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Original article

Supplementing in vitro embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulus-oocyte complexes and embryos

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ABSTRACT

In our study, we added natriuretic peptide type C (NPPC) and/or sildenafil during in vitro maturation (IVM) of bovine cumulus-oocyte complexes (COCs) followed by in vitro culture (IVC) of embryos with or without sildenafil. We evaluated the effects on the lipid content (LC) of oocytes and embryos and also verified the expression of 96 transcripts related to competence in matured COCs and 96 transcripts related to embryo quality in blastocysts. After IVM, LC was decreased in oocytes by NPPC while sildenafil did not affect LC in oocytes. The genes involved in lipid metabolism and lipid accumulation (*DGAT1*, *PLIN2*and *PLIN3*) were not affected in COCs after treatment during IVM, although the expression of *PTX3* (a cumulus cells expansion biomarker) was increased and the hatched blastocyst rate was increased by NPPC during IVM. During IVM, sildenafil increased the mRNA relative abundance of *HSF1* and *PAF1* and decreased *REST* in blastocysts. The use of sildenafil in IVC increased the LC of blastocysts. The mRNA abundance in blastocysts produced during IVC with sildenafil was changed for *ATF4*, *XBP1*, *DNMT3A, DNMT3B*, *COX2*, and *SOX2*. Although NPPC reduced the LC of oocytes after IVM and upregulated markers for cumulus expansion, embryo production was not affected and the produced blastocysts were able to regain their LC after IVC. Finally, the use of sildenafil during IVC increased the cytoplasmic LC of embryos but did not affect embryo quality, as measured by analysis of 96 transcripts related to embryo quality.

1. Introduction

The in vitro maturation (IVM) of mammalian oocytes is a crucial tool for investigating basic and applied aspects of assisted reproductive technology (ART), such as developmental biology, stem cell development, in vitro production (IVP) of embryos, cloning, and embryology [1]. However, the efficiency of IVM is still low when compared to in vivo maturation, limiting its application [2]. This inefficiency is probably caused by a drastic decrease in cyclic adenosine monophosphate (cAMP) concentration within the oocyte after the removal of cumulus-oocyte complexes (COC) from the follicular environment, causing the spontaneous resumption of meiosis during ART procedures [3,4].

Many pharmacological approaches are used in IVM to maintain meiotic arrest or delay spontaneous resumption of meiosis. One common approach consists of the use of cyclic nucleotide (cAMP and cGMP) modulators, such as dbcAMP [5] and 8-bromo-cAMP [6]; stimulators of adenylate cyclase, such as forskolin [7] and iAC [4]; and cGMP modulators, such as SNP [8,13] and SNAP [9], for modifying IVM and improving developmental competence.

Recent studies have indicated the influence of C-type natriuretic peptide (NPPC) on meiosis resumption in various species: mice [10], pig [11], cattle [12], and goat [13]. NPPC is synthetized by granulosa cells and binds to natriuretic peptide receptor 2 (NPR2) mostly present in cumulus cells. NPR2 activation induces the synthesis of cyclic guanosine monophosphate (cGMP), which is transferred via gap junctions from cumulus cells to the oocyte. There, cGMP has inhibitory action over phosphodiesterase 3A (PDE3A), maintaining high concentrations of cAMP in the oocyte and sustaining meiosis arrest [10].

Cyclic GMP synthesis is stimulated via different pathways, including through nitric oxide, natriuretic peptides (NPPA, NPPB, and NPPC),

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guanylins, uroguanylins, and guanylyl cyclase activating proteins (GCAPs), which catalyze conversion of guanosine triphosphate (GTP) into cGMP and pyrophosphate [14]. The concentration of cGMP is controlled by the balance between its synthesis and degradation, which is carried out by the PDEs themselves [15]. Sildenafil is a pharmacologic inhibitor of PDE5, which is the enzyme responsible for the hydrolysis of cGMP levels, decreasing its levels, and affecting the balance between cGMP and cAMP, which are involved in spontaneous meiosis resumption [16]. Additionally, cGMP activates cGMP-dependent protein kinase (PKG) [15]. Members of the PKG family (PKG I and II) act on specific substrates in the cells, leading to a series of metabolic effects. The signaling pathway activated by cGMP/PKG may trigger lipolytic action through phosphorylation of perilipin and lipases [17,18].

Lipids have been intensively studied in many mammalian cells. The most abundant cytoplasmic lipids in bovine oocytes and embryos are triacylglycerides (TG) [19], which are stored in lipid droplets. Intracellular lipids represent an important source of energy for oocytes and preimplantation embryos [19]. Recently, Schwarz and collaborators [20] described the influences of cGMP levels on lipid content in oocytes and embryos and on transcripts of energy metabolism. However, few studies have been conducted evaluating the effects of cGMP modulators on lipid metabolism and the few studies conducted are inconclusive [20,21]. Thus, several approaches focus on the reduction of lipid accumulation in IVP embryos by modifications of the culture media aimed at improvement in embryonic performance and quality [22–24], including enhancing their cryosurvival [22,24,25].

In this way, the manipulation of cGMP levels with NPPC (synthesized by its binding to NPR2) and sildenafil (inhibitor of the cGMP hydrolyzing enzyme PDE5A) may affect lipid metabolism in bovine COCs and embryos. The aim of this study was to determine the influence of NPPC and/or sildenafil, during IVM of bovine COCs, on lipid content, expression of markers of oocyte quality, and competence for embryonic development in cumulus cells, as well as genes involved in metabolism and the maturation process. In addition, the effects of these IVM treatments, which are used for the IVP of embryos, were assessed by the addition of sildenafil during embryo culture and measuring embryo production, lipid content, and the expression of embryo quality-related genes in bovine blastocysts.

2. Material and methods

2.1. Media and chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (USA) unless otherwise indicated.

2.2. Oocyte collection

Ovaries from *Bos Indicus* cows were collected at a commercial abattoir immediately after slaughter and transported in sterile saline solution at 30 °C. In the laboratory, 3 to 8 mm follicles were aspirated with an 18 "G" needle attached to a disposable 10 mL syringe. The aspirated follicular fluid was placed in 15 mL conical tubes and maintained for 5 min for sedimentation. The upper portion of the liquid was removed and the remaining portion was then transferred to a Petri dish (100 × 15 mm) under a stereomicroscope for selection of grade I and II cumulus-oocyte complexes. COCs were classified as grade I when presenting five or more layers of cumulus cells and homogeneous cytoplasm and grade II with 2–4 layers and homogeneous cytoplasm [26].

2.3. Experimental design

2.3.1. Experiment 1: the effects of NPPC and/or sildenafil on lipid content and gene expression in COCs after IVM

The COCs were divided into four groups during IVM: control (without supplementation), NPPC (supplemented with 100 nM NPPC, [12]), sildenafil (supplemented with 10 μM sildenafil, [27]), and NPPC plus sildenafil (supplemented with 100 nM NPPC and 10 μM sildenafil). After 24 h of IVM, 298 denuded oocytes were evaluated for the determination of lipid content using the fluorescent probe Nile Red and triplicate pools, each with 10 COCs, were evaluated by Real-time PCR (qPCR) using a Fluidigm Biomark™ HD system for relative gene expression of 96 targets related to oocyte competence and pathways involved in maturation processes.

2.3.2. Experiment 2: the effects of NPPC and/or sildenafil during IVM on lipid content and gene expression of 96 targets related to embryo quality in blastocysts

After IVM, COCs were in vitro fertilized and the embryos were in vitro cultured. The rates of embryo production were assessed on day 7 (D7). The produced 169 blastocysts or expanded blastocysts (D7, Class I and II) were analyzed for lipid content with the fluorescent probe Nile Red and quadruplicate pools with 5 blastocysts each (D7, Class I and II) were evaluated by Real-time PCR (qPCR) using a Fluidigm Biomark™ HD system regarding relative gene expression of 96 targets related to embryo quality.

2.3.3. Experiment 3: the influence of sildenafil during IVC on lipid content and gene expression of 96 targets related to embryo quality in blastocysts produced from COCs maturated with NPPC and/or sildenafil during IVM

After IVF, the embryos were cultured with 10 μM sildenafil and rates of embryo production were assessed at D5 and D7. The 210 blastocysts or expanded blastocysts produced (D7, Class I and II) were analyzed for lipid content and quadruplicate pools with 5 blastocysts each (D7, Class I and II) were analyzed by Real-time PCR (qPCR) using a Fluidigm Biomark™ HD system for relative gene expression of 96 targets related to embryo quality.

2.4. In vitro maturation

For in vitro maturation (IVM), the selected COCs were cultured in maturation medium (TCM199 with 20 mM bicarbonate) containing 0.2 mM sodium pyruvate, 0.1 IU/mL recombinant human FSH (Merck Serono; Bali, Italy), 5 mg/mL bovine serum albumin-fatty acid free and 10 μg/mL gentamicin. Groups of 50 COCs were matured in 500 μL of maturation medium under silicone oil (Quimesp Química, Guarulhos, SP, BRA), in four-well dishes (NUNC, Thermo Fisher Scientific, Rochester, NY, USA), and incubated at 38.5 °C and 5% CO2 in air and maximum humidity for 24 h.

2.5. In vitro embryo production

For embryo production in vitro, the matured oocytes were transferred to drops (25 oocytes per drop) containing 100 μL fertilization medium and covered with mineral oil in Petri dish (60 × 15 mm). Oocytes were subjected to in vitro fertilization (IVF) using frozen semen from the same bull of proven fertility. Spermatozoa were selected using the Percoll method, and the concentration was adjusted to 1×10^6 sperm cells/mL [27]. Fertilization was performed in droplets of Tyrode's Albumin-Lactate-Pyruvate (TALP) medium [28] under mineral oil, supplemented with 5 mg/mL BSA, 0.2 mM/mL pyruvate, 20 μg/mL heparin, 18 μM/mL penicillamine, 10 μM/mL hypotaurine, 1.8 μM/mL epinephrine, 100 μg/mL streptomycin sulfate and 100 IU/mL penicillin (Gibco). Oocytes and spermatozoa were co-incubated at 38.5 °C and 5% CO2 in air and maximum humidity for 18-18:30 h. The day of fertilization was defined as Day 0 (D0). Presumptive zygotes were denuded by vortexing for 2 min in slow speed, afterwards the presumptive zygotes were transferred to Petri dishes and placed in 90 μL droplets of culture medium (10–15 presumptive zygotes per drop), covered with mineral oil. The dishes were then placed in plastic bags containing a gas mixture of 5% O2, 5% CO2, and 90% N2 (White Martins, São Paulo, SP, Brazil) and incubated at 38.5 °C and maximum humidity. The culture