Public Health Nutrition: 12(5), 680–686 doi:10.1017/S1368980008002735

Maternal serum vitamin B_{12}, folate and homocysteine and the risk of neural tube defects in the offspring in a high-risk area of China

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Submitted 9 August 2007: Accepted 16 April 2008: First published online 12 June 2008

Abstract

Objective: To examine the association between the risk of neural tube defects (NTD) and maternal serum vitamin B_{12}, folate and homocysteine in a high-risk area of China.
Design: A case–control study was carried out in Luliang mountain area of Shanxi Province.
Subjects/setting: A total of eighty-four NTD pregnancies and 110 matched controls were included in the study; their serum vitamin B_{12} and folate concentrations were measured by chemiluminescent immunoenzyme assay and total homocysteine concentrations by fluorescent polarisation immunoassay.
Results: Serum vitamin B_{12} and folate concentrations were lower in NTD-affected pregnant women than in controls ($P<0·01$). Serum total homocysteine was higher in the NTD group than in controls at less than 21 weeks of gestation ($P<0·01$). Adjusted odds ratios revealed that women with lower vitamin B_{12} (adjusted OR=4·96; 95% CI 1·94, 12·67) and folate (adjusted OR=3·23; 95% CI 1·33, 7·85) concentrations had a higher risk of NTD compared to controls. Based on dietary analysis, less consumption of meat, egg or milk, fresh vegetables and fruit intake would increase the risk of NTD.
Conclusions: Lower serum concentrations of folate and vitamin B_{12} are related to the increased risk of NTD in high-risk populations. Both folate and vitamin B_{12} intake insufficiency could contribute to the increased risk of NTD. A dietary supplement, combining folate and vitamin B_{12}, might be an effective measure to decrease the NTD incidence in these areas.

Keywords
Vitamin B_{12}
Folate
Dietary pattern
Neural tube defects
Serum

Neural tube defects (NTD), as one of the most common congenital malformations, cause deaths and handicaps in nearly all affected fetuses or children, leading to tremendous social and financial burden not only to society but also to affected individuals and their families. Preconceptional supplementation of folic acid has shown a prevention of occurrence and recurrence of NTD[1,2], which led to the practice of fortifying grain products with folic acid since 1998 in the USA, and which significantly decreased the incidence of NTD. However, the mechanisms of how folate decreases NTD risk are unknown[3].

However, it is puzzling as to why a substantial proportion of women taking folic acid supplements in the preconception phase still deliver offspring with NTD. Some experimental and epidemiological data showed that the preventive effect of folic acid could be associated with other factors, such as plasma vitamin B_{12}, total homocysteine (tHcy) and methionine status[4–6]. Lower levels of serum folate and vitamin B_{12} are related to increased risk of developmental abnormalities including NTD[2]. Folate and vitamin B_{12}, as important factors for a number of metabolic pathways in cells that involve the transfer of one-carbon units and methylation reaction, have been thought to be critical mechanisms in folate-related occurrence and recurrence of NTD[7]. Hence, currently there is much interest in the possible role of vitamin deficiency and elevated tHcy concentration in the aetiology of NTD in early pregnancy. A better understanding of the relationship between altered one-carbon unit metabolism and NTD incidence could contribute to elucidate the pathogenesis of developmental abnormalities.

NTD is a major cause of stillbirth and infant mortality in China and accounts for up to one-third of all stillbirths and one-fourth of neonatal deaths[8]. Shanxi Province is located in north China and its prevalence of NTD, known to be the highest rate in the country with 105·5 per

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10 000 births in 1987[9], continued to be the highest with prevalence of 138·7 per 10 000 births in 2003[10]. These rates were over ten times higher than those reported from southern provinces of China and the United States. Epidemiological studies indicated that genetic and environmental factors contributed to the risks of NTD[11,12]. However, until now, there is a lack of reference defining the association of embryological NTD risks with serum folate, vitamin B_{12} and homocysteine status in the Chinese population.

The aim of the present study was to investigate the risk of NTD and maternal serum vitamin B_{12}, folate and homocysteine in Luliang, which is has a high prevalence of NTD in Shanxi Province in China[13]. Serum folate, vitamin B_{12} and tHcy concentrations were analysed and comparison was carried out between these affected pregnancies and matched controls.

Methods

Study area

The study was conducted in the Luliang mountain area of Shanxi Province, with an NTD prevalence of 199·38/10 000 based on local epidemiological surveillance data from January 2002 to December 2004[14]. We chose four hospitals located in Zhonyang and Jiaokou counties in Luliang area as our participating hospitals.

Case definition

For this analysis, we classified cases as anencephaly, spina bifida and encephalocele. Anencephaly was defined as isolated or multiple, occurring either alone or combined with other birth defects of various systems; spina bifida was defined as occurring either in isolation or in combination with other birth defects of various systems, except with anencephaly or spina bifida occulta; and encephalocele was defined as occurring either alone or combined with other birth defects of various systems, except with anencephaly or spina bifida.

Study subjects

From 1 March 2004 to 30 June 2005, we recruited pregnant women who were receiving prenatal healthcare or delivered in hospital and agreed to participate in our study as our subjects. The enrolled pregnant women were diagnosed by local trained clinicians and were registered in a database, which was jointly designed by WHO Collaborating Center for Research in Reproductive Health and Population Science. The study was approved by the Institutional Review Board at Capital Institute of Pediatrics, Beijing, People's Republic of China. All participates gave written informed consent.

Women who were diagnosed with NTD by B mode ultrasound scans and who electively terminated an NTD fetus or had live births or stillborns with NTD were ascertained as case women. Women who had a live-born infant with no identified structural malformation after one-year follow-up were ascertained as control women. For each case woman enrolled in the study, a matched control woman was selected by having a close date or the same month of conception compared with a case woman. These case and control women were all from the four selected hospitals and included residents and non-residents of local counties during that study period. A total of 211 pregnant women were recruited, with eighty-nine case women and 122 controls.

The exclusion criteria were variables that might confound the determination of maternal vitamin and homocysteine status (i.e. use of vitamin supplements or folate antagonists) in the six months prior to specimen collection.

Sample collection and questionnaire completion

After case and control pregnancies were identified in hospitals, samples were collected within one week of obtaining consent from both case and control pregnancies. Generally, 8 ml venous blood samples were collected into red-top Vacutainer® tubes (without anticoagulant; Becton Dickinson) by a trained staff from the local hospital. Blood samples were immediately centrifuged at 2500 rpm for 10 min; the separated serum was aliquoted without a reducing agent and stored at −20°C in local hospitals until shipped in ice boxes to study laboratories. Samples were not thawed until analysis. The assays were performed on each batch but remained double-blind about the case or control status of all specimens. The average age and gestational week at blood sample collection were 25 years (ranging from 17 to 40 years) and 21 weeks (ranging from 5 to 42 weeks), respectively.

Blood samples of controls who had live-born babies were collected when they delivered and were stored until analysis. When these controls were identified after one-year follow-up, the data from their samples would be used in study analysis.

After pregnant women were enrolled and samples were collected, a questionnaire about health status, demographic factors and dietary intake during pregnancy was filled out by a face-to-face interview between participants and trained hospital staff.

Biochemical analysis

Serum folate and vitamin B_{12} were measured with a competitive receptor binding immunoassay (Chemiluminescent Immunoenzyme Assay Access Immunoassay system; Beckman Coulter, Krefeld, Germany)[15]. The intraassay CV for serum folate and vitamin B_{12} were 3·8–6·5 % and 5–7 %, respectively.

Total serum homocysteine was measured by the AxSYM homocysteine assay (Abbott Laboratories Inc., Abbott Park, IL, USA), based on the fluorescence polarisation immunoassay technology[16]. The intraassay CV for serum tHcy were 5·0–7·05 %.

Statistical processing

Data were entered and analysed with the Epi-data 3·0 statistical package (Centers for Disease Control and Prevention, Atlanta, GA, USA) and the SPSS software package version 11·5 (SPSS Inc., Chicago, IL, USA), respectively. The distribution of maternal concentrations of serum folate, vitamin B_{12} and tHcy were positively skewed, so the raw data were transformed logarithmically. Differences in concentrations of serum parameters between groups were assessed with analysis of covariance under the control of age and gestational week at blood collections. Parameters in tables are represented as geometric mean (5th–95th percentile range) after back-transformation. Maternal serum of concentrations in NTD-affected pregnancies were dichotomised with a cut-off point at the 10th percentile (55·00 pmol/l and 7·01 nmol/l for folate and vitamin B_{12}, respectively) or 90th percentile (15·33 μmol/l for tHcy) of the values in control women and OR with 95 % CI were calculated to estimate NTD risks of the serum indices[5], and then χ^2 tests were performed to evaluate the significance of difference between the case and control groups. Partial correlations were calculated under the control of age and gestational week at blood collection. χ^2 tests were used to compare the difference between the two groups. OR values are also presented with 95 % CI to evaluate the risks of food frequencies to NTD incidence.

Table 1 Characteristics of case and control group

Characteristics of mothers	Case *n*	Case %	Control *n*	Control %
	84		110	
Age at conception (years)				
<20	2	2·38	2	1·82
20–24	41	48·81	62	56·36
25–29	28	33·33	32	29·09
≥30	13	15·48	14	12·73
Educational level (years)*				
<7	20	23·81	20	18·18
7–9	56	66·67	71	64·55
≥10	8	9·52	19	17·27
Gravidity (*n*)				
1	22	26·19	38	34·55
2	36	42·86	46	41·82
3	18	21·43	19	17·27
≥4	8	9·52	7	6·36
Gestational week (weeks)†	20	12–40	19	6–42

*χ^2 tests were performed, $\chi^2 = 7·87$; $P = 0·02$.
†Median and range.

Results

Characteristics of the subjects

A total of 211 pregnant women were recruited, including eighty-nine NTD-affected and 122 matched normal pregnant women. Five NTD-affected women and twelve controls were excluded from the research group for taking folate supplements during the six months prior to blood sampling. Among eighty-four NTD cases, seventy-nine were diagnosed by B mode ultrasound and then received elective terminations, and five were diagnosed clinically as live births. The mean age at conception of case and control pregnant women was 25 years and the age distributions were similar in both groups. There was no difference in gravidity and week of gestation between the case and control groups. The control group had a higher educational level than the NTD group (Table 1).

Concentration of vitamin B_{12}, folic acid and total homocysteine in serum

Concentrations of vitamin B_{12}, folate and tHcy were assayed and compared between the NTD and control groups, as shown in Table 2.

The mean concentration of serum vitamin B_{12} from NTD-affected subjects was significantly lower than that in the control group ($P < 0·01$). Similarly, the same trend of difference was observed in serum folate between the case and the control groups. Compared with the controls, case subjects had higher mean tHcy concentration, yet the difference was not statistically significant ($P = 0·067$). However, in the subgroup in which pregnancies were at less than 21 weeks of gestation (nearly at early second trimester), the difference of tHcy between case and control subjects became statistically significant (Table 3). NTD-affected pregnant women had higher serum tHcy than in control groups (10·05 μmol/l *v.* 7·46 μmol/l, $P < 0·01$). Similarly, serum concentrations of vitamin B_{12} and folate in NTD were significantly lower than in controls.

Assessment of neural tube defect risks

In an attempt to establish an NTD risk assessment model based on the serum level of vitamin B_{12}, folate and tHcy, we performed a statistical analysis on biochemical and clinical data in Table 4. Between the case and control groups, it is noted that low concentration of vitamin B_{12}

Table 2 Serum concentration of vitamin B_{12}, folate and tHcy, and comparision between NTD and control group

	NTD (*n* 84)		Control (*n* 110)	
	Geometric mean	P_5–P_{95}†	Geometric mean	P_5–P_{95}†
Vitamin B_{12} (pmol/l)**	73·28	30·19, 153·99	91·41	49·70, 184·35
Folate (nmol/l)**	9·55	4·17, 19·11	12·05	5·41, 38·77
tHcy (μmol/l)	8·95	4·04, 24·38	7·82	3·15, 20·71

NTD, neural tube defects; tHcy, total homocysteine.
Comparsion between case and control groups: **$P < 0·01$.
†P_5–P_{95}, 5th–95th percentile.

Table 3 Serum vitamin B_{12}, folate and tHcy levels in subgroups of pregnant women at less than 21 gestational weeks

	NTD (*n* 44)		Control (*n* 60)	
	Geometric mean	P_5–P_{95}†	Geometric mean	P_5–P_{95}†
Vitamin B_{12} (pmol/l)*	76·50	29·25, 55·91	101·39	53·01, 213·85
Folate (nmol/l)**	9·71	3·53, 23·27	13·58	7·19, 42·69
tHcy(μmol/l)**	10·05	4·28, 28·39	7·46	3·24, 20·69

NTD, neural tube defects; tHcy, total homocysteine.
Comparsion between case and control groups: $*P<0·05$, $**P<0·01$.
†P_5–P_{95}, 5th–95th percentile.

Table 4 NTD risks assessments with serum concentration of folate, vitamin B_{12} and tHcy

	NTD	Control	OR	95 % CI	Adjusted OR†	95 % CI
Vitamin B_{12} (pmol/l)‡						
$<55·0$	21	9	3·74	1·61, 8·68	4·96	1·94, 12·67
$\geq55·0$	63	101				
Folate (nmol/l)‡						
$<7·01$	18	11	2·46	1·09, 5·53	3·23	1·33, 7·85
$\geq7·01$	66	99				
tHcy (μmol/l)§						
$>15·33$	11	11	1·36	0·56, 3·30	1·50	0·54, 4·18
$\leq15·33$	73	99				

NTD, neural tube defects; tHcy, total homocysteine.
†Adjusted with age, gestation week, educational level and gravidity at blood collection using logistic regression analysis.
‡Defined 10th percentile of control group as a cut-off value.
§Defined 90th percentile of control group as a cut-off value.

(below 55 pmol/l) increased the NTD risk by about threefold (OR = 3·74; 95 % CI 1·61, 8·68). On the other hand, low concentration of folate (below 7·01 nmol/l) increased the risk of NTD by about onefold (OR = 2·46; 95 % CI 1·09, 5·53). After adjusting OR with age, gestation week, educational level and gravidity, the increasing risk of NTD in low concentration of vitamin B_{12} or folate was not changed. However, a higher serum concentration of tHcy did not statistically confer a higher risk of NTD (OR = 1·36; 95 % CI 0·56, 3·30), nor in an adjusted model (adjusted OR = 1·50; 95 % CI 0·54, 4·18). Thus, it seems that the concentrations of vitamin B_{12} and folate in serum are predictive parameters for NTD risks in pregnant women.

Dietary patterns

The dietary patterns were compared between case and control groups, to understand the potential influence on the risks of NTD. The dietary patterns between the two groups were significantly different in frequencies of consumption of meat, egg or milk, fresh vegetables, fruits and sprouted potato (Table 5).

Women with meat intake less than once weekly had a higher risk than those with meat intake more than three times per week (OR = 5·00; 95 % CI 2·16, 11·56). Similarly, compared with the reference category, egg or milk intake less than once weekly increased NTD incidence more than sevenfold. With regard to fresh vegetables or fruits, women with intakes less than three times weekly had about ten times the risk of their pregnancy being NTD-affected compared with women with fresh vegetables or fruits intake more than five times weekly in the model. When consumption frequencies of sprouted potatoes were more than one time per week, the risk of NTD increased by three times. However, there was no effect of pickled vegetable intake on NTD risk.

Discussion

It has been long established that folate is essential for neural tube closure in the fetus and recent studies suggest that multiple nutrients and nutrition-related factors may play an important role. Among them, vitamin B_{12} has received a great deal of attention in that lower levels of vitamin B_{12} in serum and in amniotic fluid of mothers have been linked to the risk of fetal NTD[17]. However, many of the previous studies were carried out in the general population and the consumption of folate and/or vitamin B_{12} supplements among study subjects could not be completely ruled out[3,4,7]. In contrast, the current study was conducted in a rural mountain area with a significantly higher frequency of NTD incidence from a historical perspective, which allowed us to enrol a large number of eligible study subjects in our study within a short period of time. In addition, local people seldom took vitamin supplements; this enabled us to accurately assess the association between vitamin B_{12} or folate and NTD in this area.

It has been hypothesised that both folate and vitamin B_{12} could have a crucial role in folate-related NTD[18]. Insufficient vitamin B_{12} may result in folate functional

https://doi.org/10.1017/S1368980008002735 Published online by Cambridge University Press

Table 5 Dietary pattern and the risks of NTD incidence

Factors	Case (*n* 80)	Control (*n* 104)	OR†,‡	95 % CI
Meat (time(s)/week)				
≥3	9	32		1·00 referent
1–2	12	30	1·42	0·53, 3·86
<1	59	42	5·00	2·16, 11·56
Egg or milk (time(s)/week)				
≥5	3	19		1·00 referent
≥3	23	41	3·55	0·95, 13·31
1–2	21	19	7·00	1·79, 27·46
<1	33	25	8·36	2·23, 31·42
Fresh vegetables (time(s)/week)				
≥5	24	68		1·00 referent
≥3	19	26	2·07	0·98, 4·40
1–2	18	5	10·20	3·41, 30·48
<1	19	5	10·7	3·62, 32·01
Fruits (time(s)/week)				
≥5	11	62		1·00 referent
≥3	14	20	3·95	1·55, 10·07
1–2	16	9	10·02	3·55, 28·30
<1	39	13	16·91	6·89, 41·48
Pickled vegetable (time(s)/week)				
<1	73	86		1·00 referent
≥1	7	18	0·46	0·18, 1·16
Sprouted potato (time(s)/week)				
<1	55	95		1·00 referent
≥1	25	9	4·798	2·09, 11·02

NTD, neural tube defects.
†The missing value was not included in statistical analysis.
‡NTD-affected women *v.* control women in modeling.

deficiency. In our study, a significantly lower serum vitamin B_{12} level was observed in the case group compared to controls. Furthermore, our study showed a significantly lower mean concentration of maternal serum folate compared with that in the control group. The interplay between folate and vitamin B_{12} was evaluated by a significant inverse correlation (partial $r = -0{\cdot}29$, $P < 0{\cdot}01$) between the serum levels of vitamin B_{12} and folate in the case group compared to that in the control group, implying that an impaired maternal and fetal folate and vitamin B_{12} metabolism could be relevant to the occurrence of NTD. Compared with the control group, we found that the case subjects with lower vitamin B_{12} (<55·00 pmol/l) and low folate levels (<7·01 nmol/l) had about 3–4 fold increased risk of NTD. This strongly suggests that vitamin B_{12} insufficiency was involved in NTD-affected pregnancy in the specific high-risk population in China. Although there are other reports from other countries showing that folate insufficiency is the major risk factor for NTD, our observation is in agreement with the findings from the study of Kirke *et al.*[19], that both vitamin B_{12} and folate are independent risk factors for NTD. The combination of measuring serum vitamin B_{12} and its metabolites would well define the interrelation between nutrition status and embryonic risks for NTD formation[20]. Most recently, it was found that a significantly low vitamin B_{12} concentration in the mother increased the risk of orofacial clefts in the offspring[21].

It is well-documented that a functional shortage of folate and vitamin B_{12} may lead to a disturbed homocysteine metabolism, affecting DNA synthesis and transcription, especially in cellular differentiation during embryogenesis[18]. Mills *et al.* found that hyperhomocysteinaemia was present in women of offspring with NTD, which indicated an abnormality in methionine synthase involving vitamin B_{12} as a cofactor in one-carbon unit metabolism[21]. Similar trend was observed in the present study, which means that the mean serum tHcy concentration in mothers of offspring with NTD was higher than that of control subjects. Moreover, a significant inverse correlation in the present study between vitamin B_{12} and tHcy concentrations was observed in controls ($r = -0{\cdot}42$, $P < 0{\cdot}01$) and a non-significant inverse correlation in case subjects ($r = -0{\cdot}15$, $P > 0{\cdot}05$). Our data indicated that not only vitamin B_{12} but also folate deficiency was negatively associated with the increased levels of tHcy, suggesting that both vitamin B_{12} and folate contribute to tHcy levels. In the aetiology of NTD, the deficiency or insufficiency of vitamin B_{12} could play a significant role compared to that of folate[22].

The pathogenesis of NTD is complex, encompassing genetic, dietary and other environmental risk factors[6,23]. Comparing the dietary frequencies between NTD and control groups, we found that with the decreasing intake frequencies of meat, egg or milk, fresh vegetable and fruit, there was an increasing risk of NTD incidence. Because a variety of dietary foods are rich in vitamin B_{12} and folate, such as fresh fruits and vegetables, which are rich sources of folate[24], and a nutritional characteristic of ruminant meat, which has high content of vitamin B_{12}[25], the dietary pattern could have an effect on serum vitamin B_{12} and folate levels. Our findings clearly showed a relatively insufficient dietary intake of folate and vitamin B_{12}, particularly in case women because of the dietary behaviours[25,26]. Differences of dietary pattern on varieties of area were exemplified, which showed that both folate and vitamin B_{12} deficiency were equally prominent in pregnancy[27–29]. The present study was in accordance with the quite early work by Grainger *et al.* that related vitamin B_{12}-deficient diet in pregnant animals to hydrocephalus and other birth defects[30]. Thus, dietary pattern was an important factor regarding the risks of NTD in this area.

In conclusion, the findings of the present study demonstrated that lower serum concentrations of folate and vitamin B_{12} are related to the increased risk of NTD in high-risk populations. Both folate and vitamin B_{12} intake insufficiency could contribute to increase the risk of NTD. Folate and vitamin B_{12} related to impaired remethylation of homocysteine were implicated as a possible mechanism for NTD formation. Our findings also suggested that a dietary supplement, combining folate and vitamin B_{12}, might be an effective measure to decrease the NTD incidence in these areas.

Acknowledgements

Funding source: The present study was funded by National '973' project on Interaction of Environment and Gene for Major Birth Defects (No. 2007CB511901), National Health Baby Promotion Program (No. FP2000NO13), Education Ministry Key Program (No. 02185), National Yang Zi Scholar Program, 211 and 985 projects of Peking University (No. 20020903).

Conflict of interest: There are no financial or other contractual agreements that might cause conflicts of interest or be perceived as causing conflicts of interest at this time.

Author contributions: All of the authors have directly participated in the planning and execution of the experiments, and all of the authors have read and approved the final submitted version. T.Z., R.X. and X.G. contributed equally to this manuscript.

Acknowledgements: The authors appreciate the technical support of Dr Liwen Wang, Dr Fangsheng Xu, Dr Jin Chen, Dr Yanli Zhu, Dr Tao Hu, Dr Haiyun Geng, *et al.* for their great efforts on the field diagnosis work and crucial roles in interviewing case and control women. The authors are grateful to the parents and their children who participated in the present study and to Dr Kouxi Li (Zhongyang Maternal Hospital), Dr Xiuqi Li (Jiaokou Maternal Hospital), Dr Baoying Gao (Luliang Hospital) for their practical assistance with blood sampling and data collecting. The authors also thank Dr Shukui Li and Ping Liu (The First Affiliated Hospital of Peking University) for the serum determination of folate, vitamin B_{12} and homocysteine concentration and thank Drs Jilei Wu and Jufen Liu for their careful work that ensured the accuracy of the data and valuable contributions to data management and statistical support.

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