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Vascularization and VEGF expression altered in bovine yolk sacs from IVF and NT technologies

Andrea Maria Mess [a,b,*], Ana Claudia Oliveira Carreira [c,d], Cláudia Marinovic de Oliveira [a], Paula Fratini [a], Phelipe Oliveira Favaron [a], Rodrigo da Silva Nunes Barreto [a], Christiane Pfarrer [b], Flávio Vieira Meirelles [e], Maria Angelica Miglino [a]

[a] Department of Surgery, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil
[b] Department of Anatomy, University of Veterinary Medicine Hannover, Hannover, Germany
[c] Biochemistry Department, Chemistry Institute, University of São Paulo, Sao Paulo, Brazil
[d] NUCEL (Cell and Molecular Therapy Center) and NETCEM (Center for Studies in Cell and Molecular Therapy), Medical Clinics Department, School of Medicine, University of São Paulo, Sao Paulo, Brazil
[e] Faculty of Animal Sciences and Food Engineering, University of São Paulo, Pirassununga, Brazil

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ABSTRACT

Reproductive technologies are widely used in cattle, although many are associated with high-embryonic mortality, especially during early gestation, when the yolk sac undergoes macroscopic changes in structure. We hypothesized that vasculogenesis and angiogenesis are affected, thereby affecting embryonic and placental differentiation. To test this, we studied yolk sac development and gene expression of the vascular endothelial growth factor system (*VEGF-A, VEGFR-1/Flt-1, VEGFR-2/KDR*). Samples from Days 25 to 40/41 of pregnancy from control cattle (n = 8) and from pregnancies established with IVF, (n = 7) or somatic cell nuclear transfer/clones (n = 5) were examined by histology, immunohistochemistry, and quantitative reverse transcriptase PCR. Yolk sacs in IVF- and nuclear transfer–derived pregnancies were immature. Development of villi was sparse in IVF yolk sacs, whereas vascularization was barely formed in clones and was associated, in part, with thin or interrupted endothelium. Transcript levels of the genes characterized exceed minimum detection limits for all groups, except in the mentioned clone with interrupted endothelium. Levels of mRNA for *VEGF-A* and *VEGFR-2* were significantly higher in IVF yolk sacs. Clones had substantial individual variation in gene expression (both upregulation and downregulation). Our data confirmed the broad range in expression of *VEGF* genes. Furthermore, overexpression in IVF yolk sacs may compensate for an immature yolk sac structure, whereas in clones, patchy expression may cause structural alterations of blood vessels. In conclusion, we inferred that disturbances of yolk sac vasculature contributed to increased early embryonic mortality of bovine pregnancies established with reproductive technologies.

1. Introduction

Assisted reproductive technologies such as IVF or cloning by somatic cell nuclear transfer (NT) are critical tools in current cattle production but are accompanied by high rates of loss, especially of clones [1–5]. Presumably, manipulation of germ cells, which alters gene expression or causes epigenetic disturbances, contributes to these increased losses [2,6–9]. Thus, cattle produced using reproductive technologies differ morphologically and physiologically from those fertilized *in vivo* [6,7,9–12]. Alterations in various organs in cloned embryos are caused by aberrant vasculature during the early intrauterine phase

* Corresponding author. Tel./fax: +55 11 30917690.
E-mail address: drandrmss@aol.com (A.M. Mess).

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[13]. In addition to offspring, the placenta [12–17] and, during early pregnancy, the yolk sac [12], are often severely altered. Macroscopically, embryos produced using reproductive technologies had small, translucent, and T-shaped yolk sacs that regressed relatively early [12]. In clones, separation of the yolk sac from the ventral body was also incomplete, with signs of necrosis [12]. Therefore, important functions that first occur in the yolk sac may be disturbed, that is, early development of the vascular system including hematopoiesis (formation of blood cell components), vasculogenesis (blood vessel formation), and angiogenesis or blood vessel growth. Moreover, yolk sac tissues form multipotent stem cells, hemangioblasts, primordial blood cells, vessels, blood islets, and unique proteins [18–25] that may also be vulnerable to developmental disturbances. The VEGF gene system is expected to have a major role in vasculogenesis and related processes in the chorioallantoic placenta [26–29], due especially to its potent mitogenic activity on endothelial cells [30]. *VEGF-A* is expressed in endothelial cells and stimulates cell proliferation, promotes cell migration, inhibits apoptosis, and induces angiogenesis and vascular developmental regulation [30–32]. In bovine placentas, *VEGF-A* is expressed in trophoblast cells and stroma of placentomes [27,29] and seems also to act locally as a modulator of steroid hormone production, with a different pattern between conventional pregnancies and those produced by cloning [33]. In the latter, expression of *VEGF-A* was higher than in controls [29].

Activation of the two specific tyrosine kinase receptor genes, *VEGFR-1* (*Flt-1*/fms-like tyrosine kinase receptor 1) and *VEGFR-2* (*KDR*/kinase insert domain-containing region), mediates the biological actions of *VEGF-A* [31,32,34,35]. In particular, *VEGFR-1* controls proliferation of endothelial cells during angiogenesis. Its soluble form binds VEGF-A and prevents activation of *VEGFR-2*, thereby acting as a negative regulator of *VEGFR-2* [26,35]. In contrast, *VEGFR-2* contributes to migration and proliferation of endothelial cells during vessel maintenance [32,34,35]. In humans and knockout-mice, disturbances of the VEGF gene system caused severe pregnancy problems or embryonic lethality [36–40]. However, similar studies of bovine yolk sacs have apparently not been reported. Therefore, the objective was to characterize vascular development and expression of VEGF genes in bovine pregnancies derived from IVF or NT, compared to abbatoir-derived controls (produced by natural breeding or artificial insemination). We focused on Days 25 to 40/41 when the yolk sac is normally evident, whereas the placenta is still immature [12,41]. Our hypotheses were that: (1) the VEGF system would be active; and (2) gene expression would be altered in IVF and/or NT yolk sacs.

2. Materials and methods

2.1. Sample collection

This research was approved by the Bioethical Committee of the School of Veterinary Medicine and Animal Science at the University of Sao Paulo (numbers: 1659/2009, 1711/2009). In total, 20 bovine yolk sacs of Days 25 to 40/41, derived from *Bos t. taurus* × *Bos t. indicus*, were investigated (Table 1). In preliminary studies, tissues from a later gestational stage (~Day 50) from controls did not yield amplification of RNA for the *VEGF* system (data not shown), consistent with the bovine yolk sac being fully involuted [41]. Samples were taken from distal areas of the yolk sac that were at least loosely attached to the chorion, but mostly free. Samples were collected from the sac-like portion that varied in size among ages, groups, and individuals. Tissues were fixed in 4% paraformaldehyde or frozen in liquid nitrogen and kept at −80 °C before RNA extraction. Material from control pregnancies (N = 8, days 25–40) was obtained from an abbatoir in Pirassununga, Sao Paulo, Brazil. Estimation of gestational age was done as described [24]. Material from IVF-derived pregnancies (N = 7) was obtained from the Sociedade Agropecuária Imaculada Conceição Ltda, Pará, Brazil [42], collected at an abbatoir at Redenção, Pará. Yolk sacs from bovine embryos produced by somatic cell nuclear transfer (N = 5) were obtained from the Faculty of Animal Sciences and Food Engineering in Pirassununga [43]. Embryos for IVF were produced using abbatoir-derived oocytes that were matured *in vitro*, fertilized with commercial bull semen, and nonsurgically transferred to previously synchronized cross-breed surrogate cows, as described [43]. For NT embryos, cultured bovine fetal fibroblast cells were used as nuclear donors. Oocytes were matured, denuded, enucleated, and transferred to surrogate cows. [43]. Ultrasonography was used to confirm the presence of an embryonic vesicle and/or heart beat and increased uterine blood flow; however, results were not considered reliable before Day 30. Therefore, these samples were collected on Days 32 to 41 of pregnancy.

2.2. Histology, immunohistochemistry, and electron microscopy

Following protocols of former studies from our lab [12,29], samples for microscopic analysis were fixed in 4% phosphate-buffered formalin for at least 48 hours, dehydrated in ethanol, cleared in xylene, embedded in Paraplast resin (Merck, Darmstadt, Germany), sectioned at 5 μm (Microtome RM 2165, Leica, Nussloch, Germany), and stained with hematoxylin-eosin or Periodic Acid Schiff. For immunohistochemistry, antigen retrieval was done by microwave treatment for 3 × 5 minutes in sodium citrate buffer (10 mM, pH 6.0). Slides were immersed in PBS

Table 1
Investigated individuals from the three groups.

Control (n = 8)	IVF (n = 7)	NT (n = 5)
Control-A_d25–30 (0.5 cm)	IVF-A_d25 (Nr. 1565)	NT-A_d32
Control-B_d25–30 (0.6 cm)	IVF-B_d25 (Nr. C77)	NT-B_d34
Control-C_d25–30 (0.8 cm)	IVF-C_d30 (Nr. 240)	NT-C_d35
Control-D_d25–30 (0.9 cm)	IVF-D_d35 (Nr. 528)	NT-D_d35
Control-E_d25–30 (0.9 cm)	IVF-E_d40 (Nr. A02)	NT-E_d41
Control-F_d30–35 (1.0 cm)	IVF-F_d40 (Nr. K748)	
Control-G_d35–40 (1.7 cm)[a]	IVF-G_d40 (Nr. CT1880)	
Control-H_d35–40 (2.1 cm)		

Abbreviations: IVF, in vitro fertilization; NT, nuclear transfer.
[a] Used for histology only.