European Review for Medical and Pharmacological Sciences 2015; 9: 1130-1140

Folic acid attenuates dexamethasone-induced placental growth restriction

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Abstract. – OBJECTIVE: Intrauterine glucocorticoid (GC) exposure is associated with disturbances in feto-placental growth. This study aimed to investigate whether folic acid supplementation can prevent dexamethasone (Dex)-induced feto-placental growth restriction.

MATERIALS AND METHODS: Female C57BL/6J mice were subject to four different treatments, respectively: normal drinking water plus saline injection (NN), normal drinking water plus Dex injection (ND), drinking water supplemented with folic acid plus saline injection (FN), and drinking water supplemented with folic acid plus Dex injection (FD). Folic acid (100 μg/L) was administrated since 2 weeks before the mating and throughout pregnancy. Dex injection (100 μg/kg•d) was performed from E12.5 to E16.5. The placentas were collected at E17.5.

RESULTS: The parameters including placental and fetal weight, the maximum placental diameter, volume of junctional and labyrinthine zones, and vascular density in the ND group were significantly smaller compared to the NN group. Except the maximum placental diameter, each of the above parameters in the FD group was significantly larger compared to the ND group. The levels of glucocorticoid receptor (GR) protein, and *endothelial growth factor A*, C (*VEGFA*, *VEGFC*) and *placental growth factor* (*PIGF*) mRNAs were significantly lower in the ND group compared to NN group. The VEGFA and PIGF mRNA level in the FD group was significantly higher than that in the ND group, as well as VEGFA and VEGFC protein level.

CONCLUSIONS: Folic acid may attenuate Dex-induced restriction on placental growth by elevating the expression of *VEGFA* and *PIGF*, and further raising vascular density.

Key Words:

Placenta, Folic acid, Glucocorticoid, Dexamethasone, Feto-placental growth, Placental vascularity.

Abbreviations

GC = glucocorticoid; Dex = dexamethasone; GR = glucocorticoid receptor; VEGF = endothelial growth factor; PIGF = placental growth factor; IUGR = intrauterine growth restriction.

Introduction

Dexamethasone (Dex), a type of synthetic glucocorticoid (GC), is widely used in obstetric practice to promote fetal lung maturation in the cases of threatened preterm labor[1]. Additionally, it is a conventional medication adopted in pregnant women with autoimmune disorders, including asthma and systemic lupus erythematous[2]. However, increasing studies illustrate that intrauterine overexposure to GC disturbs feto-placental growth, and programs the onset of metabolic, cardiovascular and mental disorders in adult offspring, which is termed "fetal programming"[3-6]. During the gestation, placenta prevents fetus from direct exposure to maternal GC[7], although the placenta itself is exposed to a high GC level equal to that of the maternal circulation system. Dex-induced placental growth restriction has been reported by Hewitt et al[8], which proposed that glucocorticoids prevent the normal increase in placental vascular endothelial growth factor (VEGF) expression and placental vascularity during late pregnancy in rats. However, no programs have been presented so far to ameliorate these undesirable adverse effects associated with Dex administration.

During pregnancy, folic acid is essential to maternal health, fetal growth and placental development[9], as it is involved in multiple physiological processes, including angiogenesis and vasculogenesis, synthesis of DNA, RNA and proteins[10-12]. In many countries, including China, it is recommend that women take 400 μg/day of folic acid prior to conception and during the first month of pregnancy[13], as is supposed to prevent neural tube defects. Considerable evidence indicates that periconceptional folic acid supplementation decreases the risk of adverse pregnancy outcomes, including intrauterine growth restriction (IUGR), preterm birth and low birth

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weight[14,15]. A previous study by Miao et al[16] has announced that folic acid prevents and partially reverses Dex-induced hypertension in rats. However, little is known on its role in Dex-induced placental growth restriction.

In the present study, a long-term Dex administration model was established using mice, and four different medication treatments were designed to investigate whether folic acid can attenuate Dex-induced placental growth restriction.

Materials and Methods

Animals and Treatments

C57BL/6J mice were purchased form Slac Laboratory Animals Inc. (Shanghai, China). The procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Guide) and were approved by the Laboratory Animal Care Committee of Tongji University. All efforts were made to minimize the number of animals used and to reduce their suffering. The animals were maintained under controlled conditions as we described previously[17]. Virgin females of 8-10 weeks were mated with experienced males (1:1) overnight. The morning when a vaginal plug was found was designated as day 0.5 of pregnancy (embryonic day, E0.5).

The following four experimental groups were designed: (1) normal drinking water + saline injection group (NN); (2) normal drinking water + Dex injection group (ND); (3) drinking water with folic acid supplementation + saline injection group (FN); and (4) drinking water with folic acid supplementation + Dex injection group (FD). Folic acid (Sigma, St. Louis, MO, USA) supplementation began 2 weeks before the mating and continued throughout the pregnancy at a concentration of 100 μg/L, which is the equivalent of the specified dosage of 400 μg/day for humans[14,15]. Dex (Sigma, St. Louis, MO, USA) was dissolved in a 4% ethanol-0.9% saline solution at a final concentration of 1.0 mg/L[18]. The injection lasted from E12.5 to E16.5 at a dose of 100 μg/kg•d as previously described[5,18]. The dams were euthanized at E17.5[18,19]. The length of major axis was measured using digital calipers with 0.01-mm resolution. The placentas were snap frozen in liquid nitrogen before being stored at -80°C or fixed in 4% (w/v) paraformaldehyde. There were six dams in each of the four experimental groups. Among all the placentae of one dam, the one whose weight approaches the mean was selected for the later experiments. So, there were six placentae used for later tests in each group.

Determination of Placental Vascular Density By Immunofluorescence

Placental vascular density was estimated according to the CD31 intensity in the labyrinthine zone by immunofluorescence. The placentas were fixed in 4% (w/v) paraformaldehyde for 1-2 days and later prepared into paraffin-embedded sections of approx. 7 μm. Afterwards, the sections were rehydrated in PBS for 1 h before blocked with 5% bovine serum albumin, followed by incubation overnight at 4°C with an anti-CD31 antibody (R&D, Minneapolis, MN, USA) diluted by 1:50 in a blocking solution. After washing, the sections were incubated with the secondary antibody (Cy3-conjugated donkey anti-goat IgG, diluted 1:200; Santa Cruz, Dallas, TX, USA) for 50 min at room temperature. The cell nuclei were stained with 4, 6-diamidine-2-phenylindole dihydrochloride (DAPI; Beyotime, Shanghai, China). The negative controls were incubated with non-immune anti-rabbit IgG. Images of the labyrinthine zone were scanned by confocal microscopy with a Nikon Eclipse microscope (Melville, NY, USA), and four randomly collected images of each section were stored. The CD31 staining intensity was calculated by manual counting.

Measurement of Placental Volume

The placentas were fixed in 4% paraformaldehyde before processed in paraffin. Next, they were cut into halves along the placental short axis passing the point at which the umbilical cord is attached to placenta, and sectioned at a thickness of 7 μm, with three consecutive sections for each placenta. The sections were stained using hematoxylin and eosin, and then coverslipped. For each section, three images at × 4 magnification were randomly collected and stored. The area of the junctional and labyrinthine zones was calculated using ImageJ software. The component volume (V) of each placenta was calculated following the formula: $V = t \times s \times \text{sum (a)}$, where t is the total placental thickness calculated by multiplying the total number of sections by the section thickness; s refers to the sampling fraction; sum (a) refers to the sum of the zonal area[19].

RNA Extraction and Real-Time RT-PCR

The total RNA was extracted from whole placentas according to the manufacturer's instruction (RNA simple Total RNA Kit, Tiangen Biotech., Beijing, China). After determination of RNA concentration, 1.0 μg of mRNA was reverse-transcribed into cDNA using the PrimeScript® RT reagent Kit (Takara BIO Inc., Dalian, China). The reverse transcription (RT) was performed for 15 min at 37°C and 5 sec at 85°C in a final reaction volume of 20 μl.

Real-time RT-PCR was performed using the power SYBR Green PCR master mix (Takara BIO, Inc., Dalian, China) with *GAPDH*, *β-actin* and *18S* as the endogenous housekeeping genes, as described by Wu et al[20]. The RT-PCR protocol was as follows: 30 sec at 95°C for the incubation, 40 cycles of 15 sec at 95°C and 20 sec at 60°C. RT-PCR was repeated three times, with three replicates each time. The specificity of primers was verified by examining the melting curve. Relative expression level was calculated using the comparative Ct method[21]. The primer sequences of the mouse *VEGFA*, *VEGFC*, *VEGF* receptor 2 (VEGFR2), placental growth factor (PIGF), GAPDH, β-actin and 18S genes are illustrated in Table I.

Protein Extraction and Western Blot

The level of proteins GR, 11β-HSD1, 11β-HSD2, VEGFA, VEGFC, VEGFR2 and PIGF in the mouse placentas was measured by Western blot, which was performed twice. The tissues were added to 400 μl of standard RIPA (Beyotime, Shanghai, China) with 1% PMSF (v/v; Beyotime, Shanghai, China) and homogenized on ice for 30 min. The homogenate was centrifuged at 15000 rpm for 5 min at 4°C. The supernatant was collected and the protein concentration was evaluated using the BCA Protein Assay Kit (Beyotime, Shanghai, China).

Next, 50 μg of protein extract from each sample was subjected to SDS-PAGE on 10% gel and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then incubated at 4 overnight with the following antibodies: anti-GR (1:1000, Cell Signaling Technology, Beverly, MA, USA), anti-HSD11B1 (1:200, Abcam, Cambridge, MA, USA), anti-HSD11β2 (1:200, Abcam, Cambridge, MA, USA), anti-VEGFA (1:1500, Proteintech, Wuhan, China), anti-VEGFC (1:500, Santa Cruz, Dallas, TX, USA), anti-VEGFR2 (1:1000, Cell Signalling Technology, Beverly, MA, USA) and anti-PIGF (1:200, Santa Cruz, Dallas, TX, USA), respectively. After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase conjugated goat IgG antibody (Proteintech, Wuhan, China) diluted 1:2000 in 5% (w/v) skimmed milk. The membranes were rinsed three times with TBST, 10 min each, and once with distilled water. The identical samples were incubated with β-actin antibodies (1:8000, Proteintech, Wuhan, China) for 2 h at room temperature to confirm protein loading. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA). Images were analyzed using ImageJ software.

Statistical Analysis

Data are presented as the means ± SEM (standard error of the mean). The results of the fetal and placental weight were analyzed using General Linear Model for the analysis of the covariance (ANCOVA), with the litter number as the covariate. The data obtained from RT-PCR, Western blot and immunofluorescence were analyzed by one-way ANOVA. Multiple comparisons were performed by least significant difference (LSD) test when ANCOVA or ANOVA revealed statistical significance ($p < 0.05$). All statistical tests

Table I. Primers for analysis of mRNA expression by real-time RT-PCR.

Gene	Forward primer(5′ to 3′)	Reverse primer(5′ to 3′)
VEGFA	CAGGCTGCTGTAACGATGAA	CACCGCCTTGGCTTGTCACA
VEGFC	GGGAAGAAGTTCCACCATCA	ATGTGGCCTTTTCCAATACG
VEGFR2	TTCTGGACTCTCCCTGCCTA	AAGGACCATCCCACTGTCTG
PIGF	CTGCTGGGAACAACTCAACAGA	CTACAGCGACTCAGAAGGACACA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCAGTGGA
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
18S	GGCCGTTCTTAGTTGGTGGAGGG	CTGAACGCCACTTGTCCCTC

were performed using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). All graphs were generated by Graph-Pad Prism Version 6.00 (Graph-Pad Prism Software, Inc., La Jolla, CA, USA).

Results

Effect of Folic Acid Supplementation on Feto-Placental Physiologic Parameters

The pregnancy characteristics of mice in the four experimental groups are shown in Table II. The mean number of delivered litters was 8 with a range of 6-11 for each dam. The overall occurrence of fetus resorption was 4.6% (9/195). The mean maternal weight was 21.0 ± 1.0 g at E0.5 and 36.0 ± 2.7 g at E17.5. At E17.5, the placental weight and fetal weight of the ND group was 18% and 6% lower than that of the NN group, respectively ($p < 0.05$; Figure 1 A, C), which was consistent with the 15% decrease in the maximum placental diameter in the ND group ($p < 0.05$; Figure 1B). There was significant difference in either the placental weight or fetal weight between the ND and FD groups (Figure 1 A, C). Meanwhile, no significant difference was observed in maximum placental diameter between the FD and ND groups.

Effect of Folic Acid Supplementation on Placental Volume and Vascular Density

The stereological analyses exhibited that the volume of the junctional and labyrinthine zones in the ND group was 22% and 30% lower than that in the NN group, respectively ($p < 0.01$; Figure 2A, C). Meanwhile, there was also significant difference between the ND and FD groups in either the junctional or labyrinthine zone.

According to the result of immunofluorescence, CD31 intensity was 23% lower in the ND group compared to the NN group ($p < 0.01$; Figure 2B, D). Meanwhile, there was also significant difference between the ND and FD groups.

Effect of Folic Acid Supplementation on GR, 11β-HSD1 and 11β-HSD2 Expression

According to the result of Western blot, the level of GR protein in the placentas in the ND group was 51% lower compared to the NN group, with significant difference ($p < 0.05$), while there was no significant difference between the FD group with either the ND or FN group (Figure 3B). Meanwhile, there was no significant difference in the level of 11β-HSD1 protein between either NN and ND groups, or ND and FD groups (Figure 3B). Similarly, there was no significant difference in the level of 11β-HSD2 protein between either NN and ND groups, or ND and FD groups (Figure 3B).

Effect of Folic Acid Supplementation on Vasculogenic Genes and Proteins

According to the result of RT-PCR, the level of placental *VEGFA, VEGFC* and *PIGF* mRNAs in the ND group were 44%, 22% and 51% lower compared to the NN group, respectively, each with significant difference ($p < 0.05$, Figure 4A). By contrast, no significant difference in the *VEGFR2* mRNA was observed between NN and ND groups. Meanwhile, significant difference in the level of either *VEGFA* or *PIGF* mRNA was observed between the ND and FD groups. According to the result of Western blot, the level of either VEGFA or VEGFC protein in the FD group was significantly higher than that in the ND group. However, there was no difference in the level of either VEGFA, VEGFC, VEGFR2 or PIGF between the NN and ND groups (Figure 4B).

Table II. Pregnancy characteristics of 24 dams in the four groups.

	NN	ND	FN	FD	*p* value
Maternal weight at E0.5 (g)	21.0 ± 0.8	20.9 ± 1.1	20.8 ± 1.5	21.1 ± 0.9	1.0
Maternal weight at E17.5 (g)	36.7 ± 3.1	37.5 ± 2.7	35.6 ± 2.9	35.1 ± 2.4	0.43
Litter number (n)	7.8 ± 1.2	8.6 ± 1.4	9.0 ± 1.1	7.0 ± 1.2	0.06
Incidence of fetus resorption (n/%)	1/2.1	4/7.7	2/3.7	2/4.7	0.60

NN: normal drinking water + saline injection group; ND: normal drinking water + Dex injection group; FN: drinking water supplemented with folic acid + saline injection group; FD: drinking water supplemented with folic acid + Dex injection group.

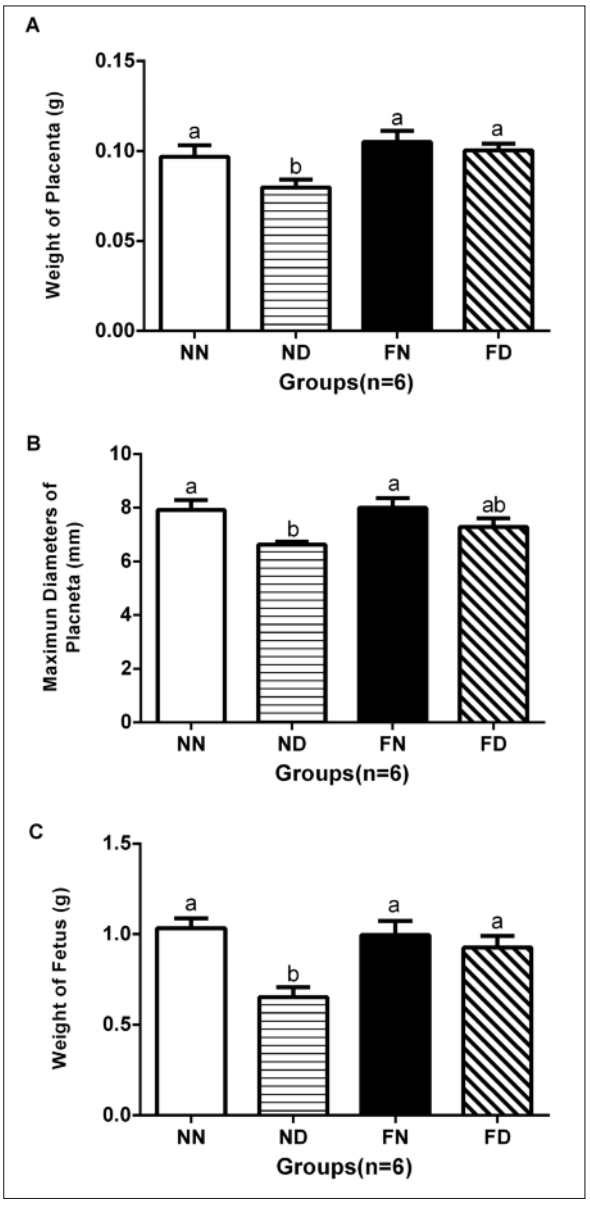

Figure 1. Effects of folic acid on placental and fetal physiologic parameters. ***A,*** Placental weight, ***(B)*** the maximum diameter of placenta, ***(C)*** fetal weight. The data represent means ± SEM from 6 dams in each group. The data are calculated as the litter average, and different small letters indicate significant differences ($p < 0.05$). NN, normal drinking water + saline injection group; ND, normal drinking water + dexamethasone (Dex) injection group; FN, drinking water supplemented with folic acid + saline injection group; FD, drinking water supplemented with folic acid + Dex injection group.

Discussion

In this study, folic acid was administrated to mice with long-term Dex injection to test whether it can attenuate Dex-induced placental growth restriction in mice by comparing feto-placental growth parameters and expression of related genes and proteins between different groups.

Dex administration was performed during the period of maximal growth rate and intense vascular remodelling in a murine placenta model at the dose that has been widely adopted in previous studies[5,18]. Significant declines were observed in the placental and fetal weight, maximum placental diameter, volume of placental junctional and labyrinthine zones after Dex administration. Thus, Dex administration during the late gestation arrests placental growth in mice. The placental growth restriction observed here coincided with the significant decrease in the levels of GR protein, *VEGFA*, *VEGFC* and *PIGF* mRNAs, as well as in the vascular density in the ND group. GC can enhance a diverse range of biological processes via binding to GR specifically[22,23]. Under normal circumstances, the level of *GR* mRNA in the labyrinthine zone almost increases by 3-fold between days 16 and 22, to facilitate a series of physiological processes, including syncytiotrophoblast differentiation and maturation and glucose transportation[22-24]. van Beek et al[25] have reported a *GR* mRNA reduction in human placental trophoblasts after Dex administration. Methylation of the promoter of *GR* gene in human placenta is also associated with low birth weight[26,27]. Since the levels of 11β-HSD (including two types 11β-HSD1 and 11β-HSD2) proteins were not significantly altered in the ND group, it seems GR has a more important role in Dex-induced growth restriction, other than 11β-HSD that are involved in GC metabolism, which is opposite to the finding that stated the elevated level of placental 11β-HSD2 protein at E17.5, while no change in GR protein in a short-term Dex-programmed mice model (1 mg/kg • h for 60 h via a mini pump beginning at E12.5)[28]. The divergence may be partially due to the long-term Dex administration in our study. Meanwhile, the levels of *VEGFA*, *VEGFC* and *PIGF* mRNAs were also significantly lower in the ND group compared to the NN group, these three genes may also have a critical role in Dex-induced feto-placental growth. Previously, Hewitt et al[8] have reported that Dex-induced restriction of fetal and placental growth is mediated, in part, via inhibition of placental *VEGFA* expression in the labyrinthine zone that leads to impaired vascularization. Our study suggests that *VEGFC* and *PIGF* may also be responsible for the reduced vascular density after Dex administration.

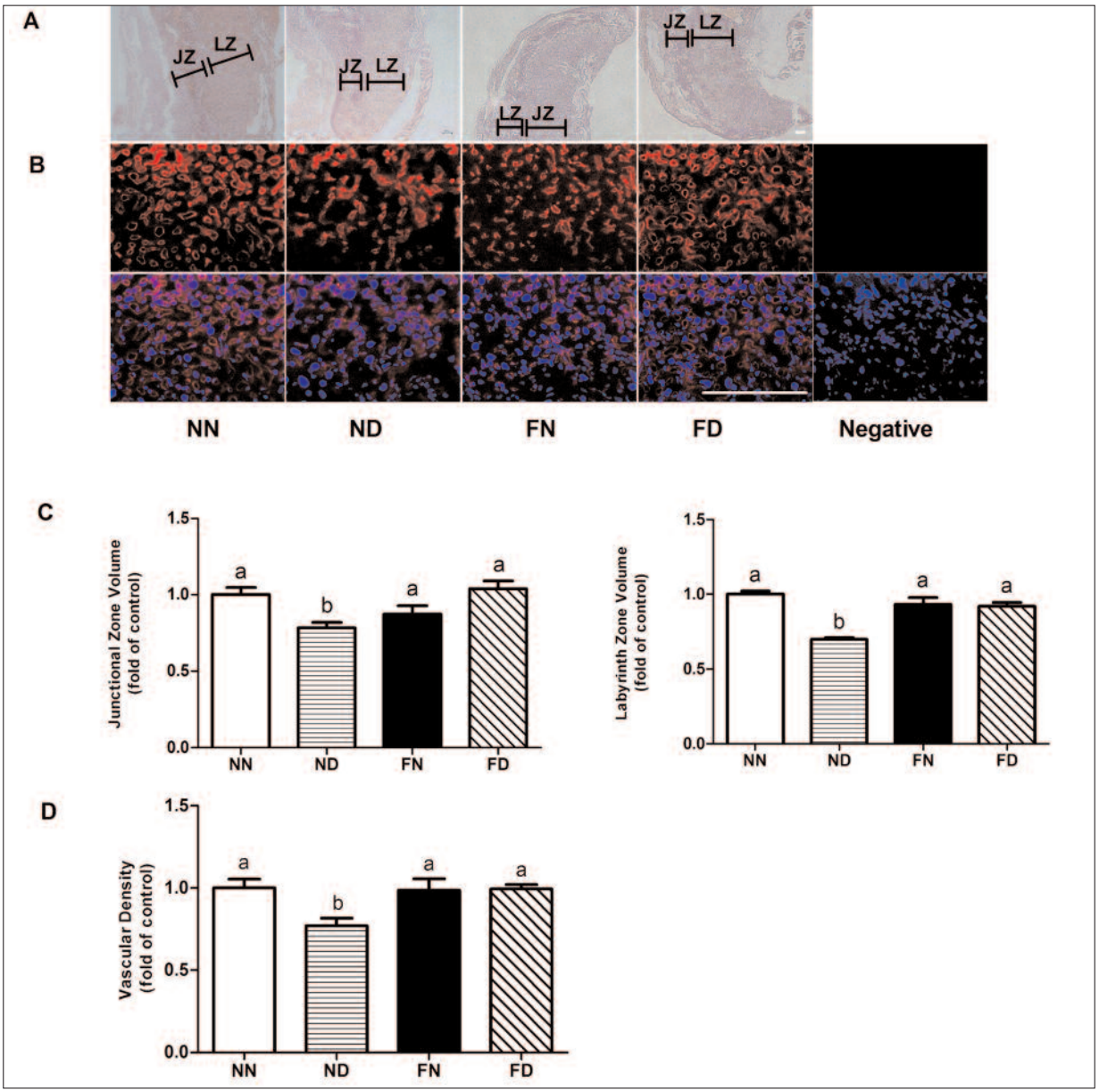

Figure 2. Placental zonal volume and vascular density. ***A,*** Hematoxylin-eosin staining of mice placenta at E 17.5. ***B,*** Immunofluorescence of the labyrinthine zone. The upper, vascular density in the labyrinth zone, as determined by CD31 immunofluorescence staining (red [Cy3]); the lower, merged images with nuclear staining (blue [DAPI]). ***C,*** Left, the placental junctional zone volume; right, the placental labyrinthine zone volume. ***D,*** Labyrinthine zone vascular density. The data represent the means ± SEM from 6 placentas of 6 dams in each group, and different small letters indicate significant differences ($p < 0.05$). Bar = 100 μm. JZ, junctional zone; LZ, labyrinthine zone; NN, normal drinking water + saline injection group; ND, normal drinking water + dexamethasone (Dex) injection group; FN, drinking water supplemented with folic acid + saline injection group; FD, drinking water supplemented with folic acid + Dex injection group.

Folic acid was administrated to mice since 2 weeks before conception and throughout the pregnancy at the dosage of 100 μg/L, which is much lower than the documented toxic dose that can result in the disruption of embryo development in mice[29,30]. After folic acid supplementation, the placental and fetal weight, and the maximum placental diameter, as well as the volume of placental junctional and labyrinthine zones of mice were significantly increased compared to those of mice administrated with Dex only. This implies that folic acid can ameliorate feto-pla-

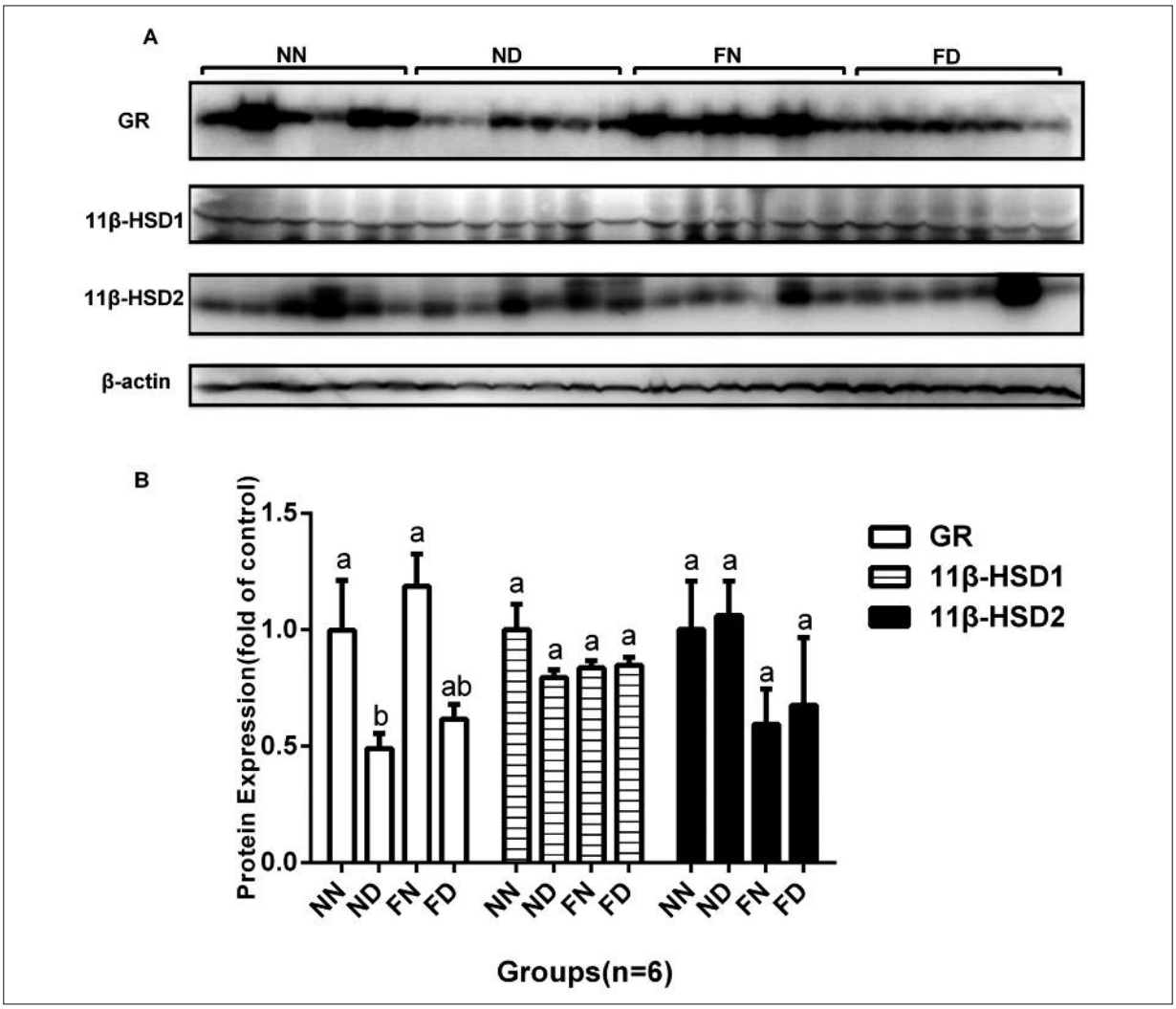

Figure 3. Expression of GR, 11β-HSD1 and 11β-HSD2 proteins in mouse placenta. ***A,*** Determination of the level of GR, 11β-HSD1 and 11β-HSD2 proteins at E17.5 by Western blot. ***B,*** Multiple comparison of GR, 11β-HSD1 and 11β-HSD2 proteins levels. Protein expression is normalized to β-actin. The data represent means ± SEM from 6 placentas of 6 dams in each group; different small letters indicate significant differences ($p < 0.05$). NN, normal drinking water + saline injection group; ND, normal drinking water + dexamethasone (Dex) injection group; FN, drinking water supplemented with folic acid + saline injection group; FD, drinking water supplemented with folic acid + Dex injection group.

cental growth restriction. Meanwhile, the vascular density of mice in the FD group was also significantly higher than that of the ND group. Since the level of *VEGFA* and *PIGF* mRNAs were significantly higher in the FD group than that of the ND group, it may be inferred that folic acid may attenuate Dex-induced feto-placental growth restriction partially by elevating the levels of *VEGFA* and *PIGF* mRNAs. Actually, it has been previously reported that folic acid restores the capillary density in Sprague-Dawley rats with hypercholesterolemia[31], and folic acid supplementation normalizes impaired vascular morphology in diabetic rats and increases the *VEGFA* mRNA level in the yolk sac of embryos[32]. Although placental vasculogenesis could be modulated by many factors, the parallel changes in *VEGFA* and *PIGF* with the administration of Dex and folic acid indicate that these two factors might be major modulators in this process.

Up to now, the detailed mechanisms by which Dex and folic acid affect the expression of VEGFs are still uncertain. Recent studies have shown that gene-specific DNA methylation might lead to alterations in feto-placental development as a result of intrauterine GC expo-

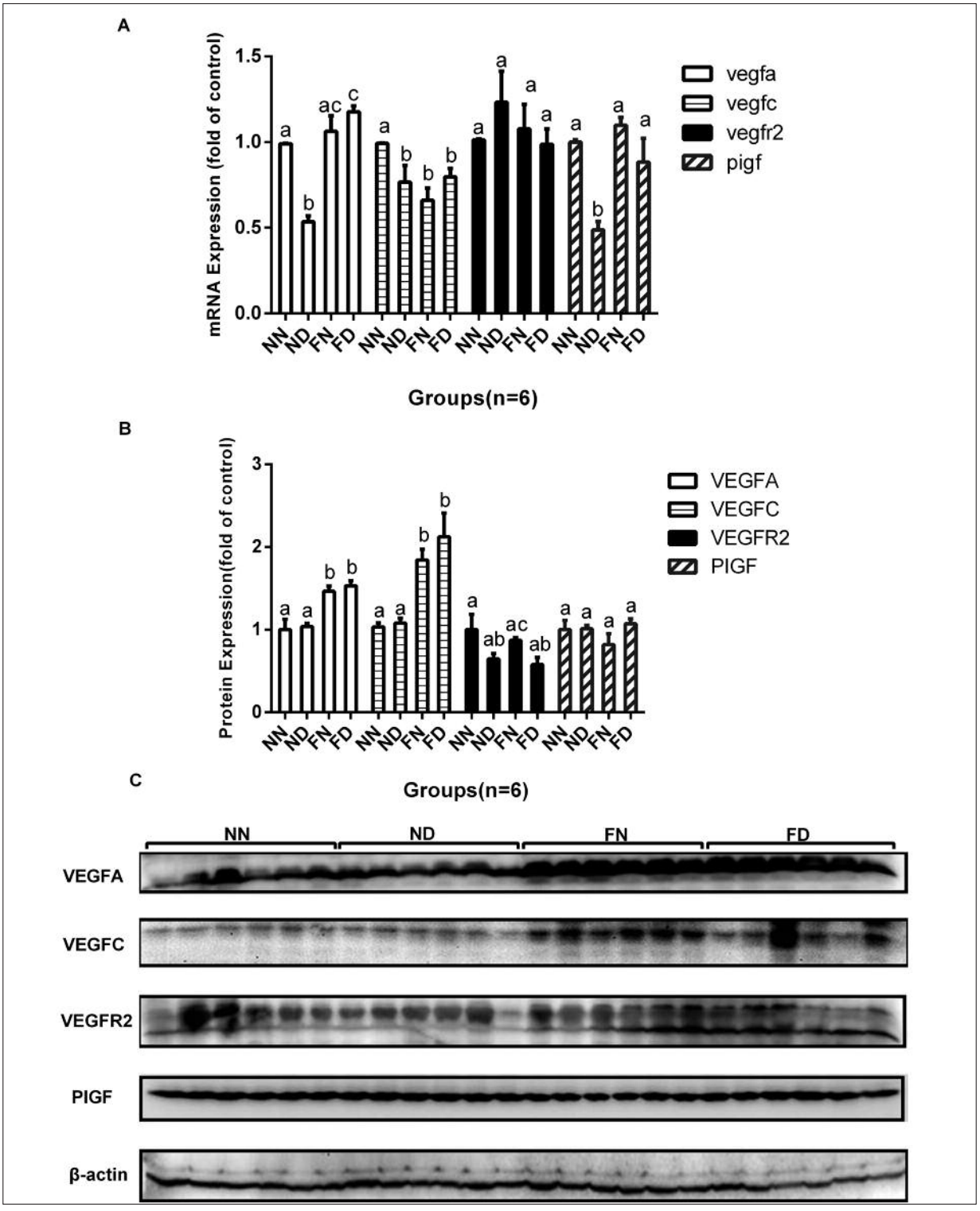

Figure 4. Expression of VEGFA, VEGFC, VEGFR2 and PIGF at E17.5 in mouse placenta. ***A,*** Multiple comparison of VEGFA, VEGFC, VEGFR2 and PIGF mRNAs levels between groups. ***B,*** Multiple comparison of VEGFA, VEGFC, VEGFR2 and PIGF proteins levels between groups. The protein expression is normalized to β-actin. ***C,*** Expression of VEGFA, VEGFC, VEGFR2 and PIGF Proteins. The data represent the means ± SEM from 6 placentas of 6 dams in each group; different small letters indicate significant differences ($p < 0.05$). NN, normal drinking water + saline injection group; ND, normal drinking water + dexamethasone (Dex) injection group; FN, drinking water supplemented with folic acid + saline injection group; FD, drinking water supplemented with folic acid + Dex injection group.

sure[33,34]. Folic acid, as a one-carbon donor, is involved in DNA methylation in the placenta[35]. Most likely, folic acid may modulate the alterations in the methylation of *VEGF* promoters that are induced by excessive intrauterine Dex. Additionally, Dex and folic acid may also affect *VEGF* expression through indirect modulation. It has been reported Dex reduces the placental expression of peroxisome proliferator-activated receptors (PPARs)[36] that up-regulate *VEGF* expression[37,38]. As well, folic acids enhances the synthesis of endothelial nitric oxide (eNO)[39], which is involved in the synthesis of peroxynitrite ($ONOO^-$)[40] that induces VEGF expression in many cell types[41,42]. Thus, the mechanisms by which Dex and folic acid affect *VEGF* expression need to be further elucidated.

Conclusions

Folic acid may attenuate Dex-induced restriction on placental growth by evaluating the expression of *VEGFA* and *PIGF,* and further raising vascular density. This study is the first one supporting the viewpoint that periconceptional folic acid might attenuate Dex-induced feto-placental growth restriction, thus providing a basis for supplementing folic acid to ameliorate the adverse effects of GC overexposure. However, since there were no significant differences in the levels of *VEGFC* and *VEGFR2* mRNAs, VEGFR2 proteins, between the FD and ND groups, and also no significant difference in VEGFA and VEGFC proteins between the NN and ND groups, more experimental proofs are required to support this conclusion. In addition, fetal gender difference was not considered here, despite gender may be an important factor influencing the effect of folic acid administration. Actually, sex-specific effects of intrauterine Dex overexposure on feto-placental growth have been reported previously[19,28]. Since the present study just aimed to preliminarily investigate the effect of folic acid administration on feto-placentas, the effect of gender on the consequence will be elaborated in future studies.

Statement of Interests

This study was supported by the National Natural Science Foundation of China (No. 81270759) and the Major State Basic Research Development Program of China (973 Program) No. 2012CB966300.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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