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Identification of bovine seminal plasma proteins with an activity to normalize endometrial epidermal growth factor concentrations in repeat breeder cows

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Abstract

Epidermal growth factor (EGF) concentrations in the bovine uterus show two peaks on Days 2-4 and 13-14 during the estrous cycle in normal cows; however, these peaks were not found in about 70% of Holstein repeat breeder cows. We have demonstrated the effect of seminal plasma (SP) to normalize the endometrial EGF concentrations on Day 3 and restore fertility in repeat breeder cows. The objective of this study was to identify SP protein(s) with the activity of normalizing the endometrial EGF concentrations. Semen was collected from 5 Holstein bulls and pooled SP obtained from 30 ejaculates were used for this study. The SP protein were separated by gel filtration and 2-dimensional electrophoresis. SP fractions with molecular weight of 16-29 kDa and pI5.8-7.0 showed an activity to normalize the endometrial EGF concentrations on Day 3. Then, protein spots in these area on electrophoresis gels were extracted and subjected to liquid chromatography with tandem mass spectrometry analysis. Twelve protein spots that include four spots of osteopontin (OPN) with different molecular weights and isoelectric points were identified. Protein extracts of one of these OPN spots normalized the endometrial EGF concentrations on Day 3 in 41.9% of repeat breeder cows. The present results indicated that OPN may be the molecule responsible for the activity normalizing the EGF concentrations on Day 3 in the endometrium of repeat breeder cows.

Key Words: epidermal growth factor, osteopontin; seminal plasma, repeat breeder cows

Introduction

In normal cows, the endometrial EGF concentrations in the uterine endometrium show two peaks on Days 2-4 and 13-14 of the estrous cycles, and the loss of these peaks of the EGF profile, even in the presence of an apparently normal estrous (ovarian) cycle,

results in reduction of fertility in dairy cows[18–20]. An alteration (*i.e.*, loss of the peaks in the EGF profile) of the endometrial EGF concentrations is found in about 70% of Holstein repeat breeder cows[19,20]. Further, a single examination of the EGF concentrations on Day 3 could appropriately determine the endometrial EGF profile. All fertile cows show an increased EGF concentration in the endometrium on Day 3[19] and loss and recovery of the two peaks of the endometrial EGF concentrations coincide in most cases[21,22].

This alteration explains, at least in part, an increased incidence of embryonic loss due to the potential role of EGF in embryonic development during the preimplantation period[17]. For this abnormality, we have reported a treatment protocol with a high dose of estradiol benzoate in combination with a progesterone releasing device[22]. This protocol normalizes the EGF profile in about 60-70% of repeat breeder cows. However, the efficacy of the treatment to normalize the endometrial EGF concentrations varies among herds from about 30% to 80% in the field[16], although the reasons for this variation of treatment efficacy are unknown. In addition, use of estrogen products in food animals has been restricted in many countries and regions. Thus, it is necessary to develop an additional treatment option.

Seminal plasma (SP) contains estrogen and testosterone, several prostaglandins and glycoprotein signaling substances, including several cytokines and growth factors[1,29]. SP has conventionally been viewed as a transport and survival medium for mammalian sperm; however, its role now extends beyond this process to actively targeting female tissues. Studies in rodents[5,40], pigs[43], horses[39] and human[48] reported that SP induces molecular and cellular changes within the endometrium or cervix following insemination. Further, the physiological response to SP in female reproductive tract suggests that SP can improve reproductive outcomes and potentially even the health and development of offspring[4,40]. For example, roles of SP in the improvement of

fertility by modulating uterine functions have been indicated in pigs[35], horses[39], hamsters[33] and mice[42]. SP infusion into the uterus stimulated an inflammatory response leading to changes of cytokines and growth factors in the uterus to facilitate pregnancy[4,23,40]. Similarly, bull SP has been suggested to contain a variety of proteins associated with fertility through the effects on sperm and uterine functions[10,26]. An earlier study reported a regression model to predict bull fertility using 4 fertility-associated protein densities[25]. However, in cattle, large-scaled studies failed to demonstrate beneficial effect of infusions of SP[38] and transforming growth factor[38], a putative fertility-associated protein, into the uterus on fertility. Our previous study[16], however, suggested that SP infusion into the vagina, but not the uterus, normalized the endometrial EGF concentrations on Day 3 and partially restored fertility in repeat breeder cows[2]. SP normalized the endometrial EGF concentrations on Day 3 and restored fertility in 60% and 50%, respectively.

Therefore, the present study aimed at identifying proteins with the activity. First, we separated SP proteins using gel filtration and 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and obtained a crude protein preparation that contains the activity to normalize the endometrial EGF concentrations in the repeat breeder cows. Then, we identified proteins in the crude protein preparation using 2D-PAGE and liquid chromatography with tandem mass spectrometry (LC-MS/MS). Finally, we examined the effect of an identified protein spot, a form of osteopontin (OPN) to normalize the endometrial EGF concentrations.

Materials and Methods

Animals

All animal experiments were conducted according to guidelines for Care and Use of the experimental animal protocol of Hokkaido University, Japan (Experimental protocol # 16-

0071). Repeat breeder cows were diagnosed by local practitioners using the criteria of failing to conceive after three or more artificial insemination (AIs) without a detectable abnormality in clinical signs, the estrous cycle and genital organs. All cows were then confirmed to meet the definition of repeat breeders with additional examinations that included transrectal ultrasonography of the genital organs, uterine cytology and oviductal patency by one of the authors before enrollment to the study. Repeat breeder cows were observed for estrus three times a Day (Day 0 = estrus) and examined for the endometrial EGF concentrations on Day 3 by using biopsy samples. These cows showing low EGF concentrations (< 4.7 ng/g tissue weight)[17] were used in this study. All repeat breeder cows were multiparous lactating Holstein cows (> 10,000 kg, 305-days fat-corrected milk) between three and six years of age and between 125 and 192 days postpartum on the Day of the first biopsy for EGF measurement. All cows did not receive any therapeutic treatment for infertility.

Biopsy of uterine endometrial tissues

Uterine endometrial tissues were obtained by biopsy using a biopsy instrument (Fujihira Industry, Tokyo, Japan) with caudal epidural anesthesia (3 ml of 2% lidocaine; 2% xylocaine, Fujisawa Pharmaceutical, Osaka, Japan) as described previously[20]. Tissues were frozen in liquid nitrogen within 10 min of collection and stored at –800C until the EGF assay.

Measurement of the endometrial EGF concentrations and judgment of the EGF profile

Concentrations of EGF in uterine tissues were determined by a double-antibody sandwich enzyme immunoassay using 96-well microtiter plates[21]. Anti-human EGF mouse monoclonal antibody (R&D Systems, Inc., Minneapolis, MN, USA) and anti-human EGF rabbit antiserum (Biogenesis Ltd., Poole, UK) used for a solid-phase and detection antibody, respectively. Both antibodies do not show significant cross-reactivity

with other cytokines tested by manufacturers. The assay system has been verified using increasing concentrations of recombinant bovine EGF. Linear regression analysis of recombinant bovine EGF concentrations and assay results gave $y = 0.96x + 0.39$, $r = 0.97$[24]. The intra- and interassay coefficients of variation were 5.6% and 7.4%, respectively. The sensitivity of the assay was 10 pg/well. The endometrial EGF concentrations on Day 3 were judged normal when the concentrations were 4.7 ng/g tissue weight or greater[17,19].

Preparation of SP samples

Semen was collected twice a week from five Holstein bulls with known fertility using artificial vagina at a commercial AI center (Genetics Hokkaido, Tokachi Shimizu, Hokkaido, Japan). Two ejaculates were collected on each day usually with a 30 min interval. SP was separated by centrifugation at 1,000 x g for 10 min. SP was frozen at -20°C and transported to the Hokkaido University. At the university laboratory, SP were thawed and centrifuged at 5,000 x g for 20 min at 4°C and the resulting supernatants from 5 bulls were pooled and used as SP samples.

Gel filtration by column chromatography

At each sample preparation, 0.5 ml of dialyzed SP that was obtained and pooled from 5 bulls was applied on a Sephadex G-200 column (0.7 x 75 cm) and eluted with 25 mM phosphate buffer containing 0.1 M NaCl (pH 7.2) at a flow rate of 2 ml/h at 4°C. Fractions at 1.25 ml/tube were collected. The column was calibrated with thyroglobulin, bovine gammaglobulin, chicken ovalbumin, equine myoglobin and vitamin B12 (molecular weight range 670,000-1,350). The void volume was determined with Blue dextran 2000. A calibration curve was shown in Fig 1.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The 2D-PAGE was performed using a protocol of O'Farrell (1975)[34]. Isoelectric-focusing gels (disc
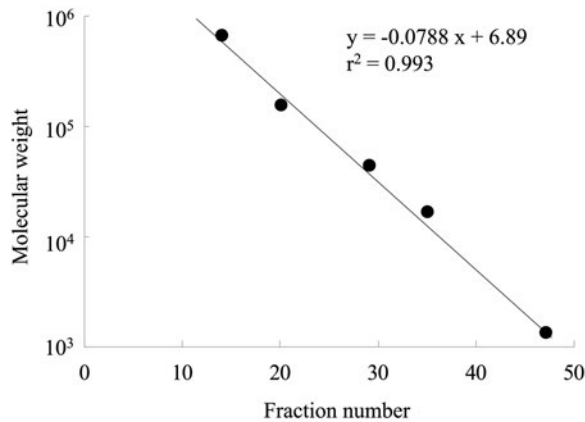
Fig.1 Calibration curve of Sephadex G-200 column (0.7 cm x 100 cm).
Elution was at 2.0 ml/hat 4°C and fractions of 1.25 ml were collected. Column was calibrated with standard proteins: thyroglobulin (670,000), bovine gammaglobulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000) and vitamin B12 (1,350). The void volume was determined with Blue dextran 2000.

Fig.2 Bovine seminal plasma (SP) proteins separated by gel filtration using Sephadex G 200 column.
SP protein (0.5 ml) was separated on Sephadex G 200 column (Fig. 1). Fractions of 1.25 ml were collected. Twenty micro liters of fractions of indicated numbers were separated by polyacrylamide gel electrophoresis (PAGE) and gels were stained with 0.1 % Coomassie brilliant blue. For infusion study (Study 1), fractions from no. 12 to no. 41 were combined into 3 pools by fraction number: high molecular weight range (fractions 12-21), medium molecular weight range (fractions 22-31) and low molecular weight range (fraction 32-41). Calculated molecular weights for fractions 21, 31 and 41 were 172, 280 and 5kDa, respectively (Fig. 1).

gel) were made in glass tubing (3.5 mm x 130 mm). Disc gel mixture were 4% of acrylamide-biz, 8 M urea, 2% of nonidet P-40 and 2% pharmalyte (broad pH range 3 to 10, GE Healthcare Bio-Sciences, Uppsala, Sweden). Pooled and dialyzed SP (25 µl) was diluted to 40 mg/ml with 0.1 M phosphate-buffered saline (PBS) (pH 7.2) and added 30 mg of urea, 1.6% nonidet P-40 and 3.2% pharmalyte and centrifuged at 10,000 x g for 10 min. The supernatant (30 µl containing about 500 µg of SP protein) was used as samples for 2D-PAGE. After prerun to establish pH gradient, samples were loaded and focused at constant voltage of 400 V for 12 h, then, 800 V for 1 h. The second dimension of electrophoresis was performed on a 12 % polyacrylamide gel (130 mm x 130 mm x 2 mm) at constant voltage of 200 V for 5 h. All procedures were performed at 4°C. When it is necessary 2D-PAGE gels were stained by 0.1% Coomassie brilliant blue R250 (Bio-Rad laboratories Inc., Hercules, CA, USA).

Infusion of SP protein into the vagina

At the time of infusion, SP fractions and eluates of protein spots (infusion samples) were prepared in a volume of 10 ml and aspirated
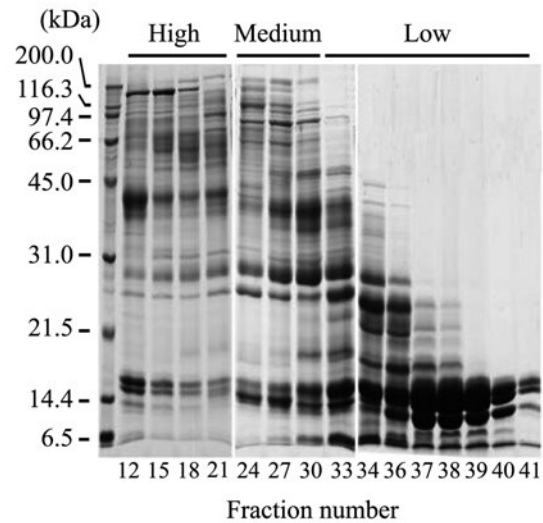
with a 10 cc syringe. A disposable plastic AI catheter was attached to the syringe and the tip of the catheters were introduced into the vagina. Infusion samples were deposited in the vagina near the external orifice of the cervix and the AI catheter was gently withdrawn after the infusion.

Identification of SP proteins

SP proteins were identified by gel based proteomic approach. SP proteins on electrophoresis gels were subjected to LC-MS/MS analysis. The data from the analysis were combined for the sequence data base search. MASCOT was used to search filtered MS/MS data against Swiss-Port (mammals-only, Swiss Institute of Bioinformatics, Geneva, Switzerland).

Study design
Study 1

The effect of SP protein fractions on the

endometrial EGF concentrations was examined using a total of 64 repeat breeder cows. Pooled SP (0.5 ml) from 5 bulls was separated on a Sephadex G-200 column. An example of SP protein separation by gel chromatography was shown in Fig. 2. Protein fractions were combined into 3 pools (high, medium and low molecular weight ranges) by fraction numbers; fractions 12-21, 22-31 and 32-41, respectively. Combined fractions of each molecular weight range were concentrated, lyophilized and stored at -20ºC. The lyophilized protein was used as infusion sample for a single cow; thus, the sample preparation was repeated for 16 times to obtain infusion samples for this study. At infusion, the lyophilized sample was reconstituted with 10 ml of PBS and infused into the vagina of repeat breeder cows at 4 h after the first detection of standing estrus (Day 0). Additional 16 repeat breeder cows were received 10 ml of PBS alone as controls. The endometrial tissues were collected on Day 3 and the EGF concentrations were determined.

Study 2

The effect of SP proteins with the molecular weight range of 16-29 kDa of different pI ranges to normalize the endometrial EGF concentrations in repeat breeders were examined using a total of 42 repeat breeder cows. Four gels of 2D-PAGE were used to prepare infusion sample for a single cow. After 2D-PAGE, 5 pieces of gels with molecular weight range of 16-29 kDa of 5 different pI ranges (pI4.0-5.8, 5.8-6.2, 6.2-6.5, 6.5-7.0 and 7.0-8.0) were dissected and the same pieces of gels from 4 replicates of 2D-PAGE were combined. Proteins in the combined 4 gel pieces were eluted and the eluate was concentrated and stored at -80ºC. The sample preparation was repeated for 6 times to obtain all infusion samples in this study. At infusion, each eluate was thawed, diluted to 10 ml with PBS and infused into the vagina 4 h after the first detection of standing estrus. Additional two groups of 6 repeat breeder cows were infused with either 0.5 ml of SP diluted to 10 ml with PBS or 10 ml of PBS alone as controls. The

endometrial tissues were collected on Day 3 and the EGF concentrations were determined.

Study 3

The effect of SP proteins (16-29 kDa and pI5.8-7.0) on fertility in repeat breeder cows was examined using a total of 118 repeat breeder cows (14 to 24 cows in 6 different dairy farms with 700 to 1,500 cows). Four gels of 2D-PAGE were used to prepare infusion sample for a single cow. After 2D-PAGE, a piece of gels with molecular weight range of 16-29 kDa and isoelectric point range of 5.8-7.0 were dissected and the pieces of gels from 4 replicates of 2D-PAGE were combined. Proteins in the combined 4 gel pieces were eluted and the eluate was concentrated, lyophilized and stored at -20ºC. The sample preparation was repeated for each cow. At infusion, the lyophilized sample was reconstituted with 10 ml of PBS and infused into the vagina of repeat breeder cows at the time of AI between 4 and 12 h after the first detection of standing estrus. The endometrial EGF concentrations were examined on Day 3 and pregnancy was diagnosed by rectal palpation between 55 and 60 days after insemination.

Study 4

SP proteins with the activity to normalize the EGF concentrations were identified. SP was separated by 2D-PAGE and proteins of the molecular weight and isoelectric point ranges of 16-29 kDa and pI5.8-7.0, respectively, were subjected to LC-MS/MS analysis.

Study 5

The effect of a SP protein (spot 11 in Table 4), that had been identified as OPN, to normalize the EGF concentrations was examined using a total of 114 repeat breeder cows. Four gels of 2D-PAGE were used to prepare infusion sample for a single cow. After 2D-PAGE, a piece of gel containing the protein spot 11 were dissected and the pieces of gels from 4 replicates of 2D-PAGE were combined. Proteins in the combined 4 gel pieces were eluted and the eluate was concentrated and stored at
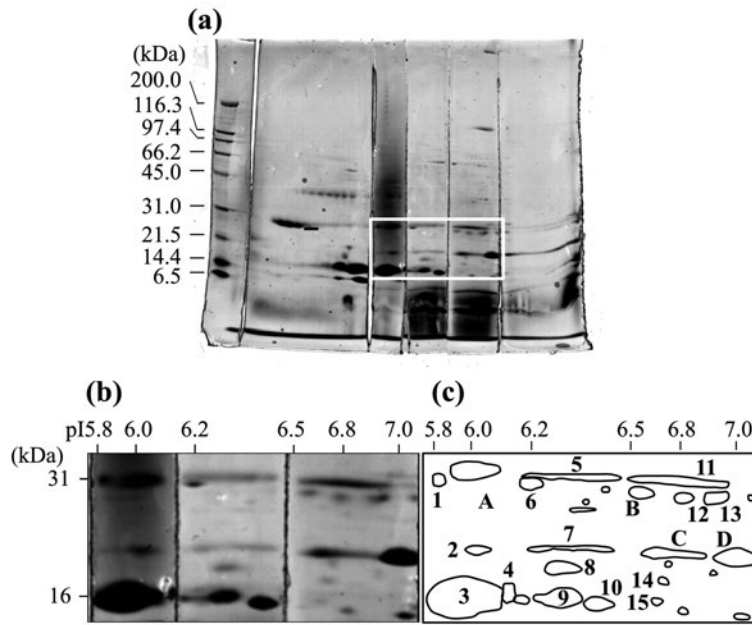
Fig.3 Bovine SP proteins separated by 2D-PAGE.
a) Proteins (500 μg) were first separated by isoelectric focusing with isoelectric point (pI) range between 3 and 10, followed by PAGE using a 12 % polyacrylamide gel. b) and c) enlarged section of gel with molecular weight range of 16-29 kDa and pI range of 5 .8-7 .0, that contain the activity to normalize the epidermal growth factor profile. In this area, maximal of 25 protein spots was found on 2D-PAGE gels and 15 protein spots constantly appearing on all gels were subjected to identification by liquid chromatography with tandem mass spectrometry analysis. Twelve protein spots were identified and the name of proteins were shown in Table 4. Four IEF standard protein markers appears on the enlarged section: (A) bovine carbonic anhydrase (31 kDa, pI6.0), (B) human carbonic anhydrase (28 kDa and pI6.5), (C) equine myoglobin (17 kDa and pI6.8) and (D) equine myoglobin (17 kDa and 7.0 pI).

-80°C. The sample preparation was repeated for each cow. At infusion, the eluate was infused into the vagina at estrus as described in Study 2. In the control group, 52 repeat breeder cows were infused with PBS into the vagina. The endometrial EGF concentrations were examined in all cows on Day 3.

Data analysis

The endometrial EGF concentrations were compared using one-way ANOVA followed by Tukey's test as post hoc. The rates of normalization of EGF profile and conception were compared by Fisher exact test. P values less than 0.05 were considered significant in all analysis.

Results

Study 1

The endometrial EGF concentrations at the first examination (before SP infusion) were similar in all groups (Table 1). The normalization rate was the highest in SP fractions of the low molecular weight range and normalized in 68.8% of repeat breeder cows. When the

endometrial EGF concentrations of cows, in which the concentrations were normalized after the infusions, were compared, the concentrations in the low molecular weight range group were at the similar levels to the medium range group but higher than those of the control (PBS) and high molecular weight range groups.

Study 2

The endometrial EGF concentrations at the first examination (before SP infusion) were similar in all groups (Table 2). Among the different pI range groups, cows infused with the SP proteins of pI6.2-6.5 showed the highest EGF concentrations after the infusion in 5 out of 6 cows (83.3%). The concentrations were at the same levels as those of the cows infused with whole SP. Further, when the endometrial EGF concentrations of cows, in which the concentrations were normalized after the infusion, were compared, the concentrations were at the similar levels in the cows infused with whole SP proteins and SP protein fractions of the three pI ranges (5.8-6.2, 6.2-6.5 and 6.5-7.0).

Study 3

The normalization rates of the EGF

Table 1. Effect of SP protein fractions with different molecule weight on the endometrial EGF concentrations on Day 3 in repeat breeder cows

Molecular weight ranges	No. of cows (n)	No. (%) of cows with the normal EGF concentrations after infusion*	The endometrial EGF concentrations (ng/g tissue weight)		
			Before infusion	After infusion	Normalized# cows
High (170 kDa <)	16	3 (18.8)[a]	2.36 ± 0.39	2.13 ± 1.04[a]	6.10 ± 1.06[a]
Medium (28-170 kDa)	16	7 (43.8)[b]	2.36 ± 0.65	4.69 ± 3.44[a]	7.31 ± 1.12[a,b]
Low (< 28 kDa)	16	11 (68.8)[c]	2.18 ± 0.92	8.44 ± 3.01[b]	11.91 ± 2.72[b]
PBS	16	3 (18.8)[a]	2.21 ± 0.81	2.31± 1.13[a]	5.98 ± 0.87[a]

Values are means ± SDs
*The normal range of the endometrial EGF concentrations on Day 3: 4.7-13.5 ng/g tissue weight[21]
#EGF concentrations of cows in which the EGF concentrations were normalized after infusion
The rates of normalization of EGF profile were compared by Fishers exact test
The endometrial EGF concentrations were compared using one way ANOVA followed by Tukey's test as post hoc
[a,b]Means with different letters within the same column differ ($P < 0.05$)

Table 2. Effect of SP protein fractions (16-29 kDa) with different isoelectric points (pI) on the endometrial EGF concentrations on Day 3 in repeat breeder cows

SP protein fractions with different pI ranges	No. of cows (n)	No. (%) of cows with the normal EGF concentrations after infusion*	The endometrial EGF concentrations (ng/g tissue weight)		
			Before infusion	After infusion	Normalized# cows
4.0 - 5.8	6	1 (16.6)	1.34 ± 0.18	2.73 ± 1.30[d]	5.38
5.8 - 6.2	6	4 (66.7)	1.39 ± 0.27	5.02 ± 3.24[b]	8.02 ± 2.26
6.2 - 6.5	6	5 (83.3)	1.37 ± 0.39	9.98 ± 1.51[a]	10.94 ± 2.27
6.5 - 7.0	6	4 (66.7)	1.34 ± 0.12	7.09 ± 2.66[bc]	8.36 ± 1.84
7.0 - 8.0	6	2 (33.3)	1.46 ± 0.34	4.00 ± 0.59[cd]	4.87, 7.31
Whole SP	6	4 (66.7)	1.38 ± 0.39	11.00 ± 2.11[a]	12.03 ± 1.76
PBS	6	2 (33.3)	1.56 ± 0.35	2.34 ± 0.69[d]	4.98, 5.16

Values are means ± SDs
*The normal range of the endometrial EGF concentrations on Day 3: 4.7-13.5 ng/g tissue weight
#EGF concentrations of cows in which the EGF concentrations were normalized after infusion.
The endometrial EGF concentrations were compared using one way ANOVA followed by Tukey's test as post hoc.
[a,b,c,d]Means with different letters within the same column differ ($P < 0.05$)

concentrations and the conception rates in all 6 herds were at the similar levels (Table 3). The normalization rates were between 55.0% and 70.6% with the mean of 61.0%. The conception rates were between 45.0% and 70.6% with the mean of 54.2%.

Study 4

An example of SP protein separation by 2D-PAGE were shown in Fig. 3. A total of 25 protein spots were found in gels with molecular weight range of 16-29 kDa and isoelectric point range of 5.8-7.0. Among those, 15 protein spots appearing on all 2D-PAGE gels were subjected to LC-MS/MS analysis and 12 out of the 15 protein spots were identified (Table 4). We found 8 proteins in this analysis: four protein spots of OPN, 2 of carbonic anhydrase and a single protein spot of bovine seminal protein A1/A2 (BSP A1/A2), BSP A3, lipocalin-type prostaglandin D synthase

(L-PGDS), tissue inhibitor of metalloproteinase 2 (TIMP-2), transforming growth factor-β1 (TGF-β1) and myoglobin (Table 4). Peptide mass data for 3 protein spots (spots 1, 12 and 15) did not match to those on the database.

Study 5

The normalization rate of the endometrial EGF concentrations on Day 3 after OPN infusion (26 cows, 41.9%) was greater than that of the controls (12 cows, 23.1%). The EGF concentrations of cows, in which the concentrations were normalized after infusion, were 7.18 ± 3.24 ng/g tissue weight.

Discussion

In the present study, we have obtained crude SP protein preparation with 16-29 kDa and pI5.8-7.0 that has an activity to normalize the endometrial EGF concentrations in repeat breeder cows. Fifteen SP protein spots were found in the crude protein preparation and 12 protein spots out of the 15 protein spots could be identified by LC-MS/MS (Table 4)[8,11,12,24,37,44,46]. The list of the proteins contains some proteins that have been linked to fertility. Those proteins include BSP A1/A2, BSP A3, carbonic anhydrases, L-PGDS, TIMP-2 and TGF-β1 and OPNs.

An earlier study reported a regression model to predict bull fertility using 4 fertility-associated protein densities[25] and 3 out of the 4 fertility-associated proteins have been identified to date. OPN has molecular weight of 55-kDa and affects sperm-oocyte binding and early embryonic development[6,31]. L-PGDS with 26 kDa reduces the production of anti-sperm antibodies in the female reproductive tract[12,25,28]. TGF-β1 upregulates pro-inflammatory cytokines and chemokines in the uterine and cervical epithelial cells, and contributes to establish pregnancy in rodent and human[41,45]. However, TGF-β1 failed to improve fertility in cattle[38]. Other proteins in the list (Table 4) also have been suggested to improve

fertility mainly by enhancing sperm function, but the link to fertility via regulation of uterine function has not been reported in cattle.

We found 4 forms of OPN with different molecule weights and isoelectric points in bovine SP. As mentioned above, OPN is one of the SP proteins related to bull fertility[6,25,31]. Similarly, in stallion[49], camel[50] and buffalo[27], OPN concentrations found to be significantly higher in the high-fertile group compared with the low-fertile group. OPN has been linked to improvement of fertility through optimizing sperm or oocyte function related to fertilization. Pre-treatment of bovine semen and oocytes with purified milk OPN enhances both in vitro fertilization and early embryo development[13]. In cattle, fertilization medium containing 10 mg/mL OPN improved in vitro embryo production and OPN positively influenced sperm capacitation in vitro[30]. Structurally, OPN is an acid single chain phosphorylated glycoprotein that ranges in length from 264 to 301 amino acids and undergoes extensive post-translational modifications that result in molecular weight variations ranging from 25 to 75 kDa[15]. The molecular mass for bovine OPN derived from bone cells, estimated from the nucleotide sequence, is 30.1 kDa[24]. OPN identified in bovine milk, a rich source of OPN, is 262 amino acids long and has an estimated molecular mass of 66 kDa[47]. Protein characterization revealed that a major form of OPN in SP showed a size of 55 kDa and was glycosylated, but not phosphorylated, consistent with the identity of the 55-kDa fertility-associated protein[6]. However, a smaller size of 30 kDa protein also found in the partially purified preparation[6]. These differences in molecular size and isoelectric point of OPN in SP could be attributed to different patterns of posttranslational modification[32] and the acidic nature of the protein, which have been shown to affect protein mobility in SDS-PAGE[9]. One of the 4 OPN found in the present study (spot 11 in Table 4) with 29 kDa and pI6.5-6.9 showed the activity to normalize the endometrial EGF concentrations in repeat breeder cows. This

Table 3. Effect of the crude SP protein preparation (16-29 kDa and pI5.8-7.0) on the endometrial EGF concentrations on Day 3 in repeat breeder cows

Farms	No. of cows	No. (%) of cows with the normal EGF concentrations after infusion*	No. (%) of cows conceived
A	20	12 (60.0)	11 (55.0)
B	20	11 (55.0)	9 (45.0)
C	17	12 (70.6)	12 (70.6)
D	14	9 (64.3)	7 (50.0)
E	23	13 (56.5)	11 (47.8)
F	24	15 (62.5)	14 (58.3)
Total	118	72 (61.0)	64 (54.2)

#All repeat breeder cows showed EGF concentrations below 4.7 ng/g tissue weight before the infusion
*The normal range of the endometrial EGF concentrations on Day 3: 4.7-13.5 ng/g tissue weight

Table 4. Identification of SP proteins with activity to normalizing the endometrial EGF concentrations

Spots (No.)	Proteins names[a]	Molecule weight (kDa)	Isoelectric point (pI)	Accession number[b].	References
1	Unknown	28.9	5.8	-	-
2	Lipocalin – type prostaglandin D synthase	17.0	6.0	O02853	Gerena et al., 1998[12]
3	Bovine seminal protein A1/A2	16.0	5.8-6.1	P02784	Esch et al., 1983[11]
4	Bovine seminal protein A3	16.2	6.1	P04557	Seidah et al., 1987[44]
5	Carbonic anhydrase	29.0	6.2-6.4	Q1LZA1	-
6	Carbonic anhydrase	28.5	6.2	Q1LZA1	-
7	Transforming growth factor-β1	17.0	6.2-6.4	P18341	Van Obberghen-Schilling et al., 1987[37]
8	Tissue inhibitor of metalloproteinases 2	16.5	6.3	P16368	DeClerck et al., 1993[8]
9	Osteopontin	16.3	6.2-6.3	P31096	Kerr et al., 1991[24]
10	Osteopontin	16.0	6.4	P31096	Kerr et al., 1991[24]
11	Osteopontin	29.0	6.5-6.9	P31096	Kerr et al., 1991[24]
12	Unknown	27.5	6.8	-	-
13	Osteopontin	27.5	6.9	P31096	Kerr et al., 1991[24]
14	Myoglobin	16.5	6.7	P02192	Shimada et al., 1989[46]
15	Unknown	16.0	6.6	-	-

[a]Names of protein are given according to the nomenclature of the database from which the sequence was sourced by LC-MS/MS analysis
[b]All accession numbers are obtained from the Swiss-Prot database

effect of OPN has not been described and may contribute to improve fertility in cattle.

Repeat breeder cows with an altered endometrial EGF profile had been treated with a high dose of estradiol benzoate in combination with a progesterone-releasing device[22]. However, efficiency of this hormonal treatment to normalize EGF differed to a large extent (30 to 80%) between herds[16]. Thus, a new treatment option is needed. In Study 3, we showed that the infusion of SP proteins normalized the endometrial EGF concentrations and improved conception rate

at the similar rates between herds. In pigs[3,36], rodents[33,42], horses[39] and women[45], SP stimulates an inflammatory response leading to changes in the cytokine and growth factor network in the uterus. The changes in the regulatory network of the endometrium by SP may regulate uterine function towards pregnancy more directly and precisely than those by exogenous ovarian steroid hormones in the hormonal treatment. This may explain the difference in the efficiency between hormonal treatment and SP proteins.

At present, the mechanism connecting the infusion of SP or OPN into the vagina to the normalization of the EGF profile in the uterus is not known. However, it is unlikely that SP or OPN is transported to the uterus to normalize the endometrial EGF concentrations since a direct infusion of SP into the uterus failed to normalize the EGF concentrations[2]. Instead, the well-known role of OPN in the regulation of immune function may be related to the mechanism by which SP or OPN normalize the EGF concentrations. OPN plays a key role in the crosstalk between innate and adaptive immunity, an important component in the establishment of pregnancy, though the regulation of cytokine expression in various cells[7]. Recently, intrauterine administration of autologous peripheral blood mononuclear cells has been shown to promote implantation rates in women with repeated failure of in vitro fertilization-embryo transfer[51]. Similar effect of activated lymphocytes to improve fertility in embryo transfer recipient cows has also been reported[14]. Activated lymphocytes are thought to enhance fertility by regulating the cytokine and growth factor network in the uterus. Together, OPN may normalize the endometrial EGF concentrations by activating immune cells in the vagina.

The results of our study confirmed that SP proteins could promote pregnancy by normalizing the endometrial receptivity in repeat breeder cows, that could be measured by the EGF profile. OPN may be responsible for this function of SP. In future, OPN could be used for infertility treatment

that targeting at the local regulatory network of uterine function by cytokines and growth factors. However, the activity of OPN on the endometrial EGF profile needs to be confirmed by infusion study using recombinant OPN or purified OPN, and by neutralization study of SP activity with antibodies against OPN. The present study used the eluate of dissected gels containing the protein spot 11, a form of OPN, for the infusion samples and the eluates may also contain other proteins that are invisible on gels by Coomassie brilliant blue staining. It is also necessary to examine the activity of remaining proteins in the crude protein preparation, that include other forms of OPN than the spot 11, before concluding the source of the activity in SP.

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