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Expression and localization of ARTEMIN in the bovine uterus and embryos

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ABSTRACT

Artemin a member of the glial cell line-derived neurotrophic factor (GDNF) family is present in mice and human preimplantation embryos, and reproductive tract, during early pregnancy promoting embryo development *in vitro*. The presence of artemin in cattle embryos and reproductive tract, however, is unknown. In the present work we identified for first time artemin in bovine uterine fluid (UF) (Western blot), endometrium (RT-PCR, Western blot and immunohistochemistry) and embryos (RT-PCR and immunohistochemistry) during early preimplantation development. In addition, GFRalpha3, a component of the artemin receptor was localized in blastocysts produced *in vitro*. Individually developing embryos released ARTEMIN in culture medium and triggered ARTEMIN mRNA down-regulation in epithelial cells from endometrial cell cultures. Our results suggest that ARTEMIN derived from early embryos and maternal reproductive tract may exert important roles during early development in cattle.
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1. Introduction

Artemin is a neurotrophic growth factor of the glial cell line-derived neurotrophic factor (GDNF) family expressed in human neural tissues and prostate, placenta, pancreas, heart, kidney, pituitary gland, lung and testis [1]. In mice and humans, artemin is expressed in preimplantation embryos and reproductive tract during early pregnancy [2,3]. Besides, addition of artemin during *in vitro* culture promotes early embryo development and decreases apoptosis [2].

Currently, in the bovine species, it is possible to produce early embryos employing a variety of *in vitro* systems. However, *in vitro* produced (IVP) embryos differ to their *in vivo* derived counterparts in morphology, developmental kinetics, gene expression, survival to cryopreservation and epigenetic alterations [4–9]. In addition animal and human embryos cultured in simple media, i.e synthetic oviduct fluid (SOF) supplemented with BSA, SOF supplemented with fatty acid free BSA, SOF supplemented with human serum or G1 Vitrolife™, show suboptimal growth and developmental abnormalities [10,11].

In an attempt to improve *in vitro* culture of embryos, a variety of growth factors (GF) has been added to embryo culture media [12]. However, some GFs promote embryo development when added during the entire *in vitro* culture period, and other GFs show time dependent effects or no effect at all [13–19]. Such controversial results may be explained because growth factors are frequently added to culture media without prior knowledge about their presence in the *in vivo* environment where the early embryo develops. Consequently, the analysis of female reproductive tract fluids during the earliest stages of mammalian embryo development can provide valuable physiological data on molecules involved in reproductive processes such as fertilization, endometrial receptivity, early embryo development and embryo-maternal communication [20]. A number of studies have examined the nature of endometrial secretions in animal species and humans [21–23]. Uterine fluid (UF) analysis overcomes many of the

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difficulties of studying the endometrium [24]. The abundant structural proteins commonly found in tissues are normally not found in UF, making it a less complex sample type that can be recovered by non invasive means. In addition, UF composition reflects general modifications of the endometrium dependent on estrous cycle phases and interactions between the embryos and the genital tract.

Proteomic analysis performed to characterize UF composition during early development stages stated that many protein changes related to cycle stage or fertility status in the endometrium are not supported by transcriptional changes [25–28]. Furthermore, post-transcriptional and post-translational regulatory mechanisms, faster than those that depend on de novo mRNA synthesis, were suggested to be better suited for supporting rapid and dynamic dialogue between the embryo and the mother during very early embryo development [29].

Recently, we have provided independent datasets that support the multiple embryo transfer (ET) model as an efficient system to help identify molecular changes during very early embryo maternal interactions in the cow [29]. In the present work we report for the first time the identification of ARTEMIN (ART) at mRNA and protein level in bovine UF, endometrium and embryos during early pre-implantation development using a multiple ET model. Specifically, we examined whether (1) ART protein is expressed in bovine UF and in the medium of single cultured bovine embryos during blastocyst formation, (2) ART mRNA and protein are present in the bovine endometrium, (3) ART mRNA and protein and GFRalpha3 protein are present in bovine embryos during the blastocyst stage, (4) endometrial and embryonic ART expression is reciprocally regulated; and (5) the presence of embryos regulates ART mRNA expression in endometrial co-cultures.

2. Materials and methods

Experimental procedures involving animals were performed according to the European Community Directive 2010/63/EU (Spanish Regulation, R.D. 53/2013), and were sanctioned by the Animal Research Ethics Committee of SERIDA (Agreement date 08 February 2012).

Ovaries were collected from cows slaughtered in a commercial abattoir and transported to the laboratory in NaCl solution (9 mg/mL) with antibiotics. All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

2.1. Embryo production

IVP embryos were obtained as described previously [30], with minor modifications. Briefly, ovarian follicles with diameters of 3–8 mm were aspirated and cumulus–oocyte complexes recovered. After *in vitro* maturation for 24 h, oocytes were subjected to IVF (day 0) using frozen/thawed semen from a single bull obtained through a swim-up procedure. Embryos were cultured in synthetic oviduct fluid (SOF) containing amino acids, citrate, and myo-inositol with 6 g L^{-1} BSA at 38.7 °C, 5% CO_2, 5% O_2 and 90% N_2 until day 8 after *in vitro* fertilization or were transferred on day 6 to the uterus of synchronized animals.

Additionally, in order to asses if bovine embryos could produce ARTEMIN, Day-6 good quality morulae (90% approximately) and early blastocysts were cultured individually in 12 µL droplets for 24 h (up to Day-7) as described [31]. Spent culture media (10 µL per droplet) and blank controls (i.e. droplets incubated without embryos) were collected on Day-7 and stored at −80 °C up to ELISA analysis.

2.2. Animals, embryo transfer and uterine flushings

Animal management, estrus synchronization and embryo production and transfer were described in detail [27]. Briefly, *in vitro* produced N = 50 Day-6 morulae were nonsurgically transferred on Day 6 to the cranial third of the uterine horn ipsilateral to CL of estrus-synchronized heifers). The transfer of multiple embryos to the uterus has been previously validated as a model to study early embryo-maternal interactions in cattle [27,28]. Control animals were sham transferred (ST) with the same volume (45 mL) of embryo holding medium (Instruments de Médecine Vétérinaire, Humeco, Huesca, Spain). Cyclic, Holstein heifers, 16–20 months old (n = 14) were housed on an experimental farm. Food was administered by an automated concentrate dispenser; 2–3 kg concentrate/day accompanied by oat straw and mixed vitamin-mineral blocks ad libitum. Body condition score was maintained on 3 ± 0.5 points over a 0–5.

Ooestrus cycles were synchronized by using an intravaginal progestagen device (PRID ALPHA, Ceva, Barcelona, Spain) for 10 days combined with a prostaglandin analogue (Dynolitic, Pfizer, Madrid, Spain) injected 48 h before progestagen removal. Animals were observed at least 3 times per 30 min a day for ooestrus detection, commencing 33 h after progestagen removal. Day 0 was considered a fixed time 48 h after progestagen removal, to coincide with the IVF onset in the laboratory. On day 0, ovaries were scanned by ultrasound and the preovulatory follicle was measured.

Synchronized recipients were allocated to either sham transfer (i.e intrauterine deposition of holding medium, ST group) or embryo transfer (ET group). On day 6, morulae (n = 50) were nonsurgically transferred to the cranial third of the uterine horn ipsilateral to the formerly detected preovulatory follicle, under epidural anesthesia. The transfer of multiple embryos to the uterus has been previously validated as a model to study early embryo maternal interactions in cattle [29]. Sham transfers were also performed with IMV (Instruments de Médecine Vétérinaire) embryo holding medium (Humeco, Huesca Spain).

Animals were regularly treated at 2–3 month intervals throughout non-consecutive ooestrus cycles, and treatments were repeated 3- to 6-times per animal. Once UF paired samples (i.e. uterine fluid recovered from an embryo-transferred recipient and uterine fluid recovered from the same recipient after a sham transfer) were obtained, recipients in another cycle were subjected to embryo transfer on Day-6 performed with 2 morulae per transferred female (2E group). Only when 1 live embryo was recovered on Day-8 the cognate UF was processed for validation purposes (Supplementary Fig. 1).

Progesterone was analyzed by ELISA (EIA- 1561, DRG Diagnostics, Germany) in blood plasma samples taken in EDTA-vacuum tubes from venycoccigeal puncture on day 0, just after ET time (day 6) and on day 8.

2.3. Uterine flushings on day- 8 and embryo collection

Recovery of uterine fluid and embryos were described in detail [28]. Briefly, the ipsilateral horns of recipients were first flushed with 45 mL of PBS, pH 7.4, with no additives and subsequently those recipients transferred with embryos were extensively flushed with PBS +1 mg/mL poly-vinyl-pyrrolidone. The embryos were identified using a stereomicroscope and processed for immunohistochemistry (IHC) or RT-PCR analysis (see below).The UF was centrifuged (2000×g) at 4 °C for 10 min, aliquoted in 1.8 mL and stored at −145 °C.

Flushed embryos and day-8 IVP blastocysts were processed for qRT-PCR and IHC as described [32]. UF with embryos contained on average n = 43.1 ± 5.2 total and n = 34.1 ± 3.7% viable embryos (as

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