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Proteomic analysis of preovulatory follicular fluid reveals differentially abundant proteins in less fertile dairy cows

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ABSTRACT

The follicular fluid (FF) proteome can provide an indication of follicular quality. High-yielding dairy cows suffer from low fertility, which could be related to follicular function. However, the proteome of preovulatory follicles has never been described in cows. Our objectives were to: 1) define the bovine preovulatory FF proteome, and 2) examine differentially abundant proteins in FF of controls (CTL, $n = 10$) and less fertile cows (LFC; failed to conceive following ≥6 inseminations, $n = 8$). Follicles ≥7 mm in diameter were aspirated in vivo, and estradiol (E_2) and progesterone (P_4) were examined. The FF from 10 preovulatory follicles ($E_2/P_4 > 1$) was analyzed; E_2 was higher and follicle diameter tended to be larger in LFC. As aspirations were conducted at a fixed time, this suggests accelerated follicular growth in LFC. The 219 identified and quantified proteins consisted mainly of binding proteins, proteases, receptor ligands, enzymes and transporters. Differential abundance of 8 relevant proteins was found in LFC compared to CTL: SERPINA1, TIMP2, ITIH1, HSPG2, C8A, COL1A2, F2, and IL1RAP. These proteins could influence follicular function—e.g., decreased SERPINA1 may be related to accelerated follicular growth—and therefore, further examination of their roles in the etiology of LFC is warranted.

Significance: High yielding dairy cows suffer from infertility that leads to major economic losses worldwide. In Israel, about 30% of dairy cows fail to conceive following ≥4 inseminations. The etiology of this low fertility is multifactorial and remains a serious challenge. Follicular fluid proteome can provide indication to follicular quality, yet the proteome of pre-ovulatory follicles has not been described in cows. This work examined the differential abundance of proteins in less fertile dairy cows compared to controls, and found 8 relevant novel proteins that could influence follicular function. The role of these proteins in the etiology of less fertile cows should be further examined.

1. Introduction

Follicular fluid (FF), secreted by granulosa cells and diffusing from the thecal capillaries, plays an essential role in the physiology of follicular growth, oocyte maturation and ovulation [1]. Follicular fluid contains a variety of soluble regulatory factors, such as estradiol (E_2), progesterone (P_4), insulin-like growth factor-1, tumor necrosis factor α, interleukin-6 (IL-6) and stem cell factor, which directly or indirectly affect the fertility competence of oocytes and their developmental potential [2,3]. A number of studies have shown that FF inhibits the binding of spermatozoa to the zona pellucida [4] and promotes hyperactivation and acrosome reaction of sperm [5,6], oocyte maturation and embryo development [7,8]. Therefore, the proteomic composition of FF from preovulatory follicles can be used as an indicator of secretory activities and metabolism of follicular cells, which regulate follicle quality [9].

In modern high-yielding dairy cows, infertility leads to major economic losses due to reproductive wastage, culling, replacement costs, and loss of genetic gain [10]. In Israel, about 30% of dairy cows fail to conceive following ≥4 artificial inseminations (AI). These cows are defined as less fertile cows (LFC; also termed "repeat breeder" cows), a situation comparable to secondary infertility in women. On an individual cow basis, an optimal strategy for treatment of LFC often remains elusive because of its multifactorial etiology, which involves cows, semen quality and inseminator or insemination technique [11]. Recently, we examined the behavioral and hormonal patterns of LFC; unexpectedly, these cows exhibited better estrus expression, similar follicular diameters, and higher plasma E_2 concentrations than control cows, suggesting that the etiology of their lower fertility lies beyond these parameters and requires further investigation [12].

Proteomic analysis is an emerging discipline that involves the global analysis of changes in protein expression [13]. There is a common consensus that the information obtained from the protein component of the cell or tissue complements genomic data, and that alterations in protein abundance depict biological processes, as proteins are vital

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elements in cell-function control [14]. In human reproductive research, the FF proteome of women with fertility problems (e.g. polycystic ovary syndrome, PCOS) has been analyzed for biomarkers and to investigate the etiology of low fertility [9,14–17]. The only previous proteomic analysis of bovine FF detected merely 40 proteins in follicular cysts [18] obtained from the slaughterhouse, leaving much to be discovered regarding the bovine FF proteome, specifically with respect to preovulatory follicles in less fertile dairy cows. In addition, the effects of specific proteins in the FF on follicular characteristics and function remain unknown. Therefore, the objectives of the present study were to: 1) define the FF proteome in preovulatory follicles of high-yielding dairy cows, and 2) examine differentially expressed proteins in FF obtained in-vivo from preovulatory follicles of LFC and control (CTL) cows, to reveal proteins that might shed light on the elusive etiology of reproductive failure in high-yielding dairy cows.

2. Materials and methods

2.1. Animals

The procedures used in this study were approved by the Volcani Center Animal Care Committee. Eighteen Israeli Holstein lactating cows (>60 d in lactation) at the Volcani Center Experimental Farm (Bet Dagan, Israel) were used in the present study, conducted during the winter to avoid effects of heat stress. All of the selected cows were normal cyclic, had normal estrus duration, and had no history of dystocia, retained placenta or metritis before starting the study. The cows were grouped into two categories: CTL ($n = 10$) and LFC ($n = 8$). The CTL cows were >60 d in lactation, cycling and not inseminated. A cow was considered a LFC if it did not exhibit any clinically detectable abnormality but did not become pregnant after at least 6 successive AI during spontaneous estrus, with normal intervals between inseminations. The average number of AI in the LFC was 7.1 ± 0.8 (range: 6 to 9). The reproductive health of the genital tracts of all participating cows was reaffirmed by two successive examinations separated by a 6- to 7-d interval by means of transrectal ultrasound examination with an Aquila 5-MHz linear array transducer (Pie Medical, Maastricht, Netherlands).

The average values (mean ± SD) in the CTL and LFC groups, respectively, were: milk, 47.4 ± 10.1 and 30.8 ± 8.0 kg/d; age, 4.7 ± 2.3 and 5.2 ± 1.8 years; lactation number, 3.0 ± 1.7 and 2.8 ± 1.6; body weight, 607 ± 73 and 734 ± 91 kg; days in lactation, 107.2 ± 27.1 and 364.0 ± 114.8 d. The differences in these parameters between groups were expected, as a result of the chosen model. The cows were fed according to National Research Council (2001) recommendations. They were housed in the same covered loose pen with an adjacent outside yard and were milked three times a day.

2.2. Estrus synchronization

Following selection, the cows' estrous cycle was synchronized using Gonadotropin-releasing hormone analogue (200 μg gonadorelin acetate; Gonabreed, Parnell Living Science, Alexandria NSW, Australia). The cows' ovaries were monitored 9 d later by transrectal ultrasound for presence of corpus luteum (CL), and only cows with a CL were injected with prostaglandin (PG, 500 μg of cloprostenol sodium; Estroplan, Parnell Living Science). From 36 h after the PG injection, the cows were visually monitored for mounting activity or other behavioral signs typical of estrus. Cows that exhibited behavioral estrus were included in the study.

2.3. Aspiration of large follicles

Out of 18 cows, 13 (7 CTL and 6 LFC) successfully responded to the estrus synchronization, exhibited behavioral estrus and were subjected to follicle aspiration. The ovaries were monitored for CL presence 14 to 15 d after behavioral estrus by ultrasound, and cows with CL on the ovaries received another PG injection; 48 h later, the FF from follicles >7 mm in diameter was aspirated according to Moallem et al. [19]. Follicles were aspirated individually with the aid of an ultrasound scanner (Pie Medical) connected to a 7.5-MHz vaginal sector transducer equipped with a needle guide and connected to a suction pump (MP86; Biometra, Goettingen, Germany) set to a flow rate of 25 to 30 mL/min. The needles were 18-gauge and were changed between follicles. After collection, the FF was centrifuged for 15 min at 3000 × *g* and the fluids were frozen at −80 °C pending analysis.

2.4. Hormone analysis

Concentrations of E_2 in the FF were determined by radioimmunoassay (RIA) (DSL4800; Beckman Coulter Inc., Brea, CA, USA) as were concentrations of P_4 (Diagnostic Products, Los Angeles, CA, USA). The androstenedione (A_4) in the FF was also determined by RIA (Diagnostic Systems Laboratories, Webster, TX, USA). Before these determinations, the FF samples were diluted 500, 100 or 30 times, respectively, to fit the detection ranges; the minimum detectable amounts were 20, 0.2 and 0.1 ng/mL for E_2, P_4, and A_4, respectively. The intra- and interassay coefficients of variation for the E_2, P_4, and A_4 assays were 3.91 and 3.7%, 8.8 and 8.3%, and 5.9 and 4.3%, respectively. Follicles were regarded as preovulatory (E_2-active) when the E_2/P_4 ratio was >1 [20], and these follicles were then subjected to further analysis.

2.5. Sample preparation for proteomic analysis

Ten samples were used for the proteomic analysis, from 5 CTL cows and 5 LFC. Each FF sample was subjected to buffer exchange with 50 mM ammonium bicarbonate using a 3-kDa molecular weight cutoff filter (Amicon, Millipore, USA). Protein concentration from each sample was determined by its reading at 280 nm in a NanoDrop spectrophotometer (Thermo Scientific, USA) and comparison to a calibration curve using bovine serum albumin in 50 mM ammonium bicarbonate. Each sample contained 100 μg total protein. Proteins were denatured using 8 M urea and incubated for 10 min at room temperature followed by addition of 5 mM dithiothreitol and incubation for 1 h at room temperature. Urea was diluted five times and proteins were alkylated with 10 mM iodoacetamide (Sigma, USA) in the dark for 30 min at 21 °C. Proteins were then subjected to digestion with trypsin (Promega, Madison, WI, USA) at a 1:50 trypsin-to-protein ratio for 16 h at 37 °C [21]. The digestions were stopped by trifluroacetic acid (1% v/v) Samples were desalted using solid-phase extraction columns (Oasis HLB, Waters, Milford, MA, USA) and stored at −80 °C until further analysis.

2.6. Liquid chromatography

Ultra performance liquid chromatography (ULC) grade solvents were used for all chromatographic steps. Protein (2 μg) from each sample was loaded using split-less nano-ULC (10 kpsi nanoAcquity, Waters). The mobile phase consisted of: (A) H_2O + 0.1% formic acid and (B) acetonitrile +0.1% formic acid. Desalting of the samples was performed online using a reversed-phase C18 trapping column (180 μm internal diameter, 20 mm length, 5 μm particle size; Waters). The peptides were then separated using a an HSS T3 nano-column (75 μm internal diameter, 250 mm length, 1.8 μm particle size; Waters) at 0.35 μL/min. Peptides were eluted from the column using the following gradient: 4% to 35% B in 150 min, 35% to 90% B in 5 min, maintained at 90% for 5 min and then back to initial conditions.

2.7. MS

The nano-ULC was coupled online through a nano-ESI emitter (10 μm tip; New Objective, Woburn, MA, USA) to a quadrupole orbitrap MS (Q Exactive Plus, Thermo Scientific, USA) using a Flex Ion nanospray apparatus (Proxeon). Data were acquired in data dependent