Protein Patterns on a Vaginal Mucus during Spontaneous and Estrus Synchronization using CIDR in Korean Native Cattle (Hanwoo)

Hak-Jae Chung*, Nam-Kuk Kim[1], Hwi-Cheul Lee[2], Hyun-Il Yoon, Suk-Dong Lee, Jin-Sung Ko, Hyeok-Jin Kwon, Hae-Ryong Oh, Yun-Ho Choy, Seong-Bok Choi, Gi-Jun Jeon, Seok-Ki Im and Myeung-Sik Lee

Hanwoo Experimental Station, National Institute of Animal Science, RDA, Pyeongchang 232-950, Korea
[1]Animal Genomics and Bioinfromatics Division, National Institute of Animal Science, RDA, Suwon 441-706, Korea
[2]Animal Biotechnology Division, National Institute of Animal Science, RDA, Suwon 441-706, Korea

ABSTRACT

The aim of the present recent study was to compare the protein patterns in the vaginal mucus of Hanwoo cattles during spontaneous and CIDR induced-estrus. Ten cattles, who had been observed in estrus, received no treatment and served as the group of cattles with normal spontaneous estrus. Thirteen cattles in the CIDR received an CIDR insert on day14 were removed and cattles were injected GnRH on day 15. Vaginal mucus samples were collected from all cattles at the same time the single AI in cattles with spontaneous estrus and the AI in cattles with induced estrus. Spontaneous and CIDR-induced estrus vaginal mucus samples were analyzed on two different array surfaces: cation-exchange (CM10), anion-exchange (Q10). In addition, using the NaCl solution by which the proteins combined after washing are 0.5, 1 and 2 M, it was fractionated and a protein was collected successively. The results are summarized as follows: 1) Ionic surfaces chemistries (Q10 and CM10) gave the best results in terms of detectable protein peaks, with more than 100 protein peaks in the two fractions and under each condition. 2) Protein mass spectrometer using 11 different proteins in protein identification of 7 were able to determine the protein. List of identified proteins as follows; Ribosome-binding protein 1, GRIP 1-associated protein 1, Katanin p60 ATPase-containing subunit A-like 1, Protein FAM44A, DUF729 domain-containing protein 1, Prolactin precursor, Dihydrofolate erductase. Conclusively, on the basis of this study, protein expression in the vaginal mucus could be used as an indicator for time of estrus manifestation in order to increase conception rates by applying AI at an optional time.

(Key words : Hanwoo, vaginal mucus estrus detection, protein expression, SELDI-TOF)

INTRODUCTION

Detection of estrus has long been known that season of the year has major impacts on beef cattle performance measures including, induce of pregnancy and artificial insemination. In addition, vaginal and cervical mucus plays an important role in the reproductive process of all mammals. In mammals, in which semen is deposited into the anterior vagina at the time of mating, such as rabbit, ruminants and primates, vaginal fluid is the first physiological medium that the spermatozoa must cross on its tour to the cranial parts of the tubular genital organs. In cattle, fertilization failure results mostly from failure of sperm to contact ova rather than from unfertilizability of ova (Hawk, 1987). Vaginal fluid is a biological product of complex composition that is mainly derived from cervical secretions. Physiological and biochemical properties of bovine vaginal mucus were determined in previous studies, but there is a paucity of reports about the activity of physiological factors (Van Klinkenberg, 1953; Boyd, *et al.*, 1972; Prasad, *et al.*, 1981). Furthermore, it is essential to determine the activity of physiological factors in bovine cervical mucus in order to compare their function in cervical mucus of cows in spontaneous and in induced estrus, and to find anomalies in cervical mucus, try to correct them, and improve pregnancy rates. The mucus constituents responsible for these biophysical properties are glycoproteins secreted mainly by the cervical and vaginal epithelium. Glycoproteins consist of a polypeptides that carries numerous heterosaccharide side chain (Doehr and Moghissi, 1973).

However, it is still unknown that the protein patterns in

† This study was supported in part by the National Institute of Animal Science, RDA.
* Correspondence : E-mail : hakjaena@rda.go.kr

spontaneous and induced-estrus vaginal mucus in Hanwoo cattles. Thus, the aim of the present study was to determine the protein expression in the vaginal mucus in Hanwoo cattles spontaneous estrus or CIDR induced-estrus.

MATERIALS AND METHODS

1. Animals

The Institutional Animal Care and Use Committee at the National Institute of Animal Science approved all protocols and procedures used in this study. Hanwoo cattles ($n = 23$) were aged 2~4 years used. All cattles had a body condition score of 4 or 5 on a 1~5 scale (Rae *et al.,* 1993). Uterine health was confirmed by cytological study of vaginal mucus smears. The cattles of all ages were assigned to one of the as following 2 groups: 1) Spontaneous estrus. Ten cattles, who had been observed in estrus, received no treatment and served as the group of cattles with normal spontaneous estrus. 2) Controlled internal drug release device (CIDR insertion = Day 0) induced estrus. Thirteen cattles in the CIDR received an CIDR insert (1.38 g of progesterone; Pfizer Animal Health, New York, NY) on day14 were removed and cattles were injected GnRH (100 μg, i. m.; Cystorelin, Merial, Athen, GA) on day 15.

2. Estrus Detection

The worker and farm personal detected estrus by direct observation of the behavior of cattles twice daily (morning and afternoon) for 1 hr each time and by observation of external genitals of cattles. Standing to be mounted, the red color and the swelling of vagina, and clear vaginal mucus were the criteria used by the investigator and the farm personal to determine estrus.

3. Collection of Vaginal Mucus

Vaginal mucus samples were collected from all cattles at the same time the single AI in cattles with spontaneous estrus and before the AI in cattles with induced estrus. Specifically, samples were collected after observed estrus, and at day 2 after the removal of CIDR-induced estrus, and at day 1 after the injection of GnRH. The vagina of the cattle was washed with disinfectant solution of povidone iodine (Yuhan Corporation, Seoul, Korea), rinse with water, and dried. Vaginal mucus samples were collected using a pipette of 10 ml. Vaginal mucus was placed into a 50 ml Falcon plastic tube and transported to the laboratory in a refrigerated box (4℃). Aliquots of 2 to 3 ml of vaginal mucus were homogenized in a homogenizer (Fisher Scientific, Pittsburgh, PA. USA) and stored at −80 ℃ for future use in measurement of protein chip analysis.

4. Artificial Insemination (AI)

After collecting the vaginal mucus samples, all cattles were inseminated with frozen semen of proven fertility once for cattles with a spontaneous estrus and for cattles with an induced estrus, at day 2 after the removal of the CIDR and at day 1 after the injection of GnRH. All estrus induced cattles were observed for estrus signs and double blind AI was performed. Pregnancy rates to AI were determined by transrectal ultrasonography (Aloka 500V equipped with a 5.0-MHz linear-array transducer; Aloka, Wallingford, CT) on day 45 after AI. Final pregnancy was diagnosed by rectal palpation 3 month AI.

5. Vaginal Mucus Fractionation

For the anion exchange fractionation, 1 μl of each vaginal mucus samples were denaturated in 9 M urea, 2% 3-[(2-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate hydrate (CHAPS), and 50 mM Tris-HCl, pH 4.0. They were separated on a 96-well filter plate containing Qhyper DF resin (BioInfra Corporation, Seoul, Korea) into two different fractions according the manufacturer's instructions. The wash buffers for the different fractions were 50 mM Tris-HCl, 0.1% n-octyl b-D-glucopyranoside (OGP), pH 4.0 each samples.

6. SELDI-TOF MS Analysis

Spontaneous and CIDR-induced estrus vaginal mucus samples were analyzed on two different array surfaces: cation-exchange (CM10), anion-exchange (Q10). Samples were randomly applies to a ProteinChip array surface (Ciphergen Biosystems, CA. USA) in a 96-well format. 5 μg of each sample was added to the binding buffer, and the arrays were incubated for 30 min with vigorous shaking. Arrays were then washed three times with 100 μl of binding buffer, followed by a final wash with water. Binding buffers used for the different arrays were 100 mM NaAC pH 4.0 for CM10; 100 mM Tris-HCl pH 9.0 for Q10. Arrays were removed from the bioprocessor and allowed to air dry. 1 μl of 50 % sinapinic acid was applied twice to the spots. Finally, arrays were analyzed on a PBSIIc ProteinChip Reader (Serise-4000, Ciphergen Biosystems, CA. USA). The data were averaged over 200 laser shots for each spot. Mass detection accuracy of PBSIIc was calibrated externally by using the All-in-1 protein II molecular mass standards

(Ciphergen Biosystems, CA. USA). To minimize experimental variation, samples were analyzed concurrently, and no sample was subjected to more than two freeze-thaw cycle. In all experiments, SELDI-TOF MS profiles were obtained in triplicate for each sample.

7. Protein Separation

To separate the protein differential expression is the same cation exchange ProteinChip CM10 Spin Columns (Bio-Rad Laboratorise, Inc. UK) were used. Spontaneous and CIDR-induced estrus vaginal mucus samples by added binding buffer 0.2 ml to 0.1 ml spin column was added to the reaction at room temperature 30 min. We separate centrifugally for 30 sec by 3,000 × g after a reaction and remove a reaction frame, and, using binding buffer 0.5 ml, it was washed 3 times. Using the NaCl solution by which the proteins combined after washing are 0.5, 1 and 2 M, it was fractionated and a protein was collected successively. The concentration of proteins recovered by Bradford *et al.* (2005) measurement methods, and 12% acryamide gel protein was in the electrophoresis. After electrophoresis, silver stain method (Hochstrasser *et al.*, 1998) to visualize the protein, the protein was confirmed by the gel obtained from the protein was used for charity.

8. Protein Identification

Visible changes in protein spot from the gel was cut and the protein used in the experiment for identification. For protein identification used trypsin enzymes which cut only the unique part of the protein and resolved with peptide. Stained by silver stain gel remove from the silver ion 30 ml potassium ferricvanide and 100 mM sodium thiosufate to 1:1 (v/v) a solution to the mixed reaction by adding 15 min to make stirring. After executing a de-stain reaction, 100 mM ammonin bicarbonate solution 300 μl and uses 100% acetonitril and of 15 min and after each 2 times reacting, completely constructed gel. Of the fried gel slice 130 ng for sequencing grade modified trypsin cheomgahan containing digestion buffer and 30℃ for 16 hr in response to peptide and protein, a small part. Move to a new tube after the reaction buffer, completely dry, then, 0.1% trifluoroacetic acid, 50% acetonitril solution melted to 2 μl was used for protein identification. Cutting through the enzyme reaction mass spectrometry of the peptide solution suffered acquired Applied Biosystems 4700 Proteomics analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems) was performed using the protein identification. Mass spectrometry of protein peptide mass value of the equipment through the Mascot program analyzed using the SWISS-Prot and NCBI database with the same mass value using to search for proteins and protein occurrence of protein score, and compare the molecular weight and pI values check the change of the protein.

RESULTS AND DISCUSSION

Conception rates for spontaneous and induced-estrus ranged from 63% and 71%, respectively. In Hanwoo, spontaneous and CIDR-induced estrus vaginal mucus samples to the investigation of the differential protein expression surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) technique was used (Fig. 1). Cation exchange between the two groups through the result of comparing the protein expression patterns by the approximately 100 types of proteins were detected (data not shown). In the present study, Hanwoo vaginal mucus collected from spontaneous estrus was abundant and clear, while that from most induced estrus cattles was demonstrated in protein expression patterns. SELDI-TOF MS has combined specially designed proteinChip array with surface ionization mass spectrometry to provide an interesting method for identifying new investigation marker in complex biological fluids. In most studies, diluted or depleted samples are spotted onto different proteinChip array and analyzed by SELDI-TOF MS. Since our goal was to maximize the number of peptides and proteins under investigation, we assessed the vaginal mucus fractionation protocol on different chromatographic surface array and analyzed the proteins by SELDI-TOF MS. Same amount of fractionated spontaneous and induced estrus vaginal mucus were applied on two surface arrays with anionic (Q10), cation (CM10) surface chemistry. A representative view of the distribution of protein/peptides in fraction 1 and 2 are shows in Fig. 1. Ionic

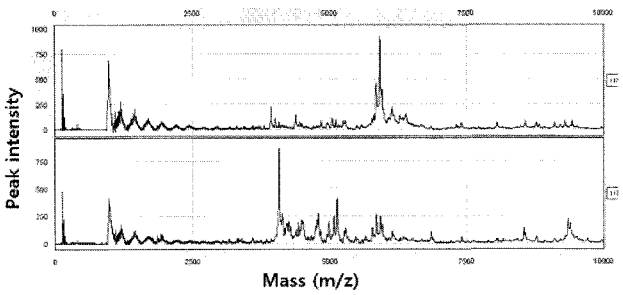

Fig. 1. SELDI mass spectrum of spontaneous (upper) and CIDR induced-estrus (under) vaginal mucus in Hanwoo cattles. Data were obtained using a CM10 array.

surfaces chemistries (Q10 and CM10) gave the best results in terms of detectable protein peaks, with more than 100 protein peaks in the two fractions and under each condition. Other studies reported, enzymes activity in cervical mucus during the spontaneous and induced estrus has been previously described (Boyd *et al.*, 1972; Linford, 1974; 1981; Al-Fknah *et al.*, 1991). Moreover, recent reported that was increased in ability of sperm to migrate in goat cervical mucus (Cox *et al.*, 2006). More than 4 folds between the two group shows the difference in protein expression to their identification with the same characteristics to take advantage of the share exchange column was the packing, 0.5, 1 and 2 M NaCl was separated by using the sequential order. Proteins obtained by the fractionation were separated in SDS-PAGE and the dye was to determine the protein. Of 1M NaCl concentration of protein expression in the most fractionated, and shows the difference between a dual-expressed was confirmed 11 types protein (Fig. 2). This result suggests indicate that both the revelation of during spontaneous and induced estrus vaginal mucus specific proteins with change of the hormones together must help the movement of amendment and the spermatozoa. In addition, the described by Chretine (2003) that aspiration of cervical mucus into capillaries maintains the microstructure that characterizes that found in the cervical channel validates it as a reliable *in vitro* assay to assess sperm-mucus interaction. Protein mass spectrometer using 11 different proteins in protein identification of 7 were

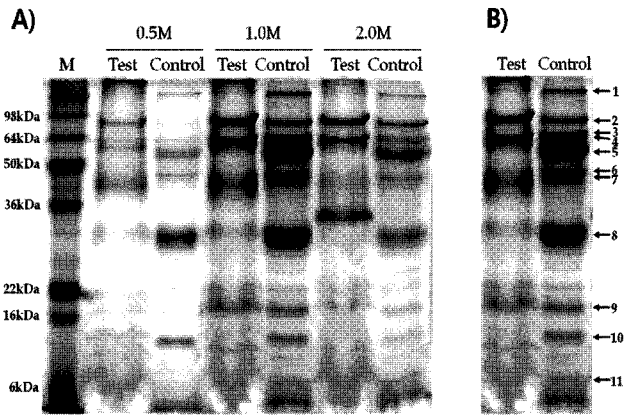

Fig. 2. SDS-PAGE profiles between spontaneous and CIDR induced-estrus vaginal mucus samples. A) Protein expression patterns of spontaneous (Test) and induced-estrus (Control) vaginal mucus samples. B) Eleven proteins were selected for mass analysis using 4700 Proteomics Analyzer (Applied Biosystems).

able to determine the protein (Table 1). List of identified proteins as follows; Ribosome-binding protein 1, GRIP 1-associated protein 1, Katanin p60 ATPase-containing subunit A-like 1, Protein FAM44A, DUF729 domain-containing protein 1, Prolactin precursor, Dihydrofolate erductase. As shown in Fig. 2 B, numbers 2, 4 and 7 proteins the strong expression is coming to seem from spontaneous (Test) and induced estrus (Control) protein expression aspects. In addition, between the two

Table 1. Characterization of differentially expressed proteins between spontaneous and induced-estrus vaginal mucus in Hanwoo cattle

Band No.	Protein name	Accession number	Entry name	Sequence coverage (%)	Mw (Da)	pI
1	Ribosome-binding protein 1	Q99PL5	RRBP1_MOUSE	32	173232	9.35
2	GRIP1-associated protein 1	Q8VD04	GRAP1_MOUSE	27	92943	5.2
3	Katanin p60 ATPase-containing subunit A-like 1	Q5XIK7	KATL1_RAT	51	55508	6.67
4	Protein FAM44A	Q8NFC6	FA44A_HUMAN	28	55196	5.17
5	Not identified			–	–	–
6	DUF729 domain-containing protein 1	Q8VED8	FA54A_MOUSE	55	41131	6.05
7	Not identified			–	–	–
8	Prolactin precursor (PRL)	P06879	PRL_MOUSE	49	25879	5.28
9	Dihydrofolate reductase	Q920D2	DYR_RAT	37	21550	6.95
10	Not identified			–	–	–
11	Not identified			–	–	–

Accession number is SWISS-PROT database number (MALDI-TOF/TOF).

groups of about 30% in the expression of protein was the difference. It's probably differences in the protein expression in the vaginal mucus among spontaneous and induced-estrus, conception rates were did not significant difference, suggesting that protein expression of the vaginal mucus is directly affect fertility in Hanwoo cattles. It is well known that in synchronization program there are substantial differences in the interval from luteolysis to estrus and ovulation (Roche *et al.*, 1981). Conclusively, on the basis of this study, protein expression in the vaginal mucus could be used as an indicator for time of estrus manifestation in order to increase conception rates by applying AI at an optional time.

REFERENCES

Al-Eknah MM, Homeida AM, Galil AKA and Elbashir AE. 1991. Oxytocin-induced biochemical changes in cervical mucus of the goat. Theriogenology 36:143-148.

Boyd LJ, Gibbons AR, Tasker JB. 1972. Characteristics of cervical mucus from progestagen-treated cattle. Br. Vet. J. 128: 260-269.

Bradford WD, Cahoon L, Freel SR, Hoopes LL and Eckdahl TT. 2005. An inexpensive gel electrophoresis-based polymerase chain reaction method for quantifying mRNA levels. Cell Biol. Educ. 4:157-168.

Chretine FC. 2003. Involvement of the glycoproteic meshwork of cervical mucus in the mechanism of sperm orientation. Acta Obstet. Gynecol. Scand. 82:449-461.

Cox JF, Alfaro V, Montenegro V and Rodriguez-Martinez H. 2006. Computer-assisted analysis of sperm motion in goat and its relationship with sperm migration in cervical mucus. Theriogenology 66:860-867.

Doehr SA and Moghissi KS. 1973. Human and bovine cervical mucins. In: Blandau RJ, Moghissi KS. Editors. The Biology of the Cervix. Chicago: The University of Chicago Press, pp. 125-142.

Hawk HW. 1987. Transport and fate of spermatozoa after insemination of cattle. J. Daily Sci. 70:1487-1503.

Hochstrasser DF, Harrington MG, Hochstrasser AC, Miller MJ and Merril CR. 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. Analy. Biochem. 173:424-435.

Linford E. 1974. Cervical mucus: an agent or a barrier to conception. J. Reprod. Fertil. 37:239-250.

Prasad A, Kalalyan NR, Bachlaus NK, Arora RC and Pandey RS. 1981. Biochemical changes in the cervical mucus of buffalo after induction of estrus with prostaglandin $F_2\alpha$ and cloprostenol. J. Reprod. Fertil. 62:583-587.

Rae DO, Kunkel WE, Chenoweth PJ, Sand RS and Tran T. 1993. Relationship of parity and body condition score to pregnancy rates on Florida beef cattle. Theriogenology 39: 1143-1152.

Roche JF, Ireland J and Mawhinney S. 1981. Control and induction of ovulation in cattle. J. Reprod. Fertil. 30:211-222.

Van Klinkenberg GA. 1953. Extremely high alkaline phosphatase activity in the vaginal mucus of the cow. Nature 172: 397.

(접수일: 2008. 11. 16 / 채택일: 2008. 11. 26)