Proteome of follicular fluid from Murrah buffaloes (*Bubalus bubalis*): normal cycling versus repeat-breeder females

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ABSTRACT

Clinical evaluations have shown that repeat breeding is a major cause of infertility in buffaloes. The follicular fluid (FF) composition reflects directly metabolic status and fertility in females. Given this scenario, this study aimed to perform quantitative proteomic analysis of the FF collected from normal cycling (NC) and compare to repeat-breeder females. According to farm records, buffaloes were divided into two groups: normal cycling (NC, $n = 7$) and repeat-breeder (RB, $n = 8$) females. After estrus synchronization and using ultrasound-guided ovum pick-up, FF was aspirated from large follicles (> 8 mm). Posteriorly, proteins were identified by the shotgun method. A total of 119 proteins were identified and, among theses, three were uncharacterized and a protein (LOC123334375) was identified only in the NC group. The protein HP-25 homolog 2 was expressed only in RB females. The LFQ (label-free quantitation)-intensity of the proteins afamin (AFM), transthyretin (TTR), clotting factor IX (F9) and Xaa-Pro dipeptidase (PEPD) was significantly ($P < 0.05$) higher in RB than in NC females. In conclusion, the use of quantitative proteomics proved to be an important tool for the study of RB in buffaloes. The identification of HP-25 homolog 2 protein only in the RB females suggests that it can be used as a biomarker for this reproductive disorder.

Keywords: Buffalo; Fertility; LC-MS/MS; Quantitative proteomic; Shotgun

1. Introduction

Interest in buffalo breeding has been growing in recent years. It is related to its characteristics such as milk and meat quality and more resistance to diseases than bovine (Minervino et al., 2020). Buffaloes are short-day seasonal polyestrous (Perera, 2011), reach late puberty, when compared to cattle, have poor estrus expression and

prolonged postpartum ovarian quiescence (Ponraj et al., 2017). Clinical evaluations have shown that anestrus and repeat breeding are the two major causes of poor reproductive efficiency and infertility in buffaloes (Perera, 2008; Saraswat and Purohit, 2016). Additionally, the Murrah breed has the highest incidence of repeat breeding in this species (Kaur et al., 2023).

A repeat-breeder is generally defined as any cow that has not conceived after three or more services associated with true estrus (Maurer and Echternkamp, 1985). Several studies have shown the proteomic approach to seek a better understanding of the molecular bases of the female reproductive physiology in different species, such as goats (Paula Junior et al., 2018), horses (Maloney et al., 2019) and cattle (Aranciaga et al., 2020). Concerning the follicular fluid (FF), studies have described the proteome in different developmental and reproductive stages (Fu et al., 2016; Itze-Mayrhofer and Brem, 2020) or associated with reproductive pathologies (Balestrieri et al., 2013).

The FF is formed from secretions of granulosa and theca cells, oocytes and trans-exudate molecules of the blood (Rodgers and Irving-Rodgers, 2010). Studies revealed that the FF is formed by substances directly involved in follicular growth and oocyte developmental competence (Walter et al., 2020). In women, FF proteins have been implicated in oocyte meiosis, ovulation, formation of corpus luteum and fertilization (Schweigert et al., 2006). Thus, FF proteins can reflect the physiological condition of the follicle and may serve as biomarkers for the reproductive health of herds (Paula Junior et al., 2018). In this context, determining the protein profile of the FF will help to establish biomarkers of oocyte quality and/or reproductive efficiency, especially in some reproductive problems. Thus, this study aimed to analyze and compare the proteome of the FF collected from normal cycling and repeat-breeder buffaloes.

2. Materials and methods

2.1. Bioethics and chemicals

This study was approved by the Ethics Committee of the State University of Ceará (# 07335339/2019). All chemical reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise described.

2.2. Location and experimental animals

The experiment was carried out at "Laguna" farm, Paracuru, Brazil (3° 25' 31" S, 39° 1' 29" W). The weather of the region is described as hot temperature (daily average is 33°C) with high humidity. Murrah buffaloes were subjected to semi-intensive management with access to pasture (*Megathyrsus maximus*, *Pennisetum purpureum* and *Saccharum barberi*) and fed concentrate with the mineral mixture, and *ad libitum* water. According to the farm´s record, the females were divided into two groups: normal cycling (NC, n=7) and repeat-breeder (RB, n=8). RB were those who failed to conceive after three or more successive services, but with normal estrus and absence of detectable clinical abnormalities (Purohit, 2008). The age (mean ± SD) was 6.02 ± 2.4 and 7.11 ± 4.3 years for NC and RB group, respectively. According to methodology described by Alapati et al. (2010), females presented an average body score of 3.34 (RB) and 3.57 (NC) and no history of dystocia, retained placenta, endometritis or metritis.

2.3. Experimental design

A schematic workflow of the experiment is presented in Fig. 1. The females received a hormonal treatment for estrus synchronization, followed by FF aspiration, and the samples were stored in an ultra-freezer until further processing (Fig. 1A). The samples were prepared for proteomics, electrophoresis SDS-PAGE, tryptic in-gel digestion, and

mass spectrometry for protein identification (Fig. 1B). Finally, the data were analyzed for functional annotation and network prediction (Fig. 1C).

2.4. Estrus synchronization

On the first day of treatment (D0), females received a CIDR (Zoetis, Argentina) simultaneously with i.m. injections of 150 mg progesterone (Sincrogest®, Ourofino, Brazil) and 1 mg estradiol benzoate (Sincrodiol®, Ourofino). On D9, the CIDR was removed, and females received an injection with 530 μg cloprostenol (Ciosin®, Intervet, Brazil) and 200 IU eCG (Novormon®, Zoetis, Argentina).

2.5. Aspiration and storage of FF

On D11, large follicles (greater than 8 mm in diameter) were observed using an ultrasound (DP-10 VetPower, Mindray Bio-Medical Electronics Co. Ltd, Shenzhen, China) equipped with a micro-convex 5 MHz transducer. The transducer was coupled to an aspiration needle-guided line system (AGS), connected to a vacuum pump (WTA, Brazil). During this procedure, all females were restrained in a chute and given epidural anesthesia of 3-5 mL 2% lidocaine hydrochloride (Anestex Fraga®, Vetoquinol, Brazil) between the last sacrum and first coccygeal vertebra. The target follicles were positioned into the trajectory course of the aspirating needle and FF was aspirated under a negative vacuum pressure of 50 mmHg. The AGS was rinsed after aspiration of each follicle in the same animal. A new aspiration needle and AGS were used for each female and each group, respectively. The FF from each female was treated with a protease inhibitor cocktail (1:100 v/v) and centrifuged at 800 × *g* for 15 min to eliminate cells and debris. The supernatant was then transferred to a new tube, centrifuged again

at 10,000 × *g* for 30 min, transferred to other tube and stored at -80°C until further analysis.

2.6. Electrophoresis and digestion of follicle fluid proteins

Samples of FF were thawed at room temperature and soluble proteins were quantified according to Bradford's method (Bradford, 1976). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 12% polyacrylamide was performed as previously described by Shevchenko et al. (2006). Briefly, a 20 µg of sample in a volume of 10 µL was used for each lane in gel electrophoresis. Electrophoresis was run at 30 mA/gel and 300 V and stopped when the samples reached the separation gel. Afterwards, each lane of the gel was excised from the gel and subjected to in-gel trypsin digestion. First, proteins were reduced using 10 mM dithiothreitol for 30 min at 56 °C followed by alkylation with 40 mM iodoacetamide at room temperature for 20 min in the dark. Proteins were digested with trypsin (12.5 ng/µL trypsin in 10 mM ammonium bicarbonate containing 10% acetonitrile) for 120 min followed by the addition of 10-20 µL of ammonium bicarbonate buffer and incubated overnight in an oven at 37 ºC. Afterwards, 5% formic acid was added to stop the digestion followed by vacuum drying of the purified sample and stored at -20ºC until further process.

2.7. Mass spectrometry analysis

Tryptic peptides were separated by Ultimate 3000 chromatograph (Thermo Scientific, Waltham, MA, USA) and analyzed in the LTQ Orbitrap XL ETD (Thermo Scientific). The runs were performed in triplicates for each sample and the gradient was run at 250 nL/min with a linear gradient from 5 to 40% in 120 min. An analytical column of 15 cm

with a 75 mm internal diameter containing C18 particles of 3 mm in diameter was used for separation. The LTQ Orbitrap XL ETD hybrid mass spectrometer (Thermo Scientific) was used for mass spectrometry. MS1 was acquired in the Orbitrap Analyzer with a resolution of 60,000.00 m/z window of 300.0 to 2000.0. MS2 was performed in an ion trap analyzer for the top 10 most intense peaks. Dynamic exclusion was enabled for the exclusion duration of 90.00 s and mass option for 401.922718 m/z. The Nanospray Ionization (NSI) voltage was 2.70 KV, current 100.00 mA, capillary temp 175.00°C, Ion Trap Full AGC Target 30000.00 and FTMS Full AGC Target 1000000.00. The MaxQuant software (www.maxquant.org.) was used for mass spectra analysis.

2.8. Data analysis

The buffalo FF protein codes were changed to bovine and human using the Uniport database system for gene ontology, searched manually from each identified protein. Further, these codes were processed using PANTHER 17.0 software (http://www.pantherdb.org/geneListAnalysis.do) and STRING (https://string-db.org) for analysis of pathways, molecular and functional classifications. The confidence score was > 0.7 for the protein-protein interaction network. At least one protein present in three animals out of five was considered for statistical analysis. The LFQ (label-free quantitation) intensity mean of proteins was compared by t-test using GraphPad software (https://www.graphpad.com/quickcalcs/). Differences were considered significant if $P < 0.05$.

3. Results

In this study, FF aspiration was performed only in females that showed signs of estrus after hormonal treatment. Thus, the results presented are those of ten females (five from each experimental group). No significant differences ($P > 0.05$) were observed between experimental groups concerning the diameter and volume of aspirated follicles (Table 1) for further proteomic analysis.

3.1 Protein profile of FF

There were 119 proteins identified in the samples of FF, with three proteins remaining uncharacterized (Supplementary Table 1). One uncharacterized protein (LOC123334375) and the protein HP-25 homolog 2 were identified only in NC and RB females, respectively. LFQ-intensity of afamin, transthyretin, coagulation factor IX and Xaa-Pro dipeptidase was significantly higher ($P < 0.05$) in RB than NC group (Fig. 2).

3.2 Analysis of gene ontology

Due to the small number of proteins identified differentially in one of the experimental groups, gene ontology analysis was performed with samples from both groups. Thus, most cellular components belonged to the cell anatomical entity (93.9%) (Fig. 3A) and the most dominant molecular functions were related to binding (41%), catalytic activity (33%) and molecular function regulator (16%) (Fig. 3B). Major biological processes were related to cellular (22%), metabolic (17%), regulations (15%) and response to stimulus (14%) (Fig. 3C). Most of the pathways were related to blood coagulation (24.4%), gonadotropin-releasing hormone receptor (15.6%) and integrin signaling (8.9%) (Fig. 3D).

3.*3 Analysis of protein-protein network*

A protein-protein interaction network was constructed by retrieving the STRING database. The interaction network (IN) of HP-25 homolog-2 is shown in Fig. 4A. In addition, the IN of transthyretin, afamin, Xaa-Pro dipeptidase and coagulation factor IX with each other as well as with others is shown in Fig. 4 B-E

4. Discussion

The FF is the microenvironment for nourishment and development of oocytes and allows molecular communication between the oocytes and granulosa cells (Dumesic et al., 2015). Also, proteomics of follicles at advanced stages of development reflects the secretory or metabolic activities of follicular cells (Da Broi et al., 2018) and can be used as a potential biomarker of oocyte competence in buffaloes (Kumar et al., 2020). In this study, we followed a shotgun approach to screen the proteome of FF from buffaloes with contrasting fertility status.

In our study, HP-25 homolog 2 was found only in the FF collected from NC buffaloes. This protein is an intracellular component that interacts with one or more other proteins, serving as a scaffold/adaptor protein. Scaffold proteins give a platform for assembling the positive or negative signal-transducing molecules for the different pathway regulations (Langeberg and Scott, 2015). Also, it can increase or decrease the threshold ability to signal molecules (Levchenko et al., 2000). According to the string database, HP-25 homolog 2 is associated with inter-alpha-trypsin inhibitor heavy chain H2 (ITIH2) and it is an acute-phase protein downregulated during the inflammatory response (Gordon et al., 2014). Therefore, the hampered inflammatory process during preovulatory follicle development further influences the ovulation processes (Duffy et al., 2019). Thus, expression of the homologous HP-25 protein 2 in the FF may probably

compromise their ovulatory cascade and subsequent embryonic development in RB buffaloes.

Four proteins were differentially more expressed in the RB when compared to the NC group: afamin, Xaa-Pro dipeptidase, coagulation factor IX and transthyretin. Afamin is an acute-phase protein that directly affects apolipoproteinA1/A2, cubilin and alpha-2-HS-glycoprotein. ApolipoproteinA1/A2 are expressed in the FF of estrus and anestrus buffaloes, respectively (Kumar et al., 2021). Cubilin plays an essential role in the normal metabolism of steroid hormones (Nykjaer et al., 2001) and, in cows, is positively related to follicle diameter (Wang et al., 2022). Although afamin is directly related to proteins involved in oocyte competence, perhaps its greater LFQ-intensity interferes negatively in this metabolic cascade. Also, Seeber et al. (2010) observed higher levels of afamin in serum and peritoneal fluid of infertile women.

Xaa-Pro dipeptidase is associated with collagen metabolism, collagen lytic activity is increased during the ovulation process (Lind et al., 2006). In addition to this protein, the higher LFQ-intensity of coagulation factor IX protein in the RB group indicated the process of ovulation is in progress. Coagulation factor IX is directly cross-linked with proteins associated with ovulation and fertilization (Shen et al., 2017; Duffy et al., 2019). In contrast, studies have revealed that higher levels of coagulation factors and decreased anticoagulant factors are associated with infertility or pregnancy loss (Ebner et al., 2008; Kwak-Kim et al., 2009). Thus, the greater LFQ-intensity of coagulation factor IX in the FF of RB buffaloes may be interfering with ovulation or oocyte competence.

Transthyretin is involved in the metabolic process of retinol and thyroid hormone transport, and it impairs the breakdown of the germinal vesicle in porcine oocytes (Ducolomb et al., 2013). Thus, it is possible to hypothesize that oocytes from RB

buffaloes have difficulty continuing their meiotic maturation, compromising fertilization. Furthermore, transthyretin is linked directly to the enzyme uricase, an enzyme related to uric acid metabolism and accumulation of uric acid, resulting in poor quality oocytes in buffaloes (Cassano et al., 1999). These points support the idea that oocytes from RB Murrah buffaloes have lower competence due to the follicular environment. Thus, increases in the LFQ-intensity of transthyretin in RB buffaloes may be indicative of the compensation process against uric acid in the FF of these females. In cattle, Kafi et al. (2021) suggest that the low oocyte maturation and fertilization rates could explain the disturbed fertility in RB females specifically with subclinical endometritis. Thus, to obtain good fertility rates, an intense release of molecules involved in a physiological cascade can interfere with the success of subsequent steps. Although afamin, coagulation factor IX and Xaa-Pro dipeptidase proteins participate in the ovulation metabolic cascade, it is hypothesized that when released profusely, these molecules interfere with events that involve receptor acquisition and energy metabolism of the oocytes. Perhaps, the greater LFQ-intensity of these proteins in RB buffaloes, when compared to NC ones, disrupts some molecular cascades that affect ovulation and metabolic processes.

In our study, gene ontology enrichment analysis revealed that most FF proteins classified in biological process presented a very similar profile to that found by Marques et al. (2022), who verified in FF of buffalo ovaries with at least one corpus luteum, the highest percentages for cellular process, metabolic process and biological regulation. Also, for gene ontology, cellular components indicates that all transcripts related to cellular organelles are active. Most proteins (93.9%) are related to the anatomical entity of the cell and, within this group, > 40% are associated with the extracellular region. These proteins contribute to cellular integrity, follicle development and ovulation

(Aranciaga et al., 2020). Molecular functions, biological processes and pathways indicated that the preovulatory cascades were active, because proteins related to binding, biological regulation, metabolic blood coagulation and gonadotropin-releasing hormone receptor were expressed prominently. Also related to gene ontology, our results are also supported by the previous studies performed on buffaloes (Purohit, 2008; Kumar et al., 2021).

5. Conclusions

The findings of this study supported our hypothesis of an inherent inferior quality of the follicle microenvironment in RB females. Additionally, a unique protein (HP-25 homolog 2) was identified only in the FF of RB females, which suggests that it can be used as a biomarker to identify this reproductive disorder in buffaloes.

Author contributions

SK: collected, analyzed and interpreted the data, and drafted the manuscript. **MSC:** assistance in experiments, data analysis, review and editing the manuscript. **MFvT:** conceptualization, data analysis, review and editing the manuscript. **AAM:** review and editing the manuscript. **AFOL:** assistance in experiments. **WGV:** supervision, experimental design, review and editing the manuscript. **STRF:** experimental design. **LMCP:** assistance in experiments, review and editing the manuscript. **AFBS:** review and editing the manuscript. **YFW:** review and editing the manuscript. **MALO:** review and editing the manuscript. **LMM:** experimental design, review and editing the manuscript**. VJFF:** conceptualization, resources, review and editing the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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448

449 **Tables**

450

451 **Table 1**

452 Parameters of aspirated follicles (> 8 mm) in normal cycling (NC) and repeat-breeder

453 (RB) buffaloes.

Parameter	**NC**	**RB**
Females (*n*)	7	8
Aspirated females (*n*)	5	5
Aspirated follicles (*n*)	5	9
Follicles used for proteomics	5	5
Diameter (mm) of aspirated follicles (mean ± SEM)	12.92 ± 1.04	12.58 ± 1.06
Volume (mL) of aspirated follicles (mean ± SEM)	0.46 ± 0.11	0.45 ± 0.09

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Figures

Fig. 1. Schematic representation of experimental procedures with the follicular fluid (FF) of normal cycling and repeat-breeder buffaloes. Collection of FF and SDS-PAGE (A). SDS-PAGE, digestion, LC-MS/MS and protein identification (B). Analysis of data for experimental groups (C).

Fig. 2. LFQ-Intensity of afamin, Xaa-Pro dipeptidase and coagulation factor IX (A) and transthyretin (B) found in follicular fluid of follicles aspirated from normal cycling (NC) and repeat-breeder (RB) buffaloes.

a,b = $P < 0.05$.

Fig. 3. Gene ontology analysis of proteins identified in follicular fluid aspirated from buffaloes. Proteins were classified according to (A) cellular components, (B) molecular functions, (C) biological process and (D) pathways. Results are displayed as percent of genes classified to a category over the total.

Fig. 4. Protein-protein interactions: HP-25 homolog 2 (XP_006073695.1) (A), afamin (AFM) (B), transthyretin (TTR) (C), coagulation factor IX (F9) (D) and interactions of AFM, TTR, F9 and PEPD proteins (E). Each ball represents a protein molecule and the line connecting the balls represent the relationship among them. The number of lines represents the strength of the relationship, and the arrows represents the target proteins.