REPRODUCTIVE PERFORMANCE OF GESTATING GILTS SUPPLEMENTED WITH RIBOFLAVIN

By

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ABSTRACT

To evaluate the effects on litter size and expression of vascular endothelial growth factor and uteroferrin, crossbreed gilts (n=24) were supplemented with 0 or 60 mg of riboflavin during gestation. Litter size and average weight of piglets were determined at birth and at weaning. In addition, samples of placenta were collected at farrowing to determine the relative expression of vascular endothelial growth factor and uteroferrin. Gilts supplemented with 60 mg of riboflavin had 11.2±0.6 total piglets born compared to the gilts not supplemented that had 8.2±0.6 of total piglets born ($P<.004$). There was also a difference between the gilts supplemented with riboflavin in piglets born alive with 10.5±0.6 when compared to 8.1±0.6 ($P<0.01$). Total piglets weaned was higher for the gilts supplemented with riboflavin when compared with the gilts not supplemented; 9.41±0.6 and 7.5±0.6, respectively ($P <0.05$). Increased differences were found between treatments on total litter weight at birth, but not at weaning. Relative expression of vascular endothelial growth factor was greater in the placenta ($P<.07$) of gilts supplemented with 60 mg of riboflavin when compared with gilts not supplemented. There were no differences between treatments in the relative expression of uteroferrin in the placenta of gilts. The results of this investigation demonstrate that daily supplementation of 60 mg of riboflavin to gilts during gestation may increase litter size, perhaps by improving vascularization of the placenta, thus enhancing embryo/fetus survival.

RESUMEN

Para evaluar los efectos en el tamaño de lechigada y la expresión del factor de crecimiento endotelial vascular y la uteroferrina, cerdas primerizas cruzadas (n=24) fueron suplementadas con 0 o 60 mg de riboflavina durante la gestación. El tamaño de la lechigada y el peso individual promedio, fue determinado al nacer y al destete. Además, se colectaron muestras de placenta al momento del parto para determinar la expresión relativa del factor endotelial de crecimiento vascular y la uteroferrina. Las cerdas primerizas suplementadas con 60 mg de riboflavina tuvieron 11.2±0.6 cerditos totales nacidos y las cerdas no tratadas tuvieron 8.2±0.6 cerditos totales nacidos (P<.004). Además, hubo diferencia en el número de cerditos nacidos vivos entre tratamientos siendo esta 10.5±0.6 y 8.1±0.6 para cerdas tratadas con riboflavina y cerdas no tratadas respectivamente (P<0.01). El número total de cerditos destetados fue mayor, 9.41±0.6, para las cerdas tratadas con riboflavina en comparación con 7.5±0.6 cerditos destetados de las cerdas no tratadas con riboflavina (P <0.05). Sí se encontraron diferencias entre los tratamientos para el peso total de la lechigada al nacer pero no al destete. La expresión relativa del factor de crecimiento endotelial vascular fue mayor (P<.07) en la placenta de cerdas primerizas suplementadas con 60 mg de riboflavina cuando se compararon con las no suplementadas. No se encontraron diferencias entre tratamientos para la expresión relativa de la uteroferrina en la placenta de las cerdas. Los resultados de esta investigación demuestran que la suplementación de 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 sobrevivencia del embrión/feto.

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1 INTRODUCTION

The swine industry of Puerto Rico contributes approximately 5.52% of the pork consumed locally (Anonymous, 2011). The consumption per capita of pork was approximately 58.62 pounds for 2011. In Puerto Rico, pork production is expensive because feed and electricity, among other production costs are high. Therefore, producers should find alternatives to increase efficiency and profitability. Some examples of reproductive metrics related to production efficiency of swine are: number of piglets alive, stillborn and mummified pigs at birth, and number of weaned piglets. The number of pigs born alive per sow, litter size, is one of the most important economic variables for this industry. The average number for litter size in the United States is approximately 10 (Anonymous, 2013). In Ireland the average litter size is 11.2, France 12.5 and Denmark 12.7 (Lawlor and Lynch, 2007). Generally, sows have high ovulation and fertilization rates; therefore, these characteristics do not represent a limitation to reproductive efficiency of female pigs. On the other hand, litter size can be limited by uterine capacity. Uterine capacity combines the ability of the uterus to provide nutrients, the ability of the placenta to transfer nutrients to the fetus, and the ability of the fetus to efficiently use those nutrients for growth and development. Regarding placenta efficiency, previous investigations have 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). Uterine capacity and placental efficiency additionally might be affected by vascularity of these tissues. Angiogenesis (development of blood vessels)

consequently will impact these tissues (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 could cross the placenta, a low amount of this mineral could result in piglets born with iron deficiency (Mahan and Vallet, 1997). Iron is actively transported through the placenta by a glycoprotein called uteroferrin. Piglets born with approximately 40 to 50 mg of Fe in their bodies and are considered iron deficient (Lipinski et al., 2010). This amount is insufficient for their rapid development and growth. The latter combined with the fact that sow's milk contains low levels of iron results in the necessity of injecting iron to piglets at birth.

Various attempts have been made to increase iron status on piglets at birth by supplementing it to the mother at gestation and lactation but without success (Wang et al., 2009). For example, sows injected 0, 2 or 10 mg of iron per pound of body weight on the 100^{th} day of gestation did not increase the iron levels in the piglets blood (Pond et al., 1961). Also in the same experiment, sows were injected iron on day six and nine of lactation but this made no significant differences in the iron content of the milk (Pond et al., 1961). Supplementation of iron in the diet throughout lactation did not increase the levels of iron on the sow's milk (Pond et al., 1965; Veum et al., 1965).

Previous investigations had demonstrated that a moderate deficiency in riboflavin impairs iron absorption (Powers, 2003). This water-soluble vitamin is also important because it is involved in energy metabolism among other functions in the body of animals and humans. Riboflavin, in occasions, is not added properly to commercial diets and could lead to a deficiency of this vitamin in swine (Frank et al., 1984).

According to some investigations, supplementing riboflavin on the diet of gestating sows did not increase litter size but increased the percentage of sows that farrow (Pettigrew et al., 1996). On the other hand, other researchers found that supplementing 100 mg/d of riboflavin during early pregnancy of gilts resulted in an increase of litter size by one piglet at birth and at weaning (Bazer and Zavy, 1988). In contrast, a similar investigation regarding reproductive performance of sows and supplementing riboflavin did not increase litter size (Tilton et al., 1991). The previous examples of riboflavin supplementation to pigs showed different effects on reproduction. Previous investigations where performed in other temperatures. To our knowledge, there are no investigations about riboflavin supplementation to pigs raised in the tropics. Therefore, the objective of this investigation was to supplement riboflavin to the diet of gestating gilts raised in Puerto Rico in an attempt to improve their reproductive performance.

2 OBJECTIVES

Evaluate the effect of riboflavin supplementation to the diet of gestating gilts on reproductive performance, and on the placental expression of vascular endothelial growth factor (VEGF) and uteroferrin.

3 LITERATURE REVIEW

A. Current situation of the swine industry in Puerto Rico

The swine industry is currently on the ninth position of the first ten agricultural industries in Puerto Rico according to 2010-2011 agricultural statistics (Anonymous, 2010). This agricultural business was positioned seven and eight in 2007 and 2008, respectively. In Puerto Rico, the consumption of pork is approximately 210.7 million pounds per year, from which only 12.7 million pound are produced locally.

The statistics in the previous paragraph represent the commercial swine production. Non-commercial (less than 6 farrowing sows and 11 growing pigs) swine production is very common in Puerto Rico. These animals are often fed with human food waste.

Regardless of the type of production, Puerto Rico's swine industry is confronting economic problems and changes. Some of the problems that this industry is facing are: medicine and feed costs, low reproductive performance, high costs of animal replacement, lower costs of imported pork, and lack of an efficient record system. The Department of Agriculture in Puerto Rico is searching for a way of improving productivity and quality of pork meat. On 2011, the Department of Agriculture and the program "Unidad de Calidad y Alto Rendimiento" (UCAR) of the swine sector, assigned $564,750 to acquire equipment, gilt replacement and boar of high genetic value, including transportation up to $200.00 per animal, gestation crates and automatic feeders. On 2012, approximately 15 swine producers that received funds from this program were given a computer and received training on the use of farm electronic records. In addition, the Department of Agriculture facilitated the purchase of digital scales, the construction of bins or water reserves, the purchase and installation of temperature and wind curtains, materials for biosecurity and high- pressure washers. The latter approach only targeted a small group of swine producers that qualified according to established regulations (i.e. commercial producers that only use concentrate feed, had oxidation ponds, among others).

Promotion of pork produced locally and control of importation is needed to help the industry. Competition with imported pork sold at a lower price is a factor that impacts the swine industry in Puerto Rico. Local producers could produce more meat to decrease the number of importations and contribute to the economy but some swine producers are closing their farms because of the lower price of imported meat and decide not to compete with them (Anonymous,

2007). Besides competition, there are also high costs of feed that not only affects Puerto Rico, but also United States and the rest of the world. Crop production declined in 2012 as a result of drought affecting meat production (Anonymous, 2012) and as a consequence the price of corn and other ingredients is now higher, which affects feed costs of pigs raised in Puerto Rico and other parts of the world.

Improving litter size has the potential to increase net economic gain for the producers of the swine industry in Puerto Rico. In Puerto Rico, only a few producers have between 10 to13 piglets born per litter and the rest are between eight and nine (local producers, personal communication). Many investigations in swine have been made in aspects such as genetics and nutrition attempting to increase productive efficiency. For example, identification of genes affecting prolificacy, selection for prolificacy and the use of Chinese lines has been explored (Rothschild et al., 1994). Companies like *Newsham Choice Genetics* and *PIC*, supply to swine producers, animals that guarantee certain characteristics including high prolificacy. These companies are not economically accessible to all producers in Puerto Rico, especially small or artisanal farms. In addition, these breeds require a specific diet so they can grow efficiently, which increases feeding costs. Swine producers are also trying new equipment like the *Birthright Deck*® to decrease pre-weaning mortality by accommodating piglets that are born from big litters. This equipment operates with a milk replacement and could also be installed on the farrowing crate. Besides equipment, local investigations may benefit the swine industry in Puerto Rico by targeting the existing problems related to efficiency and productivity at the farm.

In Puerto Rico, only a few experiments have been made regarding swine production. These investigations are related to reproduction, meat quality, and weaning and finisher performance. An investigation to evaluate reproductive performance of crossbreed pigs was conducted

demonstrating that these pigs had a better or similar performance when compared to pure breeds (Rosa and Santana-Nieves, 2009). In addition, supplementation of the diet with wastewater from a caramel production increased weight gain and carcass quality of weaning to finishing pigs (Jiménez et al., 2005). Furthermore, breadfruit is currently being evaluated to reduce feed costs by adding it to a commercial diet (Méndez and Santana, unpublished data). Furthermore, the effect of exogenous oxytocin and prostaglandin $F_{2\alpha}$ was evaluated during artificial insemination on conception rate during two seasons in Puerto Rico was evaluated and demonstrated a positive effect (Soto-Velez et al., 2007). In addition, a current research, investigates artificial insemination of pure and crossbreed gilts and sows to evaluate two types of confinement: individually *versus* in groups (Domenech and Santana, unpublished data). In Puerto Rico, there are only a few experiments relating nutritional effects on pig reproduction. For example, the effect of energy levels on the diet was evaluated on some reproductive characteristics in gilts and sows (Santana, 1988). Thus, there is a need of research in the nutritional effects on pig reproduction in tropical conditions. The temperatures in Puerto Rico are elevated almost all year, and as a consequence, animals are frequently under heat stress. Heat stress elevates the nutritional requirements for maintenance, and if the animal is in gestation or lactation the requirements for itself and the offspring become even higher (Black et al., 1993). Consequently, nutritional requirements for different stages must be evaluated to ensure efficiency of swine production. Thus, the present research investigated the effects of riboflavin supplementation on reproductive performance of gilts focusing on litter size.

B. General information of Riboflavin

Riboflavin is one of the B complex vitamins, also called vitamin B2. This compound has the formula of 7, 8-dimethyl-10-(I'-D-ribityl) isoalloxazine. Vitamin B2 is essential for cellular

growth and function (Jayapaul et al., 2012). This vitamin is associated with the metabolism of proteins, lipids, carbohydrates and alcohol. Riboflavin enters the cell by utilizing a phosphoglycoprotein transport located on the surface of cellular membranes called riboflavin carrier protein (Jayapaul et al., 2012). Riboflavin also participates as a direct precursor of either flavin adenine (FAD) or flavin mononucleotide (FMN), which are essential components of cellular biochemistry (Vogl et al., 2007). FAD is a cofactor of some of the oxido-reduction enzymes and intervenes in energy metabolism in the mitochondria (Jayapaul et al., 2012). Riboflavin is absorbed in the small intestine in the proximal region, where it is phosphorylated to FMN (Mahan and Vallet, 1997). This process has a saturable active system, but also a passive diffusion component that is used when higher concentrations of riboflavin in the body are present (Foraker et al., 2003). Collectively, riboflavin is an essential vitamin that contributes to various functions in the body and energy production.

Riboflavin is a vitamin that has been implicated in reproduction of humans and animals. In pigs, it has been related with the sow's reproductive system, gestation, birth, lactation, development of the fetuses, among other functions (Pettigrew et al., 1996). In addition, riboflavin may interact with other nutrients and improve embryo survival, which might result in bigger litters. The riboflavin carrier protein is induced during pregnancy to transport this vitamin from the mother through the placenta for the development of the embryo (Adiga and Murty, 1983). In pregnant rats, the riboflavin carrier protein was found in serum from day 4 of gestation to parturition (Muniyappa and Adiga, 1980), suggesting that it is important for embryo development.

C. Riboflavin on the reproductive system

1. Estrous Cycle

Anestrous resulted in rats deficient or with no riboflavin in their diet, and normal cycles were restored by supplementing riboflavin on their diets (Coward et al., 1942). A large amount of riboflavin secretion has been found in the uterus of sows at day 8 of the estrous cycle (Moffatt et al., 1980). Furthermore, sows receiving a diet deficient in riboflavin (0.77ppm) did not reach a fourth estrus (Esch et al., 1981). Therefore, riboflavin deficiencies might impair reproduction in animals by affecting the normal cyclicity, which in turn affects reproductive performance and productive life.

2. Gestation and Parturition

Gestation and parturition play an important role in reproduction because they will determine the number of piglets per litter at birth and at weaning. Riboflavin is one of the essential nutrients in the embryo stage because is part of the uterine secretions that increases embryonic survival (Murray et al., 1980). The uterus of sows secretes riboflavin at the time the blastocyst is beginning rapid expansion, making high concentrations of riboflavin available to embryos at an early stage of development (Murray et al., 1980). In a study with four sows, utilizing diets deficient in riboflavin, three sows did not conceive and only one farrowed but the piglets died within 48 hours (Miller et al., 1953). Similar results were observed with six gilts that were given different amounts of riboflavin in the diet; two of them did not give birth and the other four gave birth seven days earlier (Frank et al., 1984). In addition, litter weight at birth was also low compared with gilts that had adequate amounts of riboflavin in their diets (Frank et al., 1984). Additionally, sows with higher amounts of riboflavin in the diet had heavier litters at

weaning (Miller et al., 1953). On newborns, it was observed that mothers fed diets deficient in riboflavin had a lower litter weight at birth (Haggarty et al., 2009).

A deficiency of riboflavin in the diet during gestation has negative effects at parturition in mammals. A study with rats reported that litters died within 2 and 22 days of birth due to a deficiency of riboflavin found on the vaginal contents of the mother (Coward et al., 1942). A similar effect was reported in pigs; in a period of 48 hours post partum, the piglets died due to deficiency of riboflavin in the sow's diet during gestation (Ensminger et al., 1947). Furthermore, piglets born from mothers deficient in riboflavin were hairless, could not drink milk, had enlarged forelegs (due to edema in the connective tissue), and none of the pigs survived in a period of 20 to 48 hours post parturition (Ensminger et al., 1947). Post mortem irregularities have been also reported in piglets deficient of riboflavin including: pale kidneys, mottled with congested areas and yellow granular accumulations, yellow friable livers, red areas in the stomach, and excess body fluids (Ensminger et al., 1947). A severe deficiency of riboflavin during gestation could result in premature parturition and stillborn piglets (Frank et al., 1984). Consequently, diets with inadequate amounts of riboflavin can decrease litter size at birth and at weaning by affecting internal organs and physical strength.

3. Lactation

Lactation is also a critical stage for piglet growth and development. If sows are not fed properly, they could not produce quality milk neither survive this crucial phase. During pregnancy, sows' requirements for vitamins and minerals increase and if they are not provided in the diet these nutrients will be mobilized from the sow's tissue (Kim and Easter, 2001). Supplementing riboflavin to gilts during lactation decreases the number of piglets that die during

this period (Frank et al., 1988). For this reason, riboflavin is an essential vitamin on the diet of gilts and sows during this stage.

D. Riboflavin: general deficiency symptoms and anomalies

Riboflavin is essential for building and maintaining body tissues, necessary for healthy skin, prevents sensitivity of the eyes to light, and is important for the proper function of the nervous system (Perelson and Ellenbogen, 2001). Some deficiencies of riboflavin are: anorexia, weight loss, dermatitis, ocular lesions, and muscular weakness (National Research Council, 1998). A deficiency of this vitamin can cause loss of appetite in pigs (Ensminger et al., 1947; Miller et al., 1954; Frank et al., 1984). Some sows with .77ppm and 1.77ppm of riboflavin in their diet presented anorexia five to seven days pre partum (Frank et al., 1984). Stiffness (Hughes, 1940) and unsteady gait (Lehrer and Wiese, 1952) were also reported in previous studies in pigs fed diets deficient in riboflavin. Lehrer and Wiese (1952) reported alopecia, poor growth, anorexia, rough hair coat, dermatitis, light sensitivity, vomiting, scours and inflammation of anal mucosa in piglets due to riboflavin deficiency. Heavy sebaceous discharge around the eye and ear, and a dried discharge on the skin were also observed on pigs with diets containing no riboflavin or only 1 gram of this vitamin per kilogram of food (Miller et al., 1954). Lesions of the eye were observed in the lens, cornea and eyelids of pigs receiving diets deficient in riboflavin (Miller et al., 1954). Other symptoms like tail defects, syndactylism, clubfoot, short mandible and cleft palate and fusion of the ribs and shortening of the long bones have also been reported on an induced deficiency on riboflavin with galactoflavin in rats (Nelson et al., 1956). As a consequence, diets deficient on riboflavin affect a variety of external and internal organs of different species.

E. Riboflavin in other organs and systems

Riboflavin is a vitamin that contributes in various functions of the body. Among them, riboflavin plays a role in the development of the cardiovascular system, gastrointestinal system and nervous system among others.

1. Cardiovascular System

In the embryo, the cardiovascular system is the first system to develop and function (Hamilton et al., 1962). Riboflavin has been demonstrated to affect heart tissues in embryos and adult animals. For example, a riboflavin induced deficiency by supplementation of galactoflavin demonstrated defects of the interventricular septum of the heart and of the aortic arch pattern in rats (Nelson et al., 1956). In another experiment, the ventricular septal of mice embryos deficient in riboflavin were thicker than non-deficient ones (Chan et al., 2010). Without a proper development of the cardiovascular system, quality of life and survival of any specimen could be at risk. To our knowledge there are no investigations regarding this system, riboflavin deficiency and swine.

2. Gastrointestinal System

Diets deficient in riboflavin can also affect the gastrointestinal system. Rats fed a diet deficient in riboflavin, had an increase of height of the villus on the upper small intestine compared to rats fed a control diet (Williams et al., 1995). Similar results were found on weaned rats that were on diets deficient in riboflavin, were the development of the duodenum, cell proliferation, the multiplication of the intestinal crypts and the villus number were affected (Yates et al., 2001). Results like these, give relevant information because absorption of nutrients can be impaired due to different anomalies on the gastrointestinal system that affect nutrient

absorption later on. Consequently, gastrointestinal anomalies may impact weight gain in pigs or any animal.

3. Nervous System

One day old broiler chickens fed a diet deficient in riboflavin (1.8 mg/kg) exhibited peripheral nervous system anomalies as early as 11 days of age compared to the chickens fed a control diet (5.0 mg/kg) (Cai et al., 2009). These anomalies were: endoneurial edema, hypertrophic Schawnn cells, tomacula, and demyelination on the sciatic, cervical and lumbar spinal nerves. Similar results like demyelinating peripheral neuropathy were observed on broilers fed the diet deficient in riboflavin (Cai et al., 2006). This condition is responsible for the "curled toe" in chickens. Likewise, rats fed a diet deficient in riboflavin for 3 to 5 weeks presented demyelination in the sciatic nerve when compared to a control diet (Norton et al., 1976). Also, dogs presented neurological symptoms and myelin degeneration when fed a diet deficient in riboflavin (Street et al., 1941). In summary, diets deficient in riboflavin affect the nervous system and as a consequence may impair reflexes, stimuli, and coordination in different species. If such anomalies were present in pigs or any other could result in economic loss for the producers. To our knowledge there are no investigations regarding the nervous system, riboflavin deficiency and pigs.

F. Riboflavin and weight gain

Weight gain is an important trait for swine producers; they need healthy animals that can develop and grow efficiently. A deficiency of riboflavin, affects negatively weight gain on animals. Pigs that received a lower amount of riboflavin required more food to gain one pound (Miller and Ellis, 1951), and when it was increased in the diet, it induced faster and greater

weight gain (Krider et al., 1949). Young pigs receiving a diet deficient in riboflavin had slow growth rate and remained little when compared to animals receiving riboflavin in the diet (Lehrer and Wiese, 1952). It was also reported that young pigs consuming a diet containing less than 0.8 mg of riboflavin per pound ate and gained less weight when compared to the pig consuming 0.8 or 1.4 mg of riboflavin (Terrill et al., 1955). As a result, riboflavin, can improve weight gain resulting in heavier pigs.

G. Nutrient transport through the placenta

The efficiency of the placenta to transport nutrients will affect the development of the fetus. Placental insufficiency results in fetal loss, low weight at birth, stillbirth, pre-weaning mortality and poor growth (Vallet et al., 2009). Among the nutrients transported through the placenta, iron is an important mineral for pigs because they are born with iron deficiency, and consequently, it has to be supplemented intramuscularly when they are born. In addition, iron is necessary for the myelinization process in the nervous system and oxygen transport (Kroner, 2011). Iron is transported through the placenta by uteroferrin, a glycoprotein produced by the epithelial glands of the uterus (Bazer et al., 1991). Uteroferrin is transported by the areolae into the chorioallantoic capillaries and to the fetus by the umbilical vein (Renegar et al., 1982). When uteroferrin enters with iron, the iron is then transferred to transferrin and is then utilized for hematopoiesis in the fetal liver (Buhi et al., 1982). Transferrin can leave the allantoic sac intact, but uteroferrin cannot; when uteroferrin transfers the iron, it is then degenerated when it enters fetal circulation (Buhi et al., 1982). At day 60 of gestation of gilts, uteroferrin reaches the maximum reported, exceeding 1g/day (Roberts and Bazer, 1988). Placental efficiency will

affect the transport of uteroferrin and iron among other nutrients and consequently affect other organs in the body.

H. Vascular Endothelial Growth Factor (VEGF) and litter size

Angiogenesis is the process were new blood vessels are formed for normal growth and tissue development (Reynolds and Redmer, 1995). Vascular Endothelial Growth Factor is a glycoprotein (Ferrara and Henzel, 1989) that contributes to this process. The VEGF regulates normal processes such as formation of the corpus luteum (Fraser et al., 2000), wound healing (Frank et al., 1995) and bone angiogenesis (Ferrara, 2004). The gene expression of VEGF is regulated by hypoxia (Ferrara and Davis-Smyth, 1997). Furthermore, this growth factor affects angiogenesis in pathological processes such as tumor formation (Fraser et al., 2000). VEGF also stimulates proliferation of endothelial cells on blood vessels (Ferrara and Henzel, 1989) endothelial cell survival (Gupta et al., 2002) and migration (Wang et al., 2008).

Inhibition of VEGF function by blocking the protein or its encoding gene may result in physiological anomalies. For example, in neonatal mice, VEGF was partially inactivated and resulted in impaired body growth, increased mortality and abnormalities in different organs (Gerber et al., 1999). Furthermore, in adult rats, inactivation of VEGF resulted in the malformation of the corpus luteum (Ferrara et al., 1998). Moreover, the loss of a single VEGF allele can result in defective vascularization and early embryonic death (Ferrara, 2004). It has been reported that amounts of placental VEGF mRNA declined slightly from day 25 to day 36, and later demonstrated a progressive increase from day 44 to day 112 in pigs (Vonnahme et al., 2001).

Blood flow in pigs is increased at gestation by endometrial and placental angiogenesis among other factors (Edwards et al., 2011). Early in gestation, angiogenesis in the placenta will

ultimately determine its efficiency. VEGF stimulates vascular permeability in the placenta (Vonnahme et al., 2001). In addition, it has also been reported that this glycoprotein, produced by the maternal and fetal epithelial layers, promotes the growth of capillaries locally, that facilitate the transport of nutrients and waste products (Charnock-Jones et al., 2001).

Over-nourishment (Redmer et al., 2005) and restriction (Redmer et al., 2004) of nutrients during gestation can also affect the expression of angiogenic factors, such as VEGF in ewes. Feeding regimen may affect expression of VEGF during gestation and consequently development of embryo/fetus survival and ultimately litter size. VEGF is an important factor that will ultimately decide the fate of angiogenesis in the pig's placenta and piglet survival.

I. Other nutrients that could improve litter size in swine

Litter size has been increased by modification of the diet. Folic acid, also known as vitamin B9, is a water soluble vitamin utilized for decreasing the risk of birth defects at pregnancy in women (Green, 2002). Folic acid is involved with the synthesis of DNA, RNA and protein (Matte and Girard, 1989). Folic acid can improve sow's reproductive performance. Supplementation of folic acid in the diet of gilts at gestation reduces embryo mortality especially in animals with higher ovulation rates (Lindemann, 1993) and supplementation of 1ppm of folic acid at gestation/lactation increased litter size at birth (Lindemann and Kornegay, 1989). Fifteen milligrams of folic acid increased litter size when injected to sows through day 60 of gestation (Matte et al., 1984).

Vitamin A is essential for the maintenance of reproductive function and fetal development (Thompson et al., 1964). Pig's intestinal microflora can produce vitamin A; perhaps, this is why it is not investigated intensively. Deficiencies in vitamin A and β-carotene decrease certain uterine proteins, like uteroferrin, important for embryonic survival in pigs (Chew et al., 1982).

Injecting β-carotene tends to improve these uterine proteins (Chew et al., 1982). A potential role of vitamin A in gestation is the increment of progesterone concentrations in the serum which may decrease embryonic death (Whaley et al., 1997). Injection of 12,300 IU and 32.6 mg of vitamin A and β-carotene, respectively, in gilts increased the number of piglets per litter and their weight (Brief and Chew, 1985). Gilts fed a diet high in energy and injected vitamin A had an average daily gain greater than control gilts but the embryonic survival was lower (Whaley et al., 1997).

In addition to folic acid and vitamin A, arginine has also been investigated in gilts. Arginine is an amino acid that serves as a precursor of nitric oxide, which enhances blood flow during pregnancy in ovine (Mateo et al., 2007) and is also an angiogenic factor (Wu et al., 2010). Gilts receiving a diet with 1.0% L-arginine-HCl resulted in an increase of two piglets per litter compared to a control diet (Mateo et al., 2007). Similar results were observed when gilts were supplemented with 0.83% L-arginine, the number of live born and litter weight increased (Wu et al., 2010). Improving litter size can be achieved by studying and exploring numerous amino acids and vitamins.

4 MATERIALS AND METHODS

This investigation was conducted at the Swine Farm of the Department of Animal Science, University of Puerto Rico at Mayagüez (UPRM) in Lajas, Puerto Rico; during the months of May through December of 2011 (Annual temperature 89.4°F). Thirty-one crossbreed gilts (Yorkshire X Landrace), between the ages of ten and eleven months were utilized. Standing heat was detected twice daily, in the morning and in the evening with the help of a boar. Gilts were moved to gestation crates and then inseminated at 12 and 24 hours after the first sign of standing heat. Five boars (3 Yorkshire, 1 Landrace and 1 Duroc) were utilized to collect semen.

Gilts were inseminated with semen from one of the five boars according to disponibility of male pigs of the farm and avoiding consanguinity. Semen was evaluated under the microscope and the concentration was determined with the Spermacue®. For each insemination, a bottle containing approximately 3,350,000,000 spermatozoa in 50 mL of semen diluted with an extender (Modena®, International Boar Semen, Iowa, United States) was utilized. Beginning on the day of the second insemination, gilts were randomly assigned a diet supplementation of 0 or 60 mg of riboflavin throughout gestation. The basal diet was a commercially available gestation diet (15% protein) (Swine Farm Mill from UPRM), fed once daily. It has been demonstrated that the amount of 60 mg/day (NRC 1988 requirement 3.75 mg/kg of feed) of riboflavin has increased the number of sows farrowed by 22% compared to 10 mg/day (control diet) (Pettigrew et al., 1996). Riboflavin (Sigma Aldrich®, Saint Louis, MN) was weighted and put in plastic Ziploc® bags and immediately mixed with the feed. Gilts were provided 2 kg of the diet during the first 15 days post insemination. From day 16 until parturition, they were fed 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 measured: number of piglets born alive, stillborn, mummified, and birth weight. Weaning weight and number of piglets was measured at 21 days of age.

A. Tissue collection and RNA isolation:

Samples of placental tissue (approximately 2.54 cm) were collected from the region adjacent to the umbilical cord at farrowing (only from one random placenta per gilt), placed in 15 mL sterile centrifuge tubes and stored in liquid nitrogen until all the samples were collected. Then, they were utilized for isolation of total RNA by utilizing the RNAzol® RT reagent

(Molecular Research Center, Inc., Cincinnati, OH) following the basic manufacture's protocol. Briefly, samples were put on 15 mL sterile centrifuge tubes with 1 mL of RNAzol® RT per 100 mg of tissue. Tissue was 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 was put in a new tube. In this new tube, 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 then washed twice with ethanol (75%) that was 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.

B. 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) following manufacture's procedures and 400 ng of total RNA. The reaction for cDNA synthesis was performed as follows: 5 minutes at 25°C, 30 minutes at 42°C, 5 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) also following manufacture's procedures and $1/10^{th}$ of the reverse transcriptase reaction. Specific

primers for porcine transcripts of uteroferrin and vascular endothelial growth factor were obtained from previous investigations (Table 1). Final concentrations for the forward and reverse primers were 0.3 μM. Relative expression was determined by the standard curve method for vascular endothelial growth factor, and by the delta delta CT method for uteroferrin.

Table 1. Primers utilized for determining the relative expression of uteroferrin and vascular endothelial growth factor in placental tissue.

Gene	Gene sequence	Reference
Vascular endothelial growth factor	Forward 5'-CTGCTGCAACGACGAAGGTCT-3' Reverse 5'-CTCCTATGTGCTGGCCTTGGT-3'	Bloomberg et al., 2010
Uteroferrin	Forward 5'-TGCAAGCTTATGGACGTGGACG-3' Reverse 5'-GCCAAGCTTTCATCAGGCCCGTCGGTG-3'	Ling and Roberts, 1993
Cyclophilin	Forward 5'-GGGTTCCTGCTTTCACAGA-3' Reverse 5'-AGGACCCGTATGCTTCAGGA-3'	Jiménez, unpublished data

C. Statistical Analyses

Statistical analysis was performed by using SAS software (SAS software, Version 9.1, SAS Institute Inc., Cary, NC, USA). Differences between both supplementation levels (0 vs. 60 mg of riboflavin) treatments regarding litter size at birth, at weaning, VEGF and uteroferrin relative expression were determined by a completely randomized design. A nested design was utilized for individual piglet weight. The number of observations was twenty four (n=24), twelve gilts for each treatment. The variables analyzed were total piglets born, number of piglets born alive and weaned, litter weight at birth and at weaning, stillbirth, mummified, relative expression of VEGF and relative expression of uteroferrin. The breed of the boar was not included in the statistical model because there were not enough observations per boar to analyze them. For this reason, they could not be allotted in to any group. All the progeny was crossbred.

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

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 relative expression of uteroferrin

μ = population mean

T_i = effect of the treatment (0 mg riboflavin, 60 mg riboflavin)

ε_{ij} = experimental error associated with 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 relative expression of uteroferrin, boar

The weight of individual piglet was analyzed as a 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 riboflavin, 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

5 RESULTS

A. Reproductive Performance

Regardless of treatment (P>0.48), seven of the 31 gilts did not get pregnant during the experiment. Therefore, these seven gilts were eliminated from the data analysis to avoid confusing results. Therefore, there were twenty four gilts were included in the analysis (n=12, for 0 and n=12 for 60 mg of riboflavin supplementation). The number of total piglets born, born

alive and weaned were greater (11.2 ±0.6 and 9.4 ± 0.6, P<.05) in gilts supplemented with 60 mg of riboflavin when compared to gilts that were not supplemented with this vitamin (8.2 ± 0.6 and 7.5 ± 0.6) (Table 2). In this experiment, some piglets were found dead on the farrowing crate, one of them was killed by the gilt. There was a difference on total litter weight at birth, but not at weaning (Table 3). In contrast, there were no differences between treatments on neither individual piglet weight at birth or at weaning (Table 3).

Table 2. 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	9.4±0.6	0.05

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

Table 3. 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

B. Expression of VEGF and uteroferrin in the placenta

The expression of VEGF was greater in the placenta of gilts supplemented with 60 mg of riboflavin than gilts supplemented with 0 mg of riboflavin (P< .07; Figure 1). However, there were no differences (P=0.81) in the expression of uteroferrin (Figure 2).

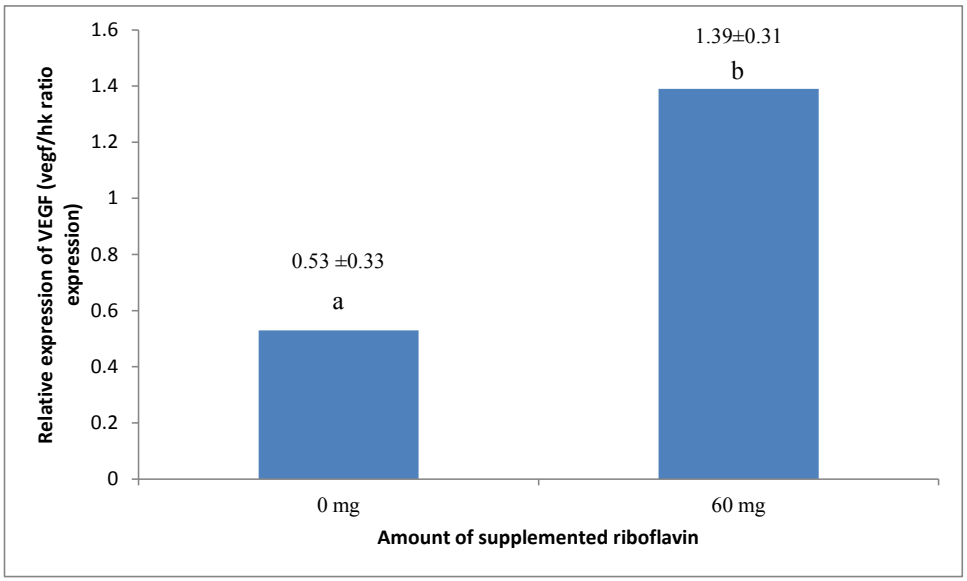

Figure 1. Relative expression of vascular endothelial growth factor (VEGF) in placenta of gilts supplemented with 0 or 60 mg of riboflavin. [a-b] columns differ. P<.07

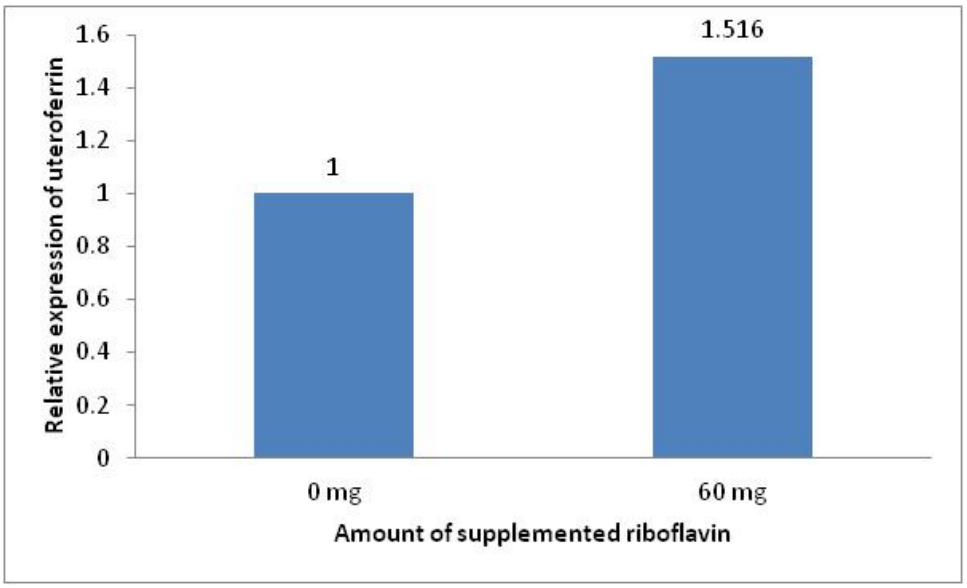

Figure 2. Relative expression of uteroferrin in placenta of gilts supplemented with 0 or 60 mg of riboflavin. No differences were observed. P=0.81

6 DISCUSSION

Bazer and Zavy (1988) reported that supplementing 100 mg of riboflavin to gilts from day 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). In addition, supplementation of riboflavin could increase the amount of the vitamin itself that can be transferred to the conceptus which may result in an increased embryonic survival (Mahan and Vallet, 1997). In this investigation, a difference of approximately two, for piglets alive at birth and at weaning, was observed on gilts supplemented daily during gestation with 60 mg of riboflavin compared to non-supplemented animals. Different to Bazer and Zavy (1988, in Pettigrew et al., 1996), in this investigation less riboflavin was supplemented but for the length of the gestation period. Perhaps, riboflavin supplementation may be important in other stages of gestation and therefore, the present investigation resulted in an increase of one more piglet as compared to Bazer and Zavy (1988, in Pettigrew et al., 1996). In another investigation the results differed from the present study, riboflavin did not increase litter size at birth from sows receiving 10 to 160 mg/d (Pettigrew et al., 1996). Some factors that might explain these differences are the length of the riboflavin supplementation, which in our investigation was throughout gestation but in Pettigrew and associates supplementation was for 21 days beginning at breeding. Perhaps, during this stage of development, supplementation of riboflavin may help to increase embryo survival and consequently an increase in litter size at birth.

The weight of each piglet at birth is important because lighter ones have a lesser percentage of survival (Milligan et al., 2002). In the present investigation, supplementing gilts with riboflavin during gestation did not affect individual piglet weight at birth, even though litter

weight at birth was higher. The latter can be explained by the greater number of piglets farrowed in the supplemented gilts. However, inadequate amounts of riboflavin (less than 6.4 mg daily on 2.0 kg of feed) could lead to low birth weight or stillborns piglets (Frank et al., 1984). On the other hand, Cockroft (1979) observed that rat embryos raised *in vitro* without riboflavin had a reduction in growth and some of them were abnormal. Therefore, riboflavin seems to be important for embryo development (Cockroft, 1979) and can improve litter weight at birth.

Litter size might be limited by vascularity because vascular endothelial growth factor contributes to angiogenesis and permeability of the placenta, which may result in a highly efficient placenta, blood flow, and a better transport of nutrients to the conceptus (Vonnahme and Ford, 2004). In this experiment, a greater expression of VEGF in the placenta of gilts supplemented with riboflavin during gestation could suggest better vascularization of the placenta, and consequently to the fetus. Therefore, these gilts perhaps improved transfer of nutrients to their embryos, which may help explain the difference in the number of total piglets born per litter on the gilts supplemented with riboflavin. To our knowledge, this is the first report relating VEGF expression and supplementation of riboflavin. An investigation with rats, it was reported that 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 defective 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 of the systems to develop and function in the embryo (Hamilton et al., 1962), and without VEGF and riboflavin anomalies can occur in its formation maybe resulting in embryonic death and consequently decreasing litter size. In pregnant mice it was reported that when blocking VEGF by the use of antibodies,

resulted in less 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 on the trophoblast at early stages of post implantation (Jakeman et al., 1993) suggesting its role on 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). Iron deficiency can cause anemia. Some signs of anemia are poor growth, rough hair coat, wrinkled skin and paleness of the mucous membranes. None of the pigs from this investigation presented any signs of anemia at birth. Possibly related, the genetic expression of uteroferrin, a protein carrier for iron, was similar in the placenta of gilts from this investigation. Although, there was no difference between treatments, uteroferrin is necessary throughout gestation to supply iron to the conceptus (Ellenberger et al., 2008). In this investigation, iron was not measured in piglets at birth; this variable could detect if riboflavin improved iron status on the piglets at birth.

7 CONCLUSIONS

Supplementing 60mg of riboflavin to the diet of gestating gilts, during the whole pregnancy, increased litter size at birth and the number of weaned piglets. Total litter weight at birth was greater for the riboflavin treatment but not litter weight at weaning. There were no differences on individual piglet weight at birth or at weaning. The expression of the VEGF gene in the placenta was higher in gilts supplemented with riboflavin, which perhaps helped to improve litter size. There were no differences in the expression of the uteroferrin gene in the

placenta. These findings suggest that riboflavin may play a role on VEGF expression and as a consequence improves reproductive performance in pigs.

8 IMPLICATIONS AND RECOMMENDATIONS

As the results from this investigation demonstrate, litter size might be improved by modifying the diet of the gilt. Also supplementation of riboflavin during the lactation period could be made to try to improve litter weight at weaning. The effect of riboflavin supplementation can be also explored in different periods of gestation to find a specific moment were is most important for gestation. Placental efficiency can also be another trait to explore to improve reproduction by measuring blood flow and weighing the placenta. Commercial diets could be evaluated under the Puerto Rican weather to evaluate the amount of riboflavin needed for adequate gestation in gilts and sows. Other experiments regarding the VEGF gene at different days of gestation with riboflavin can also be made. Iron status in piglets could be measured at birth to compare it with supplementation of riboflavin supplementation and without. In Puerto Rico, riboflavin supplementation can be implemented on commercial productions. It was estimated that the cost of supplementation per gilt was $4.00 for the length of gestation. This increased the litter by two piglets.

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