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Effect of folic acid on animal models, cell cultures, and human oral clefts: a literature review

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Abstract

Background: Folate is a naturally occurring, water-soluble B vitamin. The synthetic form of this compound is folic acid (FA), the deficiency of which is linked to neural tube disorders (NTD), which can be prevented by consuming it before, or during the early months of, pregnancy. However, the effect of FA on oral cleft formation remains controversial. The aim of the present study was to review the evidence concerning the effect of FA on the formation of cleft lip and palate (CLP) in both animals and humans, as well as its impact on different cell types. A search was conducted on various databases, including MEDLINE, EMBASE, and Central, for articles published until January 2020.

Main body: Current systematic reviews indicate that FA, alone or in combination with other vitamins, prevents NTD; however, there is no consensus on whether its consumption can prevent CLP formation. Conversely, the protective effect of FA on palatal cleft (CP) induction has been inferred from animal models; additionally, in vitro studies enumerate a cell-type and dose-dependent effect of FA on cell viability, proliferation, and differentiation, hence bolstering evidence from epidemiological studies.

Conclusions: Meta-analysis, animal models, and in vitro studies demonstrated the protective effect of FA against isolated CP; however, the heterogeneity of treatment protocols, doses, and FA administration method, as well as the different cell types used in in vitro studies, does not conclusively establish whether FA prevents CLP formation.

Keywords: Folic acid, Vitamin B complex, Cleft palate, Cleft lip, Craniofacial abnormalities, Cell proliferation

Background

Folate is a water-soluble sub-type of vitamin B, present in legumes and vegetables. It is an essential nutrient that cannot be synthesized by humans and therefore must be incorporated through the diet. Folic acid (FA) is the synthetically manufactured form of folate. It is incorporated into supplements and into fortified foods such as cold cereals, bakery products such as cookies and bread, and pastas. Folates are a group of compounds with a structure and biological properties similar to those of FA; they are involved in fundamental processes such as protein and deoxyribonucleic acid (DNA) synthesis [1].

The consumption of multivitamin or FA supplements, before pregnancy and during its early months, aids in preventing neural tube disorders (NTD). Although the exact mechanism by which FA facilitates the closure of the neural tube is not entirely known, it is estimated that up to 70% of the NTD can be prevented by supplements containing FA [1].

Systematic reviews based on clinical studies have elucidated a protective effect of FA on cleft lip and palate (CLP) formation [2–4] which is the most common congenital craniofacial malformation and the fourth most prevalent congenital anomaly [5]. However, other studies consider the use of multivitamins, but not isolated FA, as the primary factor responsible for preventing the

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formation of CLP [6]; hence, there is currently no substantive consensus on this. Animal study models and certain in vitro studies have also pointed to a protective effect exerted by FA against the cleft palate (CP) [7–9].

CLP occurs due to the lack of the fusion between palatal processes, the maxillary process, and the middle nasal process [10]. Its etiopathology is complex, with three possible groups of associated etiological factors described: environmental, teratogenic, and genetic. At present, the interaction between various epigenetic factors and its influence on the expression of different genes is considered the main etiological factor [11].

The aim of the present review was to assess the evidence regarding the effect of FA on the formation of CLP in both animals and humans, as well as its influence on different cell types.

Methods

An electronic and manual search of published articles up to January 2020 was performed in the MEDLINE, EMBASE, and Central databases, employing the following search strategy: "folic acid AND cleft lip AND cleft palate AND in vivo studies AND in vitro studies." Articles were selected according to title and abstract information. A full-text evaluation of the selected articles was conducted; relevant information in alignment with the study objective was included in the present review. The obtained results are shown in tables divided into three thematic fields: effect of FA on CLP in humans, effect of FA and orofacial clefts in animals, and FA and its effect on cell cultures.

Effect of folic acid on CLP formation in humans

Multivitamin or folate supplements taken prior to/during the first 2 months of pregnancy helps to prevent NTD. Wilcox et al. found that the consumption of FA supplements early during pregnancy (400 μg/day) was associated with a one-third reduction in the risk of suffering isolated cleft lip (CL) with/without CP. They also determined that, regardless of supplements, diets rich in fruits, vegetables, and other high-folate foods alleviated the risk. The lowest incidence of CL was found among women who consumed folate-rich diets and took FA and multivitamin supplements. However, FA did not provide any protection against isolated CP in that study [12].

In a case–control study conducted by Shaw et al., it was found that women who took periconceptional multivitamins containing FA manifested 25% ± 50% reduction in the risk of giving birth to children with orofacial clefts [13]. Tolarova and Harris, in a prospective intervention study, found that the consumption of 10 mg of FA daily decreased the recurrence of CP by 65.4% in families with a first-degree relative with a CLP and by 82.6% in families with a previous child with a unilateral cleft. However, no risk reduction was observed in families with a history of a child with a bilateral cleft [14]. Conversely, Czeizel et al. concluded that consuming FA supplements during the critical period (8–14 gestational weeks) of the lip and palate formation can prevent isolated clefts in these structures, but not CLP, following the intake of a dose lower than 1 mg of FA [15]. Prior studies have reported the incidence of oral clefts in the offspring of women who ingested folate antagonists during pregnancy, such as methotrexate, aminopterin (4-pteroylglutamic acid), and anticonvulsants [13, 16].

Although the protective effect of folate against CL and CLP is recognized, the mechanism due to which certain people possess intrinsically low levels of folate, presumably predisposing their offspring to NTD and other possible congenital pathologies, remains a contentious subject. Certain studies focused on folate pathway enzymes, such as 5, 10-methylenetetrahydrofolate reductase (MTHFR), which converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. These studies demonstrated the association between NTD and the MTHFR C/T mutation (C677T), which induces an alanine to valine substitution in the enzyme. The C677T variant produces a thermolabile enzyme with a reduced activity related to elevated plasma homocysteine (HCY) levels and decreased plasma folate. The authors proposed that homozygosity for the T or C allele of the C677T polymorphism in women is an essential susceptibility factor for the development of CLP [17, 18].

In the meta-analysis conducted by Goh et al., it was reported that the use of FA-fortified multivitamins by women before conception and during the first trimester of pregnancy is associated with a reduced incidence of several malformations such as NTD, cardiovascular abnormalities, oral clefts, urinary tract abnormalities, congenital hydrocephalus, and limb defects. However, randomized trials are necessary to determine which specific vitamins produce these protective effects [17, 19]. Wilcox et al. found that the consumption of 400 μg/day of FA supplements early during pregnancy results in a one-third reduction in the risk of isolated CL with/without CP. The consumption of FA-rich supplements and diet correlated with the lowest incidence of CL. However, no protection against isolated CP was observed in that study [12].

Although the abovementioned studies underscore the protective effects of supplements containing FA over the risks of orofacial clefts, especially for CLP, they are impeded by data and design limitations. This is particularly because they lack treatment randomization and control groups, as well as the use of interventions that combine FA with other supplements, thus obscuring the effects produced by FA-only use. The non-random assignment introduces self-selection biases in the treatment, which could potentially confound the results obtained from the study and introduce differences between the treated and untreated groups, related to the effect of the treatment.

Most previous intervention studies also employed a small sample size and were marked by statistical power limitations.

Other studies do not seem to support the protective effect of preconception FA against orofacial clefts, either in the form of tablets or fortified cereal grains [20]. Additionally, a randomized controlled clinical trial that evaluated FA supplements found no evidence of a protective effect in cases of CL, with/without CP, associated with the consumption of vitamin supplement s[21]. Hayes et al., in a case–control study, determined that FA supplements do not impact the risk of oral fissures. Their results are attributed to biological mechanisms related to the formation of the lip and palate. During neural tube closure, neural crest cells migrate from the ectoderm along the lateral margins of the neural plate. These cells experience both caudal and cephalic extensive migration. Development of the face and palate takes place over a period longer than that for neural tube development; furthermore, other physical and developmental factors are involved. Therefore, the role of neural crest cells is only one of a multitude of factors responsible for the development of oral tissues. Even though the neural crest is related to NTD and oral clefts, specific cell types, migratory mechanisms, and differentiation pathways may exhibit different susceptibilities to FA. That study did not demonstrate a protective association between the use of these vitamin supplements during the periconceptional period and the risk of occurrence of oral clefts. However, its findings do not downplay the importance of current recommendations that women of childbearing age should consume a daily supplement of FA, since its protective effect against NTD has been consistently demonstrated [22].

According to previous studies, the administration of FA supplements during the periconceptional period is well documented, resulting in a substantial reduction in the occurrence of NTD. However, whether the specific use of FA modifies the risk of other congenital anomalies, such as CLP, is inconclusive.

The available scientific evidence has been analyzed via five systematic reviews conducted between 2000 and 2019. One of these reviews concluded that there is no firm evidence of an association between the presence of oral clefts and FA-only intake; however, the use of multivitamins early in pregnancy can protect against the formation of oral clefts, specifically CLP [6]. De-Regil et al. concluded in a review that FA, alone or in combination with vitamins and minerals, prevents NTD but does not produce an apparent effect on other congenital disabilities [23]. In contrast, the most recent reviews of Blanco et al. [2], Millacura et al. [3], and Jahanbin et al. [4] indicate that FA exerts a beneficial effect on the prevention of non-syndromic CLP (Table 1).

Folic acid and orofacial cleft formation in animals

The animal model studies analyzed demonstrated the protective effect of FA against CP [7, 8, 24–28] (Table 2). The experimental design utilized varies among studies: in two of the studies, FA supplementation was enabled via feeding of female mice prior to mating [7, 29]. In certain other studies, FA was administered through injections, either intraperitoneally [8], subcutaneously during pregnancy [24], or intravenously [30]. In a few more studies, cultured ex vivo mouse embryos were treated with FA and retinoic acid [25, 26].

The FA doses employed ranged from a minimum dose of 0.09 μM/kg of the weight in FA-deficient diets [7] to 100 μM/kg of the weight [30]. Several prior studies analyzed the effect of FA in comparison with the effects generated by other substances: (a) procarbazine, which retards fetal development and delays ossification of the fetal rat skeleton, as evidenced by fetal weight reduction, extremity malformations, and CP development [24]; (b) succinyl sulfathiazole, an antibiotic that diminishes the gut microflora that synthesizes FA and results in exencephaly, developmental delay, and an 80% open secondary palate [7]; (c) all-trans retinoic acid (ATRA) as a teratogenic inducer of CP [8, 25]; and (d) methionine (Met) because of its apparent positive effect in neural tube closure [8] (Table 2).

FA reduces the incidence of CP between 69.7% and 100% in comparison with the number of cases produced by procarbazine [24], succinyl sulfathiazole [7], and ATRA [8, 25], thus illustrating the beneficial effects of FA. Additionally, concomitant therapy with FA and Met completely eliminated the prevalence of ATRA-induced CP [8]. FA treatment prior to 13-cis-retinoic acid application in rat embryos significantly alleviated cellular damage in the midfacial process, thus revealing the beneficial effects produced by FA on this tissue [26].

Folic acid and its effect on cell cultures

It has been established that FA is necessary for cell division, specifically under conditions in which cells undergo rapid proliferation; furthermore, it contributes to the methylation status of essential cell molecules such as DNA, ribonucleic acid (RNA), and proteins [31]. Certain cell types are particularly sensitive to folate, and its deficiency results in evident changes. Specifically, embryonic cells of the neural crest demonstrate an elevated degree of expression of folate receptors, thus indicating a heightened demand for it [32, 33].

Seven studies have analyzed the effect of a wide range of FA concentrations on bone tissue formation in vitro in different cell types: bone marrow mesenchymal cells [9], mouse palate [34], cells of the mouse embryonic neural crest [33], osteoblasts [35], osteoclasts [36], human

Table 1 Systematic reviews that evaluated the effect of FA on CLP formation in humans

Author, year of publication	Study population	Study conclusion	Authors' judgment
Johnson and Little, 2008 [6]	8.303 cases 119.468 controls	There is no strong evidence of the association between oral clefts and FA intake alone. However, the use of multivitamins early in pregnancy can protect against the formation of oral clefts, specifically CLP.	Most of the investigations included were case–control studies and their quality was not formally assessed, probably affecting the results of the present systematic review.
De-Regil et al. 2015 [1]	2033 cases 5.359 controls	FA, alone or in combination with vitamins and minerals, prevents NTD; however, its effect on other birth defects is not as evident.	Inferior quality of studies conducted on CL and CP may have influenced the results.
Blanco et al. 2017 [2]	928 cases 2.390 controls	High level of HCY in the maternal plasma is a risk factor for non-syndromic orofacial clefts in children.	Blanco et al. performed additional analyzes to resolve heterogeneity and small size in study samples. Results of the meta-analysis help in explaining the complex etiology of orofacial clefts.
Millacura et al. 2017 [3]	61.355 cases 59.586.433 controls	Fortification with FA produces a beneficial effect on non-syndromic CLP.	This multi-ethnic meta-analysis provides robust evidence of the beneficial effect of FA on CLP.
Jahanbin et al. 2018 [4]	27.045 cases 1.150.186 controls	FA supplements during the early stages of pregnancy mitigate the risk of non-syndromic CLP and CP in children.	Results are statistically robust based on a large sample size and thus eliminated heterogeneity.

FA folic acid, *CLP* cleft lip palate, *NTD* neural tube defects, *CL* cleft lip, *CP* cleft palate, *HCY* homocysteine

Table 2 Animal models studies that evaluated the effect of FA on CLP formation

Author, year of publication	Sample	FA dose/time of treatment	Conclusion	Authors' judgment
Paros and Beck, 1999 [27]	Pregnant mice: 19 experimental 9 controls	12 mg/24 h, during gestation days 8, 5–9, 5	FA administered continuously during the critical period of development, reduces CLP frequency.	This study shows the association between folates and the prevention of CLP.
Bienengraber et al. 2001 [24]	Pregnant rats: 11 experimental 3 controls	9.06 μM from day 14–17 post-conception, depending on the assigned group	Percentage of complete CP was significantly lower in the FA group (4 %) compared to the percentage observed in the non-folate group (53 %).	Indicates a protective effect of FA against CP.
Burgoon et al. 2002 [7]	Female mice: 21 experimental 39 controls	0.09–4.53 μM/kg diet, 4 weeks before mating	80% of the mice fed with FA-deficient diet had CP, compared with 37% of those that consumed a diet with 4.53 μM/kg.	Shows evidence of the protective effect of FA against CP.
Reynolds et al. 2003 [8]	Female mice: 30–36 experimental 10–12 control	9.06–6.79 μM/kg of body weight, on gestation day 8–11 depending on the assigned group	FA decreases the incidence of CP to 6.3%, while Met reduces the incidence to 5.7%; the FA + Met group did not present CP.	FA prevents the formation of CP.
Yao et al. 2011 [25]	Female adult mice explanted palates: 32 experimental 8 control	100 μM for 72 h	FA allows the development and fusion of the palate inhibited by retinoic acid.	FA inhibits CP formation induced by retinoic acid.
Scheller et al. 2013 [28]	150 female mice Experimental group: 761 fetuses	No FA applied. FA concentration was determined in the blood and amniotic fluid of the dams	The reduced availability of certain subgroups of vitamin B (B_1, B_5, and FA) in the amniotic fluid and serum was related to an increase in the appearance of clefts in A/WySn mice.	This finding confirms that FA is essential in preventing clefts.
Kriangkrai et al. 2017 [26]	Rat explanted embryos	100 μM for 18 h	FA decreases the teratogenic effects of 13-cis-retinoic acid in tissues of the middle facial process.	Pretreatment with FA exhibits beneficial effects on CLP prevention.

FA folic acid, *CLP* cleft lip palate, *CL* cleft lip, *HCY* homocysteine, *Met* methionine

Table 3 In vitro studies evaluating the effect of FA on bone tissue formation

Author	Cell type	FA dose	Administration method	Conclusion	Authors' judgement
Xiao et al. 2006 [34]	Mouse embryonic palate MSC	1–100 μg/mL	Directly applied to the cell-culture medium	Cell proliferation is FA dose-dependent.	FA promotes cell differentiation and could help prevent CLP.
Herrmann et al. 2007 [35]	Human osteoblasts	0–1000 μg/L	Directly applied to the cell-culture medium	Accumulation of HCY by decreasing concentrations of folate, vitamin B_{12}, and B_6 does not impact the activity of human osteoblasts.	Could not explain the effect of FA on the formation of CLP.
Herrmann et al. 2007 [36]	Osteoclasts obtained from peripheral blood-MSC	0–15 μg/L	Directly applied to the cell-culture medium	Cell cultures with low concentrations of FA, B_{12}, and B_6 demonstrate a significant increase in osteoclastic activity. Simultaneous deficiency of the three vitamins stimulates dentinal resorption and osteoclastic activity.	FA could help prevent the formation of CLP.
Kobus et al. 2009 [38]	Fertile eggs of *Gallus domesticus*	0.5 μg/0.25 μL	Injection into the yolk sac	High concentrations of FA alter neural cell differentiation in vitro.	Does not help in explaining the effect of FA on the formation of CLP.
Ahmed et al. 2016 [37]	HTR-8/SVneo cells BeWo cells Human placenta tissue explants	2–2000 ng/mL	Directly applied to the cell-culture medium	Treatment with excess FA (2000 ng/mL) resulted in reduced cellular viability in the villous trophoblast BeWo cell line and increased rates of proliferation in the HT8-8/SVneo extravillous trophoblast cell line. Deficient concentrations of FA (2 ng/mL) resulted in decreased cell viability and invasive capabilities of the HTR-8/ SVneo extravillous trophoblast cell line.	Supports the benefits of FA, which are not linked to CLP.
Melo et al. 2016 [33]	Neural crest cells of mouse embryos	0.45–90 μM	Directly on cell cultures.	High HCY concentration alters the morphogenesis of neural cells in mammals. The protective effect of folate is due to its ability to prevent HCY-induced teratogenesis.	FA could prevent HCY-induced CLP.
Santos et al. 2017 [9]	Bone marrow MSC	1–500 μg/mL	Hydroxyapatite particles loaded with FA.	Hydroxyapatite with FA particles can enhance the effect of hydroxyapatite on osteoblast differentiation.	FA promotes cell differentiation and could help prevent CLP.

MSC mesenchymal stem cells, *FA* folic acid, *CLP* cleft lip palate, *HCY* homocysteine

placenta cell lines [37], and directly on chicken embryos [38] (Table 3).

Most studies supplement vitamins in cell cultures through the culture medium; however, Santos et al. exposed cell cultures to hydroxyapatite nanoparticles loaded with FA [9]. In contrast, Kobus et al. injected substances into the yolk sac of chicken embryos [38].

The results of five of the analyzed studies reinforced the effect of FA on the proliferation, viability, and differentiation of various cell types [9, 33, 34, 36, 37]. However, Herrmann et al. found that HCY accumulation caused by low folate concentrations does not affect the activity of primary human osteoblasts [35]. Additionally, Melo et al. and Kobus et al. concluded that high concentrations of FA alter neural cell differentiation in vitro, which explains the mechanism of action of FA on NTD but does not explain the effect of FA on the formation of CLP [33, 38] (Table 3).

FA enhanced the proliferation of murine embryonic palatal mesenchymal cells restricted by the *MTHFR* gene silencing [34]. On the contrary, low FA concentrations augment human osteoclast activity. Also, combined deficiency of FA and vitamins B_{12} and B_6 and increased concentrations of HCY stimulate dentine resorption, tartrate-resistant acid phosphatase, and cathepsin activity of cultured osteoclasts causing acidification and proteolytic digestion, leading to bone matrix resorption [36]. Differences observed between studies related to HCY-induced teratogenic effects are supported by the idea that its effects are time and cell type-dependent [33], and FA effect depends also on the applied dose and cell type.

There are multiple potential mechanisms of action of FA. According to Melo et al., the protective effect of FA could be due to its ability to prevent HCY-induced teratogenesis [33]; Santos et al. found that FA serves as a methyl donor to a variety of target molecules: for instance, the methylation of HCY to Met, the latter being transformed to S-adenosylcysteine and is the first methyl donor. Consequently, FA maintains the cellular methylation status [9], necessary for DNA, RNA, protein, and neurotransmitter synthesis [19]. From a clinical point of view, Ahmed et al. reported that FA deficiency may directly affect trophoblast invasion, possibly resulting in placental dysfunction and compromising fetal development [37]. Taken together, in vitro experiments support evidence from epidemiological studies reporting that FA supplements mitigate CLP risk [9, 33, 34, 36, 37].

Conclusions

Epidemiological studies have demonstrated that periconceptional FA supplements lead to a substantial reduction in the occurrence of NTD. The meta-analysis conducted provided robust evidence of the beneficial effect of FA on CLP; however, the high degree of heterogeneity between studies compels interpreting the results with caution. Animal models studies also demonstrated the protective effect of FA against CP; additionally, in vitro studies indicate a cell-type and dose-dependent effect of FA on cell viability, proliferation, and differentiation. They also lend support to evidence derived from epidemiological studies. Nevertheless, the heterogeneity of treatment protocols, with respect to dose and FA administration method, as well as the diversity of cell types used in in vitro studies, does not establish with certainty whether FA prevents CLP formation. Taken together, the present review supports the promotion of prenatal and early-pregnancy FA supplementation, in addition to a FA-fortified diet, to help prevent CLP formation. It also recognizes the necessity of in vitro studies that analyze FA pathways to understand its mechanisms of action and its effect on CL, CP, and CLP formation.

Abbreviations

ATRA: All-transretinoic acid; CL: Cleft lip; CLP: Cleft lip and palate; DNA: Deoxyribonucleic acid; FA: Folic acid; HCY: Homocysteine; MSC: Mesenchymal stem cells; Met: Methionine; MTHF R: Methylenetetrahydrofolate reductase; NTD: Neural tube disorders; CP: Palatal cleft or cleft palate; RNA: Ribonucleic acid

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Authors' contributions

ZB contributed to the concepts, design, definition of the intellectual content, literature search, data acquisition, manuscript preparation, manuscript editing, and guarantor of the study. LE contributed to the concepts, design, definition of the intellectual content, literature search, data acquisition, manuscript preparation, manuscript editing, and manuscript review. JC contributed to the concepts, design, definition of the intellectual content, manuscript preparation, and manuscript review. MGC contributed to the concepts, design, definition of the intellectual content, manuscript preparation, and manuscript review. The authors have read and approved the manuscript.

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Competing interests

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