INGRID SOARES GARCIA

EFFECTS OF L-ARGININE SUPPLEMENTATION ON THE EXPRESSION OF GENES INVOLVED WITH OXIDATIVE STRESS IN SWINE EMBRYOS AND FETUSES

Dissertation presented to the Animal Science Graduate Program of Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Adviser: Simone Eliza Facione Guimarães

Co-adviser: Alysson Saraiva

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APPROVED: August 02, 2019.

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“No one is so wise that has nothing to learn and not so foolish that has nothing to teach”

(Blaise Pascal)

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ABSTRACT

GARCIA, Ingrid Soares, M.Sc., Universidade Federal de Viçosa, August, 2019. **Effects of l-arginine supplementation on the expression of genes involved with oxidative stress in swines conceptuses** Adviser: Simone Eliza Facioni Guimarães. Co-adviser: Alysson Saraiva.

During early gestation in highly prolific gilts, elevated oxidative stress is reported to be associated with pregnancy complications and negatively influence conceptuses development. Arginine is a conditionally essential amino acid and have a known antioxidant capability. In the present work, it was evaluated the effects of 1.0% L-arginine supplementation (ARG) on oxidative stress related genes and proteins of commercial gilts embryos (25 days) and fetuses (35 days). In 25 days of age, there was no statistical difference between the treatments in gene expression. There was a trend of two genes in fetuses from control gilts (CON), *SOD2* (P= 0.09) an antioxidant-producing gene and *EIF2AK4* (P= 0.07) related with the phosphorylation and activation of nrf2 transcription factor, with activate numerous antioxidant genes in its pathway. Protein abundance also did not present a statistically difference between treatments and in both ages for the nrf2 and sod2 proteins. These results suggest that supplementation of L-arginine in early gestation in gilts (25 and 35 days of age) with the objective to lessen ROS concentrations and oxidative stress damage, is not effective, as in both treatments (ARG and CON), most genes and proteins did not present a significant difference in its performance to maintain a redox homeostasis, and the genes with a trend in its expression did not influence the antioxidant proteins abundance.

Keywords: Antioxidants. *EIF2AK4*. *SOD2*. Early gestation.

RESUMO

GARCIA, Ingrid Soares, M.Sc., Universidade Federal de Viçosa, Agosto de 2019. **Efeito da suplementação de l-arginina na expressão de genes envolvidos com o estresse oxidativo em conceptos suínos**. Orientadora: Simone Eliza Facioni Guimarães. Coorientador: Alysson Saraiva.

Durante o período inicial da gestação em leitoas com alta prolificidade, pode ocorrer estresse oxidativo elevado, que está associado com complicações na gravidez e tem influência negativa sobre o desenvolvimento dos conceptos. A arginina é um aminoácido condicionalmente essencial e tem conhecida capacidade antioxidante. No presente estudo, foram avaliados os efeitos da suplementação com 1,0 % de L-arginina (ARG) sobre genes e proteínas relacionadas ao estresse oxidativo de embriões (25 dias) e fetos (35 dias) de marrãs comerciais. Aos 25 dias de idade, não houve diferença estatística para os valores obtidos na expressão gênica entre os tratamentos. Houve tendência de dois genes nos fetos das marrãs do grupo controle (CON), *SOD2* (P = 0,09) gene produtor de antioxidantes e *EIF2AK4* (P = 0,07) relacionado com a fosforilação e ativação do fator de transcrição nrf2, que ativa diversos genes antioxidantes que fazem parte de sua via. A abundância de proteínas também não apresentou diferença significativa entre os tratamentos e em ambas as idades para as proteínas nrf2 e sod2. Estes resultados sugerem que a suplementação de L-arginina no período inicial da gestação em marrãs (ambas de 25 e 35 dias de idade) com o objetivo de diminuir as concentrações de ROS e o dano oxidativo, não foi eficaz, já que em ambos os tratamentos, a maioria dos genes e proteínas não apresentou diferença em seu desempenho para manter a homeostase redox, e os genes que apresentaram tendência em sua expressão não influenciaram a abundância de proteínas antioxidantes.

Palavras chave: Antioxidantes. *EIF2AK4*. *SOD2*. Início da gestação.

SUMMARY

CHAPTER 1

1.1 General Introduction

1.1.1 Prenatal growth of pigs

A trait of great economic importance is the litter size of the gilts, which is the result of a number of factors such as ovulation rate, fertilization rate, embryo survival and uterine capacity (BAZER; FIRST, 1983). Ovulation rate can be regulated through genetic selection or use of exogenous gonadotrophins and fertilization rates are high in the species (around 95%). Thus, embryonic mortality is considered the main limitation for litter size in swine (BAZER et al., 1988).

Gestation of the female swine has an average duration of 114 days, which is normally divided into thirds, of which the first is characterized by maternal recognition of pregnancy and implantation of the embryos, while the second and third are characterized by fetal development (BORTOLOZZO *et al.,* 2007).

As in mammals in general, the developmental process in swines begins during oogenesis, when RNA and maternal proteins are accumulated leading to the growth and maturation of oocytes, embryogenesis will begin after fertilization, when sperm enters the ovulated oocyte triggering the development program that was started during oogenesis (TELFORD; WATSON; SCHULTZ, 1990). Embryos move from the oviduct to the uterus after fertilization and the beginning of the cleavage process, culminating with the implantation of the swine embryos in the uterus between the 12th and 18th day after fertilization (DZIUK, 1985; POPE; MAURER; STORMSHAK, 2005). The embryo begins to be considered fetus around the 30th day, when there is calcification of the skeleton and formation of organs and limbs, from the 35th day of gestation, the organogenesis is complete beginning the fetal phase (KENT et al., 2006).

1.1.2 Pregnant female nutrition

One of the main factors affecting the survivability, growth and development of embryos and fetuses is the maternal nutrition (WU et al., 2006). Different dietary levels of some amino acids and vitamins may affect the regulation of gene expression, leading to reduced incidence of disease and / or embryo defects during development (JAENISCH; BIRD, 2003; SKINNER; MANIKKAM; GUERRERO-BOSAGNA, 2010).

Several disorders in postnatal life, during childhood or in adulthood, originate during prenatal growth (BROLIO et al., 2010), Thus, embryonic survival, related to the reproductive efficiency of the sows, is closely related to the nutritional levels offered to the female swine. Then, it can be concluded that amino acid nutrition is primordial for the development of fetuses and embryos, having a positive influence on the maturation of the main systems (muscular, cardiovascular, digestive, respiratory, skeletal, among others) (FLYNN et al., 2002).

1.1.3 The importance of arginine

The main function of arginine is protein deposition, moreover, it takes part in the synthesis and secretion of hormones, it aids in vasodilation, blood pressure regulation and endothelial resilience, it also plays an important role in the development of the heart of mammalian fetuses (WU et al., 2007). Arginine also participates in the synthesis and oxidation of cellular energy of other amino acids and urea (ammoniacal detoxification), assisting in acid-base regulation, positively influencing intestinal integrity, actively participating in reproductive events (spermatogenesis, ovulation, embryo implantation, placenta development and fetal growth) and in the immune system (WU; MEININGER, 2002).

The amino acid arginine plays multiple roles in animal metabolism, serving as a substrate for synthesis of proteins and other biological molecules, including ornithine, polyamines (putrescine, spermine and spermidine), proline, glutamine, creatine, agamatine, and nitric oxide, the latter being an important vaso-relaxing factor that regulates the transfer of oxygen from the mother to the fetus during pregnancy by controlling the blood flow (WU; MORRIS, 1998). Therefore, dietary supplementation with L-arginine during gestation would improve fetal development due to the participation of this amino acid in metabolic pathways of the formation of molecules, such as polyamines and nitric oxide (WU et al., 2013).

1.1.4 Oxidative stress

The concept of oxidative stress and related defense responses were first formulated in 1985 in the book Oxidative Stress (SIES, 1985), where it is defined as

the imbalance between the production of oxidants and antioxidants in favor of oxidants, leading to a disorder in signaling and redox control of the cell, in addition to molecular damage.

Reactive oxygen species (ROS) are produced as a normal result of cellular metabolism by all living organisms, and at low concentrations are part of physiological cellular processes, but at high concentrations, they lead to oxidative stress and produce changes in cellular components, such as RNA, DNA and proteins, and can also lead to cell death. Under normal conditions of aerobic cellular metabolism, molecular oxygen (O2) undergoes tetravalent reduction, with acceptance of four electrons, resulting in the formation of H2O. During this process reactive oxygen intermediates are formed, these produced oxygen metabolites are highly reactive when compared with the original molecule and those are called ROS. The three main types of ROS that are of physiological importance are superoxide anion (O2-), hydroxyl radical (OH) and hydrogen peroxide (H2O2) and the increase in their production can be influenced by several factors, such as the environment, nutrition or even the period of life that the organism finds itself in (FINKEL; HOLBROOK, 2000; BIRBEN et al., 2012).

Studies have already been carried out demonstrating that oxidative stress affects the expression of important genes and transcription factors involved in multiple pathways of cell biology that may affect the development of the embryo, its quality, and viability (LEITE et al., 2018).

1.1.5 Antioxidants

Under normal conditions, antioxidant molecules convert ROS to H2O to prevent excessive production of oxidizing agents, preventing oxidative stress and consequently cell damage. The cell has two types of antioxidant defenses: enzymatic antioxidants and non-enzymatic antioxidants. Enzymatic antioxidants are also known as natural antioxidants and include superoxide dismutase (SOD), glutathione (GSH), GSH peroxidase (GSH-Px), GSH reductase (GR) and catalase (CAT). Non-enzymatic antioxidants, also known as synthetic antioxidants or dietary supplements include

nutrients such as vitamin C, β-carotene, selenium, taurine and glutathione (LOBO et al., 2010).

SOD present in cytoplasm (Cu-Zn SOD) and mitochondria (MnSOD) is responsible for the dismutation of the superoxide anion in hydrogen peroxide; CAT does not need an electron donor and only removes hydrogen peroxide by reaction (H2O2 + H2O2 —> 2H2O + O2); GSH-Px contains selenocysteine in its active center and plays a central role in detoxification of peroxides using the reduced form of glutathione (GSH) as an electron donor (FUJII et al., 2003).

Oxidative damage to cell membranes are inhibited by the antioxidant action of vitamin E, along with vitamin C and GSH, constituting one of the main mechanisms of the body's endogenous defense (RILEY, 1994). The cooperative effect between vitamins C and E is often mentioned in the literature, showing that their interaction is effective in inhibiting membrane lipid peroxidation and protecting DNA (GEY, 1998).

1.1.6 Genes involved in protection against oxidative stress

The increase of ROS in the mitochondria, in the energy production process, is suppressed by the action of the cellular defense system. Catalase (CAT) and superoxide dismutase (SOD) are considered the main antioxidant enzymes in mammals, being produced by the genes *CAT* and *SOD* respectively (YUAN et al., 2007). Another gene of extreme importance in the combat against oxidative stress is the Nuclear Factor Erythroid 2-Related Factor 2 (*NRF2*), whose protein (Nrf2) is a transcription factor that is responsible for the activation of several antioxidant-producing genes that are part of its pathway, including *SOD* and *CAT*, the high or the low expression of *NRF2* and the production of its protein can be influenced by several factors, such as selenium deficiency and / or high concentration of energy. Diets with these characteristics lead to a large increase in ROS production and high levels of oxidative stress, since the decrease in the expression of *NRF2* leads to a lower expression of all the genes of its pathway (YANG et al., 2017).

Transcription factors of the forkhead box family, class O (*FoxO*) is another important regulator of the cellular response to stress, promoting the cellular antioxidant defense. *FoxO* stimulate the transcription of genes encoding antioxidant proteins located in different subcellular compartments, such as in the mitochondria (ie,

superoxide dismutase-2, peroxiredoxins 3 and 5) and in peroxisomes (catalase), as well as antioxidant proteins found extracellularly in plasma (for example, P-selenoprotein and ceruloplasmin) (KLOTZ et al., 2015).

The endoplasmic reticulum (ER) is apt to sense and respond to cellular stresses, including those resulting from metabolic imbalances and / or protein folding. In response to stress originating from within the ER, PKR-like endoplasmic reticulum kinase (PERK), composed by the genes *EIF2AK1*, *EIF2AK2*, *EIF2AK3* and *EIF2AK4*, initiate a program of transcriptional and translational regulation called response to misfolded proteins. A consequence of ER stress is the accumulation of ROS, which promotes a state of oxidative stress. PERK signaling, via activation of transcription factor Nrf2, coordinates the convergence of ER stress with oxidative stress signaling. ER stress activates PERK kinase activity, leading to phosphorylation of Nrf2, which promotes the transcription of genes whose protein products promote redox homeostasis, in conclusion, Nrf2 would be a substrate of PERK (CULLINAN et al., 2003; CULLINAN; DIEHL, 2006).

1.1.7 Arginine influence over oxidative stress

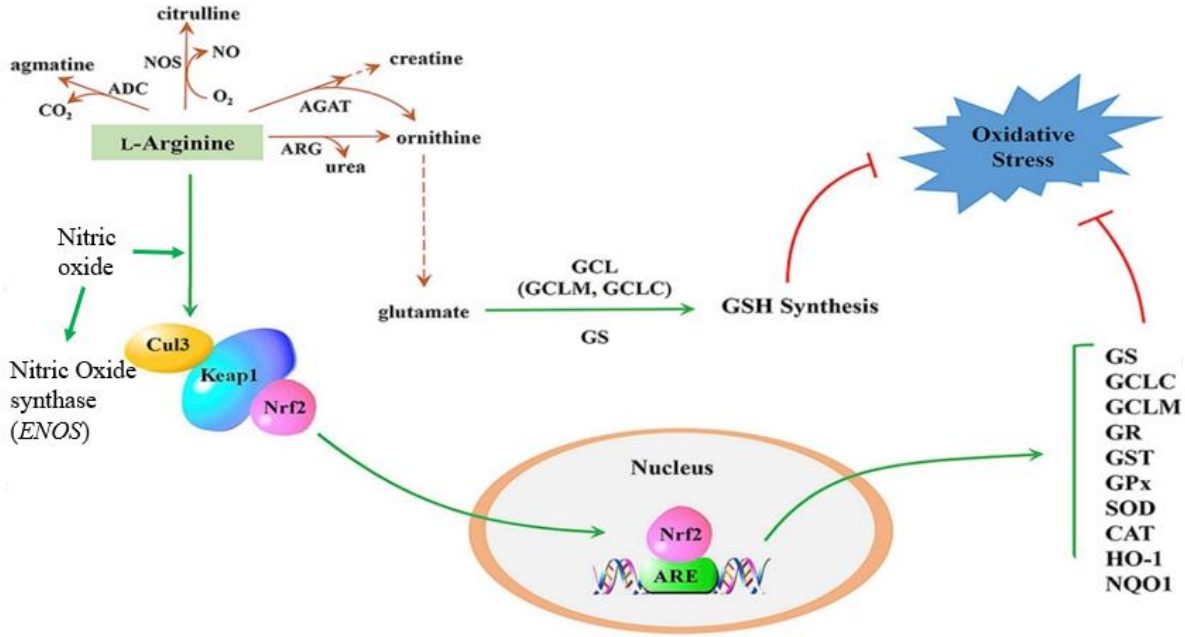
Studies with arginine have shown that it has a positive effect on different organisms in relation to the combat against oxidative stress. This occur because among the arginine metabolites, nitric oxide, ornithine and polyamines are known to effectively protect the body against damage caused by oxidative stress, which is mediated by the increased activities of antioxidant enzymes (SHI et al., 2005; DASGUPTA; HEBBEL; KAUL, 2006).

Nitric oxide (NO) is one of the signaling molecules with a participation in practically all cellular functions of the body(RAMPRASATH et al., 2012a). Nitric oxide is involved in the activation of the transcription factor nrf2, in turn, this factor activates the expression of many antioxidant-producing genes, including catalase (*CAT*), superoxide dismutase (*SOD*), UDP-glucuronosyltransferase, c-glutamylcysteine synthetase, NAD (P) H quinone oxidoreductase 1 (*HNO1*), glutathione-S-transferase (*GST*), glutathione peroxidase (*GPX*) and heme oxygenase-1 (*HO-1*) (RAMPRASATH et al., 2012b). Glutathione (GSH) is also an important antioxidant, and its synthesis

can be determined by both the action of Nrf2 and/or by glutamate cysteine ligase (GCL) and GSH synthase (GS), which originates from glutamate, originated from ornithine (one of the arginine metabolites) (LIANG et al., 2018). These processes can be observed in figure 1.

The polyamines in turn are organic polycationic unsaturated hydrocarbons having two or more primary amino groups which are involved in a variety of fundamental cellular processes, including transcription, RNA modification, protein synthesis and the modulation of enzymatic activities. Polyamines may be derived directly from ornithine by ornithine decarboxylase (ODC) or arginine, following several steps subsequently catalyzed by arginine decarboxylase (ADC) (MOSCHOU; PASCHALIDIS; ROUBELAKIS-ANGELAKIS, 2008). The study of polyamines have been shown to be effective in regulating gene expression in physiological activities under stress, causing reduction in the formation and accumulation of H2O2 and modulating the enzymatic activities (MANDAL et al., 2013).

Figure 1: Activation of the Nrf2 pathway, which is strongly regulated by Cullin3 (Cul3)/Kelch-like-ECH-associated protein 1 (Keap1) and GSH synthesis.



(LIANG et al., 2018) modified.

1.2 Objectives

We aimed with this study to evaluate the effects of supplementing commercials gilts with a diet of 1.0% L-arginine in the expression and regulation of genes that influence oxidative stress on conceptuses of 25 and 35 days, analyzing the state of these genes on early gestation, as that can influence the embryo and foetuses development.

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CHAPTER 2

EFFECTS OF L-ARGININE SUPPLEMENTATION ON THE EXPRESSION OF GENES INVOLVED WITH OXIDATIVE STRESS IN SWINES EMBRYOS AND FETUSES

2.1 Introduction

Pregnancy is suggested to be a state of oxidative stress, characterized by the placental production of reactive oxygen species including superoxide and hydrogen peroxide, the elevated oxidative stress can alter fetal formation of sows during gestation, which can influence post-embryonic animal traits (CHEN; SCHOLL, 2005; PRATER et al., 2008; BERCHIERI-RONCHI et al., 2011).

Oxidative stress is an imbalance resulted from the production of free radicals and antioxidants, part of the cell defense system. This imbalance can induce the formation of many diseases and an extensive damage of proteins, lipids and DNA (NORDBERG; ARNÉR, 2001; SEIFRIED, 2007). Reactive oxygen species (ROS; superoxide anion, $O2^{\bar{}}$; hydroxyl radical, HO; hydrogen peroxide, H2O2) are produced as a normal result of cellular metabolism by all living organisms, and at low concentrations are part of physiological cellular processes, however, excessive production of ROS may lead to oxidative stress, loss of cell function, and ultimately apoptosis or necrosis. Thus, a balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to diverse growth conditions (FINKEL; HOLBROOK, 2000; BIRBEN et al., 2012).

Arginine and its byproducts (such as ornithine, polyamines, proline, glutamine, creatine and nitric oxide) fulfill numerous functions in the body, such as protein synthesis, ammonia detoxification, cell proliferation, hormone secretion, apoptosis regulation, immune response, tissue repair, blood flow and body's antioxidant capacity, the latter being essential to animals under oxidative stress situations(WU; MORRIS, 1998; WU et al., 2007; LIANG et al., 2018).

Studies demonstrate that oxidative stress affects the expression of important genes and transcription factors involved in multiple pathways of cell biology that may affect early development of the embryo, its quality, and viability (LEITE et al., 2018).

Thus, supplementation of gilts with L-arginine during early gestation can be an interesting approach to avoid the damage caused by oxidative stress, which influence embryos and fetuses development, studies analyzing how arginine will affect this process are still needed to better understand the effects of aminoacidic supplementation for pregnant gilt. This study was aimed to evaluate the effects of supplementing commercials gilts with a diet of 1.0% L-arginine (LI et al., 2015) in the expression and regulation of genes that influence oxidative stress on conceptuses of 25 and 35 days of age.

2.2 Materials and methods

2.2.1 Animals and experimental design

The following experimental design for the animals and the conceptuses is already described by our research group at Costa et.al. (2019). Experimental protocol has followed ethical principles in animal research (CONCEA, 2016) and was approved by the Ethical Committee on Animal Use of *Universidade Federal de Viçosa* (UFV), Minas Gerais, Brazil [protocol no. 06/2017].

Summarizing, 23 commercial gilts, from 120 days of age, were housed in individual pens for better feed control of each animal. At 150 days of age, the management of puberty induction started by direct exposure of the female to an adult male twice daily (morning and afternoon). When the gilts expressed their third estrus, hormone synchronization was performed in groups of five females, using Regumate® (Merk Animal Health, USA). After identification of the fourth estrus, females were inseminated in two periods, 12 and 24 hours after the beginning of estrus. The first insemination day was considered day zero of gestation and the supply of experimental diets occurred 24 hours after the second insemination as described by (LI et al., 2010). Semen doses were collected from two commercial boars with proved reproductive performance (semen analyses) used to inseminate gilts in all treatments; semen from a single male was used to inseminate each female. The semen parameters met the requirements for use in pig artificial insemination (AI) programs recommended by Colégio Brasileiro de Reprodução Animal (2013) and used as routine at the pig farm.

Females were weighed at 120, 150, 180 and 210 days of age and on the day of the first insemination, in order to control the weight uniformity until the beginning of the supplementation. Gilts from the group of 25 days of gestation presented in the beginning of the supplementation a body weight of 154.00 ± 3.15 kg and 152.10 ± 7.12 kg for CON and ARG, respectively. Group of 35 days of gestation presented in the same period a body weight of 143.15 ± 8.96 kg and 148.55 ± 4.71 kg for CON and ARG, respectively (COSTA et al., 2019). In summary, gilts were assigned to a completely randomized design and a 2x2 factorial arrangement (two diets and two gestational ages), with five replicates per treatment on average (*n*=5). Gilts were fed either a control diet (CON), mainly composed of corn, soybean meal, mineral and vitamins supplements, formulated in order to meet the nutritional requirements of gestating sows (ROSTAGNO et al., 2011), or the CON diet supplemented with 1.0% ARG (Ajinomoto, Saga, Japan), associated with two gestational ages (25 and 35 days of gestation).

The supplementation of 1.0% L-arginine on gestation diet (ARG) was done by replacing clay filler by L-arginine. Nutritional levels for metabolizable energy (3148 kcal/kg), calcium (0.750%), phosphorus (0.395%) and digestible amino acids, lysine (0.535%), methionine and cysteine (0.381%), threonine (0.412%), tryptophan (0.113%) and valine (0.490%) were kept stable for both gestation diets. There were changes between CON and ARG diets for crude protein (12.16 vs. 14.46%) and digestible arginine (0.67 vs. 1.60%). To avoid ammonia intoxication and competition for basic amino acid transporters, 2.0% of arginine content in both diets was not exceeded, as recommended for sows (Wu et al., 2013).

Considering the 23 commercial gilts, 11 received the diet without supplementation (CON) and 12 received basal diet for pregnant animals with 1.0% L-arginine supplementation (ARG), beginning 24 hours after the second insemination. Diets were daily provided to the gilts, in equal quantities, divided into two daily feeds (9 am and 4 pm). A total of 1.8 kg/day were offered between days 1 and 3 of gestation and 2.2 kg/day were offered between days 4 and 24 (females slaughtered at 25 days of gestation), or 4 and 34 (females slaughtered at 35 days of gestation). Animals had free access to water throughout the experimental period.

From the 23 inseminated gilts, 20 became pregnant. At 25 days of gestation, five females of CON (*n*=5) and five females of ARG (*n*=5) were rendered unconscious using head-only electrical stunning (240V, 1.3A) and immediately exsanguinated. At 35 days of gestation, four females of CON (*n*=4) and six females of ARG (*n*=6) were rendered unconscious using head-only electrical stunning (240V, 1.3A) and immediately exsanguinated.

2.2.2 Conceptuses collection and experimental design

After slaughter, conceptuses (embryos: 25 days and fetuses: 35 days) were quickly collected and washed with PBS (Phosphate Buffered Saline) solution, the conceptuses from the right uterine horn were stored in liquid nitrogen for further gene expression analyses. gene expression analyses were performed within each gestational age, considering a completely randomized design with two treatments (CON and ARG) and five replicates on average per treatment (five females in CON and five females in ARG at 25 days and four females in CON and six females in ARG at 35 days). Four conceptuses were collected per female at each gestational age (two from the cranial region and two from the caudal region of the right uterine horn), totaling in average 20 conceptuses per treatment (20 conceptuses in CON and 20 conceptuses in ARG at 25 days and 16 conceptuses in CON and 24 conceptuses in ARG at 35 days).

2.2.3 Gene expression analyses from conceptuses

Total tissue RNA extraction was performed from 50 mg of embryos and fetuses samples (whole conceptuses sprayed with nitrogen) using TRIzol® (Invitrogen™) according to the protocol described by the manufacturer, and The final precipitate was rehydrated with 30 µL of UltraPure® DNase/RNase-Free water. Total RNA concentration was estimated by spectrophotometry using the NanoVue™ Plus (GE Healthcare, Freiburg, Germany), the quality integrity was determined in 1.0% agarose gel (data not shown). The cDNAs that was used in the real-time PCR analyzes was synthesized by reverse transcription reaction using the GoScript Reverse Transcriptase kit (Promega Corporation, Madison, WI, USA) following the

manufacturer's recommendations. The cDNA concentrations of the samples were estimated by spectrophotometry in the NanoVue™ Plus, and the single stranded cDNA will be stored at -20 ° C until their use in real-time quantitative polymerase chain reaction (RT-qPCR).

Primers for amplification of target and endogenous gene fragments will be designed using PrimerQuest software provided by Integrated DNA Technologies, Inc (Coralville, IA) from the nucleotide sequences obtained from the GeneBank database (http: //www.ncbi .nlm.nih.gov) (Table 1).

Table 1 - List of primers sequences for gene expression analysis by RT-qPCR for endogenous and oxidative stress related genes.

Gene	Sequence	NCBI Access code
	Housekeeping genes	
BACT	F:CTTCTAGGCGGACTGTTAGTG	XM_0031242803
	R:AGCCATGCCAATCTCATCTC	
HPRT1	F:CCAGTCAACGGGCGATATAA	NM_001032376.2
	R:GACCAAGGAAAGCAAGGTTG	
GAPDH	F:CAAAGTGGACATTGTCGCCATCA	NM_001206359.1
	R:AGCTTCCCATTCTCAGCCTTGACT	
	Oxidative Stress	
NRF2	F: GCCAGTCACTGTCTGAACTT	XM_021075132.1
	R: ATGCTGGGACTTGGGTTTAG	
SOD1	F: TGCAGGTCCTCACTTCAATC	NM_001190422.1
	R: CTTTGCCAGCAGTCACATTG	

SOD2	F: GCGGCTTGTTCAGGTATTTG	NM_214127.2
	R: CCTGGCTCTTTCCACTCTTT	
CAT	F: CTGCATCAGGTTTCCTTCC	NM_214301.2
	R: CGCCTCTCCCTTCTCATTA	
GPX	F: CGTCGCTTTCTGACCATC	AF532927.1
	R: CCCGAGAGTAGCACTGTAA	
FOXO1	F:GATCTACGAGTGGATGGTCAAG	NM_214014.2
	R: AACTTGCTGTGTAGGGACAG	
ENOS	F:CAAAGTGACCATTGTGGACCAT	NM_214295.1
	R: TGCTCGTTCTCCAGGTGCTT	
EIF2AK1	F: GGCCTGCACAGACATCATA	XM_021086083.1
	R: AGGTGACGCATACAGACAAG	
EIF2AK3	F: AGAAGAGCCCAGAATGAACC	XM_003124925.4
	R:GTCGGTCTTGGAGGAGAAATAG	
EIF2AK4	F:GGTCAAAGAGCCTCCAGAAA	XM_021097873.1
	R:GATAGGTGGGTGGGCATTTA	

BACT, B- actin; HPRT1, Hypoxanthine Phosphoribosyltransferase 1; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; NRF2, Nuclear Factor Erythroid 2-Related Factor 2; SOD1 , superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; GPX, Glutathione Peroxidase; FOXO1, forkhead box family, class O; ENOS, endothelial nitric oxide synthase;EIF2AK1, Eukaryotic Translation Initiation Factor 2 Alpha Kinase 1; EIF2AK3, Eukaryotic Translation Initiation Factor 2 Alpha Kinase 3; EIF2AK4, Eukaryotic Translation Initiation Factor 2 Alpha Kinase 4.

β-actin (*β-ACTIN*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine guanine fosforiboxiltransferase (*HPRT1*) were tested as endogenous controls. These genes were selected based on their amplification profile and dissociation curve (data not shown). None of the endogenous presented expression

difference between treatments (CON and ARG) at 25 or 35 days. Due to its greater expression and stability between treatments, the *GAPDH* was chosen as the best endogenous gene for embryo and fetal analyses (data not shown).

Gene expression was evaluated using real-time quantitative PCR (RT-qPCR), reactions were performed on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green® kit PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, USA), according to the manufacturer's recommendations. Before the expression analysis, each gene had its efficiency calculated using different concentrations of the primers (100, 200 and 400 nM) and cDNA (5, 15 and 45 ng) (SCHMITTGEN; LIVAK, 2008). *FOXO* did not present a good expression in the efficiency test, and thus, it was not evaluated in this phase, only in fetuses this gene was used. All reactions to a same target gene were done in technique duplicates. PCR reactions were submitted to the cycles protocol according to the program: 95ºC for 3 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

The data obtained in the RT-qPCR reaction will be generated by the equipment in form of thereshold cycle (Ct) values, which represents the detection of the cycle in which the fluorescence generated in the reaction reaches the threshold of the amplification curve. The values of Ct obtained were later normalized (ΔCt) based on the Ct values obtained for the endogenous control gene (*GAPDH*). The calculation of the relative gene expression levels was performed according to the $2^{-\Delta Ct}$ method, described by Livak; Schmittgen (2001).

2.2.4 Western Blotting

The total protein extract was obtained from 50mg of the fetuses and embrios from the inclusion of 500 µl of lysis buffer (10 mM Tris HCl, 100 mM NaCl, 0.5 mM DDT (dithiothreitol), 2.5 mM MgCl 2, 0.5% triton X-100 and 1% protease inhibitor cocktail, (Sigma-Aldrich®). The protein content was measured by Bradford Protein Assay (Bio-Rad, Hercules, CA) and the protein solution was stored at -80 ° C until further analysis.

A 10% SDS-PAGE gel (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) were loaded with 80µg protein per sample into each well. Protein

extracts from 5 samples from CON and 5 from ARG treatments were arranged on each gel, as well as a sample for control containing one sample from each treatment (NORM; mix of one sample from CON and one from ARG). Samples were transferred into polyvinylidene fluoride (PVDF) membranes, the membranes for the phosphorylated protein were blocked in 1% solution of BSA (Bovine Serum Albumin, Sigma-Aldrich®) and for the non-phosphorilated protein it was used a solution of 10% nonfat milk, both diluted in a TBS1x(Tris-Buffered Saline) solution, for 1 hour with gentle agitation at room temperature. Then, the membranes were incubated with nrf2 (phosphorylated protein) and sod2 (non-phosphorilated protein) primary antibodies, both obtained from Invitrogen™, overnight at 4°C, using concentrations according to the protocol described by the manufacturer. The membranes were washed with TBST (Tris-Buffered Saline e 0,1% Tween®) several times, and then incubated with a secondary antibody for 1 hour at 4°C.

Subsequently, the membranes were washed three times (five minutes each) with TBST and revealed by ECL Clarity ™ 172 substrate (Bio-Rad, Hercules, California, USA). The images were generated by C-digit (Li-Cor) and quantified using Image Studio Lite software version 5.2.

2.2.5 Gene network

For biological processes enrichment analyses the ClueGO application in Cytoscape (BINDEA et al., 2009) was used based on a unilateral hypergeometric test and Bonferroni correction, which simultaneously analyzes 1 or more sets of genes and searches for a functional Gene Ontology (GO) term or pathways that establish relationships among the genes chosen in this paper. The GO based on biological processes, in addition to Kegg where used to evaluate the genes functions.

2.2.6 Statistical analyses

Conceptuses gene expression data from each gestational age were analyzed and submitted to ANOVA using the MIXED procedure of SAS, version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). Residue normality test was performed using the UNIVARIATE procedure of SAS (SAS Institute - Cary, NC, USA).

Conceptuses were considered false replicates of the females for each treatment, represented by a nested effect in the statistical model.

Expression data of target and endogenous genes were generated as Ct (threshold cycle) values. Data were transformed in relative expression ($2^{-\Delta Ct}$), according to Livak; Schmittgen (2001), and results were to be considered significant when $P \leq 0.05$. *P*-values between 0.06 and 0.10 were considered a trend.

The following statistical model was used in the analyses for gene expression for each gestational age:

$$Y_{ijkl} = \mu + D_i + C\{D_{(i)}\}_j + B_k + \varepsilon_{ijkl}$$

where: Y_{ijkl} is the observation (relative expression) from the j-th concept; μ is the trait general mean; D_i is the effect of i-th treatment (CON or ARG); $C\{D_{(i)}\}_j$ is the random effect of concept j nested in treatment i; B_k is the random effect of k-th boar; and ε_{ijk} is the random error.

Conceptuses protein expression data from each gestational age were also analyzed and submitted to ANOVA using the MIXED procedure of SAS, version 9.0 (Statistical Analysis System Institute, Inc., Cary, NC, USA). Residue normality test was performed using the UNIVARIATE procedure of SAS (SAS Institute - Cary, NC, USA).

The following statistical model was used in the analyses for protein expression for each gestational age:

$$Y_{ijk} = \mu + D_i + B_j + \varepsilon_{ijk}$$

where: Y_{ijk} is the observation (protein abundance); μ is the trait general mean; D_i is the effect of i-th treatment (CON or ARG); B_j is the random effect of j-th boar; and ε_{ijk} is the random error.

2.3 Results

2.3.1 Gene network

To a better understanding of the functions of genes related to oxidative stress, we performed a biological process analyses, all the most enriched biological processes can be found in figure 1, while all the subprocesses can be found in supplementary table 1. These analyses demonstrate that all genes chosen where directly or indirectly related with oxidative stress process. The processes directly connected with oxidative stress are: intrinsic apoptotic signaling pathway in response to oxidative stress, cell death in response to oxidative stress, cellular response to reactive oxygen species, cellular oxidant detoxification and removal of superoxide radicals. The indirectly oxidative stress connected processes are: glutathione metabolic process, UV protection, Longevity regulating pathway, response to starvation and hemoglobin metabolic process.

Figure 1 - Functional networks showing gene interactions (triangle nodes) related to oxidative stress.

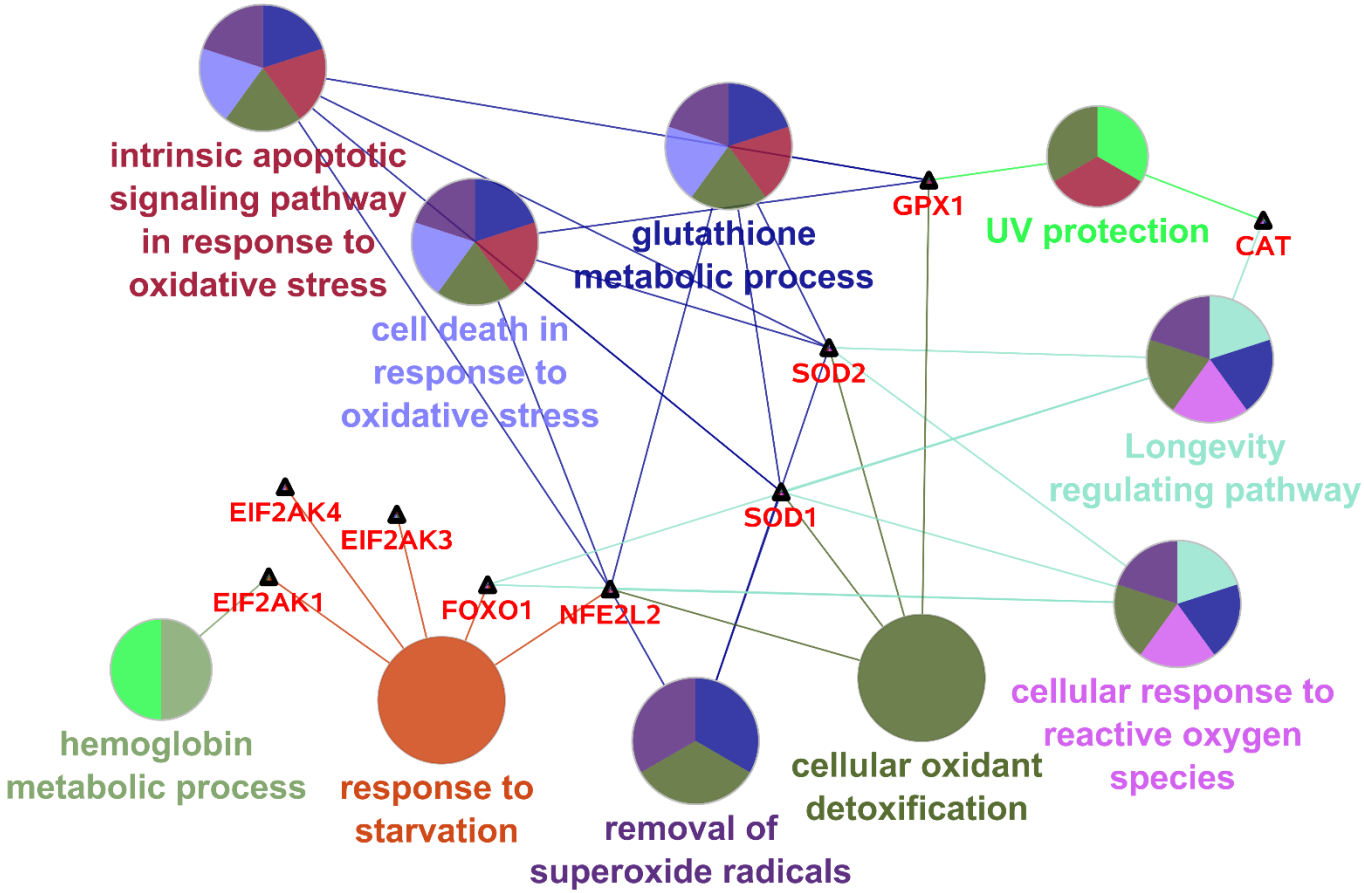

Image of own authorship

Suplementary table 1 – Biological subprocesses of the chosen genes.

Genes	Biological subprocesses
CAT	Acylglycerol metabolic process
	Amyotrophic lateral sclerosis (ALS)
	Antibiotic catabolic process
	Cellular detoxification
	Cellular oxidant detoxification
	Cholesterol metabolic process
	Foxo signaling pathway
	Glyoxylate and dicarboxylate metabolism
	Hemoglobin metabolic process
	Hydrogen peroxide catabolic process
	Hydrogen peroxide metabolic process
	Longevity regulating pathway
	Longevity regulating pathway
	Neutral lipid metabolic process
	Peroxisome
	Response to hydrogen peroxide
	Secondary alcohol metabolic process
	Sterol metabolic process
	Triglyceride metabolic process
	Tryptophan metabolism
	UV protection
EIF2AK1	Hemoglobin biosynthetic process

	Hemoglobin metabolic process
	Hepatitis C
	Macrophage differentiation
	Measles
	Negative regulation of hemoglobin biosynthetic process
	Porphyrin-containing compound metabolic process
	Protein processing in endoplasmic reticulum
	Protoporphyrinogen IX metabolic process
	Regulation of eif2 alpha phosphorylation by heme
	Regulation of hemoglobin biosynthetic process
	Regulation of translation in response to stress
	Regulation of translational initiation
	Regulation of translational initiation by eif2 alpha phosphorylation
	Regulation of translational initiation in response to stress
	Response to iron ion starvation
	Response to starvation
	Transition metal ion homeostasis
	Translational initiation
EIF2AK3	Cellular response to amino acid starvation
	Cellular response to cold
	Cellular response to glucose starvation
	Cellular response to nutrient levels
	Cellular response to starvation
	Cellular response to topologically incorrect protein

Cellular response to unfolded protein

Eif2alpha phosphorylation in response to endoplasmic reticulum stress

Endocrine pancreas development

Endoplasmic reticulum unfolded protein response

ER overload response

ER-nucleus signaling pathway

Hepatitis C

Intrinsic apoptotic signaling pathway

Intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress

Measles

Positive regulation of transcription by RNA polymerase I

Positive regulation of vascular endothelial growth factor production

Protein processing in endoplasmic reticulum

Regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway

Regulation of intrinsic apoptotic signaling pathway

Regulation of transcription by RNA polymerase I

Regulation of translation in response to endoplasmic reticulum stress

Regulation of translation in response to stress

Regulation of translation initiation in response to endoplasmic reticulum stress

Regulation of translational initiation

	Regulation of translational initiation by eif2 alpha phosphorylation
	Regulation of translational initiation in response to stress
	Regulation of vascular endothelial growth factor production
	Response to amino acid starvation
	Response to starvation
	Response to unfolded protein
	Translational initiation
EIF2AK4	Cellular response to amino acid starvation
	Cellular response to cold
	Cellular response to nutrient levels
	Cellular response to starvation
	Eif2alpha phosphorylation in response to endoplasmic reticulum stress
	Hepatitis C
	Measles
	Protein processing in endoplasmic reticulum
	Regulation of eif2 alpha phosphorylation by amino acid starvation
	Regulation of translation in response to endoplasmic reticulum stress
	Regulation of translation in response to stress
	Regulation of translation initiation in response to endoplasmic reticulum stress
	Regulation of translational initiation
	Regulation of translational initiation by eif2 alpha phosphorylation
	Regulation of translational initiation in response to stress

	Response to amino acid starvation
	Response to starvation
	Translational initiation
FOXO1	Cellular response to hyperoxia
	Cellular response to increased oxygen levels
	Cellular response to nitric oxide
	Cellular response to nutrient levels
	Cellular response to reactive nitrogen species
	Cellular response to reactive oxygen species
	Cellular response to starvation
	Foxo signaling pathway
	Longevity regulating pathway
	Longevity regulating pathway
	Negative regulation of fat cell differentiation
	Negative regulation of stress-activated MAPK cascade
	Negative regulation of stress-activated protein kinase signaling cascade
	Positive regulation of carbohydrate metabolic process
	Positive regulation of cellular carbohydrate metabolic process
	Positive regulation of complement-dependent cytotoxicity
	Positive regulation of gluconeogenesis
	Positive regulation of glucose metabolic process
	Regulation of carbohydrate metabolic process by regulation of transcription from RNA polymerase II promoter

	Regulation of gluconeogenesis
	Regulation of gluconeogenesis by regulation of transcription from RNA polymerase II promoter
	Regulation of reactive oxygen species metabolic process
	Response to hyperoxia
	Response to nitric oxide
	Response to starvation
GPX1	Acylglycerol metabolic process
	Angiogenesis involved in wound healing
	Antibiotic catabolic process
	Apoptotic mitochondrial changes
	Cell death in response to oxidative stress
	Cell redox homeostasis
	Cellular detoxification
	Cellular oxidant detoxification
	Glutathione metabolic process
	Hydrogen peroxide catabolic process
	Hydrogen peroxide metabolic process
	Intrinsic apoptotic signaling pathway
	Intrinsic apoptotic signaling pathway in response to oxidative stress
	Mammary gland epithelial cell proliferation
	Myotube cell development
	Negative regulation of cellular response to oxidative stress

Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors

Negative regulation of inflammatory response to antigenic stimulus

Negative regulation of intrinsic apoptotic signaling pathway

Negative regulation of mitochondrion organization

Negative regulation of oxidative stress-induced cell death

Negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Negative regulation of release of cytochrome c from mitochondria

Negative regulation of response to oxidative stress

Neutral lipid metabolic process

Positive regulation of blood vessel diameter

Protein oxidation

Regulation of blood vessel diameter

Regulation of blood vessel size

Regulation of cellular response to oxidative stress

Regulation of extrinsic apoptotic signaling pathway via death domain receptors

Regulation of inflammatory response to antigenic stimulus

Regulation of intrinsic apoptotic signaling pathway

Regulation of mammary gland epithelial cell proliferation

Regulation of oxidative stress-induced cell death

Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Regulation of release of cytochrome c from mitochondria

	Regulation of response to oxidative stress
	Regulation of tube diameter
	Release of cytochrome c from mitochondria
	Response to gamma radiation
	Response to hydrogen peroxide
	Response to symbiont
	Response to symbiotic bacterium
	Skeletal muscle fiber development
	Skeletal muscle tissue regeneration
	Tissue regeneration
	Triglyceride metabolic process
	UV protection
	Vasodilation
NFE2L2	Cell death in response to hydrogen peroxide
	Cell death in response to oxidative stress
	Cell redox homeostasis
	Cellular detoxification
	Cellular modified amino acid biosynthetic process
	Cellular oxidant detoxification
	Cellular response to glucose starvation
	Cellular response to laminar fluid shear stress
	Cellular response to nutrient levels
	Cellular response to oxygen radical
	Cellular response to reactive oxygen species

Cellular response to starvation

Cellular response to superoxide

Cellular response to topologically incorrect protein

Cellular response to unfolded protein

Endoplasmic reticulum unfolded protein response

ER-nucleus signaling pathway

Glutathione biosynthetic process

Glutathione metabolic process

Hematopoietic stem cell differentiation

Intrinsic apoptotic signaling pathway

Intrinsic apoptotic signaling pathway in response to oxidative stress

Negative regulation of cellular response to drug

Negative regulation of cellular response to oxidative stress

Negative regulation of endothelial cell apoptotic process

Negative regulation of hematopoietic progenitor cell differentiation

Negative regulation of hematopoietic stem cell differentiation

Negative regulation of hydrogen peroxide-induced cell death

Negative regulation of intrinsic apoptotic signaling pathway

Negative regulation of oxidative stress-induced cell death

Negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Negative regulation of response to drug

Negative regulation of response to oxidative stress

Negative regulation of response to reactive oxygen species

Negative regulation of stem cell differentiation

Nonribosomal peptide biosynthetic process

PERK-mediated unfolded protein response

Positive regulation of blood coagulation

Positive regulation of coagulation

Positive regulation of cofactor metabolic process

Positive regulation of glucose import

Positive regulation of glucose transmembrane transport

Positive regulation of glutathione biosynthetic process

Positive regulation of hemostasis

Positive regulation of reactive oxygen species metabolic process

Positive regulation of response to wounding

Positive regulation of sulfur metabolic process

Positive regulation of transcription from RNA polymerase II promoter in response to hypoxia

Positive regulation of transcription from RNA polymerase II promoter in response to stress

Positive regulation of transcription from RNA polymerase II promoter involved in cellular response to chemical stimulus

Positive regulation of wound healing

Proteasomal ubiquitin-independent protein catabolic process

Protein processing in endoplasmic reticulum

Regulation of cellular response to drug

Regulation of cellular response to oxidative stress

Regulation of cofactor metabolic process

Regulation of DNA-templated transcription in response to stress

Regulation of glucose import

Regulation of glutathione biosynthetic process

Regulation of hematopoietic progenitor cell differentiation

Regulation of hematopoietic stem cell differentiation

Regulation of hydrogen peroxide-induced cell death

Regulation of intrinsic apoptotic signaling pathway

Regulation of oxidative stress-induced cell death

Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Regulation of reactive oxygen species metabolic process

Regulation of removal of superoxide radicals

Regulation of response to oxidative stress

Regulation of stem cell differentiation

Regulation of sulfur metabolic process

Regulation of superoxide metabolic process

Regulation of transcription from RNA polymerase II promoter in response to hypoxia

Regulation of transcription from RNA polymerase II promoter in response to stress

Removal of superoxide radicals

Response to hydrogen peroxide

Response to oxygen radical

Response to starvation

Response to superoxide

	Response to unfolded protein
SOD1	Amyotrophic lateral sclerosis (ALS)
	Anterograde axonal transport
	Antibiotic biosynthetic process
	Auditory receptor cell development
	Auditory receptor cell morphogenesis
	Auditory receptor cell stereocilium organization
	Axo-dendritic transport
	Axonal transport
	Cell death in response to oxidative stress
	Cellular detoxification
	Cellular oxidant detoxification
	Cellular response to oxygen radical
	Cellular response to reactive oxygen species
	Cellular response to superoxide
	Cholesterol biosynthetic process
	Cholesterol metabolic process
	Embryo implantation
	Glutathione metabolic process
	Hair cell differentiation
	Hydrogen peroxide biosynthetic process
	Hydrogen peroxide metabolic process
	Inner ear auditory receptor cell differentiation
	Inner ear receptor cell development

Inner ear receptor cell stereocilium organization

Intermediate filament cytoskeleton organization

Intrinsic apoptotic signaling pathway

Intrinsic apoptotic signaling pathway in response to oxidative stress

Longevity regulating pathway

Muscle cell cellular homeostasis

Myelin maintenance

Myelination in peripheral nervous system

Negative reguation of sterol biosynthetic process

Negative regulation of alcohol biosynthetic process

Negative regulation of cholesterol biosynthetic process

Negative regulation of cholesterol metabolic process

Negative regulation of lipid biosynthetic process

Negative regulation of muscle contraction

Negative regulation of smooth muscle contraction

Negative regulation of steroid biosynthetic process

Negative regulation of steroid metabolic process

Neurofilament cytoskeleton organization

Ovarian follicle development

Peripheral nervous system axon ensheathment

Peripheral nervous system myelin maintenance

Peroxisome

Positive regulation of blood vessel diameter

Positive regulation of cellular response to oxidative stress

Positive regulation of intrinsic apoptotic signaling pathway

Positive regulation of oxidative stress-induced cell death

Positive regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Positive regulation of phagocytosis

Positive regulation of reactive oxygen species metabolic process

Positive regulation of response to oxidative stress

Positive regulation of superoxide anion generation

Prion diseases

Regulation of blood vessel diameter

Regulation of blood vessel size

Regulation of cellular response to oxidative stress

Regulation of cholesterol biosynthetic process

Regulation of cholesterol metabolic process

Regulation of intrinsic apoptotic signaling pathway

Regulation of oxidative stress-induced cell death

Regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Regulation of reactive oxygen species metabolic process

Regulation of response to oxidative stress

Regulation of smooth muscle contraction

Regulation of sterol biosynthetic process

Regulation of superoxide anion generation

Regulation of superoxide metabolic process

	Regulation of tube diameter
	Relaxation of smooth muscle
	Relaxation of vascular smooth muscle
	Removal of superoxide radicals
	Response to ethanol
	Response to hydrogen peroxide
	Response to oxygen radical
	Response to superoxide
	Retina homeostasis
	Retrograde axonal transport
	Schwann cell development
	Schwann cell differentiation
	Secondary alcohol biosynthetic process
	Secondary alcohol metabolic process
	Sterol biosynthetic process
	Sterol metabolic process
	Superoxide anion generation
	Transition metal ion homeostasis
	Vasodilation
SOD2	Acetylcholine-mediated vasodilation involved in regulation of systemic arterial blood pressure
	Apoptotic mitochondrial changes
	Cell death in response to oxidative stress
	Cellular detoxification

Cellular oxidant detoxification

Cellular response to oxygen radical

Cellular response to reactive oxygen species

Cellular response to superoxide

Erythrophore differentiation

Foxo signaling pathway

Glutathione metabolic process

Intrinsic apoptotic signaling pathway

Intrinsic apoptotic signaling pathway in response to oxidative stress

Longevity regulating pathway

Longevity regulating pathway

Negative regulation of blood pressure

Negative regulation of fat cell differentiation

Negative regulation of fibroblast proliferation

Negative regulation of systemic arterial blood pressure

Neurological system process involved in regulation of systemic arterial blood pressure

Peroxisome

Positive regulation of blood vessel diameter

Positive regulation of nitric oxide biosynthetic process

Positive regulation of nitric oxide metabolic process

Positive regulation of reactive oxygen species biosynthetic process

Positive regulation of reactive oxygen species metabolic process

Regulation of blood vessel diameter

Regulation of blood vessel size

Regulation of reactive oxygen species metabolic process

Regulation of systemic arterial blood pressure by acetylcholine

Regulation of systemic arterial blood pressure by neurotransmitter

Regulation of systemic arterial blood pressure mediated by a chemical signal

Regulation of tube diameter

Release of cytochrome c from mitochondria

Removal of superoxide radicals

Response to gamma radiation

Response to hydrogen peroxide

Response to hyperoxia

Response to oxygen radical

Response to superoxide

Superoxide anion generation

Transition metal ion homeostasis

Vasodilation

2.3.2 Gene expression

Embryos did not show differences of expression between treatments for any of the genes evaluated: *SOD1* (*P*=0.14), *NRF2* (*P*=0.81), *SOD2* (*P*=0.38), *CAT* (*P*=0.35), *ENOS* (*P*=0.35) *GPX* (*P*=0.88), *EIF2AK1* (*P*=0.51), *EIF2AK3* (*P*=0.27) and *EIF2SK4* (*P*=0.30) (supplementary table 2). No differences in gene expression were observed in CON and ARG fetuses for the following genes: *SOD1* (*P*=0.14), *NRF2* (*P*=0.12), *CAT* (*P*=0.31), *ENOS* (*P*=0.94), *GPX* (*P*=0.64), *FOXO* (*P*=0.33), *EIF2AK1* (*P*=0.89) and *EIF2AK3* (*P*=0.94). *SOD2* and *EIF2AK4* genes tended to be more expressed in

CON fetuses compared to ARG, presenting a *P*-value of 0.09 and 0.07 respectively (supplementary table 3).

Supplementary table 2 - Relative expression of oxidative stress genes in embryos ($2^{-\Delta Ct}$). Mean and Standard Error.

Gene	Diet	Mean	Standard Error
NRF2	CON	0.09993	0.02339
NRF2	ARG	0.09225	0.02354
SOD1	CON	2.6490	0.4378
SOD1	ARG	1.6352	0.4378
SOD2	CON	0.4823	0.08026
SOD2	ARG	0.3774	0.08026
CAT	CON	0.2443	0.04196
CAT	ARG	0.3021	0.04196
GPX	CON	0.5837	0.1960
GPX	ARG	0.6237	0.1960
ENOS	CON	0.007520	0.002171
ENOS	ARG	0.004837	0.002229
EIF2AK1	CON	0.07080	0.007080
EIF2AK1	ARG	0.07764	0.007080
EIF2AK3	CON	0.02371	0.005234
EIF2AK3	ARG	0.03102	0.005388
EIF2AK4	CON	0.02991	0.003994
EIF2AK4	ARG	0.03616	0.003994

Supplementary table 3 - Relative expression of oxidative stress genes in fetuses ($2^{\Delta Ct}$). Mean and Standard Error.

Gene	Diet	Mean	Standard Error
NRF2	CON	0.07614	0.01179
NRF2	ARG	0.05011	0.009431
SOD1	CON	0.7108	0.1695
SOD1	ARG	0.3665	0.1340
SOD2	CON	0.2706	0.03730
SOD2	ARG	0.1801	0.02957
CAT	CON	0.4322	0.09485
CAT	ARG	0.3022	0.07499
GPX	CON	2.4667	1.8321
GPX	ARG	3.5855	1.4529
FOXO	COM	0.03399	0.003266
FOXO	ARG	0.02859	0.004108
ENOS	CON	0.003954	0.001089
ENOS	ARG	0.004062	0.000875
EIF2AK1	CON	0.1416	0.02533
EIF2AK1	ARG	0.1373	0.02003
EIF2AK3	CON	0.04723	0.007424
EIF2AK3	ARG	0.04789	0.005987
EIF2AK4	CON	0.06200	0.01257
EIF2AK4	ARG	0.03300	0.01048

2.3.3 Western Blotting

No difference in protein abundance were observed in CON and ARG embryos in both nrf2 and sod2 proteins, presenting a *P*-value of 0.22 and 0.33 respectively. The treatments from fetuses also did not present any difference in protein abundance for both nrf2 and sod2, presenting a *P*-value of 0.31 and 0.94 respectively (figure 2).

Figure 2 – Protein abundance.

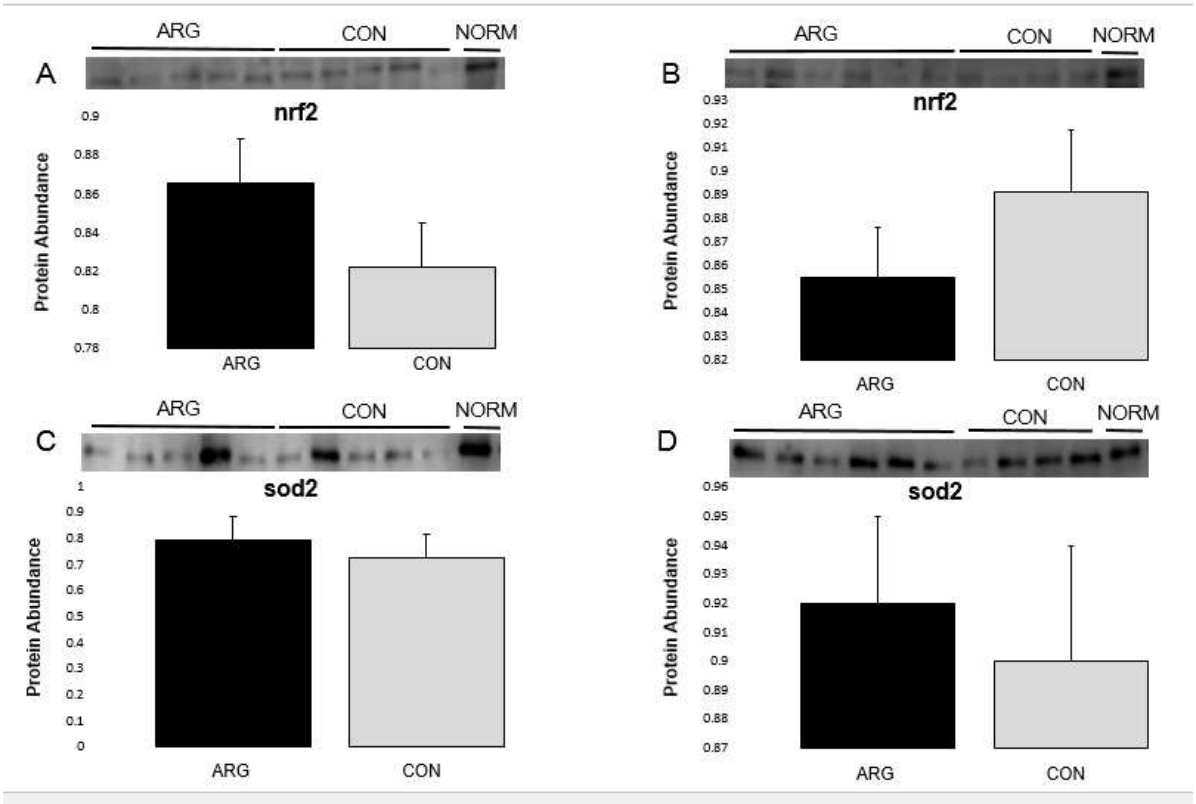

Image of own authorship. A: nrf2 protein in embryos; B: nrf2 protein in fetuses; C: sod2 protein in embryos; D: sod2 protein in fetuses. CON: control treatment; ARG: arginine treatment; NORM: control sample.

2.4 Discussion

In GO network, we could confirm that all the genes chosen in this study are involved in oxidative stress functions by biological process enrichment, such as the environment influence in stress (UV protection and response to starvation), apoptotic signaling pathways, cell response to ROS and antioxidant activity.

Although the endogenous DNA damage at around day 30 of gilts pregnancy is lower compared with other time points during the course of gestation, as the peak of oxidative stress in this period is in the final third, the damage at early gestation is not nonexistent (BERCHIERI-RONCHI et al., 2011). Morphological changes in the uterine arteries allowing free flow of maternal blood into the placenta, coincides with the rise of oxygen tension within the placental bed. This is associated with an increase in mRNA concentrations and activities of antioxidant enzymes within placental tissues during the period of normal placental differentiation, this happens to maintain a balanced and healthy uterine environment for the conceptuses, however, a burst of oxidative stress still occurs in the placenta as the maternal circulation is established (JAUNIAUX et al., 2000; DUHIG; CHAPPELL; SHENNAN, 2016).

If the rise of oxidative stress in the placenta in early gestation influence the conceptuses environment causing lipid peroxidation, oxidation of protein and DNA damage, this may lead to numerous pathologies, as well as negatively influence embryos and fetuses development and even lead to an early miscarriage (HEMPSTOCK et al., 2003). The gene that have a high influence in an early oxidative stress situation, and act as a first barrier, is the *NRF2*, and activating its pathway lead to an increase of antioxidants activity, and for that, this gene and some others that are activated by nrf2 transcription factor (*CAT, SOD1, SOD2* and *GPX*) were chosen to be evaluated during early gestation, in with there might be a lower, but still harming ROS concentration (YANG et al., 2017). Arginine byproducts have been known for influence oxidative stress related genes, proteins such as nitric oxide, are involved in the activation of the transcription factor nrf2, Glutathione (*GSH*) is also an important antioxidant, in with *GPX* depends on to be activated, and its synthesis can be determined by both the action of Nrf2 and/or by glutamate cysteine ligase (GCL) and GSH synthase (GS), which originates from glutamate, originated from ornithine (one of the arginine byproducs) (LIANG et al., 2018).

We suggested that as a first barrier these genes could have a significate difference when the animal is supplemented with L-arginine, but as it was observed by gene expression analyses, most genes did not have a significant statistical difference between treatments, both in fetuses and embryos. The trend found in 35 days fetuses *EIF2AK4* and *SOD2* genes, had a higher expression in the CON treatment. When comparing these result with Costa et.al. (2019) it makes sense, as it was found a higher

arginine plasma concentration of females from CON treatment and higher urea production in ARG treatment at 35 days of gestation. In this case, ARG treatment in 35 days could be metabolizing most of the supplemented arginine into urea via its cycle, and not into its other byproducts (MORRIS, 2002).

Nrf2 and sod2 were chosen for Protein abundance following the trend results in gene expression. Nrf2 is considerate to be a substrate of PKR-like endoplasmic reticulum kinase (PERK), in with, one of its composing genes is *EIF2AK4* (as well as *EIF2AK1* and *EIF2AK3*), cell stress activates PERK kinase activity, leading to phosphorylation of Nrf2, which promotes the redox homeostasis, as *EIF2AK4* was part of the genes that presented a trend, it was chosen to do a western blot analysis with the nrf2 phosphorylated protein (with is its active state) (CULLINAN et al., 2003; CULLINAN; DIEHL, 2006). Both proteins did not present a difference in the treatments, showing that independent of the trend results, a post-transcriptional regulation did not occur. Even without a statistical difference, nrf2 and sod2 proteins presented a higher estimate abundance in ARG treatments, probably due to a higher arginine presence in the mother plasma in embryos. In fetuses, the same explanation from the genes expression is valid, corroborating with Costa et.al. (2019), where most likely in ARG treatment there was a higher production of urea and not the byproducts. In sod2 case, the higher abundance in 35 days ARG treatment may be because this protein production can be activated by not only *NRF2* but by others transcription factor as well, also, the antioxidant protein activity can be influenced by other factor as well, and those could be independent of the arginine byproduct effects (MIAO; ST. CLAIR, 2009).

These results can lead to the theory that supplementation of L-arginine in early gestation in gilts (both until 25 and 35 days) with the objective to lessen ROS concentrations and oxidative stress damage, is not effective, as in both treatments (ARG and CON), most genes and proteins did not present a difference in its performance to maintain a redox homeostasis, and the genes with a trend in its expression did not influence the antioxidant proteins abundance. It is valid to state that in a different situation, these results may not apply, as different managements of the animals could lead to a higher oxidative stress state, activating the redox pathways.

2.5 References

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