Theriogenology 96 (2017) 118–125

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Hepatoma-derived growth factor: Protein quantification in uterine fluid, gene expression in endometrial-cell culture and effects on *in vitro* embryo development, pregnancy and birth

E. Gómez [a, *], S. Carrocera [a], D. Martin [a], M.J. Sánchez-Calabuig [b], A. Gutiérrez-Adán [c], A. Murillo [a], M. Muñoz [a]

[a] Genética y Reproducción Animal, Centro de Biotecnología Animal, SERIDA, Camino de Rioseco, 1225, 33394 Gijón, Spain
[b] Facultad de Veterinaria, Departamento de Medicina y Cirugía Animal, Universidad Complutense, Av. Puerta de Hierro, s/n, 28040 Madrid, Spain
[c] Departamento de Reproducción Animal, INIA Avda. Puerta de Hierro, nº12, local 10, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 5 February 2017
Received in revised form
20 March 2017
Accepted 4 April 2017
Available online 6 April 2017

Keywords:
Cattle
Embryo
Endometrium
HDGF

ABSTRACT

Hepatoma-derived growth factor (HDGF) is present in the endometrium of cows and other mammals. Recombinant HDGF (rHDGF) improves bovine blastocyst development *in vitro*. However, specific culture conditions and essential aspects of HDGF uterine physiology are yet unknown. In this work we quantified total HDGF protein in uterine fluid (UF) by multiple reaction monitoring (MRM), and analyzed effects of rHDGF on specific embryonic stages with Day-6 bovine embryos cultured *in vitro* with and without BSA, and on pregnancy viability and calf phenotypes after embryo transfer to recipients. In addition, mRNA abundance of *HDGF* in endometrial cells co-cultured with one male or one female embryo was quantified. In the presence of BSA, rHDGF had no effect on blastocyst development; however, in BSA-free culture rHDGF mainly promoted development of early blastocysts in contrast with morulae. As the presence of HDGF contained in commercial BSA replacements was suspected, western blot confirmed HDGF identification in BSA both with and without fatty acids. Total HDGF quantified by MRM tended to increase in UF without vs. UF with embryos ($P = 0.083$). Pregnancy and birth rates, birth weight and calf measurements did not differ between embryos cultured with rHDGF and controls without rHDGF. However, *HDGF* abundance in cultured epithelial, endometrial cells tended to increase ($P < 0.08$) in culture with one male embryo. rHDGF acts selectively on specific embryonic stages, but care should be taken with specific macromolecular supplements in culture. The endometrial expression of *HDGF* can be regulated by the embryonic sex. The use of rHDGF is compatible with pregnancy and birth of normal calves.

1. Introduction

Growth factors (GFs) produced by the reproductive tract can improve *in vitro* bovine embryo development, and/or often pregnancy rates and survival to term. Reports include Dickkopf, epidermal growth factor, insulin-like growth factor 1, granulocyte-macrophage colony-stimulating factor, hyaluronan, fibroblast growth factor 2, activin, and platelet-activating factor [1–9].

Hepatoma-derived growth factor (HDGF) is a 240 amino-acid protein isolated from supernatants of human hepatoma cells in culture [10]. Surface expressed nucleolin has recently been identified as a HDGF receptor [11], and HDGF stimulates cell proliferation in fibroblasts, endothelial cells and hepatoma cells [12]. In the cow, HDGF is present in the endometrium and uterine fluid (UF) during early pregnancy [13,14], as well as in the endometrium and conceptus in horses [15] and in the UF of the tammar wallaby [16]. Interestingly, in cattle, changes induced in the uterine HDGF by embryos [13] are not reflected as mRNA transcription and protein abundance in endometrial cells [14] by which up-regulation of HDGF in UF could be dependent on post-transcriptional modifications, as previously postulated to occur in the uterus [17]. Notably, unlike the absence of detectable transcription in response to embryos in vivo, cultured endometrial cells transcribe *in vitro* in response to one embryo [18]. Recombinant HDGF (r-HDGF)

* Corresponding author.
E-mail address: egomez@serida.org (E. Gómez).

http://dx.doi.org/10.1016/j.theriogenology.2017.04.008
0093-691X/

improves blastocyst development and cell proliferation [14]. HDGF may act by both autocrine and paracrine mechanisms to promote early embryo development, and effects of rHDGF are strictly time and/or stage dependent (i.e. it stimulates embryo growth within Day-6 but not Day-5 morulae) [14]. Within *in vitro* embryo culture, similar stage- or time-dependent effects also occur with other GFs, resulting in altered blastocyst development and quality [6,7,19,20] and, ultimately, improved pregnancy and birth rates [1,20]. In cultures with synthetic oviduct fluid (SOF), vitrified/warmed, Day-7 expanded blastocysts resulting of Day-6 morulae or early blastocysts deprived of protein yield lower miscarriage and improved birth rates upon transfer to recipients [21]. Interestingly, Day-6 morulae and early blastocysts mobilize lipid stocks differently in response to protein deprivation, and the Day-6 early blastocysts stage yields lighter calves than Day-6 morula when their cognate Day-7 vitrified expanded blastocysts are transferred [22]. Therefore, r-HDGF in culture could exert different stimulation on morulae and/or early blastocysts.

Protein supplements used in bovine embryo culture are normally impure and can induce confounding effects when testing single molecules. Serum and its extracts, as bovine serum albumin (BSA), may carry GFs, cytokines, amino-acids, steroids and a variety of contaminants [23]. Cold-precipitated BSA exists as an embryo-tested product, and more refined forms of BSA (e.g., essentially fatty-acid free –FAF-) are also used in embryo culture. Effects of exogenously added GFs can be masked not only by serum or its extracts, but also by autocrine and paracrine interactions (group embryo culture). The effects of HDGF on *in vitro* embryo culture were demonstrated both in group and single culture in chemically defined conditions [14], but it is unknown yet whether culture in medium with BSA would offer similar performance. Endometrial cells are responsive to embryonic sex early in developmentin vivo [24]. Therefore, endometrial HDGF could also show a sexually dimorphic transcription.

The objectives of this work were: 1) Total quantification of HDGF protein in UF by MRM, a technique that can be programmed to recognize peptides typically present in any protein isoform [25]; 2) Identifying the developmental effects of rHDGF on specific embryonic stages, both in semi-defined (BSA) conditions and in chemically defined conditions; 3) Analyzing the presence of HDGF in commercial BSA and BSA-FAF extracts used in embryo culture; 4) Analyzing endometrial transcription for HDGF in response to one male or female embryo in cultured endometrial cells; and 5) Determining whether embryos produced with rHDGF can establish pregnancy and reach birth with production of normal calves.

2. Materials & methods

All experimental procedures were approved by the Animal Research Ethics Committee of SERIDA (Agreement 02/02/2012), in accordance with the European Community Directive 86/609/EC. Ovaries were collected from cows slaughtered in commercial abattoirs (Matadero de Leon and Matadero de Guarnizo –Cantabria-, mostly for Holstein ovaries, and Matadero de Tineo, for Asturiana de los Valles ovaries (Spain). Ovaries were transported to the laboratory in NaCl solution (9 mg/mL) with streptomycin sulfate, 100 μg/ml, penicillin, 100 IU/mL and maintained at 25–30 °C. All reagents were purchased from SIGMA (Madrid, Spain) unless otherwise stated.

2.1. Embryo production

In vitro-produced (IVP) embryos were obtained as described [26] with minor modifications. Antral follicles (3–8 mm in diameter) were aspirated through an 18-g needle connected to a syringe. Aspirated fluid was expelled into dishes containing holding medium (HM) TCM199 (Invitrogen, Barcelona, Spain), 25 mM HEPES and BSA 0.4 g/L, and oocytes were searched under stereomicroscope. Oocytes with more than three layers of compact cumulus cells with homogenous cytoplasm were selected. For *in vitro* maturation (IVM), cumulus oocyte complexes (COCs) were washed twice in maturation medium (MM) consisting of TCM199 $NaHCO_3$ (2.2 g/L) supplemented with 10% (v/v) fetal calf serum, 1:5 μg/mL of porcine FSH-LH (Stimufol; ULg FMV, Liège, Belgium) and 1 μg/mL 17 β-estradiol. COCs were cultured (n = 30–50) into a four-well dish with 500 μL of IVM medium for 22–24 h at 38.7 °C under 5% CO_2 with saturated humidity.

After IVM, oocytes were subjected to *in vitro* fertilization (IVF; Day 0) with frozen/thawed sex-sorted or non sex-sorted spermatozoa from Holstein or Asturiana de los Valles breeds following described procedures [26,27], respectively. For experiments entirely *in vitro* or for transient embryo transfer to the uterus (Day-6 to Day-8), IVF was performed with non-sorted spermatozoa from a single bull. However, for transfer to recipients for pregnancy, embryos were produced from oocytes both from slaughterhouse ovaries or collected by Ovum-Pick-Up (OPU) following described procedures [28], and fertilized with non-sorted (N = 3 bulls) and female sex-sorted spermatozoa (N = 3 bulls). COCs were washed three times in HM in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg/mL; Calbiochem, La Jolla, CA, USA). For IVF, oocytes and sperm cells were incubated together for 18–20 h at 38.7 °C in an atmosphere of 5% CO_2 with saturated humidity. Subsequently, cumulus cells were detached using a vortex, and presumptive zygotes were cultured in modified synthetic oviduct fluid (mSOF) containing amino acids (BME Amino Acids Solution), 45 μL/mL and MEM Non-essential amino-acid Solution (SIGMA, Madrid, Spain), 5 μL/mL), citrate, myo-inositol, and BSA (6 g/L), as previously described [21]. Until Day-6, embryos were cultured in groups (N = 35–45) in droplets of mSOF (1–2 μL per embryo) layered down mineral oil. *In vitro* culture was carried out at 38.7 °C, 5%CO_2, 5%O_2 and saturated humidity. After Day-6 embryos were cultured singly using different procedures as described below.

2.2. Vitrification of embryos for transfer

Vitrification procedures have been described in detail [26]. Briefly, Day 7 excellent expanded blastocysts were vitrified in two-steps with fibre-plugs (CryoLogic Vitrification Method; CVM). Vitrification solutions contained DMSO, ethylene-glycol, and sucrose. Samples were vitrified by touching the surface of a chilled block placed in LN_2 with the fibre plug hook that carried the drop with the embryo. Embryos were warmed by direct immersion of the fibre plug end in sucrose solution in one-step [29]. Subsequently, embryos were washed twice in Embryo Preservation Medium (IMV Technologies, France) and loaded in straws for transfer to recipients. *In vitro* development of vitrified/warmed embryos cultured with and without protein was already reported [21].

2.3. Animals and embryo transfer (ET) for uterine fluid collection

Detailed procedures were described [13]. Briefly, cyclic Holstein heifers (N = 14) were synchronized in estrus with an intravaginal progestagen device combined with a prostaglandin analogue. Day 0 was considered a fixed time 48 h after progestagen removal, in coincidence with the IVF onset in the laboratory. Day-6 IVP embryos (n = 50), or vehicle (Sham transfer) were transferred into the uteri of each estrus synchronized Holstein heifer (n = 14) at non-consecutive cycles under epidural anesthesia.