Arginine enhances embryo implantation in rats through PI3K/PKB/mTOR/NO signaling pathway during early pregnancy

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Abstract

Our previous study has demonstrated that dietary arginine supplementation during early pregnancy enhanced embryo implantation in rats. However, the mechanism was not clear. The objective of this study was to determine the mechanism that arginine enhanced embryo implantation during early pregnancy. Rats were fed the basal diets supplemented with 1.3% (wt:wt) L-arginine-HCl or 2.2% (wt:wt) L-alanine (isonitrogenous control) once pregnancy. On d4 of pregnancy, rats were given intrauterine injection of L-NG-nitro arginine methyl ester (L-NAME, nitric oxide synthase inhibitor), α -difluoromethylornithine (DFMO, polyamine synthesis inhibitor), wortmannin (PI3K inhibitor), or rapamycin (mTOR inhibitor). On d7 of pregnancy, rats were killed. Intrauterine injection of L-NAME decreased the implantation sites, while dietary arginine supplementation increased the implantation sites. Intrauterine injection of DFMO decreased the pregnancy rate, which was reversed by dietary arginine supplementation. Intrauterine injection of rapamycin or wortmannin inhibited embryo implantation. However, dietary arginine supplementation did not reverse this inhibition. Western blot analysis revealed that the expression of uterine p-PKB and p-S6K1 was greater in rats fed the arginine-supplemented diet in the presence of L-NAME treatment compared with rats fed the control diet. In the presence of DFMO treatment, the expression of uterine iNOS and eNOS was significantly enhanced in the arginine group compared with the control group. Similarly, intrauterine injection of wortmannin or rapamycin decreased the expression of uterine iNOS and eNOS, which was enhanced by dietary arginine supplementation. These data indicated that dietary arginine supplementation during early pregnancy could enhance embryo implantation through stimulation of PI3K/PKB/mTOR/NO signaling pathway.

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Introduction

Amino acid signaling is required in embryo implantation, which does not only support protein synthesis and trophoblast differentiation but also regulates development of trophoblast protrusive activity (Martin *et al.* 2003). L-arginine, the conditional-essential amino acid, plays important roles in a variety of physiological conditions including embryogenesis, embryo implantation, embryonic/fetal survival, growth, and development (Fozard *et al.* 1980a, Zeng *et al.* 2008, Wu *et al.* 2010). Besides, L-arginine can also be used to prevent or treat fetal growth restriction in women and rats (Vosatka *et al.* 2005, Xiao & Li 2005). Dietary 1% L-arginine-HCl supplementation could markedly increase the live-born piglets by 2 per litter (Mateo *et al.* 2007). Daily supplementation of 26 g L-arginine to gilts from d14 to d28 of gestation enhanced fetal survival and positively affected the primary phase of myofiber formation (Bérard & Bee 2010). Besides, dietary arginine supplementation could decrease maternal abortion rate and neonatal

mortality in mice with porcine circovirus type 2 infection (Ren *et al.* 2012). i.v. injection of arginine could enhance fetal brown adipose tissue growth in nutrition-restricted sheep (Satterfield *et al.* 2011). In sows, dietary arginine supplementation could improve placental growth and reproductive performance (Kim *et al.* 2007, Liu *et al.* 2011, Gao *et al.* 2012). Our previous study demonstrated that dietary arginine or N-carbamylglutamate (an activator of arginine synthesis) supplementation during early pregnancy increased the embryonic implantation sites in rats (Zeng *et al.* 2008, 2012). All the effects following L-arginine supplementation were potentially attributed to the increase in nitric oxide production. However, arginine is involved in other metabolic pathways such as polyamines, creatine, and cell signaling pathways (Flynn *et al.* 2002, Martin *et al.* 2003).

L-arginine serves as the essential precursor for polyamines via arginase and ornithine decarboxylase (ODC; Flynn *et al.* 2002). The polyamines including putrescine, spermidine, and spermine are required for DNA and protein synthesis, as well as proliferation and

differentiation of mammalian cells (Igarashi & Kashiwagi 2000, Ishida *et al.* 2002). Inhibition of placental ODC by α -difluoromethylornithine (DFMO) induced fetal growth restriction in rats (Ishida *et al.* 2002). Intrauterine injection of DFMO on d4 of pregnancy inhibited embryonic development and embryogenesis in rats (Méndez *et al.* 1983). Previous study demonstrated that arginine stimulated proliferation of ovine trophoblast cells via mTOR signaling pathway and nitric oxide and polyamines (Kim *et al.* 2011). We hypothesize that dietary arginine supplementation during early pregnancy may activate PI3K–PKB–mTOR signaling pathway, which could increase the expression of uterine iNOS and eNOS and inhibition of polyamine synthesis could increase NO production and release. In this study, through intrauterine injection of the inhibitors of NOS, ODC, PI3K, and mTOR, we determined the signaling pathway that mediates arginine in enhancing embryo implantation.

Results

Effects of intrauterine injection of L-NG-nitro arginine methyl ester, DFMO, wortmannin, or rapamycin on embryo implantation

In rats fed the control diet, the number of implantation sites in the L-NG-nitro arginine methyl ester (L-NAME)-treated uterine horns was significantly decreased compared with that in the vehicle-treated control horns (1.2 ± 0.73 vs 6.2 ± 0.58 , $P < 0.05$, Table 1). In rats fed the arginine-supplemented diet, the number of implantation sites in the L-NAME-treated uterine horns was also decreased compared with that in the vehicle-treated control horns (4.84 ± 0.75 vs 7.83 ± 1.01 , $P < 0.05$, Table 1). However, compared with the control diet, dietary arginine supplementation could markedly increase the number of implantation sites in the L-NAME-treated uterine horns (1.20 ± 0.73 vs 4.84 ± 0.75 , $P < 0.05$, Table 1). Additionally, in rats fed the control diet, the number of implantation sites in the DFMO-treated uterine horns was drastically decreased (half of the DFMO-treated uterine horns had no embryos implanted), in comparison with that in the vehicle-treated

control horns (2.50 ± 1.15 vs 6.33 ± 0.42 , $P < 0.05$, Table 1). In contrast, in rats fed the arginine-supplemented diet, the number of implantation sites in the DFMO-treated uterine horns and vehicle-treated control horns did not significantly differ (5.50 ± 0.43 vs 6.83 ± 0.31 , $P > 0.05$, Table 1). Furthermore, dietary arginine supplementation significantly increased the number of implantation sites in the DFMO-treated uterine horns compared with the control diet (2.50 ± 1.15 vs 5.50 ± 0.43 , $P < 0.05$, Table 1), because in rats fed the control diet, half of the DFMO-treated uterine horns had no embryos implanted. In other words, dietary arginine supplementation significantly increased the pregnancy rate in the DFMO-treated uterine horns compared with the control diet (50 vs 100%). Both wortmannin (Table 1) and rapamycin (Table 1) treatment almost totally inhibited embryo implantation. Furthermore, dietary arginine supplementation could not reverse this situation compared with the control diet (Table 1).

The expression of iNOS, eNOS, p-PKB, and p-S6K1 in the presence of L-NAME, DFMO, wortmannin, and rapamycin

L-NAME treatment almost completely blocked the expression of uterine iNOS and eNOS in rats fed the control diet (Fig. 1A). However, dietary arginine supplementation during early pregnancy significantly increased the expression of uterine iNOS, eNOS, p-PKB, and p-S6K1 in the presence of L-NAME (Fig. 1B, $P < 0.05$). Under circumstance of intrauterine injection of DFMO, the expression of uterine iNOS and eNOS was much higher in rats fed the arginine-supplemented diet compared with that in rats fed the control diet (Fig. 2A and B, $P < 0.05$). With intrauterine injection of wortmannin, the expression of uterine p-PKB was almost totally inhibited in both the control diet group and the arginine-supplemented diet group (Fig. 3A). By contrast, the expression of uterine iNOS and eNOS was dramatically enhanced in arginine-supplemented group compared with that in the control group (Fig. 3B, $P < 0.05$). Similarly, with intrauterine injection of rapamycin, the expression of uterine p-S6K1 was totally

Table 1 Effects of intrauterine injection of L-NAME, DFMO, wortmannin, or rapamycin on the number of implantation sites in rats fed the control diet or arginine-supplemented diet. Data are mean $\pm$ S.E.M., $n = 6$.

Treatment	The number of implantation sites			
	Control diet		Arginine-supplemented diet	
	Left uterine horn (drugs-treated)	Right uterine horn (vehicle treated)	Left uterine horn (drugs treated)	Right uterine horn (vehicle treated)
L-NAME	1.20 ± 0.73^a	6.20 ± 0.58^b	4.83 ± 0.75^b	7.83 ± 1.01^c
DFMO	2.50 ± 1.15^a	6.33 ± 0.42^b	5.50 ± 0.43^b	6.83 ± 0.31^b
Wortmannin	0 ^a	6.16 ± 0.47^b	0 ^a	6.83 ± 0.40^b
Rapamycin	0 ^a	7.0 ± 0.57^b	0 ^a	7.66 ± 0.78^b

Means in a row with different letters indicate significance ($P < 0.05$).

Figure 1 Effects of dietary arginine supplementation on uterine iNOS, eNOS, p-S6K1, and p-PKB expression in the presence of intrauterine injection of L-NAME. (A) Representative western blots for uterine iNOS, eNOS, p-S6K1, p-PKB, and β -actin in rats. (B) The ratios of uterine iNOS, eNOS, p-S6K1, and p-PKB to β -actin. *Different from the control group, $P < 0.05$. Rats were fed the diets supplemented with 1.3% (wt:wt) L-arginine-HCl or 2.2% (wt:wt) L-alanine (isonitrogenous control) between d1 and d7 of gestation. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of L-NAME (NOS inhibitor, 2.5 mg, diluted in 50 μ l 0.9% saline) after anesthesia. The right uterine horns of the rats were received 50 μ l 0.9% saline. At the end of the supplementation, the uteri of rats were obtained for western blot analysis.

inhibited in two treatment groups (Fig. 4A). However, the expression of uterine iNOS and eNOS was increased significantly in arginine-supplemented group compared with that in the control group (Fig. 4B, $P < 0.05$).

Discussion

Amino acids are critical to pregnancy because of its influence on embryo and fetal growth and development (Kim *et al.* 2011, Wu 2010). Our previous study demonstrated that dietary arginine supplementation during early pregnancy enhanced the number of embryos implanted and the expression of iNOS and eNOS in implantation sites was increased in rats fed the arginine-supplemented diet (Zeng *et al.* 2008). However, the mechanism of the increase in iNOS and eNOS expression by dietary arginine supplementation is not yet known. NO plays important roles in embryo development and implantation (Biswas *et al.* 1998,

Ota *et al.* 1999, Chwalisz & Garfield 2000, Manser *et al.* 2004). NOS inhibition arrested blastocyst development and trophoblast outgrowth *in vitro* (Biswas *et al.* 1998, Gouge *et al.* 1998). Intrauterine administration of NOS inhibitors significantly decreased the number of implanted embryos in rats (Biswas *et al.* 1998). Similarly, in the current study, NOS inhibition during periimplantation period dramatically inhibited embryo implantation in rats. However, dietary arginine supplementation drastically increased the number of implanted embryos in the presence of NOS inhibitor. Additionally, the expression of uterine iNOS and eNOS was increased in rats fed the arginine-supplemented diet in the presence of NOS inhibitor compared with that in rats fed the control diet. Consistent with our result, arginine addition to the culture medium could reverse the inhibition of normal embryo development induced by NOS inhibitor (Gouge *et al.* 1998). Nitric oxide, a product of arginine via NOS, plays an important role in blood flow control (Cornelissen 2011). Recent studies showed that arginine supplementation could increase the uterine blood flow in women and mare potentially via nitric oxide pathway (Takasaki *et al.* 2010, Mortensen *et al.* 2011). Greene *et al.* (2012) demonstrated that dietary arginine supplementation during pregnancy could enhance vegfr2

Figure 2 Effects of dietary arginine supplementation on uterine iNOS and eNOS expression in the presence of intrauterine injection of DFMO. (A) Representative western blots for uterine iNOS, eNOS, and β -actin in rats. (B) The ratios of uterine iNOS and eNOS to β -actin. *Different from the control group, $P < 0.05$. Rats were fed the diets supplemented with 1.3% (wt:wt) L-arginine-HCl or 2.2% (wt:wt) L-alanine (isonitrogenous control) between d1 and d7 of gestation. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of DFMO (polyamine synthesis inhibitor, 0.5 mg/kg, diluted in 50 μ l 0.9% saline) after anesthesia. The right uterine horns of the rats were received 50 μ l 0.9% saline. At the end of the supplementation, the uteri of rats were obtained for western blot analysis.

Figure 3 Effects of dietary arginine supplementation on uterine iNOS, eNOS, and p-PKB expression in the presence of intrauterine injection of wortmannin. (A) Representative western blots for uterine iNOS, eNOS, p-PKB, and β -actin in rats. (B) The ratios of uterine iNOS and eNOS to β -actin. The ratio of p-PKB to β -actin is not shown here because the bands were too weak to be quantified. *Different from the control group, $P < 0.05$. Rats were fed the diets supplemented with 1.3% (wt:wt) L-arginine-HCl or 2.2% (wt:wt) L-alanine (isonitrogenous control) between d1 and d7 of gestation. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of wortmannin (PI3K inhibitor, 20 μ g, diluted in 50 μ l 0.9% saline) after anesthesia. The right uterine horns of the rats were received 50 μ l 0.9% saline. At the end of the supplementation, the uteri of rats were obtained for western blot analysis.

transcription activity in the fetoplacental tissues in mice, which is beneficial to angiogenesis or vascular development in fetoplacental units. Additionally, increase in uteroplacental blood flow may result in the increased transfer of nutrients and O_2 from mother to fetus, which may improve fetal growth and development (Wu *et al.* 2006). Therefore, it is possibly that arginine might stimulate nitric oxide production, which could increase uterine blood flow and enhance normal embryonic development, thus enhancing the embryo implantation.

However, L-arginine is a common substrate for nitric oxide and polyamines (Wu *et al.* 2009). Inhibition of NOS might increase the production of polyamines. Polyamines are essential in embryo implantation (Fozard *et al.* 1980b). ODC was strongly expressed in implantation sites. DFMO, an irreversible inhibitor of ODC, can inhibit polyamine synthesis and significantly inhibit embryo implantation (Zhao *et al.* 2008). In order to exclude the contribution of the arginine-polyamine pathway to the improvement in embryo implantation, it is speculated that inhibition of polyamine synthesis could increase the availability of arginine to release nitric oxide. In this study, the pregnancy rate of rats with intrauterine injection of DFMO was significantly decreased, while dietary arginine supplementation could increase the pregnancy rate in the presence of

DFMO. These data further indicated that dietary arginine supplementation might increase NO production, which could enhance embryo implantation under the condition that polyamine synthesis is inhibited. Additionally, the expression of uterine iNOS and eNOS was significantly increased in rats fed the arginine-supplemented diet in the presence of uterine injection with DFMO compared with the control group, indicating that the arginine-nitric oxide pathway probably played critical roles in enhancing embryo implantation.

However, there is little known about the intermediate signaling pathway between arginine and NO during embryo implantation. PI3K/PKB/mTOR signaling pathway is a critical signaling pathway for amino acids and of importance for embryo implantation (Martin *et al.* 2003). Inhibition of the PI3K/PKB pathway had an effect on the normal physiology of the blastocyst, as well as blastocyst hatching (Riley *et al.* 2005). Wortmannin, the PI3K inhibitor, prevents the phosphorylation and activation of PKB. PI3K inhibition caused fetal resorptions and poor pregnancy outcome (Riley *et al.* 2006). Similarly, we found that wortmannin completely blocked embryo implantation and dietary arginine

Figure 4 Effects of dietary arginine supplementation on uterine iNOS, eNOS, and p-S6K1 expression in the presence of intrauterine injection of rapamycin. (A) Representative western blots for uterine iNOS, eNOS, p-S6K1, and β -actin in rats. (B) The ratios of uterine iNOS and eNOS to β -actin. The ratio of p-S6K1 to β -actin is not shown here because the bands were too weak to be quantified. *Different from the control group, $P < 0.05$. Rats were fed the diets supplemented with 1.3% (wt:wt) L-arginine-HCl or 2.2% (wt:wt) L-alanine (isonitrogenous control) between d1 and d7 of gestation. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of rapamycin (20 μ g, diluted in 50 μ l 0.9% saline) after anesthesia. The right uterine horns of the rats were received 50 μ l 0.9% saline. At the end of the supplementation, the uteri of rats were obtained for western blot analysis.

supplementation could not reverse this inhibition, indicating that the improvement in embryo implantation by the increase in nitric oxide from dietary arginine supplementation could not be detected in the presence of PI3K inhibition. Previous study demonstrated that PKB activation increased nitric oxide production and release, while PKB activation deficiency decreased nitric oxide production in endothelial cells (Dimmeler *et al.* 1999). Similarly, the expression of uterine iNOS and eNOS was drastically decreased in rats fed the control diet in the presence of wortmannin. Although the expression of uterine iNOS and eNOS was increased in rats fed the arginine-supplemented diet in the presence of wortmannin, it is still not strong enough to increase the embryo implantation. These data indicated that the PI3K/PKB signaling pathway might regulate nitric oxide production and release from dietary arginine supplementation that enhanced embryo implantation.

The important downstream of PI3K–PKB signaling pathway is mTOR (Rohde *et al.* 2001). Importantly, the mTOR signaling pathway also plays important roles in embryo implantation (Martin *et al.* 2003). mTOR deficiency caused embryonic death soon after implantation (Gangloff *et al.* 2004). Intrauterine administration of rapamycin on the morning of d4 of pregnancy greatly decreased the implantation sites in mice (Chen *et al.* 2009). In porcine trophoblast cells, arginine could stimulate the mTOR signaling pathway, which could increase protein synthesis and decrease protein degradation (Kong *et al.* 2012). This may be beneficial to embryonic and fetal growth and survival. Besides, in mouse embryo, arginine could regulate trophoblast motility through mTOR signaling pathway during the preimplantation period (González *et al.* 2012). In our study, intrauterine injection of rapamycin during periimplantation period totally inhibited embryo implantation. Besides, the positive effect of dietary arginine supplementation on embryo implantation was also abolished in the presence of mTOR inhibitor. Additionally, the expression of uterine iNOS and eNOS was greater in the arginine-supplemented group than that in the control group in the presence of wortmannin or rapamycin. Interestingly, in the presence of L-NAME, the expression of uterine p-PKB and p-S6K1 was significantly increased in rats fed the arginine-supplemented diet compared with that in rats fed the control diet. These data demonstrated that nitric oxide was likely the downstream of the PI3K–PKB–mTOR signaling pathway in the process of arginine enhancing the embryo implantation.

In conclusion, dietary arginine supplementation during early pregnancy could enhance embryo implantation through the increase in nitric oxide production and release, which may require PI3K–PKB–mTOR signaling pathway. Our results provide important foundation for dietary arginine supplementation to improve embryo implantation and early embryonic survival in mammals.

Materials and Methods

Animals and experimental design

All rats used in this study were housed and handled according to the established guidelines of the China Department of Agriculture. All procedures performed on the animals were approved by the China Agricultural University Animal Care and Use Committee.

Adult female Sprague Dawley rats, weighing 220–250 g, were purchased from Beijing Laboratory Animal Center (Beijing, China). They were housed in cages in a temperature, humidity, and light-controlled room with free access to food and water. Pregnancy was produced by overnight caging of a proestrous female with a fertile male rat. The next morning, the presence of spermatozoa in the vaginal smear was defined as d1 of pregnancy. Forty-eight pregnant rats were used in the following experiments.

Experiment 1 was conducted to determine the effect of NOS inhibition on embryo implantation. Twelve pregnant rats were randomly assigned to be fed the isonitrogenous control (alanine-supplemented, 2.2% (w/w), $n=6$) or the arginine-supplemented diet (1.3% (w/w) L-arginine–HCl, $n=6$). The basal diet was a corn-, soybean meal-, flour-, and fishmeal-based rodent nonpurified diet (Science Australia United Efforts Incorporation). The content of the basal diet was the same as previous study (Zeng *et al.* 2008). On the morning of d4 of pregnancy, all the pregnant rats underwent midventral laparotomy after anesthetized with sodium pentobarbital. The fat pad that accompanies ovary and uterus was gently pulled out from the body cavity. L-NAME (2.5 mg, diluted in 50 μ l 0.9% saline) was slowly injected into the left uterine horn of each rat from the cervix toward the uterotubal junction. Each pregnant rat served as her own control, with the right uterine horn receiving 50 μ l 0.9% saline. All the rats were killed on d7 of pregnancy after anesthetized with sodium pentobarbital. After the number of implantation sites was recorded, the uteri were harvested immediately and frozen in liquid nitrogen for western blot analysis.

Experiment 2 was conducted to exclude the contribution of the arginine-polyamine pathway to the improvement in embryo implantation. Twelve pregnant rats were randomly assigned to be fed the isonitrogenous control ($n=6$) or the arginine-supplemented diet ($n=6$) as described in experiment 1. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of DFMO (polyamine synthesis inhibitor, 0.5 mg/kg, diluted in 50 μ l 0.9% saline). The right uterine horns of the rats were received 50 μ l 0.9% saline. All the rats were killed on d7 of pregnancy after anesthetized with sodium pentobarbital. After the number of implantation sites was recorded, the uteri were harvested immediately and frozen in liquid nitrogen for western blot analysis.

Experiment 3 was conducted to determine whether the PI3K/PKB signaling pathway was involved in arginine/NO improving embryo implantation. Twelve pregnant rats were randomly assigned to be fed the isonitrogenous control ($n=6$) or the arginine-supplemented diet ($n=6$) as described in experiment 1. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine

administration of wortmannin (PI3K inhibitor, 20 µg, diluted in 50 µl 0.9% saline). The right uterine horns of the rats were received 50 µl 0.9% saline. All the rats were killed on d7 of pregnancy after anesthetized with sodium pentobarbital. After the number of implantation sites was recorded, the uteri were harvested immediately and frozen in liquid nitrogen for western blot analysis.

Experiment 4 was conducted to determine whether the mTOR signaling pathway was involved in arginine/NO improving embryo implantation. Twelve pregnant rats were randomly assigned to be fed the isonitrogenous control ($n=6$) or the arginine-supplemented diet ($n=6$) as described in experiment 1. On the morning of d4 of pregnancy, the left uterine horns of the rats were received intrauterine administration of rapamycin (mTOR inhibitor, 20 µg, diluted in 50 µl 0.9% saline). The right uterine horns of the rats were received 50 µl 0.9% saline. All the rats were killed on d7 of pregnancy after anesthetized with sodium pentobarbital. After the number of implantation sites was recorded, the uteri were harvested immediately and frozen in liquid nitrogen for western blot analysis.

Western blot analysis

The uteri (because the uninvolved uterine wall did not affect the analysis of the results) were homogenized in RIPA lysis buffer containing protease inhibitor cocktails (Amresco, Solon, OH, USA). After 30-min incubation, homogenates were centrifuged at 14 000 *g* for 15 min at 4 °C. Then supernatant fluid was collected and stored at −80 °C. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins (35 µg total protein for iNOS, eNOS, p-PKB, p-S6K1, and β-actin respectively) were electrophoresed (Bio-Rad, Richmond, CA, USA) on SDS–polyacrylamide gels. Proteins were electrotransferred to a PVDF membrane (Millipore, Bedford, MA, USA) and blocked with 5% nonfat dry milk at 4 °C overnight. Prestained protein markers (Fermentas, Glen Burnie, MD, USA) were run in each gel. Samples were incubated with rabbit anti-rat polyclonal antibodies (1:1000 dilution for 2 h at room temperature or overnight at 4 °C) against iNOS, eNOS, p-PKB, p-S6K1, or β-actin (anti-iNOS, anti-eNOS, anti-p-S6K1, and anti-β-actin, Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-p-PKB, Cell Signaling Technology, Beverly, MA, USA). After being washed with Tris–Tween 20 buffer (pH 7.4) for three times, membranes were incubated with the HRP-conjugated goat anti-rabbit IgG (ZSGB-BIO, Beijing, China) for 45 min at room temperature, and wash three times as previously. Band densities were detected with the western blotting luminance reagent (Santa Cruz). The membrane was exposed to the X-ray film for 1–6 min. Band densities were quantified using Alphamager 2200 (Alpha Innotech San Leandro, CA, USA) software.

Statistical analysis

Data were analyzed using the procedures of SAS (SAS Institute) for a randomized complete block design. Pregnant dam was considered as the experimental unit. Data were analyzed using the unpaired *t*-test of SAS (Version 8.0, Cary, NC, USA:

SAS Institute). Results are expressed as mean ± S.E.M. $P < 0.05$ was considered as significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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