The Analysis of Seminal Plasma Proteins by Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE) in Hanwoo (Korean Native Cattle)

Yong-Seung Lee[1], Eun-Ji Song[1], Han-Jun Yoo[1], Joung-Jun Park[3], Hee-Tae Cheong[2], Boo-Keun Yang[1] and Choon-Keun Park[1,*]

[1]College of Animal Life Science, Kangwon National University, Chuncheon 200-701, Korea
[2]College of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea
[3]Myung-poom Hanwoo Consulting, Hoengseung 225-808, Korea

ABSTRACT

This study was to evaluate the protein profile of seminal plasma using 2-DE in Hanwoo. Seminal plasma was harvested from five mature Hanwoo, and seminal plasma protein was extracted by M-PER Mammalian Protein Extraction Reagent. Proteins were refined by clean-up kit and quantified by Bradford method until total protein was 300 μl. Immobilized pH gradient (IPG) strip was used 18 cm and 3~11 NL. SDS-PAGE was used 12% acrylamide gel. Each gels were visualized by comassie brilliant blue and silver staining. These spots were analyzed by MALDI-TOF MS and searched on NCBInr. The result, 20 proteins of 36 protein spots were searched through peptide sequencing on the NCBInr. 8 proteins profiled by 2-DE were proved through previous bovine studies and the name of each protein was albumin, nucleobindin, clusterin, TIMP-2, spermadhesin Z13, spermadhesin-1 and BSP proteins (BSP 30 kDa and BSP A1/A2). 12 new proteins were ATP synthase, protein MAK16 homolog, Transmembrane protein 214, E3 ubiquitin-protein ligase BRE1A, dual serine/threonine and tyrosine protein kinase, tissue factor pathway inhibitor 2, alpha-actinin-4, RUN domain-containing protein 3B, catenin alpha-1, protein-glutamine gamma-glutamyltransferase 2, plakophilin-1 and inter-alpha-trypsin inhibitor heavy chain H1 has not been previously described in the bovine seminal plasma study. These proteins may be contribute to define the type of proteins affecting fertility of male and improve the fertilizing ability of semen in Hanwoo.

(Key words : seminal plasma, 2D-PAGE, MALDI-TOF, Korean native cattle, proteins)

INTRODUCTION

Seminal plasma, an amorphous material existing in semen, comprises fluids from the testis and epididymis, and secretions from the sexual accessory glands of male reproductive tract. Seminal plasma contains proteins, lipids, carbohydrates, enzymes, vitamins, hormones, and trace elements, and becomes a micro-enviroment for spermatozoa survival after ejaculation (Chiu and Chamley, 2003; Miro *et al.*, 2005; Troedsson *et al.*, 2005; Cardozo *et al.*, 2006). Seminal plasma contains a variety of biochemical components, some of which are relatively specific for the regulation of sperm function (Strzezek *et al.*, 1992). The protein composition of seminal plasma has important effects on sperm motility (Sa'nchez-Luengo *et al.*, 2004), viability and fertilization (Brandon *et al.*, 1999).

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a valuable tool for the separation and characterization of proteins from complex biological samples. Many proteins from seminal plasma of various species have been described and characterized and 2-DE has been widely used for their separation and analysis from bovine (Jobim *et al.*, 2004), equine (Brandon *et al.*, 1999), ram (Jonbim *et al.*, 2005), boar (Sanz *et al.*, 1993), ovine (Cardozo *et al.*, 2006) and human (Ding *et al.*, 2007).

However, seminal plasma proteins in Hanwoo are still poorly understood. Also, in previous bovine studies with 2-DE, a total of 25 spots was found in bovine seminal plasma (Jobim *et al.*, 2004). The seminal plasma protein profile, using 2-DE, has not been described yet in Hanwoo. Therefore, the objective of this study was to evaluate the protein profile of seminal plasma using 2-DE in Hanwoo.

MATERIALS AND METHODS

† This work was supported by Grant No. 109007-3 from Agricultural R&D Promotion Center (ARPC), Republic Korea.
* Correspondence : E-mail : parkck@kangwon.ac.kr

1. Sample Collection and Processing

Semen from five mature Hanwoo were collected by artificial vagina. Seminal plasma was harvested from 1 ml of semen by centrifugation at 10,000 × g for 30 min at 4℃. The resultant supernatant was centrifuged again, and seminal plasma was kept at −80℃ for freeze drying. Seminal plasma protein was extracted from the freeze dried seminal plasma powder using freeze dryer by M-PER Mammalian Protein Extraction Reagent (Thermo, USA). Proteins were refined by clean-up kit (GE Healthcare, USA), and protein content was quantified with 300 μg using Bradford's method.

2. Two Dimension Electrophoresis (2-DE)

Isoelectrofocusing (IEF) was performed with IPGphor unit (Amersham Biosciences, USA) using precast 18 cm pH 3~11 nonlinear IPG gel strips (Amersham Biosciences). 300 μg of total proteins were mixed with the rehydration solution (7 M urea, 2 M thiourea, 4% w/v CHAPS, 50 M DTT and trace of bromophenol blue) to the total volume of 250 ml and incubated for 16 hr at room temperature (RT). Then, IEF was performed at 250 V for 2 hr, 8,000 V for 3 hr, and finally until total 60,000 V. After IEF separation, the gel strips were immediately equilibrated in the equilibrium buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, and 2% w/v SDS. The second dimension separation was carried out in 12% SDS-PAGE gels. Then, electrophoresis was performed using Protean II xi 2-D cell (Bio-Rad, USA) with 25 mA until the bromophenol blue reached the bottom of the gel.

3. Protein Stain

Gels were immersed in a solution of 0.1% Comassie Brilliant Blue R-250 (Sigma, USA), 45% methanol, 10% acetic acid and 45% water, and were stained overnight. The gels were destained in a mixture of 30% methanol, 10% acetic acid and 60% water.

Silver Staining Kit (GE Healthcare, USA) was used in 2-DE gels. The gels were fixed in 40% ethanol and 10% acetic acid for 30 min, sensitized in ethanol glutardialdehyde (25% w/v), sodium thiosulphate (5% w/v), and sodium acetate (17 g) for 30 min followed by 3 times washing with water for 15 min in each time. Then, the gels were immersed in silver nitrate (2.5% w/v) and formaldehyde (37% w/v) for 20 min, developed with sodium carbonate (6.25 g) and formaldehyde (37% w/v) for 2~5 min, and stopped in EDTA-Na_2-$2H_2O$.

4. Proteomic Analysis by MALDI-TOF

The Comassie brilliant blue stained gel was scanned with the Image Scanner (Amersham Biosciences) and analyzed with the Phoretix Expression software v.2005 (Nonlinear Dynamics, UK). Destaining and in-gel trypsin digestion of the protein spots were performed as described previously. Xcise (Shimadzu Biotech Co, Japan), the automatic sample preparation system, was used for in-gel digestion, desalting, and plating. The desalting was performed with ZipTip C18 (Millipore, USA) and the plating with the 4-hydroxy-α-cyano-cinnamic acid (HCCA) matrix solution onto a MALDI-TOF MS plate. In-gel digested peptides were analyzed with MALDI-TOF MS spectrometer, Ultraflex-TOF/TOF (Bruker Daltonics, Germany). Peptide mass fingerprinting (PMF) ion search was performed using MASCOT 2.0 software and NCBInr protein databases.

RESULTS

This study have analyzed seminal plasma protein from Hanwoo using the 2-DE method. The distribution of Hanwoo seminal plasma proteins on Comassie Brilliant Blue (CBB) and silver stained gels are shown in Fig. 1. A total of 36 CBB-stained spot and 71 silver-stained spots were identified on the each gel by image analysis.

The identified 36 CBB stained protein spots were analyzed by MALDI-TOF MS. The protein was identified using the NCBInr database, and the result is shown in Table 1. The 20 proteins of 36 protein spots were searched through peptide sequencing on the NCBInr database after MALDI-TOF MS analysis. Spot No. 1 and 3, 6 and 17, 9 and 10, 12~16, 19~21, 23 and 31, 24 and 25, 27 and 28, finally 43~45 and 60 spots were confirmed with each same protein.

DISCUSSION

This study was performed for a comprehensive protein profile of seminal plasma from Hanwoo (Korean Native Cattle) using the 2-DE. And, 2-DE spots were visualized through CBB and silver stain (Fig. 1). Most of all, we selected that CBB stained spots has relatively large protein in spot than silver stained spots for effective protein analysis by MALDI-TOF. The result, 20 proteins of 36 protein spots were searched through peptide sequencing on the NCBInr database and MALDI-TOF MS (Table 1). 8 of 20 proteins profiled by 2-DE were proved through previous bovine studies (Jobim *et al.*, 2004; Moura *et*

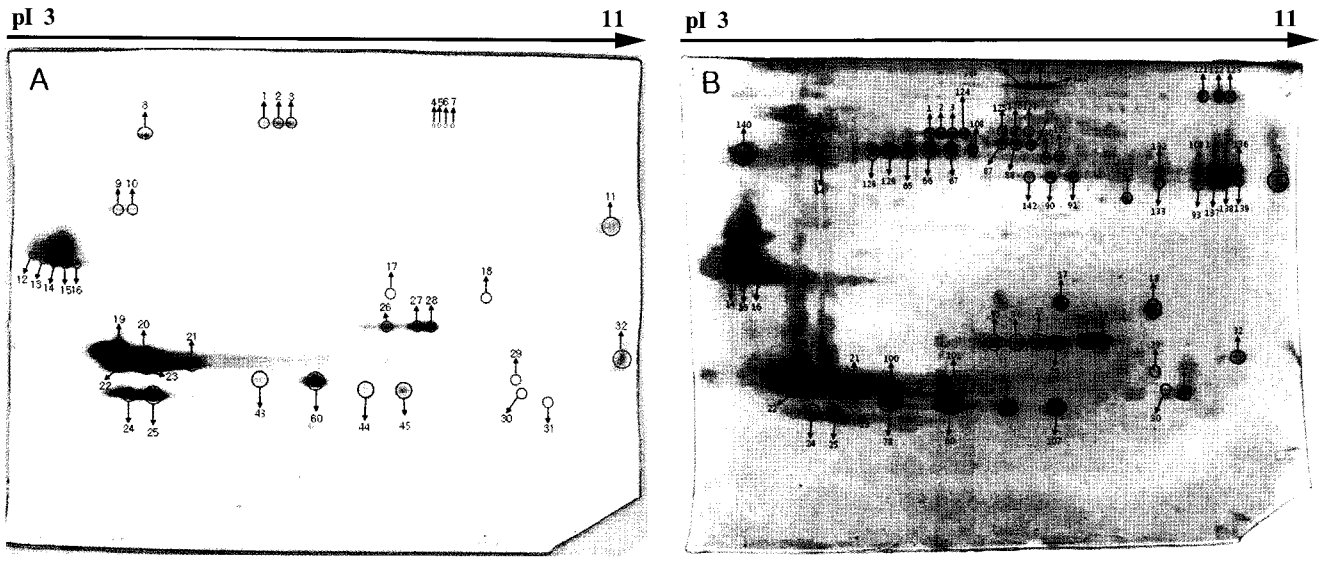

Fig. 1. Two-dimensional polyacrylamide electrophoretic gel of Hanwoo seminal plasma proteins. Two-dimensional 12% SDS-PAGE gel stained with CBB A and silver B. A: red circle was CBB stained spots; B: blue circle was only silver stained spots, red circle was indicated with spots both at CBB and silver. 300 μg of total protein was loaded to the 2-DE gel electrophoresis. First dimension used 18 cm, pI 3~11 NL strip and second dimension used 12% acrylamide gel.

al., 2007), and the name of 8 proteins was albumin, nucleobindin, clusterin, TIMP-2, spermadhesin Z13, spermadhesin-1 and BSP proteins (BSP 30 kDa and BSP A1/A2).

Protein spot 1, 3 probably corresponds to albumin, which is binds to sperm, and may also influence capacitation through its ability to modulate membrane cholesterol (Visconti and Kopf, 1998). Protein spot 12~16 and 19~21, 23, 31 corresponds to the complex of BSP proteins (BSP 30 kDa and A1/A2) present in seminal plasma, BSP 30 kDa are known to induce cholesterol efflux from the sperm membrane (Manjunath and Th'erien, 2002), and along with its ability to mediate sperm capacitation. BSP A1/A2 previously called PDC 109 is the main heparin binding protein in bovine seminal plasma; it bound specifically to sperm choline-phospholipids during ejaculation and promoted capacitation by cholesterol and phospholipids efflux (The'rien *et al.*, 1999).

Protein spot 24, 25 corresponds to acidic seminal fluid protein (aSFP) is known to inhibit oxidative stress (Schoneck *et al.*, 1996). Bovine aSFP shares identity with proteins of protein spot 43~45, 60 corresponds to the spermadhesin family (Romao *et al.*, 1997). Binding of aSFP to ejaculated sperm occurs, but it is lost after capacitation (Dostolova *et al.*, 1994), suggesting that unlike porcine spermadhesins (Caballero *et al.*, 2004, 2005), bovine aSFP does not participate in sperm-oocyte interaction. Protein spot 9 and 10 corresponds to clusterin are similar to those reported in the cauda epididymis fluid of bulls (Ibrahim *et al.*, 1999). Clusterin is a multifunctional constituent of the seminal plasma and it can prevent oxidative damage to the sperm (Reyes-Moreno *et al.*, 2002), bind and agglutinate abnormal spermatozoa in bulls (Ibrahim *et al.*, 1999) and humans (O"Bryan *et al.*, 1994) and act like a chaperone, protecting sperm from the toxic effects of protein precipitation (Humphreys *et al.*, 1999; Wilson and Easterbrook-Smith, 2000). Clusterin has the ability to inhibit complement-induced sperm lysis (Ibrahim *et al.*, 1999). Protein spot 27 and 28 corresponds to TIMP-2 associates with bovine and human sperm membranes (McCauley *et al.*, 2001; Bechman-Shaked *et al.*, 2002) and inhibitors of Metalloproteinases (MMPs) released from the sperm during acrosome reaction facilitate sperm-egg fusion in hamsters (D'ıaz-Perez and Meizel, 1992), and TIMPs interfere with gamete fusion in mice (Correa *et al.*, 2000). Protein spot 8 corresponds to nucleobindin has been found secreted by bone cells and odontoblasts and as a structural element of their respective extracellular matrices (Petersson *et al.*, 2004; Somogyi *et al.*, 2004; Moura *et al.*, 2007). However, those functions on sperm physiology are still unknown.

The spot No. 2, 4~7, 11, 17, 18, 22, 26, 29, 30 and 32 identified in the 2-DE gels has not been previously described in the seminal plasma studies. Those are new proteins expressed in the seminal plasma fluid. Protein spot 2 corresponds to the

Table 1. Information of the polypeptide analysed in seminal plasma from Hanwoo

Spots No.	Protein name	Accession No.	Coverage (%)	MW (Da)	pI
1, 3	Serum albumin	P02769	10.4	69294	5.8
2	ATP synthase subunit beta, mitochondrial	P00829	11.2	54806	6.2
4	Protein MAK16 homolog	Q1RML7	17.1	35289	5.3
5	Transmembrane protein 214	A4FV45	11.5	77017	9.4
6, 17	E3ubiquitin-protein ligase BRE1A	A2VDP1	4.9	113659	5.7
7	Dual serine/threonine and tyrosine protein kinase	Q4TVR5	4.7	104805	6.4
8	Nucleobindin-1	Q0P569	28.3	54983	5.1
9, 10	Clusterin	P17697	15.7	51114	5.7
11	Tissue factor pathway inhibitor 2	Q7YRQ8	36.8	26675	9.1
12, 13, 14, 15, 16	Seminal plasma protein BSP-30 KDa	P81019	41.5	21269	5.7
18	Alpha-actinin-4	A5D7D1	6.7	104929	5.3
19, 20, 21, 23, 31	Seminal plasma protein PDC-109	P02784	32.8	15481	4.9
22	RUN domain-containing protein 3B	Q08E29	16.1	47034	5.3
24, 25	Spermadhesin-1	P29392 (2.72e+9)	73.9	15036	5.1
26	Catenin alpha-1	Q3MHM6	8.5	100134	5.9
27, 28	Metalloproteinase inhibitor 2	P16368	30.9	24355	7.4
29	Protein-glutamine gamma-glutamyltransferase2	P51176	10.6	77113	5.1
30	Plakophilin-1	Q28161	5.5	80181	9.2
32	Inter-alpha-trypsin inhibitor heavy chain H1	Q0VCM5	4.1	101238	7.0
43, 44, 45, 60	Spermadhesin Z13	P82292	46.6	13383	5.6

ATP synthase isolated from bovine heart mitochondria (*Bos taurus*) is the best characterized ATP synthase biochemically and structurally. Beef heart is used as a source of the enyzme because of the high concentration of mitochondria in cardiac muscle (Zoleo *et al.*, 2007). The spot No. 5 corresponds to Transmembrane protein 214, these proteins are present in the inner membranes of bacterial cells or the plasma membrane of eukaryotes, and sometimes in the outer membranes, and which may reflect their common evolutionary origin and similar folding mechanism (Almén *et al.*, 2009). Protein spot 6 and 17 corresponds to E3 ubiquitin-protein ligase BRE1A, those are this is a ubiquitin ligase required for the ubiquitination of histone H2B and the methylation of histone H3 (Yamashita *et al.*, 2004). Protein spot 32 corresponds to Inter-alpha-trypsin inhibitor heavy chain H1 is function as protease inhibitors (Zhuo *et al.*, 2004). Other unknown proteins in bovine seminal plasma are the Protein spot No. 2 (Protein MAK16 homolog), 7 (Dual serine/threonine and tyrosine protein kinase), 11 (Tissue factor pathway inhibitor 2), 18 (Alpha-actinin-4), 22 (RUN domain-containing protein 3B), 26 (Catenin alpha-1), 29 (Protein- glutamine gamma-glutamyltransferase 2) and 30 (Plakophilin-1).

In conclusion, this study is protein profile of seminal plasma from Hanwoo using the 2-DE, and we found that 12 new proteins has not been previously described in the bovine seminal plasma study. So far, this the role of those proteins on the fertility of the male Hanwoo are still unknown. Therefore, we suggest that additional research are necessary to define the types of proteins affecting fertility of male and the mechanisms of their actions. Additional research may contribute to the development of strategies to improve the fertilizing ability of semen in Hanwoo.

ACKNOWLEDGEMENTS

The authors thank the Cental Laboratory and Institute of Animal Resources at Kangwon National University for support and supply of the technical assistance and protein analytic instrumentation. The authors also thank the Hoengseong Livestock Cooperative for supply of seminal plasma samples.

REFERENCES

Almén MS, Nordström KJV, Fredriksson R and Schiöth HB. 2009. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. BMC Biology 7: 50.

Bechman-Shaked O, Kraiem Z, Gonen Y and Goldman S. 2002. Presence of metalloproteinases and tissue inhibitor of metalloproteinases in human sperm. J. Androl. 23:702-708.

Blaschuk O, Burdzy K and Fritz IB. 1983. Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J. Biol. Chem. 258:7714-7720.

Brandon CI, Heusner GL, Caudle AB, Fayrer-Hosken RA. 1999. Two dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology 52:863-873.

Caballero I, Vazquez JM, Gil MA, Calvete JJ, Roca J, Sanz L, Parrilla I, Garcia EM, Rodriguez-Martinez H and Martinez EA. 2004. Does seminal plasma PSP-I/PSP-II spermadhesin modulate the ability of boar spermatozoa to penetrate homologous oocytes *in vitro*? J. Androl. 25:1004-1012.

Caballero I, Vazquez JM, Rodriguez-Martinez H, Gill MA, Calvete JJ, Sanz L, Garcia EM, Roca J and Martinez EA. 2005. Influence of seminal plasma PSP-I/PSP-II spermadhesin on pig gamete interaction. Zygote 13:11-16.

Cardozo JA, Fernandez-Juan M, Forcada F, Abecia A, Muino-Blanco T and Cebrian-Perez JA. 2006. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. Theriogenology 66:841-850.

Chiu WW and Chamley LW. 2003. Human seminal plasma antibodybinding proteins. Am. J. Reprod. Immunol. 50:196-201.

Correa LM, Cho C, Myles DG and Primakoff P. 2000. A role of TIMP-3-sensitive, Zn^{2+}-dependent metalloprotease in mammalian gamete membrane fusion. Dev. Biol. 225:124-134.

D'ıaz-Perez E and Meizel S. 1992. Importance of mammalian endoprotease activity during the acrosome reaction to subsequent sperm-egg fusion: inhibitor studies with human sperm and zona-free hamster eggs. Mol. Reprod. Dev. 31:122-130.

Ding Z, Qu F, Guo W, Ying X, Wu M and Zhang Y. 2007. Identification of sperm forward motility-related proteins in human seminal plasma. Mol. Reprod. Dev. 79:1124-1131.

Howes EA, Hurst S, Laslop A and Jones R. 1998. Cellular distribution and molecular heterogeneity of MAC393 antigen (clusterin, beta chain) on the surface membrane of bull spermatozoa. Mol. Hum. Reprod. 4:673-681.

Humphreys DT, Carver JA, Easterbrook-Smith SB and Wilson MR. 1999. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J. Biol. Chem. 274:6875-6881.

Ibrahim NM, Troedsson MH, Foster DN, Loseth KJ, Farris JA, Blaschuk O and Crabo BG. 1999. Reproductive tract secretions and bull spermatozoa contain different clusterin isoforms that cluster cells and inhibit complementinduced cytolysis. J. Androl. 20:230-240.

Jenne DE, Lowin B, Peitsch MC, Bottcher A, Schimitz G and Tschopp J. 1991. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-1 in human plasma. J. Biol. Chem. 266:11030-11036.

Jobim MIM, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F and Mattos RC. 2004. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 61:255-266.

Jobim MI, Oberst ER, Salbego CG, Wald VB, Horn AP and Mattos RC. 2005. BSP A1/A2-like proteins in ram seminal plasma. Theriogenology 63:2053-2062.

Manjunath P and Sairam MR. 1987. Purification and biochimical characterization of three major acid proteins (BSP A1, BSP A2 and BSP A3) from bovine seminal plasma. Biochem. J. 7:685-692.

Manjunath P and Th'erien I. 2002. Role of seminal plasma phospholipid-binding proteins in sperm membrane lipid modification that occurs during capacitation. J. Reprod. Immunol. 53:109-119.

McCauley TC, Zhang HM, Bellin ME and Ax RL. 2001. Identification of a heparin-binding protein in bovine seminal fluid

as tissue inhibitor of metalloproteinases-2. Mol. Reprod. Dev. 58:336-341.

Miro J, Lobo V, Quintero-Moreno A, Medrano A, Pena A and Rigau T. 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. Theriogenology 1:1706-1716.

Moura AA, Chapman DA, Koc H and Killian GJ. 2007. A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. Animal Reproduction Science 98:169-188.

O'ryan MK, Mallidis C, Murphy BF and Baker HW. 1994. Immunohistological localization of clusterin in the male genital tract in humans and marmosets. Biol. Reprod. 50:502-509.

Palmer DJ and Christie DL. 1992. Identification of molecular aggregates containing glycoproteins III, J, K (carboxypeptidade H), and H (Kex2-related proteases) in soluble and membrane fractions of adrenal medullary chomaffin granules. J. Biol. Chem. 267:19806-19812.

Pankhurst JG and Bennet CA 1998. Easterbrook-Smith SB. Characterization of the heparin-binding properties of human clusterin. Biochemistry 7:4823-4830.

Petersson U, Somogyi E, Reinholt FP, Karlsson T, Sugars RV and Wendel M. 2004. Nucleobindin is produced by bone cells and secreted into the osteoid, with a potential role as a modulator of matrix maturation. Bone 34:949-960.

Romão MJ, Kölln, I, Dias JM, Carvalho AM, Romero A, Varela PF, Sanz L, Töpfer-Petersen E and Calvete JJ. 1997. Crystal structure of acidic seminal fluid protein (ASFP) at 1.9°A resolution: A bovine polypeptide of the spermadhesin family. J. Mol. Biol. 274:650-660.

Sánchez-Luengo S, Aumüller G, Albrecht M, Sen PC, Röhm K, Wilhelm B. 2004. Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine sperm membrane Ca^{2+}-ATPase. J. Androl. 25:234-244.

Schoneck C, Braun J and Einspanier R. 1996. Sperm viability is influenced *in vitro* by the bovine seminal protein aSFP: effects on motility, mitochondrial activity and lipid peroxidation. Theriogenology 45:633-642.

Somogyi E, Petersson U, Sugars RV, Hultenby K and Wendel M. 2004. Nucleobindin- Ca^{2+}-binding protein present in the cells and mineralized tissues of the tooth. Calc. Tiss. Int. 74:366-376.

Strzezek J, Kordan W, Kostyra H and Zaborniak A. 1992. Purification and partial characterization of a 5700 Da sperm motility inhibiting factor from seminal plasma of boar. Anim. Reprod. Sci. 29:35-52.

Sylvester C, Morales R, Oko R and Griswold MD. 1991. Localization of sulfated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. Biol. Reprod. 45:195-207.

The'rien I, Moreau R and Manjunath P. 1999. Bovine seminal plasma phospholipid-binding proteins stimulate phospholipid efflux from epididymal sperm. Biol. Reprod. 59: 768-776.

Troedsson MH, Desvousges A, Alghamdi AS, Dahms B, Dow CA, Hayna J, Valesco R, Collahan PT, Macpherson ML, Pozor M and Buhi WC. 2005. Components in seminal plasma regulating sperm transport and elimination. Anim. Reprod. Sci. 89:171-186.

Visconti PE and Kopf GS. 1998. Regulation of protein phosphorylation during sperm capacitation. Biol. Reprod. 59: 1-6.

Wilson MR and Easterbrook-Smith SB. 2000. Clusterin is a secreted mammalian chaperone. TIBS 25:95-98.

Yamashita K, Shinohara M and Shinohara A. 2004. Rad6-Bre1-mediated histone H2B ubiquitylation modulates the formation of double-strand breaks during meiosis. PNAS 101 (31):11380-11385.

Zhuo L, Hascall VC and Kimata K. 2004. "Inter-alpha-trypsin inhibitor, a covalent protein-glycosaminoglycan-protein complex". J. Biol. Chem. 279 (37):38079-38082.

Zoleo A, Lippe G, Contessi S, Brustolon M, Dabbeni-Sala F and Maniero AL. 2007. Conformational role of the divalent metal in bovine heart mitochondrial F1-ATPase: An electron spin echo envelope modulation study. Biochemistry 46: 13443-13450.

(접수: 2010. 11. 18 / 심사: 2010. 11. 19 / 채택: 2010. 11. 30)