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Effects of ghrelin on developmental competence and gene expression of *in vitro* fertilized ovine embryos

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ABSTRACT

The objective was to determine the effects of various ghrelin concentrations (0, 10, 50, and 250 ng/mL) during *in vitro* oocyte maturation (IVM) and *in vitro* embryo culture (IVC) on ovine embryo development and expression patterns of genes involved in transcription regulation (*OCT4*), glucose transport (*GLUT1*), pregnancy recognition (*IFNT*), and ghrelin receptor (*GHSR-1a*). Rates of cleavage and blastocyst formation were decreased when oocytes were matured with 250 ng/mL ghrelin compared with 0, 10, or 50 ng/mL ghrelin ($P < 0.05$). Addition of 50 ng/mL ghrelin during IVC or during IVM and IVC significantly increased blastocyst rates (35.3% and 36.7% vs. 26.9% and 26.8%, respectively) and total cell numbers per blastocyst (110.4 and 108.3 vs. 81.2 and 77.4) compared with 0 and 10 ng/mL ghrelin. However, a high concentration (250 ng/mL) of ghrelin during IVM and IVC decreased cleavage, blastocyst rate, and total cell number of blastocyst compared with low concentrations ($P < 0.05$). Relative abundances of *GLUT1* and *IFNT* transcripts were higher in blastocysts treated with 50 ng/mL ghrelin during IVC compared with other concentrations ($P < 0.05$). Expression of *GHSR-1a* was higher when 10 ng/mL ghrelin was added during IVM (0.079) or during IVM and IVC (0.053) compared with other treatments. However, addition of ghrelin at higher concentrations (50 or 250 ng/mL) reduced relative abundances of *GHSR-1a* transcripts (0.032 and 0.039, $P < 0.05$). In conclusion, appropriate concentrations of ghrelin promoted ovine blastocyst formation *in vitro* and increased expression of *GLUT1*, *IFNT*, and *GHSR-1a* genes in blastocysts, although a high concentration of ghrelin suppressed embryo development.

1. Introduction

Ghrelin is a 28-amino acid peptide originally isolated from rat stomach as a natural ligand of the growth hormone secretagogue receptor type 1a (*GHSR-1a*) [1]. A striking feature of ghrelin is its widespread pattern of expression [2,3]. Ghrelin is mainly produced in the stomach of all animals investigated [2]. Expression of ghrelin has also been found in many other organs, including the gastrointestinal tract, lung, thyroid, heart, mammary gland, fat, placenta, lymph nodes, liver, kidney, adrenal gland, pancreas, and central nervous system in mammals [3]. This ubiquitous pattern of expression strongly suggests that, in addition to systemic actions of the gut-derived peptide, locally produced ghrelin might have paracrine/autocrine regulatory effects in various tissues [4–6]. This might be the case in various reproductive organs (such as endometrium, placenta, testis, and ovary [7,8]), and preimplantation embryos and blastocysts [9,10]. Although the major role of ghrelin is control of growth hormone secretion and food intake [11], ghrelin expression in reproductive organs and embryos suggest its possible effects on reproductive function [9,10]. Several studies have linked ghrelin with reproductive physiology, mainly through the modulation of the hypothalamic–hypophyseal–gonadal axis [1,9,12,13].

In vitro production of embryos provides an excellent opportunity for inexpensive and abundant embryos for

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conducting basic research and for application of emerging biotechnologies, e.g., cloning and transgenesis [14]. In sheep, techniques for *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos seem to be well established [15]. However, *in vitro* maturation (IVM) of oocytes needs to be optimized in most domestic species [16]. Oocytes undergo their nuclear maturation during the IVM period, and 85% of them are fertilized after IVF. However, only approximately 30% develop to the blastocyst stage after IVC. Therefore, the media for IVM of ovine oocytes must be optimized to improve the efficiency of *in vitro* production. A previous study suggested that ghrelin can enhance blastocyst formation of porcine *in vitro* fertilized and parthenogenetic embryos [12]. It was recently reported that inclusion of ghrelin in maturation medium at a reasonable concentration had beneficial effects on nuclear maturation of bovine oocytes [17].

Available data, although fragmentary, make it tempting to speculate that ghrelin might be a novel member of the regulatory network involved in oocyte maturation and embryonic development. However, to our knowledge, the effects of ghrelin supplementation on early *in vitro* development of ovine embryos have never been studied. The objective of the present study was to assess the effects of various concentrations of ghrelin supplemented during IVM and/or IVC on development of ovine embryos. *In vitro* development to the blastocyst stage, total cell number, and mRNA relative abundance (RA) of a panel of four genes (including *OCT4*, *GLUT1, IFNT*, and *GHSR-1a*) at the blastocyst stage were assessed.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.1. Oocyte collection and IVM

Ovine ovaries were obtained from an abattoir and transported to the laboratory within 2 hours in a 0.9% saline solution. The procedure of oocyte collection and IVM were as described by Forouzanfar et al. [18]. In brief, cumulus oocyte complexes (COCs) were aspirated from 2 to 6 mm follicles. Only oocytes with at least three layers of compact cumulus cells and homogenous cytoplasm were selected. Then, groups of 40 to 45 COCs were cultured in 500 μL maturation medium and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 for 24 hours. Maturation medium consisted of TCM199 (Gibco, Grand Island, NY, USA) supplemented with 4 mg/mL BSA, 2.5 mmol/L Na pyruvate, 1 mmol/L L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL FSH, 10 μg/mL LH, 1 μg/mL estradiol-17β, 0.1 mmol/L cysteamine, and 0, 10, 50, or 250 ng/mL ghrelin.

2.2. IVF

The IVF of ovine oocytes was carried out as described [19]. Briefly, cryopreserved semen was thawed and layered on top of a double density layer of Percoll solutions (1 mL of 45% over 1 mL of 90% in fertilization medium) in a 15 mL tube. After centrifugation, the supernatants were discarded and the pellets were suspended with fertilization medium. The concentration of the sperm suspension was adjusted to 1.0×10^6/mL live sperm. The fertilization medium was synthetic oviduct fluid containing 2% sheep serum. Drops of 450 μL of the final sperm suspension were prepared in four-well dishes, overlaid with equilibrated mineral oil, and kept in a humidified incubator until the oocytes were prepared. Matured oocytes from all groups were stripped of excess cumulus cells by gentle pipetting, and then washed once in fertilization medium. Groups of 40 to 50 oocytes were transferred into the prepared sperm drops and cultured for 20 hours at 38.5 °C in a humidified atmosphere of 5% CO_2.

2.3. In vitro culture

After fertilization, presumptive zygotes were denuded by gentle pipetting and washed three times in culture medium before being randomly assigned to treatment. The culture medium was synthetic oviduct fluid supplemented with 2% Basal Medium Eagle-essential amino acids, 1% Minimum Essential Medium nonessential amino acids, 1 mmol/L glutamine, 8 mg/mL BSA, and 0, 10, 50, or 250 ng/mL ghrelin. Zygotes (30–40) were cultured in each well containing 600 μL culture medium covered with mineral oil. Culture occurred in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C. Cleavage was assessed 48 hours after IVF and blastocyst development was recorded on Day 7 (Day 0 = day of IVF). Percentages of blastocysts were based on number of cleaved oocytes. To assess the quality of the embryos, mean cell number in Day 7 blastocysts was determined.

2.4. RNA extraction, reverse transcription, and quantification of mRNA transcript abundance

Total RNA was isolated from 30 single expanded blastocysts in each group (i.e., 30 blastocysts for 0/0 ghrelin group, 30 blastocysts for 0/10 ghrelin group, etc.) using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and dissolved in 20 μL RNase-free water. The RNA concentration and purity was determined by spectrophotometry (NanoDrop, Wilmington, DE, USA) using a 1 μL sample, in which all samples had a 260/280 ratio of absorbance between 1.8 and 2.1. Immediately after extraction, total RNA was treated for genomic DNA carryover with RNase-free DNase in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). Subsequently, the DNase-treated RNA was converted to cDNA in reverse transcription reaction using reagents and instructions supplied by Promega. Samples were quantified by real-time quantitative reverse transcription polymerase chain reaction (PCR) in 20 μL reactions with SYBR Green supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) in triplicate. Before use in quantitative PCR, cDNA was diluted 1:5 with water, with 5 μL of cDNA used as a template for each PCR run. Primer sequences, annealing temperature, and approximate sizes of the amplified fragments are listed (Table 1). The PCR amplification was carried out for one cycle, with denaturing at 95 °C for 15 minutes and subsequently 40 cycles