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Role of the Calcium-Sensing Receptor (CaSR) in bovine gametes and during *in vitro* fertilization

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ABSTRACT

Calcium Sensing Receptor (CaSR) is a G-protein coupled receptor which senses extracellular calcium and activates diverse intracellular pathways. The objective of our work was to demonstrate the presence of CaSR in bovine gametes and its possible role in fertilization and embryo development. The location of CaSR was demonstrated by immunofluorescence in bovine gametes; additionally bovine sperm were incubated with 5, 10 and 15 μM of the specific CaSR inhibitor NPS2143 in a Tyrode's Albumin Lactate Pyruvate medium (4 h). Sperm viability was maintained for all concentrations tested while total motility decreased significantly at 10 and 15 μM. Addition of 15 μM of NPS2143 during oocyte *in vitro* maturation did not alter the maturation rate. When NPS2143 (15 μM) was added to the fertilization medium during sperm-oocyte co-incubation the cleavage, morula and blastocyst rates remained unchanged. To confirm if 15 μM of NPS2143 exerted any effect on embryo development, NPS2143 was added to the embryo culture medium. Cleavage rates remained unchanged when 15 μM of NPS2143 was added to the culture medium (79.1 ± 6.8 vs. 73.7 ± 5.3; mean % ± SEM; $p > 0.05$, control vs. inhibitor). By contrast, development to the morula (46.6 ± 7.3 vs. 24.3 ± 4.3; mean % ± SEM; $p < 0.05$) and blastocyst stages (29.9 ± 9.0 vs. 9.9 ± 3.6; mean % ± SEM; $p < 0.05$) decreased (control vs. inhibitor). Our results demonstrate a key role of CaSR on sperm motility and embryo development but not on oocyte maturation or fertilization in the bovine species.

1. Introduction

The fertilization process is constituted by an intricate series of well-orchestrated events that lead to the formation of a blastocyst, eventually resulting in viable offspring. Although *in vitro* fertilization (IVF) can be achieved in the laboratory and is routinely used as a source of commercially transferable embryos in many livestock species including bovine [1–3], the extracellular signals involved in the process are still under investigation. Bovine oocytes used for IVF can be retrieved from live animals using ovum pick up or harvested directly from the ovaries post-mortem. When oocytes are retrieved from post-mortem ovaries, they are arrested in profase I of meiosis, and have to be subjected to *in vitro* maturation (IVM) to reach the metaphase II stage (MII) prior to fertilization [4]. On the other hand, ejaculated sperm have to acquire their fertilizing capacity in a process known as capacitation [5,6]. Capacitated sperm and MII oocytes are co-incubated and several events occur: the sperm crosses the zona pellucida (ZP) triggering the blockage of the ZP [7], the zygote begins to form and, after many cell divisions, embryo formation takes place [8]. Although the intracellular signaling involved in embryo formation are regulated by many extracellular molecules and ions, calcium plays a major role regulating oocyte maturation and activation [9,10], sperm capacitation [11] and embryo cleavage [12]. However, it is not just the presence or absence of calcium but also its extracellular and intracellular concentrations that play a crucial role during these events [13–16]. CaSR is a G-protein coupled receptor, that has been described in many different somatic cell types including bovine parathyroid glands, and is capable to detect and transduce subtle changes in extracellular calcium [17,18] playing a key role in numerous intracellular pathways [19]. In addition, CaSR has also been studied in germ cells [20] showing a core role in porcine and equine oocyte maturation [16,21] and in porcine and rat sperm motility [22]. Recently, CaSR has been described to play a pivotal role regulating capacitation and

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motility in stallion sperm [23]. Despite its central role in many fertilization-related events, no reports have been published in bovine gametes, and the role that CaSR may play in sperm motility, oocyte maturation and subsequent fertilization remains unknown. Thus, the present study was designed to investigate the role of CaSR during oocyte maturation, fertilization and embryo development in cattle, which included key fertilization-related events such as meiosis resumption and regulation of sperm motility.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) unless otherwise stated.

2.2. Semen collection and processing

Semen from four Holstein bulls was purchased from a commercial bull station (Centro de Recolhas de Vendas Novas, Portugal). Frozen straws (0.25 mL) were thawed at 37 °C for 1 min and semen from each bull was evaluated individually; for all bulls post-thaw total sperm motility ranged between 35 and 50%. Thawed sperm was resuspended in 5 mL of Tyrode's albumin lactate pyruvate (TALP) medium (fertilization medium), consisting of 114 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM sodium lactate, 25 mM $NaHCO_3$, 0.34 mM $NaH_2PO_4 \cdot H_2O$, 1.0 mM pyruvic acid, 2 mM $CaCl_2 \cdot 2H_2O$, 6 mg/mL of BSA, 1 μL/mL phenol red, penicillin–streptomycin (10 U/mL of penicillin and 10 μg/mL of streptomycin) and 10 μg/mL heparin. Diluted semen was centrifuged at 200 × g for 5 min and the resulting pellet was resuspended in TALP medium at 10×10^6 sperm/mL for IVF or at 50×10^6 sperm/mL for motility and viability experiments. Medium was incubated at least 3 h at 38.5 °C in a 5% CO_2/95% air incubator before the beginning of the experiment. Medium was set to an osmolality of 290–300 mOsm/Kg and adjusted at pH 7.4.

2.3. Immunofluorescence

Immunofluorescence for detection of CaSR was performed as previously reported [23]. In brief, bovine thawed sperm (n = 3, each from a different bull) were fixed with methanol cooled at −20 °C for 5 min at room temperature. On the other hand, the zona pellucida (ZP) was removed using 0.1% pronase (wt/vol) and oocytes were fixed with 4% formaldehyde (v:v) in PBS added with 0.2% of polyvinylalcohol (PBS + PVA; wt/vol) overnight at 4 °C. The next morning, the oocytes (n = 24, 7 replicates) were permeabilized with 0.2% Triton X-100 (v:v) in PBS for 30 min at room temperature. Gametes were blocked for 1 h with 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT, USA) in PBS, and incubated overnight at 4 °C with anti-goat-CaSR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:20 in PBS supplemented with 1.5% FBS (PBS + FBS; v:v). The next morning, gametes were incubated with an anti-goat IgG (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:50 in PBS + FBS. After washing in PBS + PVA the samples were mounted on a slide using glycerol and evaluated using an Olympus BX41 fluorescence microscope equipped with × 100 objective (New Hyde Park, NY).

2.4. Sperm viability

To determine the percentage of live cells the supravital eosin-nigrosin stain was used. Ten microliters of bovine sperm were mixed with an equivalent volume of the stain, and the mix was smeared on a pre-heated slide at 37 °C. The samples were air dried and examined using a light microscope (magnification × 100). One hundred sperm were counted per sample. Sperm excluding the nigrosin-eosin stain were considered alive, while sperm showing a "pinkish" colour were counted as dead. One sample per bull was evaluated (n = 4).

2.5. Motility analysis

Sperm motility was analyzed by a computer-assisted sperm analysis (CASA) system (ISAS 1.0.6; Proiser S.L., Valencia, Spain). An aliquot (6 μL) of each sample was placed in a pre-heated (37 °C) motility chamber with a fixed height of 20 μm (Proiser S.L., Valencia, Spain). A minimum of three microscopic fields and 300 total sperm were evaluated at 25 frames per second. The parameter assessed was percent of total motility (TM); sperm with an average path velocity (VAP) less than 10 μm/s were considered immotile. One sample per bull was evaluated (n = 4).

2.6. In vitro fertilization

Bovine ovaries were collected at a nearby abattoir and were maintained in saline solution (0.9% NaCl) during transport (1 h total). Upon arrival at the laboratory, the ovaries were thoroughly rinsed with PBS at 37 °C. Cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm-diameter follicles using a 10 mL plastic syringe attached to a 20-ga hypodermic needle. Oocytes with three or more layers of compact cumulus cells were washed in tissue culture medium 199 (TCM-199) and transferred to a 4-well Nunc® plate added with 500 μL of maturation medium under mineral oil and incubated at 38.5 °C for 24 h in a 5% CO_2/95% air incubator. The maturation medium consisted of TCM-199 (M2520) supplemented with 25 mM bicarbonate, 10% fetal bovine serum, 10 mU/mL of follicle-stimulating hormone (FSH; Life Technologies Corporation), 10 mU/mL of luteinizing hormone (LH; Life Technologies Corporation), and penicillin–streptomycin (10 U/mL of penicillin and 10 μg/mL of streptomycin). After oocyte maturation, COCs were washed in fertilization medium and transferred to a 90 μL droplet of fertilization medium under mineral oil. Ten microliters of thawed sperm were added to the fertilization droplet to reach a final sperm concentration of 1×10^6 sperm/mL. Gametes were co-incubated for 18 h at 38.5 °C in a 5% CO_2/95% air atmosphere. Presumptive zygotes from each group were washed in TCM-199 supplemented with 25 mM bicarbonate, penicillin–streptomycin (10 U/mL of penicillin and 10 μg/mL of streptomycin) and 10% FBS (culture medium) and transferred to 500 μL of the same medium in a 4-well Nunc® plate and covered with mineral oil. Incubation of presumptive zygotes was performed in a humidified atmosphere at 38.5 °C in a 5% CO_2/95% air incubator. Embryos from IVF experiments were evaluated by stereomicroscope on days 2, 6 and 7 post-insemination for cleavage and development to the morula and blastocyst stages respectively.

2.7. DNA evaluation

After oocyte maturation, DNA evaluation for determination of maturational stage was performed as previously reported [24]. Briefly, oocytes were denuded, and fixed in 4% formaldehyde in PBS supplemented with 0.2% PVA. Then, the oocytes (n = 74, 6 replicates for control and n = 78, 6 replicates for NPS2143 15 μM) were washed in PBS + PVA and stained with Hoechst 33342 at 2.5 μg/mL at 37 °C for 10 min in the dark. Oocytes were mounted on slides using glycerol and the DNA integrity and maturational stage were assessed using an Olympus BX41 fluorescence microscope (New Hyde Park, NY, USA) equipped with × 40 and × 100 objectives.