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Non-invasive metabolomics for improved determination of embryonic sex markers in chemically defined culture medium

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ABSTRACT

Metabolic differences between early male and female embryos can be reflected in culture medium (CM). We used a single bovine embryo culture step (24 h) supporting improved birth rates under chemically defined conditions (CDC) to investigate biomarker detection of embryonic sex in contrast to classical BSA-containing medium. In vitro matured slaughterhouse oocytes were fertilized in vitro with a single bull. Embryos were initially cultured in synthetic oviduct fluid with BSA. On day-6, morulae were cultured individually in droplets with (BSA) or without protein (CDC). On day-7, expanded blastocysts were sexed (amelogenin gene amplification) and CM was stored at −145 °C until metabolomic analysis by UHPLC-TOF MS. N = 10 embryos per group (i.e. male-protein; female-protein; male-non-protein; female-non-protein) were produced. Statistical analysis revealed N = 6 metabolites with different concentrations in CM, N = 5 in male embryos (methionine, tryptophan, *N*-stearoyl-valine, biotin and pipecolic acid), N = 1 in female embryos (threonine) ($P < 0.05$ in BSA; $P < 10^{-7}$ in CDC). Only the clear threshold between males and females in CDC allowed correct classification of 100% males and 91% females within 5 out of 6 biomarkers (one female outlier showing the male biomarker profile). The use of CDC represents a critical aspect in the efficient detection of embryonic sex biomarkers by metabolomics.

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1. Introduction

Male and female bovine embryos show contrasting differences not only in their chromosomal complement but also in their transcriptional activity [1–4] and epigenetic status [2,5]. Sexual dimorphism in the embryonic transcription affects carbohydrate and amino acid metabolism [6–9], with female embryos showing slower mitochondrial metabolism [10].

The metabolic differences between male and female embryos led to developing procedures for embryo sexing and prediction of pregnancy viability based on the direct access to culture medium (CM) as a substrate for non-invasive analysis [11]. Since products of metabolism are secreted into the culture medium as part of the secretome, differences in the composition of embryo culture medium (CM) may reflect sex and viability. In humans, female embryos incorporate more glucose than males from CM [12]. In bovine, using Fourier transform infrared spectroscopy (FTIR) analysis of CM, we determined embryonic sex [13], and also the pregnancy viability [14,15] of specific cohorts of blastocysts. Other studies in cattle CM showed that amino acid turnover is sex-specific [7], and such differences are supported by a high number of genes differentially expressed between male and female blastocysts, not only in the sex chromosomes, but also in all the somatic complement [2]. Collectively, from our studies and others, it is inferred that metabolites in the CM reflect the embryonic sex.

However, there are limitations for biochemistry and biomarker studies in cattle IVF: 1) the absence of reliable single embryo culture systems that provide suitable pregnancy and birth rates; and 2) the presence of undefined compounds in CM that negatively affect analytical performance and repeatability. 1) In bovine, attempts to create defined CM often reduced embryo development rates and pregnancy viability, with few authors reporting improvements of blastocyst development in long-term single culture compared with group culture [16]. However, a single culture step showed improved performance, as revealed by twofold hatching rates and pregnancy rates similar to those of Day-6 embryos collected in vivo from superovulated cows that were also cultured singly for 24 h [13,14]. In addition, our recent studies showed that protein (i.e.

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BSA) removal from the 24 h individual culture and replacement with polyvinyl-alcohol (PVA) led to chemically defined conditions (CDC) and embryos with improved survival after vitrification and transfer, and significant reduction in miscarriage leading to higher birth rates [17,18]. Protein removal did not affect birth weight, gestation length or morphometry of calves compared with embryos cultured with protein. This one-day individual culture system is highly efficient and potentially compatible with other previous culture steps. 2) There is a higher likelihood that interactions could occur with abundant, non-defined compounds from BSA or serum present in CM. Importantly, CDC samples can be directly analyzed upon dilution, without metabolite extraction after protein precipitation, contrary to BSA-containing samples. In culture, BSA binds fatty acids, cytokines, growth factors (GFs), metal ions, steroids, amino acids and a variety of different compounds [19]. Therefore, the albumin fraction itself can be a source of biomarkers; for this reason, it was termed the albuminome [20]. Accordingly, depletion of BSA in our work may lead to heterogeneity and alteration of concentrations in biomarkers.

The evidence of a different metabolomic fingerprinting in CM between male and female embryos individually cultured with protein strongly supports the search for metabolite biomarkers of the embryonic sex. Due to the chemically defined nature of the 24 h single culture step with PVA in contrast with BSA, we hypothesize more efficient and reliable detection of biomarkers in CDC (i.e. PVA).

In this study, with a focus on the non-invasive analysis of the secretome in the CM from bovine embryos, a non-targeted metabolomic approach has been developed to determine embryonic sex. Ultra-high performance liquid chromatography (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometer (Q/TOF MS) was used to accomplish non-targeted overview of the metabolome. Moreover, in the search to simplify sample preparation for metabolomic analysis, the effect of replacing bovine serum albumin (BSA) in a defined culture medium with PVA on sex determination has also been addressed. A panel of metabolites from spent CM samples has been unraveled to determine embryo sex outcome by means of metabolomics using a multivariate statistical model.

2. Materials and methods

2.1. Animals

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 that were slaughtered in commercial abattoirs (Matadero de León, Spain; Matadero de Guarnizo, Cantabria Spain) and transported to the laboratory in NaCl solution (9 mg/mL) with antibiotics at 25–30° during 2 h.

2.2. Chemicals

Formic acid was obtained from Riedel-de Haën (Seelze, Germany). Acetonitrile and methanol were of MS grade and purchased from Labscan (Gliwice, Poland). Other commercial standards and reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.3. In vitro embryo production

Ovarian follicles (3–8 mm in diameter) were aspirated, and oocytes enclosed in a compact cumulus (≥3 cell layers) with evenly granulated cytoplasm were selected for in vitro maturation (IVM) as described [21]. The COCs were washed in TCM-199-HEPES and matured in bicarbonate-buffered TCM 199 with FSH, LH, 17β-estradiol and 10% fetal calf serum. Approximately 50 COCs were cultured in 500 μL maturation medium in four-well dishes at 39 °C in 5% CO2 in air with high humidity for 22–24 h. After IVM, oocytes were subjected to in vitro fertilization (IVF; Day 0) with frozen/thawed spermatozoa from an Asturiana de los Valles bull following described procedures [22]. Spermatozoa separation was performed using the swim-up technique. Semen was layered down in a tube containing pre-equilibrated Sperm-TALP, and the upper layer with motile spermatozoa was collected after 1 h incubation.

2.4. Embryo culture

All CM were formulated from synthetic oviductal fluid (SOF), as shown in Supplementary Table S1, supplemented with BSA (6 g/L; SOF-BSA) or PVA (0.5 mg/mL; SOF-PVA). Day-6 morulae were collected, washed twice in the corresponding CM, and cultured individually in droplets of 12 μL under mineral oil. After 24 h of in vitro culture at 38.7 °C, 5%CO_2 and 5%O_2 in air, expanded blastocyst (XB) stage embryos were selected and processed for sexing as described in [22]. Zona pellucida was digested prior to embryo loading in PBS with a minimal CM volume amount, snap frozen in LN_2, and storage at −80 °C until PCR sexing. Embryos were collected from 3 replicates, leading to 4 groups, with N = 10 male embryos in SOF-PVA, N = 10 female embryos in SOF-PVA, N = 10 male embryos in SOF-BSA and N = 10 female embryos in SOF-BSA. Blank samples (i.e. CM incubated with no embryos being cultured in them) were also collected from SOF-BSA and SOF-PVA (N = 6 each). Supplementary Table S2 shows a summary of all samples included in this study.

2.5. Embryo sexing by PCR

The embryonic sex was determined in XB, produced with and without protein, by a built-in control PCR technique based on amelogenin amplification [22]. This method is particularly appropriate when a fully accurate embryonic sex diagnosis is necessary.

2.6. Untargeted metabolomic analysis

2.6.1. Samples

Spent CM and blank control samples were thawed on ice immediately prior to metabolic extraction. Metabolites were extracted from SOF-BSA by adding cold (−20 °C) methanol (1:5, sample-methanol, v/v). After vortex-mixing, the mixture was incubated at −20 °C for 12 h and centrifuged at 24000 × *g* for 15 min at 4 °C. Supernatant (45 μL) was collected and dried under vacuum. The vacuum-dried samples were dissolved in 22.5 μL of water and directly analyzed. SOF-PVA samples were diluted 1:3 (v/v) in water, and directly analyzed. Quality control (QC) was performed using a mixture of standards: 0.05 μM methylthioadenosine, 0.5 μM adenine, 150 μM methionine, 75 μM arginine and 7.5 μM tyramine in water.

2.6.2. UHPLC-TOF MS conditions

Analyses were performed using an UHPLC system 1290 from Agilent (Agilent Technologies, Santa Clara, CA, USA) connected to a Q/TOF Agilent 6540 equipped with an orthogonal ESI interface (Agilent Jet Stream, AJS) and operating in positive ion mode. The instrument was controlled by a PC running the Mass Hunter Workstation software 4.0 from Agilent. Reverse-phase (RP) chromatographic separation was performed using a Waters Acquity UPLC HSST3 column (2.1 × 100 mm, 1.8 μm); phase A consisted of water with 0.1% formic acid, v/v, and phase B was made up of acetonitrile with 0.1% formic acid, v/v. Each sample was analyzed in duplicate. QC was injected every 5 runs in order to gain reliable data and to assure a correct instrument intra- and inter-day variability, enabling the monitoring of performance, stability and

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