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The Role of Folic Acid in Rat Embryo Development in a Hypoxic Environment: An Experimental Study

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ABSTRACT

Objective: Folic acid (FA) is a key antioxidant with substantial metabolic roles, and research has demonstrated its effectiveness in reducing congenital anatomical development disorders. This study explores the impact of folic acid on embryo development under hypoxia-induced conditions in embryo cultures.

Materials and Methods: Female Wistar albino rats, aged 4–10 months and weighing 150–250 grams, were utilized for this research. Embryos were extracted from the maternal womb on the 9.5th day of pregnancy. We established six groups, each consisting of 10 embryos: Control (C), Hypoxia (H), 1 mmol FA (1FA), 2 mmol FA (2FA), Hypoxia + 1 mmol FA (H1FA), and Hypoxia + 2 mmol FA (H2FA). Following a 48-hour culture period, the groups were assessed morphologically.

Results: When comparing the morphological parameters of the Control and Hypoxia groups, it was statistically demonstrated that the Control group completed its development, whereas the Hypoxia group exhibited insufficient development ($p<0.05$). There were statistically significant differences between the Hypoxia group and the Hypoxia + 1 mmol FA and Hypoxia + 2 mmol FA groups ($p<0.05$). Both the Hypoxia + 1 mmol FA and Hypoxia + 2 mmol FA groups demonstrated better embryonic development compared to the Hypoxia group ($p<0.05$).

Conclusion: The study has established that FA has positive effects on embryos exposed to hypoxic conditions, which result in developmental delays.

Keywords: Folic acid, embryo culture, rat, antioxidant effects.

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INTRODUCTION

Oxygen is an essential substrate in the metabolism of living organisms. A reduction in oxygen pressure, known as hypoxia, leads to decreased cellular vital activities. Consequently, hypoxia

results in oxidative stress and the formation of free radicals.[1–3] Organisms face continuous pressure from both internal and external factors. During and after these stresses, oxidant molecules assault cells and tissues, inflicting damage. In response, organisms deploy an antioxidant system that neutralizes reactive oxygen species and other prooxidants. Antioxidants are substances that exhibit protective properties against the harmful oxidative reactions of oxygen.[4] In mammalian embryo development, certain critical periods exist when primary organ rudiments form. For rat embryos, this pivotal period spans days 9.5 to 11.5. The technique of embryo, known as "Whole Embryo Culture (WEC)," was first pioneered by New et al.[5,6] in the 1950s. This *in vitro* method was developed to yield more reliable results by avoiding the physiological and biochemical changes in the mother that could influence the experimental outcome observed with *in vivo* methods.

Folic acid (FA), a substance with antioxidant properties, plays multiple physiological roles.[7] It is the synthetic form of folate, chemically synthesized and more stable than its biological counterpart.[8] Research has shown that FA can prevent embryonic developmental disorders.[9] A healthy individual requires a minimum daily intake of 200 µg of FA. To leverage its protective properties, pregnant women are advised to consume at least 400 µg daily. For adults, FA intake should not surpass 1000 µg per day to prevent potential symptoms of vitamin B12 deficiency.[10]

Several studies have explored the role of FA in embryo culture.[7,11] However, research on the effects of FA on embryo development under hypoxic conditions remains scarce. This study therefore aims to assess the impact of FA on embryo development in a hypoxia-induced embryo culture setting.

MATERIALS AND METHODS

This is an experimental study. The design encompasses pre-experiment preparation, the experiment itself, and result analysis (Fig. 1).

Mating and Experimental Preparation

Before initiating our study, we obtained approval from the local ethics committee for animal experiments at Erciyes University. The study was approved based on the decision from meeting number 01 on January 13, 2016, and conducted between January 14, 2016, and June 14, 2018. Female Wistar albino rats, aged 4–10 months and weighing 150–250 grams, raised at the Erciyes University Experimental Animal Facility (DEKAM), were used in this study. The rats were anesthetized with 75 mg/kg of ketamine and 10 mg/kg of xylazine, and blood samples were collected from the abdominal aorta. Embryos retrieved from the maternal womb were transferred to petri dishes containing Hanks' Balanced Salt Solution (with sodium) for dissection [Hanks' Balanced Salt Solution (Sigma-Aldrich/Lot RNBD7977)].

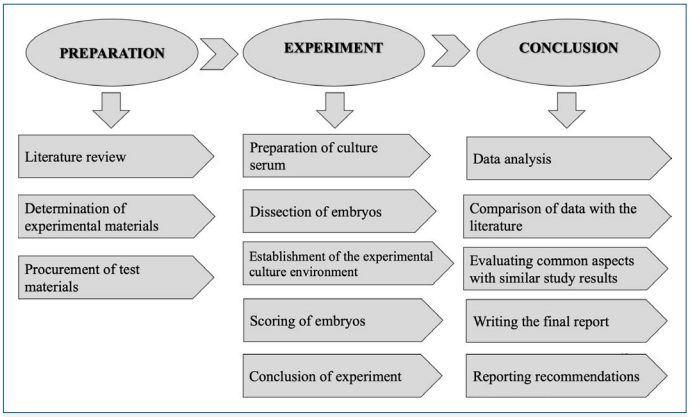

Figure 1. Study design.

Whole Embryo Culture

Preparation of Folic Acid

The dosage of FA for the study was based on the embryo culture study by Parri Wentzel et al.,[11] conducted in 2005, and was set at 1 mmol-2 mmol. The molecular weight of FA is 441.40 grams/mol.[12] To prepare the FA solution, 2.207 grams of FA were dissolved in 500 ml of sterile distilled water. Specific volumes of this solution were then added to culture bottles containing rat serum. When 555.5 microliters (µl) of the solution were mixed with 5 ml of rat serum, the final concentration of FA was calculated to be 1 mmol. When 1250 microliters (µl) of the solution were mixed with 5 ml of rat serum, the final concentration of FA in the mixture was calculated to be 2 mmol. For the study groups receiving 1 mmol of FA, 555.5 microliters (µl) of the solution were added, and for those receiving 2 mmol of FA, 1250 microliters (µl) were added [(Folic Acid (5 g) - (Merck KGaA Cas No 59-30-3 K46803884 609), Sterile Distilled Water (500 ml)].

Preparation of Rat Embryos (Embryo Dissection)

In the evening, female rats were placed in the same cage with male rats. Vaginal smears were collected at 8 AM the following morning. Females with detected sperm were considered 0.5 days pregnant and transferred to separate cages, where they were fed a standard diet for nine days. On the 9.5th day of pregnancy, under general anesthesia, the embryos were extracted from the maternal womb and prepared for culture using the technique developed by New.[5,11] While the embryos from female rats were utilized in the culture environment, both female and male rats were used to obtain the serum necessary for embryo cultivation.

Formation and Scoring of Experimental Groups

Experimental Groups and Sample Size

The number of embryos in the study groups was determined based on the *in vitro* embryo culture technique described by New D. (1978). To achieve statistical significance with a large effect size (f=0.50) between the groups at 80% power and a 0.05 type-1 error level, it was deemed appropriate to include a total of 60 subjects, with at least 10 subjects per group. Calculations were performed using G*Power 3.1 software.[5]

Six groups were established to investigate the antioxidant effects of folic acid on the anatomical development of hypoxic rat embryos: Control (C), Hypoxia (H), 1 mmol FA (1FA), 2 mmol FA (2FA), Hypoxia + 1 mmol FA (H1FA), and Hypoxia + 2 mmol FA (H2FA). Healthy embryos obtained after dissection (1 embryo/1 ml serum) were placed in culture bottles filled with normal rat serum, with each group consisting of 10 embryos. Groups C (n=10), 1FA (n=10), and 2FA (n=10) were exposed to 5% oxygen on day 1, 20% oxygen on day 2, and 40% oxygen on day 3. Groups H (n=10), H1FA (n=10), and H2FA (n=10) were exposed to 5% oxygen on days 1 and 2, and 40% oxygen on day 3.

Morphological Scoring

The 'Objective Scoring System' developed by Van Maele-Fabry et al. is a widely used scoring method designed to measure morphological development. This system evaluates 17 morphological features in rat embryos cultured *in vitro* on the 10th, 11th, 12th, and 13th days of pregnancy. Each morphological feature is categorized into six stages: vitelline sac vascularization, allantois, flexion, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, branchial arch, maxillary prominence, mandibular prominence, forelimb, hindlimb, somite count, vitelline sac diameter, head-tail length, and head length. Scores ranging from 0 to 5 are assigned to each developmental stage.[13,14]

Statistical Analysis

The normal distribution of data was assessed using histograms, q-q plots, and the Shapiro-Wilk test. Comparisons among more than two groups were conducted using one-way analysis of variance and the Kruskal-Wallis test. The Dunn-Bonferroni test was used for multiple comparisons. The relationship between quantitative variables was evaluated using Spearman and Pearson correlation analyses. Data analysis was performed using R Studio 3.2.2, and a significance level of $p<0.05$ was considered statistically significant (Table 1, 2).

RESULTS

The data obtained in this study are expressed as median (1st quartile–3rd quartile). Identical letters on the same line indicate similarity between groups, while different letters denote differences (C: Control Group, H: Hypoxia Group, 1FA: 1 mmol Folic Acid Group, 2FA: 2 mmol Folic Acid Group, H1FA: Hypoxia + 1 mmol Folic Acid Group, H2FA: Hypoxia + 2 mmol Folic Acid Group) (Table 1).

At the end of the study, embryos cultured in rat serum in the C, 1FA, and 2FA groups exhibited normal development, with no statistically significant differences among these groups ($p>0.05$). The embryos in the C group showed normal development, whereas those in the H group demonstrated lagged development. When comparing these two groups based on morphological scoring parameters, a statistically significant difference was identified ($p<0.05$). Notably, a significant difference in the total score of morphological parameters was observed between the C group and the H1FA group ($p<0.05$). Similarly, statistically significant differences were found between the C group and the H2FA group in the total score, vitelline sac diameter, head-tail length, and somite count ($p<0.05$). Furthermore, significant differences were detected between the H group and the H1FA group in several morphological parameters including vitelline sac vascularization, hindbrain, midbrain, forebrain, otic system, optic system, branchial arch, somites, total score, vitelline sac diameter, head-tail length, and somite count ($p<0.05$). Comparisons between the H group and the H2FA group revealed significant differences in vitelline sac vascularization and forebrain ($p<0.05$). However, no statistically significant differences were found between the H1FA and H2FA groups in the morphological scoring parameters ($p>0.05$). When comparing the H group with the H2FA group, a statistically significant difference was found in terms of vitelline sac vascularization and forebrain values ($p<0.05$). However, when comparing the H1FA group with the H2FA group, no statistically significant difference was observed in the values of morphological scoring parameters ($p>0.05$). In our study, the means of certain parameters were calculated among the groups. Considering the morphological scoring parameters, the C group had the highest total score and somite count. Conversely, the H group had the lowest total score and somite count. The 1FA and 2FA groups displayed higher total scores and somite counts compared to other groups, except for the C group. The H1FA and H2FA groups had higher total scores and somite counts than the H group but lower than those of the C group. Embryos in the C group showed the best development in parameters such as vitelline sac diameter, head-tail length, and caudal neural tube, whereas the H group exhibited the least developed characteristics in these parameters. Meanwhile, the H1FA and H2FA groups demonstrated better development

Table 1. Complete morphological scoring parameters (median; min–max)

	Groups						p
	C (n=10)	1FA (n=10)	2FA (n=10)	H (n=10)	H1FA (n=10)	H2FA (n=10)	
Vitelline sac vascularization	5.0 (4.0-5.0)[a]	4.0 (4.0-5.0)[a]	4.0 (3.75-5.0)[a]	4.0 (3.75-5.0)[b]	4.0 (3.75-4.25)[a]	4.0 (3.0-4.25)[a]	<0.001
Allantois	2.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[a,c]	1.0 (1.0-2.0)[b,c]	2.0 (1.75-3.0)[a,c]	2.0 (1.0-3.0)[a,c]	<0.007
Flexion	4.0 (4.0-5.0)[a]	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	2.0 (2.0-2.25)[b,c]	3.5 (3.0-4.0)[a,c]	3.5 (3.0-4.0)[a,c]	<0.001
Heart	4.0 (3.75-4.25)[a]	4.0 (3.0-4.0)[a]	4.0 (3.0-4.0)[a]	2.0 (2.0-2.25)[b,c]	3.0 (3.0-4.0)[a,c]	3.0 (3.0-3.25)[a,c]	<0.001
Caudal neural tube	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	4.0 (3.0-4.0)[a]	2.0 (2.0-3.0)[b,c]	3.0 (3.0-3.25)[a,c]	3.0 (3.0-3.25)[a,c]	<0.001
Hindbrain	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	4.0 (3.0-4.0)[a]	2.0 (1.75-2.0)[b,c]	3.0 (3.0-4.0)[a]	3.0 (3.0-3.25)[a,c]	<0.001
Midbrain	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	4.0 (3.0-4.0)[a]	2.0 (1.75-2.0)[b,c]	3.0 (3.0-4.0)[a]	3.0 (3.0-3.25)[a,c]	<0.001
Forebrain	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	4.0 (3.0-4.0)[a]	2.0 (2.0-2.25)[b]	3.5 (3.0-4.0)[a]	3.5 (3.0-4.0)[a]	<0.001
Otic system	4.5 (4.0-5.0)[a]	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	2.0 (1.0-2.0)[b,c]	3.0 (3.0-4.0)[a]	3.0 (3.0-4.0)[a,c]	<0.001
Optic system	4.0 (4.0-5.0)[a]	4.0 (3.75-5.0)[a]	4.0 (3.0-5.0)[a]	2.0 (1.0-2.0)[b,c]	3.5 (3.0-4.0)[a]	3.0 (3.0-4.0)[a,c]	<0.001
Olfactory system	2.0 (2.0-3.0)[a]	2.0 (2.0-3.0)[a,c]	2.0 (1.75-3.0)[a,c]	1.0 (1.0-2.0)[b,c]	2.0 (1.0-2.0)[a,c]	1.5 (1.0-2.0)[a,c]	<0.010
Pharyngeal arch	2.0 (1.0-2.0)[a]	1.5 (1.0-2.0)[a]	1.0 (1.0-2.0)[a]	1.0 (0.0-1.25)[b,c]	1.0 (0.75-2.0)[a]	1.0 (0.0-2.0)[a,c]	<0.242
Maxillary protrusion	3.0 (2.0-3.0)[a]	2.5 (2.0-3.0)[a]	2.0 (2.0-3.0)[a]	1.0 (0.75-2.0)[b,c]	2.0 (2.0-2.25)[a,c]	2.0 (2.0-2.0)[a,c]	<0.001
Mandibular protrusion	3.0 (2.0-3.0)[a]	2.5 (2.0-3.0)[a]	2.0 (2.0-3.0)[a]	1.0 (0.75-2.0)[b,c]	2.0 (2.0-2.25)[a,c]	2.0 (2.0-2.0)[a,c]	<0.001
Forelimb	2.0 (2.0-3.0)[a]	2.0 (1.75-3.0)[a]	2.0 (1.75-2.0)[a]	1.0 (0.75-1.0)[b,c]	1.5 (1.0-2.0)[a,c]	1.0 (1.0-2.0)[a,c]	<0.001
Hindlimb	1.5 (1.0-2.0)[a]	1.0 (1.0-2.0)[a,c]	1.0 (1.0-1.25)[a,c]	1.0 (0.0-1.0)[b,c]	1.0 (1.0-1.0)[a,c]	1.0 (0.75-1.0)[a,c]	<0.007
Somites	5.0 (4.75-5.0)[a]	5.0 (4.0-5.0)[a]	4.0 (3.75-5.0)[a]	2.0 (1.0-2.0)[b,c]	4.0 (3.75-4.0)[a]	4.0 (3.0-4.0)[a,c]	<0.001
Total score	58.0 (55.5-61.5)[a]	56.5 (53.75-57.5)[a,c]	54.5 (49.5-56.0)[a,c]	27.0 (21.0-30.25)[b]	46.5 (43.25-51.25)[b,c]	44.5 (39.25-50.25)[b,c]	<0.001
Vitelline sac diameter	3.30 (3.300-3.425)[a]	3.30 (3.275-3.425)[a,c]	3.20 (3.075-3.40)[a,c]	2.30 (2.175-2.400)[b]	3.10 (3.00-3.40)[a,c]	2.95 (2.90-3.15)[b,c]	<0.001
Crown-rump length	3.20 (3.10-3.20)[a]	3.10 (3.00-3.20)[a,c]	3.05 (2.975-3.125)[a,c]	1.95 (1.90-2.10)[b]	2.90 (2.80-3.10)[a,c]	2.80 (2.70-2.925)[b,c]	<0.001
Number of somites	28.0 (25.75-29.0)[a]	26.5 (22.75-28.0)[a,c]	23.5 (21.5-26.0)[a,c]	12.0 (8.75-14.25)[b]	22.0 (20.75-24.25)[a,b]	21.5 (19.75-25.25)[b,c]	<0.001

The letters a, b, c in the table denote the similarities and differences among the groups. Identical letters indicate similarities, whereas different letters signify differences between groups.

compared to the H group. The H group displayed delayed neural development. The C group had the best neural development among all groups, while the H1FA and H2FA groups showed better neural development than the H group (Fig. 2–4).

In the analysis results of our study, a strong positive correlation was found between vitelline sac diameter, crown-rump length, number of somites, and total score. Additionally, strong positive correlations were observed between crown-rump length and number of somites, crown-rump length and vitelline sac diameter, and number of somites and crown-rump length (Table 2).

Table 2. Correlation analyses

	Total score	Vitelline sac diameter	Crown-rump length
Vitelline sac diameter	0.871*		
Crown-rump length	0.918*	0.942*	
Number of somites	0.962*	0.868*	0.912*

*: P<0.01.

DISCUSSION

The *in vitro* embryo culture technique is an effective method for detecting the early effects of hypoxia. Rosenzweig et al.[15] examined the impact of hypoxia on embryo culture during early pregnancy and found that even brief exposure to hypoxia could disrupt embryo growth and development, negatively affecting both growth and differentiation. In our study, the morphological scoring parameters in the H group showed a statistically significant difference compared to the C group. The observation that embryos exposed to hypoxia experience developmental delays corroborates the findings of numerous studies, including those conducted by Rosenzweig et al.[15]

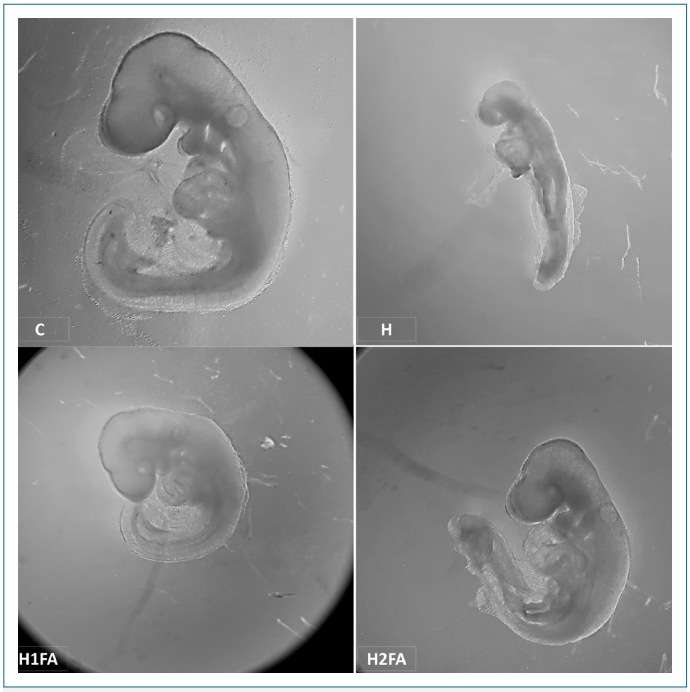

Figure 2. Images of embryos.

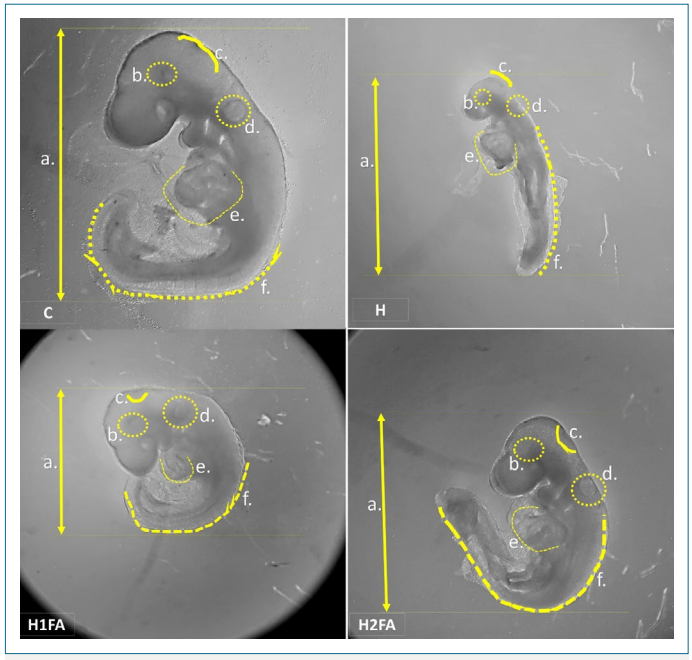

Figure 3. Measurement parameters (a: Head-tail length; b: Optic system; c: Otic system; d: Heart system; e: Somite system).

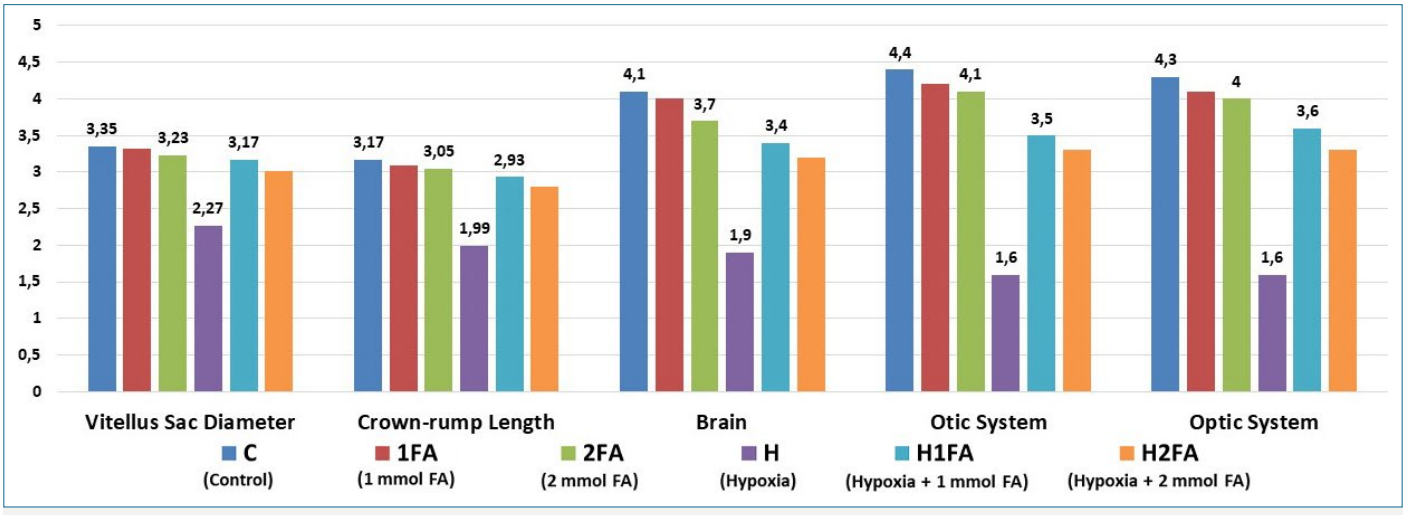

Figure 4. Morphological scoring parameters (median; min–max).

Our results indicate that embryos in the H group exhibited reduced vascularization of the vitelline sac and impaired heart development compared to those in the C group. Hypoxia appears to adversely affect vascularization, heart development, and neural tube formation.

In conclusion, our study provides evidence that hypoxia negatively impacts vascularization, heart development, and neural tube formation in embryos. These results underscore the importance of understanding the effects of hypoxia on early embryonic development and highlight the necessity for further research in this field to better understand the underlying mechanisms and potential implications for developmental abnormalities.

The aim of numerous studies on embryo development is to identify both the positive and negative effects of substances introduced into the environment, including their teratogenic

and anti-angiogenic impacts on embryonic development. These studies explore the harmful effects and beneficial contributions of these substances on embryo development. It has been observed that no conditions completely prevent embryo development, and the advantageous effects of the substances with antioxidant properties are also recognized.

Nisari et al.[16] investigated the *in vitro* effects of Interleukin-12 (IL-12) on embryonic vitelline sac vascularization and reported that IL-12 inhibited vitelline sac vascularization and heart development in a dose-dependent manner. Similarly, Uçar et al.[17] found that the addition of 1000 IU/ml Interferon Beta-1a (IFNβ-1a) and IFNβ-1b to the culture medium did not result in macroscopic teratogenic effects on embryos. However, they noted reduced measurements for vitelline sac diameter, head-tail length, and head length in the embryos in the experimental groups, suggesting that the specified doses of IFNβ-1a and IFNβ-1b caused growth retardation in embryos.

Yajun Xu et al.[18] studied the toxicity induced by ethanol during gestational days 6–15 in mice, examining the effects of vitamin B12 and FA. They administered ethanol intragastrically to CD-1 (Charles River 1) mice and co-administered separate doses of FA and B12 vitamin, as well as a combination of both. Their findings indicated that while individually administered FA and B12 vitamin could reduce the effects of ethanol, the combination of the two was more effective in mitigating the ethanol-induced toxicity.

The findings from our study reveal no significant differences in morphological scoring parameters between the embryos in the C group and those in the 1FA and 2FA groups. These results suggest that FA is not teratogenic and does not cause developmental abnormalities in embryos.

The protective effect of FA against neural tube defects (NTD) has prompted the implementation of mandatory FA fortification programs in numerous countries worldwide, including Canada, the United States, and the United Kingdom.[19] Daily FA supplementation for women of childbearing age has been proven to significantly lower the risk of NTD-affected pregnancies.[20–25] In a study by Wentzel et al.,[11] high-dose glucose was administered to rat embryos *in vitro*, forming different experimental groups: a control group (C), a group receiving only glucose, and a group receiving both glucose and FA supplementation. The results showed a significant increase in NTD incidence in the group receiving only glucose compared to the control. Additionally, embryos in the glucose-only group exhibited a decreased somite count and significantly shorter head-tail length compared to the control group. In contrast, the group supplemented with both glucose and FA exhibited a significant reduction in NTD incidence compared to the glucose-only group and had increased somite counts and longer head-tail lengths compared to the glucose-only group. However, compared to the control group (C), the glucose + FA group showed lower scores in terms of NTD incidence, somite count, and head-tail length. In a 2012 study by Koyama et al.,[7] the early embryonic development of rat embryos was investigated with a focus on blastocyst formation and cell proliferation. In this study, embryos were exposed to heat levels that did not compromise cellular viability. The experimental groups included a control group (C) and groups subjected to heat exposure, both with and without the addition of FA. It was reported that in the group without FA, heat exposure led to an accumulation of free radicals due to the heat's effect. Embryos exposed to heat demonstrated insufficient blastocyst development and a lower cell count compared to the control group (C). The developmental retardation observed in the heat-exposed embryos was attributed to the accumulation of free radicals. Conversely, the group exposed to heat and supplemented with FA exhibited improved blastocyst development and a higher cell count compared to those only exposed to heat. Koyama et al. (2012)[7] suggested that FA's antioxidant properties played a role in neutralizing the accumulated free radicals in the heat-exposed embryos, thereby supporting embryonic development. In a study by Patat et al. (2018),[26] the effects of vitamin B12 on the developmental processes of embryos exposed to hypoxia were investigated. The study highlighted the antioxidant properties of vitamin B12, showing that embryos exposed to hypoxia and supplemented with B12 exhibited better development than those in the hypoxia-only group (H group). The findings of our research align with those of Patat et al.[26]

CONCLUSION

In our study, various morphological scoring parameters of embryos—such as vitelline sac vascularization, allantois, flexion, heart, caudal neural tube, hindbrain, midbrain, forebrain, otic system, optic system, olfactory system, pharyngeal arch, maxillary protrusion, mandibular protrusion, forelimb, hindlimb, somites, total score, vitelline sac diameter, crown-rump length, and somite numbers—were assessed individually. We observed that embryos in the H1FA and H2FA groups showed improved development compared to those in the H group, according to our scoring results. It is presumed that the doses used in the experiment influenced development by affecting nutrient substrate levels. Although these findings were not statistically significant, the C group demonstrated better development compared to the 1FA and 2FA groups (Table 1).

The results of our study support previous findings on the antioxidant properties of FA. Specifically, FA has been shown to significantly prevent developmental abnormalities in embryos exposed to hypoxia.

Ethics Committee Approval: The Erciyes University Experimental Animal Ethics Committee granted approval for this study (date: 13.01.2016, number: 16/001).

Author Contributions: Concept – ED, MN; Design – ED, DÇ; Supervision – MN, TE; Resource – ED, DP; Materials – ED, SU; Data Collection and/or Processing – ED, EA; Analysis and/or Interpretation – ED, DP; Literature Search – ED, SU; Writing – ED; Critical Reviews – ED.

Conflict of Interest: The authors have no conflict of interest to declare.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

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