Effects of Diets with Different Nitrogen to Energy Ratios on Umbilical Cord Vascular Development-Related Gene Expression in Pregnant Huanjiang Xiang Pigs (Postprint)

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Abstract

This study aimed to investigate the effects of diets with different nitrogen-to-energy ratios on the expression of genes related to umbilical cord vascular development in pregnant Huanjiang Xiang pigs. Forty-eight primiparous Huanjiang Xiang pigs were selected and randomly allocated into 2 groups based on body weight, with 8 replicates (pens) per group and 3 pigs per replicate. Following mating, the pigs were fed either a high nitrogen-to-energy ratio diet [digestible energy (DE) 14.73 MJ/kg, crude protein (CP) content 13.11%, nitrogen-to-energy ratio 0.89] or a low nitrogen-to-energy ratio diet (DE 12.24 MJ/kg, CP content 9.77%, nitrogen-to-energy ratio 0.80). On gestational days 45, 75, and 110, one sow per pen was selected, euthanized, and slaughtered, and umbilical cords corresponding to the heaviest, average-weight, and lightest fetal pigs in each litter were collected. Real-time quantitative PCR was employed to determine the expression levels of vascular development-related genes—fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2), and endothelial nitric oxide synthase (eNOS). The results demonstrated that compared with the low nitrogen-to-energy ratio diet group, the high nitrogen-to-energy ratio diet significantly upregulated the expression of umbilical cord vascular development-related genes ($P<0.05$), including VEGF and VEGFR2 in the heaviest fetal pigs on gestational day 45, and VEGF in the average-weight fetal pigs; VEGF, VEGFR2, and eNOS in the heaviest fetal pigs, VEGF and eNOS in the average-weight fetal pigs, and VEGFR2 in the lightest fetal pigs on gestational day 75; and FGF, VEGFR2, and eNOS in the heaviest fetal pigs, FGF and VEGFR2 in the average-weight fetal pigs, and VEGF and eNOS in the lightest fetal pigs on gestational day 110. The expression levels of umbilical cord vascular development-related genes differed among the same weight category across different gestational stages, as well

as among different weight categories within the same gestational stage. Therefore, the high nitrogen-to-energy ratio diet may have significantly upregulated the expression of umbilical cord vascular development-related genes in pregnant Huanjiang Xiang pigs due to its higher protein and amino acid levels, and the expression of these genes also varied across different gestational stages or among different weight categories of fetal pigs within the same gestational stage.

Full Text

Effects of Different Nitrogen/Energy Diets on Expression of Genes Related to Umbilical Vessel Development in Pregnant Huanjiang Mini-Pigs

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Abstract

This study investigated the effects of different nitrogen/energy diets on expression of genes related to umbilical vessel development in pregnant Huanjiang mini-pigs. Forty-eight primiparous Huanjiang mini-pigs were randomly assigned to two groups according to body weight, with eight replicates (pens) per group and three pigs per replicate. After mating, the animals were fed either a high nitrogen/energy diet (digestible energy: 14.73 MJ/kg, crude protein: 13.11%, nitrogen/energy ratio: 0.89) or a low nitrogen/energy diet (digestible energy: 12.24 MJ/kg, crude protein: 9.77%, nitrogen/energy ratio: 0.80). At days 45, 75, and 110 of gestation, one sow per pen was selected, euthanized, and slaughtered. Umbilical cords were collected from the largest, average, and smallest fetuses in each litter, and expression levels of vessel development-related genes—fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2), and endothelial nitric oxide synthase (eNOS)—were measured using real-time quantitative PCR. The results showed that compared with the low nitrogen/energy diet group, the high nitrogen/energy diet significantly upregulated umbilical vessel development-related gene expression ($P<0.05$). Specifically, this included VEGF and VEGFR2 expression in maximal-weight fetuses at day 45; VEGF expression in average-weight fetuses at day 45; VEGF, VEGFR2, and eNOS expression in maximal-weight fetuses at day 75; VEGF and eNOS expression in average-weight fetuses at day 75; VEGFR2 expression in minimal-weight fetuses at day 75; FGF,

VEGFR2, and eNOS expression in maximal-weight fetuses at day 110; FGF and VEGFR2 expression in average-weight fetuses at day 110; and VEGF and eNOS expression in minimal-weight fetuses at day 110. Expression levels of umbilical vessel development genes differed among gestation stages for fetuses of the same standard weight, as well as among different standard-weight fetuses within the same gestation stage. These findings indicate that the high nitrogen/energy diet may significantly upregulate expression of umbilical vessel development-related genes in pregnant Huanjiang mini-pigs due to its higher protein and amino acid levels, with expression levels also varying across different gestation stages and among fetuses of different standard weights within the same gestation stage.

Keywords: Huanjiang mini-pigs; pregnancy; dietary nitrogen/energy ratio; umbilical cord; vascular development

Introduction

In intensive swine production, fetal mortality can be caused by genetic, nutritional, environmental, and disease factors, with nutrition being one of the most critical influences. Maternal nutrition affects fetal growth and development by regulating the structure of the maternal-fetal interface and placental function. The placenta serves as the organ connecting mother and fetus, and maternal-fetal exchange of nutrients occurs through placental blood vessels. The capacity for nutrient transport is closely related to vascular density, permeability, and size, with adequate nutrition and rich blood circulation being prerequisites for healthy fetal development. Impaired placental angiogenesis is closely associated with low birth weight, which reduces neonatal survival rates and has long-term negative effects on offspring growth and reproductive performance, causing substantial losses to the livestock industry. Therefore, studying placental vascular development mechanisms is important for improving fetal survival and promoting fetal growth in sows.

Existing research shows that insufficient maternal protein and energy intake during gestation and severe protein-energy imbalance can impede fetal growth and lead to low birth weight. Nutritional regulation represents an important strategy for improving organ development in low-birth-weight piglets and increasing their postnatal growth rate. For example, arginine supplementation in sow diets can stimulate nitric oxide (NO) production in the placenta and effectively alleviate low birth weight. Dietary tributyrin can improve metabolic efficiency in low-birth-weight piglets and enhance their growth during lactation, while leucine supplementation can reduce muscle atrophy. The effects of nutrients on growth and development ultimately result from regulating expression of related genes.

Our previous research found that different nitrogen/energy diets did not significantly affect litter size, litter weight, individual fetal weight, or maternal body composition in Huanjiang mini-pigs across gestation stages, but the high nitrogen/energy diet could improve metabolism and promote maternal growth

to some extent. However, whether high nitrogen/energy diets affect expression of placental and umbilical vessel-related genes remains unclear. Huanjiang mini-pigs are an excellent local breed in Guangxi, typically raised under extensive management with diets primarily composed of green forage. Their nutritional composition is often incomplete, resulting in slow growth. Therefore, improving dietary composition to promote placental vascular development is a prerequisite for increasing litter size and survival rates in Huanjiang mini-pigs and provides important reference value for preventing low birth weight. Using a low nitrogen/energy diet as control, this study investigated the effects of a high nitrogen/energy diet on expression of umbilical vessel development-related genes in pregnant Huanjiang mini-pigs and compared expression differences among fetuses of different standard weights to provide a basis for preventing low birth weight.

Materials and Methods

1.1 Experimental Animals, Grouping, and Management Forty-eight primiparous Huanjiang mini-pigs were randomly assigned to two groups according to body weight, with eight replicates (pens) per group and three pigs per replicate. After mating, the sows were fed either a high nitrogen/energy diet (digestible energy: 14.73 MJ/kg, crude protein: 13.11%, nitrogen/energy ratio: 0.89) or a low nitrogen/energy diet (digestible energy: 12.24 MJ/kg, crude protein: 9.77%, nitrogen/energy ratio: 0.80). The high nitrogen/energy diet was formulated according to NRC (1998) recommendations for energy, protein, and crude fiber, representing a high-energy, high-protein, low-fiber diet. The low nitrogen/energy diet was formulated according to Chinese standards for local pig breeds, representing a low-energy, low-protein, high-fiber diet. Dietary composition and nutrient levels are shown in Table 1 . The two experimental diets had nitrogen/energy ratios of 0.89 and 0.80, respectively, with identical 1% premix formulated according to NRC (1998) recommendations. Throughout the trial, daily feed intake per pen was 2.5% of the total body weight of the three sows. Feed was provided twice daily at 08:30 and 17:00, with free access to water. All experimental pigs received routine immunizations and management according to the farm' s standard protocols.

1.2 Sample Collection According to Johnston et al., sow gestation can be divided into early (1–30 days), mid (30–75 days), and late (75 days to parturition) stages. However, as Huanjiang mini-pigs are a small breed, to facilitate analysis of conceptus development during early gestation, this study collected samples at days 45 (early), 75 (mid), and 110 (late) of gestation. One sow per replicate (eight sows per group) was randomly selected, euthanized by cardiac exsanguination, and slaughtered. Fetuses from each litter were weighed, and umbilical cords were collected from the largest, average, and smallest fetuses in each litter (three fetuses total), snap-frozen in liquid nitrogen, and stored at –80°C for measurement of vessel development-related gene expression.

1.3 RNA Extraction and Reverse Transcription Total RNA was extracted from umbilical cord tissues using the Trizol method according to kit instructions. RNA concentration and purity were measured using a Nanodrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE), with ideal absorbance (OD) 260/280 ratios between 1.8 and 2.2. Reverse transcription was performed using the TaKaRa RR036TA kit to synthesize first-strand cDNA, which was stored at -20°C.

1.4 Gene Expression Detection Primer sequences for target genes were designed using Premier 5.0 software and synthesized by Shanghai Sangon Biotech. The target genes and primer sequences were as follows: fibroblast growth factor (FGF): F: 5 -TCAAAGGAGTGTGTGCGAAC-3 , R: 5 -CAGGGCCACATACCAACTG-3 ; vascular endothelial growth factor (VEGF): F: 5 -CCTGATGCGGTGCGGGGGCT-3 , R: 5 -TGGTGGTGGCGGCGGCTATG-3 ; vascular endothelial growth factor receptor 2 (VEGFR2): F: 5 -TACGTTGGAGCAATCCCTGT-3 , R: 5 -TACACTTTCGATGCCAAG-3 ; endothelial nitric oxide synthase (eNOS): F: 5 -ATGAAGCACCTGGAGAACGA-3 , R: 5 -ATGAAGCACCTGGAGAACGA-3 . Amplification was performed on an ABI 7900HT real-time fluorescence quantitative (RT)-PCR instrument under the following conditions to detect target gene transcription. The PCR reaction system (10 L) included: 5.0 L 2×SYBR Green PCR Master Mix, 0.4 L each of forward and reverse primers (10 mol/L), 2 L cDNA template, and ddH_2O to 10 L. The PCR program was: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. β-actin served as the internal reference, and data were analyzed using Applied Biosystem SDS 2.3 software. The 2ˆ(-ΔΔCT) method was used for data processing, with target gene expression calculated as: -ΔΔCT = (CT_{target} -CT_{reference}){treatment} -(CT{target} -CT_{reference})_{control}.

1.5 Data Processing and Analysis Independent samples t-tests were performed using SPSS 18.0 software to compare data between diet groups within the same gestation stage and standard weight. One-way ANOVA (LSD) was used to analyze data across different gestation stages within the same diet and standard weight group, and among different standard-weight fetuses within the same diet and gestation stage. Data are presented as "mean ± standard error." $P<0.05$ indicated significant difference, and $0.05 \leq P<0.10$ indicated a trend.

Results

2.1 Effects of Different Nitrogen/Energy Diets on Umbilical Vessel FGF Gene Expression As shown in Table 2 , compared with the low nitrogen/energy diet group, the high nitrogen/energy diet group showed significantly increased FGF gene expression in umbilical vessels of maximal- and average-weight fetuses at day 110 of gestation ($P<0.05$). Within the high nitrogen/energy diet group, FGF expression in maximal-weight fetuses at day 45 was significantly higher than in the other two standard weight groups ($P<0.05$).

2.2 Effects of Different Nitrogen/Energy Diets on Umbilical Vessel VEGF and VEGFR2 Gene Expression As shown in Table 3 , compared with the low nitrogen/energy diet group, the high nitrogen/energy diet group exhibited significantly elevated VEGF gene expression in umbilical vessels of maximal- and average-weight fetuses at days 45 and 75, and in average- and minimal-weight fetuses at day 110 ($P<0.05$). VEGF expression in maximal-weight fetuses at day 110 showed an increasing trend ($P=0.087$). The high nitrogen/energy diet also significantly increased VEGFR2 expression in maximal-weight fetuses at days 45, 75, and 110, in minimal-weight fetuses at day 75, and in average-weight fetuses at day 110 ($P<0.05$). In the low nitrogen/energy diet group, VEGF expression in maximal-weight fetuses at day 45 was significantly higher than at day 110 ($P<0.05$), while expression in minimal-weight fetuses at day 45 was significantly higher than at days 75 and 110 ($P<0.05$). VEGFR2 expression in minimal-weight fetuses in the low nitrogen/energy diet group first increased and then decreased significantly ($P<0.05$). Within the low nitrogen/energy diet group at day 75, VEGFR2 expression in maximal-weight fetuses was significantly lower than in average- and minimal-weight fetuses ($P<0.05$).

2.3 Effects of Different Nitrogen/Energy Diets on Umbilical Vessel eNOS Gene Expression As shown in Table 4 , compared with the low nitrogen/energy diet group, the high nitrogen/energy diet group showed significantly increased eNOS gene expression in umbilical vessels of maximal- and average-weight fetuses at day 75, and in maximal- and minimal-weight fetuses at day 110 ($P<0.05$). Expression in maximal-, average-, and minimal-weight fetuses at day 45 showed increasing trends ($P=0.052$, $P=0.065$, and $P=0.057$, respectively). In the high nitrogen/energy diet group, eNOS expression in maximal-weight fetuses tended to first increase and then decrease across gestation ($P=0.055$).

Discussion

FGF can stimulate proliferation of uterine artery and fetal placental artery endothelial cells. Basic FGF in blastocysts may amplify the angiogenic response in the endometrium and induce mesodermal cell differentiation into angioblasts. FGF can also act directly on vascular cells, inducing vascular endothelial cell growth and promoting vessel formation. Well-developed placentas have high vascular density and strong capacity for oxygen and nutrient transport, resulting in larger fetal weights. Our results indicate that the high nitrogen/energy diet significantly upregulated FGF gene expression, thereby affecting vascular development and fetal growth during late gestation.

VEGF serves as the primary pro-angiogenic growth factor, promoting division and migration of vascular endothelial cells, enhancing capillary permeability, and regulating angiogenesis and neovascularization. During vascular development, VEGF exerts its biological effects primarily through vascular endothelial growth factor receptors 1 (VEGFR1) and VEGFR2, with VEGFR2 being the

main receptor that promotes vascular development. Chen et al. reported reduced VEGF expression in placentas of intrauterine growth retardation (IUGR) animals. Our findings demonstrate that the high nitrogen/energy diet significantly upregulated VEGF and VEGFR2 expression, promoting vascular development and increasing blood flow in the placenta and umbilical cord. As gestation progressed, VEGF expression in maximal- and minimal-weight fetuses in the low nitrogen/energy diet group was highest at day 45, consistent with previous reports that vascular development begins in early gestation. Placental vascular networks are generally considered mature by mid-gestation. At day 75, minimal-weight fetuses in the low nitrogen/energy diet group showed the highest VEGFR2 expression, with average- and minimal-weight fetuses having significantly higher expression than maximal-weight fetuses, possibly because average- and minimal-weight fetuses require more angiogenesis to maintain their growth during mid-gestation.

eNOS plays an important role in regulating vascular function. Under physiological conditions, NO synthesized by eNOS relaxes smooth muscle cells and promotes vascular growth. Our study showed that the high nitrogen/energy diet significantly upregulated eNOS expression in fetal umbilical vessels, increasing NO production and promoting vascular development. VEGF induces angiogenesis and promotes blood circulation by increasing NO levels. NOS is the primary source of NO in the vascular system, and VEGF can increase eNOS expression through VEGFR2 activation. In our study, changes in eNOS expression in maximal-weight fetuses during late gestation were consistent with those of VEGF and VEGFR2, suggesting that NO produced by eNOS may be involved in VEGF-mediated pro-angiogenic effects.

The high nitrogen/energy diet used in this study contained 34.19% more crude protein and higher levels of amino acids including lysine (33.73%), methionine + cysteine (25.00%), threonine (30.00%), tryptophan (38.46%), arginine (114.81%), histidine (80.00%), isoleucine (100.00%), leucine (55.36%), phenylalanine (87.50%), valine (75.00%), aspartic acid (84.62%), cysteine (37.50%), glutamic acid (60.16%), glycine (59.26%), and proline (68.42%) compared with the low nitrogen/energy diet. The significant upregulation of FGF, VEGF, VEGFR2, and eNOS expression by the high nitrogen/energy diet is related to its higher amino acid content. For example, arginine can serve as a substrate for synthesizing factors related to vascular development, thereby benefiting fetal vascular development. Wu et al. demonstrated that dietary arginine supplementation upregulated VEGF and eNOS expression in placental vessels, improving placental vascular function and promoting fetal growth. Liu et al. found that arginine supplementation significantly increased total nitric oxide synthase (TNOS) and constitutive nitric oxide synthase (cNOS) activities in placentas. Therefore, compared with the low nitrogen/energy diet, the high nitrogen/energy diet provides sufficient functional amino acids to meet the needs of placental vascular development.

In conclusion, the high nitrogen/energy diet significantly upregulated expression

of genes related to umbilical vessel development in pregnant Huanjiang mini-pigs, promoting fetal umbilical vessel development through its higher protein and amino acid content. Expression levels of these genes differed across gestation stages and among fetuses of different standard weights within the same gestation stage, which may be related to the occurrence of low birth weight.

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Note: Figure translations are in progress. See original paper for figures.