Original Article

Serological Differences in Folate/Vitamin B_{12} in Pregnancies Affected by Neural Tube Defects

Irina Cech, PhD, and Keith D. Burau, PhD

Background: Laboratory evidence is presented of significant associations between reduced maternal serum folate and vitamin B_{12} levels and neural tube birth defects (NTD) compared to referents.

Methods: This was an incident case-control study. Cases of neural tube defects (including anencephaly and open spina bifida) diagnosed in residents within 100 miles of the US-Mexico border from January 1993 to October 2000 were eligible. Most cases were diagnosed in utero upon visits to clinics, obstetrical or genetic expert offices. Cases identified upon hospital admission or at delivery were also eligible. Cases identified after discharge were not. Controls were matched on geographic region, maternal age, race/ethnicity, gestational age, and type of health insurance (including none).

Results: Three hundred eighty-two border area residents (107 cases and 275 individually matched controls) provided biological specimens. Median folate concentrations for case mothers were 36% lower than controls (9.8 ng/mL vs. 15 ng/mL). Maternal serum folate concentrations in quartiles above 9.5 ng/mL indicated significantly reduced risk (OR = 0.4, OR = 0.3, and OR = 0.2). Likewise, the risk for NTD decreased (OR = 0.4, OR = 0.3, and OR = 0.2) in quartiles of sera B_{12} concentrations above 246 pg/mL.

Conclusions: Physician attention is invited to significantly lower concentrations of serum folate and vitamin B_{12} in women with NTD-affected pregnancies. This study assayed sera samples from women while still pregnant or immediately after delivery. The confounding effect of reduced folate and B_{12} levels with other biological and chemical exposures will be addressed in subsequent communications.

From the University of Texas Health Science Center at Houston, School of Public Health, Houston, TX.

Reprint requests to Irina Cech, PhD, the University of Texas Health Science Center at Houston, School of Public Health, RAS E1005, 1200 Hermann Pressler, Houston, TX 77030. Email: irina.cech@uth.tmc.edu

Supported in part by the ATSDR grants H75/ATH682909-03-01.

Authors have no commercial or proprietary interests in any of the methods, equipment, or computer software used for data analysis.

This study was approved by the Institutional Review Boards of the University of Texas Health Sciences Center at Houston (UTSPH) and by each of the participating border area hospitals.

Accepted September 24, 2009.

0038-4348/0−2000/X00-0001

Key Words: neural tube defects, pregnancy, serum folate, serum vitamin B_{12}, Texas-Mexico border

In response to an anencephaly cluster and the border community's health concerns about environmental pollutants, a case-control study of neural tube defects (NTD) was conducted.[1] One of several research questions addressed by the study was if there was a difference in serum folate and vitamin B_{12} concentrations between case mothers with NTD-afflicted pregnancies and unaffected controls.

Methods

This was an incident-matched case-control study investigating causes of NTD births along the Texas-Mexico border. All women who delivered or terminated an NTD-afflicted pregnancy diagnosed in a border-area prenatal care clinic, genetic clinic, or delivery hospital between January 1993 and October 2000, and who resided within 100 miles of

Key Points

- Direct serological evidence shows that low serum folate and low vitamin B_{12} in women residing at the time of pregnancy within 100 miles of the Texas-Mexico border was associated with neural tube defect (NTD).
- Based on categorical analyses presented in this study, a threshold of 9.5 ng/mL is suggested as a realistic reference concentration for normal serum folate in pregnant women.
- A threshold of 246 pg/mL is suggested as a realistic reference concentration for normal serum vitamin B_{12} in pregnant women.
- Folate concentrations in cord sera were highly correlated with maternal results, except for a few outliers, but the sample number was smaller than for the maternal sera samples.
- A lack of correlation was found between serological findings on folate/vitamin B_{12} and maternal recall of the folate/vitamin supplementation collected by the face-to-face questionnaire.

the border at the time of pregnancy, were eligible for the study. A mother was ineligible if the NTD was first diagnosed after release from the delivery hospital. Also excluded from the control series were fetal losses, stillbirths, and births with congenital anomalies.

Controls were individually matched to cases on maternal age, race/ethnicity, gestational age, and type of health insurance. The resulting matching ratio ranged from 1:1 to 1:4, depending on the availability of controls. The controls were enrolled from the same prenatal care clinic or delivery hospital as the case. Pregnant women who were diagnosed as carrying NTD infants and individually matched controls were invited to participate in the study by their doctors and project coordinators. Women who were diagnosed as having an NTD-affected infant upon delivery were also asked to participate. All participants signed an informed consent prior to participation in the study.

Maternal biological specimens were obtained at the time of NTD diagnosis for cases and at matching gestational ages for controls. Cord blood was collected at delivery or termination of the pregnancy. A strict specimen collection and processing protocol was developed to minimize contamination. Blood samples were drawn by venipuncture, centrifuged, and the sera frozen at –20°C within one hour of the phlebotomy. The frozen specimens were sent on dry ice to the Centers for Disease Control and Prevention (CDC) laboratory, where they were kept at –70°C until analyzed.

For prenatally recruited participants, a doctor or other healthcare provider placed a notice of participation in the patients' prenatal care records. The note prompted that when a woman delivered her baby, a healthcare provider or a project representative would arrange for collecting cord blood and for shipping specimens to a CDC laboratory.

Specimen collection kits were delivered to clinics that agreed to participate in the study. The kits consisted of two tiger-top tubes for serum, one purple-top tube for blood samples, hair collection kit, urine collection kit, and instructions for labeling specimens. Centers for Disease Control laboratories provided containers with appropriate preservatives. The University of Texas School of Public Health (UTSPH) office assembled these containers into boxes, with instructions for use and shipment to the CDC. If a clinic was not equipped to collect maternal specimens, a UTSPH coordinator called a local phlebotomist collaborating with the study. The phlebotomist used a UTSPH kit to collect specimens and was responsible for arranging transport to the CDC.

For participants who were enrolled at delivery, a healthcare provider or our field representative arranged for collecting specimens into UTSPH kits. Cord blood for NTD cases was shared between the Texas Department of Health (TDH) and our study, according to a memorandum of understanding between the TDH and UTSPH. Texas Department of Health representatives collected cord blood and divided it according to availability and mutually agreed priorities. Samples of cord blood were then shipped on dry ice to the CDC.

Upon arrival at the CDC, samples were entered into a log and distributed to participating specialty laboratories. Information about samples and the assigned field identification number was forwarded to the project office at UTSPH, where a study number was assigned according to a predetermined sequence. Samples were processed as soon as possible and were kept frozen until the analyses. At the CDC/National Centers for Environmental Health laboratory, both serum folate and vitamin B_{12} were measured using the Bio-Rad Laboratories' Quantaphase II Folate/Vitamin B_{12} radioassay kit.[2]

The UTSPH field coordinator abstracted delivery hospital records to confirm information contained in the clinics' medical records. Hospital records for control participants were reviewed to exclude those with a birth defect in the newborn. Data on the use of vitamins and folic acid supplements were obtained as part of a face-to-face interview with participating family members. Control subjects were interviewed before they knew the status of their pregnancy, to avoid influencing their responses.

Results

Three hundred and eighty-two border area residents (107 women with anencephaly and open spina bifida pregnancies and 275 individually matched controls) participated in this study and provided biological specimens. The median age for NTD case mothers (and matched referent mothers) was 23 years and most of the women were of Hispanic descent. More than 60% of participants were born outside the US, but the majority, 80 to 85%, resided in the US at the time of conception of index babies. More case mothers (33%) than controls (23%) changed residence between conception and enrollment. Forty-seven percent of case mothers were enrolled from the Lower Rio Grande Valley; 38% from El Paso-Ciudad Juarez area, and 15% from Laredo-Nuevo Laredo. There was no significant difference in educational attainment of cases and control families.

Serological Evidence

Three hundred and twenty samples were collected for serum folate and vitamin B_{12} analyses. Several samples were not processed for various analytical reasons (ie, insufficient quantity). Valid serum folate results were obtained for 310 samples (265 maternal sera and 45 cord samples). Results on vitamin B_{12} were obtained for 306 samples (261 maternal and 45 cord sera).

Serum Folate

Per the CDC definition, normal ranges for serum folate are approximately 2.6–12.2 ng/mL for both sexes and all ages, with values lower in females than in males. Serum levels of 1.4–2.6 ng/mL are usually called "indeterminate" because of an overlap

Table 1. Folate concentrations (ng/mL) and vitamin B_{12} concentrations (pg/mL), maternal and cord sera in the present study

Compound	Observed	Median	Mean	Standard deviation	Min	Max
Cases						
Serum folate, mother	64	9.8	11.7	6.8	1.0	35.8
Serum folate, cord	23	17.2	18.6	13.2	5.7	59.7
Vitamin B_{12}, mother	61	272	485	1148	85	8957
Vitamin B_{12}, cord	23	242	413	465	132	2223
Controls						
Serum folate, mother	197	15.3	16.4	10.6	1.00	79.1
Serum folate, cord	22	17.6	17.3	7.93	6.60	37.2
Vitamin B_{12}, mother	197	315	395	369	105	3803
Vitamin B_{12}, cord	22	315	525	746	165	2293

Analytical limits of detection: serum folate = 0.2 ng/ml and vitamin B_{12} = 20 pg/ml.

between deficient and normal ranges. Serum folate <1.4 ng/mL are taken to indicate inadequate folate intake, while elevated values are attributed to supplementation.[2]

In this study, the folate concentration in serum samples of case mothers was lower than controls (median: 9.8 vs. 15.3 ng/mL; mean: 11.7 vs. 16.4 ng/mL) (Table 1). There were nonmissing data for fewer cord samples than maternal sera samples, but none of the former (cases or controls) had folate concentrations below 5 ng/mL. Figure indicates cord vs. maternal serum samples were highly correlated, but the reported measures of central tendency (means and medians) were strongly influenced by outliers.

Vitamin B_{12}

Roughly 26% of case mothers had serum vitamin B_{12} concentrations at or below the CDC laboratory normal reference value of 200 pg/mL (Table 1). Half as many maternal controls had low vitamin B_{12} concentrations (13%). Similarly, a greater proportion of cord samples for the case babies had vitamin B_{12} concentrations at or below 200 pg/mL compared to controls (21.7 vs. 13.4%). At the other extreme, 4.9% of the case mothers and 3.5% of controls had serum vitamin B_{12} concentrations above 800 pg/mL, which was the upper normal limit of the expected reference range. About 8.7% of the case cord samples, but only 4.5% of the control samples, had concentrations above 800 pg/mL. The maternal serum vitamin B_{12} concentration for the cases was lower than controls (median: 272 vs. 315 pg/mL; mean: 485 vs. 395 pg/mL). This was also true for case babies (median: 242 vs. 315 pg/mL; mean: 413 vs. 485 pg/mL) (Table 1). Due to a positively skewed distribution in case mothers (85 pg/mL–

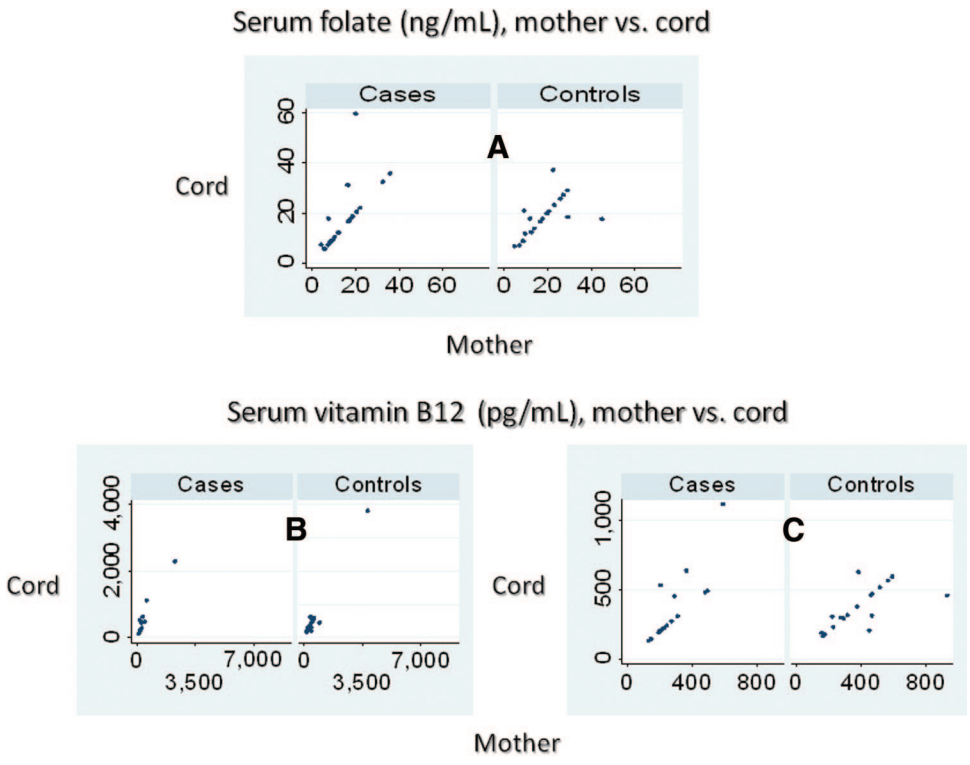

Fig. Cord vs. maternal concentrations, separately by case and control status of: *A*, serum folate, ng/mL; *B*, serum vitamin B_{12}, pg/mL; *C*, the same as (*B*) but, with outliers (one case and one control) removed, cord >2,000 pg/mL.

Table 2. Odds ratios for NTD associated with folate (ng/L) and vitamin B_{12} (pg/L) concentrations in maternal sera[a]

Concentration Quartiles	Cases	Controls	OR	95% CI	*P*
Serum folate, mother					
1st quartile of controls (≤9.5 ng/L)	31	51	1.0	Reference	
2nd quartile of controls (>9.5 to ≤15.3 ng/L)	16	48	0.4	(0.15–0.99)	0.048[b]
3rd quartile of controls (>15.3 to ≤19.6 ng/L)	10	52	0.3	(0.11–0.88)	0.037[b]
4th quartile of controls (>19.6 ng/L)	7	46	0.2	(0.08–0.62)	0.002[c]
Vitamin B_{12}, mother					
1st quartile of controls (≤246 pg/L)	29	50	1.0	Reference	
2nd quartile of controls (>246 to ≤315 pg/L)	10	50	0.4	(0.13–0.94)	0.038[b]
3rd quartile of controls (>315 to ≤433 pg/L)	10	48	0.3	(0.10–0.85)	0.024[b]
4th quartile of controls (>433 pg/L)	12	49	0.2	(0.07–0.72)	0.012[b]

[a]*NTD, neural tube defect.*

[b]$P = <0.05$.

[c]$P = <0.01$.

8,957 pg/mL) vs. controls (105 pg/mL–3,083 pg/mL), the mean vitamin B_{12} in maternal case sera was higher, 485 vs. 395 pg/mL for the controls (Fig).

Serum folate and vitamin B_{12} were stratified by quartiles, based on the concentration levels in the controls (Table 2). For both variables, the increase in serum folate concentrations resulted in significant declines in the odds ratios for NTD. Odds ratios progressively decreased in the top three quartiles, compared to the reference first quartile (OR = 0.3, OR = 0.3, and OR = 0.2), all being statistically significant. Vitamin B_{12} results indicated the same trend (OR = 0.4, OR = 0.3, and OR = 0.2) with all three being statistically significant.

Self-Recall Results

Eighty-seven case mothers (81.3%) and 225 control mothers (81.8%) completed a face-to-face survey questionnaire (Table 3), which indicated low agreement between maternal self-recall concerning the periconceptual use of vitamins and folic acid supplements and laboratory serum concentrations of serum folate and vitamin B_{12}. About 65% of case mothers and 35% of control subjects recalled using at least some vitamins and minerals in the periconceptional period (+/−3 months conception), resulting in an odds ratio equal to 1.8 (95% CI 0.97–3.18). When participants were asked specifically if they had taken prenatal vitamins, multivitamins, a single vitamin or folic acid in this period, there were no significant differences between case and control mothers in their responses.

Table 3. Vitamin and folic acid supplements in periconceptual period

Variable	Cases (n = 88) (%)	Controls (n = 87) (%)	OR[a] (95% CI)	*P*
Vitamins and minerals between (−3) and (+3)				
Yes	57 (64.8)	107 (47.6)	1.8 (0.97–3.18)	0.062
No	30 (34.5)	113 (50.2)	Reference	
Prenatal vitamins between (−3) and (+3)				
Yes	47 (53.4)	89 (39.6)	1.4 (0.79–2.65)	0.227
No	40 (45.4)	128 (56.9)	Reference	
Multivitamins between (−3) and (+3)				
Yes	16 (18.2)	37 (16.4)	1.0 (0.49–1.92)	0.930
No	71 (80.7)	179 (79.6)	Reference	
Folic acid between (−3) and (+3)				
Yes	4 (4.6)	13 (5.8)	0.6 (0.17–1.89)	0.359
No	83 (94.3)	207 (92.0)	Reference	
Levels of folic acid supplementation between (−3) and (+3)				
None[b]	29 (33.0)	111 (49.3)	Reference	
Regular vitamins[c]	11 (12.5)	23 (10.2)	1.6 (0.66–3.86)	0.271
Prenatal vitamins[d]	47 (53.4)	89 (39.6)	1.6 (0.86–3.13)	0.197

Total percentile may not add up to 100% because of missing data.

[a]*OR, odds ratio.*

[b]*Not exposed: women who did not take vitamins pre- or postconception and women who took vitamins only postconception (between +1 and +3).*

[c]*Took regular multivitamins or single vitamin of folic acid (between −3 and +3), which are assumed to contain 400 μg of folic acid.*

[d]*Took prenatal vitamins between −3 and +3, which are assumed to contain 1000 μg of folic acid.*

Some reports[3] suggest prenatal vitamins contain roughly 1,000 μg of folic acid, while multivitamins and single folic acid vitamins contain 400 μg. Accordingly, a variable was generated corresponding to these levels of folate intake, as (a) none, (b) 400 μg, and (c) 1,000 μg of folic acid. Using this classification, questionnaire responses indicated 33% of our study case mothers and 49% of control subjects were classified as having no folic acid supplementation in the periconceptional period. Approximately equal percentages of women (13% cases and 10% controls) took multivitamins and/or a single folic acid vitamin (400 μg of folic acid), and 54% of case mothers vs. 40% of control subjects took prenatal vita-

mins (corresponding to 1,000 μg of folic acid). There were no significant differences in odds ratios for NTD associated with recalled levels of supplementation (Table 3).

Discussion

The prevalence of NTDs has declined following the folic acid fortification of the food supply in the US and other countries,[4–9] although investigators caution that factors other than fortification might have contributed to this decline.[10,11] On the other hand, several case-control studies were unable to show decreased mean folate concentrations in pregnant women carrying fetuses with NTD.[12–14] Studies that investigated the dietary folate intake specifically among Mexican-Americans living along the Texas-Mexico border and on the west coast in California suggested that folic acid supplements might not sufficiently protect these populations from NTD.[3,15]

Much of the earlier data on the relationship between folate and birth defects in south Texas women were collected via questionnaire. Also, studies which measured serum folate concentrations several months postpartum run a risk of missing low folate status during the critical early gestation period. The present study was designed to evaluate differences in serum folate and vitamin B_{12} among NTD mothers and matched controls at an early stage, using an early assessment of cord blood concentrations. Individual-level matching was done to ensure that differences with respect to outcomes were due to factors other than matching criteria. Otherwise, maternal age, race/ethnicity, and type of insurance would have been either independent risk factors or surrogates of independent risk factors.

Gestational age was also a biological age, both maternal and fetal. Great effort was made to enroll participants as soon as possible after the NTD diagnoses, before evidence of antenatal exposure diminished over time. Given that NTD occurs early in pregnancy, matching on gestational age had the same purpose as matching on time that passed since exposure. Matching on geographic area was important to ensure that controls represented the population given. The Texas-Mexico border is 4,000 miles long, and an attempt was made to minimize geographic differences between cases and controls due to mobility, access to health care, training of health-care providers, dialects, diets, etc. While matching was done on geographic residence at enrollment, residence around conception was one of the independent variables investigated in this study. Although not an explicit matching criterion, the date of conception was implicitly matched because controls were selected and enrolled in parallel with cases as close to the date of conception as possible. This helped to minimize differences in temporal exposures, holding time, and sample processing in general.

The findings agree with results reported elsewhere, although our study had a strong advantage over earlier studies since the samples were taken when most of the pregnancies were still underway. Compared with women in the lowest serum folate quartile, women in the highest quartile showed a significantly decreased risk for NTD (OR = 0.2) and this risk monotonically decreased in the second and third quartiles (OR = 0.4 and OR = 0.3). Likewise, compared with women in the lowest vitamin B_{12} quartile, women in the highest quartile showed a significantly decreased NTD risk (OR = 0.2) with a monotonic decrease in the second and third quartiles (OR = 0.4 and OR = 0.3). There were no significant odds ratios associated with the dichotomy of serum folate as >5 and ≤ 5 ng/mL, the threshold currently accepted as the normal concentration. However, when categorical concentration intervals were computed based on the frequency range of our data, an increase in the maternal serum folate concentrations above 9.5 ng/mL resulted in odds ratios monotonically and significantly decreased to one-fifth of the reference odds ratio. Consequently, we suggest that this threshold of 9.5 ng/mL is possibly a more realistic reference concentration for normal serum folate in pregnant women (at least on the Texas-Mexico border) than the currently used 5 ng/mL. Likewise, the odds ratios for NTD progressively decreased to one-fifth in the second, third, and fourth quartiles, as the vitamin B_{12} concentrations in the maternal sera exceeded 246 pg/mL.

The rigorous design in our study required the enrollment of participants as soon as possible after the NTD diagnoses. With the extensive transborder migration in the US-Mexico border area, the probability of locating, verifying, and successfully recontacting families with NTD births several years after the fact was remote. This consideration weighed heavily in our decision to gather evidence as cases occurred. The incident design used in our study offered tremendous research advantages into the genesis of birth defects, but was a very difficult design to execute. Specifically, there was a short time window between notification of an incident case occurrence and pregnancy resolution. This required timely efforts to enroll the study participants, and then to arrange for collection and shipment of biological specimens. If the case was first detected at delivery, the time for arranging the enrollment and the specimen collection before the mother left the hospital was measured in hours, not days. The study protocol dictated that once the mother left the hospital, she was no longer eligible for the specimen draw. Some potential enrollees were lost to the study in this manner. In addition, the body chemistry may have changed dramatically once the pregnancy was resolved. With this in mind, the advantages of the design were considered to outweigh the disadvantages and judged to be well worth the extra effort.

By design, the gestational ages of the controls were individually matched to the gestational ages of the cases and, by the time the needed gestational age was reached, some previously enrolled controls might have moved away or failed to keep appointments for the specimen draw or the face-to-face interview. Other difficulties may have occurred in uni-

formly handling the blood samples, which were collected at numerous participating facilities along the Texas-Mexico border. To prevent this from happening, all participating clinics and maternity wards were provided uniform specimen collection kits, with appropriate preservatives and shipping instructions.

The authors readily acknowledge the problems of the small sample sizes for the cord sera specimens. The reason was not the refusal to provide the specimen, but rather, insufficient quantities of cord blood available at collection time, due to the short gestational age at time of resolution for most of the NTD-affected pregnancies. For comparison pregnancies, a bright color notification of the participation was placed in the prenatal care records, requesting that the cord blood sample be collected at delivery. However, the time interval of several months between the enrollment at the prenatal care facility and delivery introduced uncertainty beyond our control, in that a delivery hospital could have been outside of the study area. An interesting observation made in our study was that the self-reported folic acid supplementation (obtained from the survey questionnaire) failed to identify significant differences between case and control mothers. The issue of folic acid supplementation received substantial media attention during case ascertainment. However, the difference in findings between the survey and serology may or may not entirely be related to recall bias. It has been postulated[16] that the genetic makeup of an individual may not allow an optimal assimilation of the dietary folate.

Acknowledgments

We acknowledge the contributions of Dr. Elaine Gunter, Division of Environmental Health Laboratory Science, Centers for Disease Control, who performed the analyses for serum folate and vitamin B_{12}. We are grateful to the project staff, community partners, volunteers, and especially participating families.

References

1. Cech I, Burau KD, Al-Hashimi R, et al. *Chemical and biological factors associated with neural tube defect births along Texas-Mexico border. Report to the Agency for Toxic Substances and Disease Registry, December 2005*. Atlanta, GA.
2. Gunter EW, Miller DT. *Laboratory Procedures Used by the Division of Environmental Health Laboratory Sciences, Center for Environmental Health, Centers for Disease Control, for the Hispanic Health and Nutrition Examination Survey (HHANES) 1982–1984*. Atlanta, GA, Centers for Disease Control, 1986, pp 24–26.
3. Suarez L, Hendricks KA, Cooper SP, et al. Neural tube defects among Mexican Americans living on the US-Mexico border: effects of folic acid and dietary folate. *Am J Epidemiol* 2000;152:1017–1023.
4. Moore LL, Bradlee ML, Singer MR, et al. Folate intake and the risk of neural tube defects: an estimation of dose-response. *Epidemiology* 2003; 14:200–205.
5. Centers for Disease Control. *Preventing Neural Tube Defects: A Prevention Model and Resource Guide*. Atlanta, Georgia, US Department of Health and Human Services, CDC, 1998.
6. Czeizel AE, Tóth M, Rockenbauer M. Population-based case control study of folic acid supplementation during pregnancy. *Teratology* 1996; 53:345–351.
7. Persad VL, Van den Hoff MC, Dubé JM, et al. Incidence of open neural tube defects in Nova Scotia after folic acid fortification. *CMAJ* 2002; 167:241–245.
8. Hertrampf E, Cortés F. Folic acid fortification of wheat flour: Chile. *Nutr Rev* 2004;62:S44–S48; discussion S49.
9. Stevenson RE, Allen WP, Pai GS, et al. Decline in prevalence of neural tube defects in a high-risk region of the United States. *Pediatrics* 2000; 106:677–683.
10. Honein AM, Paulozzi LJ, Mathews TJ, et al. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA* 2001;285:2981–2986.
11. Shaw GM, Lammer EJ, Zhu H, et al. Maternal periconceptional vitamin use, genetic variation of infant reduced folate carrier (A80G), and risk of spina bifida. *Am J Med Genet* 2002;108:1–6.
12. Wald NJ, Hackshaw AD, Stone R, et al. Blood folic acid and vitamin B_{12} in relation to neural tube defects. *Br J Obstet Gynaecol* 1996;103:319–324.
13. Bower C, Stanley FJ. Dietary folate as a risk for neural-tube defects: evidence from a case-control study in Western Australia. *Med J Aust* 1989;150:613–619.
14. Wild J, Schorah CJ, Sheldon TA, et al. Investigation of factors influencing folate status in women who have had a neural tube defect-affected infant. *Br J Obstet Gynaecol* 1993;100:546–549.
15. Shaw GM, Schaffer D, Velie EM, et al. Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. *Epidemiology* 1995;6:219–226.
16. Ratan SK, Rattan KN, Pandey RM, et al. Evaluation of the levels of folate, vitamin B_{12}, homocysteine and fluoride in the parents and the affected neonates with neural tube defect and their matched controls. *Pediatr Surg Int* 2008;24:803–808.