Effects of milk osteopontin on the normalization of endometrial epidermal growth factor profile and restoration of fertility in repeat breeder dairy cows

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Contents

Abbreviations

AI: Artificial insemination

ANOVA: Analysis of variance

BSA: Bovine serum albumin

CBB: Coomassie brilliant blue

cc: Cubic centimeter

EGF: Epidermal growth factor

E_2: Estradiol

GnRH: Gonadotropin-releasing hormone

g: Gram

g: Gravity

h: Hour

kDa: Kilodalton

kg: Kilogram

L: Liter

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

min: Minutes

mL: Milliliter

mg: Milligram

mM: Millimolar

M: Molar

mOPN: Milk osteopontin

NaCl: Sodium chloride

ng: Nanogram

nm: Nanometer

OPN: Osteopontin

P_4: Progesterone

PG: Prostaglandin

pg: Picogram

SP: Seminal plasma

SDS-PAGE: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

%: Percentage

µL: Microliter

µg: Microgram

V: Voltage

Notes

The contents of Chapter 1 have been published in Theriogenology journal:

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The contents of Chapter 2:

Kyaw HM, Tagami T, Yanagawa Y, Katagiri S. Effects of milk osteopontin of repeat breeder dairy cows on the endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows (*In preparation*)

Preface

Reproductive efficiency and performance of dairy cattle have declined over the last 30 years [1]. The reasons for this decline include changes in increased milk production and monitoring fertility [1]. Since mid-twentieth century to the present day, milk production of dairy cattle has been increasing from 4,000 kg to >12,000 kg of milk in a 305-day period [2, 3]. Maintaining the high reproductive efficiency in modern dairy herds is a big challenge and of great importance, because it has a great impact on herd profitability [4].

One of the reasons related to reduced fertility is the presence of repeat breeder cows in the dairy herds. Repeat breeder cows are defined as cows failing to conceive after three or more inseminations without detectable abnormalities in their genital organs, the estrous cycle and clinical signs. Frequency of embryonic loss increases in the repeat breeder cows [5, 6]. The incidence of repeat breeding in cattle has been reported between 9 to 22% [7–10]. The causes of infertility in repeat breeder cows remain unclear, but may include environmental, management, and animal factors. Changes of circulating ovarian steroid hormone concentrations and the resulting alterations in growth factors and cytokines in the uterus are examples of animal factors [6, 11]. Slow increases and low peak levels of estradiol (E_2) and progesterone (P_4) which characterize endocrine changes found both in high producing cows and repeat breeder cows [11–13]. Among growth factors and cytokines, epidermal growth factor (EGF) seems one of the most important regulatory components of uterine function and embryonic development [14, 15]. Estrogen stimulates the EGF production in the mouse [16, 17] and rat uterus [18]. EGF acts as an estrogen-inducible physiological mediator for the growth and differentiation in the mouse uterus and the vagina [19, 20]. The presence of ligands of EGF family, e.g., epidermal growth factor (EGF), transforming growth factor α (TGF-α), heparin binding EGF (HB-EGF), betacellulin (BTC), amphiregulin (ARGE), epiregulin (ERGE), and their receptors in the uterine endometrium have been reported in cattle [21–23], sheep [24], goat[25], and pig [26]. In cattle, EGF shows an increase on days 13-14 [27] when the embryo initiates elongation [28]. EGF from the uterus seems an important maternal signal to embryos at this stage since cattle embryos express the EGF receptor, but not EGF itself [29]. Furthermore, the endometrial EGF has the ability to increase the production of prostaglandin (PG), E_2 and $PGF_{2\alpha}$ [22], and the PGE_2: $PGF_{2\alpha}$ ratio in pigs [30] and rats [31]. These effects of EGF on endometrial PG production should enhance CL function in the cattle [32, 33].

In fertile cattle, the endometrial EGF concentrations exhibited a cyclic change with apparent peaks on days 2-4 and 13-14 (day 0 = estrus). However, repeat breeder cows exhibit an abnormality in the profile of endometrial EGF concentrations during the estrous cycle [34]. Treatments to normalize the endometrial EGF profile and restore fertility in repeat breeder

cows have been reported in the previous studies. Hormonal treatment with a high dose of estradiol benzoate in combination with an intravaginal P_4-releasing device normalized the endometrial EGF profile in approximately 70% and restored fertility in approximately 60% of repeat breeder cows [35]. Another treatment option, an infusion of seminal plasma (SP) proteins into the vagina, normalized the endometrial EGF profile and restored fertility in approximately 60% and 40% of repeat breeder cows, respectively [36]. Moreover, one of the SP proteins, osteopontin (OPN) in a low molecular mass fraction of less than 29 kDa [37], may have been found to be responsible for the SP activity [36]. SP contains different molecular variants of OPN and one of the variants with 55 kDa molecular mass has been described as fertility-associated protein [38, 39].

OPN, also known as secreted phosphoprotein 1, is a highly acidic calcium-binding glycosylated phosphoprotein containing the arginine-glycine-aspartic acid binding, heparin, thrombin and calcium binding zones [40–42]. It was initially isolated from the mineralized matrix of bovine diaphyseal bone [43, 44], and later ubiquitously detected in the epithelial cells and secretions of the oviduct, uterus, and placenta [41], SP [39], blood [45], urine [46], and milk [47]. OPN is present in various forms with different molecular weights due to post-translational modifications that include enzymatic cleavage, glycosylation, phosphorylation, oxidation, and sulfation, depending on the tissue and cell types [40, 41, 48]. It is a multifunctional depending on the expressed tissues or body fluids and its highest concentrations are found in milk [49]. Moreover, milk OPN (mOPN) is the most phosphorylated form and contains between 25 to 30 phosphate groups [50]. Human OPN, the most studied OPN, consists of 298 amino acids [48], with different molecular mass variants ranging between 25 and 75 kDa while bovine OPN consists of 262 amino acids with variants molecular mass ranging between 16 and 60 kDa [37, 48, 51].

In Chapter 1, I prepared mOPN in a large-scale using 1 L of milk samples and used for treatment of multiple repeat breeder cows. I have demonstrated that the infusion of mOPN normalized the endometrial EGF profile and restored fertility in repeat breeder cows. However, this protocol may allow viral or bacterial infection spreading via treatment. Therefore, use of mOPN preparation from her own milk sample of individual repeat breeder cow may be an alternative strategy to avoid the risk of spreading infections. In Chapter 2, to examine feasibility of this treatment option, I first examined changes in OPN contents in milk at different lactation stages in different cow types and to estimate milk sample volume that is necessary to obtain OPN of minimum quantity enough for treatment since contents of OPN may vary. Repeat breeder cows are usually diagnosed at the mid or late stage of lactation, at which OPN content may be low [45]. Further, OPN contents had not been examined in repeat breeder cows. Then,

OPN was prepared using milk sample of individual repeat breeder cow and examined its effect on the EGF profile and fertility.

Chapter 1

Effects of milk osteopontin on the endometrial epidermal growth factor profile and restoration of fertility in repeat breeder dairy cows

Introduction

Growing evidence that ovarian steroid hormones primarily regulate the timing and levels of local factors that include EGF in the uterine endometrium of mice, rat [14, 15], and cattle [35]. Alteration of endometrial EGF profile may be due to the changes in circulating ovarian steroid hormones reported in repeat breeder cows [13, 52, 53]. In fertile cow shows two peaks of endometrial EGF concentrations on days 2-4 and 13-14 (day 0 = estrus) of estrous cycle [34] though those peaks were absent or lower in approximately of 70% of repeat breeder cows [34, 54] and approximately 25% of high yielding cows (>10,000 kg of milk yield) at approximately 60 days postpartum [55]. Previous studies demonstrated that a single measurement of the concentration of EGF in endometrial tissue obtained by biopsy on day 3 may reveal the endometrial EGF profile because the loss and recovery of the two peaks simultaneously occurred in most cases [55, 56]. Alterations of endometrial EGF concentrations have been linked to reduced fertility after artificial insemination (AI) in repeat breeder cows [27] and failed pregnancy after embryo transfer in apparently normal recipient cows [56].

The normalizing effects of SP on the endometrial EGF profile has been demonstrated in repeat breeder cows. The infusion of SP into the vagina of repeat breeder cows at estrus normalized the endometrial EGF profile in approximately 60% and restored fertility than the controls (44.4 vs. 19.4%) [36]. In addition, a 29 kDa OPN variant normalized the endometrial EGF profile in approximately 41.9% (n = 62) of repeat breeder dairy cows [37], which was higher than the normalization rate of 23.1% (n = 52) in the control group. The previous results indicated that a form of OPN in SP may replace SP activity [37].

OPN plays multiple roles in different physiological systems [48], including the immune, reproductive, and nervous systems. For example, OPN functions as a Th1 cytokine, promotes cell-mediated immune responses, and contributes to chronic inflammatory and autoimmune diseases [57, 58]. In reproduction, OPN is involved in sperm capacitation and the acrosome reaction [59, 60], blastocyst formation, the implantation of embryos, and placentation in different species [40, 43, 59]. Moreover, dietary supplementation with OPN elicited changes in the exploratory behavior of young pigs [61] and promoted cognitive development in mice [62].

Bovine mOPN supplemented into fertilization medium promoted not only sperm

capacitation, but also cleavage, blastocyst formation, and embryo development [38, 46]. Moreover, bovine mOPN enhanced the proliferation and activity of alkaline phosphatase in the osteoblasts of rat [63]. These effects of mOPN in cells and tissues, which are different from the original target, prompted me to examine its ability to normalize the endometrial EGF profile. Despite wide variations in OPN molecules, mOPN, a rich source of OPN both in humans and cattle [48], may replace the action of OPN in various *in vitro* systems. Therefore, in Chapter 1, I prepared the purify mOPN from bovine milk and its ability to normalize the endometrial EGF profile and restore fertility in repeat breeder dairy cows with an altered EGF profile was examined.

Materials and Methods

Animals

Thirteen commercial dairy farms in Hokkaido prefecture, Japan participated in the present study. All animal experiments were conducted according to guidelines for the Care and Use of the Experimental Animals protocols of Hokkaido University, Japan (Experimental protocols # 16-0071 and 19-0030).

Repeat breeder cows were diagnosed by local practitioners using the criteria of failing to conceive after three or more AI without a detectable abnormality in the estrous cycle, clinical signs, and genital organs (Fig.1-1). All cows were then confirmed to meet the definition of repeat breeder cows through additional examinations [36], including uterine morphology by transrectal ultrasonography [64], uterine cytology by cytobrush [65] and oviductal patency by tubal insufflation [66]. Endometrial EGF concentrations were examined on day 3 in all cows and repeat breeder cows showing low EGF concentrations (< 4.70 ng/g tissue weight) were used in the present study (Fig. 1-1). All repeat breeder cows were multiparous lactating Holstein cows (> 10,000 kg of 305-day fat-corrected milk) younger than 10 years of age and between two and five in parity.

Synchronization of cows and timed insemination

For initial screening, cows were synchronized for estrus or ovulation using a single intramuscular administration of $PGF_{2\alpha}$ (20 or 25 mg of dinoprost tromethamine, Pronalgon F, Zoetis Japan, Tokyo, Japan) or an Ovsynch protocol. In the Ovsynch protocol [67], cows received the first gonadotropin-releasing hormone (GnRH) treatment (100 μg of fertirelin acetate, Conceral, MSD Animal Health, Tokyo, Japan), followed by a treatment with $PGF_{2\alpha}$ (25 mg of dinoprost tromethamine) 7 days later and a second GnRH treatment (100 μg of fertirelin acetate) approximately 55 ± 2 h after the $PGF_{2\alpha}$ treatment. Estrus was detected by

either observations twice daily after the $PGF_{2\alpha}$ treatment or using an automated activity monitor (estrus = day 0). Cows failed to show estrus within five days after the $PGF_{2\alpha}$ treatment were excluded from the study. When the cows were synchronized by the Ovsynch protocol, the next day of the second GnRH was considered day 0. All selected cows for the study were synchronized for treatment with mOPN using the Ovsynch protocol starting with the first GnRH between 7 and 9 days of the estrous cycle. When cows were inseminated, they were subjected to AI on day 0 with frozen semen by professional AI technicians approximately 16 to 20 h after the second GnRH treatment.

Biopsy of uterine endometrial tissues

Uterine endometrial tissues were obtained by biopsy on the contralateral side to recent ovulation or CL development from the uterine intercaruncular area using a biopsy device (3050100, Fujihira Industry, Tokyo, Japan) with caudal epidural anesthesia (3 mL of 2% lidocaine; 2% xylocaine, AstraZeneca, Osaka, Japan) as previously described [54]. The caruncle region was distinguished from the intercaruncle region as fluffy cut surface due to rich blood vessels. If the biopsy sample contained caruncle region, it was dissected out and the rest of the tissue was used [34]. Endometrial tissue biopsy samples for EGF assay were frozen in liquid nitrogen within 10 min of tissue collection and stored below -30°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 previously described. Endometrial EGF concentrations in tissue extracts were measured with a double-antibody sandwich enzyme immunoassay using 96-well microtiter plates (Costar 3590, Corning, NY, USA) [34]. An anti-human EGF mouse monoclonal antibody (MAB636, R & D Systems, Minneapolis, MN, USA) and anti-human EGF rabbit antiserum (5022-100, Biogenesis, Poole, UK) were used as the solid-phase antibody and detection antibody, respectively. Neither antibody showed significant cross-reactivity with other cytokines in tests conducted by the manufacturers. The assay system was verified using increasing concentrations of recombinant bovine EGF [36]. A linear regression analysis of the assay results with recombinant bovine EGF gave $y = 0.96x + 0.39$, $r = 0.97$. Assay sensitivity was 10 pg/well. The intra- and interassay coefficients of variation at 50 pg/well were 4.2 and 6.8%, respectively.

In the study, endometrial EGF concentrations were measured on day 3 of the estrous cycle and the EGF profile was considered to be normal when concentrations were within the normal range for day 3 (4.70-13.50 ng/g tissue weight) [54, 56].

Preparation of mOPN

OPN was purified from bovine milk according to a previously described method [68] with minor modifications. Briefly, 1 L of fresh bovine milk was collected from the university farm (Experimental farm of Hokkaido University, Japan), and centrifuged at 6,000 x g at 10°C for 10 min to remove the fat portion. Acetic acid was added to skim milk to adjust pH to 4.6, and the precipitate was removed by centrifugation at 1,500 x g at 15°C for 15 min. The resultant acidic whey was applied to a 100 mL of DEAE-Sepharose Fast Flow column (ø 2.5 × 20 cm) (Cytiva, Tokyo, Japan) equilibrated with 100 mM sodium acetate buffer (pH 5.0). The column was washed with 0.1 M and 0.2 M NaCl in equilibration buffer, and mOPN was then eluted with 500 mL of 0.3 M NaCl in equilibration buffer. Purified mOPN was dialyzed against PBS (Takara Bio, Kusatsu, Japan) for 48 h. Column chromatography and dialysis were performed at 4°C. The elution profile of the protein was monitored with absorbance at 280 nm and confirmed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analyses.

To calculate the extinction coefficient of mOPN, 50 mL of purified mOPN (absorbance at 280 nm, A280 = 0.115) was dialyzed against distilled water and lyophilized, and the resultant solid mOPN was weighed. The ratio between the concentration of mOPN (0.52 mg/mL) and A280 was measured as its extinction coefficient. Therefore, the concentration of mOPN was 4.50 mg/mL when the predicted A280 was 1.0. The concentrations of other batches of purified mOPN were also estimated according to the extinction coefficient.

Gel electrophoresis was performed on a 12% TGX polyacrylamide gel (Bio-Rad laboratories Inc., Hercules, Ca, USA), according to the manufacturer's instructions manual, at a constant voltage at 200 V. Proteins were stained with Coomassie Brilliant Blue G-250 (CBB G-250) (Nacalai Tesque Inc., Kyoto, Japan). OPN from bovine milk (O3514, Sigma-Aldrich. Inc. USA,) was used as an authentic mOPN. Aliquots of purified samples were lyophilized with 1% trehalose as a cryoprotectant agent and stored at 4°C for later infusion into repeat breeder cows.

Confirmation of the identity of OPN

The identity of purified OPN was confirmed by Western blotting and peptide mass fingerprinting analyses. In Western blotting, fractions showing protein bands that matched those of the authentic mOPN were separated by SDS-PAGE and transferred to a ProBlott PVDF membrane by a semi-dry blotting method using transfer buffer (30 mM Tris, 17 mM boric acid, 0.055% SDS, and 20% methanol). The resultant membrane was incubated in 1% BSA in TBS-T buffer (10 mM Tris pH 7.5, 150 mM NaCl, and 0.05% Tween-20) at room

temperature for 1 h for blocking. After washing the membrane with TBS-T buffer, the membrane was incubated with an anti-cow SPP1/OPN polyclonal antibody (Abbexa Ltd., Cambridge, UK) at 4°C for 16 h. The unbound primary antibody was washed out using TBS-T, and the secondary antibody (anti-IgG-horseradish peroxidase (HRP): SouthernBiotech, Birmingham, AL) was bound at room temperature for 2 h. After washing the unbound secondary antibody, peroxidase was detected using 3,3′-diaminobenzidine staining (Fujifilm Wako Pure Chemical Industries Ltd., Japan).

In the peptide mass fingerprinting analysis, the in-gel digestion of proteins was performed according to a previously reported method [69]. Briefly, protein bands cut from the SDS-PAGE gel were washed 4 times in 50% methanol with sonication on ice, gradually dehydrated using 50 and 100% acetonitrile, and swollen with 50 mM ammonium bicarbonate. Dehydration by 100% acetonitrile and swelling by 50 mM ammonium bicarbonate were repeated. The gel pieces were sequentially dehydrated by 50% acetonitrile containing 50 mM ammonium bicarbonate and 100% acetonitrile, and dehydrated gels were completely dried using a spin vacuum for 5 min. Dried gels were swollen using 15 μL of trypsin solution (25 μg/mL dissolved in 50 mM ammonium bicarbonate) and incubated at 37°C for 24 h. Tryptic peptides were extracted twice by 45% acetonitrile containing 0.1% trifluoroacetic acid with sonication on ice. Extracts (total volume of 40 μL) were dried in vacuo and dissolved in 20 μL of 0.1% formic acid containing 2% acetonitrile. The resultant peptides were subjected to the nanoflow LC-MS/MS system (LTQ-OrbitrapXL; ThermoFisher Scientific, Waltham, MA) at the Instrumental analysis service of Hokkaido University. The MS/MS values detected were analyzed by a Mascot MS/MS ion search [69].

SP preparation

SP was prepared according to a previously reported method [36]. At a commercial AI center (Genetics Hokkaido, Tokachi Shimizu, Hokkaido, Japan), semen was collected twice a week from seven fertile Holstein bulls using an artificial vagina. Two ejaculates were collected on the same day, generally with an approximately 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 Hokkaido University. At the university laboratory, SP was thawed and centrifuged at 5,000 x g at 4°C for 20 min and the resulting supernatants from seven bulls were pooled and used as SP samples throughout the present study. Aliquots of 0.5 mL of SP were stored and transported at -20ºC.

Infusion of samples into vagina of repeat breeder cows

Lyophilized mOPN (1.3 mg) was reconstituted with 10 mL of PBS immediately before

use. An aliquot of frozen SP was thawed by adding 9.5 mL of PBS immediately before use. All infusion samples including vehicle (PBS) were prepared in the same volume of 10 mL with PBS immediately before infusion. Samples were loaded into a 10-cc syringe and infused into the vagina as previously described [36]. Briefly, a disposable plastic catheter for AI was attached to the syringe loaded with infusion samples. The tip of the catheter was introduced into the vagina and guided deep into the vagina adjacent to the external orifice of the cervix with the hand inserted into the rectum in the manner of AI. Samples were then deposited by pushing the plunger of the syringe and the AI catheter was gently withdrawn.

Study design: effects of mOPN on the normalization of the endometrial EGF profile and restoration of the fertility of repeat breeder cows

A diagrammatic timeline of the screening, synchronization and treatment sequences of repeat breeder cows for the present study were shown in Fig. 1-1. The effects of mOPN on the normalization of endometrial EGF concentrations were assessed using 317 repeat breeder cows showing low endometrial EGF concentrations (< 4.70 ng/g tissue weight) on day 3 in the previous estrous cycle. Repeat breeder cows were synchronized with Ovsynch protocol and infused with 1.3 mg of mOPN (171 cows), 0.5 mL of SP (62 cows), or PBS (84 cows) into the vagina approximately 16 to 20 h after the second GnRH treatment, corresponding to the timing of AI. Endometrial EGF concentrations were measured on day 3 for the second time to assess the normalizing effects of treatments on the endometrial EGF profile.

Some repeat breeder cows treated with the mOPN (46 cows), SP (50 cows), or PBS (45 cows) infusion were subjected to AI immediately before the treatment. Pregnancy was diagnosed by transrectal palpation between days 60 and 65 after AI.

Data analysis

Data were analyzed using the computer software JMP Pro 14 (SAS Institute Inc., Tokyo, Japan). Endometrial EGF concentrations were compared between groups using a two-way analysis of variance (ANOVA) with repeated measurements, followed by Tukey's test or the paired t-test. Normalized endometrial EGF concentrations after the infusion were compared using a one-way ANOVA followed by Tukey's test. The rates of normalization of the EGF profile and pregnancy were compared between the groups using the Chi-squared test and Fisher's exact test, respectively. P values less than 0.05 were considered to be significant in all analyses.

Results

Purification of mOPN

In six batches of mOPN preparations, between 6.3 and 23.4 mg of mOPN was obtained from 1 L of bovine milk. OPN preparations showed three major bands with apparent molecular masses of approximately 61 kDa (peptide I), 37 kDa (peptide II), and 31 kDa (peptide III) by SDS-PAGE. The three bands accounted for approximately 85% of the total protein content. All three major bands were confirmed to be OPN by the Western blotting analysis (Fig. 1-2. A, B). In the peptide mass fingerprinting analysis, the tryptic peptide masses of peptides I, II, and III were matched at approximately 50, 40 and 10%, respectively, to the amino acid sequence of bovine OPN with partial phosphorylation (Uniprot ID: P31096) (Fig. 1-3).

Effects of mOPN on the endometrial EGF profile and restoration of fertility in repeat breeder cows

Endometrial EGF concentrations on day 3 in the first examination for the recruitment of repeat breeder cows were similar in all groups (Table 1-1). The normalization rates of the endometrial EGF profile were similar in the SP and mOPN groups (58.1 and 56.1%, respectively), and were higher than that of the control (PBS) group (23.8%) ($P < 0.05$). Mean endometrial EGF concentrations on day 3 were also similar after the SP and mOPN infusions and, were higher than that of the control group ($P < 0.05$). Furthermore, levels of EGF concentrations in cows showing normalized EGF concentrations after the infusion were similar between mOPN and SP groups, and were higher than that of the PBS group.

The conception rate after the infusion of mOPN was similar to that of the SP group and higher than that of the control (PBS) group ($P < 0.05$) (Table 1-2). In cows showing a normalized EGF profile after the mOPN and SP infusions, the conception rate was higher than that of cows with an altered EGF concentration ($P < 0.05$).

Discussion

This is the first study to demonstrate that OPN may restore fertility in repeat breeder cows with an altered endometrial EGF profile. Previous studies demonstrated that SP normalized the EGF profile in repeat breeder cows [36] and showed that a form of OPN with a molecular weight of 29 kDa found in SP was responsible, at least in part, for SP activity [37]. The present results confirmed the normalizing effects of OPN on the EGF profile, which resulted in improved fertility. The present study also revealed that mOPN may replace truncated OPN of 29 kDa found in SP to normalize the endometrial EGF profile in repeat breeder cows. The capacity of mOPN to normalize the EGF profile (56.1%) and improve fertility (43.5%) was similar to those of SP (58.1 and 40.0%, respectively) in the present study and hormone therapy (66.7 and 40.0%, respectively) in the previous study [35]. Milk is an abundant source of OPN in cattle [48], and OPN can be separated by a simple procedure. Due to the limited supply of SP, mOPN is a promising source for the preparation of therapeutic agents.

OPN shows different forms in different tissue and cell types. However, mOPN has been used in many studies and shown to exert some effects that include the promotion of tissue repair and enhancements in *in vitro* embryo production [38, 46]. The present study showed that purified mOPN exerted similar effects to OPN in SP despite its molecular forms (61, 37, and 31 kDa) differing from those of OPN in SP (29 kDa or less) [37]. Differences in molecular masses may be attributed due to post-translational modifications in specific tissues. The present mOPN forms appear to be phosphorylated. The peptide mass analysis detected many tryptic peptides containing phosphorylated serine or threonine residues (Fig.1-3). Purified mOPN, particularly peptide I, may be strongly phosphorylated and, thus, observed as 61 kDa, which was consistent with previous findings [70, 71], however, its theoretical molecular weight calculated from its amino acid sequence was 29 kDa [51]. A high content of acidic residues, including phosphorylated serine and threonine, inhibits the migration of a protein on SDS-PAGE.

The mechanisms by which an infusion of OPN into the vagina normalizes EGF levels in the uterus 3 days later currently remain unknown. However, a possible scenario is that OPN sensitizes immune cells in the vagina, causing uterus-targeted or systemic changes through immune cell activation in the attached lymph nodes. The site of action of OPN appears to be the vagina because SP infused into the vagina, but not the uterus, normalized the EGF profile in repeat breeder cows [36]. A receptive mechanism may exist that mediates the effects of OPN by vaginal cells and/or cells migrating into the vaginal mucus. A previous study demonstrated that, OPN plays a key role in the crosstalk between innate and adaptive immunity through the

Th1/Th2 balance and macrophage immune responses [72]. Among inflammatory cytokines, interleukin-1α (IL-1α) [73] , IL-1ß [74], and tumor necrosis factor-α (TNF-α) [75, 76] play roles in the regulation of bovine endometrial functions and embryo development. TNF-α may activate estrogen signaling pathways via estrogen receptor α in endometrial epithelial cells [77]. This effect of TNF-α may stimulate the production of EGF in the uterus [17].

The results of the present study cannot be applied to all so-called repeat breeder cows. In the present study, repeat breeder cows were recruited based on strict selection criteria to exclude cows with any potential causes of reduced fertility. Cows were recruited as repeat breeders based on breeding and health records by practitioners and a thorough examination for uterine morphology by transrectal ultrasonography [64], uterine cytology by cytobrush [65], and oviductal patency by tubal insufflation [66]. The EGF profile was then examined in cows with no abnormalities in these examinations. Repeat breeder cows selected by strict definitions and additional examinations account for 8% of the entire herd [55], and 70% have an EGF abnormality [34, 54]. In other words, between 5 and 6% of all cows are the subjects of this study. This percentage itself is, however, by no means small for dairy farming, which is under strict financial management.

Previous study demonstrated that approximately 25% of cows at 60 days postpartum showed similarly altered EGF profiles to those in repeat breeder cows [55]. If a treatment with mOPN before the start of AI exerts similar effects on the restoration of a normal EGF profile and fertility in these cows, the time from delivery to conception of the herd will be shortened, and dairy farming will achieve great economic benefits. However, further study with a large number of animals is needed to confirm the present results and elucidate the mechanisms by which the intra-vaginal infusion of OPN normalizes the EGF profile in the uterus of repeat breeder cows.

In Chapter 1, I have demonstrated that OPN purified from bovine milk normalized the endometrial EGF profile in repeat breeder dairy cows. The present results confirmed the effect of seminal OPN on the normalization of the EGF profile that is found in the previous study [36]. To my best knowledge, this is the first example that have demonstrated the effect of seminal factor to improve fertility through the regulation of uterine function or environment by specific pathway in cattle.

Tables and Figures

Table 1-1. Effects of a milk osteopontin (mOPN) infusion on the normalization of the endometrial epidermal growth factor (EGF) profile in repeat breeder cows

Groups (n)	No. (%) of cows with the normal EGF profile after infusion	Endometrial EGF concentrations (ng/g tissue weight)		
		Before infusion	After infusion	
			All cows	Normalized cows (n)
PBS (84)	20 (23.8)^{A}	2.05 ± 0.74 ^{a}	3.19 ± 1.85 ^{bA}	6.07 ± 0.88 ^{A} (20)
SP (62)	36 (58.1)^{B}	2.18 ± 0.90 ^{a}	5.14 ± 2.24 ^{bB}	6.86 ± 0.97 ^{B} (36)
mOPN (171)	96 (56.1)^{B}	1.98 ± 0.73 ^{a}	4.99 ± 2.29 ^{bB}	6.76 ± 1.11 ^{B} (96)

SP: Seminal plasma

Values are means ± SDs.

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

^{A,B}Values with different letters within the same column differ significantly ($P < 0.05$).

Table 1-2. Effects of a milk osteopontin (mOPN) infusion on the restoration of fertility in repeat breeder cows

Groups	No. (%) of cows with the indicated endometrial EGF profile		No. (%) of cows conceived after treatment
PBS	Normalized	11 (24.4)^{A}	5 (45.4)
	Unnormalized	34 (75.6)	5 (14.7)
	Total	45 (100)	10 (22.2)^{A}
SP	Normalized	25 (50.0)^{B}	15 (60.0)^{a}
	Unnormalized	25 (50.0)	5 (20.0)^{b}
	Total	50 (100)	20 (40.0)^{B}
mOPN	Normalized	30 (65.2)^{B}	17 (56.7)^{a}
	Unnormalized	16 (34.8)	3 (18.7)^{b}
	Total	46 (100)	20 (43.5)^{B}

^{a,b}Values with different letters between different EGF profiles within the same infusion group differ significantly ($P < 0.05$).

^{A,B}Values with different letters within the same column differ significantly ($P < 0.05$).

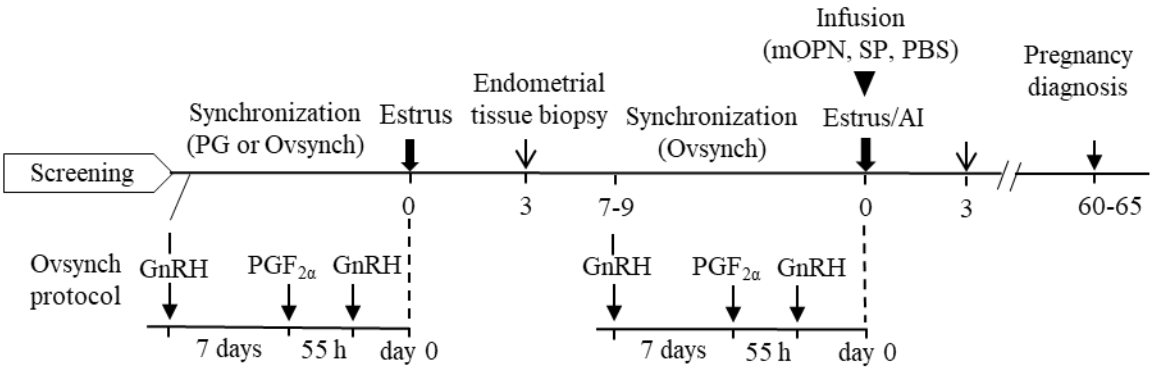

Fig. 1-1. A diagrammatic timeline of the screening, synchronization, and treatment sequences of repeat breeder cows

Repeat breeder cows meeting the criteria of failing to conceive after three or more AI without a detectable abnormality in the estrous cycle, clinical signs, genital organs were subjected to additional examinations including uterine ultrasonography, cytology and oviductal patency, the examination for the EGF profile. Repeat breeder cows showing no sign of abnormality in the additional examinations were synchronized by giving a signal administration of PGF $_{2\alpha}$ (20 or 25 mg) or an Ovsynch protocol [67]. They were examined for the endometrial EGF concentrations on day 3 (4.70-13.50 ng/g tissue weight) [54, 56] and those cows showing the low EGF concentrations lower than the lower limit of the normal range were used for the present study. All selected cows were synchronized for treatment with mOPN using the Ovsynch protocol starting with the first GnRH between 7 and 9 days of the estrous cycle. When cows inseminated, they were subjected to AI and then samples were infused into the vagina. To evaluate the effect of mOPN, endometrial EGF concentrations were examined on day 3 for the second time. Pregnancy was diagnosed between days 60 and 65 after AI.

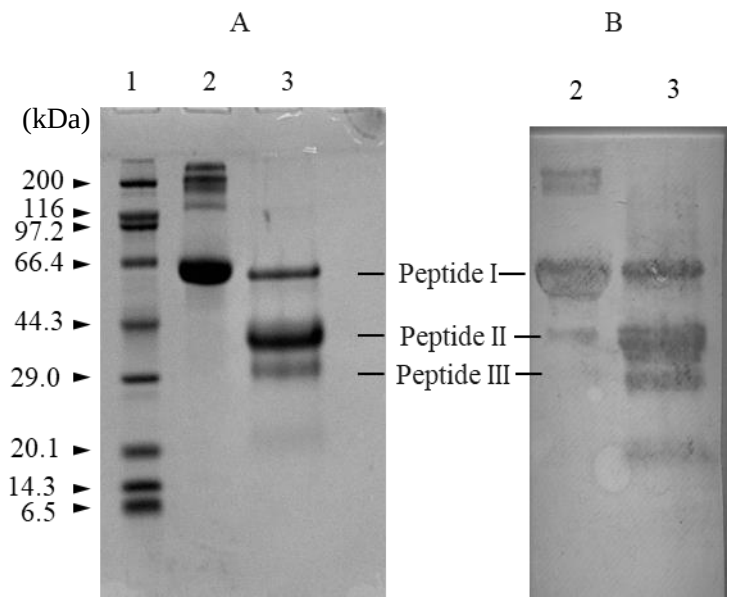

Fig. 1-2. Verification of mOPN by Western blotting

(A) Detection of mOPN on SDS-PAGE followed by CBB-G250 staining; Lane 1: Molecular mass marker, Lane 2: Commercial bovine mOPN (positive control), and Lane 3: Purified mOPN in this study. Three mOPN bands migrated with different post-translational modifications as 61 kDa (peptide I), 37 kDa (peptide II), and 31 kDa (peptide III), respectively. Purity was approximately 85% with a few minor bands. (B) Identification of mOPN by Western blotting; Lane 2: Commercial bovine mOPN (12 µg), Lane 3: Purified mOPN (10 µg) in this study.

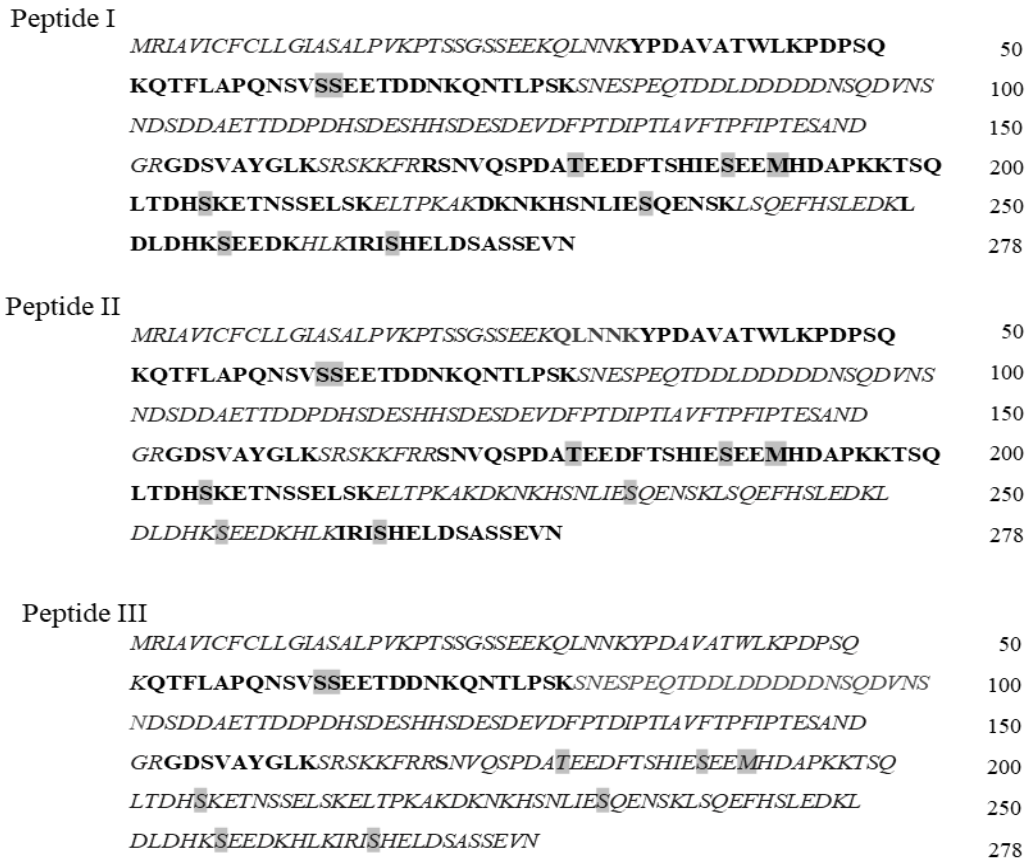

Fig. 1-3. Verification of mOPN by a peptide mass fingerprinting analysis

All three major bands were identified as OPN in bovine milk. Their tryptic peptide masses were matched approximately 50% (peptide I), 40% (peptide II), and 10% (peptide III), respectively, to the amino acid sequence of bovine OPN (Uniport ID; P31096). Matched or not matched peptides are indicated with bold characters and gray characters with an italic font, respectively. Phosphorylation at serine and threonine (S/T) and oxidation at methionine (M) sites identified in bovine milk were shown as light shading.

Summary

Endometrial EGF shows a cyclic change with two peaks on days 2-4 and 13-14 during the estrous cycle. An altered profile (i.e., loss of the two peaks) has been linked to reduced fertility in repeat breeder cows. Previous study demonstrated that a form of OPN, with a molecular weight of 29 kDa and found in bull SP, normalized the EGF profile and restored fertility in repeat breeder cows. OPN has many molecular forms due to post-translational modifications and is abundant in bovine milk. The purpose of the present study was to investigate whether mOPN normalizes the endometrial EGF profile and restores fertility in repeat breeder dairy cows with an altered EGF profile.

OPN was separated by one-step anion-exchange column chromatography from the whey of bovine milk. Purified mOPN was verified by Western blotting and peptide mass finger printing analyses. The OPN fraction showed three major protein bands of 61, 37 and 31 kDa (peptide I, II and III, respectively) on SDS-PAGE. All the three major bands were detected as OPN by Western blotting and their tryptic peptide masses were matched at approximately 50, 40, and 10%, respectively, to the bovine amino acid sequence by a peptide mass finger printing analysis. The three bands accounted for approximately 85% of the total protein content and 6.3 to 23.4 mg of OPN was obtained from 1 L of bovine milk. A lyophilized eluate containing 1.3 mg of mOPN, 0.5 mL of frozen SP, and PBS was infused at estrus into the vagina of repeat breeder cows with an altered EGF profile. Some of the cows treated with mOPN, SP, and PBS (46, 50, and 45 cows, respectively) were inseminated immediately before infusion and then examined for pregnancy between days 60 and 65. The rate at which mOPN to normalize the EGF profile (56.1%, n = 171) was similar to that of SP (58.1%, n = 62) and higher than that of PBS (23.8%, n = 84) ($P < 0.05$). The conception rate after the infusion of mOPN (43.5%, n = 46) was similar to that of SP (40.0%, n = 50) and higher than that of PBS (22.2%, n = 45) ($P < 0.05$). The present results indicate that the infusion of mOPN into the vagina is a treatment option for repeat breeder cows with an altered EGF profile.

Chapter 2

Effects of milk osteopontin of repeat breeder dairy cows on the endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows

Introduction

The role of OPN in milk is not well defined, but studies suggest that it plays a role in immunological processes and development in infants [78]. OPN has been shown to possess cytokine-like properties and regulate the Th1/Th2-type cytokine balanced immune response [57]. In addition, OPN plays an important role in brain development and behavior in infancy [61], possibly by promoting myelination [79]. Milk OPN may play a role at the gut mucosal surface of infants, because OPN can induce the expression of interleukin-12 from intestinal mononuclear cells [47]. *In vitro* experiments have indicated that human and bovine milk OPN are in part resistant to proteolysis in the infant intestinal tract, which makes OPN a potentially bioactive component of human milk [80].

OPN has also been reported to be involved in mammary gland development and differentiation [81, 82]. The information about OPN content in cattle milk at different lactation stages is extremely limited. OPN contents are reported most abundant in colostrum which contains relatively high levels of proteins that confer immunological defense in newborns [81]. In cattle, OPN has been suggested to be an important regulatory protein during lactation that specifically plays a role for milk protein gene expression [83].

OPN from milk can be separated by the method that is relatively simple and efficient [68, 70, 84] and purified OPN from bovine milk are commercially available. However, bovine milk may contain pathogens including viral [85–89], bacterial [88–90] and protozoal agents [88] (Table 2-1). It may be possible that mOPN preparation could become a source of disease transmission. More importantly, the risk of spreading diseases by infection of unknown pathogens in milk sample is inevitable, when mOPN preparations from infected donor cows are used for treatment of repeat breeder cows.

Therefore, in this study, I examined the feasibility of treatment scheme of a repeat breeder cow with mOPN prepared from her own milk sample to avoid the risk of spreading diseases. First, OPN contents in milk samples from apparently normal (control) cows at different lactation stages and those from repeat breeder cows were examined to determine the minimal volume of milk sample for OPN preparation from the individual cow. Then, as a treatment trial, milk samples of repeat breeder cows were sent to the university laboratory by

carrier service at 4°C and mOPN preparation from the milk samples of individual cows were sent back to the practitioners for treatment.

Materials and Methods

Animals

In this study, apparently normal cows were kept in the experimental farm of Hokkaido University and all repeat breeder cows were kept in one of thirteen commercial dairy farms in Hokkaido prefecture. Repeat breeder cows were diagnosed by local practitioners using the criteria of failing to conceive after three or more AI without a detectable abnormality in the estrous cycle, clinical signs, and genital organs and then confirmed through additional examinations (Fig. 2-1). Thereafter, the endometrial EGF profile was determined by the endometrial EGF concentration on day 3 (day 0 = estrus).

Synchronization and timed insemination in repeat breeder cows

Animals were synchronized and inseminated as described in Chapter 1. Briefly, for initial screening, cows were synchronized either estrus or ovulation with a single administration of $PGF_{2\alpha}$ or using Ovsynch protocol (Fig.2-1). Cows were subjected to timed AI by local AI technicians.

Endometrial tissues biopsy

Tissue collection was performed according to the method in Chapter 1. Uterine endometrial tissues were obtained by biopsy on the contralateral side to recent ovulation or CL development. Endometrial tissue samples were frozen in liquid nitrogen within 10 min of collection and stored below -30°C until the EGF assay.

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

The concentrations of EGF in endometrial tissues were determined by enzyme immunoassay as described in Chapter 1. The assay sensitivity was 10 pg/well. The intra- and inter-assay coefficients of variation at 50 pg/well were 5.3% and 7.3%, respectively.

Milk sample collection

Fresh milk samples (1 L) were obtained from apparently normal Holstein cows at the Hokkaido University experimental farm. Colostrum was obtained from cows within two days after parturition. Milk samples were collected in a 50 mL plastic tube (352070, Falcon, Mexico) by hand milking prior to machine milking. In addition, milk samples (100 mL) were obtained

from repeat breeder cows at the commercial dairy farms and transported to the university laboratory by using courier service at -20℃. All milk samples were collected during diestrus.

Protein identification

Western blotting was performed as mentioned in Chapter 1. Proteins on the SDS-PAGE were transferred to a ProBlott PVDF membrane and then membrane was incubated with an anti-cow SPP1/OPN polyclonal antibody. After detection with the secondary antibody, membrane was imaged using a scanner (GT-X700, Seiko Epson Corp., Japan). For the protein quantification, image was analyzed using Image J software.

Preparation of milk OPN (mOPN)

Acidic whey was prepared as described in Chapter 1 and mOPN was purified using two different volumes of milk samples as a large-scale (1 L) and small-scale (100 mL) purifications. For the large-scale preparation, mOPN was prepared following the procedures described in Chapter 1 using DEAE-Sepharose column.

For small-scale preparation, milk samples were thawed at 4°C and mOPN was separated as above by using 10 mL of DEAE-Sepharose Fast Flow column (ø 1.5 × 12 cm) (Bio-Rad Laboratories). The column was washed by 0.2 M NaCl prepared in the 100 mM sodium acetate buffer and mOPN was then eluted with 70 mL of 0.3 M NaCl prepared in the same sodium acetate buffer. Aliquots of purified mOPN samples were lyophilized with 1% trehalose as a cryoprotectant agent and stored at 4°C.

Infusion of samples into the vagina of repeat breeder cows

Lyophilized mOPN (1 mg) from control and repeat breeder cows and infused into the vagina as described in Chapter 1.

Study design

All animal experiments were conducted according to the guidelines for the Care and Use of the Experimental Animals protocols of Hokkaido University, Japan (protocols # 16-0071 and 19-0030). The screening, synchronization, and treatment protocol of repeat breeder cows were same as in Chapter 1 and a diagrammatic timeline was shown in Fig. 2-1. Protein was identified using Western blotting analysis and then quantified using Image J software.

Study 2-1. Comparison of OPN contents at different lactation stages

A total of 62 milk samples from control (n = 32) and repeat breeder (n = 30) Holstein

dairy cows were obtained at different lactation stages. Lactation stages were divided into four stages: colostrum (within two days after parturition, n = 9), early (from 5 to 100 days postpartum, n = 13), mid (101 to 200 days postpartum, n = 5), and late (from 201 to 300 days postpartum, n = 5). In repeat breeder cows, milk samples were collected at mid (n = 15) and late stages (n = 15). All milk samples were prepared to acidic whey (pH 4.6). Targeted protein in whey samples was detected using the SDS-PAGE and Western blotting analyses. The content and proportion of three major OPN bands were determined at different lactation stages of the different cow types.

Study 2-2. Effects of mOPN from individual repeat breeder cows on the normalization of the endometrial EGF profile and restoration of fertility in repeat breeder dairy cows

The effect of mOPN infusion on the endometrial EGF profile and fertility was assessed using 95 repeat breeder cows showing low endometrial EGF concentrations (< 4.70 ng/g tissue weight) on day 3. Cows were synchronized for ovulation and subjected to AI on day 0. mOPN from repeat breeder cows (1 mg, n = 30), mOPN from the control cows (1 mg, n = 30, positive control) or PBS (n = 35, negative control) were infused into the vagina immediately after AI. Endometrial EGF concentrations were measured on day 3 for the second time to assess the effect of treatments on the normalization of the endometrial EGF profile. Pregnancy was diagnosed by transrectal palpation between days 60 and 65 after AI.

Data analysis

Data were analyzed using the computer software JMP Pro 16 (SAS Institute Inc., Tokyo, Japan). OPN quantification at different lactation stages was compared using the one-way ANVOA, followed by Tukey's test. Proportion of the three major OPN bands at different lactation stages in the control cows were analyzed with two-way ANOVA, followed by Tukey's test. The proportion of the bands at different lactation stages and in different cow types were analyzed using the three-way ANOVA, followed by Tukey's test. Endometrial EGF concentrations were compared between groups using a two-way ANVOA with repeated measurements, followed by Tukey's test or paired t-test. The normalized endometrial EGF concentrations after the infusion were compared by using one-way ANOVA, followed by Tukey's test. The rates of normalization of the EGF profile and pregnancy were compared by Fisher's exact test. P values less than 0.05 were considered to be significant in all analyses. Data were shown as mean ± SD.

Results

Study 2-1. Comparison of mOPN contents at different lactation stages

OPN content was higher in the colostrum than the other lactation stages in the apparently normal (control) cows (Table 2-2, Fig. 2-2). OPN contents in the early, mid and late lactation stages were at the similar levels. All repeat breeder cows were at the mid or late stages and OPN content was similar in both stages. OPN contents of the repeat breeder cows were also similar to those of the control cows at both lactation stages (Table 2-2, Fig. 2-2). Within control cows, proportion of the three major OPN bands differed between the lactation stages ($P < 0.05$) (Table 2-2). The proportion of bands I and II were similar and higher than band III in colostrum and milk samples at the early and mid stages while the proportion of three bands were similar in milk samples at the late stage. When the proportion of the three bands were compared between the control and repeat breeder cows, the proportion of the bands were not different by the lactation stages. Repeat breeder cows and the control cows showed different proportion of the three bands (Table 2-2).

Study 2-2. Effects of mOPN from individual repeat breeder cows on the normalization of the endometrial EGF profile and restoration of fertility in repeat breeder dairy cows

In the small-scale preparation, between 1.0 and 3.3 mg of mOPN was obtained from 100 mL of individual milk samples while, in the large-scale preparation, between 6.3 and 23.4 mg of mOPN was obtained. Both OPN preparation from the different scales showed the same three major protein bands with apparent molecular masses of 61 kDa, 37 kDa, and 31 kDa by SDS-PAGE and Western blotting analyses (Fig. 2-3. A, B).

Repeat breeder cows in the three treatment groups showed similar EGF concentrations at the initial examination (Table 2-3). The normalization rates of the endometrial EGF profile were similar in both mOPN groups (63.3% and 60.0%) and higher than that of the PBS group (25.7%) (Table 2-3). As a result, endometrial EGF concentrations on day 3 after treatment were higher at similar levels in both mOPN groups and higher than those of controls. However, EGF concentrations in the normalized cows of all three groups were similar. The conception rates after the infusion of mOPN preparation from control cow milk tended to be higher than that of the PBS group ($P = 0.07$). The conception rate after infusion of the mOPN preparation from repeat breeder cows were at the intermediate level of the other two groups and was not different from the two groups.

Discussion

I determined OPN contents at different lactation stages of apparently normal cows and repeat breeder cows. In addition, two preparation protocols with different starting material volume, namely the large-scale (1 L) and small-scale (100 mL) preparation, by using an anion-exchange column chromatography. The concentrations of mOPN in milk samples from apparently normal cows (large-scale preparation) and individual repeat breeder cows (small-scale preparation) range from 6.3 to 23.4 mg and 1.0 to 3.3 mg, respectively. Then I demonstrated that mOPN (1 mg) from individual repeat breeder cows normalized the endometrial EGF profile (60.0%) while fertility was not improved by mOPN from repeat breeder cows.

The conception rate was not improved by mOPN from repeat breeder cows. It is most likely that a small number of animals may be responsible for this result and a study with an increased number of animals is needed to confirm the activity of mOPN on the restoration of fertility. Another possibility is the different proportions of the three OPN bands between control and repeat breeder cows. The proportion of three bands of mid and late lactation stages differed between cow types. In the future, it is of interest to compare the capacity of three OPN molecules in bovine milk to normalize the EGF profile and improve fertility in repeat breeder dairy cows with an altered endometrial EGF profile.

To date, only a few studies have focused on the quantification of milk OPN at different lactation stages of Holstein dairy cows. In the present study, the mean concentration of OPN in colostrum was higher than those in the rest of the stages (Table 2-2). However, the mean concentration of OPN between the early (5 to 100 days postpartum), mid (101 to 200 days postpartum), and late (201 to 300 days postpartum) of lactation stages of control cows as 161.61 ± 67.38 mg/L, 159.49 ± 37.34 mg/L, and 137.63 ± 25.54 mg/L, respectively, was not significantly difference. In repeat breeder cows, OPN concentration was 164.07 ± 57.84 mg/L at mid (101 to 200 days postpartum) and 151.54 ± 60.06 mg/L at late lactation stages (201 to 300 days postpartum). The results were similar in both groups and those were similar to those of control cows.

In the previous reports, OPN concentrations in colostrum was high around 1000 mg/L [45] while protein was measured as < 200 mg/L in the bovine colostrum [51]. At the early lactation stage (5 days of postpartum), OPN concentration was < 200 mg/L in bovine milk [51]. OPN concentrations in my study differed in colostrum from the other reports and it might be related to the different protein quantification methods. In the present study, protein concentrations at lactation stages were determined using Western blotting analysis with reference to a known quantity of purified mOPN and then measured the protein (OPN) bands

area by Image J software. However, in other reports, OPN concentrations were measured by ELISA kit [45] or Protein assay method [51] at lactation stages.

In this study, I purified mOPN from individual repeat breeder cows to avoid the risks of infections from infected donor cows. Bovine milk may contain pathogens including viral [85–89], bacteria [88–90] and protozoal agents [88] (Table 2-1). Then, I determined the small-scale preparation of milk sample (100 mL) is the minimal volume to obtain the 1 mg of OPN for treatment since contents of OPN has shown to vary between lactation stages. In conclusion, the present study indicated that mOPN from individual repeat breeder cows also normalized the endometrial EGF profile in repeat breeder dairy cows. Due to the clear effect of mOPN, its high content and availability, OPN from milk sample of the repeat breeder themselves is a promising therapeutic option for the treatment of repeat breeder cows with an altered endometrial EGF profile.

Tables and Figures

Table 2-1. Importance of pathogens from the source of milk

	Pathogens	Diseases
Virus	*Bovine viral diarrhea virus (BVDV)*	Immune suppression, Fertility reduction
	Bovine herpesvirus (BHV) Type-1 Type-4, Type-5	Rhinotracheitis, Metritis and Endometritis
	Bovine leukemia virus (BLV)	Immune suppression, Mastitis,
	Bovine immunodeficiency virus (BIV)	Immune suppression, Mastitis
Bacteria	*Salmonella dublin, typhimurium*	Early embryonic loss, Abortion,
	Brucella abortus	Diarrhea, Retained placenta, Metritis,
	Leptospira borgpetersenii	Reproductive failure, Mastitis
	Campylobacteria fetus	Reproductive failure, Mastitis
	Streptococcus aureus	Mastitis, Metritis
	Bacillius megaterium	Diarrhea, Metritis, Abortion
	Escherichia coli	Diarrhea, Metritis, Abortion
	Mycoplasma paratuberculosis	Johne's disease
Protozoa	*Trichomonas fetus*	Early embryonic loss, Abortion
	Neospora caninum	Abortion
Parasite	*Ostertagia ostertagi*	Fertility failure
	Dictyocaulus viviparus	Reduction of fertility

Table 2-2. Concentrations of mOPN in control and repeat breeder cows at different lactation stages

Lactation stages (n)	OPN concentrations (mg/L)		Proportion of OPN band signals					
	Control cows	Repeat breeder cows	Control cows			Repeat breeder cows		
			I	II	III	I	II	III
Colostrum (9)	255.22 ± 44.38^{A} (9)	-	0.42 ± 0.09^{a}	0.37 ± 0.07^{a}	0.21 ± 0.05^{bCD}	-	-	-
Early (13)	161.61 ± 67.38^{B} (13)	-	0.36 ± 0.05^{a}	0.43 ± 0.07^{a}	0.21 ± 0.09^{bC}	-	-	-
Mid (20)	159.49 ± 37.34^{B} (5)	164.07 ± 57.84 (15)	0.35 ± 0.04^{ab}	0.42 ± 0.08^{a}	0.23 ± 0.05^{bCD}	0.36 ± 0.11	0.43 ± 0.05	0.21 ± 0.08
Late (20)	137.63 ± 25.54^{B} (5)	151.54 ± 60.06 (15)	0.33 ± 0.05	0.33 ± 0.05	0.32 ± 0.07^{D}	0.40 ± 0.06	0.41 ± 0.08	0.19 ± 0.09
Subtotal$^{\#}$	-	-	0.34 ± 0.05^{ab}	0.39 ± 0.06^{a}	0.27 ± 0.07^{b}	0.38 ± 0.09^{a}	0.42 ± 0.07^{a}	0.20 ± 0.08^{b}

Values are means ± SDs.

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

^{A,B} Values with different letters within the same column differ significantly ($P < 0.05$).

^{C,D} Values with different letters within the same column differ significantly ($P = 0.08$).

I: 61 kDa, II: 37 kDa and III: 31 kDa molecular weight of OPN detected on SDS-PAGE and Western blotting at different lactation stages.

$^{\#}$Subtotal of OPN proportion band signals at mid and late lactation stages.

Table 2-3. Effects of mOPN from individual repeat breeder cows on the normalization of endometrial EGF profile and fertility

Groups	No. of cows	Sources of milk	No. (%) of cows with normal EGF concentrations on day 3 after infusion	No. (%) of cows conceived after infusion	Endometrial EGF concentrations (ng/g tissue weight)		
					Before infuision	After infusion	
						All cows	Normalized cows
mOPN	30	Apparently normal cows	19 (63.3)^{A}	15 (50.0)^{C}	1.91 ± 0.69^{a}	5.06 ± 2.15^{bA}	6.54 ± 0.80
mOPN	30	Repeat breeder cows	18 (60.0)^{A}	12 (40.0)^{CD}	1.92 ± 0.63^{a}	4.96 ± 2.15^{bA}	6.52 ± 1.01
PBS	35	-	9 (25.7)^{B}	10 (28.6)^{D}	1.91 ± 0.68^{a}	3.48 ± 1.81^{bB}	6.07 ± 0.83

Values are means ± SDs.

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

^{A,B} Means with different letters within the same column differ significantly ($P < 0.05$).

^{C,D} Means with different letters within the same column differ significantly ($P = 0.07$).

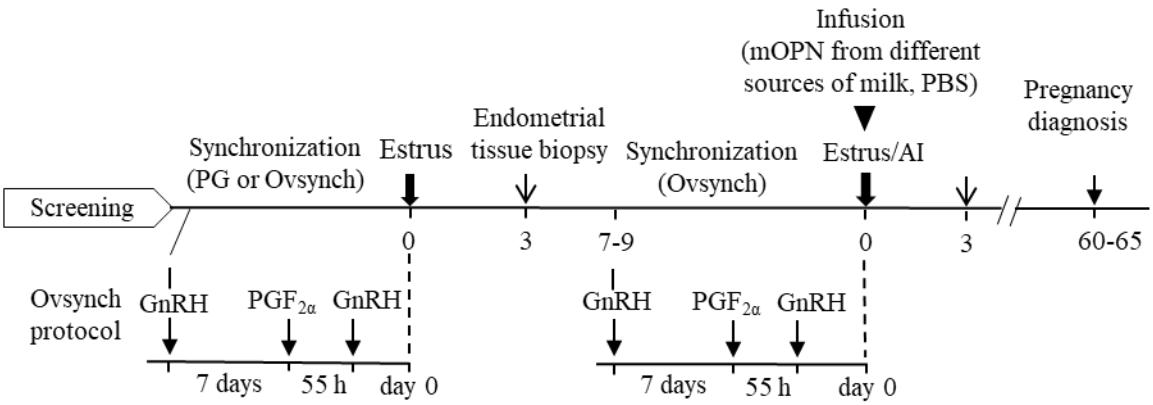

Fig. 2-1. A diagrammatic timeline of screening, synchronization, and treatment of animals

We used the same protocol for the screening and synchronization of animals in the studies of Chapter 1. Briefly, repeat breeder cows meeting the criteria of failing to conceive after three or more AI without a detectable abnormality in the estrous cycle, clinical signs, genital organs were subjected to additional examinations. Repeat breeder cows were synchronized by giving a signal administration of $PGF_{2\alpha}$ (20 or 25 mg) or an Ovsynch protocol [67]. All selected cows were synchronized for treatment with mOPN using the Ovsynch protocol starting with the first GnRH between 7 and 9 days of the estrous cycle. When cows were inseminated, they were subjected to AI (16 to 20 h after the second GnRH) and then samples were infused into the vagina. To evaluate the effect of mOPN, endometrial EGF concentrations were examined on day 3 for the second time. Pregnancy was diagnosed between days 60 and 65 after AI.

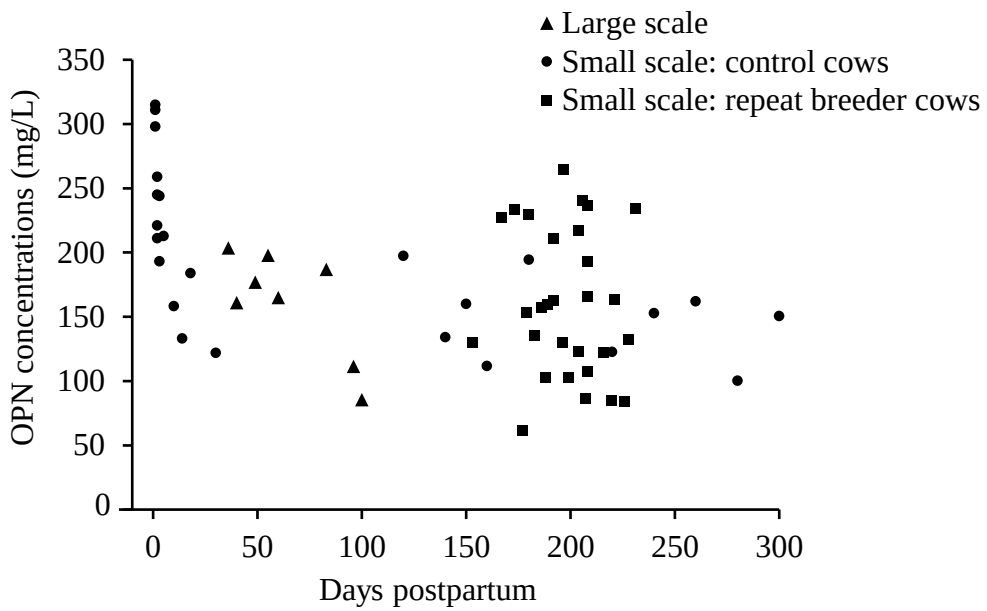

Fig. 2-2. Bovine milk osteopontin (mOPN) contents at different lactation stages

mOPN contents were measured from the apparently normal (control) cows in two protocols (n = 32, large-scale and small-scale preparation) and repeat breeder dairy cows (n = 30, small-scale preparation alone). Colostrum was obtained within two days after parturition which showed the higher OPN concentrations than the other stages. Milk samples of early (from 5 to 100 days postpartum), mid (from 101 to 200 days postpartum) and late (201 to 300 days postpartum) lactation stages were collected to determine the OPN content.

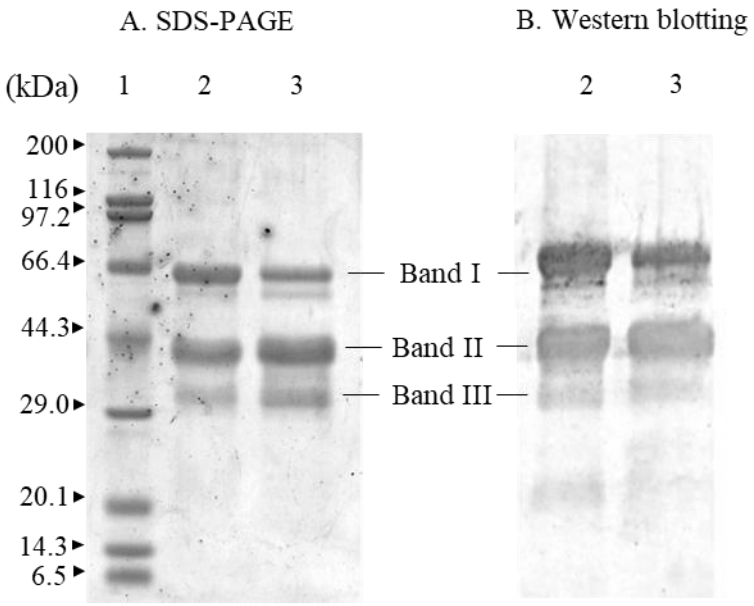

Fig. 2-3. Detection of bovine mOPN by SDS-PAGE and Western blotting analysis

(A) 12% of SDS-PAGE followed by CBB-G250 staining and (B) Western blotting. Lane 1: Molecular mass marker, Lane 2: mOPN purified from control cow and Lane 3: mOPN purified from repeat breeder cow. Three major bands of OPN were detected in milk samples of repeat breeder cows and were with the same molecular masses of 61, 37 and 31 kDa as those found in milk samples of apparently normal (control) cows.

Summary

The objective of the study in Chapter 2 was to examine the feasibility of treatment protocol for repeat breeder cows with mOPN prepared from individual repeat breeder cows to avoid the risk of disease transmission via milk samples. Firstly, I have determined OPN contents in milk samples at different lactation stages. OPN content was higher in the colostrum (n = 9) than the early (from 5 to 100 days postpartum, n = 13), mid (from 101 to 200 days postpartum, n = 5), and late (from 201 to 300 days postpartum, n = 5) lactation stages of apparently normal cows. Repeat breeder cows were at either mid (n = 15) or late (n = 15) lactation stage. OPN contents in repeat breeder cows were similar in both stages, and were similar to those of the control cows at the same lactation stages.

One liter of milk samples from apparently normal cows (large-scale purification) and 100 mL of milk samples from repeat breeder cows (small-scale purification) were used for OPN preparation. OPN was obtained between 6.3 and 23.4 mg from 1 L of milk and between 1.0 and 3.3 mg from 100 mL of milk. Within control cows, proportion of the three bands was differed between the lactation stages ($P < 0.05$). The proportion of bands I and II were similar and higher than band III in the colostrum and milk samples at the early and mid stages while proportion of three bands were similar in milk samples at the late stage. When the proportion of the three bands were compared between the control and repeat breeder cows, the proportion of the bands differed only by the type of cows but not by the lactation stages.

Then lyophilized 1 mg of mOPN from apparently normal (control) cows and mOPN from individual repeat breeder cows were infused into repeat breeder cows on the day of estrus and endometrial EGF concentrations were determined on day 3 of estrous cycle. Pregnancy was diagnosed between day 60 and 65. The normalization rates of the endometrial EGF profile in both mOPN groups prepared from control cows (63.3%, n = 30) and repeat breeder cows (60.0%, n = 30) were similar to each other, and higher than that of the PBS group (25.7%, n = 35) ($P < 0.05$). The conception rate of repeat breeder cows infused with mOPN from control cows tended to be higher than that of the PBS group ($P = 0.07$). The conception rate of repeat breeder cows infused with mOPN from repeat breeder cows own milk were at the intermediate level of the other two treatment groups.

The present study suggests that mOPN preparation from individual repeat breeder cows can be an alternative strategy to normalize the EGF profile without risk of disease transmission via milk samples of donor cows. However, it is necessary to confirm the effect of this treatment protocol on fertility in a study with an increased number of animals.

Summary and Conclusions

Endometrial EGF concentrations show a cyclic change with two peaks on days 2-4 and 13-14 during the estrous cycle. These peaks are absent or lowered in approximately 70% of Holstein lactating repeat breeder cows. The altered profile has been linked to reduced fertility. A form of OPN in bull SP normalized the EGF profile and restored fertility in the repeat breeder cows. The purpose of this study was to examine effects of OPN from milk, a rich source of this protein, on the normalization of the endometrial EGF profile and restoration of fertility in repeat breeder cows. The study is composed of two parts, the first part (Chapter 1) examined effects of mOPN to normalize the endometrial EGF profile and restore fertility in repeat breeder cows. The second part (Chapter 2) examined the feasibility of the treatment protocol using mOPN of the repeat breeder cows own milk samples. OPN contents in milk samples of apparently normal cows and repeat breeder cows at different lactation stages were examined. Then, 1 mg of mOPN was prepared using milk samples of individual repeat breeder cows and examined its effect on the EGF profile and fertility in repeat breeder cows.

In Chapter 1, effects of mOPN to normalize the endometrial EGF profile and restore fertility were examined in repeat breeder cows. OPN was purified from 1 L of bovine milk by one-step of anion-exchange column chromatography. The OPN fractions showed three major protein bands of 61, 37 and 31 kDa on SDS-PAGE. All three bands were identified as OPN by Western blotting, and their tryptic peptide masses matched approximately 50, 40 and 10%, respectively, to bovine OPN amino acid sequences by peptide mass finger printing analysis. Sum of the three detected bands accounted for approximately 85% of total protein contents of the OPN preparation. OPN was obtained between 6.3 and 23.4 mg from 1 L of bovine milk. A lyophilized OPN preparation containing 1.3 mg of mOPN, 0.5 mL of SP (positive control), and PBS (negative control) were infused immediately after AI into the vagina of repeat breeder cows with an altered EGF profile. The normalization rate of EGF profile (56.1%, $n = 171$) was similar to that of SP (58.1%, $n = 62$) and higher than that of PBS (23.8%, $n = 84$) ($P < 0.05$). Similarly, the conception rate after the infusion of mOPN (43.5%, $n = 46$) was similar to that of SP (40.0%, $n = 50$) and higher than that of PBS (22.2%, $n = 45$) ($P < 0.05$). The present results confirmed the effect of seminal OPN on the normalization of EGF profile. However, using OPN prepared from raw milk for the treatment of many other cows carries the risk of spreading the infection. Thus, an alternative strategy to use mOPN for the treatment of repeat breeder cows is needed.

In Chapter 2, therefore, I have examined the feasibility of treatment protocol for the repeat breeder cow with mOPN prepared from her own milk to avoid the risk of disease transmission through the treatment. Firstly, OPN content was measured in milk samples from

apparently normal cows and repeat breeder cows with different lactation stages: colostrum (within two days after parturition, n = 9), early (5 to 100 days postpartum, n = 13), mid (101 to 200 days postpartum, n = 5) and late (201 to 300 days postpartum, n = 5) lactation stages to estimate milk sample volume that is necessary to obtain OPN enough (1 mg) for treatment. OPN contents were higher in the colostrum than the other lactation stages ($P < 0.05$). OPN contents of the other stages were at similar levels. The contents of mOPN in repeat breeder cows were similar to those of the control cows. Milk OPN of 1.0 to 3.3 mg was obtained from 100 mL of milk. Three major bands of 61, 37 and 31 kDa were detected on SDS-PAGE and all three bands were identified as OPN by Western blotting. Within control cows, the proportion of the three bands differed between the lactation stages ($P < 0.05$). However, proportion of band I and II were similar and higher than band III at the early and mid stages while the proportion of three bands were similar in the milk sample at the late stage. When the proportion of the three bands were compared between the control and repeat breeder cows, the proportion of the bands differed by the type of cows but not by the lactation stages.

Then, 1 mg of OPN prepared from milk samples of the normal and individual repeat breeder cows was used for the treatment. Infusions of the mOPN of normal and repeat breeder cows normalized the endometrial EGF profile at similar rates of 63.3% (n = 30) and 60.0% (n = 30), respectively, and the normalization rates were higher than that of the PBS group. However, effects of mOPN from repeat breeder cows own milk remained to be confirmed since the conception rate were at an intermediate level of the PBS and mOPN of the normal cow groups.

The present study indicated that use of mOPN from individual repeat breeder cows could be an effective treatment option to enabling normalization of the endometrial EGF profile and avoiding the risk of spreading infection. However, the effect of mOPN from repeat breeder cows on the restoration of fertility remained to be confirmed in a study with an increased number of animals. Nevertheless, high content of OPN and its clear effect on the normalization of the EGF profile make the mOPN a promising candidate source of OPN preparation for the treatment of repeat breeder cows with an altered endometrial EGF profile.

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