Effects of recombinant osteopontin expressed in *Escherichia coli* on the recovery of the endometrial epidermal growth factor profile and fertility in repeat breeder dairy cows

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Abstract

Endometrial epidermal growth factor (EGF) shows a cyclic change with two peaks on days 2–4 and days 13–14 of the estrous cycle. In repeat breeder cows, loss of the peaks has been associated with reduced fertility. By infusing seminal plasma (SP) and osteopontin (OPN) derived from SP and milk into the vagina, their EGF profile and fertility are restored. However, SP is difficult to obtain, and both SP and OPN can transmit infectious diseases. While OPN can be sourced from recombinant protein without this risk, recombinant bovine OPN (rOPN) expressed in *Escherichia coli* should be examined for its effects on the EGF profile, since it does not undergo posttranslational modification, which is important for its biological activity. In study 1, PBS, SP (0.5 ml), and rOPN (0.3 mg) were infused into the vagina at estrus (day 0) in 74, 37, and 105 repeat breeder Holstein cows, respectively, with an altered EGF profile. The endometrial EGF concentrations were measured on day 3. Some cows (n = 58, 20, and 83, respectively) were inseminated immediately before the infusion and then diagnosed for pregnancy between days 30 and 35. The normalization rate of the EGF profile and conception rate in the rOPN group (58.1% and 47.0%, respectively) were not significantly different from those in the SP group (62.2% and 45.0%, respectively) but higher than those in PBS group (29.7% and 28.1%, respectively) ($P < 0.05$). In study 2, repeat breeder cows with an altered EGF profile were infused with PBS (n = 18) and rOPN (n = 17), while fertile controls with a normal EGF profile (n = 18) were infused with PBS. Two or three embryos were transferred into cows on day 7 and then recovered on day 14. Embryo recovery rates of the rOPN and fertile groups were comparable (58.7% vs. 58.3%) but higher than that of the PBS group (58.7% vs. 32.0%) ($P < 0.05$). The embryo recovery rate of cows with normalized EGF profile was higher than that of cows with unnormalized EGF profile (64.4% vs. 16.7%) ($P < 0.05$). The embryo sizes of cows in the rOPN and fertile groups were comparable but larger than those in the PBS group ($P < 0.05$). However, the embryo size was not correlated to the corresponding endometrial EGF concentrations. In conclusion, rOPN without posttranslational

48 modifications normalized the EGF profile in repeat breeder cows. Improved fertility by
49 normalization of the EGF profile could be attributed partly to the increased embryo viability up to
50 day 14.
51
52 *Keywords*: Repeat breeder cows, Epidermal growth factor, Endometrium, Embryo development,
53 Osteopontin
54

55 *1. Introduction*

56 Repeat breeder cows are major sources of economic loss in the dairy industry [1]. Repeat breeder
57 cows are generally defined as cows that fail to conceive after at least three inseminations without
58 detectable abnormalities in their genital organs during an estrous cycle [1,2]. The modest changes
59 in circulating ovarian steroid hormone concentrations [3–6] have associated with their reduced
60 fertility. Plasma progesterone (P_4) concentrations rise slowly after ovulation and show lower levels
61 during the luteal phase [3–5]. Moreover, repeat breeder cows have lower plasma estradiol (E_2)
62 concentrations around the day of ovulation [6]. These modest changes in the ovarian steroid
63 hormone concentrations of repeat breeder cows may be amplified as an alteration of growth factors
64 and cytokines in the endometrium [7] since these factors are primarily regulated by E_2 and P_4 [8,9].

65 Epidermal growth factor (EGF) is a growth factor that controls the endometrial function
66 [8,10–12] and embryo development [8,13]. Alteration in the endometrial EGF profile reduces
67 fertility in embryo transfer (ET) recipients [14] and repeat breeder cows [7,15–17]. In fertile cows,
68 endometrial EGF concentrations peak twice on days 2–4 and days 13–14 during the estrous cycle;
69 in approximately 70% of Holstein lactating repeat breeder cows, these peaks decrease [15–17]. In
70 most cases, the loss and recovery of the endometrial EGF peak on day 3 is accompanied by those
71 on day 14 [16,18], thus making the endometrial EGF concentration on day 3 an indicator of the
72 endometrial EGF profile [14,19].

73 Infusion of seminal plasma (SP) into the vagina restores the normal endometrial EGF
74 profile and fertility in repeat breeder dairy cows [20]. This SP function is characterized by a form
75 of osteopontin (OPN) in SP [21]. OPN purified from bovine milk (mOPN) restores the normal
76 endometrial EGF profile and fertility in repeat breeder dairy cows [22]. OPN is an acidic protein in
77 cattle consisting of 262 amino acids; it also called secreted phosphoprotein 1 (SPP1) [23,24]. This
78 protein is secreted in various tissues [25] in the reproductive organs such as the ovary [26,27],
79 oviduct [28], uterus [29–31], testis [32], and epididymis [32]. OPN has multiple roles in different

physiological systems, including tumor progression [33], inflammation [34], and reproductive systems [35–39]. In the reproductive system, OPN has a role in sperm capacitation [38] and acrosome reaction [36], preimplantation embryo development [35,36], conceptus attachment [37,40,41] and invasion into the endometrium [42], and corpus luteum (CL) formation [39].

As regards the application of OPN to fertility improvement technology at production sites, SP and raw milk may not be suitable as raw materials to prepare OPN. SP is difficult to obtain in quantity and both SP [43] and raw milk [44–46] may transmit infectious diseases. An alternative source of OPN without these disadvantages may be recombinant protein [47], which can be cost-effectively produced in *Escherichia coli* (*E. coli*)[47]. However, such recombinant proteins do not undergo posttranslational modification, which is considered important for biological activity of various proteins [48].

For OPN, posttranslational modifications have shown to be crucial in the expression of its biological activity [34,49–51], but some functions of OPN do not depend on this modification [40,41,49,50,52,53]. OPN enhances the lung cancer cell growth depending on glycosylation in human [51]. OPN induces the expression of interleukin-12 [49,50] and tumor necrosis factor α (TNFα) [50] in macrophage depending on phosphorylation, while it induces the chemotaxis of macrophages [50] and suppress the expression of interleukin-10 [49] in a phosphorylation independent manner in mice. In sheep and pigs, recombinant rat OPN produced in *E. coli* without posttranslational modification binds to integrins in uterine luminal epithelial cells [40], trophectoderm cells [40,41], endothelial progenitor cells [52] and the placental tissue [53], leading to promote trophectoderm cell adhesion and migration [40,41], endothelial progenitor cell incorporation into vasculature [52], and ion transport across the placenta [53].

Therefore, in the present study, we investigated the effect of full-length recombinant OPN (rOPN) expressed in *E. coli*, which has no posttranslational modification [48], on the normalization of the endometrial EGF profile and the recovery of fertility in repeat breeder dairy

105 cows. Fertility was evaluated with respect to the conception rate after artificial insemination (AI)
106 and by the survival and size of ET embryo recovered on day 14.
107

2. Materials and methods

2.1. Recombinant OPN preparation

A gene-encoding bovine OPN (Uniprot ID: P31096) was synthesized by GeneArt (Thermo Fischer Scientific, Waltham, MA, USA) with codon optimization for *E. coli* expression. The DNA encoding was performed to predict the mature region of OPN (Leu17–Asn278) and amplified by PCR using primers [5′-GCCGCGCGGCAGCCATATGCTGCCGGTTAAACCGACCAG-3′ (sense; *Nde*I-site underlined) and 5′-CGAGTGCGGCCGCAAGCTTTTAATTCACTTCACTGCTTG-3′ (antisense; *Hind*III-site underlined)]. PCR was performed using KOD Plus Neo™ DNA polymerase (Toyobo, Osaka, Japan), purified with QIAEX II Gel Extraction kit (Qiagen, Hilden, Germany), and inserted into *Nde*I–*Hind*III sites of pET-28a (Merck Millipore, Darmstadt, Germany) using the seamless ligation cloning extract [54]. The resultant plasmid vector was designated as SPP1/pET-28a.

The *E. coli* Rosetta (DE3) (Merck Millipore) cells harboring SPP1/pET-28a were cultured overnight on LB agar plates containing 50 μg/mL kanamycin and 30 μg/mL chloramphenicol at 37°C. A single colony was inoculated into 150 ml LB medium containing 50 μg/ml kanamycin and 30 μg/ml chloramphenicol, and to grow the cells, a seed culture was agitated overnight at 37°C and inoculated into 3000 mL LB medium containing 50 μg/mL kanamycin. When OD_{600} of the culture medium reached to between 0.4 and 0.8, the culture medium was cooled in ice for 10 min, and to induce protein expression, 0.1 mM isopropyl β-D-thiogalactopyranoside was added. Then, the cells were cultured at 30°C for 20–22 h, harvested by centrifugation (10,000 × *g* at 25°C for 5 min), and frozen at −80°C until rOPN was purified according to a previous report [55]. Briefly, the cells were resuspended in Buffer-A (20 mM sodium phosphate buffer, pH 7.0 and 0.3 M NaCl) at 4°C and disrupted by sonication. Cell-free lysate was obtained by centrifugation (6,000 × *g* at 4°C for 20 min) and applied to a Ni^{2+}-charged Chelating Sepharose Fast Flow column (Cytiva, Marlborough, MA, USA), which was washed with

133 20 mM imidazole in Buffer-A. Then, rOPN was eluted with a 20–500 mM imidazole linear
134 gradient in Buffer-A. Purified rOPN was dialyzed against the phosphate-buffered saline (PBS)
135 (Takara Bio, Kusatsu, Japan) with cellulose membrane [cellulose tubing (30/32), Viskase
136 Companies, Illinois, USA]. The purification and dialysis of rOPN were performed at 4°C. The
137 dialyzed proteins containing rOPN and 1% (w/v) trehalose as a cryoprotectant was filtrated (pore
138 size of the filter: 0.22 μm), and the solution containing 0.3 mg rOPN was aliquoted in 10 mL vials
139 and lyophilized. The lyophilized rOPN were stored at 4°C until infusion into repeat breeder cows.
140 Concentrations of rOPN in final preparation were calculated based on total protein
141 concentrations and purities of rOPN. The total protein concentration was measured based on the
142 absorbance at 280 nm ($E^{1\%}_{280\,nm,\ 1\,cm} = 10$) [56], and the purity of rOPN calculated by the
143 densitometry using the gel image of sodium dodecyl sulfate-polyacrylamide gel electrophoresis
144 (SDS-PAGE) by ImageJ (https://imagej.net/ij/index.html).
145 The identity of rOPN was confirmed by western blotting according to previous
146 methods [22], with slight modification. Briefly, in the western blotting, the purified rOPN was
147 subjected to SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membrane
148 (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Inc., Berkeley, California, USA). The
149 membrane was blocked by incubating it in PBS with 5% bovine serum albumin at room
150 temperature for 1 h. The membrane was washed with Tris-buffered saline with Tween 20 (TBS-T)
151 (pH 7.5) and then incubated in the TBS-T with the anti-OPN polyclonal antibody (LB-4225,
152 Cosmo bio, Cambridge, UK) at a 1:1,000 dilution for 3 h at room temperature. The unbound
153 primary antibody was washed out using TBS-T, and the membrane was incubated in the TBS-T
154 with the secondary antibody (anti-IgG-horseradish peroxidase: 4050-05, SouthernBiotech,
155 Birmingham, AL) at a 1:4,000 dilution for 2 h at room temperature. The unbound secondary
156 antibody was washed using TBS-T, and the peroxidase was detected using 50 mM Tris (pH7.0)
157 dissolved DAB tablet (Fujifilm Wako Pure Chemical Industries Ltd., Japan).

158

159 *2.2. SP preparation*

160 SP was prepared as reported previously [20]. Briefly, semen was collected from seven fertile
161 Holstein bulls using an artificial vagina at a commercial AI center (Genetics Hokkaido, Tokachi
162 Shimizu, Hokkaido, Japan). Two ejaculates were collected on the same day twice a week. SP was
163 separated from the semen by centrifugation (1000 × *g* for 10 min), stored at −20°C, and
164 transported to Hokkaido University. Frozen SP was thawed and centrifuged (5,000 × *g* at 4°C for
165 20 min). The resulting supernatant from SP collected from seven bulls were pooled and used as the
166 SP sample, which was aliquoted in 0.5 mL and stored at −30°C until infusion into repeat breeder
167 cows.

168

169 *2.3. In vitro embryo production and freezing for direct transfer*

170 In the present study, ovaries were obtained from Japanese black or F1 crossbred (Japanese black ×
171 Holstein) cattle slaughtered at a local abattoir located within 1 h from the laboratory and
172 transported to the laboratory within 4 h after collection. All media (BO-IVM, BO-IVF, BO-
173 SemenPrep, and BO-IVC) used in these experiments were purchased from IVF Bioscience
174 (Cornwall, UK).

175 Cumulus-oocyte complexes (COCs) were aspirated from follicles (2 to –8 mm in
176 diameter) of slaughterhouse-derived ovaries were submitted to *in vitro* maturation (IVM), *in vitro*
177 fertilization (IVF) and *in vitro* culture (IVC) as described previously [57]. For IVM, COCs were
178 washed twice in TCM199 (Invitrogen; Grand Island, NY, USA) supplemented with 0.1% polyvinyl
179 alcohol, 25 mM HEPES, 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation
180 medium, pH 7.4 at 37°C), as described elsewhere [58], and once in BO-IVM media before culture
181 in 50 μL BO-IVM droplets (approximately 10 COCs/50 μL droplets) covered with paraffin oil at
182 39°C for 20–24 h in 5% CO_2 in humidified air. After IVM, COCs were subjected to IVF and IVC.

183 Commercial frozen semen (Genetics Hokkaido) derived from a Holstein bull was used for IVF.
184 The sperms were washed twice by centrifugation (300 × *g* for 5 min) in BO-SemenPrep media, and
185 2×10^6 cells/mL sperm was coincubated with oocytes in 500 µL of BO-IVF medium (day 0) in a
186 4-well dish without an oil overlay at 39°C for 18–24 h in 5% CO_2 humidified air for IVF. After
187 IVF, inseminated oocytes were denuded of the surrounding cumulus cells and excess spermatozoa
188 by vortexing. For IVC, presumptive zygotes were washed three times in BO-IVC media and then
189 cultured in a 30 µL BO-IVC droplet (approximately 10–25 zygotes/droplet) with an overlay of
190 paraffin oil at 39°C for 144–150 h in 5% O_2, 5% CO_2, and 90% N_2. An inverted microscope was
191 used to examine the development of the embryos at days 6 and 7. Grade 1 blastocysts according to
192 the International Embryo Technology Society classifications [59] were then frozen in 1.5 M
193 ethylene glycol in Dulbecco's PBS (DPBS) supplemented with 0.1 M sucrose and 20% calf serum
194 (freezing medium) in a program freezer (ET-1, Fujihira, Tokyo). The embryos were directly
195 transferred into the freezing medium and then loaded into a 0.25 mL straw to equilibrate for 15–20
196 min. The straws were then placed in a methanol bath of a programmable freezer at −7°C for 1 min,
197 seeded at −7°C, maintained at −7°C for 9 min, cooled to −30°C at the rate of 0.3°C/min, and
198 plunged into liquid nitrogen [60].
199
200 *2.4. Animals*
201 All animal experiments were performed in accordance with the Japanese Act on Welfare and
202 Management of Animals and approved by the Institutional Animal Care and Use Committee of
203 Hokkaido University (Approval No. 16-0071 and 19-0030). In the present study, 269 lactating
204 Holstein cows were used and housed in 16 commercial farms in Hokkaido, Japan. They were
205 multiparous lactating cows (>9500 kg of 305-day fat-corrected milk) and between two and six in
206 parity. We excluded cows with problems at calving and during the postpartum period requiring
207 three or more veterinary visits.

208 Repeat breeder cows (n = 251) were selected by local veterinarians according to the
209 criteria for failure to conceive after three or more AIs without a detectable abnormality during an
210 estrous cycle, clinical signs and genital organs by rectum palpation, vaginal examination, and
211 ultrasonography [20,22]. The absence of any abnormality in the uterine morphology was
212 confirmed by transrectal ultrasonography [61] and the uterine cytology by cytobrush [62] before
213 the enrollment in the study.
214 Fertile controls (n = 18) were cows 120 days postpartum on the day of ET and
215 between two and six in parity before the first service after parturition. They were also confirmed to
216 have no detectable abnormality in the estrous cycle, clinical signs and genital organs by rectum
217 palpation, vaginal examination, ultrasonography, and uterine cytology before the enrollment of the
218 study. They became pregnant within two inseminations after the study period.
219
220 *2.5. Synchronization of estrus and ovulation*
221 Estrus was synchronized by giving a single administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (20 or 25
222 mg of dinoprost tromethamine im, Pronalgon F, Zoetis Japan, Tokyo, Japan) or an Selectsynch
223 protocol [63] in cows. In the Selectsynch protocol, gonadotropin-releasing hormone (GnRH) (100
224 μg fertirelin acetate im, Conceral, MSD Animal Health, Tokyo, Japan) was treated between day 7
225 and day 9 of the estrous cycle, and $PGF_{2\alpha}$ (25 mg of dinoprost tromethamine im) was administrated
226 7 days after the GnRH treatment. All cows synchronized by a single administration of $PGF_{2\alpha}$ and
227 the Selectsynch protocol showed estrus within 5 and 4 days after $PGF_{2\alpha}$ treatment, respectively,
228 detected either by observation twice daily after the $PGF_{2\alpha}$ treatment or by using an automated
229 activity monitor. The day of estrus was defined as day 0.
230 Ovulation was synchronized by an Ovsynch protocol [64], in which the cows were
231 treated with the first GnRH (100 mg of fertirelin acetate im) followed by $PGF_{2\alpha}$ (25 mg dinoprost
232 tromethamine im) 7 days later and the second GnRH (100 mg fertirelin acetate im) 55 ± 2 h after

233 $PGF_{2\alpha}$ treatment. The next day of the second GnRH treatment was considered as day 0.

234

235 *2.6. Endometrial tissue collection, EGF assay, and the analysis of the endometrial EGF profile*

236 Endometrial tissues were collected for the EGF assay as described previously [15]. Tissues were
237 weighed and frozen in liquid nitrogen within 10 min and then stored at −20°C until the EGF assay.

238 Endometrium tissue samples were processed, and the tissue extracts were used for the
239 EGF assay [16,20]. An antihuman EGF mouse monoclonal antibody (MAB636, R & D Systems,
240 Minneapolis, MN, USA) and antihuman EGF rabbit antiserum (5022-100, Biogenesis, Poole, UK)
241 were used as the solid-phase antibody and detection antibody, respectively. The assay system was
242 verified for the measurement of bovine EGF using recombinant bovine EGF [20]. Assay sensitivity
243 was 10 pg/well. The intra and interassay coefficients of variation at 50 pg/well were 4.2% and
244 6.8%, respectively.

245 In the present study, endometrial EGF concentrations were measured on day 3, and a
246 normal endometrial EGF profile was defined by concentrations within the normal range for day 3
247 (4.70–13.50 ng/g tissue weight) [16,17].

248

249 *2.8. AI and ET*

250 AI was performed with commercial frozen-thawed Holstein semen by professional AI technicians
251 within 4–12 h after estrus detection. For ET, commercial, *in vivo* derived, frozen-thawed Japanese
252 black embryos or *in vitro* derived, frozen-thawed embryos were transferred on day 7 into the
253 uterine horn ipsilateral to the side of the ovaries with a CL by transcervical method using a flexible
254 transfer catheter (Mo–NO.4; Misawa Medical Industry, Kasama, Japan).

255

256 *2.9 Infusion of samples into the vagina*

All samples were prepared in 10 mL PBS, loaded into a 10 mL syringe, and infused into the vagina as previously described [20,22]. Briefly, the tip of a disposable plastic catheter for AI, which was attached to the syringe loaded with an infusion sample, was introduced into the vagina and guided deep into the vagina adjacent to the external orifice of the cervix. The plunger was pushed to deposit the samples.

2.10. Embryo collection and measurement of embryo length

Transferred embryos were collected on day 14 (i.e., 7 days after ET) via the balloon catheter either 18 Fr, two eyes (01209300, Nipro, Osaka, Japan) or 20 Fr, two eyes (0120752520, Nipro) flushing the uterus nonsurgically with a commercial medium for embryo collection (Embryotech; Zenoaq, Fukushima, Japan). Side holes of the balloon catheter were enlarged by removing the rubber between two side holes next to each other. Embryo length was determined as the length of the long axis of collected embryos, or twice the length of fragmented filamentous embryos with embryonic disc that were recovered.

2.11. Study design

2.11.1. Study 1: The effect of rOPN on the restoration of the endometrial EGF profile and fertility

After the screening the repeat breeder cows for any detectable abnormality in the uterine morphology and cytology, estrus or ovulation was synchronized by a single administration of $PGF_{2\alpha}$ or the Ovsynch protocol. The endometrial EGF concentration was determined on day 3. The cows with an altered EGF profile were used for the study (Fig. 1A). Repeat breeder cows were synchronized for estrus according to the Selectsynch protocol and infused with 10 mL PBS (negative control, n = 74), 0.5 mL frozen-thawed SP (positive control, n = 37), or 0.3 mg rOPN (n = 105) into the vagina within 4–12 h after estrus detection, corresponding to the timing of AI. To examine the effects of the treatments on the endometrial EGF profile, the endometrial EGF

concentration was measured on day 3 for the second time. Some cows infused with PBS (n = 58), SP (n = 20), or rOPN (n = 83) were subjected to AI immediately before the samples were infused. Pregnancy was confirmed by transrectal ultrasonography between days 30 and 35 after AI.

2.11.2. Study 2: The effect of the rOPN on the embryo survival and elongation

After the screening for any detectable abnormality in the uterine morphology and cytology in repeat breeder cows and fertile control cows, the endometrial EGF concentration was measured on day 3 on the same schedule as in study 1 (Fig. 1B). Repeat breeder cows with an altered EGF profile (n = 35) and fertile controls with normal EGF profile (n = 18) were synchronized for estrus and infused with rOPN or PBS, as in study 1. Repeat breeder cows were infused with PBS (n = 18, PBS group) or rOPN (n = 17, rOPN group), while fertile controls were infused with PBS (n = 18, fertile control group). To examine the endometrial EGF profile, the endometrial EGF concentration was measured on day 3 for the second time. On day 7, the cows were transferred either *in vivo* derived embryos or *in vitro* derived embryos (two embryos in each cow: PBS group, n = 4; rOPN group, n = 5; fertile control group, n = 6; three embryos in each cow: PBS group, n = 14; rOPN group, n = 12; fertile control group, n = 12). In total, 50, 46, and 48 embryos were transferred in the PBS, rOPN, and fertile control group, respectively. On day 14, the embryos were recovered, and the embryo lengths were measured.

2.12. Data analyses

Data were analyzed using the computer software JMP Pro 16 (SAS Institute Inc., Tokyo, Japan). Fisher's exact test with the Benjamini-Hochberg method was performed to compare the normalization rates of the endometrial EGF profile, conception rates, the proportion of cows from which embryos were collected, and embryo recovery rates between groups. The Steel–Dwass test was used to compare endometrial EGF concentrations between groups. The Wilcoxon signed-rank

307 test was conducted to compare endometrial EGF concentrations before and after treatment.
308 Embryo lengths were denoted by the sinh-arcsinh normal distribution, and the transformed data
309 were evaluated by a two-way analysis of variance (ANOVA) with groups and embryo types as
310 factors. Tukey's test was used to compare embryo lengths transformed into the sinh-arcsinh normal
311 distribution between groups. The Pearson's rank correlation coefficient was used to determine the
312 correlation between the endometrial EGF concentration and the embryo length transformed into
313 the sinh-arcsinh normal distribution. P values less than 0.05 were considered for statistical
314 significance.
315

316 *3. Results*

317 *3.1. Preparation of rOPN*

318 The predicted mature OPN, where a predicted signal peptide was truncated, was produced in *E.*
319 *coli* as N-terminally His6-tagged recombinant protein. By western blotting, OPN was confirmed as
320 the band with the apparent molecular mass of approximately 45,000 on the SDS-PAGE gel (Fig.
321 2). In eight batches of rOPN preparations, the bands of rOPN accounted for 49.0% ± 11.8% (range:
322 from 34.6% to 65.3%) of the total protein content in the final preparation. The yields of rOPN
323 were 1.9 ± 1.0 mg/L of culture.

324

325 *3.2. Study 1: The effect of rOPN on the restoration of the endometrial EGF profile and fertility*

326 Endometrial EGF concentrations before treatment were comparable in all groups ($P > 0.1$) (Fig. 3).
327 After treatment, endometrial EGF concentrations in the rOPN and SP groups were comparable but
328 higher than those in the PBS group ($P < 0.05$). The normalization rates of the endometrial EGF
329 profile of the rOPN and SP groups were comparable (58.1% vs. 62.2%) but higher than that of the
330 PBS group (29.7%) ($P < 0.05$, Table 1).

331 The conception rate of inseminated cows with normalized endometrial EGF profile after
332 treatment with rOPN and PBS infusion was higher than that of inseminated cows with
333 unnormalized endometrial EGF profile ($P < 0.05$, Table 1), while the conception rate of cows after
334 SP infusion was not different between cows with normalized and unnormalized endometrial EGF
335 profile. The overall conception rate of the rOPN group was higher than that of the PBS group
336 (47.0% vs. 28.1%) ($P < 0.05$). The conception rate of the SP group was intermediate between PBS
337 and rOPN groups.

338

339 *3.3. Study 2: The effect of the rOPN on the embryo survival and elongation*

340 The proportion of cows with a normal endometrial EGF profile after treatment of PBS and rOPN
341 groups were 44.4% (8/18) and 58.8% (10/17), respectively. The fertile control group has the
342 highest proportion of cows with a normal endometrial EGF profile after treatment (94.4%, 17/18),
343 which was higher than those of the PBS (44.4%) and rOPN groups (58.8%) ($P < 0.05$). One cow in
344 the fertile control group had an altered endometrial EGF profile after treatment (endometrial EGF
345 concentration: 4.39 ng/g tissue). Endometrial EGF concentrations after treatment of the PBS and
346 rOPN groups were similar but lower than those of the fertile control group ($P < 0.05$, Fig. 4).
347 The number of filamentous embryos that lost one of the distal ends and the number of
348 distal end fragments without embryo discs were equal. Therefore, the number of embryo discs
349 represented the number of embryos. The proportion of cows from which embryos were collected
350 and embryo recovery rates were similar between *in vitro* and *in vivo* derived embryos within
351 groups (Table 2, $P > 0.1$). Embryo recovery rates of the rOPN and fertile control groups were
352 comparable (58.7% vs. 58.3%) and higher than that of the PBS group (32.0%) ($P < 0.05$). The
353 proportion of cows from which embryos were collected and embryo recovery rates in cows with a
354 normal endometrial EGF profile after treatment were higher than those in cows with an altered
355 endometrial EGF profile after treatment ($P < 0.05$, Table 3). In most of cows from which embryos
356 were collected, multiple embryos were recovered.
357 The two-way ANOVA revealed the effect of treatments but not embryo types, without
358 correlation ($P > 0.1$). Embryo lengths of all recovered embryos and the largest embryos in each
359 cow from the fertile control and rOPN groups were comparable and greater than those from the
360 PBS group ($P < 0.05$, Table 2). Endometrial EGF concentrations was not correlated to the embryo
361 lengths of all recovered embryos ($r = -0.01$) or the largest embryos in each cow ($r = -0.09$) (Fig.
362 5, $P > 0.1$).
363

364 *4. Discussion*

365 In the present study, rOPN affected the normalization of the endometrial EGF profile and recovery
366 of fertility in repeat breeder cows with an altered EGF profile. Despite the role of posttranslational
367 modifications in some activities of OPN [49–51], these modifications were not essential and the
368 peptide or protein moiety without the modifications can restore the normal endometrial EGF
369 profile and fertility in repeat breeder cows. In study 1, rOPN normalized the endometrial EGF
370 profile to a similar extent as SP in repeat breeder cows. Furthermore, the effect of normalizing the
371 endometrial EGF profile by rOPN (58.1%) was similar to that of milk OPN (56.1%) [22]. The
372 present results also showed that the normalization of the endometrial EGF profile after rOPN
373 infusion resulted in the recovery of fertility in repeat breeder cows. The conception rate of cows
374 infused with rOPN (47.0%) was similar to those that underwent hormone therapy (40.0%) [18], SP
375 (44.4%) [20] and mOPN (43.5%) [22]. However, further study including a larger number of
376 animals is needed to confirm the effect of rOPN on fertility.

377 It remains unknown how OPN infused into the vagina restores the normal endometrial
378 EGF profile. However, it is unlikely that SP (i.e., OPN) ejaculated into the vagina reaches the
379 uterus [65] because of the low volume of semen ejaculated from bull (around 5 ml) [66], the length
380 of the cervix [67] and the high viscosity of cervical mucus [68] although sperm have the potential
381 to act as a vehicle of SP factors into the uterus [65,69]. Accordingly, our previous report showed
382 that SP restored the normal endometrial EGF profile and fertility by infusion into the vagina, but
383 not into the uterus, in repeat breeder cows [20]. Furthermore, intrauterine infusion of SP at the
384 time of AI had no effect on pregnancy rates in cows [70,71]. These reports indicate that the site of
385 action of SP and OPN to restore the normal endometrial EGF profile and fertility in repeat breeder
386 cows is the vagina.

387 Hypothetically, OPN activates the immune cells in the vagina, causing uterus-targeted or
388 systemic changes by the activation of immune cells in the adjacent lymph nodes [22]. These

activated immune cells in the vagina and lymph nodes may recirculate and infiltrate into the uterus, causing the normalization of the endometrial EGF profile in repeat breeder cows. OPN causes proinflammatory response in the immune cells and activate them, including macrophages, dendritic cells, and lymphocytes [34]. Proinflammatory cytokines, such as interleukin-6 (IL-6) [72] and TNFα [73], play roles in the regulation of endometrial functions. In proinflammatory cytokines, TNFα has been hypothesized to be involved in the normalization of the EGF profile by OPN infusion [22] since EGF synthesis is primary regulated by estrogen in the endometrium [8,74] and TNFα activates estrogen receptor signaling pathways in endometrial epithelial cells [73]. The role of immune cells and the proinflammatory cytokines (e.g., IL-6 and TNFα) in normalizing the endometrial EGF profile after intravaginal OPN infusion is needed to be investigated. In addition, the active site of OPN to normalize the endometrial EGF profile and receptive mechanism of OPN in the vagina also need to be studied to understand the mechanism of this function of OPN.

Although the normalization of the endometrial EGF profile in repeat breeder cows increases embryo survival or promotes embryo development, thereby recovering the conception rate [7,16], this hypothesis had not been tested. The proportion of cows from which embryos were collected and embryo recovery rate by day 14 of pregnancy reflects the embryo survival in the uterus [75–78]. In the present study, the normalization of the endometrial EGF profile after OPN infusion increased the embryo survival to day 14 in the uterus in repeat breeder cows. Since EGF controls the endometrium function [8,10–12], EGF secreted by the endometrium on days 2–4 would regulate the uterine environment to suit embryo development in cattle [15]. Furthermore, the recovery of the endometrial EGF peak on day 3 of the estrous cycle is accompanied by that on day 14 in most cases [18]. Bovine elongating embryos (days 13–16 of pregnancy) express EGF receptors, but not ligand [79], thus EGF secreted by the endometrium on days 13–14 may act on the embryo and stimulate embryo development [15,79].

Alternatively, we could not find the relationship between embryo size and the endometrial EGF concentration on day 3. Endometrial EGF concentrations on days 3 and 14 have been correlated to embryo length on day 16 of pregnancy [80]. Thus, embryo elongation might become apparent after the exposure of the EGF peak on days 13–14. The elongation of a bovine embryo begins between days 12 and 14 [81], and mean values of embryo length indicated logarithmic growth between day 10 and 16 [82]. To confirm that the normalization of the endometrial EGF profile may promote embryo elongation in repeat breeder cows, further investigation should evaluate embryo lengths later than day 14 (i.e., day 16).

Interestingly, the present results suggest that in addition to the normalization of the endometrial EGF profile in repeat breeder cows, OPN infusion into the vagina might enhance embryo development in the uterus by an unknown mechanism independent of the endometrial EGF concentration on day 3. The embryo recovery rate was comparable between the rOPN group and the fertile control group despite lower proportion of cows with a normal endometrial EGF profile and endometrial EGF concentrations on day 3 after treatment of rOPN group than those of the fertile control group. Furthermore, although the embryo length was not correlated to the endometrial EGF concentration on day 3 after treatment, embryo lengths on day 14 were comparable between the rOPN group and the fertile control group but higher than those of the PBS group. Although OPN has been shown to directly promote adhesion, migration and cytoskeletal remodeling of trophectoderm cells that is essential for elongation and attachment of trophectoderm cells to uterine luminal epithelium for implantation in sheep [41] and pigs [40], as discussed above, it seems unlikely that rOPN infused into the vagina directly enhance the development of transferred embryos in the uterus in repeat breeder cows. OPN infusion into the vagina may help in controlling the endometrial function by activating immune cells in the vagina and adjacent lymph nodes [22], which in repeat breeder cows, may regulate the uterine environment via a pathway independently on the endometrial EGF concentration on day 3. Furthermore, the formation of CL

438 has been described as an inflammation involving various immune cells [83,84]. In cattle, P_4
439 secreted from CL enhances embryo elongation in the uterus by regulating the secretion of
440 histotrophic substances and growth factors from the endometrium [85–88]. Thus, immune cells
441 activated by OPN infusion into the vagina might support CL formation and enhance progesterone
442 secretion from a CL, thus independently promoting embryo development in the uterus with
443 endometrial EGF concentration on day 3.
444

5. Conclusion

In this study, rOPN infused into the vagina restored the normal endometrial EGF profile and fertility in repeat breeder cows without posttranslational modifications. The normalization of the endometrial EGF profile by OPN infusion improved embryo survival in the uterus. However, whether the endometrial EGF concentration on day 3 is directly correlated to the embryo size on day 14 remains unclear. Further study must confirm the relationship between the normalization of the endometrial EGF profile and embryo elongation.

CRediT authorship contribution statement

Takashi Tanida: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - original draft. **Takayoshi Tagami**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing. **Hiroko Sato**: Data curation, Investigation, Methodology, Visualization. **Hay Mar Kyaw**: Investigation, Methodology. **Takeshi Fujikawa**: Investigation, Methodology. **Masashi Nagano**: Methodology, Writing - review & editing. **Kenji Momozawa**: Methodology. **Yojiro Yanagawa**: Methodology, Writing – review & editing. **Seiji Katagiri**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - review & editing.

Declarations of interest

None

Acknowledgement

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 19H00964 and 22J20415, 23H00353) and by Grants-in-Aid for the Promotions of Livestock Industry from the Japan Racing Association. We acknowledge the support of the practitioners and veterinarians who assisted with the present study in commercial dairy farms. We thank Frontier Science Co., Ltd. for their technical assistance.

References

[1] Levine HD. The repeat breeder cow. Bov Pract 1999;33:97–105. https://doi.org/https://doi.org/10.21423/bovine-vol33no2p97-105.

[2] Casida LE. Present status of the repeat-breeder cow problem. J Dairy Sci 1961;44:2323–9. https://doi.org/10.3168/jds.S0022-0302(61)90063-7.

[3] Kimura M, Nakao T, Moriyoshi M, Kawata K. Luteal phase defeciency as a possible cause of repeat breeding in dairy cows. BritVetJ 1987;143:560–6. https://doi.org/10.1016/0007-1935(87)90047-9.

[4] Shelton K, Gayerie De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE. Luteal inadequacy during the early luteal phase of subfertile cows. J Reprod Fertil 1990;90:1–10. https://doi.org/10.1530/jrf.0.0900001.

[5] Båge R, Gustafsson H, Larsson B, Forsberg M, Rodríguez-Martínez H. Repeat breeding in dairy heifers: Follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. Theriogenology 2002;57:2257–69. https://doi.org/10.1016/S0093-691X(02)00840-3.

[6] Erb RE, Garverick HA, Randel RD, Brown BL, Callahan CJ. Profiles of reproductive hormones associated with fertile and nonfertile inseminations of dairy cows. Theriogenology 1976;5:227–42. https://doi.org/10.1016/0093-691X(76)90235-1.

[7] Katagiri S, Moriyoshi M. Alteration of the endometrial EGF profile as a potential mechanism connecting the alterations in the ovarian steroid hormone profile to embryonic loss in repeat breeders and high-producing cows. J Reprod Dev 2013;59:415–20. https://doi.org/10.1262/jrd.2013-048.

[8] Brigstock DR. Growth factors in the uterus: Steroidal regulation and biological actions. Baillieres Clin Endocrinol Metab 1991;5:791–808. https://doi.org/10.1016/S0950-351X(10)80015-1.

497 [9] Paria BC, Song H, Dey SK. Implantation: Molecular basis of embryo-uterine dialogue. Int J
498 Dev Biol 2001;45:597–605.
499 [10] Nelson KG, Takahashi T, Bossert NL, Walmer DK, McLachlan J a. Epidermal growth
500 factor replaces estrogen in the stimulation of female genital-tract growth and
501 differentiation. Proc Natl Acad Sci U S A 1991;88:21–5.
502 https://doi.org/10.1073/pnas.88.1.21.
503 [11] Johnson DC, Chatterjee S. Embryo implantation in the rat uterus induced by epidermal
504 growth factor. J Reprod Fertil 1993;99:557–9. https://doi.org/10.1530/jrf.0.0990557.
505 [12] Johnson DC, Chatterjee S. Epidermal growth factor (EGF) replaces estradiol for the
506 initiation of embryo implantation in the hypophysectomized rat. Placenta 1993;14:429–38.
507 https://doi.org/10.1016/S0143-4004(05)80463-1.
508 [13] Paria BC, Dey SK. Preimplantation embryo development in vitro: cooperative interactions
509 among embryos and role of growth factors. Proc Natl Acad Sci U S A 1990;87:4756–60.
510 https://doi.org/10.1073/PNAS.87.12.4756.
511 [14] Katagiri S. Relationship between endometrial epidermal growth factor and fertility after
512 embryo transfer. J Reprod Dev 2006;52(suppl):133–7.
513 [15] Katagiri S, Takahashi Y. Changes in EGF concentrations during estrous cycle in bovine
514 endometrium and their alterations in repeat breeder cows. Theriogenology 2004;62.
515 https://doi.org/10.1016/j.theriogenology.2003.08.019.
516 [16] Katagiri S, Takahashi Y. Potential relationship between normalization of endometrial
517 epidermal growth factor profile and restoration of fertility in repeat breeder cows. Anim
518 Reprod Sci 2006;95:54–66. https://doi.org/10.1016/j.anireprosci.2005.09.001.
519 [17] Katagiri S, Moriyoshi M, Yanagawa Y. Endometrial epidermal growth factor profile and its
520 abnormalities in dairy cows. J Reprod Dev 2016;62:465–70.
521 https://doi.org/10.1262/jrd.2016-039.

[18] Katagiri S, Takahashi Y. A progestin-based treatment with a high dose of estradiol benzoate normalizes cyclic changes in endometrial EGF concentrations and restores fertility in repeat breeder cows. J Reprod Dev 2008;54:473–9. https://doi.org/10.1262/jrd.20078.

[19] Katagiri S, Moriyoshi M, Takahashi Y. Low incidence of an altered endometrial epidermal growth factor (EGF) profile in repeat breeder Holstein heifers and differential effect of parity on the EGF profile between fertile Holstein (dairy) and Japanese Black (beef) cattle. J Reprod Dev 2013;59:575–9. https://doi.org/10.1262/jrd.2013-067.

[20] Badrakh D, Yanagawa Y, Nagano M, Katagiri S. Effect of seminal plasma infusion into the vagina on the normalization of endometrial epidermal growth factor concentrations and fertility in repeat breeder dairy cows. J Reprod Dev 2020;66:149–54. https://doi.org/10.1262/jrd.2019-148.

[21] Badrakh D, Shirasawa A, Yanagawa Y, Nagano M, Katagiri S. Identification of bovine seminal plasma proteins with an activity to normalize endometrial epidermal growth factor concentrations in repeat breeder cows. Jpn J Vet Res 2020;68:91–103. https://doi.org/10.14943/jjvr.68.2.91.

[22] Kyaw HM, Sato H, Tagami T, Yanagawa Y, Nagano M, Katagiri S. Effects of milk osteopontin on the endometrial epidermal growth factor profile and restoration of fertility in repeat breeder dairy cows. Theriogenology 2022;184:26–33. https://doi.org/10.1016/j.theriogenology.2022.02.008.

[23] Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. FASEB J 1993;7:1475–82. https://doi.org/10.1096/fasebj.7.15.8262332.

[24] Icer MA, Gezmen-Karadag M. The multiple functions and mechanisms of osteopontin. Clin Biochem 2018;59:17–24. https://doi.org/10.1016/j.clinbiochem.2018.07.003.

[25] Sodek J, Ganss B, Mckeel D. Osteopontin. Crit Rev Oral Biol Med 2000;11:279–303. https://doi.org/https://doi.org/10.1177/10454411000110030101.

547 [26] Brunswig-Spickenheier B, Mukhopadhyay AK. Expression of osteopontin (OPN) mRNA in
548 bovine ovarian follicles and corpora lutea. Reprod Domest Anim 2003;38:175–81.
549 https://doi.org/10.1046/j.1439-0531.2003.00413.x.
550 [27] Poole DH, Ndiaye K, Pate JL. Expression and regulation of Secreted phosphoprotein 1 in
551 the bovine corpus luteum and effects on T lymphocyte chemotaxis. Reproduction
552 2013;146:527–37. https://doi.org/10.1530/REP-13-0190.
553 [28] Gabler C, Chapman DA, Killian GJ. Expression and presence of osteopontin and integrins
554 in the bovine oviduct during the oestrous cycle. Reproduction 2003;126:721–9.
555 https://doi.org/10.1530/rep.0.1260721.
556 [29] Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. Ovine
557 Osteopontin: II. Osteopontin and αv β3 Integrin Expression in the Uterus and Conceptus
558 During the Periimplantation Period. Biol Reprod 1999;61:892–9.
559 https://doi.org/10.1095/biolreprod61.4.892.
560 [30] Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. Analysis of
561 osteopontin at the maternal-placental interface in pigs. Biol Reprod 2002;66:718–25.
562 https://doi.org/10.1095/biolreprod66.3.718.
563 [31] Joyce MM, González JF, Lewis S, Woldesenbet S, Burghardt RC, Newton GR, et al.
564 Caprine uterine and placental osteopontin expression is distinct among epitheliochorial
565 implanting species. Placenta 2005;26:160–70.
566 https://doi.org/10.1016/j.placenta.2004.05.009.
567 [32] Erikson DW, Way AL, Chapman DA, Killian GJ. Detection of osteopontin on Holstein bull
568 spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in
569 bovine fertilization. Reproduction 2007;133:909–17. https://doi.org/10.1530/REP-06-0228.

570 [33] Castello LM, Raineri D, Salmi L, Clemente N, Vaschetto R, Quaglia M, et al. Osteopontin
571 at the crossroads of inflammation and tumor progression. Mediators Inflamm 2017;2017.
572 https://doi.org/10.1155/2017/4049098.
573 [34] Lund SA, Giachelli CM, Scatena M. The role of osteopontin in inflammatory processes. J
574 Cell Commun Signal 2009;3:311–22. https://doi.org/10.1007/s12079-009-0068-0.
575 [35] Liu Q, Xie QZ, Zhou Y, Yang J. Osteopontin is expressed in the oviduct and promotes
576 fertilization and preimplantation embryo development of mouse. Zygote 2014;760:622–30.
577 https://doi.org/10.1017/S0967199414000483.
578 [36] Monaco E, Gasparrini B, Boccia L, De Rosa A, Attanasio L, Zicarelli L, et al. Effect of
579 osteopontin (OPN) on in vitro embryo development in cattle. Theriogenology 2009;71:450–
580 7. https://doi.org/10.1016/j.theriogenology.2008.08.012.
581 [37] Johnson GA, Burghardt RC, Bazer FW, Spencer TE. Osteopontin: Roles in implantation
582 and placentation. Biol Reprod 2003;69:1458–71.
583 https://doi.org/10.1095/biolreprod.103.020651.
584 [38] Boccia L, Di Francesco S, Neglia G, De Blasi M, Longobardi V, Campanile G, et al.
585 Osteopontin improves sperm capacitation and invitro fertilization efficiency in buffalo
586 (Bubalus bubalis). Theriogenology 2013;80:212–7.
587 https://doi.org/10.1016/j.theriogenology.2013.04.017.
588 [39] Kuwabara Y, Katayama A, Tomiyama R, Piao H, Kurihara S, Ono S, et al. Gonadotropin
589 regulation and role of ovarian osteopontin in the periovulatory period. J Endocrinol
590 2015;224:49–59. https://doi.org/10.1530/JOE-14-0203.
591 [40] Erikson DW, Burghardt RC, Bayless KJ, Johnson GA. Secreted phosphoprotein 1 (SPP1,
592 osteopontin) binds to integrin alphavbeta6 on porcine trophectoderm cells and lntegrin
593 alphavbeta3 on uterine luminal epithelial cells, and promotes trophectoderm cell adhesion

and migration. Biol Reprod 2009;81:814–25.
https://doi.org/10.1095/biolreprod.109.078600.

[41] Kim J, Erikson DW, Burghardt RC, Spencer TE, Wu G, Bayless KJ, et al. Secreted phosphoprotein 1 binds integrins to initiate multiple cell signaling pathways, including FRAP1/mTOR, to support attachment and force-generated migration of trophectoderm cells. Matrix Biol 2010;29:369–82. https://doi.org/10.1016/j.matbio.2010.04.001.

[42] Kramer AC, Erikson DW, McLendon BA, Seo H, Hayashi K, Spencer TE, et al. SPP1 expression in the mouse uterus and placenta: Implications for implantation†. Biol Reprod 2021;105:892–904. https://doi.org/10.1093/biolre/ioab125.

[43] Givens MD. Review: Risks of disease transmission through semen in cattle. Animal 2018;12:s165–71. https://doi.org/10.1017/S1751731118000708.

[44] Vasavada PC. Pathogenic Bacteria in Milk — A Review. J Dairy Sci 1988;71:2809–16. https://doi.org/10.3168/jds.S0022-0302(88)79876-8.

[45] Herlekar DA, Shashikant CS, Gurjar AA, Jayarao BM. Presence of viral and bacterial organisms in milk and their association with somatic cell counts. J Dairy Sci 2013;96:6336–46. https://doi.org/10.3168/jds.2013-6631.

[46] Kuczewski A, Orsel K, Barkema HW, Mason S, Erskine R, van der Meer F. Invited review: Bovine leukemia virus—Transmission, control, and eradication. J Dairy Sci 2021;104:6358–75. https://doi.org/10.3168/jds.2020-18925.

[47] Overton TW. Recombinant protein production in bacterial hosts. Drug Discov Today 2014;19:590–601. https://doi.org/10.1016/j.drudis.2013.11.008.

[48] Sahdev S, Khattar SK, Saini KS. Production of active eukaryotic proteins through bacterial expression systems: A review of the existing biotechnology strategies. Mol Cell Biochem 2008;307:249–64. https://doi.org/10.1007/s11010-007-9603-6.

618 [49] Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al.
619 Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity. Science (80-)
620 2000;287:860–4. https://doi.org/10.1126/science.287.5454.860.
621 [50] Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H, Ashkar S. Phosphorylation-
622 dependent interaction of osteopontin with its receptors regulates macrophage migration and
623 activation. J Leukoc Biol 2002;72:752–61. https://doi.org/10.1189/jlb.72.4.752.
624 [51] Minai-Tehrani A, Chang SH, Park SB, Cho MH. The O-glycosylation mutant osteopontin
625 alters lung cancer cell growth and migration in vitro and in vivo. Int J Mol Med
626 2013;32:1137–49. https://doi.org/10.3892/ijmm.2013.1483.
627 [52] Wing TT, Erikson DW, Burghardt RC, Bazer FW, Bayless KJ, Johnson GA. OPN binds
628 alpha V integrin to promote endothelial progenitor cell incorporation into vasculature.
629 Reproduction 2020;159:465–78. https://doi.org/10.1530/REP-19-0358.
630 [53] Wu G, Li X, Seo H, McLendon BA, Kramer AC, Bazer FW, et al. Osteopontin
631 (OPN)/secreted phosphoprotein 1 (SPP1) binds integrins to activate transport of ions across
632 the porcine placenta. Front Biosci - Landmark 2022;27:117.
633 https://doi.org/10.31083/j.fbl2704117.
634 [54] Okegawa Y, Motohashi K. Evaluation of seamless ligation cloning extract preparation
635 methods from an Escherichia coli laboratory strain. Anal Biochem 2015;486:51–3.
636 https://doi.org/10.1016/j.ab.2015.06.031.
637 [55] Tagami T, Miyano E, Sadahiro J, Okuyama M, Iwasaki T, Kimura A. Two novel glycoside
638 hydrolases responsible for the catabolism of cyclobis-(1→6)-α-nigerosyl. J Biol Chem
639 2016;291:16438–47. https://doi.org/10.1074/jbc.M116.727305.
640 [56] Noble JE, Bailey MJA. Quantitation of protein. Methods Enzymol 2009;463:73–95.
641 https://doi.org/10.1016/S0076-6879(09)63008-1.

642 [57] Chelenga M, Sakaguchi K, Abdel-Ghani MA, Yanagawa Y, Katagiri S, Nagano M. Effect
643 of increased oxygen availability and astaxanthin supplementation on the growth, maturation
644 and developmental competence of bovine oocytes derived from early antral follicles.
645 Theriogenology 2020;157:341–9. https://doi.org/10.1016/j.theriogenology.2020.07.023.
646 [58] Harada M, Miyano T, Matsumura K, Osaki S, Miyake M, Kato S. Bovine oocytes from
647 early antral follicles grow to meiotic competence in vitro: Effect of FSH and hypoxanthine.
648 Theriogenology 1997;48:743–55. https://doi.org/https://doi.org/10.1016/S0093-
649 691X(97)00298-7.
650 [59] Robertson I, Nelson RE. Certification and identification of embryos. In: Stringfellow DA,
651 Givens MD, editors. Man. Int. Embryo Transf. Soc. 4th ed, Champaign: International
652 Embryo Transfer Society, Champaign; 2010, p. 86–105.
653 [60] Dochi O, Takahashi K, Hirai T, Hayakawa H. The use of embryo transfer to produce
654 pregnancies in repeat-breeding dairy cattle 2008;69:124–8.
655 https://doi.org/10.1016/j.theriogenology.2007.09.001.
656 [61] Mee JF, Buckley F, Ryan D, Dillon P. Pre-breeding ovaro-uterine ultrasonography and its
657 relationship with first service pregnancy rate in seasonal-calving dairy herds. Reprod
658 Domest Anim 2009;44:331–7. https://doi.org/10.1111/j.1439-0531.2008.01079.x.
659 [62] Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Definitions and diagnosis of
660 postpartum endometritis in dairy cows. J Dairy Sci 2010;93:5225–33.
661 https://doi.org/10.3168/jds.2010-3428.
662 [63] Larson JE, Lamb GC, Stevenson JS, Johnson SK, Day ML, Geary TW, et al.
663 Synchronization of estrus in suckled beef cows for detected estrus and artificial
664 insemination and timed artificial insemination using gonadotropin-releasing hormone,
665 prostaglandin F2α, and progesterone. J Anim Sci 2006;84:332–42.
666 https://doi.org/10.2527/2006.842332x.

667 [64] Wiltbank MC, Pursley JR. Theriogenology The cow as an induced ovulator : Timed AI after
668 synchronization of ovulation. Theriogenology 2014;81:170–85.
669 https://doi.org/10.1016/j.theriogenology.2013.09.017.
670 [65] Fernandez-Fuertes B. Review: The role of male reproductive tract secretions in ruminant
671 fertility. Animal 2023;17:100773. https://doi.org/10.1016/j.animal.2023.100773.
672 [66] Murphy EM, Kelly AK, O'Meara C, Eivers B, Lonergan P, Fair S. Influence of bull age,
673 ejaculate number, and season of collection on semen production and sperm motility
674 parameters in holstein friesian bulls in a commercial artificial insemination centre. J Anim
675 Sci 2018;96:2408–18. https://doi.org/10.1093/jas/sky130.
676 [67] Perkins JR, Olds D, Seath DM. A Study of 1,000 Bovine Genitalia. J Dairy Sci
677 1954;37:1158–63. https://doi.org/10.3168/jds.S0022-0302(54)91384-3.
678 [68] Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Hum Reprod
679 Update 2006;12:23–37. https://doi.org/10.1093/humupd/dmi047.
680 [69] Chu TM, Nocera M, Flanders KC, Kawinski E. Localization of seminal plasma
681 transforming growth factor-β1 on human spermatozoa: An immunocytochemical study.
682 Fertil Steril 1996;66:327–30. https://doi.org/10.1016/s0015-0282(16)58461-1.
683 [70] Odhiambo JF, Poole DH, Hughes L, DeJarnette JM, Inskeep EK, Dailey RA. Pregnancy
684 outcome in dairy and beef cattle after artificial insemination and treatment with seminal
685 plasma or transforming growth factor beta-1. Theriogenology 2009;72:566–71.
686 https://doi.org/10.1016/j.theriogenology.2009.04.013.
687 [71] Ortiz WG, Rizo JA, Carvalheira LR, Ahmed BMS, Estrada-Cortes E, Harstine BR, et al.
688 Effects of intrauterine infusion of seminal plasma at artificial insemination on fertility of
689 lactating Holstein cows. J Dairy Sci 2019;102:6587–94. https://doi.org/10.3168/jds.2019-
690 16251.

691 [72] Campanile G, Baruselli PS, Limone A, D'Occhio MJ. Local action of cytokines and
692 immune cells in communication between the conceptus and uterus during the critical period
693 of early embryo development, attachment and implantation – Implications for embryo
694 survival in cattle: A review. Theriogenology 2021;167:1–12.
695 https://doi.org/10.1016/j.theriogenology.2021.02.020.
696 [73] Gori I, Pellegrini C, Staedler D, Russell R, Jan C, Canny GO. Tumor necrosis factor-α
697 activates estrogen signaling pathways in endometrial epithelial cells via estrogen receptor α.
698 Mol Cell Endocrinol 2011;345:27–37. https://doi.org/10.1016/j.mce.2011.06.043.
699 [74] Huet-Hudson YM, Chakrabortyt C, De SK, Suzuki Y, Andrews GK, Dey SK. Estrogen
700 regulates the synthesis of epidermal growth factor in mouse uterine epithelial cells. Mol
701 Endocrinol 1990;4:510–23. https://doi.org/10.1210/mend-4-3-510.
702 [75] O'Callaghan E, Sánchez JM, McDonald M, Kelly AK, Hamdi M, Maicas C, et al. Sire
703 contribution to fertilization failure and early embryo survival in cattle. J Dairy Sci
704 2021;104:7262–71. https://doi.org/10.3168/jds.2020-19900.
705 [76] Lonergan P, Woods A, Fair T, Carter F, Rizos D, Ward F, et al. Effect of embryo source
706 and recipient progesterone environment on embryo development in cattle. Reprod Fertil
707 Dev 2007;19:861–8. https://doi.org/10.1071/RD07089.
708 [77] Sánchez JM, Passaro C, Forde N, Browne JA, Behura SK, Fernández-Fuertes B, et al. Do
709 differences in the endometrial transcriptome between uterine horns ipsilateral and
710 contralateral to the corpus luteum influence conceptus growth to day 14 in cattle? Biol
711 Reprod 2019;100:86–100. https://doi.org/10.1093/biolre/ioy185.
712 [78] Mateo-Otero Y, Sánchez JM, Recuero S, Bagés-Arnal S, McDonald M, Kenny DA, et al.
713 Effect of exposure to seminal plasma through natural mating in cattle on conceptus length
714 and gene expression. Front Cell Dev Biol 2020;8. https://doi.org/10.3389/fcell.2020.00341.

715 [79] Kliem A, Tetens F, Klonisch T, Grealy M, Fischer B. Epidermal growth factor receptor and
716 ligands in elongating bovine blastocysts. Mol Reprod Dev 1998;51:402–12.
717 https://doi.org/10.1002/(SICI)1098-2795(199812)51:4<402::AID-MRD7>3.0.CO;2-9.
718 [80] Katagiri S, Takahashi Y. Relationship between endometrial concentrations of epidermal
719 growth factor (EGF) and preimplantation embryo development in dairy cattle. Biol Reprod
720 2004;SI:211.
721 [81] Lonergan P, Forde N. Maternal-embryo interaction leading up to the initiation of
722 implantation of pregnancy in cattle. Animal 2014;8:64–9.
723 https://doi.org/10.1017/S1751731114000470.
724 [82] Betteridge KJ, Eaglesome MD, Randall GCB, Mitchell D. Collection, description and
725 transfer of embryos from cattle 10-16days after oestrus. J Reprod Fertil 1980;59:205–16.
726 [83] Fair T. The contribution of the maternal immune system to the establishment of pregnancy
727 in cattle. Front Immunol 2015;6:1–8. https://doi.org/10.3389/fimmu.2015.00007.
728 [84] Alrabiah NA, Evans ACO, Fahey AG, Cantwell N, Lonergan P, Mccormack J, et al.
729 Immunological aspects of ovarian follicle ovulation and corpus luteum formation in cattle.
730 Reproduction 2021;162:209–25. https://doi.org/10.1530/REP.
731 [85] Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, et al.
732 Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an
733 indirect effect via the endometrium? Reproduction 2009;138:507–17.
734 https://doi.org/10.1530/REP-09-0152.
735 [86] Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, et al. Effects of low
736 progesterone on the endometrial transcriptome in cattle. Biol Reprod 2012;87:1–11.
737 https://doi.org/10.1095/biolreprod.112.103424.
738 [87] Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, O'Gaora P, et al. Changes in the
739 endometrial transcriptome during the bovine estrous cycle: Effect of low circulating

progesterone and consequences for conceptus elongation. Biol Reprod 2011;84:266–78. https://doi.org/10.1095/biolreprod.110.085910.

[88] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J Dairy Sci 2016;99:5941–50. https://doi.org/10.3168/jds.2015-10070.

747 *Figure captions.*

748 Fig.1. The outline of activities during study 1 (A) and study 2 (B). (A) Repeat breeder cows, which
749 failed to conceive after three or more artificial inseminations (AI) without a detectable abnormality
750 in the estrous cycle, clinical signs and genital organs, were subjected to the examination of uterine
751 ultrasonography and cytology (Screening). The cows showing no abnormality at screening were
752 synchronized estrus or ovulation, and the endometrial EGF concentration was determined on day 3
753 (day 0 = the day of estrus or the next day of the second GnRH treatment of Ovsynch program) and
754 cows with an abnormal EGF profile were used for the further study. Cows were synchronized
755 estrus and infused with samples into the vagina 4 to 12 h after estrus detection. The endometrial
756 EGF concentration was determined on day 3 for the second time. Some cows were subjected to AI
757 immediately before the infusion with samples. Pregnancy was diagnosed between day 30 and 35
758 after AI. (B) Repeat breeder cows and fertile control cows were subjected to the screening and the
759 endometrial EGF concentration was determined on day 3 same as study 1. Repeat breeder cows
760 with an altered EGF profile and fertile control cows with a normal EGF profile were synchronized
761 estrus and infused with samples into the vagina 4 to 12 h after estrus detection. The endometrial
762 EGF concentration was determined on day 3 for the second time. Thereafter, cows were transferred
763 embryos on day 7. Embryos were recovered on day 14 and embryo lengths were measured.
764
765 Fig.2. Verification of recombinant osteopontin (rOPN) by Western blotting. The band with the
766 apparent molecular mass of approximately 45,000 on the SDS-PAGE gel (lane 2) was identified as
767 OPN by Western blotting (2.4 μg of total protein per lane). Lane 1: molecular mass marker, lane 2:
768 final preparation.
769
770 Fig.3. The effect of recombinant osteopontin (rOPN) on endometrial epidermal growth factor
771 (EGF) concentrations in repeat breeder dairy cows in study 1. Repeat breeder cows with an altered

772 EGF profile were infused with PBS (n = 74), seminal plasma (SP, n = 37) or rOPN (n = 105) into
773 the vagina at the estrus. Endometrial EGF profiles of 22, 23 and 61 cows were normalized in PBS,
774 SP and rOPN groups, respectively. Endometrial EGF profiles of 52, 14 and 44 cows were
775 unnormalized in PBS, SP and rOPN groups, respectively. a, b Values with different letters between
776 before and after treatment within the same group differ significantly ($P < 0.05$). A, B Values with
777 different letters between groups within all cows before treatment or all, normalized or
778 unnormalized cows after treatment differ significantly ($P < 0.05$).
779
780 Fig.4. The effect of recombinant osteopontin (rOPN) on endometrial epidermal growth factor
781 (EGF) concentrations in repeat breeder dairy cows in study 2. Repeat breeder cows with an altered
782 EGF profile were infused with PBS (PBS group, n = 18) or rOPN (rOPN group, n = 17) into the
783 vagina at the estrus. Fertile cows with a normal EGF profile (Fertile control group, n = 18) were
784 infused with PBS into the vagina at the estrus. a, b Values with different letters between before and
785 after treatment within the same group differ significantly ($P < 0.05$). A, B Values with different
786 letters between groups within cows before or after treatment differ significantly ($P < 0.05$).
787
788 Fig.5. The relationship between the endometrial epidermal growth factor (EGF) concentration and
789 the embryo length. Embryos were transferred to repeat breeder cows infused with PBS (white
790 squares) or recombinant OPN (gray triangles) or to fertile cows infused with PBS (black circles).
791 There were no correlations between the endometrial EGF concentration and the embryo length of
792 all recovered embryos ($r = -0.01$) or that of the largest embryos per cows ($r = -0.09$), respectively
793 ($P > 0.1$. The correlation was evaluated between the data of endometrial EGF concentrations and
794 that of embryo lengths transformed into the sinh-arcsinh normal distribution).
795
796

797 Table 1. The effect of recombinant osteopontin (rOPN) on the endometrial epidermal growth factor

798 (EGF) profile and fertility in repeat breeder cows.

799

Groups (n)	No. (%) of cows with a normal EGF profile after treatment	Endometrial EGF profile after treatment	No. of inseminated cows	No. (%) of conceived cows
PBS (74)	22 (29.7) ^{A}	Normalized	19	9 (47.4) ^{C}
		Unnormalized	39	7 (18.4) ^{D}
		Total	58	16 (28.1) ^{A}
SP (37)	23 (62.2) ^{B}	Normalized	9	6 (66.7)
		Unnormalized	11	3 (27.3)
		Total	20	9 (45.0) ^{AB}
rOPN (105)	61 (58.1) ^{B}	Normalized	47	32 (68.1) ^{C}
		Unnormalized	36	7 (19.4) ^{D}
		Total	83	39 (47.0) ^{B}
Total (216)	106 (49.1)	Normalized	75	47 (62.7) ^{C}
		Unnormalized	86	17 (19.8) ^{D}
		Total	161	64 (39.8)

800

801 SP: Seminal plasma.

802 A, B Normalization rates of the endometrial EGF profile and conception rates with different letters

803 in the same column differ significantly ($P < 0.05$).

804 C, D Conception rates with different letters between different endometrial EGF profiles within the

805 same group differ significantly ($P < 0.05$).

806 Table 2. The effect of recombinant osteopontin (rOPN) on the embryo survival and elongation

807

Groups	Embryo type	No. of cows	No. (%) of cows from which embryos were collected	No. of transferred embryos*	No. (%) of recovered embryos	Embryo lengths of all recovered embryos (mm)	Embryo lengths of the largest embryos in each cow (mm)
PBS	In vitro	10	4 (40.0)	26	9 (34.6)	9.6 ± 11.4	17.5 ± 13.5
	In vivo	8	3 (37.5)	24	7 (29.2)	14.8 ± 17.0	16.5 ± 21.5
	Total	18	7 (38.9)	50	16 (32.0) [A]	11.9 ± 13.8 [A]	17.1 ± 15.7 [A]
rOPN	In vitro	10	7 (70.0)	25	16 (59.5)	31.6 ± 25.9	46.7 ± 26.9
	In vivo	7	4 (57.1)	21	11 (52.3)	27.1 ± 27.1	57.0 ± 16.4
	Total	17	11 (64.7)	46	27 (58.7) [B]	29.7 ± 26.0 [B]	50.4 ± 23.3 [B]
Fertile control	In vitro	10	7 (70.0)	24	13 (54.2)	53.3 ± 26.8	70.3 ± 18.2
	In vivo	8	6 (75.0)	24	15 (62.5)	30.9 ± 23.8	48.4 ± 22.4
	Total	18	13 (72.2)	48	28 (58.3) [B]	41.3 ± 27.2 [B]	60.1 ± 22.4 [B]

808

809 Embryo lengths are described as means ± standard deviations.

810 * Two or three embryos were transferred into the uterus in each cow.

811 A, B Embryo lengths and embryo recovery rates with different letters in the same column differ significantly ($P < 0.05$).

812 Table 3. The relationship between the endometrial EGF profile after treatment and the embryo survival

813

Endometrial EGF profile after treatment	Groups	No. of cows	No. (%) of cows from which embryos were collected	No. of transferred embryos*	No. (%) of recovered embryos	No. of cows transferred three embryos and recovered indicated number of embryos			No. of cows transferred two embryos and recovered indicated number of embryos	
						Three	Two	One	Two	One
Normal	PBS	8	6 (75.0) ^{A}	23	14 (60.9) ^{A}	2	3	0	1	0
	rOPN	10	9 (90.0) ^{A}	27	21 (77.8) ^{A}	4	2	0	2	1
	Fertile control	17	12 (70.6)	46	27 (58.7)	3	4	1	4	0
	Total	35	27 (77.1) ^{A}	96	62 (64.6) ^{A}	9	9	1	7	1
Altered	PBS	10	1 (10.0) ^{B}	27	1 (3.7) ^{B}	0	0	0	0	1
	rOPN	7	2 (28.6) ^{B}	19	6 (31.6) ^{B}	2	0	0	0	0
	Fertile control	1	1 (100.0)	2	1 (50.0)	0	0	0	0	1
	Total	17	4 (22.2) ^{B}	48	8 (16.7) ^{B}	2	0	0	0	2

814

815 * Three or two embryos were transferred into the uterus per cows.

816 A, B The proportion of cows from which embryos were collected and embryo recovery rates with different letters within the same group in the same

817 column differ significantly ($P < 0.05$).

818

819

Fig. 1.

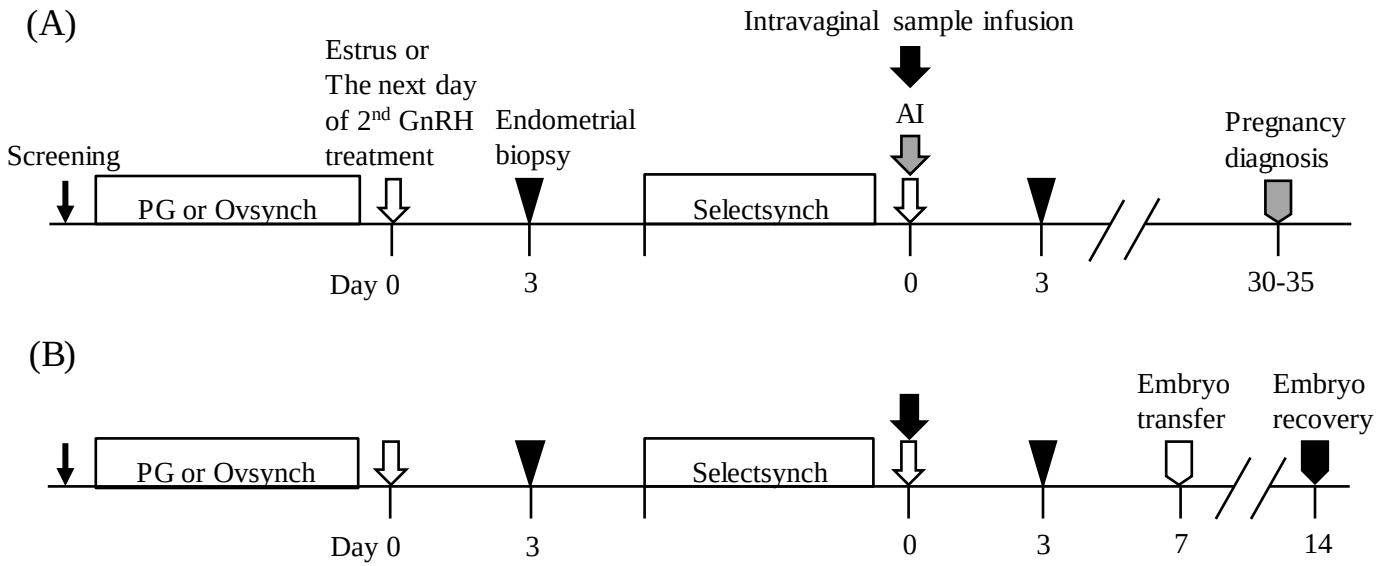

(A)
Intravaginal sample infusion
Estrus or
The next day
of 2nd GnRH
treatment
Endometrial
biopsy
AI
Screening
Pregnancy
diagnosis
PG or Ovsynch
Selectsynch
Day 0
3
0
3
30-35
(B)
Embryo
transfer
Embryo
recovery
PG or Ovsynch
Selectsynch
Day 0
3
0
3
7
14

Fig. 2.

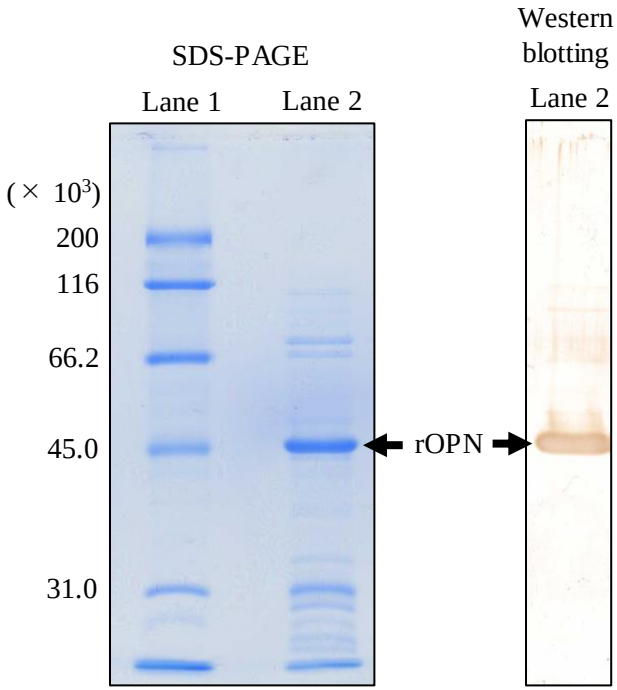

SDS-PAGE
Western blotting
Lane 1
Lane 2
Lane 2
(× 10³)
200
116
66.2
45.0
31.0
rOPN

Fig. 3.

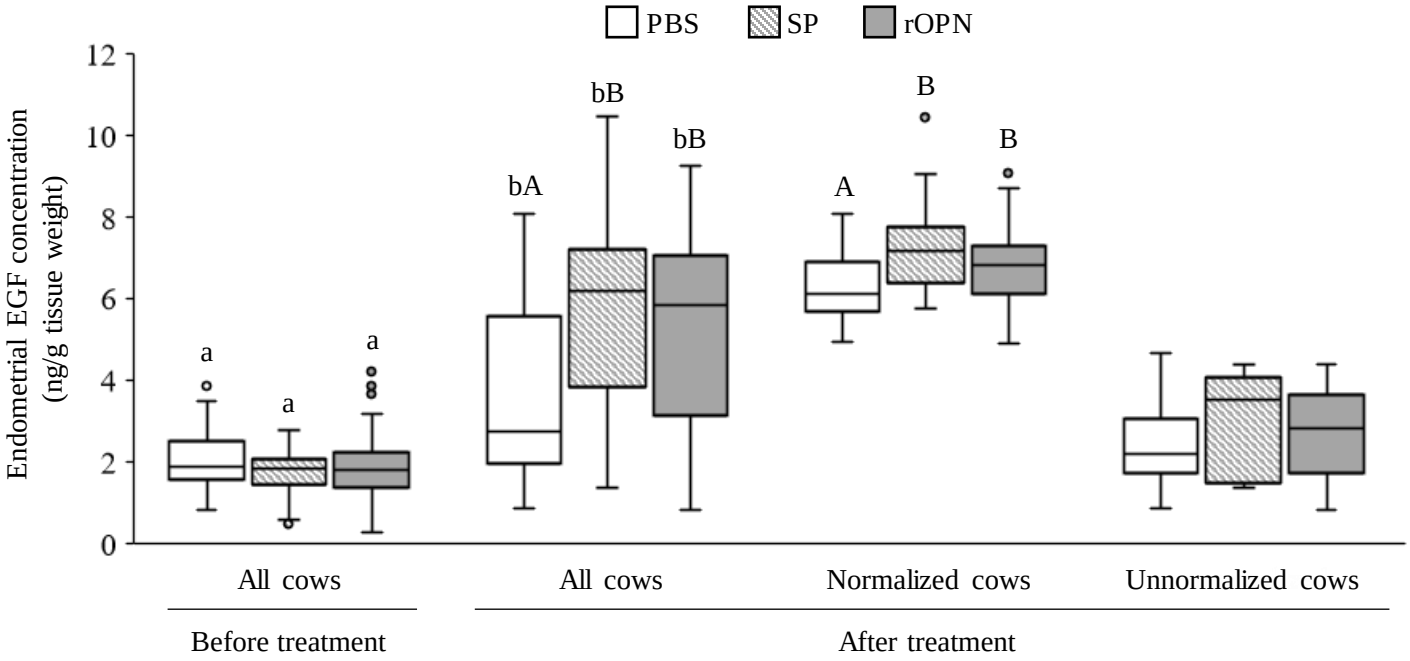

PBS
SP
rOPN
Endometrial EGF concentration (ng/g tissue weight)
12
10
8
6
4
2
0
a
a
a
bA
bB
bB
A
B
B
All cows
All cows
Normalized cows
Unnormalized cows
Before treatment
After treatment

Fig. 4.

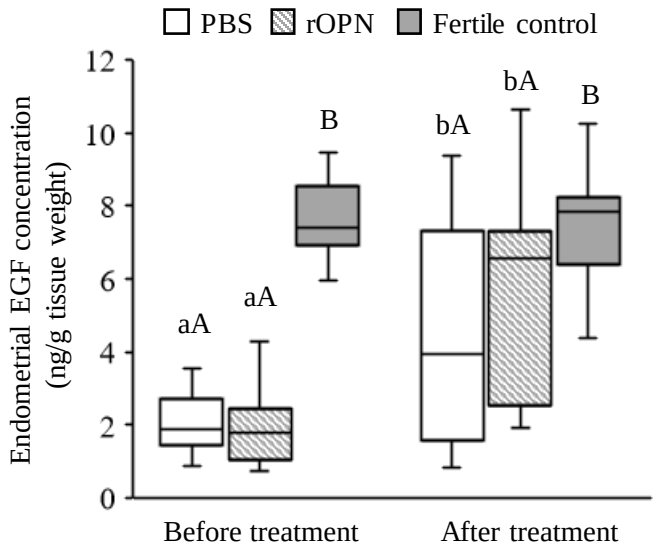
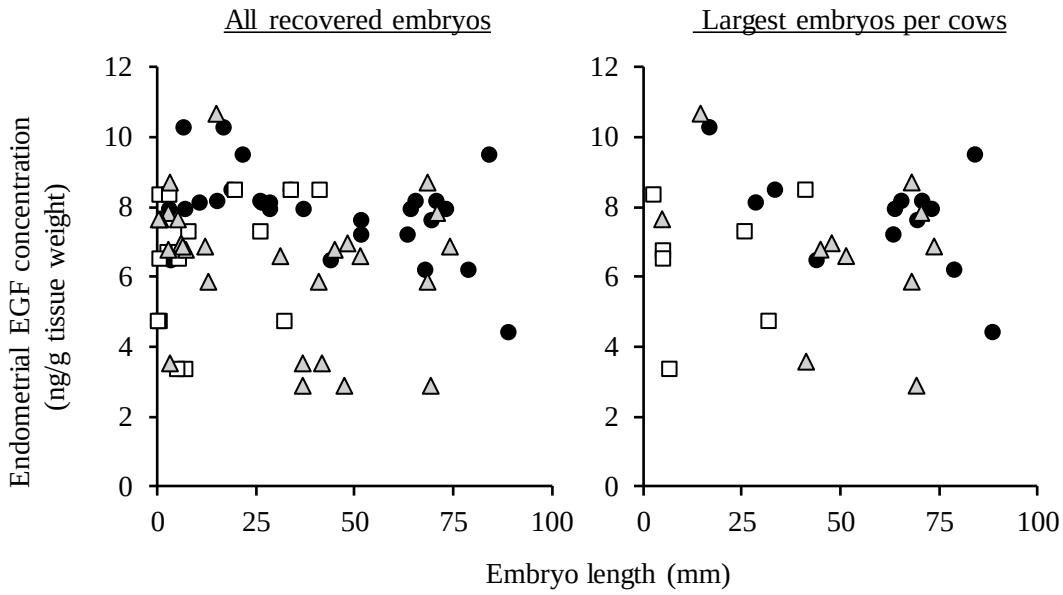

PBS
rOPN
Fertile control
Endometrial EGF concentration
(ng/g tissue weight)
12
10
8
6
4
2
0
aA
aA
B
bA
bA
B
Before treatment
After treatment

Fig. 5


All recovered embryos
Largest embryos per cows
Endometrial EGF concentration (ng/g tissue weight)
Embryo length (mm)
0
2
4
6
8
10
12
0
25
50
75
100