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ORIGINAL ARTICLE

# Effects of dietary L-arginine or *N*-carbamylglutamate supplementation during late gestation of sows on the miR-15b/16, miR-221/222, VEGFA and eNOS expression in umbilical vein

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**Abstract** Placental vascular formation and blood flow are crucial for fetal survival, growth and development, and arginine regulates vascular development and function. This study determined the effects of dietary arginine or *N*-carbamylglutamate (NCG) supplementation during late gestation of sows on the microRNAs, vascular endothelial growth factor A (VEGFA) and endothelial nitric oxide synthase (eNOS) expression in umbilical vein. Twenty-seven landrace × large white sows at day (d) 90 of gestation were assigned randomly to three groups and fed the following diets: a control diet and the control diet supplemented with 1.0% L-arginine or 0.10% NCG. Umbilical vein of fetuses with body weight around 2.0 kg (oversized), 1.5 kg (normal) and 0.6 kg (intrauterine growth restriction, IUGR) were obtained immediately after farrowing for miR-15b, miR-16, miR-221, miR-222, VEGFA and eNOS real-time PCR analysis. Compared with the control diets, dietary Arg or NCG supplementation enhanced the reproductive performance of sows, significantly increased ($P < 0.05$) plasma arginine and decreased plasma VEGF and eNOS ($P < 0.05$). The miR-15b expression in the umbilical vein was higher ($P < 0.05$) in the NCG-supplemented group than in the control group. There was a trend in that the miR-222 expression in the umbilical vein of the oversized fetuses was higher ($0.05 < P < 0.1$) than in the normal and IUGR fetuses. The expression of eNOS in both Arg-supplemented and NCG-supplemented group were lower ($P < 0.05$) than in the control group. The expression of VEGFA was higher ($P < 0.05$) in the NCG-supplemented group than in the Arg-supplemented and the control group. Meanwhile, the expression of VEGFA of the oversized fetuses was higher ($P < 0.05$) than the normal and IUGR fetuses. In conclusion, this study demonstrated that dietary Arg or NCG supplementation may affect microRNAs (miR-15b, miR-222) targeting VEGFA and eNOS gene expressions in umbilical vein, so as to regulate the function and volume of the umbilical vein, provide more nutrients and oxygen from the maternal to the fetus tissue for fetal development and survival, and enhance the reproductive performance of sows.

**Keywords** L-Arginine · *N*-Carbamylglutamate · Umbilical vein · VEGFA · ENOS · MicroRNAs

**Abbreviations**

| | |
|---|---|
| Arg | L-Arginine |
| eNOS | Endothelial nitric oxide synthase |
| iNOS | Inducible nitric oxide synthase |
| IUGR | Intrauterine growth restriction |
| NAG | *N*-Acetylglutamate |
| NCG | *N*-Carbamylglutamate |
| NO | Nitric oxide |
| RT-PCR | Real-time polymerase chain reaction |
| VEGFA | Vascular endothelial growth factor A |

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## Introduction

High embryonic loss and fetal deaths during gestation limited the number of piglets born at farrowing in sows (Pope 1994; Mateo et al. 2007). Higher fetal growth rates may require an increased provision of nutrients for supporting the metabolic needs of both the sow and her fetuses (Wu et al. 2004; Kim et al. 2005). Adequate vasculogenesis and angiogenesis of the maternal vasculature is important for providing adequate maternal oxygen/nutrients and blood flow to the placenta. Placental angiogenesis supports the required blood flow on the fetal side necessary for fetal growth and development. Therefore, vasculogenesis and angiogenesis are essential for proper placental development (Wu et al. 2004; Demir et al. 2007; Arroyo and Winn 2008). Umbilical venous blood flow is crucial for fetal growth and development (Barbera et al. 1999; Ferrazzi et al. 2000; Boiti et al. 2002).

The vascular endothelial growth factor (VEGF) proteins are mostly known to regulate the processes of vasculogenesis and angiogenesis (Hanahan 1997; Otrock et al. 2007; Demir et al. 2007; Arroyo and Winn 2008; Yao et al. 2011). As a potent endothelial survival factor, VEGF induces vasodilation and facilitates blood flow by increasing nitric oxide (NO) production (Hood et al. 1998; Otrock et al. 2007). MicroRNAs (miRNAs), about 22-nucleotide, non-coding RNAs, have been shown to be involved in various biological processes in animals (Ambros 2004; Kloosterman and Plasterk 2006), including angiogenesis regulation (Kuehbacher et al. 2008; Anand et al. 2010). Recently, it is reported that miR-15b, miR-16, miR-221 and miR-222 target VEGFA (Hua et al. 2006; Karaa et al. 2009) and eNOS (Poliseno et al. 2006; Suárez et al. 2007) expressions in angiogenesis.

Arginine (Arg) can enhance the reproductive performance of pigs (Mateo et al. 2007; Wu et al. 2007) and also regulate angiogenesis (Raghavan and Dikshit 2004). In addition, *N*-carbamylglutamate (NCG) increases the endogenous synthesis of Arg (Frank et al. 2007; Wu et al. 2010).

Therefore, we hypothesized that dietary supplementation with Arg or NCG may enhance the reproductive performance of sows and the potential mechanisms are that microNRAs (miR-15b, miR-16, miR-221 and miR-222) target VEGFA and eNOS gene expression in fetal umbilical vein so as to regulate the function and volume of the umbilical vein, thereby providing more nutrients and oxygen from the maternal to the fetus tissue for fetal development and survival.

## Materials and methods

This study was performed in accordance with the Chinese guidelines for animal welfare and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, the Chinese Academy of Sciences (Yin et al. 1993).

### Animals and experimental design

A total of 27 Large White × Landrace crossbred sows at d 90 of gestation with initial body weight (BW) of 187 ± 5 kg, parity of 3.2 ± 0.7 and similar reproductive performance at last parity were chosen and assigned to three groups randomly: a control group (fed a corn- and soybean meal-based diet) and two treatment groups (fed a corn- and soybean meal-based diet supplemented with 1.0% L-Arg·HCl (Arg) or 0.1% NCG) (Table 1). L-Arg·HCl was obtained from Ajinomoto Inc. (Tokyo, Japan) and NCG was provided by the Institute of Subtropical Agriculture, the Chinese Academy of Sciences.

The dose of Arg was based on previous study (Mateo et al. 2007), and the dose of NCG was based on our own study.

The sows were housed individually in gestation crates (2.0 × 0.6 m, concrete floor) and transferred to individual farrowing crates (2.2 × 1.5 m) at d 107 of gestation. The sows were provided 2 kg diet (on an as-fed basis) daily as two equal-sized meals (08:00 and 16:30 h) during the entire gestation period. All the diets provided 13.5 MJ metabolizable energy/kg and 14.7 crude protein (on as-fed basis). All the sows had free access to drinking water.

### Sample collection

Blood samples were collected 2 h after feeding via jugular venepuncture into heparinized tubes on d 110 of gestation. Samples were centrifuged at 2,000×*g*, 15 min at 4°C (Mateo et al. 2007; Yin et al. 2010). Plasma was transferred to 1.5 microcentrifuge tubes and stored at −20°C until analysis (Geng et al. 2011). The total number of piglets and their BW at birth were recorded. The piglets were classified as born alive or dead as previously described (Mateo et al. 2007). The number of mummified fetuses (early or middle gestation deaths) was neglected.

The umbilical veins of piglets with BW of about 2.0 kg (oversized), 1.5 kg (normal) and 0.6 kg (IUGR) were obtained immediately after farrowing. Samples were of length about 5 cm, 10 cm from the body, and washed with 4°C PBS (RNA free). Then the samples were collected into 1.5 microcentrifuge tubes (RNA free) with RNAlater (Applied Biosystems, Valencia, CA, USA) in it and stored at −20°C for RT-PCR analyses (Wu et al. 2010).

### Chemical analyses

Plasma samples were assayed for biochemical indices using Beckman Coulter CX4 Pro. (Beckman, USA),

**Table 1** Composition of gestation diets, on an as-fed basis (%)

| Item | Treatment | | |
|---|---|---|---|
| | Control | Arg | NCG |
| Ingredient | | | |
| Corn | 70.30 | 70.30 | 70.30 |
| Soybean meal | 12.00 | 12.00 | 12.00 |
| Wheat middling | 4.00 | 3.00 | 3.90 |
| Rice bran meal | 3.00 | 3.00 | 3.00 |
| Puffed soybean | 3.00 | 3.00 | 3.00 |
| Salt | 0.35 | 0.35 | 0.35 |
| Potassium chloride | 0.75 | 0.75 | 0.75 |
| Vitamin–mineral premix[a] | 3.00 | 3.00 | 3.00 |
| Dicalcium phosphate | 2.20 | 2.20 | 2.20 |
| Limestone | 0.50 | 0.50 | 0.50 |
| Soy oil | 0.50 | 0.50 | 0.50 |
| Mold-inhibitor | 0.09 | 0.09 | 0.09 |
| L-Lys·HCl | 0.20 | 0.20 | 0.20 |
| DL-Met | 0.06 | 0.06 | 0.06 |
| L-Thr | 0.05 | 0.05 | 0.05 |
| L-Arginine·HCl | – | 1.00 | – |
| NCG | – | – | 0.10 |
| Nutrient composition | | | |
| DM (%) | 88.2 | 88.2 | 88.2 |
| ME (MJ/kg) | 13.5 | 13.5 | 13.5 |
| CP (%) | 14.7 | 14.7 | 14.7 |
| Lys (%) | 0.78 | 0.78 | 0.78 |
| Met + Cys (%) | 0.69 | 0.69 | 0.69 |
| Ca (%) | 0.92 | 0.92 | 0.92 |
| Total P (%) | 0.67 | 0.67 | 0.67 |
| Available P (%) | 0.45 | 0.45 | 0.45 |

[a] The premix provided the following per kg of diet: Fe ($FeSO_4 \cdot H_2O$) 80 mg, Mn ($MnSO_4 \cdot 5H_2O$) 45 mg, Zn (ZnO) 100 mg, Cu ($CuSO_4 \cdot 5H_2O$) 20 mg, I (KI) 0.70 mg, Se ($Na_2SeO_3 \cdot H_2O$) 0.25 mg, vitamin A 10,000 IU, vitamin $D_3$ 2,500 IU, vitamin E 100 IU, vitamin K 10 IU, vitamin $B_2$ 10 mg, vitamin $B_6$ 1 mg, vitamin $B_{12}$ 50 μg, biotin 80 μg, folic acid 5 mg, nicotinic acid 15 mg, choline chloride 1,500 mg

standards obtained from Beckman (Beckman, USA) (Tang et al. 2005). Plasma concentrations of free amino acids were analyzed by Amino Acid Analyzer, Hitachi L8800 (Hitachi, Japan), and amino acid standards were obtained from Sigma Chemical (Kong et al. 2009). Plasma concentrations of VEGF and eNOS were analyzed using enzyme-linked immunosorbent assay (ELISA) from R & D system (Minneapolis, MN, USA) and an ELISA plate reader (BioTek, USA) (Deng et al. 2010). Concentrations of hormones were analyzed by radioimmunoassay (Jiuding, China).

## Real-time PCR analyses

### *RT-PCR for VEGFA and eNOS in fetal umbilical vein*

Total RNA was isolated by Trizol (Invitrogen, USA, Karaa et al. 2009) and treated with DNase. Reverse transcription was performed using AMV Reverse Transcriptase Kit (Promega, USA). mRNA levels for VEGFA and eNOS were determined by a standard real-time polymerase chain reaction (RT-PCR) method. RT-PCR was performed with the total RNA using TaKaRa one-step RNA PCR Kit (TaKaRa Bio Inc, Japan). The primer pairs for VEGFA, eNOS and GAPDH are presented in Table 2. GAPDH was used as the housekeeping gene, whose mRNA levels in the fetal umbilical vein did not differ among the groups. The RT-PCR conditions were: 10 min pre-denaturation at 95°C, and then 15 s denaturation at 94°C, and 30 s annealing at 60°C for 40 cycles. The relative quantification of gene amplification by RT-PCR was performed using cycle threshold ($C_T$) values. The comparative $C_T$ value method was employed to quantitate expression levels for VEGFA and eNOS relative to those for GAPDH. The final PCR product was visualized in a 2% agarose gel.

### *RT-PCR for miR-15b, miR-16, miR-221 and miR-222*

Expression of mature miRNAs was measured using miScript PCR System (Qiagen, Hilden, Germany) (Chen et al.

**Table 2** Primers for VEGFA, eNOS and GAPDH

| Target gene | Primer sequences | Products | Annealing temperature (°C) |
|---|---|---|---|
| VEGFA | F: CAACGACGAAGGTCTGGAGTG | 155 | 60 |
| | R: GCCTCGCTCTATCTTTCTTTGG | | |
| eNOS | F: ACGCCCGTCTTCCACCA | 193 | 60 |
| | R: ACGCCTTCACTCGCTTCG | | |
| GAPDH | F: GTGCTGAGTATGTCGTGGAGTC | 196 | 60 |
| | R: CAGTTGGTGGTACAGGAGGC | | |

*F* forward, *R* reverse

2005). The miScript PCR System comprises the following components: miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, miScript Primer Assay.

Total RNA was extracted as described above. After cDNA synthesis, the cDNA serves as the template for real-time PCR analysis using a miScript Primer Assay in combination with the miScript SYBR Green PCR Kit. Mature miRNAs are amplified using the miScript Universal Primer together with the miRNA-specific primer (the miScript Primer Assay). Primers for sus scrofa miR-15b, miR-16, miR-221 and miR-222 (miRBase, http://www.mirbase.org) were designed by Qiagen. 5S rRNA (forward primer: 5′-gcccgatctcgtctgatct-3′, reverse primer: 5′-agcct acagcacccggtatt-3′) was used as the referent for miRNAs expression for its constant expression level across all samples and suitable size. The amplification protocol was as follows: 95°C for 5 min, 50 cycles of denaturation at 94°C/15 s, annealing temperature of 55°C/30 s, and extension at 70°C/30 s. Real-time analysis of PCR amplification was performed on an Applied Biosystems 7900HT Sequence Detection System and analyzed with an SDS 2.3 Software (Applied Biosystems).

The final PCR product was visualized in a 2% agarose gel. All the procedures above followed the instructions of each manufacturer.

### Statistical analyses

The relative quantification of gene amplification by RT-PCR was performed using cycle threshold ($C_T$) values. The comparative $C_T$ value method was employed to quantitate expression levels for VEGFA and eNOS relative to those for GAPDH. The $\Delta\Delta C_T$ method is used for relative quantification when working with the miScript PCR System. This comparative method relies on comparing the differences in $C_T$ values obtained with normal versus experimental samples. The threshold cycle ($C_T$) obtained with the miScript PCR Control (5S rRNA) is used to normalize the data.

Values are presented as the mean ± SEM. Data of gene and miRNAs expression were analyzed using the GLM and the others using the one-way ANOVA (SAS 9.1.3, SAS Inc., USA). In case of a $P$ value $< 0.05$, the result was regarded as statistically significant, while $0.05 \leq P < 0.1$ was considered as a trend.

## Results

### Gestation performance

The reproductive performance of sows fed diets supplemented with Arg or NCG can be seen in Table 3. The total number of piglets born, birth weight of all piglets born or born alive, and litter birth weight of all piglets born did not differ between the three groups of sows. However, there was a trend ($0.05 < P < 0.1$) toward an increase in the number of piglets born alive for sows fed the Arg or NCG-supplemented diet compared with sows fed the control diet. The litter birth weight of all piglets born alive were 15% higher ($P < 0.05$) for Arg-supplemented sows and 14% ($P < 0.05$) higher for NCG-supplemented sows, both

**Table 3** Reproductive performance of sows

| Items | Control | Arg | NCG | SEM |
|---|---|---|---|---|
| Total piglets born per litter (*n*) | 11.22 | 11.38 | 11.33 | 0.26 |
| Total piglets born alive per litter (*n*) | 9.78 | 10.87 | 10.77 | 0.24 |
| Birth weight of all piglets born (kg) | 1.44 | 1.50 | 1.49 | 0.02 |
| Birth weight of all piglets born alive (kg) | 1.45 | 1.51 | 1.49 | 0.02 |
| Litter birth weight of all piglets born (kg) | 16.09 | 16.87 | 16.83 | 0.25 |
| Litter birth weight of all piglets born alive (kg) | 14.12b | 16.26a | 16.04a | 0.29 |
| Piglets born dead per litter (*n*) | 1.44a | 0.50b | 0.56b | 0.14 |

In the same row, values with different letter mean significant difference ($P < 0.05$)

**Table 4** Plasma biochemical indices

| Items | Control | Arg | NCG | SEM |
|---|---|---|---|---|
| Glucose (mmol/L) | 3.51 | 3.77 | 3.89 | 0.14 |
| Ammonia (μmol/L) | 85.42 | 85.09 | 85.73 | 0.16 |
| Urea nitrogen (mmol/L) | 5.83 | 5.19 | 5.41 | 0.14 |
| Albumin (g/L) | 26.29 | 28.03 | 26.87 | 0.61 |
| Total protein (g/L) | 62.09 | 65.96 | 67.12 | 1.59 |
| Phosphorus (mmol/L) | 1.52a | 1.74b | 1.76b | 0.04 |
| $Ca^{2+}$ (mmol/L) | 2.09 | 2.28 | 2.19 | 0.05 |
| $Cu^{2+}$ (μmol/L) | 33.84 | 32.40 | 32.22 | 0.79 |
| $Mg^{2+}$ (mmol/L) | 1.73 | 1.71 | 1.65 | 0.20 |
| $Zn^{2+}$ (mmol/L) | 3.12a | 4.94b | 4.27b | 0.26 |

In the same row, values with different letter mean significant difference ($P < 0.05$)

compared with the control group. The number of piglets born dead were 65% lower ($P < 0.05$) for the Arg-supplemented sows and 61% lower ($P < 0.05$) for the NCG-supplemented sows, both compared with the control group. The days from weaning to estrus of sows did not differ between the three groups (data not shown).

## Plasma biochemical assays

Concentrations of glucose, ammonia, albumin, total protein, $Ca^{2+}$, $Cu^{2+}$ and $Mg^{2+}$ in plasma did not differ between the three groups (Table 4). Concentrations of phosphorus and $Zn^{2+}$ were both higher ($P < 0.05$) in Arg or NCG -supplemented sows than in the control group of sows (Table 4). There was a trend ($0.05 < P < 0.1$) toward the decrease in the concentrations of urea nitrogen for Arg-supplemented sows compared with the control group of sows.

## Plasma-free amino acids concentration

Concentrations of the most measured free amino acids in plasma did not differ among the three groups of sows at d 110 of gestation. Compared with the control group, dietary supplementation with Arg ($P < 0.01$) or NCG ($P < 0.05$) increased the concentrations of arginine in the plasma of sows (Fig. 1). Compared with the control diet and Arg-supplemented diet, NCG increased ($P < 0.05$) the concentrations of aspartate in the plasma of sows and decreased ($P < 0.05$) the concentrations of proline (Fig. 1).

## Plasma hormone concentrations

Concentrations of estriol and progesterone did not differ among the three groups (Table 5). Plasma growth hormone in Arg or NCG-supplemented sows were higher ($P < 0.05$) compared with sows fed the control diets. Concentrations of estradiol were lower ($P < 0.05$) in NCG-supplemented sows than in the other two groups. In addition, there was a trend ($0.05 < P < 0.1$) for sows fed the NCG-supplemented diet to have increased hormone concentrations of insulin-like growth facter-1 compared with sows in the control group.

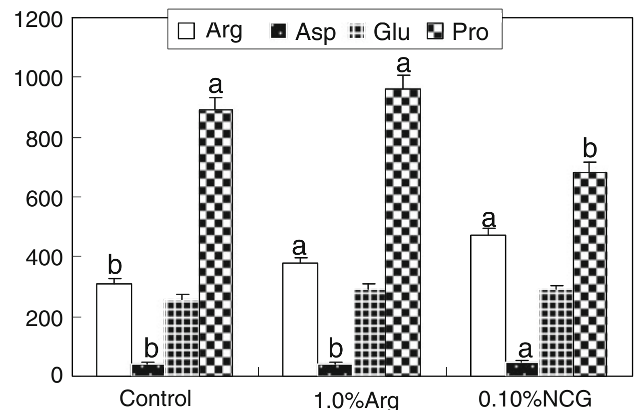


**Fig. 1** Concentrations of free amino acids in plasma (μmol/L). For the *same grayscale* of the *bar*, values with different letter mean significant difference ($P < 0.05$), the same as given below

## Plasma concentrations of VEGF and eNOS

Protein concentrations of VEGF in plasma were 11 and 10% lower in Arg-supplemented sows ($P < 0.05$) and NCG-supplemented sows ($P < 0.05$) than in the control group of sows, respectively (Table 6). Protein concentrations of eNOS were 17 and 23% lower in Arg-supplemented sows ($P < 0.05$) and NCG-supplemented sows ($P < 0.01$) than in the control group of sows (Table 6).

## VEGFA and eNOS gene expression

The expression of eNOS in both Arg-supplemented and NCG-supplemented group was lower ($P < 0.05$) than in the control group (Fig. 2). The expression of VEGFA was higher ($P < 0.05$) in the NCG-supplemented group than in

**Table 5** Concentration of hormones in plasma

| Items | Control | Arg | NCG | SEM |
|---|---|---|---|---|
| Insulin (μg/dl) | 37.92 | 40.53 | 35.97 | 2.46 |
| Growth hormone (ng/ml) | 1.21a | 1.70b | 1.68b | 0.10 |
| Insulin-like growth factor-1 (ng/ml) | 30.52 | 43.68 | 46.22 | 3.91 |
| Estradiol (pg/ml) | 135.72b | 138.44b | 67.02a | 13.60 |
| Estriol (pg/ml) | 4.38 | 4.98 | 4.21 | 0.66 |
| Progesterone (ng/ml) | 2.74 | 2.48 | 2.11 | 0.18 |

In the same row, values with different letter mean significant difference ($P < 0.05$)

**Table 6** Plasma concentration of VEGF and eNOS

| Items | Control | Arg | NCG | SEM |
|---|---|---|---|---|
| VEGF (mg/L) | 177.38a | 158.03b | 159.57b | 3.85 |
| eNOS (U/L) | 10.61a | 8.78b | 8.15b | 0.37 |

In the same row, values with different letter mean significant difference ($P < 0.05$)

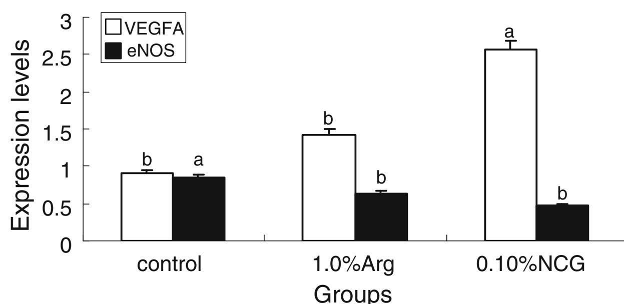


**Fig. 2** Effects of Arg and NCG on the VEGFA and eNOS gene expression levels in the umbilical vein

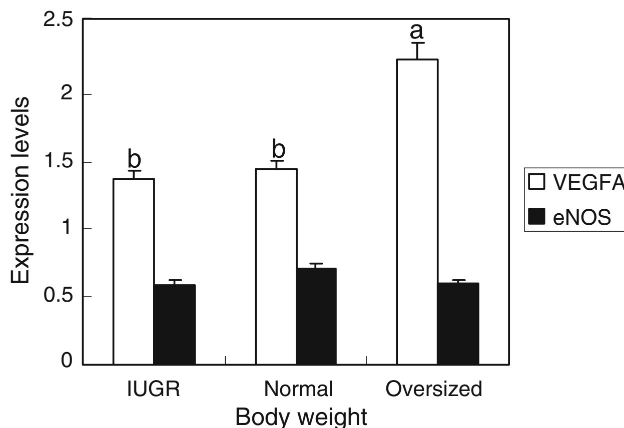


**Fig. 3** Effects of body weight on the VEGFA and eNOS gene expression levels in the umbilical vein

the Arg-supplemented and the control group (Fig. 2). Meanwhile, the expression of VEGFA of the oversized fetuses was higher ($P < 0.05$) than the normal and IUGR fetuses (Fig. 3). There was no effect of the diet × BW interaction on VEGFA and eNOS gene expression.

## MiR-15b, miR-16, miR-221 and miR-222 expression

The miR-15b expression in the umbilical vein was higher ($P < 0.05$) in the NCG-supplemented group than in the control group (Fig. 4). There was a trend toward the miR-222 expression in the umbilical vein of the oversized fetuses being higher ($0.05 < P < 0.1$) than the normal and IUGR fetuses (Fig. 5). There was no effect of diet × BW interaction on these miRNAs expression.

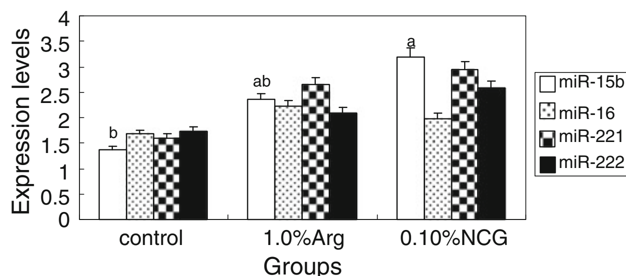


**Fig. 4** Effects of Arg and NCG on the miR-15b, 16, 221 and 222 expression levels in the umbilical vein

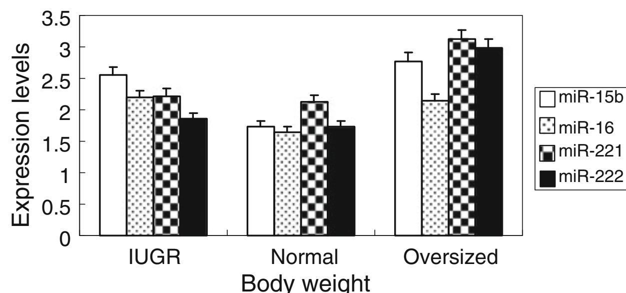


**Fig. 5** Effects of body weight on the miR-15b, 16, 221 and 222 expression levels in the umbilical vein

## Discussion

Maternal nutrition and oxygen play a key role in regulating fetal survival, growth and development (Wu et al. 2004). Malnutrition is known to be a major cause of pregnancy complications, such as intrauterine growth restriction (IUGR) or even worse, such as embryonic loss and fetal deaths during gestation. Thus, providing the pregnant dam with proper nutrition is vital for the fetus (Snoeck et al. 1990; Hoet and Hanson 1999; McPherson et al. 2004). Various evidences have substantiated the importance of arginine in the survival, growth, and development of fetal pigs (Wu et al. 2004, 2007). Furthermore, amino acid malnutrition in gestating sows results in lower concentrations of arginine in the placenta and fetal plasma (Wu et al. 1998), as well as reduced the synthesis of NO (the endothelium-derived relaxing factor) from L-arginine (Wu et al. 2009) and the synthesis of polyamines (Pegg 1986). Impaired placental synthesis of both NO and polyamines is considered a major factor contributing to IUGR (Wu et al. 2004, 2006). Additionally, previous studies showed that uterine uptake of arginine may not be sufficient to meet fetal growth requirements during late gestation in pigs (Wu et al. 1999). NCG is a safe and metabolically stable analog of NAG (Wu et al. 2009; Gessler et al. 2010) and increases the endogenous synthesis of Arg (Frank et al. 2007; Wu et al. 2009).

The results of this study showed that Arg or NCG supplementation to gestation diets for late pregnant sows improved pregnancy outcome, decreased plasma urea concentrations and increased the plasma concentrations of free arginine of sows at d 110 of gestation. Mateo et al. (2007) also reported the similar results. This suggested that both Arg and NCG supplementation provided better nutrients to sows, and therefore probably improved the uterine environment for fetal growth and development. Additionally, arginine is not only required for protein synthesis and ammonia detoxification, but is also a precursor of many metabolically important molecules, including proline, ornithine, polyamines and NO (Wu and Morris 1998; Kim et al. 2007).

However, proper nutrition for sows cannot guarantee good reproductive efficiency. The placenta is responsible for the exchange of nutrients and oxygen from the mother to the fetus. Adequate vasculogenesis and angiogenesis of the maternal vasculature are important for providing adequate maternal nutrients/oxygen and blood flow to the placenta. Placental vascular formation and function are important for fetal growth and development. Proper development of the placenta is critical for a successful pregnancy, mediates important steps, such as maternal blood flow to the placenta and delivery of nutrients to the fetus, and ensures the exchange of nutrients/oxygen and blood flow necessary for fetal growth (Arroyo and Winn 2008).

Also, umbilical venous blood flow is crucial for fetal growth and development (Barbera et al. 1999; Ferrazzi et al. 2000; Boiti et al. 2002). Pathologic umbilical vein leads to pregnancy complications too (Klaritsch et al. 2008; Koech et al. 2008). Vascular growth is necessary to increase placental fetal blood flow over gestation. Poor vascular development is known to cause intrauterine embryonic death characterized by low vascular density in the placental villi along with fibrosis and other deficiencies. The VEGF proteins are the most studied family of growth factors known to regulate the processes of vasculogenesis and angiogenesis. VEGFA (also known as VEGF), aside from being a potent endothelial survival factor, is also known to induce vasodilation by increasing nitric oxide (NO) production, another function which facilitates blood flow. ENOS is critical in the regulation of vascular function (Lu et al. 2011) and can generate both nitric oxide (NO) and superoxide ($O_2^-$), which are key mediators of cellular signaling (Chen et al. 2010).

MicroRNAs (miRNAs), about 22-nucleotide, non-coding RNAs, have come into focus as a powerful mechanism to regulate angiogenesis (Dews et al. 2006; Urbich et al. 2008). It has been demonstrated that miR-221 and miR-222 block endothelial cell migration, proliferation and angiogenesis and indirectly regulate the expression of endothelial nitric oxide synthase (Poliseno et al. 2006). In addition, miR-221 and miR-222 inhibit cell proliferation and reduce the expression of c-Kit in hematopoietic progenitor cells—a process that can contribute to vessel growth (Kuehbacher 2007). Two other miRNAs that might be involved in angiogenesis are miR-15b and miR-16. MiR-15b and miR-16 have been shown to control the expression of VEGF (Hua et al. 2006). Data indicate that hypoxia-induced reduction of miR-15b and miR-16 contributes to an increase in VEGF. Some other miRNAs also regulate angiogenesis and vascular function (Anand et al. 2010).

In this study, the levels of gene expression of VEGFA and eNOS in umbilical vein and decrease in the plasma concentrations of VEGFA and eNOS in both the Arg- and NCG-supplemented groups may be a feedback regulatory mechanism of arginine-produced NO in the fetal umbilical vein and placenta compared with the control group. This is supported by high expression of miR-15b and miR-222 in the umbilical vein of dietary Arg- or NCG-supplemented groups.

The arginine treatment may enhance placental angiogenesis and growth during early-to mid-gestation, thereby promoting an optimal intrauterine environment throughout pregnancy (Wu et al. 2004). Therefore, it is possible that dietary supplementation with arginine increases the synthesis of NO in the placenta and fetus, as reported for adult

rats (Wu and Morris 1998; Kohli et al. 2004). The outcome would be to enhance placental angiogenesis and growth (including vascular growth), utero-placental blood flow, the transfer of nutrients from mother to fetus, and, therefore, fetal survival, growth and development (Kwon et al. 2004; Wu et al. 2004, 2006).

Although the majority of the conceptus loss occurs during the peri-implantation period, there is evidence that significant losses also occur during later gestation (Wilson 2002). Piglets born dead per litter were significantly decreased both in Arg-supplemented and NCG-supplemented groups in this study, which is similar to the study reported previously (Mateo et al. 2007). This may suggest that dietary Arg or NCG supplementation also regulated placenta vascular functions.

Notably, we found that plasma concentrations of phosphorus and $Zn^{2+}$ were higher both in sows of the Arg-supplemented and NCG-supplemented groups, indicating that Arg or NCG supplementation increases protein synthesis of fetus (Castillo-Durán and Weisstaub 2003; Frank et al. 2007). This is in agreement with the findings reported previously (Mateo et al. 2007). This study showed that dietary Arg or NCG supplementation increased litter piglets born alive and litter birth weight of all piglets born alive, while there were not differences in the average birth weights of all piglets born or of piglets born alive between groups. Furthermore, plasma concentrations of growth hormone were higher in sows of Arg-supplemented and NCG-supplemented groups than in sows of the control group.

In summary, supplementing dietary Arg or NCG during late gestation enhanced the reproductive performance of sows. Also, Arg or NCG treatment improved efficiency in the utilization of dietary nutrients; we propose that Arg or NCG treatment may effect the expression of miRNA-15b and miRNA-222, thereby controlling its target, VEGFA and eNOS, respectively, gene expression in the umbilical vein. Thus, Arg may regulate angiogenesis and vascular development and functions of umbilical vein and placenta, providing more nutrients and oxygen from mother to fetuses for fetal survival, growth and development. However, it is necessary to determine how arginine regulate fetal survival, growth and development through microRNAs.

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