Reproductive performance of gestating gilts supplemented with riboflavin[1]

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ABSTRACT

To evaluate the effects on litter size and expression of vascular endothelial growth factor (VEGF) and of uteroferrin, crossbreed gilts (n = 24) were supplemented with 0 or 60 mg daily of riboflavin during gestation. Litter size and average weight of piglets were determined at birth and at weaning. Samples of placenta were collected at farrowing to determine the relative expression of VEGF and uteroferrin. Supplemented and not supplemented gilts had 11.2 ± 0.6 and 8.2 ± 0.6 of total piglets born, respectively ($P < 0.004$). There were also corresponding increases in piglets born alive with 10.5 ± 0.6 versus 8.1 ± 0.6 ($P < 0.01$) and in total piglets weaned by the gilts supplemented with riboflavin (9.41 ± 0.6 and 7.5 ± 0.6, $P < 0.05$). A difference between treatments was found for total litter weight at birth, but not at weaning. Relative expression of VEGF was greater ($P < 0.07$) in the placenta of gilts supplemented with riboflavin than in those not supplemented, but no differences between treatments were observed in the relative expression of uteroferrin. The present results demonstrate that daily supplementation with 60 mg of riboflavin to gilts during gestation may increase litter size, perhaps by improving vascularization of the placenta, thus enhancing embryo/fetus survival.

Key words: riboflavin, VEGF, litter size, gilts

RESUMEN

Desempeño reproductivo de cerdas primerizas suplementadas con riboflavina durante la gestación

Para evaluar los efectos en el tamaño de lechigada y la expresión del factor de crecimiento endotelial vascular (VEGF; por sus siglas en inglés) y de la uteroferrina, cerdas primerizas cruzadas (n = 24) se suplementaron con 0 o 60 mg de riboflavina diariamente durante toda la gestación. El tamaño de la lechigada y el peso individual de las crías se determinó al nacer y al destete. Se colectaron muestras de placenta al momento del parto para determinar la expresión relativa del VEGF y de la uteroferrina. Las cerdas suplementadas y no suplementadas parieron 11.2 ± 0.6 y 8.2 ± 0.6 cerditos totales, respectivamente ($P < 0.004$). Hubo diferencias análogas en el número de cerditos nacidos vivos de 10.5 ± 0.6 contra 8.1 ± 0.6 ($P < 0.01$) y en el número total de cerditos destetados (9.41 ± 0.6 y 7.5 ± 0.6, $P <$

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0.05). Se encontró diferencia entre los tratamientos para el peso total de la lechigada al nacer pero no al destete. La expresión relativa del VEGF fue mayor (P < 0.07) en la placenta de cerdas suplementadas con riboflavina que en las controles, pero los tratamientos no difirieron en la expresión relativa de la uteroferrina. Los resultados presentes indican que la suplementación con 60 mg diarios de riboflavina a cerdas primerizas durante la gestación puede incrementar el tamaño de la lechigada, quizás mediado por una mejor vascularización de la placenta y por consiguiente mejorando la supervivencia del embrión/feto.

Palabras clave: riboflavina, VEGF, lechigada, cerdas

INTRODUCTION

The swine industry of Puerto Rico contributes only 5.52% of pork consumed locally (Anonymous, 2011); per capita consumption being approximately 27 kg in 2011. Local pork production is expensive because of high costs of feed, electricity, and other inputs. Therefore, producers need alternatives to increase their efficiency and profitability. Some examples of reproductive metrics related to production efficiency of swine are: number of piglets born alive, stillborn and mummified at birth, and number of weaned piglets. The number born alive per sow (litter size) is one of the most important economic variables for this industry. Generally, sows have high ovulation and fertilization rates; therefore, these characteristics do not represent a limitation to female reproductive efficiency. On the other hand, litter size can be limited by uterine capacity. Uterine capacity combines the abilities of the uterus to provide nutrients, the placenta to transfer nutrients to the fetus, and the fetus to efficiently use those nutrients for growth and development. Regarding placenta efficiency, previous research has demonstrated that the transport capacity of the placenta correlates positively with fetal growth (Reynolds and Redmer, 1995). Placental efficiency depends on placental size, morphology, blood flow, and abundance of nutrient transporters (Fowden et al., 2006).

Vascularity is important for the delivery of oxygen and nutrients to the conceptus (Vonnahme et al., 2001). In addition, uterine capacity and placental efficiency may be affected by vascularity of these tissues, thus angiogenesis (development of blood vessels) is important (Vonnahme and Ford, 2004). Angiogenesis in turn depends on the expression of vascular endothelial growth factor (VEGF) (Ferrara, 2004).

Pig fetuses depend on the iron that crosses the placenta and a low level of transfer could result in piglets born deficient in that mineral (Mahan and Vallet, 1997). Iron is actively transported through the placenta by a glycoprotein called uteroferrin. Piglets are naturally born deficient in iron with only 40 to 50 mg in their body (Lipinski et al., 2010).

Previous research had demonstrated that a moderate deficiency in riboflavin impairs iron absorption (Powers, 2003). This water-soluble vitamin is also important to energy metabolism, among other body functions of animals and humans. Riboflavin is not always properly supplied by commercial diets which could lead to a deficiency in swine (Frank et al., 1984).

To our knowledge, there are no reports of research on riboflavin supplementation to pigs raised in the tropics. The objective of the present study was to supplement riboflavin to gestating gilts in an attempt to improve their reproductive performance.

MATERIALS AND METHODS

This research was conducted at the Swine Farm of the Department of Animal Science, University of Puerto Rico-Mayagüez (UPRM) in the municipality of Lajas, during the months of May through December of 2011. Thirty-one crossbreed gilts (Yorkshire x Landrace), between the ages of 10 and 11 months were used. Standing heat was detected twice daily, in the morning and evening, with the help of a boar. Gilts were moved to gestation crates and then inseminated twice at 12 and 24 hours after the first sign of standing heat. Five boars (Yorkshire, Landrace and Duroc) were utilized to collect semen. Gilts received randomly semen from one of the five boars according to the availability of male pigs, but avoiding consanguinity. Semen was evaluated under the microscope and the spermatozoa concentration was determined with the Spermacue®.[4] Each insemination utilized a bottle containing approximately 3.35 x 10^9 spermatozoa in 50 mL of semen diluted with an extender (Modena®, International Boar Semen, Iowa, United States). Beginning on the day of the second insemination, gilts were randomly assigned a dietary supplementation of 0 or 60 mg of riboflavin daily throughout gestation. The basal diet was a commercial gestation concentrate mix (15% crude protein), fed once daily. This level of supplementation was chosen based on evidence that 60 mg/day of riboflavin increased the number of piglets farrowed by 22% compared to 10 mg/day (control diet) (Pettigrew et al., 1996). Riboflavin (Sigma Aldrich®, Saint Louis, MN) was weighted, placed in plastic Ziploc® bags and immediately mixed into the feed. Gilts were provided with 2 kg of the diet daily during the first 15 days post insemination, then from day 16

[4]Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

until parturition, with 2.2 kg of the same diet. This feeding regimen was recommended by Dr. Donald C. Mahan, Department of Animal Science, The Ohio State University (personal communication). A week before parturition, gilts were moved to farrowing stalls. At farrowing the following variables were recorded: number of piglets born alive, stillborn and mummified; and birth weight. Weaning weight and number of piglets surviving were recorded at 21 days of age.

Tissue collection and RNA isolation

Samples of placental tissue (approximately 2.54 cm length) were collected adjacent to the umbilical cord at farrowing (one per gilt), placed in 15 mL sterile centrifuge tubes and stored in liquid nitrogen until all of the samples were collected. The samples were subjected to isolation of total RNA by utilizing the RNAzol® RT reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. Samples in 15 ml sterile centrifuge tubes with 1 mL of RNAzol® RT per 100 mg of tissue were homogenized for one minute at the highest speed of a Polytron® Homogenizer and then 0.4 mL of molecular grade water (MGW) was added. Each tube was stirred for 15 seconds and then left to rest for 15 minutes at room temperature. Tubes were centrifuged for 15 minutes at 4° C and 12,000 X *g*. The supernatant (phase containing RNA) was collected and placed in a new tube, to which a 1:1 ratio of 100% isopropanol per milliliter of supernatant was added. After mixing and waiting 10 minutes, the tube was centrifuged at 12,000 X *g* for 10 minutes. The isopropanol mix was then discarded. The resulting RNA pellet was washed twice with ethanol (75%) added in a ratio of 0.5 mL per initial milliliter of isopropanol. The final RNA pellet was diluted with 50 µl of MGW. Concentration of total RNA was determined by utilizing an Eppendorf BioPhotometer Plus®. The tubes were then stored at -80° C. To eliminate contamination with genomic DNA, the RNA solution was then treated with DNAse 1 (Sigma Aldrich®, Saint Louis, MN) prior to cDNA synthesis.

cDNA synthesis and real-time PCR analysis

Twenty microliters of a reverse transcriptase reaction was performed with the Quanta Biosciences qScript cDNA SuperMix® (Gaithersburg, MD) and 400 ng of total RNA. The reaction for cDNA synthesis was performed as follows: five minutes at 25° C, 30 minutes at 42° C, five minutes at 85° C, and then kept at 4° C. Real time PCR was performed with Quanta Biosciences PerfeCta SYBR Green Fast Mix® (Gaithersburg, MD), following the manufacture's procedures, and 1/10th of the reverse transcriptase reaction. Specific primers for porcine transcripts of VEGF were: Forward 5'-CTGCTGCAACGACGAAGGTCT-3' Reverse

5'-CTCCTATGTGCTGGCCTTGGT-3' (Blomberg et al., 2010) and for uteroferrin: Forward 5'-TGCAAGCTTATGGACGTGGACG-3'Reverse 5'-GCCAAGCTTTCATCAGGCCCGTCGGTG-3' (Ling and Roberts, 1993). Cyclophilin, a housekeeping gene was also utilized and designed manually: Forward 5'-GGGTTCCTGCTTTCACAGA-3'Reverse 5'-AGGACCCGTATGCTTCAGGA-3'. Final concentrations for the forward and reverse primers were 0.3 µM. Relative expression was determined by the standard curve method for VEGF, and by the delta delta CT method for uteroferrin.

Statistical Analysis

Statistical analysis was performed using SAS software (Version 9.1, SAS Institute Inc., Cary, NC). Differences between treatments regarding litter size at birth and at weaning; and VEGF and uteroferrin relative expression, were determined for a completely randomized design. A nested design was utilized for individual piglet weight. The number of observations was twenty-four (n = 24), twelve gilts per treatment. The treatments were control (0 mg) and supplementation of riboflavin (60 mg). The dependent variables were numbers of total piglets born, piglets born alive and weaned, stillbirth, and mummified, litter weight at birth and at weaning, relative expression of VEGF and of uteroferrin. Breed of the boar was not included in the statistical model due to their limited availability at the farm. Only a few boars, not related to the gilts under study, could be used, thus they could not be allotted in predetermined fashion. Theoretically, all the progeny had hybrid vigor/heterosis. The statistical model had a completely randomized design:

Y_{ij} = total piglets born, number of piglets born alive and weaned, litter weight at birth and at weaning, stillbirth, mummified, or relative expression of VEGF and of uteroferrin
μ = population mean
T_i = effect of the treatments (0 and 60 mg riboflavin)
ε_{ij} = experimental error associated with the dependent variable in question

Statistical model for nested design:

$$Y_{ijk} = \mu + \alpha_i + \beta_{j(i)} + \varepsilon_{ijk}$$

Y_{ijk} = individual piglet weight
μ = population mean
α_i = effect of the treatment (0 mg and 60 mg riboflavin)
$\beta_{j(i)}$ = effect of the *j*th gilt within *i*th treatment
ε_{ijk} = experimental error associated with individual piglet weight

RESULTS AND DISCUSSION

Independent of treatment ($P > 0.48$), seven of the 31 gilts inseminated did not get pregnant during the experiment. These were eliminated from the data analysis. As a result, the remaining twenty-four gilts provided the experiment data ($n = 12$, for each of the two treatments). The numbers of total piglets born, born alive and weaned were greater ($P < 0.004$, $P < 0.01$ and $P < 0.05$, respectively) in gilts supplemented with riboflavin than in those not supplemented (Table 1). A few piglets were found dead in the farrowing crate, one of which is known to have been killed by the gilt. The treatments differed in total litter weight at birth, but not at weaning (Table 2). In contrast, there were no differences between treatments in individual piglet weight either at birth or at weaning (Table 2).

Expression of VEGF and uteroferrin in the placenta

The expression of VEGF was greater in the placenta of supplemented gilts than in the controls (1.39 ± 0.31 versus 0.53 ± 0.33, respectively; $P < 0.07$). However, there were no differences (1 versus 1.516, $P = 0.81$) in the expression of uteroferrin.

Bazer and Zavy (1988) reported that supplementing 100 mg of riboflavin to gilts from days 4 to 7 postcoitum increased litter size by one piglet compared to a control diet. Approximately at day 8 of gestation, riboflavin is found in uterine secretions of gilts, coinciding with the expansion of the trophoblast (Murray et al., 1980). Possibly, supplementation with riboflavin could increase the amount transferred to the conceptus, which may enhance embryonic survival (Mahan and Vallet, 1997). In this study, a difference of approximately two piglets alive at birth and at weaning was observed in gilts supplemented daily during gestation with 60 mg of riboflavin compared to non-supplemented animals. In contrast to Bazer and Zavy (1998), in the present research daily supplementation level of riboflavin was lower, but it continued for the length of the gesta-

TABLE 1.—*Mean number of total piglets born, born alive, stillborn, mummified and weaned from gilts supplemented with 0 or 60 mg of riboflavin.*

	0 mg	60 mg	P Value
Total piglets born	8.2 ± 0.6	11.2 ± 0.6	0.004
Born alive	8.1 ± 0.6	10.5 ± 0.6	0.01
Stillborn	0.1 ± 0.2	0.5 ± 0.2	0.33
Mummified*	0	0.2 ± 0.1	0.30
Total piglets weaned	7.5 ± 0.6	9.41 ± 0.6	0.05

*Only one gilt in the riboflavin treatment had two mummified piglets.

TABLE 2.—*Mean individual weight of piglets and total litter weight at birth and at weaning from gilts supplemented with 0 or 60 mg of riboflavin.*

	0 mg	60 mg	P Value
Total litter weight (kg)			
Birth	13.96 ± 1.37	18.12 ± 1.29	0.04
Weaning	35.56 ± 4.34	44.34 ± 4.09	0.16
Individual piglet weight (kg)			
Birth	1.71 ± 0.03	1.66 ± 0.02	0.63
Weaning	4.73 ± 0.06	4.56 ± 0.04	0.63

tion period. Perhaps, riboflavin supplementation is also important in the later stages of gestation; thus, in this study one more piglet was born per litter compared to the experiment by Bazer and Zavy (1988). However, Pettigrew et al. (1996) reported that increased riboflavin supplementation did not increase litter weight of sows receiving levels from 10 to 160 mg/d. The latter study and the present one differed in the duration of riboflavin supplementation, being for 21 days beginning at breeding versus throughout gestation, respectively. Perhaps, both embryo and fetus survival are benefited by riboflavin supplementation, which may lead to increased litter size at birth.

The weight of each piglet at birth is important because lighter ones have a lesser probability of survival (Milligan et al., 2002). In the present research, supplementing gilts with riboflavin during gestation did not significantly affect individual piglet weight at birth or at weaning, but tended to favor the control. The latter can be explained because gilts not treated with riboflavin had fewer piglets at birth. In contrast, total litter weight at birth of supplemented gilts was greater, although litter weight at weaning was not. Inadequate riboflavin intake (less than 6.4 mg daily in 2.0 kg of feed) can lead to low birth weight or stillborn piglets (Frank et al., 1984). In rats, Cockroft (1979) observed that embryos grown *in vitro* without riboflavin showed reduced growth and some were abnormal. Therefore, this author concluded that riboflavin is important for embryo development and a generous intake seems to improve litter weight at birth.

Litter size might be limited by a mechanism of vascularity (Vonnahme and Ford, 2004). Since VEGF contributes to angiogenesis and permeability of the placenta, it may foster improved blood flow, and transport of nutrients to the conceptus (Vonnahme and Ford, 2004). In this experiment, the greater expression of VEGF observed in the placenta of gilts supplemented with riboflavin during gestation could sug-

gest better vascularization of the placenta, and consequently flow of nutrients to the fetus, which may help to explain the increased number of total piglets born per litter to these gilts. We have not found other reports relating VEGF expression to supplementation with riboflavin in any animal species. In a study on rats, folic acid supplementation increased VEGF mRNA levels in the yolk sac (Zabihi et al., 2007).

Without VEGF, the efficiency of the placenta can be compromised, resulting in defects in vascularization (Ferrara, 2004), transport of nutrients (Bell et al., 1987) and formation of organs and systems (Gerber et al., 1999). The cardiovascular system is one that develops and functions in the embryo (Hamilton et al., 1962), and without VEGF and riboflavin anomalies can occur in its formation, perhaps resulting in embryonic death and consequently decreasing litter size. Blocking VEGF in pregnant mice by utilizing antibodies, resulted in fewer embryos implanted (Zhang et al., 2001), suggesting that VEGF also plays a role in implantation and consequently in litter size. In addition, hybridization of VEGF mRNA was found in the trophoblast at early stages of post implantation (Jakeman et al., 1993), again suggesting a role in implantation.

The embryo, before implantation, receives endometrial secretions important for embryonic survival, including uteroferrin (Ellenberger et al., 2008). Riboflavin deficiency may interfere with the utilization of iron and its absorption (Powers, 2003) and thus cause anemia. Some signs of anemia are poor growth, rough hair coat, wrinkled skin and paleness of the mucous membranes. None of the pigs used in this investigation presented any signs of anemia at birth, a typical symptom of iron deficiency. This finding is consistent with the fact that expression of uteroferrin, a protein carrier for iron, was similar in the placenta of all gilts in the current study independent of treatments. Uteroferrin is necessary throughout gestation to supply iron to the conceptus (Ellenberger et al., 2008). In this study, serum iron concentration was not measured in piglets at birth; this variable could be used to determine if riboflavin supplementation improves iron status in piglets at birth.

In conclusion, supplementing 60 mg of riboflavin daily to the diet of gestating gilts, during the whole course of pregnancy, increased litter size at birth and the number of weaned piglets. Total litter weight at birth, but not litter weight at weaning, was greater for the riboflavin treatment. Differences in individual piglet weight at birth and at weaning were not significant but favored the non-supplemented treatment. Expression of the VEGF gene in the placenta was higher in gilts supplemented with riboflavin, which, perhaps, contributed to improve litter size. There were no differences between treatments in the expression of the uteroferrin gene in the placenta. These findings suggest that riboflavin may play a role in VEGF expression and as a consequence improve

reproductive performance in pigs. Further research regarding the interaction of riboflavin and VEGF during gestation in gilts is needed.

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