Quantitative Proteomic Analysis of buffalo ovaries reveals the regulation of kinase and phosphatase during the Formation and Regression of Corpus Luteum

Ming Zhang (✉ mingzhang@gxu.edu.cn)

animal reproduction institute, Guangxi University <https://orcid.org/0000-0002-7840-5038>

Zhe Ni

College of Life Science and Technology, Guangxi University; State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources

Qiang Fu

State Key Laboratory of Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University

Liping Pu

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University

Pengfei Zhang

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University

Dongrong Chen

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University

Shun Yao

Institute of Microbiology Chinese Academy of Sciences

Fengling Huang

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University

Fumei Chen

Department of Histology&Embryology, School of Pre-clinical Medicine, Guangxi Medical University

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Abstract

Background: Reproductive characteristics are made up of many complicated physiology procedures that have significant effects on the growth of the buffalo industry and are affected by numerous factors. The ovary is the most important sexual organ of female mammals, and during the oestrous cycles, its corpus luteum (CL) plays a significant part in mammalian reproduction. During the development and regression of corpus luteum, however, the differentially expressed proteins are less defined. In this study, we used a 6-plex tandem mass tag (TMT) strategy for quantitative proteomic comparison of three distinct ovary phases (corpus hemorrhagicum, corpus luteum and corpus hemorrhagicum) in buffalo. **Results:** A total of 148 differentially expressed proteins were identified, 32 of these proteins were identified as differentially expressed in the group CH (corpus hemorrhagicum) and 116 were identified as differentially expressed in the group CF (corpus fibrosum), with the group CL (corpus luteum) serving as the control group. Notably, we discovered that quite some enzymes such as kinase and phosphatase, are upregulated in the ovary CL phase, and three upregulated enzymes and proteins in the CL phase (PLK1, PGP, and HGS) were verified using Western blotting, quantitative RT-PCR, and immunohistochemistry analysis. The results of these validations were consistent with the quantitative analysis of the TMT-label, which indicated that they could play a crucial role during the CL's reproductive cycle. These buffalo results during the formation and regression of the corpus luteum are also shown to be a substantial reference value for comparable human and mouse studies after analyzing homologous BLAST cross-species. The expression information is accessible with the PXD009957 identifier via ProteomeXchange. **Conclusions:** Our research gives a deeper understanding of CL formation and regression during the oestrous cycles, which shows kinase and phosphatase regulation, and indicates some potential enzymes and proteins that may influence buffalo fertility. **Keywords:** corpus luteum, quantitative proteomic, oestrous cycles, ovary, cross-species

Background

Buffalo (*Bubalus bubalis*) is a specie with a single ovule, in its heat phase. Generally, only one follicle matures and ovulates, leading to a sharp decrease in fertility rates, which limits the buffalo industry to flourish. Reproductive characteristics are quantitative traits that are influenced by different factors.

The ovary is female mammals' most significant sexual organ. Its corpus luteum (CL) plays a vital role in mammalian reproduction during its formation and regression. The oestrous cycles of female mammals are mainly the cyclic adjustment method of follicles and corpus luteum and reflect the periodicity of modifications in follicles under the regulation of gonadal hormones. However, there are few types of research conducted on water buffalo during the oestrous cycles on the creation and regression of CL and associated protein concentrations.

With the implementation of proteomic approaches in numerous physiological procedures in husbandry animals such as pigs, cows and sheep, many critical proteins connected with reproductive features are under consideration. Chiaradia E et al. provided the outcomes of the 2-DE maps of sheep's blood serum indicating a noticeable reduction in protein spots, i.e. transthyroxine, apolipoprotein A1, and a substantial boost in places marked as binding to globin, endorphin 1B, and other 1B glycoproteins, and explained the use of PROT. Archaeology assumed by biomarkers in early diagnosis[1]. Research on the proteomic characterization of bovine follicular granulosa cells conducted by Hao et al. has presented a theory analyzing that of these, 259 proteins have a distinct expression in DF (dominant follicle) and SF (subordinate follicle). In 259, an aggregate quantity of 26 proteins was upregulated in DF (fold change ≥ 2), and 233 proteins were down-regulated in DF (fold change ≤ 0.5), pointing out that the research findings will provide valuable data to explore the follicle's growth and function[2].

Similar research methods were also used for bovine follicular liquid (bFF) and porcine follicular fluid (pFF). Ferrazza RdA et al. evaluated the FFs in a proteomic unit gathered from nine cows. They found that 143 proteins were recognized and split into multiple biological procedures that included responses to stimuli and metabolic processes. In his research, they outlined the various expression of proteins that provide inward-looking protein modifications that rely on size variables that may influence follicular function in the ovarian follicular microenvironment [3]. Bijtteleier J et al. carried out a comparative proteome analysis of porcine follicular fluid (pFF). Their trials showed that they were prevalent to both liquids, of which 13 were considerably distinct in abundance ($p < 0.05$), and they discovered that serum (2)-macroglobulin depletion in the serum could substantially compensate for the fluid development that is affected by the mature oocyte in the serum [4].

In recent years, sensitive proteome technique (nano HPLC tandem mass spectrometry) has enabled us to access the proteome from scarce materials, such as oocytes and blastocysts, which are isolated by micromanipulation. The Proteome analysis of pig oocytes during in vitro maturation indicated that UCH-L1 played a significant part in completing the first meiotic cycle and transitioning it to the subsequent stage [5]. Gupta MK et al. conducted studies on the global protein profiles of parthenogenetic activated (PA) and in vitro fertilized (IVF) zygotes. They discovered comparable protein profiles, but the distinction in individual protein expression was also outstanding [6]. Hou L et al. evaluated that in a method of mass spectrometry the peptides (47405), proteins (14701) and proteomes (7634) in the ovaries of 8-week-old and 32-week-old Banna miniature pigs by tandem mass tag and explained the protein expression pattern of post-birth pig ovaries. It was first discovered that steroid hormone metabolism, lipid metabolism, and power metabolism pathway were rich in protein in a 32-week-old unit that relates to the physiological characteristics of sexual maturity [7].

The analysis was performed to enable people to determine the differentially expressed proteome during the formation and regression of CL in water buffalo through quantitative proteomic analysis of ovaries from the phases of CH, CL, and CF. The aim was to define the various proteome expression during CL formation and regression and to discover alternative proteins that could play significant roles in buffalo oestrous cycles.

Methods

Sample Preparation

All ovary buffalo specimens (*Bubalus bubalis*) were acquired from a local commercial slaughterhouse. Two 3.8–4.0 g ovaries were harvested from female buffalo (age 4.0–6.0 years) in the corpus hemorrhagicum (CH) phase, two 4.1–4.5 g ovaries were harvested from female buffalo (age 4.5–5.5 years) in the corpus luteum (CL) phase, and two 3.3–3.9 g ovaries were harvested from female buffalo (age 5.0–6.5 years) in the corpus fibrosum (CF) stage. In the months of September and October of the same year, all ovaries were collected. Detailed ovary sample data was shown in Additional File 5: Table S5 and Additional File 6: Figure S1. Figure 5 shows the workflow of these studies. Six ovaries from three phases (CH, CL, CF) were used for sample preparing, and the six ovaries were split into two distinct samples for two biological replicates. The ovaries were transported in precooled physiological saline to the laboratory. The lysis buffer (7 M urea, 3 per cent CHAPS, 1.5 M thiourea, 1 per cent DTT, and 1 per cent v / v protease inhibitor cocktail) was used to remove the ovarian protein after liquid nitrogen grinding. After incubation with vortexing every 15 min for 60 min on ice, the samples were centrifuged at 4 ° C at 12,000 / g for 30 min, and the supernatants of lysates were collected for the next experiment. The Bradford assay kit quantified the levels of the extracted proteins.

Digestion of Protein and TMT-labeling

One hundred micrograms of protein from each sample was precipitated overnight in the precooled acetone at -20°C and then centrifuged at 13,000 g for 30 min at 4°C . The supernatant was removed, and precipitation was dissolved in a combination of 100 mM TEAB (triethylammonium bicarbonate), 5 μL 2 percent SDS, and 55 μL of ultrapure water. The protein reduction alkylation was performed at 55°C with the 5 μL of 200 mM trichloroethyl phosphate for 60 min, and then 5 μL of 375 mM iodoacetamide was added in the dark and incubated at room temperature for 30 min. The sample was then precipitated for 3 hours in cold acetone (1:6) at -20°C and then placed in 100 μL of 100 mM triethyl ammonium bicarbonate. Finally, for 16-18 hours, each sample was digested with 2.5 μL of trypsin (1 μg / μL) at 37°C . The TMT reagents were used to label the peptides in accordance with the manufacturer's instructions. Briefly, 41 μL of anhydrous acetonitrile was added to each TMT label tube (0.8 mg), and each sample was marked by adding 20 μL of TMT label and incubated at room temperature for 60 min. After that, 8 μL of 5 per cent hydroxylamine was added to each sample and incubated for 15 min to end the reaction. Samples of CH, CL, and CF buffalo ovaries were marked with 126, 127, and 130, respectively, for the first biological replicate; samples of CH, CL, and CF buffalo ovaries were marked with 128, 129, and 131, respectively, for the second biological replicate. The six samples with six tags were blended by equal quantity before downstream separation after labelling.

High pH Reverse Phase Separation and Nano-LC-MS/MS Analysis

Each sample was evaporated in a vacuum and resuspended in 50 μL buffer A (98% ddH₂O, 2% acetonitrile; pH 10.0) and separated by high-pH reversed-phase liquid chromatography (RP-HPLC) column (2.1 ± 100 mm, three μm , 150 $\text{\AA}$, C18). We used buffer A and buffer B (98 per cent acetonitrile, 2 per cent ddH₂O; pH 10.0) for a 60-min linear gradient (4 per cent – 20 per cent buffer B for 30 min, 20 percent – 95 percent buffer B for 25 min, and 95 percent buffer B for 5 min). The fractions eluted from the column were collected every 1.5 min, and according to the maximum intensity, 20 fractions were gathered. The gathered fractions were desalted using a column of ZipTips C18 (Millipore, Billerica, MA, USA). The fractions were evaporated with a vacuum after desalination, and 10 μL of solvent A (2 per cent acetonitrile and 0.1 per cent formic acid) was used to resuspend the samples. In a trap column (PepMap RSLC C18 column, 50 μm 15 cm, 2 μm nanoViper, Thermo Fisher Scientific, Bremen, Germany), a volume of 2 μL from each sample was loaded, and a maximum pressure (600 bar) was applied and a column for analysis (Acclaim® PepMap100C18 column, 0.075 about 150 mm, 3 μm , 100 $\text{\AA}$, Thermo Fisher Scientific, Bremen, Germany) was used for elution at a rate of 300 nL / min. Next, we used buffer A (2 percent acetonitrile and 0.1 percent formic acid) and buffer B (98 percent acetonitrile and 0.1 percent formic acid), followed by a gradient of 60 min (5 percent – 40 percent buffer B for 45 min, 40 percent – 100 percent buffer B for 10 min, 100 percent buffer B for 5 min) to separate peptides. Finally, an LTQ-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) linked to an Easy-nLC 1,000 nano fluid chromatography scheme (Thermo Fisher Scientific, Odense, Denmark) was used to analyze all peptide fractions online.

For mass spectrometry (MS) analysis, a data-dependent mode in the scan range of 350–1,800 m / z was performed, and the survey scans were captured by the Orbitrap analyzer at a mass resolution of 60,000 at 400 m / z. In the linear ion trap, under the mode of high-energy collision dissociation, 10 of the most intense precursor ions were selected for secondary mass spectrometry analysis (MS²). The dynamic exclusion parameter included a two exclusion count and a 40 s exclusion moment. Siloxane ions have been used for inner calibration (m / z = 445.1200).

Proteomic Data Analysis and Bioinformatics

The raw information files were processed and quantified using Proteome Discoverer v1.3.0.339 (Thermo Fisher Scientific, Massachusetts, USA) and searched using the SEQUEST algorithm against the UniProt Bos Taurus protein database (UP000009136, 24078 sequences, release 2017_03). The search parameters used were as follows: fixed changes, including cysteine carbamidomethylation (+ 57.02146 Da); TMT reagent adducts (+ 229.162932 Da) on lysine and amino peptide termini; and variable changes, including methionine oxidation (+ 15.99492 Da). Precursor ion mass tolerance was 20 ppm, and fragment ions were set to ± 0.5 Da, enzyme specificity, trypsin. The false discovery rate (FDR) was calculated using the peptide validator and based on a search for the decoy database. A p-value < 0.05 and a fold change > 1.5 were regarded important.

The GO (gene ontology) was used to annotate the recognized proteins, which consisted of BP (biological processes), CC (cellular elements) and MF (molecular functions), and the analysis was based on the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>). The pathway analysis of differently expressed proteins was based on the KEGG (Encyclopedia of Genes and Genomes) database. The online KEGG Automatic Annotation Server (KAAS) service instruments were used to annotate the KEGG database description for each protein and to display the annotation outcomes using other online KEGG service instruments and the KEGG mapper. The interaction between groups of differential expression protein (DEPs) was extracted from the database of Search Tools for the Retrieval of Interacting Genes / Proteins (STRING). A high trust (0.7) was selected to draw the protein-protein interaction map using Cytoscape 3.2.1 for the necessary interaction score and the active interaction sources, including text mining, tests, and databases.

The IDs conversion of identified proteins and homologous BLAST cross-species

All buffalo protein IDs were transformed to Uniprot database mouse (*Mus musculus*) IDs and human (*Homo sapiens*) IDs using blast software (Blastall v2.2.26). The program name (comparison technique) is blastp, and the screening criteria is the expectation value < 1e-5. A similarity comparison of cross-species (buffalo, mouse and human) was also performed with the amino acid sequences of DEPs found in buffalo. In addition, the analysis of the GO and KEGG pathway was used to annotate the converted IDs from DEPs, including mouse and human.

Verification of Differentially Expressed Proteins Using Quantitative qRT-PCR

Total RNA obtained from ovaries at separate phases (CH, CL, and CF, n=3) was performed by Trizol reagent (Invitrogen). To reverse-transcribe and collect the cDNAs, a Takara RNA polymerase chain response (PCR) kit was used. A Light Cycler 96 scheme (Roche, Switzerland) performed quantitative inverse transcription PCR (qRT-PCR) analyzes. The reaction solution (20 μ L) is a composition of 1.0 μ L of cDNA, ten μ L of SYBR® Premix Ex Taq™ II (Takara), 2 μ L of primer mix (2 nM), and 7.0 μ L of ddH₂O. The 2- $\Delta\Delta$ CT technique was used to calculate the relative expression concentrations of targeted genes. Each gene was evaluated using different oocyte sets and tests were carried in three replicates. The primer sequences used for qRT-PCR analyses are provided in Additional File 4: Table S4.

Verification of Differentially Expressed Proteins Using Western Blotting

A western blot assay (n=3) evaluated the protein expression concentrations of PLK1, PGP, and HGS. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was used to separate equal amounts of proteins from each sample (CH, CL and CF), and these samples were then transferred via a semidry western blot system (Trans-Blot® Turbo™ System, Bio-Rad, Singapore) to a polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked for two h in 5 per cent nonfat milk at 37 ° C and incubated for 16-18 hours at 4 ° C with main antibiotics

(1:1,000–5,000 dilution). Membranes were washed three times using TBST buffer (a mixture of TBS [Tris-buffered saline] and Tween 20) and then incubated for two h at 37 ° C with secondary antibodies in the TBST buffer. Bands were visualized using an alkaline phosphatase detection kit (C3206, Beyotime Biotechnology Inc., Shanghai, China). The following commercially accessible antibodies were used as primary antibodies: anti-PGP9.5 mouse monoclonal antibody (ab72911 ; Abcam, Cambridge, MA, USA), anti-HGS rabbit polyclonal antibody (ab72053 ; Abcam), anti-actin (loading control) rabbit polyclonal antibody (bs-17654R ; Bioss biotechnology Inc., Beijing, China) and anti-Phospho-PLK1 (Thr210) rabbit polyclonal antibody (bs-3344R ; Bioss Biotechnology Inc., Beijing, China)

Immunohistochemistry (IHC)

IHC was conducted on ovaries fixed with a polyoxymethylene (n=3). The ovaries embedded in paraffin were dewaxed and dehydrated, and 30 minutes of endogenous peroxidase activity was quenched with methanol comprising 3% H₂O₂. The paraffin parts were subjected to antigen extraction using microwave heating in the 0.01 M sodium citrate buffer (pH 6.0). These sections were blocked at room temperature for 2 hours using the 5 per cent BSA and then incubated overnight at 4 ° C with primary antibodies against PLK1, PGP, and HGS. The parts were then cleaned for 15min by PBS-Tween-20, and this step was repeated three times. The parts were then incubated with secondary HRP-conjugated antibodies (CWBIO). The immunoreactive sites were visualized in brown after staining with diaminobenzidine (CWBIO), and the parts were stained with hematoxylin and assembled for observation using a bright-field microscope (Olympus, Japan). Instead of a primary antibody, negative checks were incubated with ordinary IgG.

Statistical Analysis

IBM SPSS Statistics version 17.0 and GraphPad Prism version 5.0 were used for statistical analysis. The research information was displayed as mean ± standard deviation (SD). A student t-test was used to compare the qRT-PCR outcomes of two groups ($p < 0.05$ was considered statistically significant).

Results

Differentially expressed protein identification using TMT combined with Nano-LC-MS / MS analysis

We used the Bradford method to determine protein concentration and performed SDS-PAGE electrophoresis to check the impact of protein extraction. The findings showed that the electrophoretic bands had better differentiation degrees and were noticeable, indicating that protein extraction could meet the criteria of the downstream test (Fig. 1A). We then used the TMT sixplexTM Isobaric Label Reagent Set (Thermo ScientificTM, 90061, Labels from 126 to 131) to conduct this experiment. Samples of CH, CL, and CF buffalo ovaries were marked with 126, 127, and 130, respectively, for the first biological replicate; samples of CH, CL, and CF buffalo ovaries were marked with 128, 129, and 131, respectively, for the second biological replicate. After labelling, the six samples with six tags were mixed into a new pipe by equal quantity before downstream separation, which can decrease machine mistake (such as mass spectrometer and high pH inverse phase liquid chromatography tool) and experimental operation error. A graphic table of contents for this study is shown in Fig.5.

The specimens were then divided by high-pH reverse-phase fluid chromatography (RP-HPLC), and the figure of peaks is shown in Additional File 7: Figure S2-I (Repeat 1) and Additional File 7: Figure S2-II (Repeat 2). After separation, an LTQ-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to an Easy-nLC 1,000 nano liquid chromatography system (Thermo Fisher Scientific, Odense, Denmark) was used online to analyze

all peptide fractions (Additional file 7: Figure S2 -III) and each TMT tag was obviously recognized (Additional file 7: Figure S2-IV). Analysis of the hierarchical cluster showed that the three distinct phase ovaries had an evident differential protein expression that emerged in the two biological replicates (Fig. 1B-I and 1B-II).

In all groups, a total of 2,103 proteins have been quantified. Taking CL as a control group, in the CH and CF groups respectively, proteins with TMT ratios > 1.5 or < 0.67 and P-values < 0.05 were regarded to be expressed differently. Through comparative study, 32 proteins with fold changes of ± 1.5 ($\log_2 0.58$) were recognized as differentially expressed in the CH group (Fig. 1C-I) and upregulated 21 proteins and downregulated 11 proteins (Additional File 1: Table S1). Of 116 proteins with a fold change of $\geq \pm 1.5$ ($\log_2 0.58$) recognized as differentially expressed in the CF group (Fig. 1C-II), 64 proteins were upregulated, and 52 proteins were downregulated (Additional File 1: Table S1).

Bioinformatic Analysis of Differentially Expressed Proteins

CH versus CL group differentially expressed proteins and CF versus CL group were categorized by gene ontology annotation based on three classifications: biological process, cellular component, and molecular function. The proteome of the GO annotation was obtained from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>) (additional file 2: table S2). After analyzing GO differentially expressed proteins from the CH vs CL group, we discovered that the biological procedures included wound healing, epidermal cell spreading, collagen biosynthetic process, endodermal cell differentiation, primary germ layer formation, and steroid biosynthetic process (Fig. 2A). The molecular function of these proteins is essential in hydrolyase activity, carbon-oxygen lyase activity, and transferase activity (Fig. 2A). The findings of the cellular component analysis showed that these proteins were primarily a component of banded collagen fibril, a complex of collagen trimers, and mitochondrial nucleoid (Fig. 2A).

A GO analysis was also performed on the CF versus CL group (Fig. 2B), and we discovered that the amount of GO conditions in this group was much higher than the CH versus CL group, which may imply that differentially expressed CF versus CL group proteins are more crucial than the CH vs CL group during the creation and regression of CL in water buffalo. An analysis of biological mechanisms recognized many mechanisms such as regulation of endodermal cell differentiation, cellular amine metabolic process, cytokine manufacturing involving the immune response and lipopolysaccharide-mediated signalling pathway, and prostanoid biosynthetic process (Fig. 2B). The molecular function of these proteins plays a significant part in the operation of motor microfilament and cargo receptors and in the binding of the protein complex, actin, cytoskeletal protein and antigen (Fig. 2B). In addition, the differentially expressed proteins from this group are primarily an element of collagen type V trimmer, fibrillar collagen trimmer, photoreceptor exterior section, and neuronal cell body (Fig. 2B). Interestingly, we discovered that many distinct kinds of enzymes appeared extremely expressed in the CL stage (Table 1), including kinase and phosphatase, which indicates that phosphorylated protein modification may play a significant role during ovarian CL formation. We performed validations for three proteins to further test our hypothesis: serine / threonine-protein kinase PLK1 (PLK1), glycerol-3-phosphate phosphatase (PGP), and hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), all of which were upregulated in the CL stage.

Indeed, we evaluated differentially expressed proteins based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Additional File 3: Table S3). We discovered that the CH vs CL group's differentially expressed proteins were primarily enriched in autoimmune thyroid disease; hematopoietic cell lineage; valine, leucine and isoleucine degradation; PPAR signalling pathway; and phagosome (Fig. 2C). These pathways may be connected with CL formation. In addition, the associated processes were primarily engaged in the absorption and digestion of vitamins, protein, fat, and minerals in the CF vs CL group. Meanwhile, phagocytosis, cell cycle, steroid biosynthesis,

and DNA replication also emerged during this era (Fig. 2D), indicating that these pathways may be involved in regulating regression in CL.

Analysis and Visualization of Identified Proteins

We also used the Cytoscape 3.2.1 mapping software to visualize the relationship between the 2,103 proteins recognized. The interaction was extracted from the database Search Tools for the Retrieval of Interacting Genes / Proteins (STRING). Experiments are used for the input file in the Cytoscape 3.2.1 mapping software for high confidence (0.7) for the necessary interaction score and active interaction sources, including text mining. In addition, we performed KEGG's study online through the STRING database and exported significant pathways including actin cytoskeleton regulation, carbon metabolism, PI3K-Akt signalling pathway, oxidative phosphorylation, glycolysis/gluconeogenesis, and citrate cycle (TCA cycle). These pathway interactions were visualized using Cytoscape 3.2.1 mapping software (Fig. 3A). The BinGO package was used for gene ontology (GO) analysis for these recognized proteins and showed that the proteins were primarily involved in establishing protein localization (Fig. 3B), oxidation-reduction (Fig. 3C), and vesicle-mediated transport (Fig. 3D). Notably, we discovered that six proteins were expressed differently. (Detailed data on these proteins appears in Table 2).

The homology and bioinformatic analysis of identified proteins among buffalo, human and mouse

To evaluate the conservation of the molecular system during the formation and regression of the corpus luteum in the ovary, we used homology and bioinformatic analysis to extend our outcomes to mouse and human. The GO and KEGG analysis was performed on the converted human IDs (Additional file 8: Figure S3A-B) and mouse (Additional file 8: Figure S3C-D), and we discovered that many biological procedures are involved in both species, such as metabolic process, reproductive process, cell aggregation, hormone secretion and cellular component organization or biogenesis. In addition, many pathways are enhanced in the converted protein IDs from humans and mice, such as actin cytoskeleton regulation, MAPK signalling pathway, PI3K-Akt signalling pathway, PPAR signalling pathway and valine, leucine and isoleucine degradation. The findings of GO and KEGG pathway study disclosed that these pathways and biological procedures might be connected with human and mouse CL formation and regression. The visualization of protein-protein interaction analysis from mouse and human transformed IDs was obtained from the database of Search Tools for Retrieving Interacting Genes / Proteins (STRING). High confidence (0.7) was selected to draw the protein-protein interaction map using Cytoscape 3.2.1 for the necessary interaction score and the active interaction sources, including text mining, tests, and databases. Also, we discovered that the same protein-protein interaction style of actin cytoskeleton regulation, oxidative phosphorylation and buffalo carbon metabolism were discovered both in humans (Additional file 8: Figure S3E) and mouse (Additional file 8: Figure S3F). We also examined the homologous of upregulated enzymes and proteins in the CL phase of ovary cross-species (Table 3), and it is essential to remember that these proteins demonstrate an elevated sequence identity (from 77.78 per cent to 95.65 per cent, Table 3) after homologous BLAST cross-species, indicating that these buffalo results appear during the creation and regression of corpus luteum.

Validation of Differentially Expressed Proteins

The three proteins (PLK1, PGP, HGS) that were upregulated in the CL phase were chosen for western blotting, RT-qPCR, and IHC. The findings of the three proteins' western blot analysis are provided in Fig. 4B. Using Image J software, we evaluated the gray value of each western blot lane, and the findings were consistent with TMT quantification and transcription level analyses (Fig. 4C). All analyses used the CL group as control. We used immunohistochemistry localization analysis to investigate the prospective functions of the three further

differentially expressed proteins because there is limited data about their location in the ovary at the CH, CL, and CF phases. As shown in the figure. 4A, PLK1 was articulated primarily around the follicle and in the ovary medulla ministry. PGP was expressed primarily in the follicle, particularly in the CL phase. PGP and HGS were both primarily situated in the surroundings of ovarian follicles and medulla ministry. The fluorescence intensity of the three proteins in the CL phase was higher compared to the CH and CF phase, particularly in the CL versus CF category, which suggests that the three proteins may play a significant part in the creation and regression of CL in the ovary, particularly for phosphorylated alteration during this biological process.

Discussion

In this research, a quantitative proteomics method based on TMT was implemented to screen differentially expressed proteins connected with CL formation and regression in the ovary of water buffalo. Three upregulated proteins in the CL phase (PLK1, PGP, and HGS) were verified using western blotting, quantitative RT-PCR, and ICH, indicating that they could play a vital part in the regular CL reproductive cycle. Progesterone generated by the corpus luteum (CL) controls the synthesis of multiple endometrial proteins needed for embryonic implantation and growth [8]. The CL plays a vital role in regulating the oestrous cycle and maintaining pregnancy [9].

One of the most significant modifications during CL formation and regression is the shift in cellular reaction to external signals that enable the luteal cells to react to a fresh set of hormones. The most researched hormones are follicle-stimulating hormones, LH, prolactin (PRL), estrogen, and progesterone.

Fundamental understanding of the contribution of antioxidant enzyme mechanisms in progesterone synthesis and in maintaining luteal cell integrity in relation to pregnancy is still relatively limited. The processes engaged in CL formation, growth and regression are complicated and diverse in different mammalian species [8].

Proteomics can provide physicians and biologists with a better knowledge of the fundamental biological mechanisms engaged in maintaining CLs structural integrity and functional activity during the pre-implantation era [8]. In this research, we discovered that many kinds of enzymes, including kinase and phosphatase, emerged in the ovary CL phase (Table 1), which may imply that the formation and regression of CL are controlled by distinct enzymes, which may play a part in the activation of many signalling pathways. Many studies focused on activating signalling pathways during the formation and regression of CL.

The luteinization of luteal cells and cumulus cells mainly relies on the activation of the LH receptor (LH-R). Activation of LH-R binds to boost guanine nucleotide-binding protein Gs and emit adenylate cyclase (AC) signal, and cAMP is enhanced, and then cAMP-dependent PKA is activated [10]. Mitogen-activated protein kinases (MAPKs) comprise a superfamily kinase [11] recognized in multiple species CL. MAPK was involved in CREB phosphorylation. ERK1 and ERK2 have been recorded in porcine, bovine, rat, and human CL, and p38/MAPK has been shown to be expressed in rat and cow CL [12]. Jun N-terminal kinase was mostly researched in bovine CL [13]. PGF2 α is essential for inhibiting progesterone synthesis in CL [14]. Although the uterus generates PGF2 α , several studies reveal that the CL also generates PGF2 α [15]. The expression levels of luteal COX-2 [16] and PGF2 α [17] increase quickly and transiently in bovine animals after injection of PGF2 α . This finding is also validated by the rise of COX-2 in mRNA levels [16]. Considering that PGF2 α stimulates its own synthesis in CL, it can be thought that releasing PGF2 α from the pulse of the uterus improves the production of PGF2 α in CL, leading to an intensification in the luteolysis system.

In this research, we recognized many upregulated enzymes (Table 1) that are engaged in oxidation-reduction, such as 7-dehydrocholesterol reductase, prostaglandin E synthase, cytochrome c oxidase subunit four isoform 1, NADH

dehydrogenase [ubiquinone] 1 beta subunit 7, isocitrate dehydrogenase [NADP] cytoplasmic, isovaleryl-CoA dehydrogenase, carnitine O-palmitoyltransferase 2. And prior trials have also discovered substantial proof that reactive oxygen species (ROS) are essential factors in determining the lifespan of CL[18] and that antioxidants play essential roles in CL physiology during the oestrous/menstrual cycle [19–21]. The rare in-vivo studies studying rat CL [22], women [19] and sheep [23, 24] showed the significance of antioxidant enzymes in controlling CL function during the peri-implantation period. NADH and NADPH are essential components in controlling ROS development and maintaining cellular redox state [25]. The mitochondrial NADP⁺-dependent isocitrate dehydrogenase produces NADPH through the oxidative decarboxylation of isocitrate [26]. Meanwhile, many of the upregulated enzymes recognized in this research are engaged in the process of protein phosphorylation and dephosphorylation, such as glycerol-3-phosphate phosphatase, hepatocyte growth factor-regulated tyrosine kinase substratum, serine / threonine-protein kinase PLK1. The research identified phosphorylated CREB mainly located in the soluble compartment in luteinized granulosa cells in primary cell culture [27], a compartment in which phosphorylated CREB could not satisfy its role as a transcription factor. The growth factors insulin-like growth factor-1 (IGF-I) and fibroblast growth factor (FGF) may each result in p38 MAPK / MAPKAPK activation leading to CREB phosphorylation in other experimental structures [28, 29], indicating that these growth factors could serve as stimuli during luteal maturation. CREB phosphorylation's first and second peaks correspond to earlier described cAMP-responsive PKA-mediated occurrences [27, 30, 31]. By comparison, the third luteal-phase CREB phosphorylation constitutes what is believed to be a cAMP-nonresponsive event [27]; therefore, a CREB kinase other than PKA is anticipated to catalyze this stage of phosphorylation.

Finally, we produced a validation of three recognized enzymes (PLK1, PGP and HGS) (Fig. 4A-C) and they were upregulated in the luteal phase, and these enzymes may be engaged in regulating hormone secretion during luteal formation. We discovered that their expression modifications were compatible with proteomics outcomes, suggesting that during the formation and degradation of corpus luteum there was not only the involvement of different hormones earlier reported, but also that many enzymes play a part in this phase.

One differentially expressed kinase was PLK1, which was upregulated in the ovary's CL phase. PLK1 is a serine / threonine-protein kinase that has several significant tasks throughout the M phase of the cell cycle, including regulation of centrosome and spindle processing, removal of chromosome arm adhesions, inactivation of anaphase-promoting complex / cyclosome (APC / C) inhibitors, and regulation of mitotic manufacturing and cytokinesis [32]. Analysis of biological procedures linked to this protein showed that this kinase was involved in the G2/M transition during the mitotic cell cycle and in the negative regulation of cyclin-dependent protein serine/threonine kinase activity. It has long been thought that regulatory oocyte-derived molecules behave in the follicle to inhibit premature luteinization and limit progesterone biosynthesis. TGF β superfamily members have the antiproliferative impacts of oocytes reported in vitro research [33]. The TGF β superfamily has been proposed as luteinization inhibitors including activins and inhibins, TGF β , BMPs (bone morphogenetic proteins), GDFs (growth/differentiation factors), and anti-müller hormone [34]. Of these, the oocyte produces MP-15[33] and GDF-9 [35]. Thus, we speculate that PLK1 upregulation may help with TGF β -related enzymes by inhibiting luteinization during CL regression. Another chosen phosphatase in this study was PGP (phosphoglycolate phosphatase), which is a phosphatase engaged in such processes as glyoxylate and dicarboxylate metabolism. PGP acts as a particular Gro3P phosphatase (G3PP) and plays the central role in controlling glucose and lipid metabolism and signalling as well as a metabolic stress response in mammalian cells [36]. In CL, PGF2 α works with the Gq-coupled receptor, which has been commonly discovered in mammalian species[37]. After activation, the Gq-coupled receptor creates PLC-mediated inositol triphosphate and diacylglycerol generation [38], followed by enhanced free intracellular calcium and PKC activity [39]. In addition, PGF2 α activates the ERK1/2 signalling pathway in luteinized rat cells, which is dependent on

enhanced free intracellular calcium [40]. The activation of PGF2 α -and calcium-dependent ERK1/2 also results in the transcription factor JunD phosphorylate [40], which is mainly expressed in luteal cells [41]. Some studies report that, in relation to diacylglycerol and the MAPK signalling cascade, PGF2 α can activate phospholipase D in luteal cells, generating phosphatidic acid [42]. These results indicate that PGP may play a significant role in the regression phosphorylation and dephosphorylation that happens during CL growth. HGS (hepatocyte growth factor-regulated tyrosine kinase substratum) was also a differentially expressed protein that was upregulated in the CL group in the current research. HGS is an evolutionarily preserved vesicular sorting protein belonging to ESCRT (an endosomal-sorting protein needed for transportation) [43]. HGS allows the molecule to anchor the inner surface of the lipid bilayer by having a FYVE domain [44] and by having a UIM domain it binds monoubiquitinated receptors [43]. There are reports that HGS can regulate hepatocyte growth factor or epidermal growth factor signalling through the endosomal/lysosomal pathway by associating with ubiquitinated receptors such as MET or EGFR [45]. HGS is also active in the transduction of cytokine-mediated intracellular signals and growth factors and is involved in BMP signalling through the phosphorylation of SMADs and TAK1 in early mouse embryos [46]. Targeted mutations in HGS cause immediate lethality to the early embryo after gastrulation [47]. Indeed, the connection between ACTR1 and SMAD2 is improved by HGS for ACTIVIN signalling [48]. These results show that HGS can be involved in a wide variety of extremely complex and multifaceted biological procedures connected with CL, ensuring a normal ovary reproductive cycle. We also performed the analysis of sequence similarity comparison and homology Blast acquired from buffalo, human and mouse, and discovered that the findings of this research in buffalo might have a substantial reference value for further study of the mechanism during the creation and regression of corpus luteum in human and mice ovaries. We speculate that regulatory processes for the creation and regression of corpus luteum in mammals may be conservative. Therefore, further studies are still needed to determine the role of antioxidant enzymes and protein phosphorylation-related enzymes in the function and integrity of CL during the peri-implantation era.

Conclusions

Our research findings give fresh insight into the molecular processes of CL formation and regression and may provide some future novel biomarkers for further research of mammalian estrus.

Abbreviations

DEPs: differentially expressed proteins

TMT: tandem mass tag

CH: corpus hemorrhagicum

CL: corpus luteum

CF: corpus fibrosum

GO: Gene ontology;

KEGG: Kyoto Encyclopedia of Genes and Genomes

RT-qPCR: Quantitative reverse transcription PCR

Declarations

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Availability of data and materials

Data are available via the ProteomeXchange with the identifier PXD009957.

Authors' contributions

F.C. and M.Z. Designed research; Z.N. and Q.F. performed research; F.C. and Z.N. wrote the paper; Q.F. analyzed data; L.P. and P.Z. Collected buffalo ovaries and performed the proteomics experiments; D.C. and S.Y. analyzed the protein-protein interaction network and carried out western blot experiments; F.H. carried out qRT-PCR experiments.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author Details

¹College of Life Science and Technology, Guangxi University, Nanning, Guangxi 530004, China. ²State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, Guangxi 530004, China. ³Department of Histology & Embryology, School of Pre-clinical Medicine, Guangxi Medical University, Nanning, Guangxi 530021, China

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Tables

Table 1.Up-regulated enzymes in CL stage of ovary identified by TMT based quantitative proteomics approach

Uniprot ID	Protein name	Gene name	P.Value	MW [kDa]	PI	Fold Change
Q32KR6	Squalene synthase	FDFT1	0.00036	48.27	6.47	1.92245
G8JKY2	7-dehydrocholesterol reductase	DHCR7	0.03641	54.23	8.46	3.06128
Q95L14	Prostaglandin E synthase	PTGES	0.00619	17.29	9.50	2.68775
Q71SP7	Fatty acid synthase	FASN	0.00235	274.38	6.70	2.13043
P00423	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	COX4I1	0.03470	19.56	9.31	1.65144
Q02368	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	NDUFB7	0.00227	16.39	8.18	1.58914
Q2T9S4	Glycerol-3-phosphate phosphatase	PGP	0.01197	34.30	5.34	1.61235
A0A140T8A5	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	0.00291	46.73	6.55	1.50291
Q0V8S0	Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	0.01425	85.73	6.19	1.61779
Q3SZI8	Isovaleryl-CoA dehydrogenase, mitochondrial	IVD	0.01189	46.47	7.42	1.55649
G3N2Y7	NADPH:adrenodoxin oxidoreductase, mitochondrial	FDXR	0.02662	55.00	8.51	1.99474
Q2TA25	Serine/threonine-protein kinase PLK1	PLK1	0.04132	67.96	8.85	1.70863
F1N1M7	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2	0.04198	74.38	8.25	1.64666

Table 2.Information of differentially expressed proteins involved in the establishment of protein localization, oxidation reduction and vesicle-mediated transport

Biological Process	Uniprot ID	Protein name	GN	P.Value	Fold Change	MW [kDa]	PI
the establishment of protein localization	Q3ZCC9	Protein SEC13 homolog	SEC13	0.01473	1.84 (CF_vs_CL)	35.45	5.48
	P01888	Beta-2-microglobulin	B2M	0.00041	1.84 (CF_vs_CL)	13.67	8.00
oxidation reduction	Q3SZI8	Isovaleryl-CoA dehydrogenase	IVD	0.01189	0.64 (CF_vs_CL)	46.47	7.42
	Q3SZ00	HADHA protein	HADHA	0.04876	1.53 (CH_vs_CL)	83.20	9.06
vesicle-mediated transport	E1BNR0	Uncharacterized protein	APOB	0.00708	0.66 (CF_vs_CL)	515.44	6.77
	Q0V8S0	Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	0.01425	0.62 (CF_vs_CL)	85.73	6.19

Table3. The results of homologous BLAST cross-species from up-regulated enzymes and proteins in CL stage of ovary

Uniprot ID (Buffalo)	Gene name	P.Value	Mapping ID (Human/Mouse)	Identity(%) (Human/Mouse)	E_value (Human/Mouse)	Score (Human/Mouse)
Q32KR6	FDFT1	0.00036	P37268/P53798	91.61/86.92	0/0	822/780
G8JKY2	DHCR7	0.03641	Q9UBM7/A0A140LIT2	88.63/88.00	0/0	867/858
Q95L14	PTGES	0.00619	O14684/Q9JM51	84.97/77.78	2E-93/1E-83	268/243
Q71SP7	FASN	0.00235	A0A0U1RQF0/A0A0U1RNJ1	79.20/78.30	0/0	3837/3821
P00423	COX4I1	0.0347	P13073/P19783	82.84/84.62	3E-108/1E-107	306/305
Q02368	NDUFB7	0.00227	P17568/Q9CR61	86.86/84.67	6E-73/3E-71	215/210
Q2T9S4	PGP	0.01197	A6NDG6/Q8CHP8	90.97/89.72	0/0	516/546
A0A140T8A5	IDH1	0.00291	O75874/O88844	95.65/94.20	0/0	835/825
Q0V8S0	HGS	0.01425	O14964/Q3UMA3	92.85/91.35	0/0	1184/1168
Q3SZI8	IVD	0.01189	A0A0A0MT83/Q9JHI5	91.31/88.73	0/0	818/789
G3N2Y7	FDXR	0.02662	P22570-2/Q61578	87.71/87.08	0/0	831/800
Q2TA25	PLK1	0.04132	P53350/Q07832	95.02/91.71	0/0	1026/1004
F1N1M7	CPT2	0.04198	P23786/P52825	86.78/83.59	0/0	1216/1177

Figures

Figure 1

(A) SDS-PAGE electrophoresis of the protein obtained from CH, CL and CF groups. (B) Heatmap of the three phases (CH, CL and CF) of the two biological replicates, repeat 1(I) and repeat 2(II). (C) Differentially expressed proteins in

CH versus CL and CF versus CL groups. Protein volcano plot modifications in group CH versus CL group (I) and CF versus CL group (II). The fold changes of > 1.5 or < 0.67 and p-values of < 0.05 were considered as significantly dysregulated.

Figure 2

(A) Gene ontology (GO) analysis of the CH versus CL group of differentially expressed proteins. (B) Gene ontology (GO) analysis of the CF versus CL group of differentially expressed proteins. (C) Regulated enrichment statistics from KEGG pathway analysis in the CH versus CL group. (D) Regulated enrichment statistics from KEGG pathway analysis in the CF versus CL group.

Figure 3

The interaction between the recognized proteins was studied using Cytoscape 3.2.1 software. (A) Visualization of KEGG analysis through the online STRING database and six pathways including actin cytoskeleton regulation, carbon metabolism, PI3K-Akt signalling pathway, oxidative phosphorylation, glycolysis/gluconeogenesis, and citrate cycle (TCA cycle) were discovered. (B-D). Visualization of the analysis of gene ontology (GO) using the BinGO package supplied by the Cytoscape 3.2.1 software. Three biological procedures have been discovered, including the establishment of protein localization (B), oxidation-reduction (C), and vesicle-mediated transport (D). Six differentially expressed proteins were discovered, and a red box marked them respectively.

Figure 4

Validation of three differently expressed proteins (PLK1, PGP, and HGS). (A) IHC validation of the three proteins expressed differently. PLK1 was articulated primarily around the follicle and in the medulla ministry (red arrows). PGP was expressed primarily in the follicle, particularly in the CL phase. PGP and HGS were both primarily situated in the surroundings of ovarian follicles and the ministry of the medulla (red arrows). (B) Analysis of the Western blot. β-actin was used as load control. (C) The combination graph of the tandem mass tag (TMT) quantitative information (blue pillar), the expression concentrations of mRNA (gray pillar) and the gray value of the western blot (black pillar). The western blot fold shift depends on the gray value of each row from the three groups (CH, CL, and CF) evaluated by the Image J software.

Figure 5

A graphic table of contents for this study.

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