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Early embryonic and endometrial regulation of tumor necrosis factor and tumor necrosis factor receptor 2 in the cattle uterus

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ABSTRACT

Tumor necrosis factor (TNF) alpha likely mediates embryomaternal communication in mammals. In bovine, we have previously found that the uterine fluid of heifers that carried early embryos shows downregulation in the TNF and nuclear factor κB system. In this work, we assessed the expression of TNF and its receptor TNFR2 in the bovine endometrium and embryos during blastocyst development. Moreover, to explore the endometrial immune response to early embryos, we analyzed the number of CD45 leukocytes in the bovine endometrium. Day 8 endometrium and blastocyst recovered from animals after transfer of Day 5 embryos showed TNF and TNFR2 mRNA transcription and protein colocalization. The presence of embryos increased endometrial TNF and TNFR2 protein, whereas endometrial leukocytes decreased. Blastocysts exposed to the uterine tract had undetectable levels of *TNF* and lower levels of *TNFR2* mRNA. These results suggest that the endometrium might lower the TNF concentration in the blastocyst by (1) regulating TNF secretion into the uterine fluid and (2) inducing decreased *TNF* and *TNFR2* mRNA transcription in the embryo. Thus, TNF and TNFR2 might participate in early embryomaternal communication.

1. Introduction

Embryomaternal communication is essential for embryo implantation and successful pregnancy to term [1]. Failure to replicate the maternal environment surrounding the embryo hampers the *in vitro* reproduction procedures [2], making research in this field a priority for reproductive biology. In natural conditions, communication is governed by growth factors produced and secreted by the embryo and the endometrium [3]. One of such factors might be tumor necrosis factor (TNF), a proinflammatory cytokine that mediates cell differentiation, survival, renewal, and tissue homeostasis [4]. Tumor necrosis factor activates several intracellular pathways through its binding to two distinct receptors, TNFR1 and TNFR2 [5]. Receptor TNFR1 associates with apoptosis and TNFR2 is more versatile leading to apoptotic or proliferation processes depending on the stimulus.

Some evidence supports the participation of TNF and TNFR2 in normal reproduction. Thus, TNF and TNFR2 endometrial expression vary throughout the estrous cycle in humans and domestic animals [6–9], and both seem to exert a role during pregnancy in humans, mice, and dogs [8,10–12]. In bovine, TNF and TNFR2 mRNA and protein have been detected in the cyclic and pregnant endometrium [7,13]. Expression of

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the *TNF* and *TNFR2* genes is not regulated by pregnancy [13], although their mRNA levels change throughout the estrous cycle, being regulated by TNF itself, other cytokines (interleukin 1alpha), and sexual hormones such as oxytocin [7]. Similarly, TNF and TNFR2 protein and mRNA synthesis occurs at specific developmental stages in embryos from humans, rats, and mice [14], and also in cattle [15,16]. Uterine fluid (UF) contains TNF in cyclic cows, humans, and mice [16–19] as well as in cows and monkeys that have carried embryos [16,20]. Tumor necrosis factor release has been observed from human endometrial cells cultured *in vitro* [6] and embryos [21]. Collectively, the previously mentioned data indicate that the cytokine is secreted by the endometrium and or embryos. In the embryo-carrying bovine endometrium, we have detected the 78 kDa TNF isoform downregulated in the UF [16]. Most of the previously mentioned studies provided significant knowledge on the TNF system in the embryo and maternal tissues, particularly during implantation [8,10,11,13]. However, little is known about the endometrial expression of TNF system during earlier stages. Furthermore, in pregnant bovine endometrium, only mRNA transcriptions of *TNF* and its receptor, but not protein expression, have been studied [13]. Similarly, in the bovine embryo only the mRNA for *TNFR2* has been found [15].

During blastocyst formation, ungulate embryos downregulate uterine nuclear factor κB at the protein and gene expression levels [16,22], probably depressing the immune response in favor of embryo tolerance. Other studies have described changes in the immune endometrial cells around implantation in response to embryos (Days 12, 15, 16, and 18) [23,24]. However, to our knowledge, the protein expression of immune cells in the bovine uterus in response to very early embryos has not been studied yet.

The aim of this study was to assess the regulation of TNF system and CD45 leukocytes by the embryo and the maternal tract as likely contributors to early embryomaternal communication in cattle. We tested the hypothesis that the embryo and the endometrium may reciprocally regulate TNF, TNFR2, and CD45 leukocytes at the time of blastocyst formation. Specifically, we examined whether (1) TNF and TNFR2 mRNA and protein are present in the bovine endometrium and embryos during the blastocyst stage; (2) their expression is reciprocally regulated; and (3) the presence of embryos affects the expression of endometrial CD45 leukocytes.

2. Materials and methods

Reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated. Experiments involving animals complied with the Directive 2010/63/EU (Spanish RD 53/2013) and were sanctioned by the Servicio Regional de Investigación y Desarrollo Agroalimentario Animal Research Ethics Committee. Animal management, estrus synchronization, and embryo production and transfer procedures are previously described [16].

2.1. Embryo production

In vitro–produced (IVP) embryos were obtained as described [25], with minor modifications. Ovaries were obtained from a local abattoir, and from them antral follicles (from 3 to 8 mm) were aspirated. The recovered cumulus-oocyte complexes (COCs) were rinsed three times in a holding medium (HM) consisting of Medium 199 (Invitrogen, Barcelona, Spain), 25 mM HEPES, and BSA 0.4 g/L. Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for maturation. Groups of approximately 50 COCs were placed in 500 μL maturation medium consisting of TCM199 $NaHCO_3$ (2.2 g/L), fetal calf serum (10% v:v), porcine FSH-LH (1:5 μg/mL, Stimufol; ULg FMV, Liège, Belgium, France), and 17 β-estradiol (1 μg/mL). Cumulus-oocyte complexes were matured for 24 hours at 38.7 °C in 5% CO_2 and saturated humidity.

For IVF (Day 0), sperm was prepared by the swim-up procedure [26]. Briefly, semen from one frozen straw corresponding to one bull was thawed in a water bath and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm-TALP (Tyrode's albumin lactate pyruvate). After 1 hour of incubation, the upper layer of supernatant containing motile sperm was recovered. The sperm were centrifuged for 7 minutes at 200× *g* and the supernatant aspirated to leave a pellet containing the sperm in which the concentration was determined with a hemocytometer. Meanwhile, COCs were washed twice in HM and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg/mL; Calbiochem, La Jolla, CA, USA). Spermatozoa were added at a concentration of 2×10^6 cells/mL in 500 μL of medium per well containing a maximum of 100 COCs. The IVF was accomplished by incubating oocytes and sperm cells together for 18 to 20 hours at 39 °C in an atmosphere of 5% CO_2 with saturated humidity.

For IVC, cumulus cells were detached using a vortex, and presumptive zygotes were cultured in synthetic oviduct fluid modified with amino acids (BME Amino Acids Solution (SIGMA, Madrid, Spain), 45 μL/mL and MEM Non-essential Aminoacid Solution (SIGMA, Madrid, Spain), 5 μL/mL), citrate, myo-inositol, and BSA (6 g/L), as previously described [27]. Droplets of synthetic oviduct fluid modified (1–2 μL per embryo) were layered under mineral oil and embryos cultured in groups of 35 to 45. *In vitro* culture was carried out at 39 °C, 5% CO_2, 5% O_2, and saturated humidity. Culture medium was renewed on Days 3 and 6 by transferring the embryos to fresh droplets. Cleavage (Day 3) and development (from Days 6 to 8) rates were recorded following reported criteria [28].

2.2. Embryo transfer

Embryos were *in vitro* cultured until Day 8 or were nonsurgically transferred on Day 5 to the cranial third of the CL ipsilateral uterine horn of synchronized animals (50 morulae per embryo transferred [ET] cow). The transfer of multiple embryos to the uterus has been previously validated as a model to study early embryomaternal interactions in cattle [16,29]. A control group of animals was sham transferred (ST) with same volume (45 mL) of embryo HM (Instruments de Médecine Vétérinaire, Humeco, Huesca, Spain). Age-matched heifers and uniparous cows (n = 3 heifers + 2 cows for ET; 3 heifers + 3 cows for ST) were sacrificed in a nearby slaughterhouse (Matadero de Pravia, Asturias) on Day 8.