Nutrition ■■ (2021) 111250

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Gestational folic acid deficiency alters embryonic eye development: Possible role of basement membrane proteins in eye malformations

Ouafa Sijilmassi XX [a,*], Aurora Del Río Sevilla XX [a,b], Estela Maldonado Bautista XX [b], María del Carmen Barrio Asensio XX [a,b]

[a] Universidad Complutense de Madrid, Faculty of Optics and Optometry, Anatomy and Embryology Department, Madrid, Spain
[b] Universidad Complutense de Madrid, Faculty of Medicine, Anatomy and Embryology Department, Madrid, Spain

ARTICLE INFO

Article History:
Received 16 January 2021
Received in revised form 8 March 2021
Accepted 19 March 2021

Keywords:
Folic acid deficiency
Collagen IV
Laminin-1
Eye defects

ABSTRACT

Objectives: Folic acid (FA) is crucial before and during early pregnancy. FA deficiency can occur because dietary FA intake is low in mothers at the time of conception. Likewise, various ocular pathologies are related to the alteration of extracellular matrices. The present study aimed to investigate the association between maternal FA deficiency and congenital eye defects. We also investigated whether maternal diet deficient in FA alters the expression of collagen IV and laminin-1 as a possible mechanism responsible for the appearance of ocular malformations. Both proteins are the main components of the basal lamina, and form an interlaced network that creates a relevant scaffold basement membrane. Basal laminae are involved in tissues maintenance and implicated in regulating many cellular processes.

Methods: A total of 57 mouse embryos were classified into the following groups: Control group, (mothers were fed a standard rodent diet), and D2 and D8 groups (mothers were fed FA-deficient [FAD] diet for 2 or 8 wk, respectively). Female mice from group D2 were fed a FAD diet (0 mg/kg diet + 1% succinyl sulfathiazole used to block the synthesis of FA) for 2 wk from the day after mating until day 14.5 of gestation (E14.5). On the other hand, female mice from group D8 were fed a FAD diet for 8 wk (6 wk before conception and during the first 2 wk of pregnancy). For the data analysis, we first estimated the incidence of malformations in each group. Then, the statistical analysis was performed using IBM SPSS Statistics, version 25.0. Expression patterns of collagen IV and laminin-1 were examined with the immunohistochemical technique.

Results: Our results showed that mice born to FA-deficient mothers had several congenital eye abnormalities. Embryos from dams fed a short-term FAD diet were found to have many significant abnormalities in both anterior and posterior segments, as well as choroidal vessel abnormalities. However, embryos from dams fed a long-term FAD diet had a significantly higher incidence of eye defects. Finally, maternal FA deficiency increased the expression of both collagen IV and laminin-1. Likewise, changes in the spatial localization and organization of collagen IV were observed.

Conclusions: A maternal FAD diet for a short-term period causes eye developmental defects and induces overexpression of both collagen IV and laminin-1. The malformations observed are probably related to alterations in the expression of basement membrane proteins.

Introduction

Folic acid (FA), also known as vitamin B9 or folate, is a synthetic water-soluble vitamin crucial for the nervous system and craniofacial structure development [1,2]. Folate is found naturally in many foods. Dark-green leafy vegetables (e.g., asparagus, broccoli, Brussels sprouts, collard greens, turnip greens) and cereal grains (e.g., wheat germs, wheat flour, rye flour) are an important dietary source for folate, as well as Baker's yeast, chickpeas (dried), garlic, citrus fruit, several nuts, seafood, egg yolk, legumes, and liver. FA is the synthetic form of folate, which is found in fortified and supplemental foods [3]. This vitamin is essential for fetal development due to its important participation in several biochemical reactions, including biosynthesis of purine, pyrimidine, and methionine that are fundamental components for both DNA and RNA biosynthesis [4]. The roles played by folate in the metabolism of nucleic acid explains its importance during embryonic development. For this reason, many health authorities recommend for women planning pregnancy to take FA during the periconceptional period to

*Corresponding author. Tel.: +34 913946874; fax: xxx.
E-mail address: o.sijilmassi@ucm.es (O. Sijilmassi).

https://doi.org/10.1016/j.nut.2021.111250
0899-9007/

decrease offspring risk of neural tube defects [5,6], a group of congenital malformations that represent the second most-common birth defect in humans. Therefore, women of childbearing age should take 0.4 mg per d of FA when planning a pregnancy, starting at least 1 mo before conception [6]. The FA supplementation should continue up to 12 wk during pregnancy [7].

In vertebrates, the development of the eye and nervous system are closely linked temporally and spatially, and have many shared factors and expressed genes [8,9]. During embryological development, as the neural tube (NT) is closing at the rostral end, two small bumps appear on the NT on either side. These optic pits are the beginning of the eyes [10], and occurs in mice around the 8th gestational d (E8). In humans, the complete closure of the ends of the NT occurs around the end of week 4 of embryogenesis. Failure of NT closure leads to several congenital malformations, such as neural tube defects [11]. Folate-binding protein 1 has been established to play an important role in embryogenesis, and is closely associated with anterior NT closure. This receptor is also expressed in mouse eye tissues at the beginning of the development. The first folate-binding protein 1 expression in the optic vesicles was observed at E10.5 in mice [12,13].

Currently, anophthalmia and microphthalmia are the most common congenital eye malformations among those diagnosed at birth. Epidemiological studies suggest that congenital eye abnormalities can be related to chromosomal abnormalities, syndromes [14], gene defects, or mutations [15]. However, the etiology of the most prevalent congenital eye malformations, such as anophthalmia, microphthalmia, congenital cataract, iris coloboma, retinochoroidal coloboma, corneal opacity, and congenital glaucoma, have an unknown cause [14]. For this reason, we studied the effect of 2 and 8 wk of an induced folic acid deficient (FAD) diet in female mice on the development of the eye in their offspring, to check for a possible etiological factor for congenital eye malformations. This study aimed to evaluate the frequency of congenital eye abnormalities associated with a maternal FAD diet, and verify if there is a statistically significant relationship between malformations in offspring of FAD dams compared with controls.

On the other hand, various studies have found several mechanisms involved in the failure of the normal closure of the NT due to FA deficiency. Cell polarity, for example, is essential during NT formation and for epithelial maintenance [16,17]. Besides, cell polarity plays a decisive role in numerous morphogenetic processes [18]. The main components of the basal lamina are collagen IV and laminin-1. Both proteins form an interlaced network that creates a relevant scaffold basement membrane (BM). Basal lamina components are involved in tissues maintenance and implicated in regulating many cellular processes, including cell adhesion, polarity, migration, proliferation, survival, and differentiation [19]. Extracellular matrix (ECM) is synthesized and secreted by embryonic cells beginning at the earliest stages of development. The basal laminae in the developing eye appear to be associated with the neuroectoderm, epithelium, and blood vessels. Basal laminae in the eye are found surrounding the lens (lens capsule), in the neural retina (internal limiting membrane [ILM]), retinal pigment epithelium (RPE), corneal epithelium and endothelium, and palpebral and conjunctival epithelium. As a highly dynamic structure, the ECM is constantly undergoing a remodeling process by which components are degraded and modified to maintain tissue homeostasis [20]. Thus, basal laminae alterations during development can lead to serious alterations [21]. In a recent study, we found alterations in the composition within the interstitial ECM of mouse lenses and retinas due to maternal deprivation of FA at early gestation [22]. However, the effect of a FAD diet on the basal laminae has not been explored. For this reason, we also examined the expression of ocular BM proteins, collagen IV, and laminin-1. Both proteins are the major structural components of ocular BMs. Therefore, alterations in the expression of one or both proteins may lead to the disruption of basal laminae. Therefore, we assessed the impact of FA deficiency on the expression of collagen IV and laminin-1. Our goal was to search for some molecular cause of ocular malformations.

Methods

Animals and diets

Experiments were performed on 8-wk-old C57/BL/6J female mice (Harlan Laboratories, Barcelona, Spain). Female mice had free access to water and were divided into two groups according to the gestational FA diet: Control group (female mice fed standard rodent diet [2 mg FA/kg diet; SAFE A04/A03 Harlan] or FAD diet [0 mg FA/kg diet + 1% succinyl sulfathiazole] for 2 or 8 wk), and D2 and D8 groups. Both diets were purchased from Harlan Laboratories, Inc. (Indianapolis, IN). Female mice from group D2 were fed a FAD diet (0 mg/kg diet + 1% succinyl sulfathiazole to inhibit microbial folate synthesis in the intestines) for 2 wk from the day after mating until day 14.5 of gestation (E14.5). Female mice from group D8 were fed a FAD diet for 8 wk (6 wk prior to conception and during the first 2 wk of pregnancy).

Female mice mated only one night with a male mouse, with maximum two female mice in a male mouse's cage. In the morning, the female mice were checked for the presence of a vaginal plug. Day 0.5 of pregnancy (E0.5) is the day when the vaginal plug is first observed. At this moment, the female mice were started on the FAD diet for 2 wk (D2 group). Female mice from group D8 were fed a FAD diet for 8 wk (6 wk before pregnancy and during the first 2 wk of pregnancy). On day 14.5 of pregnancy (E14.5), the female mice were killed by cervical dislocation, and the embryos were removed by Caesarean section. This is the optimal period because in mice, the great majority of eye structures are already formed and can be examined at a very early stage. This period, E14.5, is equivalent to approximately 7 wk of human gestation [23].

Embryos were fixed in 4% paraformaldehyde and then decapitated. The heads were severed and kept separately from the bodies, and later embedded in paraffin. Histological sections were cut into serial sections 5 μm thick using a rotary microtome. Some sections were stained with hematoxylin and eosin by using standard procedures, and others were labelled with anticollagen IV or antilaminin-1.

Immunohistochemistry

The epitope was unmasked using a 0.2% solution of pepsin (Sigma-Aldrich, Inc., St. Louis, MO) in hydrochloric acid 0.1N (for anticollagen IV) or 1mM ethylenediaminetetraacetic acid (Sigma-Aldrich; for antilaminin). Sections were then incubated for 2 h at room temperature with either 1:200 polyclonal rabbit immunoglobulin G antimouse laminin (Sigma-Aldrich) or 1:200 polyclonal rabbit immunoglobulin G antihuman collagen IV (ICN Biomedical Inc., Aurora, OH). Labelling was developed using the Rabbit/Mouse EnVision Peroxidase System, a peroxidase-conjugated dextran polymer (Dako Corp., Carpinteria, CA), and 3,30 diaminobenzidine (DAB kit) as chromogen (Dako Corp.). The sections were observed using a Leica DMR microscope and photographed with a Leica DFC 320 digital camera (Leica Geosystems AG, St. Gallen, Switzerland).

The mice were kept in the animal house of the Faculty of Medicine at the Complutense University of Madrid. Manipulation of the animals was performed following the European Union Normative (2003/65/CE). The experimental protocol used was reviewed and ethically approved by the Animal Welfare Ethics Committee of the Hospital Clínico San Carlos of the Complutense University of Madrid (Code 08/19-18; 2009). The study sample consisted of 57 embryos, with 19 controls, 19 in the D2 group, and 19 in the D8 group. For the statistical analysis, we first estimated the incidence of malformations in each group. Then, the data analysis was performed using IBM SPSS, version 25 (SPSS, Inc.). The association between qualitative variables was analyzed with a χ^2 test and probability values of $P < 0.05$ were considered significant.

Quantifying protein expression: Image analysis

Using MATLAB R2020b (MathWorks, Inc., Natick, MA), all color images were converted into grayscale images and normalized to have the same range of the signal. Then, the pixel intensity values for any color range from 0 to 1, with 0 being the darkest shade of color and 1 the lightest. For each image, five ocular BMs were analyzed: Corneal epithelium BM, lens capsule, palpebral conjunctival, ILM, vitreous (BM of blood vessels), and choroid (BM of blood vessels). For each BM, we selected seven pixels, and these values were used to calculate a mean value for each region of interest. The next step was assigning a score for high positive (3+), positive (2+), and low positive (1+). To facilitate interpretation, the control group was scored as 1+.

Results

Morphological findings and statistical analysis

In this study, we obtained complete data from 19 embryos of mothers with FAD diet during the first two weeks of gestation (D2), 19 embryos of mothers with FAD diet eight weeks, six weeks before pregnancy and two after (D8), and 19 control embryos. Maternal FAD diet causes ocular malformations in both groups studied (D2 and D8).

In the group fed a FAD diet from the beginning of pregnancy (D2), numerous alterations in the eyeball affecting both the anterior and posterior ocular segments were detected in addition to a high frequency of microphthalmia. On the other hand, for the D8 group, the malformations were more severe and affected the anterior and posterior segments in the same way.

All ocular congenital abnormalities detected in the D2 group were statistically significant. Several mice with microphthalmia were observed, and 42.1% of the embryos had microphthalmia of at least one eye (Fig. 1B) compared with the control group (Fig. 1A). As a result, the major alteration observed was a thick cornea (57.9%). An examination of these corneas revealed that the stroma became disorganized (Fig. 1E) compared with those of the control group (Fig. 1D). The second most frequent alteration was retinal lens adhesion affecting 52.6% of the embryos (arrow in Figs. 1E and H) compared with those in the control group (Figs. 1D and G). Moreover, large choroidal blood vessels were observed in 47.4% of the embryos (Fig. 1K; large arrows), and the vessels were larger than those of the control group (Fig. 1J).

Morphological alterations of the lens had the same prevalence (47.4%), with some lenses exhibiting defects in shape and size (Fig. 1E), which was associated with the presence of vacuoles in some cases. The ocular malformation frequencies of the rest of the abnormalities were cornea attached to the lens (36.8% of embryos), absent anterior chamber in many cases, adhesion of the eyelid to the eyeball (31.6%; Fig. 1N) when there usually is a space between the eyelid and conjunctiva in a normal eye (Fig. 1M), and reduced anterior chamber (26.3%; Fig. 1H). Finally, in some cases, a reduced vitreous chamber (Fig. 1H) and retinal detachment were also found (21.05%).

All embryonic eyes from the D8 group were highly affected, and the malformations became much more severe. Like group D2, abnormalities in the anterior and posterior segments were statistically significant (Table 1). The most frequent anomalies were unilateral or bilateral microphthalmia (Fig. 1C) and large choroidal blood vessels (Fig. 1L) that affected 68.4% of the embryos compared with those of the control group (Figs. 1A and J, respectively). In addition to ocular alterations, several developmental disorders of the central nervous system (Fig. 1C) were found.

Likewise, lens morphological alterations were observed in 63.1% of cases (Figs. 1F and I; defects in shape and size). On the other hand, augmented corneal thickness (Fig. 1F), adhesion between the retina and lens (Fig. 1I) and between the eyelids and eye (Fig. 1O) were seen in 52.6% of the mouse embryos. Furthermore, the vitreous volume of the D8 group was significantly lower than that of the control group (Figs. 1F and G, respectively). In some cases, its size was dramatically reduced (Fig. 1I). Moreover, corneal lens adhesions (Fig. 1I) and a decrease of the anterior space between the lens and cornea affected 36.8% of the embryos. Finally, in four cases (21.05%), at least one eye was rotated (Fig. 1C).

On the other hand, we also observed retinal detachments in many cases that generally did not affect the entire eye. Figure 2A shows that the inferior half of the neural retina is attached to the RPE. However, the superior half of the neural retina is detached from the RPE, because a large empty space separates the neurosensory retina from the underlying RPE (Figs. 2A and 2a$_1$ [*]). A relationship can be established between the region of the neurosensorial retina, separated or not from its epithelium, and choroidal vessels. The size of choroidal blood vessels was the same as those of the control group when a retinal detachment occurs (Figs. 2A and a$_1$ [*]). In contrast, in the area that does not undergo detachment (neural retina is attached to the pigment epithelium), the choroidal vessels had a caliber with lumens larger (Figs. 2A and a$_2$) than those of the control group.

The results of the statistical analysis are summarized in Tables 1 and 2. Figure 3 serves as a comparison between the D2 and D8 groups, and shows that the highest frequency of defects occurs in group D8.

Expression of collagen IV and laminin-1 in basement membrane

Immunohistochemistry for collagen IV reveals that already in a short period of the FAD diet (D2), this protein was overexpressed in all BMs of the eye compared with the control group. Thus, the intensity of marking increased in D2 embryos (Fig. 3; row 2) compared with the control group (Fig. 3; row 1). Likewise, we observed that the type IV collagen labelling pattern began to curl, which was better appreciated in the corneal epithelial BM (Fig. 3B), lens capsule (Fig. 3E; black arrows), ILM (Fig. 3H; red arrows), and epithelial BM of the eyelids compared with the control group (Figs. 3A and D). Moreover, a fragmented expression of collagen IV in the corneal epithelial BM was observed (Fig. 3B; double arrow shows discontinuities of the BM).

Regarding the expression of laminin-1, there was also a modification in the labelling pattern. In normal ocular BM, a thin and typically continuous line of laminin-1 is noted in the corneal epithelial BM, ILM of the retina, lens capsule, and conjunctival epithelium. With a FAD diet, laminin-1 exhibited a strong pattern of staining in the BMs of the eye, as shown in the ILM (Fig. 3H; double arrow) and conjunctival epithelium (Fig. 3K; double arrow). The increase in intensity of labelling also affected lens epithelial cells, and the intensity of labelling increased in group D8 (Fig. 3; row 3) compared with the control group (Fig. 3; row 1). In addition, the basal lamina of the hyaloid vessels endothelium of both the D2 and D8 groups was strongly positive for collagen IV (Figs. 3E and F) and laminin-1 (Figs. 3H and I) than the control group (Figs. 3G and J). The results obtained by image analysis for the quantitative comparison of immunohistochemical scoring between the control and D2 and D8 groups are presented in Table 3.

Fig. 4.

Discussion

FA deficiency can result from many factors, including low intake of sources rich in folates (e.g., legumes and green leafy vegetables), impaired absorption (e.g., folate malabsorption is common in untreated diseases, such as celiac disease, tropical sprue, short bowel syndrome, amyloidosis, or mesenteric vascular insufficiency), increased demand (e.g., due to pregnancy or lactation), and the consumption of alcohol and drug abuse [24]. Furthermore, the retention of folate in various foods is highly dependent on both the method and duration of cooking. Folates are destroyed when exposed to heat for a prolonged period of time. Moreover, extensive losses of folate in boiled vegetables have been reported [25]. For these reasons, during pregnancy, increased folate intake is required. The recommended daily amount of FA for women is 400 mg per d [6].

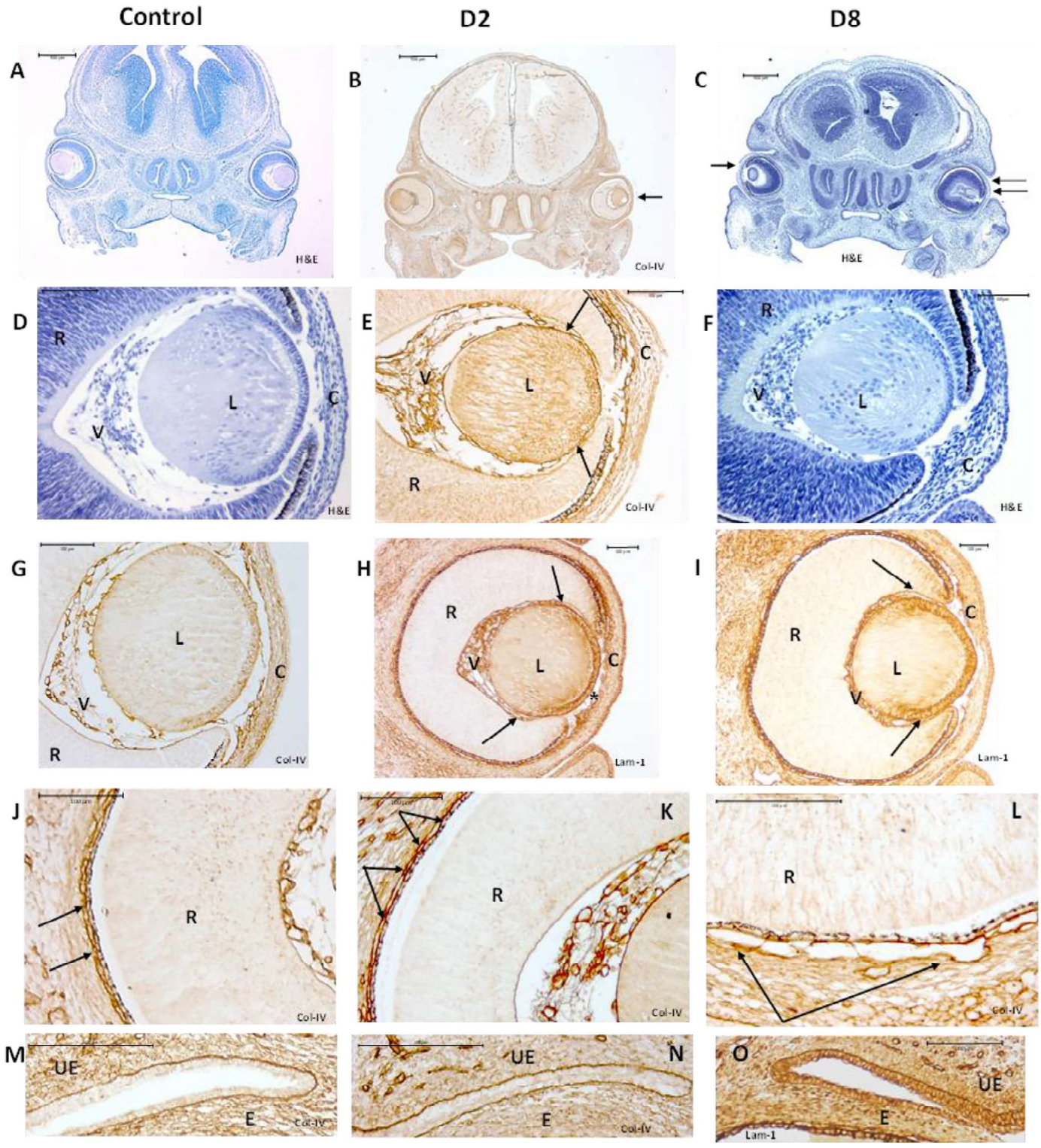

Fig. 1. Comparison of frontal sections of the eye at E14.5 for the (A), (D), (G), (J), (M) control group; (B), (E), (H), (K), (N) D2 group; and (C), (F), (I), (L), (O) D8 group. (B) Head overview documenting the eye microphthalmia (arrow). (C) Microphthalmia (arrow) and ocular alterations (eye is rotated and lens is absent [double arrow]) with developmental nervous system abnormalities. (D) Control with well-developed cornea and lens. (E) and (F) Cornea appear abnormally thickened, corneal stroma is disorganized, retina adherent to the lens (arrow), lens exhibit defects in shape and size. (G) Control with a well-developed anterior chamber and vitreous. (H) Microphthalmia, with retina adherent to the lens (arrow), reduced anterior chamber is shown (*),vitreous cavity is reduced. (I) Microphthalmia, with retina adherent to the lens (arrow), anterior chamber and vitreous cavity are absent, lens exhibit defects in shape and size. (J) Well-developed choroidal vasculature. (K) and (L) Large choroidal blood vessels (arrows). (M) Normal distance between upper eyelid and eye. (N) and (O) Adhesion between eyelid and eyeball. C, cornea; D2, mice mothers were fed folic acid-deficient diet for 2 wk; D8, mice mothers were fed folic acid-deficient diet for 8 wk; E, eye; L, lens; R, retina; UE, upper eyelid; V, vitreous. Original magnifications are (A), (B), (C) × 250; (H), (I) × 100; (D), (E), (F), (G), (J), (K), (O) × 200; (L), (M), (N) × 400. (A, C, D, F) Hematoxylin and eosin-stained slides.(B, E, G, J, K, L, M, N) Labelled with anticollagen IV. (H, I, O) Labelled with antilaminin-1.

Table 1
Types of major congenital eye malformation observed in D8 group

D8 group	Malformation/alteration										
	Corneal lens adhesion	Retinal lens adhesion	Eyelids–eye adhesion	Thickened cornea	Alterations of the lens	Reduced anterior chamber	Reduced vitreous chamber	Large choroidal blood vessels	Microphthalmia	Retinal detachment	Eye rotated downward
N	7	10	10	10	12	7	8	13	13	12	4
%	36.8	52.6	52.6	52.6	63.1	36.8	42.1	68.4	68.4	63.1	21.05
χ^2	8.581	13.571	13.751	13.571	17.538	8.581	10.133	19.760	19.760	17.538	4.471
P-value	0.003	0.001	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.034

D8, mice mothers were fed folic acid-deficient diet for 8 wk.
Statistical significance: $P < 0.05$ compared with control group.

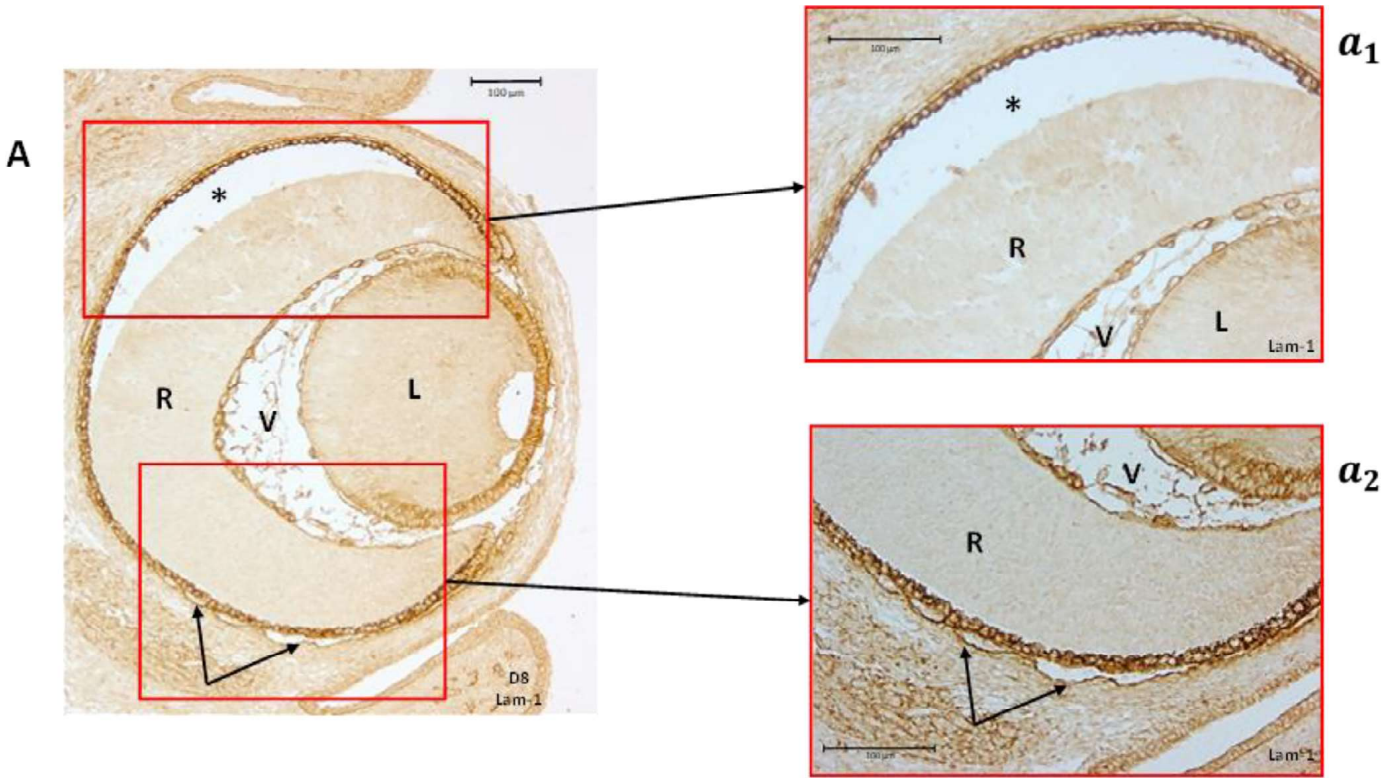

Fig. 2. Retinal detachment of a D8 E14.5 embryo. In A and a_1, the neural retina is clearly separated from the pigment epithelium (*). a_1, retinal detachment (*) associated with small size choroidal vessels. a_2, large choroidal blood vessels are observed (arrows) where there is no retinal detachment. A, original magnification × 100. a_1 and a_2, original magnification × 200. (A) a_1 and a_2 are labelled with antilaminin-1. D8, mice mothers were fed folic acid-deficient diet for 8 wk; L, lens; R, retina; V, vitreous.

Table 2
Types of major congenital eye malformation observed in the D2 group

D2 group	Malformation/alteration									
	Corneal lens adhesion	Retinal lens adhesion	Eyelid–eye adhesion	Thickened cornea	Morphological alterations of lens	Reduced anterior chamber	Reduced vitreous chamber	Microphthalmia	Retinal detachment	Large choroidal blood vessels
N	7	10	6	11	9	5	4	8	4	9
%	36.8	52.6	31.6	57.9	47.4	26.3	21.05	42.1	21.05	47.4
χ^2	8.581	13.571	7.125	15.481	11.793	5.758	4.471	10.133	4.471	11.793
P-value	0.003	0.001	0.008	0.001	0.001	0.016	0.034	0.001	0.034	0.001

D2, mice mothers were fed folic acid-deficient diet for 2 wk.
Statistical significance: $P < 0.05$ compared with control group.

The present study examined the effect of a maternal FAD diet during early pregnancy on eye development in mouse embryos. The deficient diet lacked FA, in addition to neutralizing the bacterial flora of the gastrointestinal tract with 1% succinyl sulfathiazole to prevent endogenous folate synthesis. In a FAD diet for 2 wk, female mice had their folate level reduced by half compared with those in the control group [26]. In this case, FA insufficiency could be considered when embryonic development begins. On the other hand, for dams who were fed a deficient diet for 8 wk, embryonic development began with a drastic reduction in liver reserve about six times compared with those in the control group, which reveals a sever FA-deficiency. Therefore, the present study reveals the importance of folate for ocular development since embryos from both groups manifested ocular alterations.

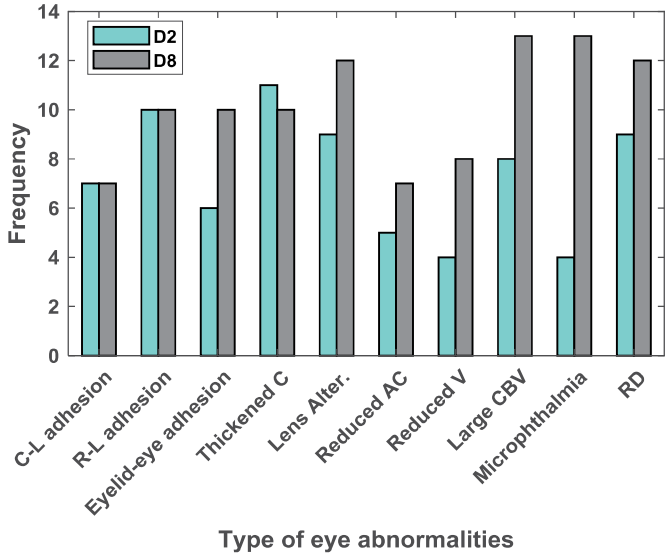

Fig. 3. Comparative analysis of frequencies of diverse congenital eye defects between groups D2 and D8. AC, anterior chamber; C, cornea; CBV, choroidal blood vessels; D2, MICE mothers were fed FOLIC ACID-deficient diet for 2 wk; D8, MICE mothers were fed FOLIC ACID-deficient diet for 8 wk; L, lens; RD, retinal detachment; V, vitreous.

Our results indicate that embryos from mothers subjected to a FAD diet exhibited morphological abnormalities of the eye in both anterior and posterior segments compared with control embryos. The FAD group embryos developed microphthalmia (reduction in eye size), absence of the anterior chamber of the eye, reduced-size vitreous, fusion of the lens and cornea or lens and retina, detachment of the retina, and marked abnormality of the lens or the entire eye. There is very little research on congenital ocular abnormalities due to a maternal FAD diet [27,28], and very drastic FA deprivation before conception appears necessary to detect ocular alterations [28]. However, in this work, we present statistical evidence that the eye is highly susceptible to the lack of FA because after only 2 wk of maternal FA deficiency, congenital malformations in both anterior and posterior segments were statistically significant. The present study found a statistically significant association between a maternal FAD diet and congenital eye malformations, and shows that maternal deprivation of FA at early gestation leads to a failure of normal eye development that involves both the anterior and posterior segments. The data obtained from group D2 embryos correspond to FA deprivation during the first 7 wk of human gestation, and evidence the vulnerability of ocular development to an inadequate dietary intake of FA. Considering that the worldwide rate of unplanned pregnancies in 2012 was approximately 53% [29], pregnancy could begin with low maternal folate status. Abnormal maternal folate metabolism during early pregnancy could be a risk factor for eye defects. Our findings further show that the risk of congenital eye defects augments with the increase of FA deficiency in the mother.

Table 3
Quantitative comparison of immunohistochemical scoring between the control and D2 and D8 groups

Basement membrane	Score					
	Collagen IV			Laminin-1		
	Control	D2	D8	Control	D2	D8
Corneal epithelium	1+	2+	3+	1+	2+	3+
Lens capsule	1+	2+	3+	1+	2+	3+
Palpebral conjunctival	1+	3+	3+	1+	2+	3+
Internal limiting membrane (retina)	1+	2+	3+	1+	2+	3+
Vitreous (blood vessels)	1+	2+	3+	1+	2+	3+
Choroid (blood vessels)	1+	2+	3+	1+	2+	3+

D2, mice mothers were fed folic acid-deficient diet for 2 wk; D8, mice mothers were fed folic acid-deficient diet for 8 wk.

In our study, we also found that a FAD diet during pregnancy alters the expression of some ECM molecules, collagen IV, and laminin-1 in the basal laminae of the developing eye. Both molecules were overexpressed, and there was a greater deposition in the basal laminae. Furthermore, the distribution of collagen IV was different from the control group. The combination and concentration of components is well established to be those that define a basal lamina [30]. Besides, there must be a correct assembly of components that guarantee the precise architectural integrity of basal lamina and its interaction with receptors [31,32]. The maternal FAD diet evidently modified the concentration of laminin-1 and collagen IV, and assembly appears to fail due to the disruption in the arrangement of collagen IV. Consequently, the functions that basal laminae perform during development, such as proliferation, adhesion, and migration, the organization of tissues was altered. This disturbance of basal laminae could trigger the large number of ocular malformations we recorded.

The role of collagen IV and laminin-1 in the development of the eye is well known [33,34]. Several studies have suggested that an alteration of one or both molecules causes defects in the eye like those observed in our study. For example, null mutations of the mouse genes COL4A1 and COL4A2 result in corneal lens adhesion, thickened cornea, and a reduction in anterior chamber depth [35]. Anterior segment dysgenesis was also observed in zebrafish lama-1 eyes [36]. Furthermore, morphological lens abnormalities were probably originated from the alteration of collagen IV and laminin-1. Alport syndrome is the result of collagen IV mutations. The presence of the anterior lenticonus, as well as abnormalities of several eye structures like those observed in the current study, were also seen in patients with Alport syndrome [37].

Symblepharon is a term used in human ophthalmic pathology to indicate adhesion between the palpebral conjunctiva of the eyelid and bulbar conjunctiva of the eye. Our findings reveal that FA deficiency in a mother's diet produces this congenital abnormality. Regarding the retina, retinal detachments were also observed. Mutations in collagen IV or laminin-1 in mice are associated with numerous congenital retinal anomalies [38,39]. Besides, various embryos had microphthalmia and a reduced vitreous chamber that could also be related to changes in the BM. Other studies have also related these abnormalities with alterations in the BMs [36,40].

Abnormality in choroidal and hyaloid vasculature is clearly visible in D2 and D8 embryos. The size and density of choroidal and hyaloid blood vessels are increased in numerous embryos. Furthermore, the blood vessels show an alteration in the expression of collagen IV and laminin-1, and both molecules are overexpressed in the basal laminae surrounding blood vessels. FA is also required during embryogenesis for vasculogenesis. Severe hyperhomocysteinemia was associated with an alteration of retinal vasculature, including ischemia concomitant with neovascularization [41]. Under hypoxic conditions, astrocytes release a vascular endothelial growth factor (VEGF) to stimulate endothelial cell migration, differentiation, and proliferation. Elevated retinal VEGF levels cause pathophysiological changes in the choroid [42]. Under hypoxic conditions, cerebral capillary blood vessel diameter has been shown to increase (larger vessel diameters occur) during vasculogenesis, leading to aberrantly large vascular sinuses [43,44]. Our results are consistent with this finding. High levels of VEGF appear to play a significant role in altering vascular patterning [45,46]. VEGF has been shown to induce ECM proteins in the umbilical

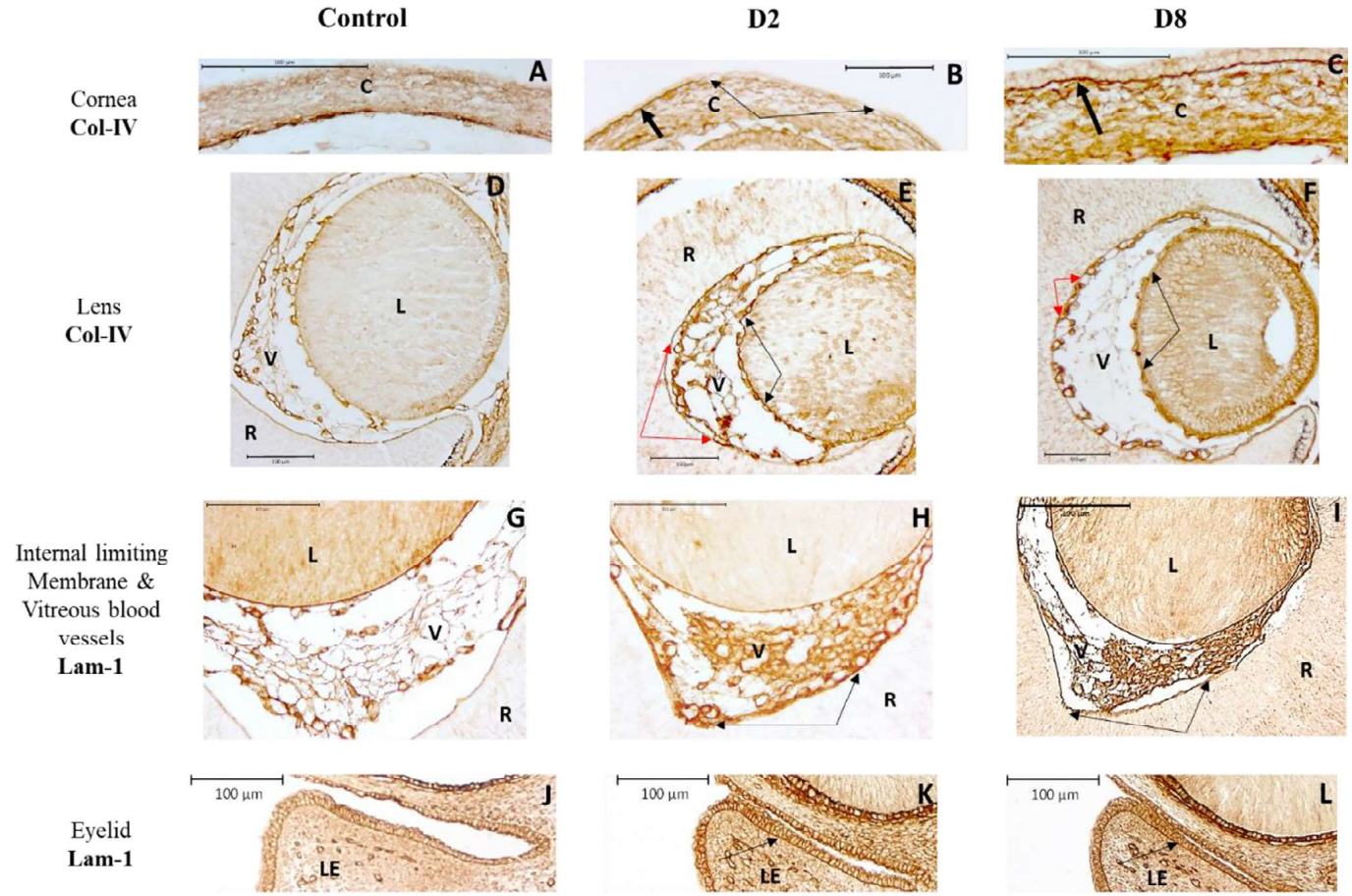

Fig. 4. Expression analysis of collagen IV and laminin-1 in embryonic eyes, with (A, D, G, J) normal E14.5 eyes; (B, E, H, K) the D2 group; and (C, F, I, L) the D8 group. In (A) and (D), normal collagen IV distribution in the BM of the corneal epithelium, lens capsule, and ILM. (B, C) Greater intensity of labelling pattern of collagen IV in the corneal epithelium, with labeling also curly. (B) Double arrow shows the discontinuities of the BM. (E, F) Greater intensity of labelling pattern of the collagen IV in the lens capsule (double black arrow) and ILM of the retina (double red arrow). (G) Normal laminin-1distribution in the BM of the lens capsule and ILM. (H, I) Greater intensity of labelling pattern of laminin-1 the lens capsule and ILM of the retina (double black arrow). (J) Normal laminin-1 distribution in the BM of the palpebral and bulbar conjunctiva. (K, L) Spatial distribution pattern of laminin-1 distinctly different from the control (arrow). Original magnification for (J), (K), (L) × 100; (B), (D), (E), (F), (I) × 200; and (A), (B), (G), (H) × 400. BM, basement membrane; C, cornea; ILM, internal limiting membrane; L, lens; LE, lower eyelid; R, retina; V, vitreous.

artery since collagen IV and laminin-1 increase after treatment with VEGF [47]. Thus, FA deficiency perhaps alters vasculogenesis in the embryos, which could be related to the increase in the labelling intensity of collagen IV and laminin-1 shown in our study.

Conclusions

The results presented herein indicate that the eye is affected by a maternal FAD diet. Moreover, a maternal FAD diet alters the expression of collagen IV and laminin-1, which means changes in BM protein expression, which might lead to ocular malformations. Some studies have found irregularly thickened BMs, amorphous deposition in BMs, and perivascular areas in cerebral microvessels as a result of folate deficiency [48]. On the other hand, a study from our group [49] showed that a maternal FAD diet affects collagen IV and laminin-1 expression within the lens. This alteration in expression observed in eye BMs may be associated with the various ophthalmologic developmental anomalies observed in our study. The eye is an organ that is very susceptible to a lack of FA, even when mothers have liver reserves at the beginning of pregnancy. Therefore, dietary supplements of FA might help prevent some congenital eye diseases if used early enough at adequate doses.

Declaration of Competing Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgments

The authors acknowledge Dolores Arroyo and Alicia Cerro from the Anatomy and Embryology Department for their excellent technical assistance. This study was supported by grants to the Complutense Research Group 920202 from the Spanish Ministry of Health (PI06/0184 and PS09/01762). This article was done with a fellowship awarded by Complutense University of Madrid and Santander bank (CT27/16–CT28/16). The animal facilities and experimental protocol used were reviewed and ethically approved by the Animal Experimentation Committee of the Complutense University of Madrid-Spain.

References

[1] Maddox DM, Manlapat A, Roon P, Prasad P, Ganapathy V, Smith SB. Reduced-folate carrier (RFC) is expressed in placenta and yolk sac, as well as in cells of the developing forebrain, hindbrain, neural tube, craniofacial region, eye, limb buds and heart. BMC Dev Biol 2003;3:6.

[2] Tang LS, Santillano DR, Wlodarczyk BJ, Miranda RC, Finnell RH. Role of Folbp1 in the regional regulation of apoptosis and cell proliferation in the developing neural tube and craniofacies. Am J Med Genet C Semin Med Genet 2005;125C:48–58.
[3] Witthöft CM, Forssén K, Johannesson L, Jägerstad M. Folates-food sources, analyses, retention and bioavailability. Näringsforskning 1999;43:138–46.
[4] Stover PJ, Field MS. Trafficking of intracellular folates. Adv Nutr 2011;2:325–31.
[5] Eurocat. Eurocat Folic Acid Working Group Special Report: Prevention of neural tube defects by periconceptional folic acid supplementation in Europe. Ulster: University of Ulster, Eurocat Central Registry; 2003.
[6] Wald N, Sneddon J, Densem J, Frost C, Stone R. Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. Lancet 1991;338:131–7.
[7] Norsworthy B, Skeaff CM, Adank C, Green T. Effects of once-a-week or daily folic acid supplementation on red blood cell folate concentrations in women. Eur J Clin Nutr 2004;58:548–54.
[8] Graw J. Genetic aspects of embryonic eye development in vertebrates. Dev Genet 1996;18:181–97.
[9] Klann M, Seaver EC. Functional role of pax6 during eye and nervous system development in the annelid Capitella teleta. Dev Biol 2019;456:86–103.
[10] Sadler TW. Langman's medical embryology. Philadelphia, PA: Lippincott Williams & Wilkins; 2011.
[11] Harris MJ, Juriloff DM. Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects. Birth Defects Res A Clin Mol Teratol 2007;79:187–210.
[12] Saitsu H, Ishibashi M, Nakano H, Shiota K. Spatial and temporal expression of folate-binding protein 1 (Fbp 1) is closely associated with anterior neural tube closure in mice. Dev Dyn 2003;226:112–7.
[13] Saitsu H. Folate receptors and neural tube closure. Congenit Anom (Kyoto) 2017;57:130–3.
[14] Bermejo E, Martínez-Frías ML. Congenital eye malformations: Clinical-epidemiological analysis of 1,124,654 consecutive births in Spain. Am J Med Genet 1998;75:497–504.
[15] Graw J. The genetic and molecular basis of congenital eye defects. Nat Rev Genet 2003;4:876.
[16] Balashova OA, Visina O, Borodinsky LN. Folate action in nervous system development and disease. Dev Neurobiol 2018;78:391–402.
[17] Eom DS, Amarnath S, Fogel JL, Agarwala S. Bone morphogenetic proteins regulate hinge point formation during neural tube closure by dynamic modulation of apicobasal polarity. Birth Defects Res A Clin Mol Teratol 2012;94:804–16.
[18] Andrew DJ, Ewald AJ. Morphogenesis of epithelial tubes: Insights into tube formation, elongation, and elaboration. Dev Biol 2010;341:34–55.
[19] Wiradjaja F, DiTommaso T, Smyth I. Basement membranes in development and disease. Birth Defects Res C Embryo Today 2010;90:8–31.
[20] Rozario T, DeSimone DW. The extracellular matrix in development and morphogenesis: A dynamic view. Dev Biol 2010;341:126–40.
[21] Sekiguchi R, Yamada KM. Basement membranes in development and disease. Curr Top Dev Biol 2018;130:143–91.
[22] Sijilmassi O, López-Alonso JM, Barrio Asensio MDC, Del Río Sevilla A. Alteration of lens and retina textures from mice embryos with folic acid deficiency: Image processing analysis. Graefes Arch Clin Exp Ophthalmol 2018:1–13.
[23] Otis EM, Brent R. Equivalent ages in mouse and human embryos. Anat Rec 1954;120:33–63.
[24] Khan KM, Jialal I. Folic acid deficiency. Treasure Island, FL: StatPearls Publishing; 2020.
[25] McKillop DJ, Pentieva K, Daly D, McPartlin JM, Hughes J, Strain J, et al. The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. Br J Nutr 2002;88:681–8.
[26] Maldonado E, Murillo J, Barrio C, del Rio A, Perez-Miguelsanz J, Lopez-Gordillo Y, et al. Occurrence of cleft-palate and alteration of Tgf-beta(3) expression and the mechanisms leading to palatal fusion in mice following dietary folic-acid deficiency. Cells Tissues Organs 2011;194:406–20.
[27] Armstrong RC, Monie I. Congenital eye defects in rats following maternal folic-acid deficiency during pregnancy. Development 1966;16:531–42.
[28] Maestro-de-las-Casas C, Pérez-Miguelsanz J, López-Gordillo Y, Maldonado E, Partearroyo T, Varela-Moreiras G, et al. Maternal folic acid-deficient diet causes congenital malformations in the mouse eye. Birth Defects Res A Clin Mol Teratol 2013;97:587–96.
[29] Sedgh G, Singh S, Hussain R. Intended and unintended pregnancies worldwide in 2012 and recent trends. Stud Fam Plann 2014;45:301–14.
[30] Hausman RE. Ocular extracellular matrices in development. Prog Retin Eye Res 2007;26:162–88.
[31] Roediger M, Miosge N, Gersdorff N. Tissue distribution of the laminin β1 and β2 chain during embryonic and fetal human development. J Molec Histol 2010;41:177–84.
[32] Kruegel J, Miosge N. Basement membrane components are key players in specialized extracellular matrices. Cell Mol Life Sci 2010;67:2879–95.
[33] Bai X, Dilworth DJ, Weng YC, Gould DB. Developmental distribution of collagen IV isoforms and relevance to ocular diseases. Matrix Biol 2009;28:194–201.
[34] Byström B, Virtanen I, Rousselle P, Gullberg D, Pedrosa-Domellöf F. Distribution of laminins in the developing human eye. Invest Ophthalmol Vis Sci 2006;47:777–85.
[35] Favor J, Gloeckner CJ, Janik D, Klempt M, Neuhauser-Klaus A, Pretsch W, et al. Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: An extension of the Col4 alpha 1 allelic series and the identification of the first two Col4a2 mutant Alleles. Genetics 2007;175:725–36.
[36] Semina EV, Bosenko DV, Zinkevich NC, Soules KA, Hyde DR, Vihtelic TS, et al. Mutations in laminin alpha 1 result in complex, lens-independent ocular phenotypes in zebrafish. Dev Biol 2006;299:63–77.
[37] Savige J, Sheth S, Leys A, Nicholson A, Mack HG, Colville D. Ocular features in Alport syndrome: Pathogenesis and clinical significance. Clin J Am Soc Nephrol 2015;10:703–9.
[38] Alavi MV, Mao M, Pawlikowski BT, Kvezereli M, Duncan JL, Libby RT, et al. Col4a1 mutations cause progressive retinal neovascular defects and retinopathy. Sci Rep 2016;6.
[39] Edwards MM, Mammadova-Bach E, Alpy F, Klein A, Hicks WL, Roux M, et al. Mutations in Lama1 disrupt retinal vascular development and inner limiting membrane formation. J Biol Chem 2010;285:7697–711.
[40] Firtina Z, Danysh BP, Bai X, Gould DB, Kobayashi T, Duncan MK. Abnormal expression of collagen IV in lens activates unfolded protein response resulting in cataract. J Biol Chem 2009;284:35872–84.
[41] Tawfik A, Al-Shabrawey M, Roon P, Sonne S, Covar JA, Matragoon S, et al. Alterations of retinal vasculature in cystathionine-Beta-synthase mutant mice, a model of hyperhomocysteinemia. Invest Ophthalmol Vis Sci 2013;54:939–49.
[42] Tee L, Penrose M, O'Shea J, Lai C, Rakoczy E, Dunlop S. VEGF-induced choroidal damage in a murine model of retinal neovascularisation. Br J Ophthalmol 2008;92:832–8.
[43] Drake CJ, Little CD. Exogenous vascular endothelial growth factor induces malformed and hyperfused vessels during embryonic neovascularization. Proc Natl Acad Sci U S A 1995;92:7657–61.
[44] Zhang H, Rzechorzek W, Aghajanian A, Faber JE. Hypoxia induces de novo formation of cerebral collaterals and lessens the severity of ischemic stroke. J Cereb Blood Flow Metab 2020;40:1806–22.
[45] Ogunshola OO, Stewart WB, Mihalcik V, Solli T, Madri JA, Ment LR. Neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain. Dev Brain Res 2000;119:139–53.
[46] Vempati P, Popel AS, Mac Gabhann F. Extracellular regulation of VEGF: Isoforms, proteolysis, and vascular patterning. Cytokine Growth Factor Rev 2014;25:1–19.
[47] Infanger M, Grosse J, Westphal K, Leder A, Ulbrich C, Paul M, et al. Vascular endothelial growth factor induces extracellular matrix proteins and osteopontin in the umbilical artery. Ann Vasc Surg 2008;22:273–84.
[48] Kim JM, Lee H, Chang N. Hyperhomocysteinemia due to short-term folate deprivation is related to electron microscopic changes in the rat brain. J Nutr 2002;132:3418–21.
[49] Sijilmassi O, López Alonso JM, Barrio Asensio MC, Del Río Sevilla A. Collagen IV and laminin-1 expression in embryonic mouse lens using principal components analysis technique. J Microsc 2018;271:207–21.