-nutrient interaction in the homocysteine metabolism cycle increasing the incidence of NTD. Nutritional FA deficiency in particular or otherwise because of the enzymatic and genetic defects ultimately affect the homocysteine metabolism cycle, which plays a key role in the etiopathogenesis of the NTD. [2, 14] Though maternal FA supplementation is known to prevent 50-70% of NTD, the occurrence of NTD in sequential pregnancies despite use of FA and even no apparent benefit of its additional supplementation in some individuals suggest that a proportion of NTD are unresponsive to FA. Folic acid is thought to act through one carbon folate metabolism which transfers single carbon units for methylation and nucleotide biosynthesis. Hence suboptimal performance of the intervening reactions can limit the efficacy of folic acid in some individuals. [15] Folate deficiency can occur due to inadequate dietary intake, malabsorption, altered hepatic and peripheral metabolism, or an increased elimination of folate, some of these may not be amenable to rectification with supplemental FA. [16]

Various studies have documented that maternal body mass index (BMI) of > 30 is associated with twice higher odds of having an NTD-affected pregnancy as compared to the normal weight women with BMI between18-24.9. [17, 18] However, in our study; most of the mothers (85%) in NTD as well in control

group were having BMI of < 24.99 and a poor overall nutritional status. Certain risk factors e.g. antenatal medication in the form of anti-epileptics, vitamin A preparations, febrile illnesses, maternal diabetes, maternal smoking & alcohol consumption, history of NTD in the previous pregnancy have been described in the past by Kondo et al (2009), Wallingford et al (2013) and Pulikkunnel et al (2005) in the etiopathogenesis of NTD. [2, 14, 19] However, in our study, there was no mother with history of such risk factors except two mothers, one in each group. These two mothers were addicted to tobacco chewing and smoking respectively.

Parental professions such as farmers and labourers are considered as known causative factors in the etiopathogenesis of NTD as the nutritional deficiency of important vitamins as well as contact with various pesticides may affect homocysteine metabolism cycle. [11, 20] In our study also, the risk of NTD was found to be 6.8 times more if parents were indulging in these kinds of field works.

Serum homocysteine levels were significantly raised in neonates and their mothers in NTD group. Hyperhomocysteinemia has been described as an independent risk factor for the NTD occurrence by Vanderput et al (2001), Felkner et al (2009) and Mills et al (1996) in their studies. [2. 21. 22] The observed elevated homocysteine levels in both NTD neonates and their mothers still keep us in the dark in terms of the underlying mechanism(s) that result in a defective closure of neural tube. The direct teratogenic role of homocysteine causing NTDs has been demonstrated in chick and avian embryos in previous studies, though this evidence still lacks its importance in human population. [23, 24]

Serum vitamin B12 levels were lower in the study group though the difference was not statistically significant. As a cofactor for the enzyme methionine synthase in homocysteine metabolism cycle, vitamin B12 helps in conversion of homocysteine to methionine preventing accumulation of the teratogenic homocysteine in the maternal blood. In addition, vitamin B12 influences incorporation of folates into the cellular pool as well as flux of folate derived 1-carbon units destined for DNA synthesis or for methylation reactions essential for embryonic development. [25] Ray et al (2007) in their study noted a three-fold risk of NTD development if mothers had low vitamin B12 status. Thus, addition of vitamin B12 to the fortified grains or by supplementing it in its synthetic form may be an acceptable way to further reduce the prevalence of NTD. [26]

In the NTD group, the neonates had low serum FA levels and interestingly their mothers were noted to have significantly higher levels as compared to the control mothers. It may not be incorrect to speculate that maternal FA is not reaching the target tissue, which in this case, is fetal neural tube. Daly et al (1999) reported that placental MTHFR activities were related to C677T MTHFR variants, suggesting a possible association with NTD development. [27] In addition, three types of receptors/transporters: folate receptor α (FOLR1), proton coupled transporter (PCFT) and reduced folate carrier (RFC1) have been described by Farkas et al (2013) which in their diseased states can hinder trans-placental transfer of FA. [28] Thus, it can be predicted that folate deficiency, which can lead to megaloblastosis and cell death, particularly of highly proliferating somatic cells, will result in adverse consequences to the fetus during gestation. Embryonic neural tube and neural crest cells have a short doubling time and folate deficiency has the most profound effect on these rapidly dividing cells resulting in neural tube defects and neurocristopathies. [29, 30]

Despite considerable research, no specific polymorphisms or mutations of the human

folate receptor gene have been identified. Overcoming the receptor barrier with higher supplementation dose of FA might be an explanation for decrease in incidence of NTD occurrence with folic acid supplementation. Administration of higher doses of FA has been recommended for peri-conceptional intake especially when there has been previous pregnancy with neural tube defect. [31]

Conclusions

Maternal and neonatal hyperhomocysteinemia were independent risk factors in the etiopathogenesis of NTD. Maternal FA levels were significantly higher suggesting defective utilization. Vitamin B12 levels were also lower in the NTD group. However, the levels of these biochemical markers did not have any impact on the severity of the neural tube defect.

A longitudinal study estimating the levels of FA serially during the course of pregnancy may answer as to why FA levels were high in NTD mothers in spite of low or no intake of folic acid. There may be a role for supplementation of a combination of FA with Vitamin B12 to increase the efficacy of preventive therapy in occurrence of NTD. Evaluation of defects in FA receptors and transporters at the placental level should be taken in consideration in human population.

Limitations of the study- Estimation of levels of folic acid in the antenatal period, during the time when events leading to occurrence of NTD are happening, would be ideal; hence postnatal estimation of FA levels is the limitation of this study. However the control group was also subjected to similar conditions and still there were differences between the NTD and control group.

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