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Iberian pig early pregnancy: Vascular endothelial growth factor receptor system expression in the maternofetal interface in healthy and arresting conceptuses

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ABSTRACT

In Iberian pigs, a high conceptus loss occurs during the first 30 days of gestation. Although the exact causes for these losses have not been determined to date, the importance of blood vessel development during early pregnancy has been noted. The aim of this study was to analyze the messenger RNA (mRNA) and protein expression of VEGF-rs (vascular endothelial growth factor, the VEGFR1, and the VEGFR2 receptor system) and elucidate a possible relationship with the conceptus status (healthy or arrested) on gestational Days (gd) 22 and 32. Both mRNA and protein expression for VEGF-rs molecules were consistently expressed in conceptuses and endometrium during the pregnancy period analyzed. In endometrium, a significant increase in VEGF mRNA and VEGFR2 mRNA expression in healthy sites was observed as pregnancy advances ($P < 0.001$ and $P < 0.05$, respectively), whereas VEGFR1 mRNA expression was maintained at a constant level. Interestingly, a significantly elevated VEGFR2 mRNA expression ($P < 0.05$) was observed on gd 22 in endometrium from arrested conceptuses. Furthermore, VEGF mRNA and VEGFR1 mRNA expression in trophoblasts from healthy conceptuses decreased as pregnancy proceeded ($P < 0.001$). Arrested trophoblasts on gd 32 showed higher VEGFR2 mRNA expression than healthy conceptuses ($P < 0.05$). Although, in endometrium attachment sites, the pattern of VEGF-rs immunostaning was not affected by conceptus status, the immunoexpression of VEGF-rs in healthy attachment sites increased slightly but consistently as gestation proceeded. In arresting trophoblasts, VEGF and VEGFR2 staining decreased from gd 22 to 32. Moreover, the number of VEGF and VEGFR1-positive capillaries in the subepithelial vascular plexus of endometrium was related to the conceptus status, showing a moderate increase in healthy sites as pregnancy advances. In conclusion, it appears that VEGF-rs is expressed and related to vascular development in Iberian pigs between gd 22 and 32. The upregulated expression of VEGF mRNA and VEGFR2 mRNA in healthy uterine sites suggests a significant role for these angiogenic factors in early pregnancy.

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1. Introduction

The Iberian pig is an autochthonous breed from the Iberian Peninsula known worldwide for the production of a cured meat product of high quality, namely, the Iberian ham, and characterized in reproduction by a low prolificacy [1]. Recently, it has been reported that more than 30% of

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oocytes fertilized and conceptuses successfully attached were lost during the first 30 days of gestation in the Iberian breed [2]. These facts confirm the relevance of the phenomena that occur during early gestation, between the periattachment period (gestation Days [gd] 12–18) and the complete development of the placenta (gd 35), a window stage in which most embryo losses occur in pigs (review in Edwards et al. [3]). Although the exact causes for these losses have not been determined yet, the development of blood vessels in endometrial and placental tissues has been described as a significant event [4–7].

Angiogenesis is a physiological process characterized by the growth of new blood vessels from preexisting ones, a process that is controlled by a delicate network of factors. The most potent activator of angiogenesis is VEGF (vascular endothelial growth factor), which exerts its effects after binding to homologous membrane tyrosine kinase receptors, VEGFR1 (Flt1) and VEGFR2 (KDR/Flk1), that are expressed mainly by blood vessel endothelial cells [8].

Vascular endothelial growth factor binds with the highest affinity to VEGFR1 playing a direct role in the proliferation of the endothelial cells of blood vessels [9]. The binding of VEGF to VEGFR2 induces a cascade of different signaling pathways, resulting in the upregulation of genes involved in the proliferation and migration of endothelial cells and promoting their survival and vascular permeability. Vascular endothelial growth factor receptor 2 signaling is therefore considered as essential for the differentiation of endothelial precursor cells into vascular endothelial cells and for their proliferation [10,11].

During pig early pregnancy, the expression pattern of VEGF and its receptors has shown spatial and temporal differences, with upregulation of expression in both the maternal interface area [7,12,13] and the conceptus [14]. This upregulation has been related to the successful development of the embryo, as has been previously reported in human trophoblast cells [15]. In early arresting pig fetuses (gd 21–23), a reduced vascularity of the placental membranes is characteristic and has been associated with lower or null VEGF expression [4,5,16]. The relevance of vascular density in placental efficiency has been recognized in the Chinese Meishan pig, a prolific breed in which attachment sites are more vascularized than those in other commercial pigs [17]. A relationship between the placental VEGF mRNA expression and the number of blood vessels in the placental–endometrial interface has been observed in white swine crossed breeds [18]. In porcine pregnancy, VEGF-rs has been immunohistochemically localized in the luminal epithelium, in the uterine glands and blood vessels in the maternal placenta, and in trophoblasts [6,19].

To date, limited data have been published regarding the expression of VEGF-rs and its possible relationship with the vascular development at the fetal–maternal interface during Iberian pig pregnancy. In a recent study, the level of VEGFA gene expression in fetuses on gd 42 has been positively correlated with placental efficiency [20]. Moreover, in a Colombian Criollo breed of pig, named Zungo Pelao (related to Iberian pigs), persistent immunoexpresion of VEGF-rs was observed at the embryo–maternal interface between gd 18 and 26 and was associated with epitheliochorial placentation in early pregnancy [21].

Thus, the aims of the present study were to evaluate, in Iberian pig, the pattern of mRNA VEGF-rs expression and the localization of VEGF, VEGFR1, and VEGFR2 proteins in healthy and arresting conceptuses and uterine attachment sites on gd 22 and 32, a well-known critical period of embryo loss.

2. Materials and methods

2.1. Animals and tissue collection

Forty multiparous Iberian sows, with an average age of 27 months and an average of 3.5 farrowings, were selected for this study. The average animal weight was 180 kg, and they were housed indoors in individual pens with automatic ventilation control in a commercial facility in Guijuelo (Salamanca, Spain). Pigs were fed with 1.6 kg/day of a commercial cereal diet and had access to water *ad libitum*. The animals were inseminated at their first postweaning estrus after parity according to farm standard operating protocols and placed in two different groups, gd 22 ($n = 20$) and 32 ($n = 20$). The day of the last insemination was considered gd 0. The experimental protocol was performed after approval from the Scientific Ethic Committee of Complutense University in accordance with current European regulations.

The entire reproductive tract from each sow was collected at a local commercial abattoir and transported on dry ice to the laboratory. Normal CL were morphologically evaluated in the ovaries to assess ovulation rate. The uterus was opened, and all of the conceptuses from each sow were weighed (weight of the isolated fetus) and measured (crown–rump length). They were grouped as healthy or arresting according to fetal length, weight, and vascularity of the placental membranes, according to commonly accepted criteria [4,16]. On gd 22, conceptuses that showed a crown–rump length 0.9 cm or lesser, a weight 0.09 g or lesser, and white placental membranes due to poor vascularity were categorized as arrested. On gd32, conceptuses showing a crown–rump length 1.2 cm or lesser, a weight 0.44 g or lesser, and scarce blood vessels in placental membranes were categorized as arrested (Fig. 1).

For each pregnant uterus, a small portion of four healthy trophoblasts (near the umbilical cord) and their corresponding endometrium attachment sites at the mesometrial area were taken. Thus, 80 healthy trophoblasts and endometrium sites on gd 22 as well as 80 healthy sites (trophoblasts and endometrium) on gd 32 were collected. Miduterine implantation sites were selected to avoid differences in embryo development caused by the fetal position in the uterus. All arresting conceptuses and their attachment sites on both gd 22 ($n = 9$) and 32 ($n = 9$) were collected. The samples recovered were divided into two pieces. One piece was immediately placed in RNAlater Tissue Collection (Applied Biosystems; Life Technologies, Foster City, CA, USA) to stabilize and protect the RNA in fresh specimens and

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