Effect of seminal plasma proteins on the normalization of endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows

（精漿タンパク質がリピートブリーダー牛の
子宮内膜における上皮成長因子濃度変化
および受胎性の正常化に及ぼす効果）

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Abbreviations

AI: Artificial insemination

BSP: Bovine seminal protein

EGF: Epidermal growth factor

E_2: Estradiol

h: Hour

kDa: Kilodalton

kg: Kilogram

L-PGDS: Lipocalin-type prostaglandin D synthase

LC-MS/MS: Liquid chromatography with tandem mass spectrometry

min: Minute

ml: Milliliter

mg: Milligram

µl: Microliter

µl: Microgram

OPN: Osteopontin

pI: Isoelectric point

PBS: Phosphate-buffered saline

PMN: Polymorphonuclear neutrophil

P_4: Progesterone

PG: Prostaglandin

SP: Seminal plasma

TGF: Transforming growth factor

2D-PAGE: Two dimensional polyacrylamide gel electrophoresis

Contents

Preface

The performance of dairy herds is affected mainly by factors related to genetic selection of cows and herd management practices. Since mid-twentieth century to the present day, milk production of dairy cattle has been increasing from 5,000 kg per year to 9,000 kg per year [1]. In contrast, dairy cows have become delicate and fragile animals that require high levels of nutritional and cow comfort management to realize their genetic potential [2–4]. These changes inevitably results in the reduction of fertility rate in modern dairy herds.

Repeat breeder cows are defined as cows failing to conceive after several (usually three or more) inseminations without clinical signs and abnormalities in their genital tracts, and with apparently normal estrous cycles [5–8]. The presence of the repeat breeder cows in herds is an important source of economic loss for dairy producers due to reduced milk production, extend calving intervals, increased costs of insemination, treatment and replacement cattle [9, 10]. The incidence of repeat breeding in lactating dairy cows has been reported between 10 and 24% [9, 11].

Causes of infertility in repeat breeder cows are usually unclear, but include environmental, management, and animal factors. From the physiological aspect, fertilization failure and embryonic loss are the causes of infertility in repeat breeder cows [4, 9, 10, 12, 13]. Particularly, the incidence of early embryonic loss increased during the last 40 years while fertilization rate has been kept at the high level (about 90%) [2, 12]. The incidence of high quality embryos at 6-7 days after artificial insemination (AI) decreases in lactating cows (between 33% and 53%) compared with heifers (72%) and dry cows (83%) [14]. Together, the retarded embryonic development may be common in modern high yielding cows and, thus, become a major cause of infertility in repeat breeder cows [15]. Interestingly, a reciprocal embryo transfer study that exchanged embryos between fertile and repeat breeder cows showed that uterine environment, but not quality of embryo, is the factor increasing embryo loss in repeat breeder cows [16].

Increased levels of milk production have a negative impact on the endocrine environment of the uterine function due to decreased progesterone (P_4) [14, 17] and estradiol (E_2) concentrations in circulation [14]. To meet greater energy requirements, the high yielding cows uptake a larger amount of food and this leads to an increase in liver blood flow that, in turn, increases the clearance of both P_4 and E_2 from the circulation. Repeat breeder cows may not necessarily be high yielders but exhibit the similar alterations in the steroid hormone profiles to those found in high yielding cows [18–21].

The alterations in the steroid hormone profiles in high yielding and repeat breeder cows could be amplified and become detectable in the endometrium as alterations in growth factor and cytokine expression since ovarian steroid hormones regulate expression of these local factors [22, 23]. Epidermal growth factor (EGF) seems one of the most important regulatory components of uterine function and embryonic development [22, 23]. Estrogen stimulates EGF production in the uterus [24–26]. EGF replaces estrogen in the uterine and vaginal growth, lactoferrin (a major estrogen-inducible secretory protein) in mice [27] and a nidatory estrogen surge that initiates blastocyst attachment to the endometrium in rats [28]. Drastic negative effects on the inner cell mass [29], placenta formation and viability of offspring [29, 30] have been reported in EGF receptor knockout mice.

The presence of EGF and its receptor in the endometrium has been reported in many farm species, including the cattle [31–33], sheep [34], goat [35] and pig [36]. Further, EGF increases endometrial production of prostaglandin (PG) E_2 and the PGE_2:PGF_2 ratio in pigs [37] and rats [38]. These effects of EGF on PG synthesis should enhance corpus luteum function and support the survival of embryos in the cattle [39, 40]. Therefore, an alteration of EGF action in the endometrium may cause uterine dysfunction that, in turn, causes early embryo loss in cattle.

In normal cows, endometrial EGF concentrations peak twice on Days 2-4 and 13-14 with low EGF concentrations around Day 7 during the estrous cycle [33, 41, 42]. The loss of these peaks in the EGF profile has been found in about 70% of Holstein repeat breeder cows and linked to infertility in

repeat breeder and high yielding cows [33, 41–43]. Recipient cows for embryo transfer with low EGF concentrations on Day 3 showed a lower conception rate than those with EGF concentrations within the normal range (33.3% vs. 76.9%) [44]. In treatment studies, the normalization of the EGF profile resulted in the restoration of fertility in repeat breeder cows [41, 45].

Seminal plasma (SP) has been shown to improve fertility by modulating uterine function and environment in the rodent [46, 47], pig [48–50] horse [51] and human [52]. In mice, SP factors stimulate the synthesis of inflammatory cytokines and induce inflammatory response, when they interact with estrogen-primed uterine epithelial cells [47, 53, 54]. This function of SP has been linked to improvement of fertility in mice [55]. In pigs, conception and farrowing rates were greater in females that have received AI with SP than AI with extender alone [49, 55]. In horses, SP reduces sperm binding to polymorphonuclear neutrophils (PMN)s after AI [51]. This SP action may improve fertility when spermatozoa are inseminated into inflamed uterus typically during the early postpartum period in horses. In cattle, a large-scaled studies that examined the effect of SP infusion into the uterus on fertility failed to show an beneficial effect on fertility [56, 57], although SP has shown to induce inflammatory response in the bovine uterine cells [58–60]. In the natural mating, semen is deposited into the uterus or the cervix in mice, pigs and horses which SP shows an effect on fertility, while into the vagina in cattle. Thus, the site of SP deposition in the fertility studies is questionable and the effect of SP on the cytokine expression in cultured uterine cells may not reflect physiological role of SP in cattle.

Bull SP has been suggested to contain a variety of proteins associated with fertility through the effects on sperm and uterine functions [61, 62]. An earlier study reported a regression model to predict bull fertility using 4 fertility-associated protein densities [63]. To date 3 out of the 4 proteins, osteopontin (OPN), lipocalin-type prostaglandin D synthase (L-PGDS) and transforming growth factor-β_1 (TGF-β_1) have been identified. These proteins have been suggested to facilitate the establishment of pregnancy. OPN enhances sperm-oocyte binding and early embryonic development in cattle [64, 65].

L-PGDS reduces the production of anti-sperm antibodies in the female reproductive tract in human [63, 66, 67]. TGF-β_1 up-regulates growth factors and cytokines in the uterus and contributes to establish pregnancy in the rodent, pig and human [68–71]. However, the role of these proteins has not been investigated in relation to the regulation of uterine growth factor expression and uterine function to support embryonic development in cattle.

In Chapter I, I have aimed at examining the effect of SP to normalize the endometrial EGF profile and restore fertility in repeat breeder cows with an altered EGF profile. In Studies I-1 and I-2, I examined the effect of the deposition site of SP and the effect of SP volume, respectively, on the endometrial EGF profile in repeat breeder cows. In Study I-3, I have demonstrated that SP infusion normalizes the endometrial EGF profile and restores fertility in repeat breeder cows.

In Chapter II, I have identified SP protein(s) with an activity of normalizing the endometrial EGF profile. SP fractions were separated by gel filtration and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the effect of the SP fractions on the EGF profile in repeat breeder cows were examined. A crude SP protein preparation with molecular weight range of 16-29 kDa and isoerectric point (pI) range of 5.8-7.0 was found to have an activity to normalize the EGF profile and restore fertility. Then, proteins in the crude SP protein preparation were identified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis. Among those proteins that were identified in the preparation, a form of OPN was tested for the activity to normalize the EGF profile in repeat breeder cows and found to normalize the EGF profile in the repeat breeders cow.

Chapter I

Effect of seminal plasma infusion into the vagina on the normalization of the endometrial epidermal growth factor concentrations and fertility in repeat breeder dairy cows

Introduction

Endometrial EGF is one of the key factors that regulate uterine function and embryonic development [22, 23]. Although most of our knowledge on the regulation of EGF in the uterus was obtained using mice and rats, estrogen is the primary regulator of EGF production in the endometrium [23–26]. 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 [33, 42, 43]. These peaks are not found in about 70% of Holstein repeat breeder cows and 25% of Holstein cows at 60 days postpartum [33, 42]. Further, a single examination of the EGF concentrations on the day of the first peak (Day 3) could appropriately determine the endometrial EGF profile since loss and recovery of the two peaks of the endometrial EGF concentrations coincide in most cases [42, 44]. The loss of the peaks in the endometrial EGF concentrations during the estrous cycle may impair the regulation of uterine functions and, in turn, causes asynchrony between the uterus and the blastocyst when the blastocysts enters the uterus [18, 19, 39]. Maternal supply of EGF from the endometrium also seems to play a role in elongation of bovine embryos (Days 14-16) [32]. Accordingly, an alteration of the endometrial EGF concentrations has been linked to an increased frequency of embryo loss after AI in repeat breeder cows [41, 72] and a failure of pregnancy after embryo transfer in apparently normal recipients [44].

Some endocrine changes have been described in repeat breeder cows and are a slow rise or

low peak of plasma E_2 concentrations, a delayed luteinizing hormone surge in the peri-ovulatory period, and a slow rise or low plasma P_4 levels during the luteal phase [18, 20]. These changes may cause an alteration of the endometrial EGF profile in the uterus since the synthesis of EGF is regulated by estrogen together with P_4 [22–24]. Therefore, we developed a treatment protocol with a high dose of estradiol benzoate in combination with a progesterone releasing device in repeat breeder cows. The treatment normalized the endometrial EGF profile and restored fertility in about 70% and 60% of repeat breeder cows, respectively [45]. However, the efficacy of treatment to normalize the endometrial EGF concentrations varies to a large extent between 30% and 85% depending on farms by unknown reasons. Further, use of estrogen products in food animals has been restricted in many countries and regions. Thus, an additional treatment option is needed.

Previous studies have indicated that SP may improve fertility by modulating uterine function and environment in rodents, pigs, horses and human [46, 48, 51, 52, 55]. In mice, SP factors initiate inflammatory response and activate the synthesis of inflammatory cytokines, when they interact with estrogen-primed uterine epithelial cells [47, 53, 54]. These cytokines activated by SP induce influx of inflammatory cells into the uterine endometrium, which have roles in remodeling of the endometrial tissue, activating maternal immune accommodation of pregnancy, and promoting development of pre-implantation embryos [73–75]. In pigs, conception and farrowing rates were greater in females that have received AI with SP than AI with extender alone [49]. Exposure of the uterus to SP resulted in an increase in the proportion of viable embryos from 77% to 91% (control vs. SP) in gilts, when embryo recovery data on Days 5 and 9 were combined, leading to a 58% increase in the number of viable embryos recovered from gilts after SP treatment [50]. In horses, SP reduces sperm binding to PMNs after AI [51]. This SP action may improve fertility when spermatozoa are inseminated into the uterus with mild inflammation typically during the early postpartum period.

These circumstantial evidences in different species prompted us to examine if SP affects the

endometrial regulatory network, which includes EGF, and normalizes the EGF profile in repeat breeder cows. A large scaled trial, in which SP was infused into the uterus, has failed to improve pregnancy rate in both dairy and beef cows [56]. In natural copulation, semen is deposited into the vagina in cattle while into the uterus or the cervix in abovementioned other species in which SP has been indicated to facilitate pregnancy. The deposition site of semen at natural copulation should be taken in consideration when the effect of SP in the female genital tracts is evaluated. Therefore, the present study firstly examined the effect of the deposition sites of SP, the uterus and vagina, on the EGF concentrations in repeat breeder cows (Study I-1). Then, the effect of SP volume on the normalization of the EGF concentrations was examined (Study I-2). Finally, the effect of SP infusion into the vagina on fertility of repeat breeder cows was examined (Study I-3).

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, 19-0030). The present study used 4 apparently normal cows and 107 repeat breeder cows with low endometrial EGF concentrations on Day 3 in the previous estrus cycle. These repeat breeders were diagnosed by local practitioners using the criteria of failing to conceive after three or more 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 breeder cows with additional examinations that include uterine morphology by transrectal ultrasonography [76], uterine cytology by cytobrush [77] and oviductal patency by tubal insufflation [78] by one of the authors before enrollment to the study. We used repeat breeder cows with the normal uterine morphology and oviductal patency and proportion of polymorphonuclear leucocyte in uterine cytology < 6% [79]. The uterine morphology was examined

using a real-time B-mode ultrasound scanner (HS-1500, Honda electronics Co., Ltd., Toyohashi, Japan) and judged as normal according to the criteria of no fluid or small volume of black fluid in a spoke wheel-shaped lumen with infoldings of the endometrium during the luteal phase [76]. Oviductal patency was tested using Atom PA-200（Atom Medical Corporation, Tokyo, Japan）and judged as normal when the air begins to escape from the abdominal os at 100-120 mmHg and continues to escape till 60 mmHg [78].

All repeat breeder cows selected as above were examined for endometrial EGF concentrations on Days 3 by using biopsy samples and cows showing low EGF concentrations (< 4.7 ng/g tissue weight) were used in this study. All repeat breeder cows were multiparous lactating Holstein cows (> 10,000 kg of 305-days fat-corrected milk) between three and nine years of age and between 145 and 250 days post-partum 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 [33]. Tissues were frozen in liquid nitrogen within 10 min of collection and stored at –80^{o}C until the EGF assay.

Measurement of endometrial EGF concentrations and judgment of the EGF profile

Uterine endometrial tissues were processed for the EGF assay as described previously [80] with a modification to increase the ratio of the extraction solution (ml) to the tissue weight (g) from 1:5 to 1:15 [41]. Concentrations of EGF in uterine tissue extracts were determined by a double-antibody sandwich EIA using 96-well microtiter plates [41]. 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 $y = 0.96x + 0.39$, $r = 0.97$. The intra- and interassay coefficients of variation were 4.8% and 6.2%, respectively. The sensitivity of the assay was 10 pg/well.

In Studies I-1 and I-2, endometrial EGF concentrations were determined by a single examination on Day 3 and the profile was judged to be normal when the concentrations were 4.7 ng/g tissue weight or greater [41, 42]. While, in Study I-3, endometrial EGF profile was determined by three examinations (Days 3, 7 and 14) and judged to be normal when the concentrations on all three days were within the normal range (Day 3: 4.7 to 13.5, Day 7: 1.8 to 4.6, Day 14: 4.9 to 14.9 ng/g tissue weight, respectively) [41].

Preparation of SP samples

Semen was collected twice a week from nine 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 -20ºC and transported to the Hokkaido University. At the university laboratory, all frozen SP were thawed and centrifuged 5,000 x g for 20 min at 4℃ and the resulting supernatants were used as SP samples. In Study I-1, SP from three out of the nine bulls were pooled and kept in 0.5 ml aliquots and stored at -80℃. In Studies I-2 and I-3, SP from the nine bulls (six to ten ejaculates per bull) were pooled and kept in either 0.5 ml or 10 ml aliquots and stored at -80℃.

Infusion of SP into the vagina and the uterus

At the time of infusion, SP was thawed on farms and, SP alone or diluted SP with phosphate-buffered saline (PBS) was aspirated with either 5.0 cc or 10 cc syringe. A disposable plastic AI catheter was attached to the syringe with either SP samples or PBS (control). The AI catheter was introduced into either the vagina or the uterus through the cervix. The SP or PBS was deposited in the vagina near the external orifice of the cervix or the uterine body. The AI catheter was gently withdrawn after the infusion.

Study design

All studies were conducted at commercial dairy farms in Hokkaido prefecture, Japan, between April to June (Studies I-1 and I-2) and between September and December (Study I-3). Conception rates of cows in all farms were between 46.7% and 57.4% and were at the similar levels during the study period.

Study I-1

Repeat breeder cows were observed for estrus three times a day (Day 0 = estrus) and examined the endometrial EGF concentrations on Day 3. Eight repeat breeder cows with a low endometrial EGF concentration (< 4.7 ng/g tissue weight) were used during the next estrus cycle. In four repeat breeder cows, 0.5 ml of SP was infused into the vagina at 4 h after the first detection of standing estrus while, in the other four repeat breeder cows, SP was infused into the uterus. In all repeat breeder cows, the endometrial EGF concentrations were determined on Day 3. Apparently normal cows were identified by measuring the endometrial EGF concentrations on Day 3 before recruiting for the study and four cows with the normal EGF concentrations ($\geq$ 4.7 ng/g tissue weight) on Day 3 were used as fertile controls. Cows in the fertile control group were infused with 0.5 ml of SP into the vagina on the day of

the next estrus and were used for the examination of the EGF concentrations on Day 3.

Study I-2

The effect of volume of SP on the endometrial EGF concentrations was examined using 32 repeat breeder cows with a low endometrial EGF concentration on Day 3. Repeat breeder cows were observed for estrus three times a day. SP alone (0.5 ml or 10 ml), 0.5 ml of SP diluted to 10 mL with PBS or 10 ml of PBS were infused into the vagina of eight repeat breeder cows each at 4 h after the first detection of standing estrus. In all cows, the endometrial EGF concentrations were determined on Day 3 for the second time.

Study I-3

The effect of SP infusion on the endometrial EGF profile and fertility was examined using 67 repeat breeder cows with a low endometrial EGF concentration on Day 3. Cows were observed for estrus three times a day. Thirty-six and thirty-one repeat breeder cows were infused with 0.5 ml of SP diluted to 10 ml with PBS and 10 ml of PBS (control), respectively, into the vagina at 4 h after the first detection of standing estrus (Day 0). The endometrial EGF concentrations were determined on Days 3, 7 and 14. All repeat breeder cows were subjected to AI at the next estrus and pregnancy was diagnosed between Days 50 to 60.

Data analysis

Data were analyzed using a computer software JMP Pro 14 data (SAS Institute Japan Inc, Tokyo, Japan). In all studies, the endometrial EGF concentrations were compared using two-way ANOVA with repeated measure and followed by Tukey's test (for three or more means) or Student's t-test (for two means) as post-hoc tests. The endometrial EGF concentrations of cows in which EGF

became at the normal levels after infusion were compared using one-way ANOVA followed by Tukey's test. The rates of normalization of the EGF profile and pregnancy were compared by Fisher exact test.

Results

Study I-1

In all four apparently normal (fertile) cows, the endometrial EGF concentrations at the first examination were at the normal levels (≥ 4.7 ng/g tissue weight) and the EGF concentrations did not change after SP infusion (Table I-1). All the four cows conceived by up to two AIs after the study. In repeat breeder cows, the endometrial EGF concentrations at the first examination (before SP infusion) were at the same levels in both groups with SP infusion. When SP was infused into the vagina, the endometrial EGF concentrations increased to the normal levels in 3 out of 4 cows and were at the same levels as the normal cows. However, the endometrial EGF concentrations of all four repeat breeder cows remained low when SP was infused into the uterus.

Study I-2

The endometrial EGF concentrations at the first examination (before SP infusion) were similar in all four groups (Table I-2). In three groups with SP infusion, the endometrial EGF concentrations on Day 3 after the infusion increased to the normal levels and were higher than those of the control group (PBS alone). However, the endometrial EGF concentrations of the normalized cows, in which the EGF concentrations became at the normal levels after the infusion, differed between the SP infusion groups. The EGF concentrations of the two groups with 10 ml of SP and 0.5 ml of SP diluted to 10 ml were higher than those of 0.5 ml of SP.

Study I-3

The endometrial EGF concentrations of both SP and control groups before infusion were at the same levels (Table I-3). The normalization rate of the endometrial EGF profile in SP infusion group was higher than that of controls (58.3% vs. 22.6%, $P < 0.05$). Accordingly, the endometrial EGF concentrations on Days 3 and 14 after SP infusion were greater than those after SP infusion ($P < 0.05$). However, the endometrial EGF concentrations in cows showing the normal EGF profile after infusions of SP and PBS were at the similar levels on both Days 3 and 14. The conception rate after SP infusion was greater than that of controls (44.4% vs. 19.4%, $P < 0.05$). Among those in which the EGF profile was normalized after SP infusion, the conception rate of the cows showing the normal EGF profile was greater than that of the cows remaining with an altered EGF profile (61.9% vs. 20.0%, $P < 0.05$).

Discussion

In the present study, we have demonstrated that SP normalized the endometrial EGF profile during the estrous cycle and improved fertility in repeat breeder cows with an altered EGF profile, when it was infused into the vagina, but not the uterus. In general, SP induces an inflammatory response leading to changes in the endometrial cytokine and growth factor network in the uterus and may improves fertility in pig [50, 81], rodent [75], horse [82] and human [69]. However, in cattle, large scaled studies failed to demonstrate beneficial effect of SP infusion into the uterus on fertility [56, 57]. The results of these studies seem to be inconsistent with our findings. However, our study design is different in two points from these studies [56, 57]. Firstly, we have used a specific group of cows, namely repeat breeder cows with an abnormal EGF profile in the endometrium while the previous studies targeted apparently normal cows or all cows in herds. The results of Study I-3 and those of previous studies [44, 72] indicate that improvement of fertility in the repeat breeder cows could be attributed to the normalization of the endometrial EGF concentrations. This effect of SP on fertility may not be detectable

in the previous studies [56, 57] since the majority of apparently normal cows show the normal EGF profile before SP infusion.

The second point in our study design that differs from the previous studies [56, 57] is the site of SP deposition. In the Study I-1, we examined the effect of SP deposition sites (the uterus and vagina) and found that SP should be deposited in the vagina to normalize the endometrial EGF concentrations. In the natural mating, semen is deposited in the uterus or the cervix in abovementioned species in which uterine SP infusion has been linked to improvement of fertility. While semen is deposited in the vagina in cattle. The present study demonstrated that SP infusion into the vagina, but not the uterus, could increase the endometrial EGF concentrations in repeat breeder cows. Therefore, if we want to examine the effect of SP on fertility in apparently normal cows, SP should be infused into the vagina.

We have examined the effect of the volume of SP on the normalization of the endometrial EGF concentrations in Study I-2. In Study I-1, we used 0.5 ml of SP by following the previous study [56, 57] and also due to the limited volume of SP since we collected SP from young bulls that provide 2-3 ml of SP per ejaculate. However, bull ejaculates contain a larger volume (4-6 ml) of SP. Thus, we examined the effect of SP with an increased volume of 10 ml in Study I-2. The increased volume of SP showed greater effect on the endometrial EGF concentrations than 0.5 ml of SP. Interestingly, when 0.5 ml of SP was diluted to 10 ml, the effect on the endometrial EGF concentrations became at the same levels as 10 ml of SP. These results indicate that 0.5 ml of SP contains the activity enough to exhibit a maximal effect of normalizing endometrial EGF concentrations. The results also indicate that volume of infusing samples is important to obtain the maximal effect. For example, area of vaginal mucosa being exposed to SP may affect degree of the endometrial response in the EGF production.

The mechanism by which SP infusion into the vagina changes EGF concentrations in the uterus is unknown. It is unlikely that SP is transported to the uterus to normalize EGF concentrations since SP infusion into the uterus failed to normalize the EGF concentrations. At present, evidence that SP protein

improves fertility seems related to sperm function and viability or direct effect on the uterus found in other species [75, 83, 84] and cultured cells [59, 60]. Further, little information is available function of the vagina in the establishment of pregnancy. Further study to identify the seminal factors responsible for the activity to normalize the endometrial EGF profile would give us an insight into the elucidation of the mechanism.

To our knowledge, this is the first report demonstrating that SP infusion into the vagina can modulate uterine growth factor expression associated with uterine function and improve fertility. Function of SP found in the present study may explain, in part, the difference in conception rate between natural mating and AI. This SP function seems beneficial in cows with reduced fertility that include repeat breeders and early postpartum high yielding cows, but the effect of SP infusion into the vagina in apparently normal cows with the normal EGF profile remained to be examined. Nevertheless, the SP function is not utilized in the current AI based reproductive management in cattle and could contribute to improve fertility of dairy cows by moving repeat breeder cows back to the pool of fertile cows.

Tables

Table I-1. Effect of SP infusion into the vagina and uterus on the endometrial EGF concentrations

Cows[#]	(n)	Sites of SP infusion	No. (%) of cows with the normal EGF concentrations on Day 3 after infusion	Endometrial EGF concentrations (ng/g tissue weight)	
				Before infusion	After infusion
Repeat breeders	4	Uterus	0 (0)	1.86 ± 0.64^{A}	1.74 ± 0.71^{A}
	4	Vagina	3 (75)	2.01 ± 0.81^{aA}	6.31 ± 2.25^{bB}
Fertile	4	Vagina	4 (100)	7.18 ± 1.75^{B}	7.43 ± 0.75^{B}

Values are means ± SDs.

[#]All repeat breeder cows showed low EGF concentrations before the infusion while all four cows in the fertile group showed EGF concentrations within the normal range before the infusion. The normal range of endometrial EGF concentrations on Day 3 is 4.7-13.5 ng/g tissue weight [14].

[a,b]Means with different letters within the same row differ ($P < 0.01$).

[A,B]Means with different letters within the same column differ ($P < 0.01$).

Table I-2. Effect of the volume of SP infusing into the vagina on the endometrial EGF concentrations

SP (ml)	PBS (ml)	(n)	No. (%) of cows with the normal EGF concentrations on Day 3 after infusion	Endometrial EGF concentrations (ng/g tissue weight)		
				Before infusion	After infusion	
					All cows	Normalized cows*
0.5	0	8	4 (50.0)	1.83 ± 0.94^{a}	4.84 ± 1.54^{bB}	6.01 ± 0.76^{A} (4)
10	0	8	5 (62.5)	1.83 ± 1.36^{a}	5.93 ± 1.83^{bB}	7.21 ± 0.61^{B} (5)
0.5	9.5	8	5 (62.5)	1.84 ± 1.34^{a}	5.91 ± 2.29^{bB}	7.46 ± 0.76^{B} (5)
0	10	8	2 (25.0)	1.95 ± 1.24	2.55 ± 1.92^{A}	5.35, 5.86 (2)

Values are means ± SDs.

*The numbers in the parentheses are the number of cows with the normalized EGF concentrations after infusions. PBS infusion (control) group was excluded from statistical analysis due to small numbers.

^{a,b}Means with different letters within the same row differ ($P < 0.01$).

^{A,B}Means with different letters within the same column differ ($P < 0.01$).

Table I-3. Effect of SP infusion on the normalization rate of the EGF profile and fertility in repeat breeder cows

Groups	No. (%) of cows with the indicated EGF profile after infusion		Conception rate (%)	Endometrial EGF concentrations (ng/g tissue weight)			
				Before infusion (Day 3)	After infusion		
					Day 3	Day 7	Day 14
SP	Normal	21 (58.3)^{A}	13 (61.9)^{C}	1.97 ± 1.02	8.21 ± 1.08^{a}	1.39 ± 1.10^{b}	8.41 ± 1.50^{a}
	Altered	15 (41.7)	3 (20.0)^{D}	1.95 ± 0.77	2.18 ± 0.97	1.81 ± 1.73	2.97 ± 1.68
	Total	36 (100)	16 (44.4)^{A}	1.96 ± 0.81	6.08 ± 1.96^{aA}	1.89 ± 0.09^{b}	6.18 ± 1.41^{aA}
PBS	Normal	7 (22.6)^{B}	3 (42.9)	1.81 ± 0.97	7.24 ± 1.93^{a}	1.47 ± 1.41^{b}	6.86 ± 1.88^{a}
	Altered	24 (77.4)	3 (12.5)	1.87 ± 1.14	1.99 ± 0.87	2.37 ± 1.36	2.07 ± 1.55
	Total	31 (100)	6 (19.4)^{B}	1.86 ± 1.01	2.67 ± 0.98^{B}	1.92 ± 0.16	2.88 ± 0.86^{B}

Values are means ± SDs.

^{A,B}Means with different letters within the same EGF profile of SP and PBS groups differ ($P < 0.05$).

^{C,D}Means with different letters between the different EGF profiles within the same infusion groups differ ($P < 0.05$).

^{a,b}Means with different letters within the same row differ ($P < 0.05$)

Summary

Endometrial EGF concentrations in the uterus show two peaks on Days 2-4 and 13-14 during the estrous cycle in normal cows. Loss of the two peaks has been linked to reduced fertility in repeat breeder cows. This study aimed to examine the effect of SP on normalizing endometrial EGF concentrations and restoring fertility in repeat breeder cows with low EGF concentrations on Day 3. In Study I-1, we examined the effect of the deposition sites (the vagina and uterus) of SP on the endometrial EGF concentrations in the repeat breeder cows. SP infusion into the vagina, but not uterus, on the day of estrus (Day 0) normalized the endometrial EGF concentrations (≥ 4.7 ng/g tissue weight) on Day 3. In Study I-2, the effect of volume of SP (0.5 ml of SP, 10 ml of SP and 0.5 ml of SP diluted to 10 ml) on EGF concentrations were examined. All groups with SP infusion increased EGF concentrations on Day 3 and cows with 10 ml of SP and 0.5 ml of SP diluted to 10 ml showed the highest levels of EGF concentrations. In Study I-3, we examined the effect of SP infusion on fertility. SP infusion normalized two peaks of endometrial EGF concentrations in about 60% of repeat breeder cows and produced more pregnancies than controls (44.4% vs. 19.4%). Therefore, we concluded that SP may contain an activity to normalize EGF profile and restore fertility in repeat breeder cows with an altered EGF profile.

Chapter II

Identification of bovine seminal plasma proteins with an activity to normalize endometrial epidermal growth factor concentrations in 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 [33, 42, 43]. 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 [33, 42]. 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 [42] and loss and recovery of the two peaks of the endometrial EGF concentrations coincide in most cases [41, 45].

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 [44]. For this abnormality, we have reported a treatment protocol with a high dose of estradiol benzoate in combination with a progesterone releasing device [45]. This protocol normalizes the EGF profile in about 60-70% of repeat breeder cows. However, the efficacy of the treatment varies among herds from 30% to 85% in the field [85], 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.

SP contains estrogen and testosterone, several PGs and glycoprotein signaling substances,

including several cytokines and growth factors [86, 87]. 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 [75, 88], pigs [49], horses [82] and human [52] 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 [75, 84]. For example, roles of SP in the improvement of fertility by modulating uterine functions have been indicated in pigs [89], horses [82], hamsters [46] and mice [47]. SP infusion into the uterus stimulated an inflammatory response leading to changes of cytokines and growth factors in the uterus to facilitate pregnancy [75, 83, 84]. Similarly, bull SP has been suggested to contain a variety of proteins associated with fertility through the effects on sperm and uterine functions [61, 62]. An earlier study reported a regression model to predict bull fertility using 4 fertility-associated protein densities [63]. However, in cattle, large-scaled studies failed to demonstrate beneficial effect of infusions of SP [56] and TGF-β_1 [56], a putative fertility-associated protein, into the uterus on fertility. Our previous study [85], 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 that had lost a peak of the endometrial EGF concentrations on Day 3 in the previous estrous cycle. 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 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 LC-MS/MS. Finally, we examined the effect of an identified protein spot, a form of OPN to normalize the endometrial EGF concentrations.

Materials & 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 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) [44] 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 [33]. Tissues were frozen in liquid nitrogen within 10 min of collection and stored at -80^{0}C 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 [41]. 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 $y = 0.96x + 0.39$, $r = 0.97$. 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 [42, 44].

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 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℃. 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 II-1.

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

The 2D-PAGE was performed using a protocol of O'Farrell (1975) [90]. Isoelectric-focusing gels (disc 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 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℃. 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 II-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. II-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 -20℃. 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 II-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 -80°C. 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 II-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 -20°C. 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 h 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 II-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 II-5

The effect of a SP protein (spot 11 in Table II-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 -80℃. The sample preparation was repeated for the number of animals (62 cows) used for the infusion. At infusion, the eluate was infused into the vagina at estrus as described in Study II-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 Fishers exact test. P values less than 0.05 were considered significant in all analysis

Results

Study II-1

The endometrial EGF concentrations at the first examination (before SP infusion) were similar in all groups (Table II-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 II-2

The endometrial EGF concentrations at the first examination (before SP infusion) were similar in all groups (Table II-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 II-3

The normalization rates of the EGF concentrations and the conception rates in all 6 herds were at the similar levels (Table II-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 II-4

An example of SP protein separation by 2D-PAGE were shown in Fig. II-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 II-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, L-PGDS, tissue inhibitor of metalloproteinase 2 (TIMP-2), TGF-β_1 and myoglobin (Table II-4). Peptide mass data for 3 protein spots (spots 1, 12 and 15) did not match to those on the database.

Study II-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 II-4) [66, 91–96]. 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-β_1 and OPNs.

An earlier study reported a regression model to predict bull fertility using 4 fertility-associated protein densities [63] 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 [64, 65]. L-PGDS with 26 kDa reduces the production of anti-sperm antibodies in the female reproductive tract [63, 66, 67]. TGF-β_1 upregulates pro-inflammatory cytokines and chemokines in the uterine and cervical epithelial cells, and contributes to establish pregnancy in rodent and human [68, 69]. However, TGF-β_1 failed to improve fertility in cattle [56]. Other proteins in the list (Table II-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 [63–65]. Similarly, in stallion [97], camel [98] and buffalo [99], 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 [100]. In cattle, fertilization medium containing 10 mg/mL OPN improved in vitro embryo production and OPN positively influenced sperm capacitation in vitro [101]. 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 [102]. The molecular mass for bovine OPN derived from bone cells, estimated from the nucleotide sequence, is 30.1 kDa [93]. OPN identified in bovine milk, a rich source of OPN, is 262 amino acids long and has an estimated molecular mass of 66 kDa [103]. 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 [64]. However, a smaller size of 30 kDa protein also found in the partially purified preparation [64]. These differences in molecular size and isoelectric point of OPN in SP could be attributed to different patterns of posttranslational modification [104] and the acidic nature of the protein, which have been shown to affect protein mobility in SDS-PAGE [105]. One of the 4 OPN found in the present study (spot 14 in Table II-4) with 29 kDa and pI6.5-6.9 showed the activity to normalize the endometrial EGF concentrations in repeat breeder cows. This 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 [45]. However, efficiency of this hormonal treatment to normalize EGF differed to a large extent (30-85%) between herds [85]. Thus, a new treatment option is needed. In Study II-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 [50, 81], rodents [46, 47], horses [82] and women [69], 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 [106]. 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 [107]. 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 [108]. Similar effect of activated lymphocytes to improve fertility in embryo transfer recipient cows has also been reported [109]. 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 improve fertility 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.

Tables and figures

Table II-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[4)]

#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 II-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	Normalize# 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 ($P < 0.05$)

Table II-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 II-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[66]
3	Bovine seminal protein A1/A2	16.0	5.8-6.1	P02784	Esch et al., 1983[91]
4	Bovine seminal protein A3	16.2	6.1	P04557	Seidah et al., 1987[92]
5	Carbonic anhydrase	29.0	6.2-6.4	Q1LZA1	-
6	Carbonic anhydrase	28.5	6.2	Q1LZA1	-
7	Transforming growth factor- β_1	17.0	6.2-6.4	P18341	Van Obberghen-Schilling et al., 1987[95]
8	Tissue inhibitor of metalloproteinases 2	16.5	6.3	P16368	DeClerck et al., 1993[96]
9	Osteopontin	16.3	6.2-6.3	P31096	Kerr et al., 1991[93]
10	Osteopontin	16.0	6.4	P31096	Kerr et al., 1991[93]
11	Osteopontin	29.0	6.5-6.9	P31096	Kerr et al., 1991[93]
12	Unknown	27.5	6.8	-	-
13	Osteopontin	27.5	6.9	P31096	Kerr et al., 1991[93]
14	Myoglobin	16.5	6.7	P02192	Shimada et al., 1989[94]
15	Unknown	16.0	6.6	-	-

[a]Protein names 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

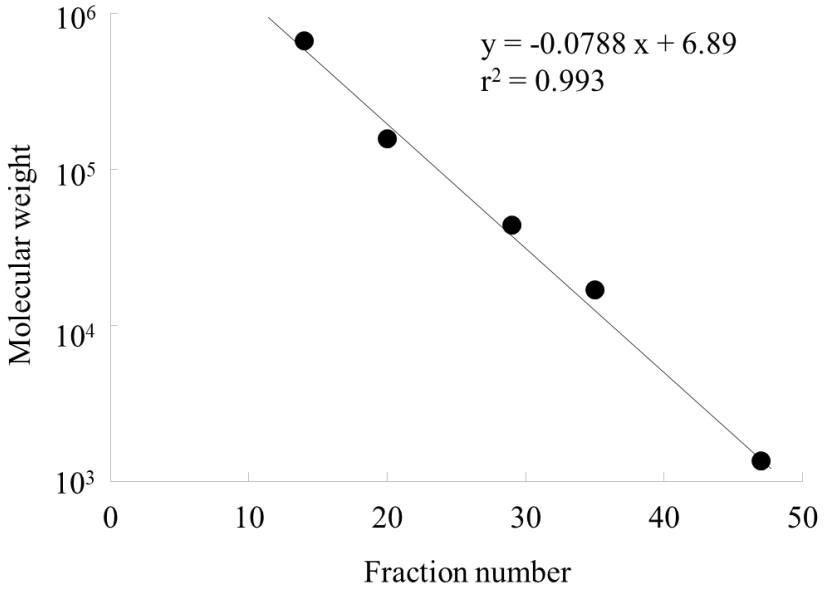

Fig. II-1. Calibration curve of Sephadex G-200 column (0.7 cm x 75 cm). Elution was at 2.0 ml/h at 4°C and fractions of 1.25 ml were collected. Column was calibrated with standard proteins: thyroglobulin (670,000), bovine gammaglobulin (158,000), chicken ovalbumin (44,000), equine myoglobin (17,000) and vitamin B_{12} (1,350). The void volume was determined with Blue dextran 2000.

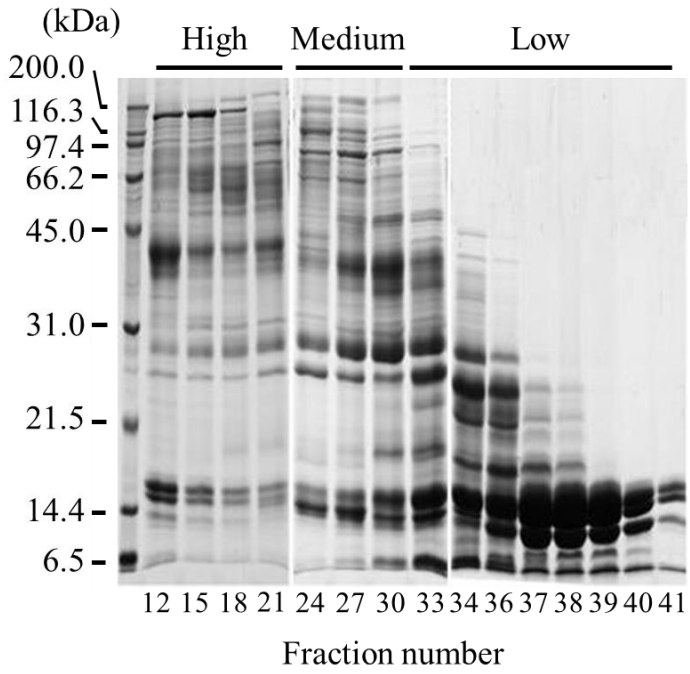

Fig II-2. SP proteins separated by gel filtration using Sephadex G 200 column. SP protein (0.5 ml) was separated on Sephadex G 200 column (Fig. II-1). Fractions of 1.25 ml were collected. Twenty microliters of fractions of indicated numbers were separated by polyacrylamide gel electrophoresis (PAGE) and gels were stained with 0.1% Coomassie brilliant blue. For infusion study (Study II-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 5 kDa, respectively (Fig. II-1).

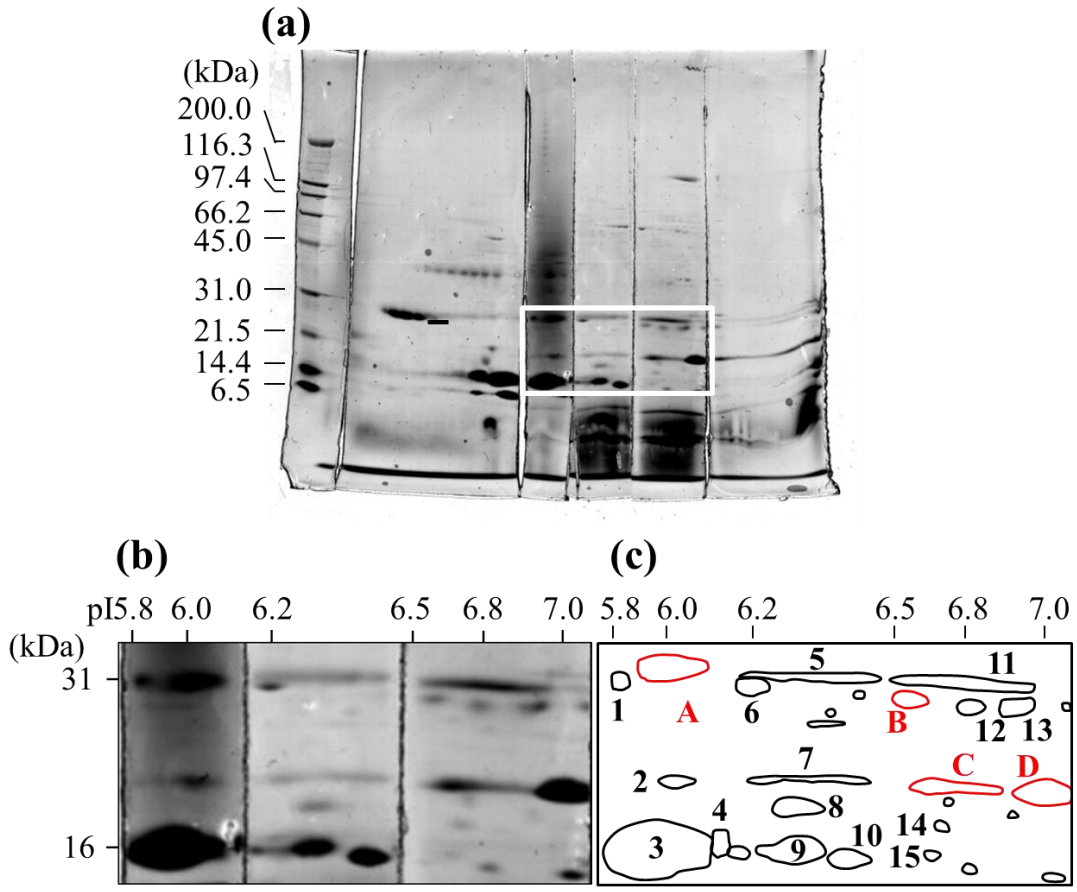

Fig II-3. Bovine SP proteins separated by 2D-PAGE. a) Proteins (500 µg) were first separated by isoelectric focusing (IEF) with isoelectric point (pI) range between 3 and 10, followed by PAGE using a 12 % polyacrylamide gel. b) and c) enlarged section of gel with molecular weight range of 16-29 kDa and 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 LC-MS/MS. Twelve protein spots were identified and the name of proteins were shown in Table II-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).

Summary

Endometrial 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 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 2D-PAGE. 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 LC-MS/MS analysis. Twelve protein spots that include four spots of 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 26 out of 62 (41.9%) 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.

Summary and Conclusions

In this study, I have examined the effect of SP or SP proteins on fertility of repeat breeder cows by evaluating the capacity of SP to normalize the endometrial EGF concentrations on Day 3, an established marker for recovery of fertility in repeat breeder cows. The study also included a small-scaled fertility study in repeat breeder cows. The study was conducted in two parts: the first part (Chapter I) demonstrated that SP contains an activity to normalize the endometrial EGF profile and restores fertility in repeat breeder cows, and the second part (Chapter II) identified the protein with the activity to normalize the EGF concentrations in repeat breeder cows.

In Chapter I, the effect of SP to normalize the endometrial EGF profile was examined. Firstly, to determine the effect of the site of SP deposition, SP was infused into the uterus or vagina of the repeat breeder cows with an altered endometrial EGF profile at estrus (Day 0) and examined the EGF concentrations on Day 3. I found that SP normalized the endometrial EGF concentrations when SP was deposited in the vagina, but not in the uterus. This finding is very important in the arena of physiological study on the role of SP in the regulation of uterine function since all previous studies have used SP infusion into the uterus or cultured uterine cells.

Then, the effect of SP volume was examined in two volumes, 0.5 ml and 10 ml. In repeat breeder cows, 10 ml of SP showed higher activity than 0.5 ml of SP. Interestingly, the activity of 0.5 ml of SP became at the similar levels to 10 ml of SP when it was infused after dilution to 10 ml with PBS. The results indicate that 0.5 ml of SP contains the activity enough to exhibit a maximal effect of normalizing endometrial EGF concentrations. The results also indicate that volume of infusing samples is important to obtain the maximal effect. For example, area of vaginal mucosa being exposed to SP may affect degree of the endometrial response in the EGF production.

Finally, the study examined the effect of SP infusion into the vagina on fertility in repeat breeder

cows. The SP infusion group showed a higher normalization rate of the EGF profile than the controls (58.3% vs. 22.6%). Since pregnancy rate of the cows after normalization of the EGF profile was high (> 60%), pregnancy rate after SP infusion in repeat breeder cows were higher than the controls (44.4% vs. 19.4%).

These results indicate that the bull SP contains an activity to normalize the endometrial EGF profile and restore fertility of repeat breeder cows with an altered EGF profile.

In Chapter II, SP protein with the activity to normalize the endometrial EGF concentrations were identified. Firstly, pooled SP from different bulls was separated by gel filtration with a Sephadex G-200 column and 2D-PAGE to obtain crude SP protein fractions. A crude protein fraction with molecular weight range of 16-29kDa and isoelectric point range of 5.8-7.0 was found to contain the activity. Proteins in this area was identified using LC-MS/MS. I found 15 protein spots on 2D-PAGE gel and could identify 12 spots that include 3 putative fertility-associated proteins: OPN, TGF- β_1 and L-PGDS. The list of identified protein spots contains four forms of OPN and one form of OPN with 29 kDa was tested for its activity to normalize the endometrial EGF concentrations. Eluate of the OPN spot of 2D-PAGE gel showed a higher normalization rate of the EGF profile than the controls (41.9% vs. 23.1%).

These results indicate OPN with molecular weight of 29 kDa may be responsible for the activity of SP to normalize the endometrial EGF profile in repeat breeder cows.

In my study, I have demonstrated that SP proteins could promote pregnancy in repeat breeder cows by normalizing the endometrial receptivity that could be measured by the endometrial EGF profile. A form of OPN seems responsible for the activity of SP, although the present study could not provide direct evidence for OPN to improve fertility.

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