ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
SCHOOL OF GRADUATE STUDIES
DEPARTMENT OF ANATOMY

PROJECT PAPER ON EFFECTS OF MATERNAL FOLIC ACID SUPPLEMENTATION ON THE DEVELOPMENT OF NEURAL TUBE AND CARDIOVASCULAR SYSTEM OF THE OFFSPRINGS IN HUMAN AND ANIMAL MODELS

PROJECT PAPER SUBMITTED TO ADDIS ABABA UNIVERSITY, SCHOOL OF MEDICINE, DEPARTMENT OF ANATOMY IN PARTIAL FULFILMENT OF MASTER OF SCIENCE DEGREE IN HUMAN ANATOMY

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Acronyms and Abbreviations

ASD: Atrial septal defect

BMI: Body mass index

CHDs: Congenital heart defects

CI: Confidence interval

CP: Cleft palate

FAF: Folic acid fortification

GD: Gestational day

NTDs: Neural tube defects

POR: Prevalence odd ratio

RFC1: Reduced folate carrier

VSD: Ventricular septal defect

Summary

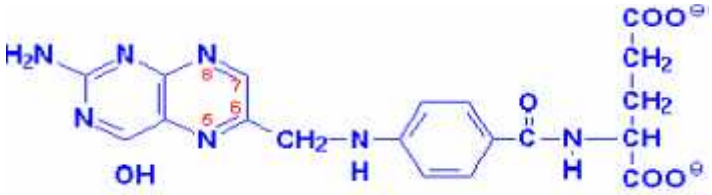
Folate is a water-soluble vitamin B present in legumes (e.g. beans, peas and lentils), leafy green vegetables (e.g. spinach and asparagus), liver and certain fruits (e.g. banana, cantaloupe and strawberry). Folic acid supplementation to pregnant women had no acute and long-term adverse effects on the health status of mothers as well as the new born infants. Maternal folic acid supplementation had no significant association with multiple births.

Maternal folic acid supplementation had a protective effect for neural tube defects (NTDs) especially spina bifida and anencephaly. Concomitant administration of maternal folic acid and methionine may also prevent retinoic acid induced cleft palate than use of folic acid alone. Maternal obesity before pregnancy with body mass index (BMI) ≥30 kg/m^2 was significantly associated with an approximately two fold increased risk of NTDs in offspring. The NTD protective association of folic acid was also stronger in overweight/ obese women BMI ≥ 25 kg/m^2 than in normal/underweight women BMI < 25 kg/m^2. Food fortification with folic or maternal supplementation of folic acid may have a protective effect for coarctation of aorta and left ventricular outflow tract obstruction, but no significant association was observed for tetralogy of Fallot and d-transposition of the great arteries.

High doses of daily maternal folate supplementation (50 mg/kg/day) during embryonic/fetal development are necessary for early post-implantation embryonic viability, chorioallantoic fusion, hematopoiesis, and the development of neural tube and heart. Maternal supplementation of multivitamin containing folic acid had more effective in preventing NTDs and congenital heart defects (CHDs) than use of folic acid alone, if it starts two months before conception and continues until completion of the second month of pregnancy and the frequency should be higher than five times per week. Use of vegetable and fruit during pregnancy also has a beneficial effect in preventing NTDs.

Despite the protective effect of folic acid in NTDs by facilitating the neural tube closure, additional investigation is required to understand the exact mechanism of action of folic acid in neural tube.

***Key words**: folic acid; maternal; supplementation; NTDs; CHDs*


H2N
N
N
N
6
7
5
5
N
CH2
N
H
OH
O
C
N
H
COO⊖
CH2
CH2
CH
COO⊖

1.1.1. Folate cycles

In a cell, 5-methyl tetrahydrofolate can function as a methyl donor for homocysteine remethylation with subsequent formation of tetrahydrofolate, which can directly be converted into 5, 10-methylene tetrahydrofolate by the action of serine hydroxyl methyl transferase. Serine hydroxyl methyl transferase is a vitamin B6–dependent enzyme that uses serine as a one-carbon donor. In humans, serine hydroxyl methyl transferase has both a cytosolic and mitochondrial isoform (Blom, 2009).

The methyl tetrahydrofolate reductase enzyme is of great importance for the regulation of available 5-methyl tetrahydrofolate for homocysteine remethylation. Homocysteine remethylation to methionine is catalyzed by the methionine synthase enzyme and links the folate cycle with the homocysteine metabolism (Blom, 2009).

1.1.2. Methylation Cycles

In the methylation cycle, the methyl group of 5-methyl tetrahydrofolate is transferred to homocysteine to produce methionine. The reaction of methionine with adenosine triphosphate affords serine adenosylmethionine, which is the principal donor of methyl groups in cells (Blom *et al.*, 2006). Loss of a methyl group from serine adenosylmethionine generates serine adenosylhomocysteine, which is a strong inhibitor of methyltransferases. If an inadequate amount of 5-methyl tetrahydrofolate is available, homocysteine accumulates in the cell. Consequently, accumulation of homocysteine leads to the build up of serine adenosylhomocysteine, which might lead to dysregulation of gene expression, protein function, and lipid and neurotransmitter metabolism through inhibition of putative methyltransferases (Blom *et al.*, 2006).

There are multiple indications that methylation especially of DNA or histones are robust contributors to neurulation. Neurulation defects were induced in wild type rat embryos when cultured in reduced levels of methionine. Methionine deficiency was associated with a failure of the neural folds to turn medially, suggesting a deficit in microfilaments and diminished cytoskeletal contractility (Coelho and Klein, 1990). Experiments in chick and mouse embryos indicate that exposure to homocysteine or inhibitors of the methylation cycle delayed neural tube closure in a dose-dependent manner (Dunlevy *et al.*, 2006).

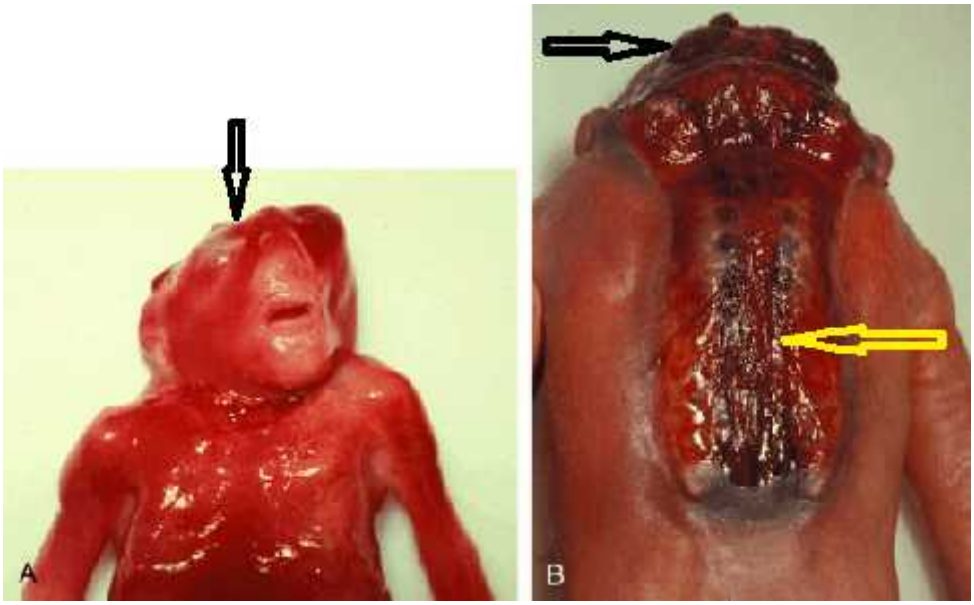
1.2. Embryology of neural tube and neural tube defects

The nervous system develops from neural plate ectoderm which gives rise to the neural tube and neural crest which in turn form all parts of the central and peripheral nervous system. In mouse, starting around embryonic day 7.5 the neural groove begins to form along the midline of the neural plate. While the primitive streak is regressing and the neural plate extending posteriorly, the neural groove deepens and the neural folds develop. As the folds become higher, the edges start to approach each other and finally meet and fuse to form the neural tube which underlies the surface ectoderm. Closure of the neural tube starts around day 8.25 at the position of the 4^{th} to 5^{th} somite and progresses anteriorly and posteriorly. The open ends of the neural tube are called the anterior and posterior neuropores. The anterior neuropore closed around embryonic day 9 while closure of the posterior neuropore is not completed until the 10^{th} embryonic days (Kispert and Gossler, 2004).

Cells from the edge of the neural folds between neuroectoderm and surface ectoderm give rise to the neural crest. The neural crest cells disperse rapidly and migrate through the embryo, which give rise to spinal ganglia, ganglia of cranial nerves V, VII, IX and X, the peripheral nervous system, the adrenal medulla, the melanocytes of the epidermis, pigment cells, Schwann cells, leptomeninges, carotid body, parafollicular cells, odontoblasts, pharyngeal arch cartilage, bulbar and conal ridges in heart, head mesenchyme and connective tissue (Kispert and Gossler, 2004; Moore and Persuad, 2008).

In humans, formation of the neural tube, the embryonic precursor to the brain and spinal cord, occurs during the fourth week of gestation with the formation of the neural plate from specialized ectodermal cells, the neuroectoderm. On approximately the 18th day, the neural plate invaginates along its central axis to form a longitudinal median neural groove, which has neural folds on each side. By the end of the third week, the neural folds have begun to move together and fuse, converting the neural plate into a neural tube, the primordium of the central nervous system. These processes are completed by the end of the fourth week after closure of the anterior neuropore at approximately day 25 (18- to 20-somite stage) and the posterior neuropore at day 27 (25-somite stage) (Moore and Persuad, 2008; Sadler, 2012).

Failure of the neural tube to close will result in neural tube defects (NTDs). Failure of anterior regions of the neural tube to close results in anencephaly, whereas failure of closure of the posterior regions of the neural tube results in spina bifida, the most common NTD in humans (Botto *et al*., 1999; Boulet *et al*., 2008).


A
B

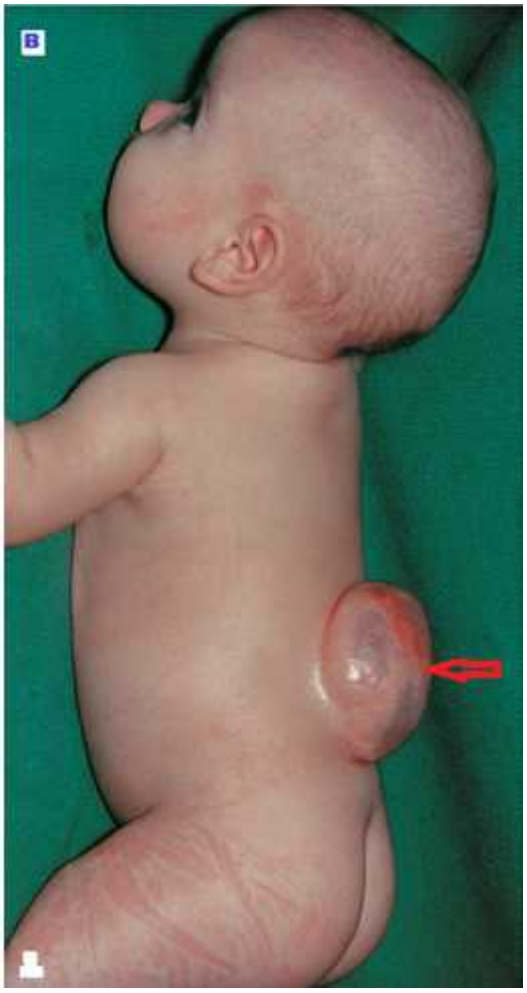
Spina bifida is a general term for NTDs affecting the spinal region. It consists of a splitting of the vertebral arches and may or may not involve underlying neural tissue. Two different types of spina bifida occur (Sadler, 2012).

1) Spina bifida occulta is a defect in the vertebral arches that is covered by skin and usually does not involve underlying neural tissue. It occurs in the lumbosacral region and is usually marked by a patch of hair overlying the affected region (fig. 3A) (Moore and Persuad, 2008).

2) Spina bifida cystica is a severe NTD in which neural tissue and/or meninges protrude through a defect in the vertebral arches and skin to form a cyst like sac (fig. 3B). Most lie in the lumbosacral region and result in neurological deficits, but they are usually not associated with mental retardation (Moore and Persuad, 2008).

When the sac contains meninges and cerebrospinal fluid, the anomaly is called spina bifida with meningocele. The spinal cord and spinal roots are in their normal position, but there may be spinal cord abnormalities. If the spinal cord and/or nerve roots are included in the sac, the anomaly is called spina bifida with meningomyelocele. Hydrocephaly develops in virtually every case of spina bifida cystica because the spinal cord is tethered to the vertebral column. Spina bifida cystica can be diagnosed prenatally by ultrasound and by determination of α-fetoprotein levels in maternal serum and amniotic fluid (Sadler, 2012).


A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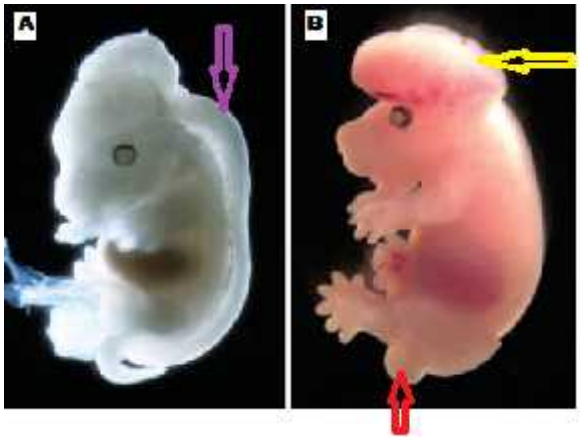
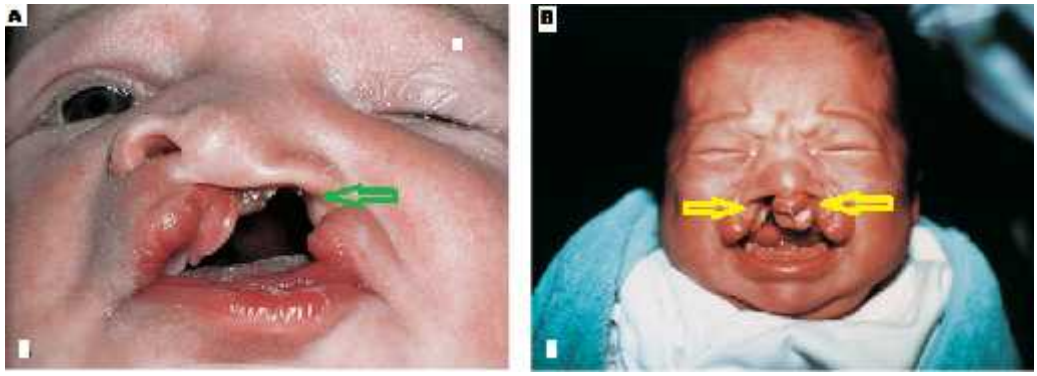

A
B

In mouse, failure of initiation of closure at the upper spinal level results in the severe defect craniorachischisis (fig. 4A); in which most of the brain and the entire spinal cord remain open. The commonest defect of spinal closure, however, involves the lower spinal neural tube, which produces open spina bifida (fig. 4B). Unlike the cranial defects, which are usually lethal at or before birth, spina bifida is compatible with postnatal survival. However, affected individuals can suffer from motor and sensory defects in the legs, urinary and faecal incontinence, vertebral curvature defects and hydrocephalus (Copp *et al*., 2003).

Neural tube defects and oral clefts are embryologically related because facial and tooth tissues develop from neural crest cells that originate from the dorsolateral aspect of the developing neural tube. Unilateral cleft lip results from failure of the maxillary prominence on the affected side to unite with the merged medial nasal prominences (fig. 5A). Bilateral cleft lip results from failure of the mesenchymal masses in both maxillary prominences to meet and unite with the merged medial nasal prominences (fig. 5B and D). Cleft palate *(CP)* with or without a cleft lip may involve only the uvula or extend through the soft and hard regions of the palate (fig. 5 A, B, and F). In severe cases associated with a cleft lip, the cleft in the palate extends through the alveolar part of the maxilla and the lips on both sides (Copp *et al*., 2003; Moore and Persusd, 2008; Sadler, 2012).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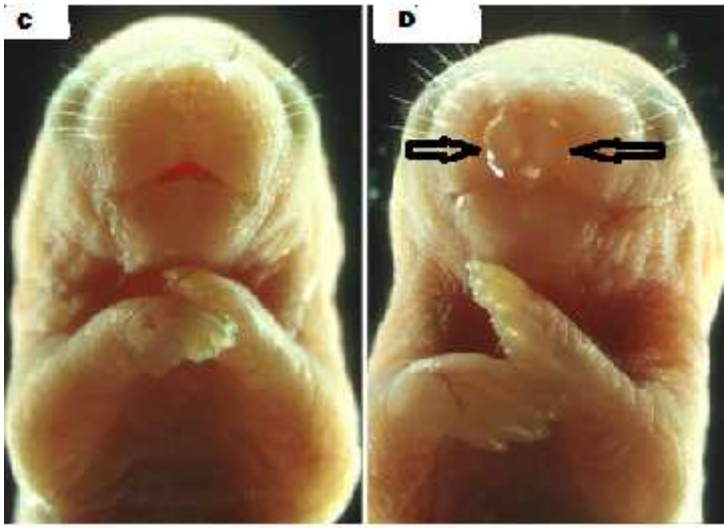
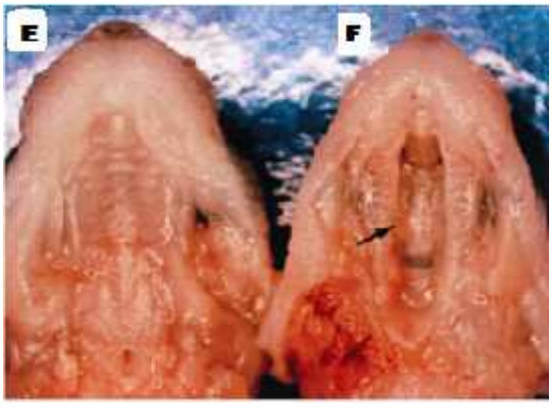

C
D

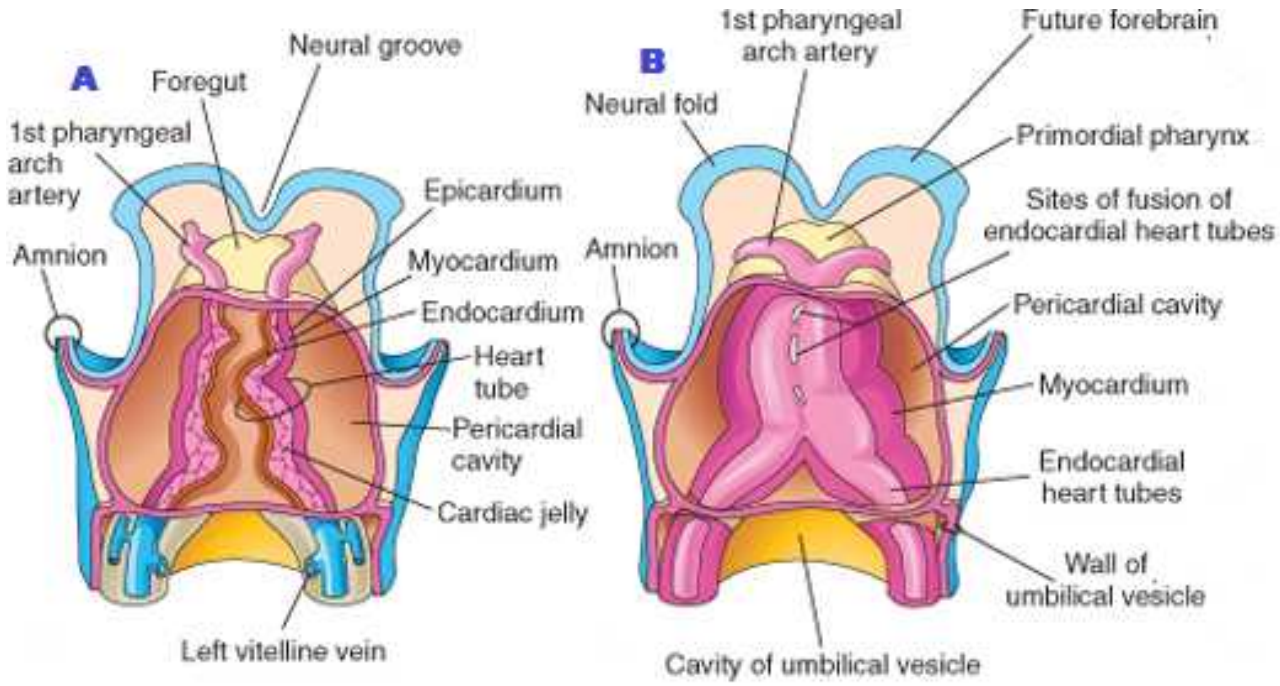

E
F

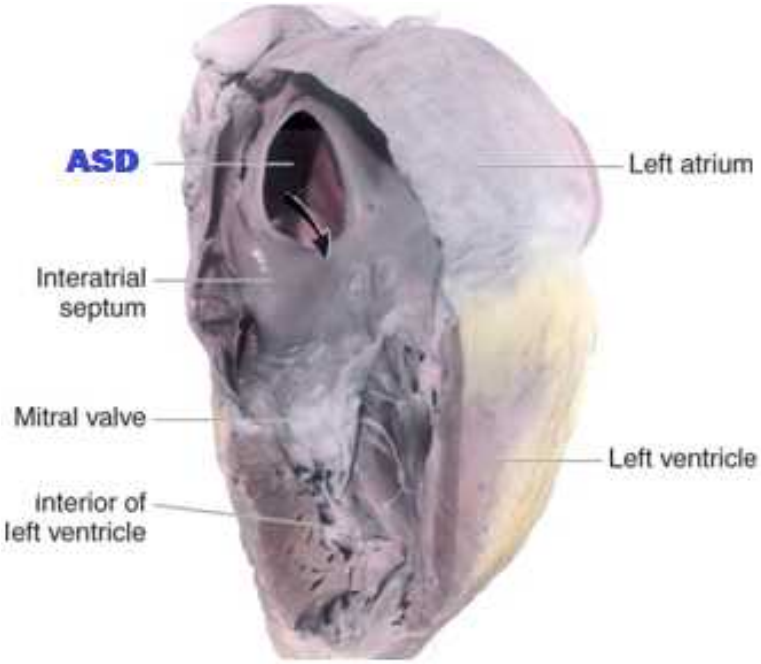
1.3. Embryology of cardiovascular system and cardiovascular congenital anomalies

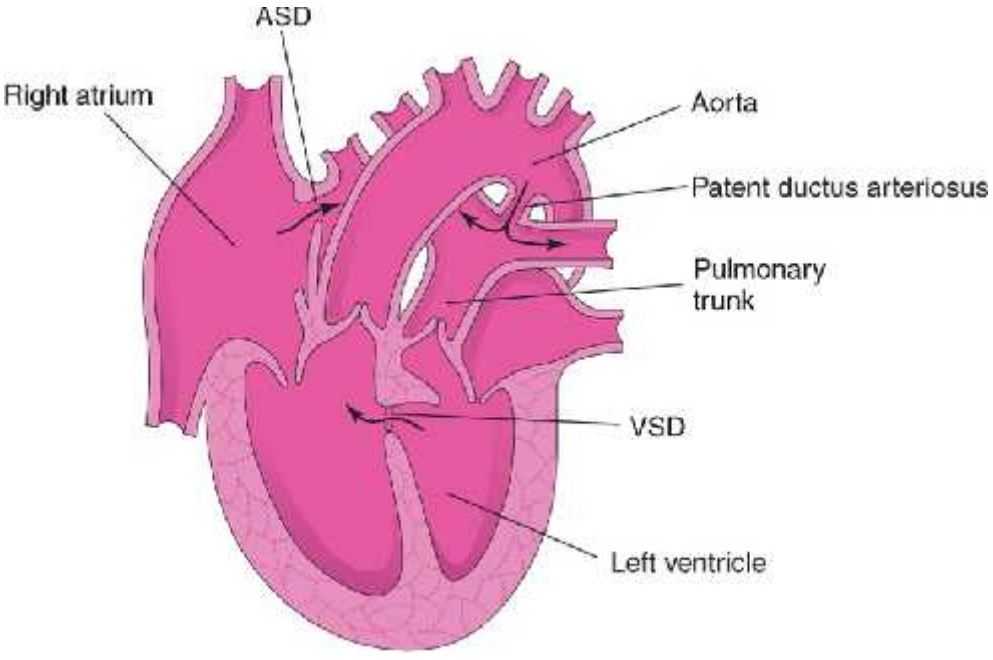
The cardiovascular system is the first organ system to reach a functional state. In mouse, the lateral plate mesoderm splits into the dorsal (somatic) mesoderm underlying the ectoderm and the ventral (splanchic) mesoderm underlies the endoderm. Between these layers the coelom forms which will later be subdivided into the separate pleural, pericardial and peritoneal cavities. Lateral plate mesoderm cells form tissues such as the heart, blood vessels, connective tissues of the viscera and cartilage and bone of the limbs (Kispert and Gossler, 2004).

In mouse, extraembryonic mesoderm of the yolk sac is the first site of hematopoiesis in the developing embryo. From the 7^{th} day of gestation onwards blood islands appear on the inner side of the visceral yolk sac. These are condensations of mesenchymal cells which form an irregular girdle around the exocoelom. The inner cells of these condensations become embryonic red blood cells, whereas the peripheral cells differentiate and form the endothelium of blood vessels of the yolk sac. Following the process known as primitive hematopoiesis, the major hematopoietic site shifts to the fetal liver around day 12 of gestations, and thereafter to the bone marrow by embryonic day of 15–16, definitive hematopoiesis (Kispert and Gossler, 2004; Erb, 2006).

In humans, heart and great vessels form from mesenchymal cells in the cardiogenic area. Paired, longitudinal endothelial-lined channels (fig. 6A); the endocardial heart tubes develop during the third week and fuse to form a primordial heart tube (fig.6B). The fusion begins at the cranial ends of the heart tubes and extends caudally until a single tubular heart is formed. Blood cells develop from the endothelial cells of vessels as they develop on the umbilical vesicle and allantois at the end of the third week. Blood cells continue to form in various parts of the embryonic mesenchyme, mainly the liver and later in the spleen, bone marrow, and lymph nodes. By the end of the third week, the blood is circulating and the heart begins to beat on the 21^{st} or 22^{nd} day (Moore and Persuad, 2008).


A
Neural groove
Foregut
1st pharyngeal arch artery
Epicardium
Amnion
Myocardium
Endocardium
Heart tube
Pericardial cavity
Cardiac jelly
Left vitelline vein
B
1st pharyngeal arch artery
Future forebrain
Neural fold
Primordial pharynx
Sites of fusion of endocardial heart tubes
Amnion
Pericardial cavity
Myocardium
Endocardial heart tubes
Wall of umbilical vesicle
Cavity of umbilical vesicle


ASD
Left atrium
Interatrial septum
Mitral valve
Left ventricle
interior of left ventricle


ASD
Right atrium
Aorta
Patent ductus arteriosus
Pulmonary trunk
VSD
Left ventricle

Ventricular septal defects (VSDs) are the most common type *of CHD*, accounting for approximately 25% of heart defects. In this condition, an opening occurs in the ventricular septum (fig.8). Because of this opening, blood from the left ventricle flows back into the right ventricle, due to higher pressure in the left ventricle. This causes an extra volume of blood to be pumped into the lungs by the right ventricle, which can create congestion in the lungs. VSDs occur more frequently in males than in females (Erb, 2006; Moore and Persuad, 2008).

In transposition of the great arteries, the aorta originates from the right ventricle, so most of the blood returning to the heart from the body is pumped back without going to the lungs, whereas the pulmonary artery originates from the left ventricle; therefore, most of the blood returning from the lungs goes back to the lungs again (fig. 8). Transposition of the great arteries is the most common cause of cyanotic heart disease in newborn infants. Tetralogy of Fallot is a classic group of four cardiac defects which consists of pulmonary artery stenosis, VSD, overriding aorta and right ventricular hypertrophy (Erb, 2006; Sadler, 2012).

2. Objective

2.1. General objective

To review and analyze published articles on effects of maternal folic acid supplementation on the development of neural tube and cardiovascular system of offspring in human and animal models.

2.2. Specific objective

- To review and analyze published articles on effects of maternal folic acid supplementation on the development of neural tube of offspring in human models.
- To review and analyze published articles on effects of maternal folic acid supplementation on the development of neural tube of offspring in animal models.
- To review and analyze published articles on effects of maternal folic acid supplementation on the development of cardiovascular system of offspring in human models.
- To review and analyze published articles on effects of maternal folic acid supplementation on the development of cardiovascular system of offspring in animal models.

3. Review and analysis of published articles

3.1. Effects of maternal folic acid supplementation on the development of neural tube of offspring in human models

Simmons *et al*. (2004) evaluated the effect of folic acid fortification on the prevalence of birth defect.

Materials and methods

Birth defect cases were identified from the Arkansas reproductive health monitoring system for the birth years 1993 to 2000. Live births, stillbirths, elective terminations, and spontaneous abortions were included as cases for this analysis. Exposure to folic acid fortification was classified by year of birth or pregnancy completion. Authorization of folic acid fortification began in March of 1996 and became mandatory beginning in January of 1998. Birth years from 1993 to 1995 were identified as "pre-fortification." Births from 1996 through 1998 were classified as occurring during a "transition period," because the timing and amount of folic acid exposure through fortification was difficult to specify. The "post-fortification" period includes the birth years 1999 and 2000, when all conceptions would have occurred after fortification became mandatory.

Prevalence rates were calculated using data for all non-Hispanic white or African-American live births to Little Rock, Arkansas residents during 1993–2000 as the denominator. Odds were computed using cases as the numerator and non-Hispanic white or African American Arkansas live births that did not link to any birth defect cases. Logistic regression analysis was used to compute crude and adjusted prevalence odds ratios comparing the identified time periods, "pre-fortification versus transition," "transition versus post-fortification" and "pre-fortification versus post-fortification". Statistical significance was evaluated at $\alpha = 0.05$ level.

Result

The results suggest a decline in the prevalence of the specific defects evaluated in this study during the 1990s (table 1). For spina bifida, a statistically significant decrease was observed when comparing the odds of the pre- and post-fortification periods [prevalence odds ratio (POR), 0.56; 95% confidence interval (CI), 0.37, 0.83], as well as between the transition and post-fortification periods (POR, 0.57; 95% CI, 0.38, 0.86). Prevalence odds ratios for anencephalus were not statistically significant during these periods (POR, 0.95; 95% CI, 0.59, 1.54 and POR, 0.88; 95% CI, 0.55, 1.41, respectively).

Table 1: Prevalence rates per 10,000 live births of selected defects in Arkansas during specified periods relative to folic acid fortification

Defect groups	Pre-fortification (1993–1995)		Transition (1996–1998)		Post-fortification (1999–2000)	
	No.	Rate	No.	Rate	No.	Rate
Neural tube defects	114	10.9	127	11.6	61	8.2
Anencephalus	40	3.8	46	4.2	28	3.8
Spina bifida	81	7.8	83	7.6	33	4.4
Oral-facial cleft defects	204	19.6	215	19.6	126	16.9
Cleft lip with and without palate	119	11.4	141	12.9	75	10.1
Cleft palate without cleft lip	85	8.2	74	6.7	51	6.8
Conotruncal heart defects	85	8.2	97	8.8	67	9.0
Tetralogy of Fallot	37	3.5	39	3.6	31	4.2
Transposition of great arteries	40	3.8	43	3.9	32	4.3
Limb defects	56	5.4	62	5.7	34	4.6
Abdominal wall defects (Gastroschisis and Omphalocele)	66	6.3	87	7.9	53	7.1

The crude prevalence odds ratios for Down syndrome and cleft lip with and without cleft palate decreased during the post-fortification period, but showed non-significant associations with each of the periods when folic acid fortification was implemented. Prevalence also decreased for cleft palate without cleft lip, limb defects, and omphalocele, but the odds ratios were not statistically significant for these data (table 1). For gastroschisis, tetralogy of Fallot, transposition of the great arteries, and diaphragmatic hernia, prevalence increased during this study period, but none of the odds ratios were statistically significant.

3.2. Effects of maternal folic acid supplementation on the development of neural tube of offspring in animal models

Reynolds *et al.* (2003) evaluated the effect of combination therapy with folic acid and methionine in the prevention of retinoic acid-induced cleft palate in mice.

Materials and methods

In this study, virgin female Swiss-Webster mice, 6–9 weeks of age were obtained from Sysco laboratories weighting 21–26g. The mice were isolated for two weeks for environmental conditioning, and then mated with males from the same strain with similar weights and ages. Two female mice were assigned randomly to a single male mouse. Presumed pregnant animals were isolated after detection of a vaginal plug [gestational day (GD) 0] and randomly assigned to one of four treatment groups comprising 10–12 animals each. The room was maintained at a temperature of approximately 72°F with a 12 hours light-dark cycle.

Dose materials were organized into two categories, teratogenic treatment and therapy. All-*trans* retinoic acid was given as the teratogenic inducer of cleft palate and the therapy included folic acid, methionine, or a combination of the two. The dose solution of the retinoic acid was prepared by thorough mixing in corn oil until a suspension was reached. Folic acid, methionine, and folic acid + methionine dose solutions were prepared by attaining adequate solvation in distilled water. Prepared solutions were stored at 64.8°F and brought gradually to room temperature at the time of dose delivery.

Experiment A

Ten to twelve presumed pregnant Swiss–Webster mice were assigned randomly to each of four treatment groups. Each mouse received 50 mg/kg body weight of retinoic acid with vehicle on GD 10 via an intraperitoneal injection. Therapy was also administered on GD 8 to 11 by intraperitoneal injection. The experiment was a two-way factorial design in which group 1 received water, group 2 received folic acid (4 mg/kg body weight), group 3 received methionine (187 mg/kg body weight), and group 4 received both folic acid and methionine at the same dose levels as in groups 2 and 3. The volume administered was 0.01 ml/gm body weight twice daily during the treatment period. Around GD 18, the dams were observed for clinical signs of toxicity. Body weights were determined on alternate days from GD 0–18. On GD 18, each female was euthanized with ether and examined for clinical effects including external body surfaces and orifices. For *uteri* that appeared non-gravid, the animals were

killed by cervical dislocation and stained with a 5% ammonium sulphide solution to determine implantation sites. If no implantation sites were observed, the animal was considered not pregnant, and was not included in the data. Both uterine horns were opened and the number and position of implantations, early and late resorptions, and live and dead fetuses were recorded. The weight, gender, and presence of external alterations, including CP of live fetuses were noted. Approximately one-half of the fetuses from each litter were selected randomly and prepared for skeletal evaluations, whereas the remainder were selected for visceral examinations. The occurrence of fetuses showing CP was recorded at the time of external evaluation. Fetuses assigned for skeletal examination were fixed initially in 70% ethanol for 5 days, and then placed in 2% potassium hydroxide and 0.004% alizarin red solution for approximately 24 hours. This was followed by placing the fetuses in increasing concentrations of glycerin to clear the tissues. All fetuses were examined under a Wild dissection microscope for skeletal or visceral malformations.

Experiment B

With the exception of decreased therapy volumes and concentrations, this trial was conducted according to the protocol outlined in experiment A. While keeping the level of retinoic acid at 50 mg/kg, the therapy dose concentrations were decreased by 25%. The levels of therapy introduced to the dams were 3 mg/kg folic acid and 140 mg/kg methionine. This change was made to investigate further the apparent interaction between folic acid and methionine in the rescue of retinoic acid induced CP.

Result

Maternal Effects

Experiment A

Neither maternal deaths nor clinical symptoms were observed in any of the dams.

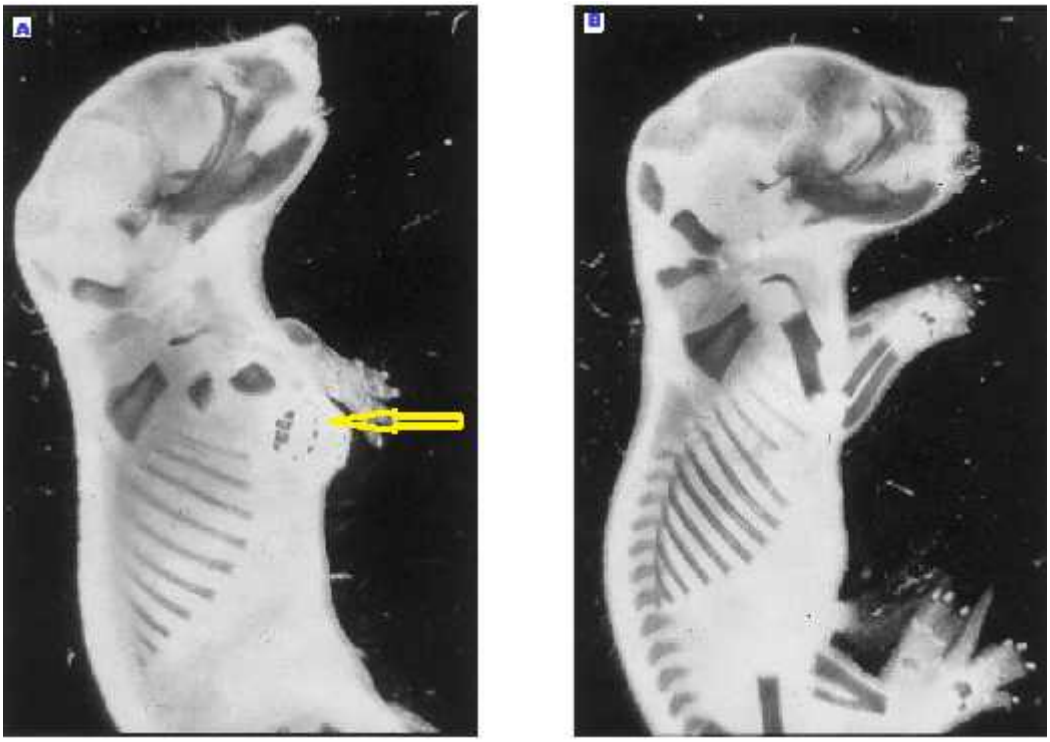
Experiment B

Neither deaths nor signs of clinical toxicity were observed in any of the study groups.

Developmental Effects

Experiment A

Retinoic acid exposure with no therapy produced a greater number of resorptions per litter (5.3%), but the differences in resorption frequency across all groups were not significant.


A
B

Skeletal evaluation revealed under ossified cranial bones, truncated tails, and micromelia in all groups (fig. 9A). In group 1, 28.7% of the pups showed reduced (or delayed) ossification of at least one of the cranial bones. Although this frequency decreased in group 2 to 11.8%, only groups 3 ($p \leq 0.05$) and 4 ($p \leq 0.01$) showed significantly lower rates of reduction relative to group 1, with percentage of delayed cranial bone ossification at 6.0% and 2.7%, respectively. However, it should be noted that there was a marginally significant ($p \approx 0.06$) reduction in the delay of cranial bone ossification in group 2. A significant decrease in the frequency of skeletal defects caused by retinoic acid was observed in all groups receiving therapy, demonstrating the success in folic acid, methionine, and folic acid + methionine mediated prevention (fig. 9 B).

Experiment B

A 25% decrease in dosage therapy yielded a reduction in prevention of retinoic acid induced CP. Pups in group 1 manifested 85.7% CP in the absence of therapy. Pups in the folic acid group exhibited a frequency of 83.2% (not significant) and methionine reduced the occurrence to 57.7% (marginally significant, $p \approx 0.06$). The combination of folic acid and methionine was shown to lessen significantly the frequency of CP pups to 46.4% ($p \leq 0.05$).

External evaluation of the pups revealed malformations of the limbs and tail in each group. Skeletal examination showed that 27.9% of the pups in group 1 had delays in cranial bone ossification. Although not eliciting a significant decrease, 16.2% of the pups in group 2 had ossification delays. Significant reductions ($p \leq 0.05$) in cranial bone under ossification were seen in groups 3 and 4 where the frequencies were 7.7%, and 7.5%, respectively.

Micromelia was noted to occur at the rate of 81.4% in the control and at 87.3% in group 2. Differences in the frequency of limb defects were significant in group 3 and 4 (50.2% and 41.3%, respectively; $p \leq 0.05$). The rate of tail truncations observed in group 1 (46.3%) was reduced 31.6%, 16.2%, and 22.5% in the three therapeutic groups.

3.3. Effects of maternal folic acid supplementation on the cardiovascular system of offspring in human models

Bedard *et al*. (2013) evaluated the effect of folic acid fortification on the birth prevalence of congenital heart defect.

Materials and methods

Cardiac cases were coded and classified with assistance from paediatric cardiologist and with pathology details provided by computed tomography. Review of coding and classification of CHD cases into isolated and multiple groups were done by a pediatrician and clinical geneticist. Birth prevalence with 95% CI was calculated for CHD cases and is reported as per 1000 total births (live births and stillbirths). POR were calculated comparing pre-folic acid fortification (FAF), 1995–1997 and post-FAF, 1999–2002.

Cases with CHDs were ascertained using the Alberta congenital anomalies surveillance system. Additional data sources were actively searched and include both pediatric cardiology centers in Alberta, autopsy records including terminations of pregnancy for fetal anomalies and hospital records. Cardiac diagnoses were verified by echocardiography, cardiac catheterization, surgery, and/or autopsy.

Live births, stillbirths (≥20 weeks or ≥500 grams), and terminations of pregnancy (<20 weeks) diagnosed with a CHD and born in Alberta to Alberta residents between 1 January 1995 and 31 December 2002 were included. Cases were excluded if they had an isolated patent foramen ovale or isolated patent foramen ovale versus ASD and the precise diagnosis was not confirmed, a patent foramen ovale that was not followed up to assess persistence, or a patent foramen ovale and/or ASD and were premature (<37 weeks). Fetal deaths with a CHD diagnosed prenatally but not confirmed by autopsy were also excluded.

CHD cases were classified into conotruncal (truncus arteriosus, d-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, and double outlet left ventricle); septal (ASD and VSD); atrioventricular septal defect; left ventricular outflow tract obstruction (hypoplastic left heart syndrome, coarctation of aorta, interrupted aortic arch, aortic valve stenosis and bicuspid aortic valve) and right ventricular outlflow tract obstruction (pulmonary atresia, pulmonary stenosis and tricuspid atresia). Each case was classified with one CHD type and counted once. Cases were further classified into isolated and multiples. The “multiples” group included cases with associated noncardiac anomalies and known

etiologies (chromosomes, syndromes, Mendelian disorders, and teratogens). Cases without noncardiac anomalies and without a known etiology were classified as isolated.

Result

The number of births (live births and stillbirths) in the pre-FAF period totalled 113,286 and in the post-FAF period, 150,898. There were 2826 isolated CHD cases born between 1 January 1995 and 31 December 2002. The number of isolated CHD cases included in the analyses was 2214. The prevalence rate for CHDs overall was 9.34 (95% CI, 8.79–9.92) pre-FAF and 9.41 (95% CI, 8.93–9.91) post-FAF with most prevalence rates for the CHD subgroups comparable between the pre-FAF and post-FAF periods. The prevalence rates for tetralogy of Fallot were equal for both the pre-FAF and post-FAF periods (POR, 0.99; 95% CI, 0.53–1.87) as was the prevalence of d- transposition of great artery (POR, 1.00; 95% CI, 0.55–1.83). The prevalence of left ventricular outflow tract obstruction was significantly lower in the post-FAF period (POR, 0.76; 95% CI, 0.61–0.94) with coarctation of the aorta substantially contributing to this reduction (POR, 0.55; 95% CI, 0.32–0.92). The prevalence of septal defects as a group increased in the post-FAF period with odds ratio of 1.13 (95% CI, 1.02–1.26). It appears that cases with an ASD or an ASD with VSD accounted for this increase (ASD POR, 1.42; 95% CI, 1.13–1.80 and ASD with VSD POR, 1.52; 95% CI, 1.10–2.10).

3.4. Effects of maternal folic acid supplementation on the cardiovascular system of offspring animal models

Gelineau-van Waes *et al*. (2008) evaluated the effect of maternal folate supplementation on the embryonic development of reduced folate carrier (RFC1) of knockout mouse.

Materials and methods

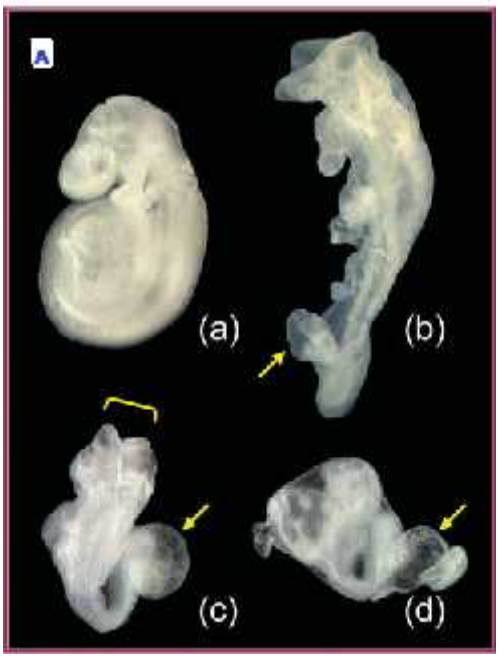
In this study, pregnant dams received vehicle (control) or folic acid (25 or 50 mg/kg) by subcutaneous injection (0.1 mL/10g body weight), beginning on the first embryonic day and continuing throughout gestation until the time of sacrifice. Fifteen litters were collected for each of the three treatment groups. At the desired gestational time point, pregnant RFC1 dams were killed by cervical dislocation, the abdomen opened, and the uterine contents removed. The location of all viable fetuses and resorption sites were recorded. Embryos/fetuses were dissected free of the decidual capsule, including its chorion and amnion, and examined for the presence of gross abnormalities. Following the gross morphological examination, fetuses were processed for histological analysis. Tissues were fixed in 4% paraformaldehyde, transferred to 70% ethanol, dehydrated, and embedded in paraffin. Ten micrometer thick sections were cut, affixed to plus charged slides, and stained with hematoxylin and eosin.

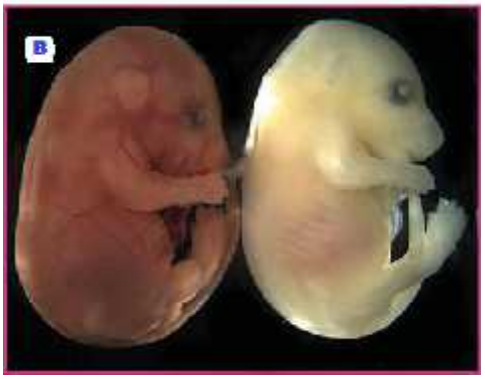
Result

The result shows that, without additional maternal folate supplementation, all RFC1 nullizygous embryos died shortly after implantation (seventh embryonic day), and were subsequently resorbed.

Supplementation of pregnant dams with low dose folic acid (25 mg/kg/day subcutaneous) prolonged survival of 27/49 (55%) RFC1 nullizygous embryos until embryonic day 10–11. The surviving RFC1 mutant embryos were smaller, dysmorphic, and developmentally delayed for their gestational age relative to their heterozygous and wild-type littermates (Fig. 10A). RFC1 knockout embryos harvested from dams receiving low dose folic acid supplementation displayed a failure of chorioallantoic fusion (Fig. 10A: [a] RFC1 wild-type embryo; [b–d] RFC1 nullizygous embryos), significantly reduced numbers of erythrocytes in the yolk sac blood islands and embryonic vasculature and multiple malformations, including craniofacial abnormalities, small (absent) limb buds, and delayed heart development.

Maternal folate supplementation with an even higher dose of folic acid (50 mg/kg/day subcutaneous) resulted in ‘‘rescue’’ or survival of 7/32 (22%) of the RFC1 nullizygous fetuses examined on embryonic day 19. Although the surviving near-term reduced folate carrier fetuses appeared grossly morphologically normal, only two of the seven fetuses appeared pink in color, while the remaining five fetuses were extremely pale in comparison to heterozygous and wild-type littermates (Fig. 10B). All seven of the surviving RFC1 null fetuses had varying degrees of cardiac and lung malformations, open eyelid defects, and skin abnormalities.


A
(a)
(b)
(c)
(d)


B

4. Discussion

According to Adugna and his colleagues (2004), the average daily requirement of folic acid is 0.1 mg/day (during lactation and pregnancy it should increase to 0.5- 0.8 mg/day). On the other hand, Pitkin (2007) advised women planning pregnancy to take 0.4 mg of folic acid per day (for high risk women it should increase to 4 mg/day) for at least one month before conception and during the first trimester of pregnancy in order to get an optimal prevention efficacy. The above assertions are more or less similar, but the recommendation by Pitkin (2007) is slightly lower than the daily requirement of folic acid for pregnant mothers' asserted by Adugna *et al.* (2004). This difference may probably because of Pitkin (2007) considered slight folate produced by intestinal bacteria (Tran *et al*., 2002).

According to Czeizel and Tomcsik, 1999), neither acute nor long-term adverse effects of high doses (120– 150 mg) of folic acid during pregnancy were detected at the birth of their newborn infants and some years later when the health status of both mothers and children was checked. Inadequate intake, impaired absorption, increased demand during pregnancy and lactation, impaired metabolism and drugs like anticonvulsants and oral contaceptives may cause folate deficiency, which in turn leads to megaloblastic anemia (Adugna *et al*., 2004).

Exposure to higher levels of folic acid could increase the risk of multiple gestations (Ericson *et al*., 2001). They reported that the multiple birth rates were significantly higher in the multivitamin containing folic acid group (3.8%) than in the control group (2.7%). However, the difference between the two groups was not explained by a difference in the use of ovulation-inducing drugs which may predispose to multiple gestations. On the other hand, Li *et al*. (2003) and Shaw *et al*. (2003) found no increased risk of multiple gestations after food fortification with folic acid. The study conducted by Lawrence *et al. (*2004) also showed no relationship between food fortification with folic acid and the rates of multiple births. An increase in multiple gestations during pregnancy may be associated with the use of ovulation-inducing drugs like clomiphene citrate and menotropins (Humegon and Pergonal) and anti retroviral therapy (Lawrence *et al.,* 2004). Therefore, maternal folic acid supplementation has no association with multiple gestations.

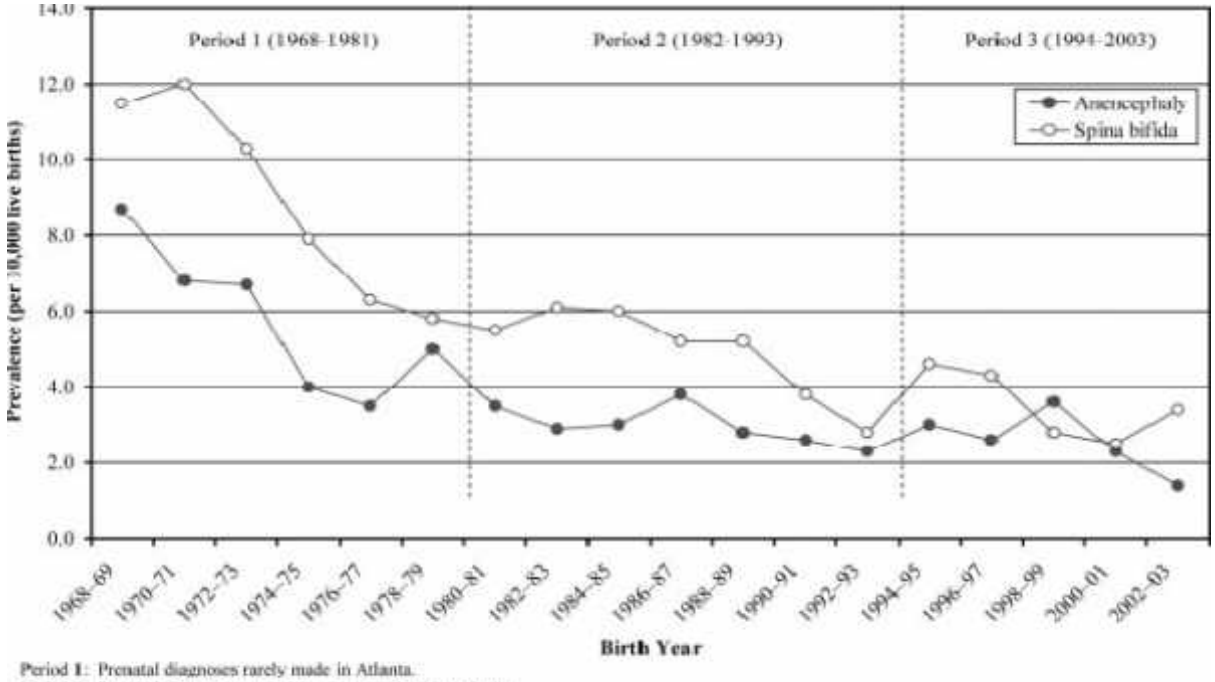
According to Frey and Hauser (2003), NTDs are believed to have a multifactorial etiology with interplay of both genetic and environmental factors. The higher recurrence rate of NTDs within families, the preponderance of NTDs in monozygotic twins and the association

between NTDs and ethnicity imply genetic factors are involved in NTD etiology. Differences in NTD prevalence in time, between seasons, geographical areas and socioeconomic status indicate that environmental factors are also involved in NTD etiology (Frey and Hauser, 2003).

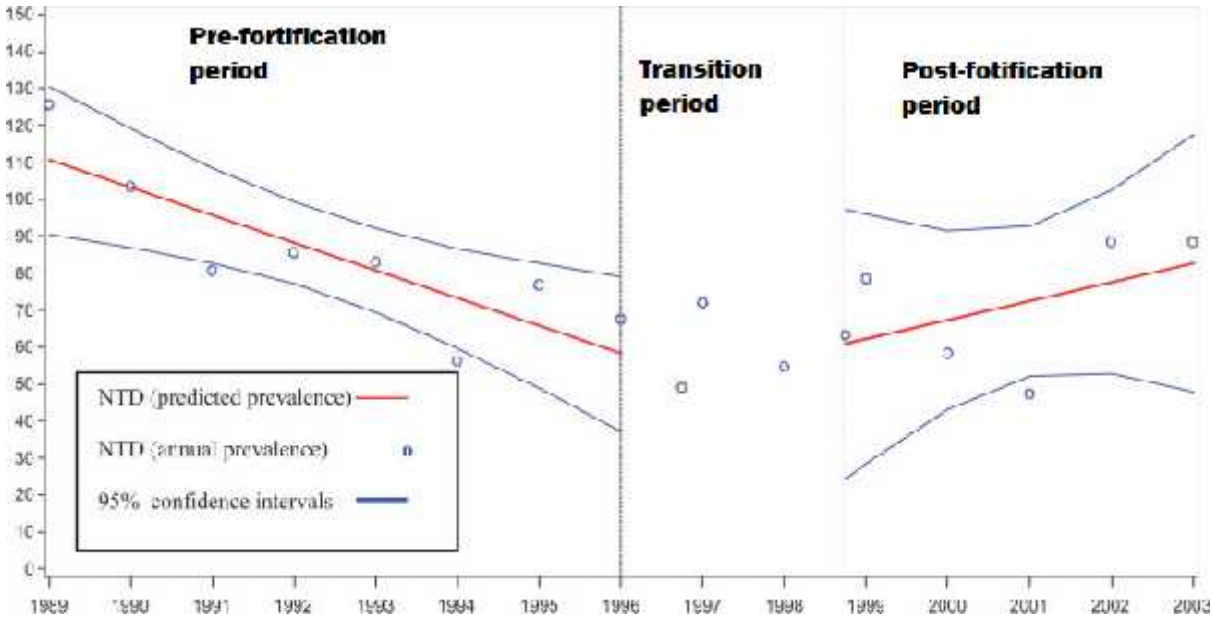
Specific risk factors for NTDs that have been identified include maternal diabetes mellitus (Becerra *et al*., 1990; Loeken, 2005), maternal obesity (Mc Mahon *et al*., 2013), maternal use of antiepileptic drugs such as valproic acid (Lammer *et al*., 1987), hyperthermia and hypervitaminosis A (Li *et al*., 2007), and parental occupation (Blatter *et al*., 1996; Shaw *et al.,* 2002). Lack of folic acid supplement, familial history of spina bifida, use of antiepileptic drugs and low birth weight ≤ 2500g were also associated with increased risk of spinal bifida (Kondo *et al.,* 2013). Maternal obesity before pregnancy with BMI ≥30 kg/m^2 was significantly associated with an approximately two fold increased risk of NTDs in offspring. The NTD-protective association of folic acid was stronger in overweight/ obese women BMI ≥ 25 kg/m^2 than in normal/underweight women BMI < 25 kg/m^2 (Mc Mahon *et al.,* 2013). Hence, maternal supplementation of folic acid is important during pregnancy especially for overweight women.

Simmons *et al*. (2004) stated that a statistically significant decrease in prevalence rate of spina bifida was observed when comparing the pre- and post-fortification of folic acid (from 7.8 to 4.4 per 10,000 live births in pre-fortification and post-fortification period, respectively). This finding is in agreement with the finding that folic acid fortification reduces the prevalence rate of spina bifida from 8.8 per 10,000 live births during pre-fortification period to 4.3 per 10,000 live births during post-fortification period (Berry *et al*., 1999; De Villarreall *et al*., 2002 and De Wals *et al*., 2007; 2008). Similar studies also suggested a significant decline in prevalence of spina bifida by 40% (Sayed *et al.,* 2008) and by 41.6% (Orioli *et al*., 2011) following folic acid fortification. The results mentioned above may be related with maternal supplementation with methyl donor precursor's folate leads to hypermethylation, which in turn facilitates proliferation of cells in the neural tube and surrounding mesenchyme (Wolff *et al.,* 1998). Therefore, food fortification with folic acid had a significant effect in prevention of spina bifida.

De Wals and his colleagues (2007) asserted that food fortification with folic acid reduces the prevalence of anencephaly by 38%. This finding is consistent with the finding of food fortification with folic acid reduces the prevalence of anencephaly by 32% (Bower *et al*., 2009). These results may be associated with methylation by folic acid which was crucial for



Period 1: Prenatal diagnoses rarely made in Atlanta.
Period 2: Prenatal diagnoses made but not ascertained in Atlanta.
Period 3: Prenatal diagnoses ascertained in Atlanta.


Pre-fortification period
Transition period
Post-fotification period
NTD (predicted prevalence)
NTD (annual prevalence)
95% confidence intervals

According to Chen *et al*. (2008b), periconceptional multivitamin supplementation containing folic acid during pregnancy reduces the prevalence rate of NTD from 1.80/1,000 pregnancies in those mothers who had not received periconceptional multivitamin supplementation to 0.35/1,000 pregnancies in those mothers who received periconceptional multivitamin supplementation containing folic acid during pregnancy. The protective rate which was 80.4% reached 87% when pregnant women started taking a multivitamin before pregnancy, ending two months after pregnancy and the frequency should be higher than five times per week (Chen *et al*., 2008b). This finding agreed with the previous finding that supplementation of multivitamins containing folic acid reduces the prevalence of NTDs by 93% (Czeizel and Dudas, 1992; Berry *et a*l., 1999). Therefore, supplementation of periconceptional multivitamin containing folic acid is more effective in preventing NTD cases compared to use of folic acid alone.

The lowest NTD incidence rate was 0.18/1,000 pregnancies for the women who complied fully with the multivitamin supplementation and who consumed more vegetables and fruits. Those women who did not take multivitamins and who consumed relatively few vegetables and fruits had the highest NTD rate 3.48/1,000 pregnancies. Those women who didn't take multivitamins but ate more vegetables and fruits had a relatively lower NTD rate, 1.35/1,000 pregnancies (Chen *et al*., 2008b). These indicate that intake of folic-acid-enriched food also improves prevention of NTD in offspring.

Czeizel *et al*. (1996); Mitchell *et al*. (2003) and Van Rooij *et al*. (2003) found a statistically significant protective association between the use of folic acid and the risk of oral clefts. Badovinac *et al*. (2007) also supported the hypothesis of a protective effect of folic acid containing supplement during pregnancy for oral clefts. This finding may be related to poor maternal nurture of folate which could be a possible cause of oral clefts, because some embryonic tissues of the face are derived from cephalic neural crest cells and folate plays an important role in neural tube formation (Wald and Sneddon, 1991). Therefore, maternal folic acid supplementation may reduce the risk of having a baby with oral cleft.

On the other hand, Simmons *et al*. (2004) found a statistically non-significant decrease in prevalence of cleft lip with and without cleft palate 11.4 per 10,000 live births during pre-fortification to 10.1 per 10,000 live births following folic acid fortification. Munger *et al*. (2004); Yazdy *et al*. (2007) and Sayed *et al. (*2008) also supported the hypothesis of no association between folate and cleft lip/palate. This result is inconsistent with the previous findings that a statistically significant protective association between the use of folic acid and

the risk for oral clefts (Czeizel *et al*., 1996); Mitchell *et al*., 2003) and Van Rooij *et al*., 2003). The failure to match may be due to maternal smoking (Yazdy *et al*., 2007), maternal use of anticonvulsants, such as phenobarbital and diphenylhydantoin and maternal age (Moore and Persuad, 2008; Sadler, 2012). Genetic disruption of folate intracellular transport in folate binding protein 1 also known as folate receptor 1 may also contribute to the above discrepancies (Gelineau-van Waes *et al*., 2008).

According to Yazdy *et al*. (2007), women who were reported smoking during pregnancy had a higher prevalence of infants with orofacial clefts than women who didn`t smoke cigarette during pregnancy both before fortification (11.9 versus 8.22 per 10,000 live births) and after fortification (12.53 versus 7.83 per 10,000 live births). Therefore, women should avoid cigarette smoking especially during pregnancy.

The Hungarian randomized control trial and cohort control trial failed to show a reduction in the birth prevalence of cleft palate and cleft lip with or without cleft palate following supplementation with a multivitamin preparation containing a low dose (0.8 mg) of folic acid (Czeizel, 2009). On the other hand, Badovinac *et al*. (2007) stated that, supplementation of multivitamin containing folic acid was effective in reducing oral cleft. The study by Johnson and Little (2008) also found no strong evidence pertaining the association between oral clefts and folic acid use, but multivitamins may protect against oral clefts. Thus, supplementation of multivitamin containing folic acid may protect against oral cleft than use of folic acid alone.

According to Reynolds *et al.* (2003), neither maternal deaths nor signs of clinical toxicity were observed with administration of folic acid to pregnant mice. Retinoic acid increased the frequency of CP occurrence from the normally observed spontaneous rate of 1–3% to 76%. With the addition of folic acid, the percentage of pups with CP decreased to a level of 6.3%. Likewise, methionine was active therapeutically, lessening CP occurrence to 5.7%. Pups that were given a concomitant administration of folic acid and methionine had no CP (Reynolds *et al.,* 2003). This result may be associated with the fact that methionine facilitates the neural folds to turn medially and methylation by folic acid was crucial for proper closure of the neural tube (Afman *et al*., 2005 and Coelho and Klein, 1990). This result suggests concomitant administration of maternal folic acid and methionine had a significant effect in preventing retinoic acid induced cleft palate than use of folic alone.

Administration of maternal retinoic acid resulted in micromelia at a frequency of 80.0%. This defect was reduced by administration of folic acid to 27.6%, methionine to 20.4%, and folic

acid + methionine to 9.5%. Retinoic acid administration also increased the incidence of under ossified cranial bones, truncated tails, and skeletal defects to 28.7%. This defect was also reduced by administration of folic acid to 11.8%, methionine to 6%, and folic acid + methionine to 2.7%. However, a 25% decrease in dosage therapy yielded a reduction in prevention of retinoic acid induced CP, micromelia, under ossified cranial bones, truncated tails, and skeletal defects (Reynolds *et al.,* 2003). This implies that the preventive effect of folic acid and methionine in retinoic induced cleft palate may depend on the dose administered for pregnant mothers.

According to Naitoh *et al.* (1998), methionine and folic acid (in the form of tetrahydrofolate) interact through the folate and amino acid metabolism pathways. Methionine serves as methyl group (CH3) donor, whereas folic acid as methyl transfer cofactor. With folic acid acting as a methyl group cofactor, folic acid is degraded for the synthesis of various compounds such as cysteine, whereas homocysteine is produced as an intermediate. The folic acid cofactor donates its methyl group to methionine synthase, which then recycles homocysteine back to methionine before toxic levels are reached. The nutrients used in methylating the proteinaceous retinoic acid receptors, thus prevent the active teratogen from binding and elicit developmental alterations in epithelial cells or mesenchyme.

Because such methylating reactions are pivotal to DNA synthesis and subsequent expression, it is possible that the therapeutic substances act on various signaling pathways involved with normal palatal closure. With folic acid acting as a cofactor and methionine as a methyl group donor, the mechanistic pathway may also involve the release of increased tetrahydrofolate, the active form of folic acid in biological reactions. This tetrahydrofolate release counteracts hyperhomocysteinemia, which has been shown to cause orofacial defects (Wong *et al.,* 1999).

Gefrides *et al*. (2002) asserted that treating wild-type dams with folic acid did not increase the incidence of embryo/fetal death or induce any NTDs. In addition, when these vitamin supplements were administered to *Splotch* heterozygous litters, neither the embryo/fetal death nor the incidence of spontaneous NTDs was significantly reduced. This result may be related with defect in folate metabolism of *Splotch* mice because of genetic background (Copp *et al*., 2003). It has also been suggested that *Splotch* homozygous mouse embryos have a metabolic deficiency (i.e methylenetetrahydrofolate reductase deficiency) in the supply of folate for pyrimidine biosynthesis (Fleming and Copp, 1998). However, folic acid supplementation significantly reduced the incidence of arsenic-induced NTDs in the wild-type litters compared to the group that received arsenic alone. This may be because methylation pathway

detoxifies arsenic and therefore, folate levels in the embryo may be a likely factor that may influence the detoxification of this metal (Gefrides *et al*., 2002).

Burren and his colleagues (2010) stated that under folate deficient conditions, cranial NTDs (exencephaly) were seen at embryonic day 11.5 among wild type mice embryos whereas this strain has never been found to develop NTDs under normal dietary condition. Curly tail mutant embryos displayed a low frequency (11%) of cranial NTDs under normal dietary conditions. However, folate deficiency caused a dramatic increase in the frequency of cranial NTDs in curly tail embryos to more than 50%. This result may be related to the genetic background of curly tail mutant embryo high sensitivity to folic acid deficiency. It was also hypothesized that cranial NTDs in folate-deficient conditions occur as a result of reduced proliferation associated with growth retardation (Burren *et al*., 2008). Maternal folic acid supplementation reduces the frequency of cranial NTDs in curly tail mutants, but it was statistically non-significant (Burren *et al*. (2010). Although statistically non-significant, the result indicates the importance of maternal folic acid supplementation in prevention of NTDs.

On the other hand, Tran *et al*. (2002) suggests that a low-folate diet had no effect on NTD frequency in curly tail mutant embryos. However, the diets used in the study contained minimal levels of folic acid (0.3 mg/kg) and no antibiotics administered, suggesting that residual folate produced by intestinal bacteria may be available. The lack of folic acid effect on NTD incidence thus correlates with the finding that availability of even low levels of dietary folate appears sufficient to enable neural tube closure and dramatically enhance embryonic folate content (Burren *et al*., 2008; 2010).

According to Burren *et al. (*2010), folate deficiency also had a deleterious effect on reproductive success of wild type and curly tail mice pregnancy as well as a significant reduction in the number of implantations per litter and increases the number of resorptions**.** Among litters collected at embryonic day 13.5 (n = 5 wild type and 6 curly tail), very few viable embryos were observed whereas the resorption rate was very high (5.5 ± 0.6 and 5.8 ± 1.3 per litter, respectively). This finding may be because of folate was important for remethylation of homocysteine, which has a toxic effect on the developing embryo. In folate deficient condition, the concentration of homocysteine in blood increases and brings toxic effect to the embryo (Blom *et al.,* 2009). This result suggests the importance of folic acid for implantation embryo in addition to prevention of NTDs.

According to Bedard *et al.* (2013), the prevalence rates for tetralogy of Fallot was equal in both the pre-FAF and post-FAF periods as was the prevalence of d-transposition of great arteries. This result agreed with the previous finding that the prevalence rates of tetralogy of Fallot and d- transposition of the great arteries remain the same in the pre-FAF and post-FAF periods (Simmons *et al*., 2004). The prevalence of left ventricular outflow tract obstruction was significantly lower in the post-FAF period with coarctation of the aorta substantially contributing to this reduction. The prevalence of septal defects (ASD and VSD) as a group increased in the post-FAF period. The prevalence rate for CHDs overall was 9.34 per 1,000 births pre-FAF and 9.41 per 1,000 births post-FAF period (Bedard *et al*., 2013). Hence, food fortification with folic acid may reduce the prevalence of CHDs especially left ventricular outflow tract obstruction and coarctation of the aorta.

Botto *et al. (*2000) and Czeizel (2009) demonstrated that folic acid containing multivitamin supplementation prevent cardiovascular congenital anomalies, mainly conotruncal defects e.g., common truncus, transposition of the great vessels, tetralogy of Fallot and certain types of VSD. They suggested that periconceptional multivitamin supplementation was associated with an approximately 40% reduction in risk for cardiovascular congenital anomalies (Botto *et al.*, 2000; Czeizel, 2009). Another study also showed that periconceptional multivitamin supplementation protects against some congenital anomalies of the cardiovascular system, principally VSD, and obstructive congenital anomalies of the urinary tract, particularly stenosis of the pelvicoureteric junction (Czeizel, 2009). Thus, multivitamin containing folic acid reduces the concentrations of homocysteine in the adult population and the reductions in homocysteine prevents 10% of heart attacks and strokes (Jacques *et al*., 1999). Hence, use of multivitamin containing folic acid is a beneficial in preventing CHDs than use of folic acid alone.

According to Meijer *et al*. (2006), maternal folic acid use was not associated with any of the four cardiac subgroups; conotruncal defects, VSD, ostium secundum type ASD and endocardial cushion defects among individual with Down syndrome. On the other hand, Bean *et al*. (2011) asserted that, lack of maternal folic acid supplementation was associated with an approximately 1.7-fold increased frequency of atrial ventricular septal defects and of ASD II, but not VSD among individual with Down syndrome. This discrepancy may probably because of Meijer *et al*. (2006) included primarily white mothers, whereas Bean *et al*. (2011) included a more racially and ethnically diverse population. In addition, the ascertainment period was not the same; Meijer *et al*. (2006) identified probands prior to the 1998 mandate

for dietary folic acid fortification (1978–1997), whereas Bean *et al*. (2011) sample was ascertained after mandatory folic acid fortification (2001–2004). Therefore, variability of folic acid exposure and racial or ethnic differences might have contributed to these conflicting findings.

According to Gelineau-van Waes *et al*. (2008), high doses of daily maternal folate supplementation of greater than 50 mg/kg/day during embryonic/fetal development are necessary for early postimplantation embryonic viability of RFC1 nullizygous embryos, and play a critical role in chorioallantoic fusion, erythropoiesis, and proper development of the neural tube, limbs, lungs, heart, and skin. Supplementation of pregnant dams with low dose of folic acid (25 mg/kg/day) prolonged survival of 27/49 (55%) RFC1 nullizygous embryos until embryonic days of 9.5–10.5. Nevertheless, the surviving RFC1 mutant embryos died during midgestation due to a failure of chorioallantoic fusion, and displayed a marked absence of erythropoiesis. Without maternal folate supplementation, all RFC1 nullizygous embryos died shortly after implantation (embryonic day 6.5), and were subsequently resorbed (Gelineau-van Waes *et al.,* 2008). This result agreed with the finding that folate deficiency had a deleterious effect on reproductive success as well as a significant reduction in the number of implantations per litter and increase the number of resorption (Burren *et al.,* 2010). Hence, maternal supplementation of folic acid does not only prevent NTDs, but it also prevents embryonic loss.

According to Gelineau-van Waes *et al. (*2008), adequate uptake of folate and nutrients by the yolk sac visceral endoderm was also necessary for proper development of the adjacent yolk sac mesenchyme, formation of the allantois, and initiation of primitive erythropoiesis. It also plays a critical role in the survival, proliferation, and differentiation of mesodermal progenitors involved in placentation, erythropoiesis, gastrulation, neural tube closure, fetal heart development and organogenesis. RFC1 nullizygous embryos on low dose maternal folate supplementation fail to initiate primitive erythropoiesis and die during midgestation due to failure of chorioallantoic fusion (Gelineau-van Waes *et al.,* 2008).

According to Pickell *et al*. (2009), methylenetetrahydrofolate reductase and folate deficiencies resulted in increased developmental delays, increased embryonic losses and smaller embryos. This result is related with the previous study of high doses of daily maternal folate supplementation during embryonic/fetal development are necessary for early postimplantation embryonic viability and low dose maternal folate supplementation fail to initiate primitive erythropoiesis and die during midgestation due to failure of chorioallantoic

fusion (Gelineau-van Waes et al., 2008). Pickell *et al*. (2009) also asserted that both maternal methylenetetrahydrofolate reductase and folate deficiencies were significantly associated with decreased numbers of somite pairs, and decreased crown-rump lengths and weights. This result is positively correlated with the previous findings that folate deficiency resulted in a significant reduction in crown-rump length and number of somites in both wild type and curly tail strains (Burren *et al*., 2010).

Folate-deficient mice also had significant decrease in placental weight and total placental area due to folic acid deficient diet as well as severe placental defects, including placental abruption and disturbed patterning of placental layers. The result also shows a variety of embryonic defects in both maternal methylenetetrahydrofolate reductase and folate deficient groups, such as neural tube, heart looping, and turning defects (Pickell *et al.,* 2009). These results may be associated with the fact that folate appears to play a critical role in the survival, proliferation, and differentiation of mesodermal progenitors involved in placentation and its deficient condition leads to developmental defect of the placenta (Gelineau-van Waes *et al*., 2008). Thus, folic acid supplementation is important in the formation of placenta as well as early heart development.

On the other hand, Mikael *et al*. (2013) suggest that provision of folic acid supplemented diet of 20 mg/kg/day was associated with a higher incidence of embryonic loss and increased the number of delayed embryos. This finding is inconsistent with the finding that 50mg/kg/day maternal folate supplementation during embryonic/fetal development necessary for early postimplantation embryonic viability (Gelineau-van Waes *et al*., 2008). The above discrepancies may be because of Mikael *et al*. (2013) included all pregnant mice with or without methylenetetrahydrofolate reductase deficiency, but Gelineau-van Waes *et al*. (2008) used knockout mouse, which have methylenetetrahydrofolate reductase enzyme for homocysteine methylation. The methylenetetrahydrofolate reductase enzyme also has a unique function that regulates the availability of methyl groups for methylation reactions, which plays a critical role in fetal heart development (Gelineau-van Waes *et al*., 2008). Mouse with methylenetetrahydrofolate reductase deficiency had metabolic defect in the supply of folate for homocysteine methylation and increase blood homocysteine, which have toxic effect on developing embryo (Naitoh *et al.,* 1998). Therefore, embryonic loss and increased number of delayed embryos suggested by Mikael *et al*. (2013) were because the differences in genetic background of the animals used were for experimentation rather than for folic acid supplementation.

5. Conclusion

Folic acid is generally not toxic for pregnant women. No acute and long-term adverse effects of folic acid during pregnancy were detected at the birth of their newborn infants as well as on the health status of mothers. Maternal folic acid supplementation or food fortification with folic acid had no relationship with the rates of multiple births while an increment in multiple births was caused by the use of ovulation-inducing drugs and antiretroviral therapy.

Maternal folic acid supplementation has a protective effect for neural tube defect especially spina bifida and anencephaly in both human and animal models. This may be because of the fact that folic acid facilitates the closure of neural tube. The NTDs protective association of folic acid was stronger in overweight/obese women BMI $\geq$ 25 kg/m^2 than in normal/underweight women BMI < 25 kg/m^2.

For orofacial cleft; cleft lip/palate, there is conflicting evidence of the effect of folic acid fortification on human models. However, maternal folic acid supplementation has importance in reducing orofacial cleft; cleft lip/palate in experiment tested on animal models. Women who reported smoking during pregnancy had a higher prevalence of infants with orofacial clefts than did women who did not report smoking during pregnancy before as well as after folic acid fortification.

Maternal supplementation of multivitamin containing folic acid had a significant effect in preventing NTDs if it starts two months before conception and continues until the completion of the second month of pregnancy, and the frequency was higher than five times per week. The lowest NTD incidence was also registered in women who consumed more vegetables and fruits. These indicates that intake of folic acid enriched food may improve prevention of NTD in the offspring.

When a multivitamin containing folic acid was used, the reduction of NTD cases was high compared to high dose of folic acid used alone. There are also evidences that showed the protective effect of maternal supplementation of folic acid against NTDs, if provided 0.4 mg/day to 0.8mg/day (for recurrence cases it should increased to 4.0 mg/day) starting at least one months before conception and continued to the end of the 1st trimester of pregnancy. Concomitant administration of maternal folic acid and methionine had a significant effect in preventing retinoic acid induced cleft palate than use of folic alone.

Maternal folic acid supplementation had a protective effect for coarctation of aorta and left ventricular outflow tract obstruction, but no association was found for tetralogy of Fallot (except VSD) and d-transposition of the great arteries. For ventricular septal defect, ostium secundum type atrial septal defect and endocardial cushion defects, there is conflicting evidence of an effect of folic acid fortification on human models. Supplementation of periconceptional multivitamin containing folic acid may have more protective effect against CHDs than use of folic acid alone.

High doses of daily maternal folate supplementation (50 mg/kg/day) during embryonic/fetal development are necessary for early post implantation embryonic viability, chorioallantoic fusion, hematopoiesis and the development of neural tube, limbs, lungs, heart, and skin. Folate metabolic pathway is crucial for implantation and many aspects of embryonic development, and severe impairment of the pathway is incompatible with survival beyond early embryogenesis. Folate deficiency can impair implantation and early embryogenesis. Folic acid deficiency also leads to severe placental defects, including placental abruption and disturbed patterning of placental layers in experiment tested in animal models.

6. Recommendation

In this review of the effects of maternal folic acid supplementation on the development of neural tube and cardiovascular system of the offsprings in human and animal models, the following important concerns are recommended.

Despite the protective effect of folic acid in NTDs by facilitating the neural tube closure, additional investigation is required to understand the exact mechanism of intracellular utilization of folic acid on the neural tube.

Maternal folic acid supplementation has a conflicting evidence in protection of orofacial cleft; cleft lip/palate especially in human models; so that, more research is required to understand the association between folic acid and orofacial cleft; cleft lip/palate.

Because of maternal obesity before pregnancy BMI ≥30 kg/m^2 was significantly associated with an approximately two fold increased risk of NTDs in offspring and the NTDs protective association folic acid was stronger in overweight/ obese women BMI ≥ 25 kg/m^2 than in normal/underweight women BMI < 25 kg/m^2, strong attention should be given to overweight (obese) women during prenatal care.

When a multivitamin containing folic acid is used, the reduction of cases of NTDs and CHDs were high compared to a high dose of folic acid alone. Therefore, it is better if the health care providers give multivitamin containing folic acid for pregnant mothers starting from two months before conception and continue until completion of the second month of pregnancy at frequency of higher than five times per week.

The lowest incidence of NTDs were observed among women who were consumed more vegetables and fruits. Therefore, a woman should be encouraged to eat more fruit and vegetable than the usual intake of fruit and vegetable during pregnancy.

Maternal folic acid supplementation has conflicting evidence in protection of congenital heart defect; therefore, more investigation is required to understand the association between folic acid and congenital heart defects.

In Africa including Ethiopia, literature on effects of maternal folic acid supplementation on the development of neural tube and cardiovascular system of the offspring is limited. Therefore, additional investigation is required because ethnicity and environmental factor may have impact in folate metabolism.

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