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The Two Dimensional Electrophoresis and Mass Spectrometric Analysis of Differential Proteome in Yak Follicular Fluid

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Abstract: Proteome refers to the entire protein set encoded by a single genome. To understand the mechanisms underlying the seasonal reproduction of yak at the protein level and to examine the changes of protein composition in the yak follicular fluid and plasma, the differential protein and its composition in the yak follicular fluid and plasma have been identified by two dimensional electrophoresis (2DE) and Mass Spectrometry (MS). The follicular fluid and plasma from yak on Qinghai Plateau were subjected to 2DE and silver staining. The gel image was analyzed by image master 2D Platinum Software followed by Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF/TOF)™ MS identification. The proteins of high abundance were removed using ProteoExtract Albumin/Immunoglobulin G (IgG) Removal kit. Gel images with high resolution, clear background and good reproducibility from follicular fluid and plasma were obtained by 2DE. Comparison between images from follicular fluid and plasma showed 24 differentially expressed proteins, of which two were up-regulated and twenty two were down-regulated. MS analysis identified eight proteins and five unknown proteins. The proteomic images were successfully separated and identified differential proteins between follicular fluid and plasma in the yak which provided experimental evidence for the mechanisms of yak follicular development and the understanding of microenvironment for oocyte development.

Key words: Yak, follicular fluid, plasma, differential proteome, two dimensional electrophoresis, mass spectrometry identification

INTRODUCTION

Proteome refers to the entire protein set encoded by a single genome (Nowak, 1995). Proteomics is the study of proteome which includes the study of protein composition and its changing patterns from cellular and systematic levels that leads to better understanding of physiological and pathological processes (Blackstock and Weir, 1999). Proteomics contains a series of high throughput methods that can simultaneously examine all proteins in cells, organisms or biological fluid including protein sample preparation, two dimensional electrophoresis (2DE), image analysis, protein separation and identification by Mass Spectrometry (MS) and database search. Proteomics is the milestone indicating that life science research has entered the post genome era and is the key content. Follicular fluid is composed of the plasma components across the blood-ovary barrier and the metabolic products of the granulosa cells which serves as the microenvironment during oocyte maturation. It contains materials involved in oocyte mitosis, ovulation, differentiation of ovarian cells into functional corpus luteum and fertilization. Therefore, the content of follicular fluid reflects the developmental stage of oocytes and the maturation level of follicles (Eppig *et al*., 2005; Sugiura *et al*., 2005). Follicular fluid is the culture medium for oocyte development and differentiation which directly or indirectly affects the development potential of the oocytes (Coleman *et al*., 2007). Growth factors and cytokines such as the growth hormone, macrophage inflammatory protein and macrophage colony stimulating factor may serves as the markers for oocyte maturation (Kawano *et al*., 2004). Spitzer *et al*. (1996) compared the protein expression between the matured and immature follicular fluid in human by 2DE and found that the differential expression patterns during different stages reflected the physiological condition to some extent which could be considered as critical markers for follicular fluid

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maturation. Huang *et al*. (2013) successfully separated and identified several differentially expressed proteins between matured and immature follicular fluid in buffalo by 2DE-MS.

Yaks (*Bos grunniens*) reach late sexual maturity and their reproduction is obviously seasonal. The reproductive mechanism is distinct from other bovine species (Sarkar *et al*., 2008). Yaks have low fecundity and their reproductive performance is mainly determined by follicle growth and development in the ovaries and is also affected by nutrition, feeding and management. Although, people tried to improve yak reproductive performance by controlled reproduction (Zi *et al*., 2006), embryological technology (Yu *et al*., 2010) and molecular biology methods (Ding *et al*., 2012; Qiu *et al*., 2012), in addition to the reinforcement of feeding and management, the results are unsatisfactory which is mainly because the regulatory mechanisms of yak follicular development are still poorly understood. Moreover, it has been reported that 6 months old yaks differ from adult yaks in the ovarian and follicular development including the weight and size of the ovary and number of follicles and oocytes (Xu *et al*., 2012). The development potential of *in vitro* yak oocytes is different during the reproductive and non-reproductive season (Guo *et al*., 2012). Hence, the protein composition change during follicular development in yak needs further investigation.

Researcher planned to study the follicular fluid and plasma from yaks during the reproductive season by proteomic approaches. The comparison of protein composition between follicular fluid and plasma and the establishment of 2DE protein images could help us to screen the key proteins involved in follicular development and oocyte maturation which could provide reference for the research on the expression, quantification and localization of these proteins during follicular development. The study findings could elucidate the mechanisms underlying the seasonal reproduction in yak which could technically increase the efficiency of yak reproduction and accelerate yak breeding as well as theoretically improve the *in vitro* culture system for yak oocytes.

The study examined the changes of protein composition in the yak follicular fluid and plasma based on the proteomic strategy to screen the key proteins involved in follicular development and oocyte maturation. The study aimed to elucidate the underlying regulatory mechanisms and understand the pattern of seasonal reproduction in yak from proteomic perspective. The findings would shed light on the improvement of ovarian reserve and reproductive performance in yak.

MATERIALS AND METHODS

Collection and purification of follicular fluid: The ovaries and blood samples were collected from the yak slaughter in Qinghai Plateau. Follicular fluid was drawn from follicles with a diameter >8 mm using sterile syringes followed by centrifugation twice (1st, 5000 rpm for 15 min at room temperatrue, collect supernatant; 2nd, 12000 rpm for 10 min at room temperatrue, collect supernatant) to completely remove granulosa cells, oocytes and debris. The blood samples which contained ethylenediaminetet-raacetic acid anticoagulant were centrifuged at 5000 rpm for 20 min. The supernatant plasma was collected. Both follicular fluid and plasma samples were then aliquoted into 1.5 mL EP tubes and stored at -80°C for future use.

Protein extraction and quantification: Follicular fluid and plasma samples (200 μL each) were placed in sample tubes with added protein extraction buffer for grinding. Then, samples were placed on ice for 1 h and centrifuged at 13000 rpm for 20 min at 4°C. Supernatant was collected. ProteoExtract Albumin/IgG Removal kit (Calbiochem Company, Massachusetts and United States of America) was used to remove proteins of high abundance per manufacturer's manual. Protein concentration was measured by Bradford Method (Bradford, 1976). Samples were stored at -80°C and total protein extraction was tested by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Two DE

1st dimensional Isoelectric Focusing (IEF): Protein samples (100 μg) were loaded into the 13 cm, pH 3-10 non-linear gel strips [Immobilized pH Gradient (IPG), Amersham Bioscience Inc., Amersham and United Kingdom (UK)] to perform the 1st dimensional IEF in the solid phase with pH gradient [Ettan IPGphor IEF System, GE Amersham (GE Healthcare Life Sciences) Buckinghamshire and UK]. The electrophoresis condition was: 30 V for 12 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 8 h and 500 V for 4 h.

2nd dimensional SDS-PAGE: After IEF, the IPG gel strip was transferred to the 2nd dimensional SDS-PAGE (12.5%). The electrophoresis was run under constant current 15 mA/gel for 30 min and then 30 mA/gel until the bromophenol blue reached 0.5 cm from the bottom of the gel [Hofer SE 600, GE Amersham (GE Healthcare Life Sciences, Buckinghamshire and UK)].

Gel staining and image analysis: The gel was stained with silver nitrate and scanned by Image Scanner [Umax

Powerlook 2110XL, GE Amersham (GE Healthcare Life Sciences), Buckinghamshire and UK] (Chevallet *et al*., 2006). Image Master Software was used to analyze the images and match the protein spots. Protein spots with fold change in light density >1.5 were considered as differentially expressed proteins.

MS identification: The differentially expressed protein spots were cut out from the gel and analyzed by Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI)-TOF/TOF™ MS (4800 proteomics analyzer, Applied Biosystems Inc., California and USA). The sequence reads were searched in the primary and secondary MS database using Mascot Software.

RESULTS

The 2DE images from yak follicular fluid and plasma after proteins of high abundance were removed: Both plasma and follicular fluid samples contained protein of high abundance at 66.2 ku as there were dark bands in the SDS-PAGE (Fig. 1). Hence, to obtain 2DE images with high resolution, good quality and reproducibility, the ProteoExtract Albumin/IgG Removal kit was used to remove the protein of high abundance in plasma and follicular fluid. After the highly abundant protein was removed, the band at 66.2 ku became significantly lighter in both plasma and follicular fluid SDS-PAGE (Fig. 1). The efficiency was ideal.

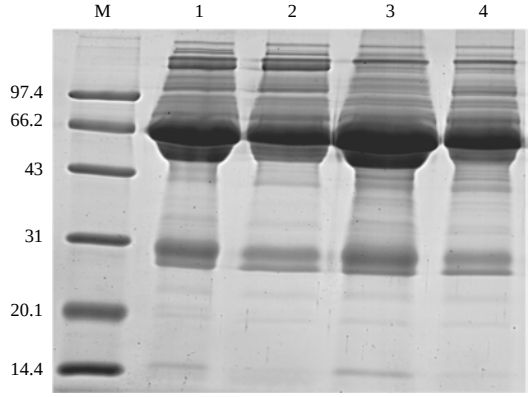

Fig. 1: SDS-PAGE of yak follicular fluid and plasma samples; M: Marker; 1: plasma sample before highly abundant protein was removed; 2: plasma sample after highly abundant protein was removed; 3: follicular fluid sample before highly abundant protein was removed and 4: follicular fluid sample after highly abundant protein was removed

The establishment of 2DE images from yak follicular fluid and plasma samples: After the protein of high abundance was removed, yak plasma and follicular fluid samples were subjected to 2DE (Fig. 2).

Analysis of differentially expressed proteins in yak follicular fluid and plasma: The 2DE were performed three times. Twenty four protein spots with fold change >1.5 and 16 protein spots with fold change >2 were obtained. Using plasma samples as control, two protein spots were found in the follicular fluid samples with fold change >1.5 were up-regulated and the rest 22 were down-regulated. Part of the enlarged differentially expressed protein spots was shown (Fig. 3).

Identification of differentially expressed proteins by MS: The 16 differentially expressed protein spots were cut

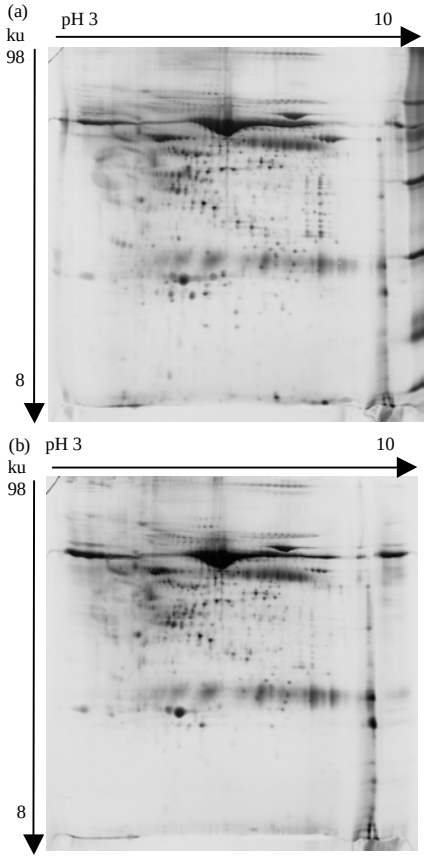

Fig. 2: Comparison between yak plasma and follicular fluid 2DE plasma image after highly abundant protein was removed; a) plasma 2DE image after highly abundant protein was removed and b) follicular fluid 2DE image after highly abundant protein was removed

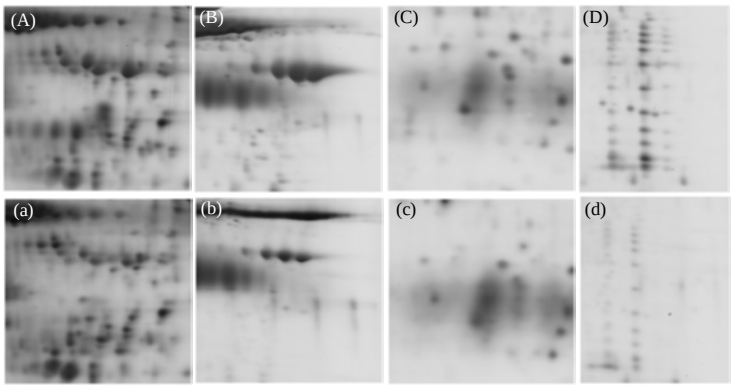

Fig. 3: Part of the enlarged differentially expressed protein spots between follicular fluid and plasma; A-D) Part of the enlarged 2DE images of differentially expressed protein spots on the yak plasma; a-d) the corresponding positions of the enlarged 2DE images of yak follicular fluid

Table 1: Identification of differentially expressed proteins by MS

Protein names	NCBI accession No.	Molecular weight/u	PI	Protein score	Protein score C.I.%	Differences multiples
Fibrinogen alpha chain precursor	gi|75812954	67484.2	6.73	122	100	2.92477
PR domain zinc finger protein 5	gi|300798423	75021.4	8.95	68	98.01	2.80851
C4b-binding protein alpha chain	gi|76677514	22393.3	6.34	215	100	2.72175
Fibrinogen gamma-B chain precursor	gi|27806893	50839.5	5.54	213	100	2.65529
C-type lectin domain family 9 member A-like isoform X2	gi|528950702	28015.1	9.27	68	99.31	2.50686
Transferrin	gi|602117	79869.5	6.75	79	100	2.49628
Protein gamma, GTP binding	gi|224267	8119.1	4.76	68	98.27	2.39829
TPA: keratin 6A-like	gi|296487880	63341.2	8.41	85	99.96	2.14482

from the gel and digested by enzymes. The peptide sequences were analyzed and identified by MS. By searching National Center for Biotechnology Information (NCBI) *Bos taurus* database using Mascot, the study successfully identified eight protein spots whose protein scores were >65 and confidence were above 95% (Table 1). Three spots failed to be identified and five were unknown proteins.

DISCUSSION

Proteomics is an effective method to study follicular fluid and plasma, among which 2DE/MS is a classic strategy of follicular fluid and plasma protein analysis and identification (Granier, 1988; Huang *et al*., 2014). Protein sample preparation is the basis and prerequisite for proteomic research and is the key step of 2DE which directly affects the quality of image and the reliability and reproducibility of the experiment (Simpson, 2003). With the development of follicles, the follicular fluid, made up by the secretion of granulosa cells in the lumen and the substances selectively across the blood-ovary barrier, gradually accumulates. Its composition is similar to plasma which mainly includes proteins, enzymes, amino polysaccharides, steroid hormones and other small molecule metabolites (Eppig *et al*., 2005; Fortune, 1994).

Follicular fluid and plasma contain large amount of impurity and highly abundant proteins which could inevitably affect protein IEF. ProteoExtract Albumin/IgG Removal kit was used to remove proteins of high abundance in plasma and follicular fluid and obtained protein samples of high quality which was tested by SDS-PAGE. The experimental platform composed of IEF (pH 3-10; 13 cm IPG gel strip) and SDS-PAGE (12.5%) for the analysis of follicular fluid and plasma was established. The 2DE images with high resolution and clear background from yak follicular fluid and plasma were obtained when the sample loading quantity was 100 μg.

The analysis of follicular fluid and plasma proteins by proteomic approaches not only can screen for differentially expressed proteins between the two samples but also can validate or correct the previous conclusions. Angelucci *et al.* (2006) analyzed human follicular fluid by proteomic approaches and identified 695 protein spots between 10-200 kDa and pH 3-10 of which only 625 were present in the plasma (Angelucci *et al*., 2006). MALDI-TOF-MS identified 183 proteins shared by follicular fluid and plasma and 27 specifically expressed proteins in either sample. Schweigert *et al.* (2006) analyzed protein and peptides in human follicular fluid and plasma by surface enhanced laser desorption/ionization TOF-MS and found that haptoglobin (α1 and α2 chains),

haptoglobin 1 and transthyretin could serve as the candidate markers for oocyte maturation and quality diagnosis (Schweigert *et al*., 2006). The comparison of yak follicular fluid and plasma by 2DE showed compositional similarity as well as differentially expressed protein spots between the two samples. Hence, the study found 24 differentially expressed protein spots whose fold change was >1.5, of which 16 had fold change >2. Of which, 2 were up-regulated and 22 were down-regulated in follicular fluid. Eight protein spots were successfully identified by MS which played regulatory roles in follicular fluid and plasma and greatly affected oocyte maturation (Table 1). The expression of the differential proteins was in accordance with its function. However, the detailed mechanisms require further investigation.

Novel proteins in follicular fluid can be identified by proteomic approaches and database search. These proteins might be relevant to the development and maturation of follicles or oocytes. Anahory *et al.* (2002) analyzed the protein content in the matured human follicular fluid and identified new proteins in it. They run 2D PAGE using matured human follicular fluid and obtained around 600 protein spots on the gel. According to the expression levels, four spots were picked and analyzed by direct sequencing or MALDI-MS of which three were identified and for the first time they were reported as the components of follicular fluid. Lee *et al.* (2005) identified novel proteins in the matured follicular fluid by proteomic approaches (2DE and MALDI-MS). All together 180 protein spots were found and of which 10 were identified. Database search suggested that six proteins had already been reported by reverse transcription-polymerase chain reaction, genes that encoded the four newly identified proteins were found to be expressed in human primary granulosa cells. In this study, 16 differentially expressed protein spots were picked for MS analysis of which eight were successfully identified by Mascot search (NCBI_*Bos taurus*) and three failed to be identified. The rest five proteins could not be matched by Mascot search which suggested that there were novel yak-specific proteins during follicular development. However, its structure and function need to be further investigated and validated.

CONCLUSION

The proteins of high abundance were removed by ProteoExtract Albumin/IgG Removal kit and then the 2DE images were obtained with plentiful protein spots, clear background, high resolution and good reproducibility from yak follicular fluid and plasma. These images were analyzed by Image Master 2D Platinum Software and 24 differentially expressed proteins were found of which eight were successfully identified by MS. These proteins might be relevant to the regulatory mechanisms underlying the development of follicular fluid in yak.

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