eISSN 1303-1775 • pISSN 1303-1783

Neuroanatomy (2007) 6: 12–16

Original Article

Effects of folate supplementation on cleft palate induced by lamotrigine or cyclophosphamide: an experimental study in mice

Published online 28 February, 2007 © http://www.neuroanatomy.org

PRAKASH [1] ✦
Latha Venkatraya PRABHU [1]
Gajendra SINGH [2]

Department of Anatomy [1] Kasturba Medical College Mangalore Karnataka INDIA; Institute of Medical Sciences [2] Banaras Hindu University Varanasi INDIA.

✦ Dr. Prakash,
Assistant Professor,
Department of Anatomy, Centre for Basic Sciences,
Kasturba Medical College, Bejai, Mangalore,
Karnataka 575004—INDIA.
☎ 91-9480229670
91-8242428183
✉ prakashrinku@rediffmail.com

Received 5 July 2006; accepted 20 February 2007

ABSTRACT

This study aims to elucidate the preventive role of folate supplementation on induction of cleft palate in mice by drugs of two separate categories i.e. lamotrigine (newer antiepileptic and antipsychotic) and cyclophosphamide (anticancer and immunosuppressive).

10 pregnant swiss white mice (C) received normal saline intraperitoneally on day 10 of gestation. Two groups of 10 pregnant mice (T1) and (T2) each received lamotrigine or cycloposphamide respectively 10 mg/kg body weight (bw) intraperitoneally on day 10 of gestation. Folate was supplemented 0.8 μg/kg bw intraperitoneally along with lamotrigine or cyclophosphamide to two more groups of 10 pregnant mice (T3) and (T4) each respectively on the same day 10, of gestation. Fetuses were collected by Caesarian Section on day 18 of gestation. Fetuses collected from all the groups were examined macroscopically with stereomicroscope for palatal malformations. Coronal sections of fetal head were taken for histological study of palatine defects. Cleft palates were detected in 42 out of 70 (60.00%) fetuses of lamotrigine treated group (T1) and 49 out of 61 (80.33%) fetuses of cyclophosphamide treated group (T2). Folate supplementation resulted in different response; 15 out of 72 (20.83%) fetuses in T3 group and 51 out of 64 (79.69%) fetuses in T4 group had cleft palate. The difference was highly significant ($p<0.001$) when folic acid was administered with lamotrigine (T3) and was not significant ($p>0.05$) when it was administered with cyclophosphamide (T4) as compared to only lamotrigine (T1) or cyclophosphamide (T2) treated groups respectively.

The preventive efficacies of folate supplementation for cleft palate vary considerably and in the same subject under identical conditions, depend primarily on the mechanism of action of the inducing agent. ***Neuroanatomy; 2007; 6: 12–16.***

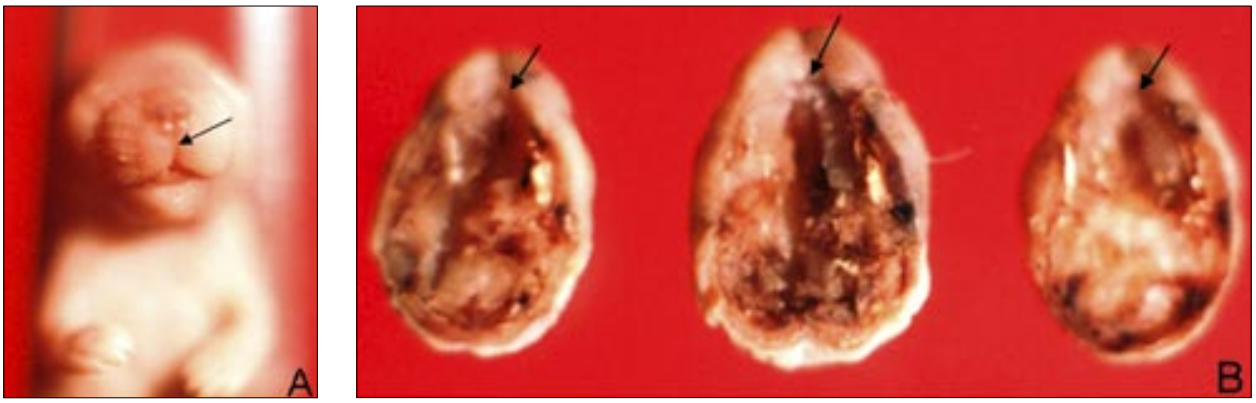
Key words [cleft palate] [cyclophosphamide] [folic acid] [lamotrigine] [mice]

Introduction

Cleft palate is one of the most common congenital anomaly affecting the human beings. It has an approximate incidence of 1 in 700 people who later manifest dysfunctional speech resulting in communication impairment. The etiology of congenital cleft palate is almost always related to the developmental life. Primary palate formation starts with the beginning of the sixth week of intrauterine life. By the end of the sixth week lip development is completed, which is followed by the palatal fusion. From both the sides three derivatives of the frontonasal process namely, medial nasal, lateral nasal, and maxillary processes are involved in the formation of primary palate. After its formation the development of secondary palate begins which will form the major portion of the adult palate. From both the sides two palatine shelves (outgrowth from the maxilla) grow vertically downwards, on either side of tongue, from the maxillary processes have to become horizontal and subsequently the two shelves fuse in the midline forming secondary palate, As the initial position of the shelves are lateral to tongue, which is positioned high in between the two shelves, the tongue has to descend down enabling the rotation of shelves to attain horizontal position. Palatal closure involves a delicate balance between shelf elevating force on the one side and tongue resistance on the other [1,2]. Failure of the fusion of palatine shelves is the most common mechanism underlying cleft palate.

Lamotrigine was approved by FDA for use as mood stabilizer in bipolar disorders in 2003 and as an antiepileptic drug in 1994. It is a phenyl triazine derivative, initially developed as an antifolate agent. Although structure activity studies indicate that its effectiveness as an antiseizure drug is unrelated to its antifolate activities [3]. The mechanisms underlying its broad spectrum of actions are incompletely understood [4]. Lamotrigine is thought to inhibit neuronal sodium channels and the release of excitatory amini acids, glutamate, and aspartate [5,6]. The antiseizure drugs introduced after 1990 have teratogenic effects but whether such effects occur in humans is yet uncertain [4]. Padmanabhan et al [7] reported cleft palate among malformations produced by intrauterine exposure to lamotrigine in mice.

Cyclophosphamide is a broad spectrum alkylating agent and according to Goodman and Gilman [4], lethality of DNA alkylation depends on the recognition of the adduct, the creation of DNA strand breaks by repair enzymes, and an intact apoptotic response. Cells thus blocked in the G1/S interface either repair DNA alkylation or undergo apoptosis [4]. It is widely used as anticancer and immunosuppressive agent. Cycloposphamide is classified as a pregnancy risk factor D drug. Besides being teratogenic to experimental animals its human teratogenicity has also been reported by various authors [4,8–11]. Kirshon et al [12] reported cleft palate along with other anomalies in human studies, when



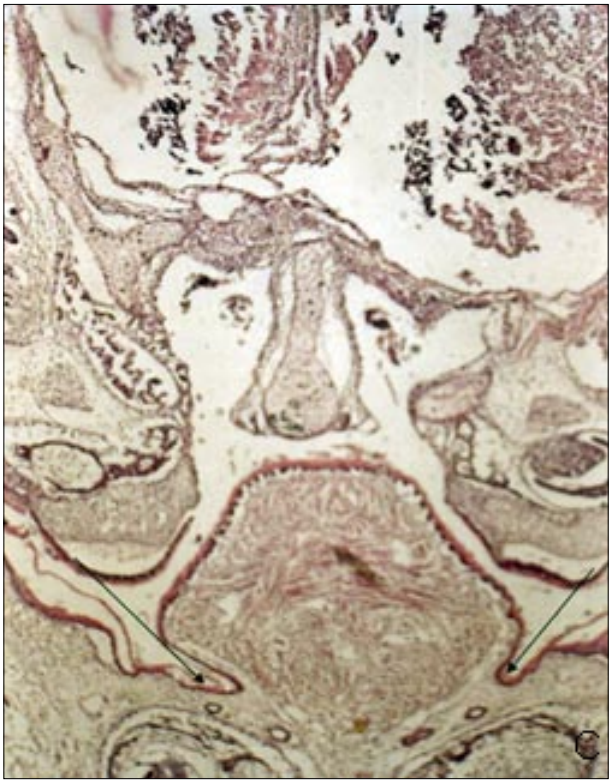


Figure 1. Photographs and photomicrographs of pups exposed to 10 mg cyclophosphamide on day 10 and collected on day 18 of gestation. Color version of figure is available online. *(**A:** Ventral view of pup showing cleft lip (**arrow**). The pup had cleft palate also. **B:** Dissected upper jaws from cyclophosphamide treated pups cleft lip (**arrow**) and cleft palate; **C:** Histological section of the palate and cranium in coronal plane showing wide complete cleft with tongue interposed between the two side maxillary processes. The palatine shelves are not developed. The nasal septum is approaching the dorsum of tongue (H and E × 42). **Arrows** indicate location occupied by palatine shelves if they had developed.*

cyclophosphamide was administered in the first trimester of pregnancy.

Folic acid is an essential vitamin used in nucleotide synthesis and DNA methylation [13]. Humans have to dependent on dietary sources as they can not synthesize folic acid. To enter the circulation dietary folate should be monoglutamated and reduced. Many enzymes are involved in this process, among which folypolyglutamate hydrolase, 5,10-methylenetetrahydrofolate reductase and dihydrofolate reductase are the most important [14,15]. Rapidly growing and developing embryos have an increased requirement for folate. An abnormal pattern of folate metabolism would result in a decreased rate of DNA synthesis and gene methylation, with deleterious effects on the developing embryo [16]. Lamotrigine decreases fetal folate levels in rats, therefore, folic acid supplementation should be considered for all women of child bearing potential who are taking lamotrigine [17]. Rats receiving upto 0.5 times an equivalent human dose of 500 mg/day lamotrigine, produced offspring with decreased fetal folate concentrations, an effect known to be associated with teratogenicity in humans and animals [18]. Folate supplementation (0.4 mg/day) has been recommended by the US Public Health Service for all women of child bearing age to reduce the likelihood of neural tube defects, and this is appropriate for epileptic women as well [4].

Material and Methods

Approval of the Animal Ethical Committee of the college was obtained prior to the present work. Sexually mature Swiss white female mice (of 8 to 10 weeks age) weighing 16-36 g were used in this study. Female mice during their proestrous phase of estrous cycle were caged overnight with the males of the same stock (female:male=1:1). The vaginal smear was examined next morning at 8.00 a.m. Presence of spermatozoa in the smear was taken as day 'zero' of pregnancy. A total of 50 pregnant mice divided into 5 groups of 10 each were studied, details are given in Table 1. Control group (C) with 10 pregnant mice received normal saline 0.5 ml per 20 g body weight intraperitoneally on day 10 of gestation. Two treated groups of 10 pregnant mice each was formed, first received lamotrigine (T1) whereas the second cyclophosphamide (T2), 10 mg/kg body weight in 0.5 ml saline per 20 g body weight intraperitonealy on day 10 of gestation. Two more treated groups of 10 pregnant mice (T3) and (T4) were supplemented with folate 0.8 μg/kg body weight again in 0.5 ml saline per 20 g body weight intraperitoneally along with lamotrigine or cyclophosphamide respectively on day 10 of gestation. The pregnant mice were sacrificed with overdose of ether anesthesia on day 18 of pregnancy i.e. two days prior to full term. The uterine horns were exteriorized after opening the abdomen by midline incision. The sacs were

Table 1. Table shows the drugs which is maternally exposured in different study groups with the intraperitoneal doses. (Gestational day of collection of fetuses=18)

Groups	Drug [a]	Dose [b]
Control (C)	Normal Saline	0
Treated (T1)	Lamotrigine	10 mg
Treated (T2)	Cyclophosphamide	10 mg
Treated (T3)	Lamotrigine + Folate	10 mg + 0.8 µg
Treated (T4)	Cyclophosphamide + Folate	10 mg + 0.8 µg

[a] The drug exposured on gestational day 10. [b] Intraperitoneal dose of drug per kg in 0.5 ml normal saline per 20 g body weight.

inspected foe sites of resorption and viable fetuses. The fetuses were removed from the uterus and were dried by wiping on a blotting paper. Fetuses collected from all the groups were first examined for gross malformations, and then they were examined macroscopically with stereomicroscope for palatal malformations. Bouin's solution was used for fixation and they were prepared for light microscopic study by paraffin sections, serially cut at 8 μm in coronal plane and stained with haematoxylin and eosin. Photographs of gross malformations and photomicrographs of histological findings were taken and studied.

Different form of data was tested for statistical significance by using 'Z' (normal test). Any value of $p<0.05$ or Z (normal deviate) > 1.96 was recorded as significant.

Results

Reduction in body weight and stunting in size along with gross malformations like brachygnathia, open eyes, limb deformities and digital anomalies of the fetuses resulted in all the treated groups. Although in the present study they were not in the focus of the specific observations for the cleft palate.

In the lamotrigine treated group (T1) cleft palates both partial and complete, with or without cleft lips were detected in 42 out of 70 (60.00%) fetuses, isolated cleft palate in 20 (28.57%) fetuses out of which 10 (14.29%) each had total and partial cleft palate, 22 (31.43%) fetuses had both cleft palate and cleft lip (Table 2, Fig. 1). Whereas in cyclophosphamide treated group (T2) cleft palates both partial and complete with or without cleft lips were detected in 49 out of 61 (80.33%) fetuses, isolated cleft palate in 20 (32.79%) fetuses out of which 16 (26.20%) had total and 4 (6.56%) partial cleft palate, 29 (47.54%) fetuses had both cleft palate and cleft lip (Table 2, Fig. 1). As compared to the control group (C) the difference of cleft palates both partial and complete, with or without cleft lips were highly significant ($p<0.001$) in both (T1) and (T2) treated groups.

Preventive response after folate supplementation was different in lamotrigine (T3) and cyclophosphamide (T4) treated groups. Both partial and complete cleft palate with or without cleft lips were observed in 15 out of 72 (20.83%) fetuses, isolated cleft palate in 13 (18.06%) fetuses out of which 3 (4.17%) had total and 10 (13.89%) partial cleft palate, 2 (2.78%) fetuses had both cleft palate and cleft lip when folic acid was supplemented along with lamotrigine administration in (T3) group (Table 2, Fig. 1). The difference of cleft palates both partial and complete, with or without cleft lips was highly significant ($p<0.001$) as compared to only lamotrigine (T1) treated group. On the contrary 51 out of 64 (79.69%) fetuses exhibited cleft palate either partial or complete with or without cleft lip, isolated cleft palate in 21 (32.81%) fetuses out of which 18 (28.16%) had total and 3 (4.69%) partial cleft palate, 30 (46.88%) fetuses had both cleft palate and cleft lip, even after folic acid supplementation along with cyclophosphamide administration in (T4) group (Table 2, Fig. 1). The difference of cleft palates both partial and complete, with or without cleft lips was not significant ($p>0.05$) as compared to only cyclophosphamide (T2) treated groups.

In T1, T2, and T4 treated groups complete cleft palate associated with cleft lip were predominant findings, whereas isolated partial cleft palate was predominant finding in T3 treated groups (Table 2). The preventive efficacy of folate supplementation for cleft palate is a highly sensitive phenomenon and is influenced in the same subject by a number of factors the most important of which is the modus operandi and the mechanism of action of the inducing agent.

Table 2. Incidence of cleft palate in different study groups.

Groups	Maternally exposured drug	N	Fetuses with cleft palate ± cleft lip	Fetuses with isolated cleft palate	Fetuses with isolated total cleft palate	Fetuses with isolated partial cleft palate	Fetuses with both cleft palate and cleft lip
Control (C)	Normal Saline	72	0	0	0	0	0
Treated (T1)	Lamotrigine	70	42 (60.00%)	20 (28.57%)	10 (14.29%)	10 (14.29%)	22 (31.43%)
Treated (T2)	Cyclophosphamide	61	49 (80.33%)	20 (32.79%)	16 (26.20%)	4 (6.56%)	29 (47.54%)
Treated (T3)	Lamotrigine + Folate	72	15 (20.83%)	13 (18.06%)	3 (4.17%)	10 (13.89%)	2 (2.78%)
Treated (T4)	Cyclophosphamide + Folate	64	51 (79.69%)	21 (32.81%)	18 (28.16%)	3 (4.69%)	30 (46.88%)

N=Number of fetuses studied.

Table 3. Various inducing agents and their modus operandi manifesting cleft palate.

	Modus operandi	Inducing agent	Mechanism of action
1	Delay in palatine shelves elevation and this delays promoted by fetal membranes and the tongue (interfere with programmed cell death	Antiepileptic drugs including Phenytoin	Episodes of embryonic hypoxia due to embryonic cardiac arrhythmia and generation of reactive oxygen species during the reperfusion phase
		Glucocorticoids	Inhibits growth of palatal mesenchymal cells
		Dioxins	Alters the terminal cell differentiation of the medial palatal cells
2	Palatine shelves remain shorter in size, not sufficient to close the gap between them	Anticancer drugs like cyclophosphamide and hydroxyurea	Increased programmed cell death or apoptosis
3	Palatine shelves are well formed and full in size but are unable to move because the tongue is unable to move down	Amniocentesis	Mechanically induced amniotic bands
4	Delay in the descent of the tongue from its position between palatal shelves or from a delay in the cephalic flexion of embryo	Pierre Robin malformation (microretrognathia)	History of perinatal respioratory and feeding difficulties
5	Opposing palatine shelves fail to fuse in the midline	Excess vitamin A	Controlling influence over both the ostoeblasts and osteoclasts in the epithelial cartilage

Discussion

Birth defects can result from genetic abnormalities and multifactorial environmental conditions. Table 3 displays various inducing agents and their modus operandi manifesting cleft palate. Antiepileptic drug administration during pregnancy causes delay in palatine shelves elevation whereas anticancer drug like cyclophosphamide result in the palatine shelves shorter in size, not sufficient to close the gap between them.

Folate deficiency is most important among teratogenicity related to altered endogenous metabolism. Various hypothesis regarding the pathogenic mechanism of decrease in folate manifesting as birth defects exist. When the concentration of 5-methyltetrahydrofolate is reduced, remethylation of homocysteine into methionine consequently will be diminished, leading to fewer methyl groups being available for DNA methylation. Hypomethylation can change the transcription and suppression of genes involved in formation of the lip, alveolus, and or palate [19]. An elevated homocysteine level is itself teratogenic [20–22]. Rooij et al [19] reported that low periconceptional folate intake increases the risk of cleft lip with or without cleft palate in the offspring. Several studies reported the protective role of maternal periconceptional use of folic acid against cleft lip with or without cleft palate [23–26]. Munger [27] concluded that folate antagonists are associated with increased risk of oral clefts in humans. In another study Munger et al [28] reported that prevalence at birth of oral cleft did not change following the introduction of food fortification with folic acid. Bienengraber et al [29] reported that folate has a partial ameliorating effect on the teratogenicity of procarbazine given to rats. Whereas in our study folate had extremely different responses for prevention of cleft palate induced by two drugs of separate categories tested in identical conditions. Paros and Beck [30] in their study in mice concluded that folinic (tetrahydrofolic) acid can prevent cleft lip. Numerous studies with periconceptional folate supplementation reported inconsistent different results of orofacial clefts in different species [24,25,31–33].

A single high dose (10 mg/kg body weight) of lamotrigine or cyclophosphamide was administered as it is considered to have a longer lasting profound and persistent teratogenic effect compared to smaller doses given at short intervals which might lead to repair of tissue damage and cellular repair. Preventive efficacy of folate supplementation was seen in T3 treated group with lamotrigine administration where isolated partial clefts was predominant finding. This finding is in accordance with report of Tolavora and Harris [24], who concluded that prevention of isolated cleft palate is more difficult than cleft lip with or without cleft palate, because in cleft palate, the genetic components play a leading role in multifactorial etiology.

In conclusion, the preventive efficacies of folate supplementation for cleft palate vary considerably and in the same subject under identical conditions, depend primarily on the mechanism of action of the inducing agent. In human beings majority of the cleft disorders are idiopathic and the cause could not be determined. Study in animal models can help only partially in determining risk factors. More randomized clinical trials are needed to establish the hidden facts.

References

[1] **Mohanty C.** A correlative study of cleft palate induced by amniocentesis and its spontaneous prenatal repair in rat. **Thesis submitted for the degree of Master of Surgery (Anatomy), Banaras Hindu University, 1987.**

[2] **Singh G.** Teratogenic effect of amniocentesis in rat. **Thesis submitted for the degree of Master of Surgery (Anatomy), Banaras Hindu University, 1974.**

[3] **Macdonald RL, Greenfield LJ Jr.** Mechanisms of action of new antiepileptic drugs. **Curr. Opin. Neurol. 1997; 10: 121–128.**

[4] **Goodman A, Gilman L.** The Pharmacological Basis of Therapeutics. **11th Ed., The MacGraw Hill Companies. 2006; pp 1324–1328, 1415.**

[5] **Harrison TR.** Principal of Internal Medicine. **16th Ed., The MacGraw Hill Companies. 2005; pp 2388–2368, 2557.**

[6] **Tierny LM Jr, McPhee SJ, Papadakis MA.** Current Medical Diagnosis and Treatment. **45th Ed., The MacGraw Hill Companies. 2006; pp 984, 1078.**

[7] **Padmanabhan R, Abdulrazzaq YM, Bastaki SM, Shafiullah M, Chandranath SI.** Experimental studies on reproductive toxicologic effects of lamotrigine in mice. **Birth Defects Res. B Dev. Reprod. Toxicol. 2003; 68: 428–438.**

[8] **Zemlickis D, Lishner M, Erlich R.** Teratogenicity and carcinogenicity in a twin exposed in utero to cyclophosphamide. **Teratog. Carcinog. Mutagen. 1993; 13: 139–143.**

[9] **Bergstrom SK, Altman AJ.** Pregnancy during therapy for childhood acute lymphoblastic leukemia: two case reports and a review of the literature. **J. Pediatr. Hematol. Oncol. 1998; 20: 154–159.**

[10] **Enns GM, Roeder E, Chan RT, Ali-Khan Catts Z, Cox VA, Golabi M.** Apparent cyclophosphamide (cytoxan) embryopathy: a distinct phenotype? **Am. J. Med. Genet. 1999; 86: 237–241.**

[11] **Lucia MBID, Azoubel R.** Cyclophosphamide effects on the epithelial covering of rats fetus's tongue. A morphometric study. **Int. J. Morphol. 2005; 23: 105–109.**

[12] **Kirshon B, Wasserstrum N, Willis R, Herman GE, McCabe ER.** Teratogenic effects of first trimester cyclophosphamide therapy. **Obstet. Gynecol. 1988; 72: 462–464.**

[13] **Hansen DK, Billings RE.** Phenytoin teratogenicity and effects on embryonic and maternal folate metabolism. **Teratology. 1985; 31: 383–371.**

[14] **Rosenberg I, Selhub J.** Intestinal absorption of folates. **In: Blakely RL, editor. Folates and pterins; nutritional, pharmacological, and physiological aspects. New York, Wiley. 1986; pp 147–176.**

[15] **Hansen DK.** Alterations in folate metabolism as a possible mechanism of embryotoxicity. **In: Kavlock RJ, Datson GP, editors. Drug toxicity in embryonic development I. Handbook of experimental pharmacology. New York, Sringer-Verlag. 1987; pp 407–432.**

[16] **Finnell RH, Dansky LV.** Parental epilepsy, anticonvulsant drugs, and reproductive outcome: epidemiologic and experimental findings spanning three decades; 1: Animal studies. **Reprod. Toxicol. 1991; 5: 281–299.**

[17] **Rambeck B, Kurlemann G, Stodieck SR.** Concentrations of lamotrigine in a mother on lamotrigine treatment and her newborn child. **Eur. J. Clin. Pharmacol. 1997; 51: 481–484.**

[18] **Iqbal MM,Gundlapalli SP, Ryan WG, Ryals T, Passman TE.** Effect of antimanic mood-stabilizing drugs on fetuses, neonates, and nursing infants. **South Med. J. 2001; 94: 305–322.**

[19] **van Rooij IA, Vermeij-Keers C, Kluijtmans LA, Ocke MC, Zielhuis GA, Goorhuis-Brouwer SM, van der Biezen JJ, Kuijpers-Jagtman AM, Steegers-Theunissen RP.** Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphism affect the risk of cleft lip with or without cleft palate? **Am. J. Epidemiol. 2003; 157: 583–591.**

[20] **Rosenquist TH, Ratashak SA, Selhub J.** Homocysteine induces congenital defects of the heart and neural tube: effect of folic acid. **Proc. Natl. Acad. Sci. USA. 1996; 93: 15227–15232.**

[21] **Andaloro VJ, Monaghan DT, Rosenquist TH.** Dextromethorphan and other N-methyl-D-aspartate receptor antagonists are teratogenic in the avian embryo model. **Pediatr. Res. 1998; 43: 1–7.**

[22] **Rosenquist TH, Schneider AM, Monogham DT.** N-methyl-D-aspartate receptor agonists modulate homocysteine-induced developmental abnormalities. **FASEB J. 1999; 13: 1523–1531.**

[23] **Czeizel AE.** Prevention of congenital abnormalities by periconceptional multivitamin supplementation. **BMJ. 1993; 306: 1645–1648.**

[24] **Tolarova MM, Harris J.** Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. **Teratology. 1995; 51: 71–78.**

[25] **Shaw GM, Lammer EJ, Wasserman CR, O'Malley CD, Tolarova MM.** Risks of orofacial clefts in children born to women using multivitamins containing folic acid periconceptionally. **Lancet. 1995; 346: 393–396.**

[26] **Itikala PR, Watkins ML, Mulinare J, Moore CA, Liu Y.** Maternal multivitamin use and orofacial clefts in offspring. **Teratology. 2001; 63: 79–86.**

[27] **Munger R.** Maternal nutrition and oral clefts. **In: Wyszynski DF, ed. Cleft lip and palate: From origin to treatment. New York, Oxford University Press. 2002; pp 170–192.**

[28] **Munger RG, Sauberlich HE, Corcoran C, Nepomuceno B, Daack-Hirsch S, Solon FS.** Maternal vitamin B-6 and folate status and risk of oral cleft birth defects in the Philippines. **Birth Defects Res. A Clin. Mol. Teratol. 2004; 70: 464–471.**

[29] **Bienengraber V, Malek FA, Moritz KU, Fanghanel J, Gundlach KK, Weingartner J.** Is it possible to prevent cleft palate by prenatal administration of folic acid? An experimental study. **Cleft Palate Craniofac J. 2001; 38: 393–398.**

[30] **Paros A, Beck SL.** Folinic acid reduces cleft lip in A/Wyn SN mice. **Teratology. 1999; 344–347.**

[31] **Czeizel AE, Tóth M, Rockenbauer M.** Population-based case control study of folic acid supplementation during pregnancy. **Teratology. 1996; 53: 345–351.**

[32] **Hayes C, Werler MM, Willett WC, Mitchell AA. Case-control study of periconceptional folic acid supplementation and oral clefts. Am. J. Epidemiol. 1996; 143: 1229–1234.**

[33] **Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Folic acid antagonists during pregnancy and the risk of birth defects. N. Engl. J. Med. 2000; 343: 1608–1614.**