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Effects of deep-horn AI on fertilization and embryo production in superovulated cows and heifers

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ABSTRACT

The primary objective of this study was to determine the effect of site of semen deposition on fertilization rate and embryo quality in superovulated cows. The hypothesis was that deposition of semen into the uterine horns would increase the fertilization rate compared with deposition of semen into the uterine body. The secondary objective was to evaluate the effect of uterine environment on fertilization rate and embryo quality. It was hypothesized that subclinical endometritis at the onset of superstimulation would decrease the fertilization rates and embryo quality. In experiment 1, 17 superovulated heifers were randomly assigned to receive artificial insemination (AI) into the uterine body or uterine horns. The total number of fertilized structures and fertilization rate from superovulated heifers was increased ($P = 0.04$ and $P = 0.02$, respectively) when semen was deposited into the uterine horns compared with the uterine body. Other embryo characteristics did not differ based on the site of semen deposition. In experiment 2, 14 lactating dairy cows were superovulated twice and were randomly assigned to receive AI into the uterine body or deep into the uterine horns using a crossover design. Neither fertilization rate nor any other embryo characteristics were improved when semen was placed deep into the uterine horns compared with the uterine body. In experiment 3, 72 superovulated lactating dairy cows were randomly assigned to receive AI into the uterine body or uterine horns. Before initiation of superstimulatory treatments, an endometrial cytology sample was collected from each cow. Ova/embryos were collected by a nonsurgical technique at 70 ± 3 days in milk. Similar to experiment 2, neither fertilization rate nor any other embryo characteristics differed based on the site of semen deposition in experiment 3. The percentage of cows with subclinical endometritis did not differ between treatments. Interestingly, there was a tendency ($P = 0.09$) for a reduction in embryo recovery rate and a reduction ($P = 0.01$) in the fertilization rate for cows with subclinical endometritis. In conclusion, deposition of semen into the uterine horns rather than into the uterine body did not improve the fertilization rate or embryo quality in superovulated cows. Subclinical endometritis decreased the fertilization rate in superovulated cows.

1. Introduction

Successful fertilization *in vivo* depends on coordinated transport of viable male and female gametes into the oviduct [1–3]. Although artificial insemination (AI) with frozen semen has facilitated dramatic genetic progress

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throughout the dairy and beef cattle industries, it may also produce reduced fertility if the technician improperly deposits semen into the female reproductive tract. For example, deposition of frozen semen into the cervix rather than the uterine body increased retrograde loss of semen into the vagina with up to 60% of semen lost after placement in the cranial portion of the cervix [4]. Indeed, >20% of inseminations resulted in semen being deposited into the cervix rather than the uterine body [4–6]. Deposition of semen into the uterine horns has been proposed as an alternative method to the traditional deposition into the uterine body. However, comparison of these two techniques has produced surprisingly variable results with reports of deep-horn AI having no effect [7–13] or improving [14–16] fertility in cattle.

Fertilization rates in superovulated cows are generally lower than in nonsuperovulated cows [17]. Ova from superovulated cows, fertilized or unfertilized, have fewer accessory sperm, suggesting that low fertilization rates are a consequence of low numbers of viable sperm at the site of fertilization or altered ability for sperm penetration into oocytes of superovulated cows [17–20]. Many factors may contribute to a reduced fertilization rate in superovulated cows, including greater retrograde semen loss due to increased mucus, increased velocity of ova transport [21] suboptimal timing of semen deposition [22], changes in maturation of superovulated ova [23], an inappropriate hormonal environment altering gamete transport [21,23], dose of FSH used [24], LH content in the FSH preparation (Bender et al., unpublished data), FSH batch [25], stage of the estrous cycle when the superstimulation treatment begins [26], intervals after calving [18], AI technician skill, semen fertility [27], and type of semen [28–30]. Fertilization rate and therefore embryo production in superovulated cattle may be improved by optimizing the physiological conditions and techniques used during superstimulation protocols.

Thus, our primary objective was to determine the effect of site of semen deposition in superovulated cows on fertilization rates and embryo quality. Our hypothesis was that fertilization rates of superovulated lactating dairy cows would increase when semen is deposited into the uterine horns compared with the uterine body. Our secondary objective was to evaluate the impact of uterine environment (presence of subclinical endometritis) on fertilization rate and embryo quality. We hypothesized that cows with subclinical endometritis at the beginning of the superstimulation would have decreased fertilization rates and embryo quality.

2. Materials and methods

2.1. Supplies and semen

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, 25 mg of Dinoprost tromethamine/dose) was from Pfizer Animal Health, NY, USA—experiment 1 and 2; or Estrumate (500 μg of cloprostenol sodium/dose) was from Schering-Plough Animal Health, Union, NL, USA—experiment 3. The GnRH (100 μg/dose; Cystorelin) was from Merial (Duluth, GA, USA). FSH (NIH-FSH-P; Folltropin-V) was from Bioniche Life Sciences (Belleville, ON, Canada). Intravaginal progesterone (P4) implants (Eazi-Breed controlled internal drug release [CIDR]; containing 1.38 g of progesterone) were from Pfizer Animal Health. Lidocaine (Lidocaine Hydrochloride Injectable 2%; 5 mL/dose) was from Phoenix Pharmaceutical Inc. (St. Joseph, MO, USA). Embryo filters (MiniFlush Embryo System), y-tubing (Y-Junction Tubing), catheters (Silicon ET catheter CH16/CH18, two-way Foley, 30 mL balloon), medium for embryo recovery (BoviPro Recovery Medium with PVA, 2L), holding medium (BoviPro Holding Medium, with BSA), ethylene glycol (BoviPro Ethylene Glycol with Sucrose), embryo straws (MiniStraw, 0.25 mL), and plugs were from Minitube of America Inc. (Verona, WI, USA). The RIA kit (Coat-A-Count) was from Diagnostic Products Corporation (Los Angeles, CA, USA). Uterine smears were stained with Diff-Quick/Wright-Giemsa (Dade Behring, Newark, DE, USA).

Nonsexed frozen semen (20×10^6 sperm/straw) from 8 and 11 sires from several AI companies and of proven fertility (high estimated relative conception rate [ERCR]) were used in experiments 1 and 2, respectively. In experiment 3, all breeding were performed with nonsexed frozen semen (20×10^6 sperm/straw) from two Holstein sires with high genetic merit, proven outstanding field fertility (sire conception rate [SCR] USDA scores $\geq$ 1.5), and produced from a single ejaculate/sire. Sperm motility (sire 1 = 53% and sire 2 = 56% at 0 hour) was performed objectively by a computer-assisted semen analyzer (CEROS, Hamilton Thorne). Sperm abnormalities (sire 1 = 9% and sire 2 = 14%) and intact acrosome (sire 1 = 61% and sire 2 = 56%) were evaluated under differential interference contrast optics (× 600).

2.2. Animals and treatment protocol

All procedures were approved by the Animal Care Committee of the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

Experiment 1—Seventeen nulliparous Holstein heifers (12–16 months old) were housed at the Dairy Cattle Research Center (UW-Madison, WI, USA) in loose housing with headlocks. Heifers were fed *ad libitum* a total mixed ration (TMR) once a day consisting of corn silage, alfalfa silage, and grass hay, supplemented with vitamins and minerals, and balanced to meet or exceed the minimum requirements. For superstimulation, heifers on Day 7 of a cycle received two $PGF_{2\alpha}$ treatments, 12 hours apart, and a GnRH treatment at 24 hours after the first $PGF_{2\alpha}$ treatment to synchronize ovulation. A CIDR device was inserted 6 days after GnRH, and 2 days later, 10 decreasing doses of FSH were given at 12-hours intervals for 5 days with a total equivalent of 150 or 300 mg NIH-FSH-P1 (half of the animals in each group were treated with either the higher or lower dose of FSH). Treatment with $PGF_{2\alpha}$ was done at the seventh FSH treatment and the CIDR was removed at the time of the ninth FSH treatment. Twelve hours after the last FSH, ovulation was induced with GnRH, and 18 hours after GnRH, one dose of semen was deposited in the body of the uterus or in the greater curvature of uterine horns (half dose in each horn). All heifers were inseminated, and embryos were recovered by the same technician (R. Sartori).

Seven days after GnRH, heifers had ova/embryos recovered using a nonsurgical shallow uterine horn technique as described by Sartori et al. [31], and each uterine horn was flushed separately with 1 L of embryo recovery medium. Structures were collected into an embryo filter, immediately washed into a petri dish, searched under a stereo microscope, and classified for quality (1 = excellent, 2 = good, 3 = fair, 4 = poor, and 5 = degenerate) as previously described [32,33]. Embryos graded as 1, 2, and 3 were defined as viable embryos, whereas only grades 1 and 2 were classified as freezable embryos. All embryo searching and grading procedures were performed by a single treatment-blind technician.

Experiment 2—Fourteen lactating Holstein cows (>40 DIM) were housed at the Dairy Cattle Research Center (UW-Madison, WI, USA) in individual tie-stalls. Cows were fed individually *ad libitum* a TMR once a day consisting of corn silage (9.98 kg DM/day), alfalfa silage (5.44 kg DM/day), and grass hay (0.9 kg DM/day) as forage, and roasted whole soybean (2.13 kg DM/day), high moisture corn (4.54 kg DM/day), canola meal (1.0 kg DM/day) as concentrated, supplemented with vitamins and minerals, and balanced to meet or exceed minimum requirements for lactating dairy cows (CNCPS-6.1).

Cows were superstimulated twice 28 days apart. For superstimulation, cows were presynchronized with an Ovsynch protocol (Day 0, GnRH; Day 7, $PGF_{2\alpha}$; Day 9.5, GnRH). Thirty-six hours after the last GnRH of the presynchronization period, a CIDR device was inserted, and 10 decreasing doses of FSH were given at 12-hours intervals for 5 days with a total equivalent of 300 mg NIH-FSH-P1. $PGF_{2\alpha}$ was administered at the ninth and tenth FSH treatments, and CIDR was removed at the time of the ninth FSH. Twelve hours after last FSH, ovulation was induced with GnRH, and 18 hours after GnRH, one dose of semen was deposited in the body of the uterus or in the tip of uterine horns (half dose in each horn, deposited after the greater curvature at the cranial end of the uterine horns) in a crossover design. Therefore, each cow was inseminated with deep AI at one replicate and in the uterine body at the other replicate. For each cow, the same sire was used in both replicates. All cows were inseminated and embryos were recovered by the same technician (R. Sartori). Seven days after GnRH, heifers had embryos and graded as described for experiment 1. Immediately after embryo recovery and 12 hours later, the cows were treated with $PGF_{2\alpha}$. Six days after the first $PGF_{2\alpha}$, Ovsynch was performed and a second superstimulation was done similar to the first replicate.

In experiments 1 and 2, ovarian ultrasound evaluations (Aloka 500-V; Corometrics Medical Systems Inc., Wallingford, CT, USA, equipped with a 7.5-MHz linear array transducer) were performed at the time of embryo recovery to estimate CL number.

Experiment 3—Seventy-two lactating Holstein cows (primiparous = 28 and multiparous = 44) were housed in a single farm at US Dairy Forage Research Center (WI, USA) in individual tie-stalls from calving to 70 ± 3 DIM and fed similarly to what was described for experiment 2. Cows were superstimulated with a modified 5d-double Ovsynch protocol, as follows (Fig. 1): 50 ± 3 DIM cows were presynchronized with an Ovsynch protocol with the addition of a CIDR between first GnRH and $PGF_{2\alpha}$ (Day 0, GnRH plus CIDR insert; Day 7, $PGF_{2\alpha}$ and CIDR removal; Day 9, GnRH). Seven days after the last GnRH of the presynchronization period, all cows had follicles >5 mm ablated by ultrasound-guided aspiration (Aloka 900 equipped with a 13-MHz convex array transducer) as previously described [34]. Approximately 36 hours after follicle ablation, a new CIDR was inserted and eight decreasing FSH treatments were performed at 12-hours intervals. Treatment with $PGF_{2\alpha}$ was done at the fifth and seventh FSH treatments, and CIDR was removed at the time of the last FSH. Twenty-four hours after the last FSH and CIDR removal, ovulation was induced with GnRH (Fig. 1).

All cows were inseminated and embryos were recovered by one of the two technicians (P.D. Carvalho or A.H. Souza). Cows were blocked by parity and calving date and randomly assigned to one of the two treatments, deposition of semen into the uterine body or deposition of semen at the greater curvature of the uterine horn, similar to experiment 1. Cows were inseminated twice at 12 and 24 hours after GnRH with semen from the same sire and by the same technician, both equally distributed between treatments. Seven days after GnRH, cows had embryos recovered using the same technique as previously described. Structures were collected into an embryo filter, immediately washed into a petri dish, searched under a stereo microscope, and classified for quality according to International Embryo Transfer Society standards [35].

Blood samples (~8 mL) were collected into vacutainer collection tubes containing K3 EDTA by puncture of the coccygeal vein or artery before the administration of each GnRH injection (G1 and G2) of the presynchronization and

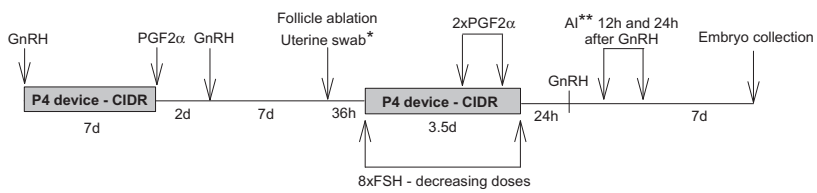

Fig. 1. Experimental design for experiment 3. Intervals between treatments are not scaled for time. Single asterisk indicates uterine swab sample collected to count proportion of cells with PMN. Double asterisks indicate that during AI, semen was deposited in the uterine body or equally split between horns and deposited in the greatest curvature.

just before follicular ablation to evaluate if cows responded to the protocol. Blood samples were kept refrigerated after collection and centrifuged within 20 minutes after collection at 3000 × *g* for 20 minutes. Plasma samples were stored at −20 °C until assayed for progesterone using an antibody-coated RIA kit as described by Garbarino et al. [36]. The sensitivity and coefficient of variation for the assay were 0.04 ng/mL and 2.2%, respectively.

Endometrial cytology samples were collected immediately before follicular ablation using a disposable cytobrush connected to the tip of a regular AI applicator. The coupled AI applicator and brush were then covered with an AI sheath to prevent contact with the vagina and cervix. When inside the uterus, the brush was pushed forward, gently rolled three times clockwise, pulled back into the AI sheath, and removed from the uterus. The brush was then rolled over a microscope slide. Endometrial smears were air-dried and transported to the laboratory for staining [37]. A single treatment-blind technician evaluated all the slides by counting a minimum of 100 cells at × 400 magnification and determined the number and percentage of polymorphonuclear cells (PMNs) in the endometrial smear. Subclinical endometritis was considered positive for cows with >5% PMN.

2.3. Definitions and statistical analysis

Animals with three or more CL at the time of embryo collection assessed by ultrasound were considered to have responded to the superstimulation. Because we wanted to test our hypothesis in superovulated cattle, heifers or cows with less than three CL on the day of the embryo recovery (experiment 1 = two heifers; experiment 2 = four cows; experiment 3 = one cow) were excluded from further analyses. The percentage of fertilized structures was calculated by dividing the total number of cleaved structures by the total number of structures. The percentage of freezable embryos was calculated by dividing the total number of embryos grade 1 and 2 by the total number of structures. The percentage of transferable embryos was calculated by dividing the total number of embryos grade 1, 2, and 3 by the total number of structures. The percentage of degenerated embryos was calculated by dividing the total number of degenerated embryos by the total number of structures. The percentage of degenerated embryos of the total fertilized embryos was calculated by dividing the number of degenerated embryos by the total number of fertilized structures. All values are expressed as least square means ± SEM. All statistical analyses were performed using SAS computational software, version 9.3, of the SAS system for windows [38]. The GLIMMIX procedure was used to compare embryo characteristics using heifer or cow as the experimental unit. The final logistic regression models for each trial were as follows: experiment 1, type of AI with heifer as a random effect; experiment 2, type of AI, replicate, and one-way interaction between type of AI and replicate as fixed effects, with cow within replicate as a random effect in a crossover model structure; experiment 3, type of AI and percentage of PMN (for ova/embryo recovery rate and fertilization rate models only) as well as the one-way interaction was used in the model, with cow used as a random effect. The univariate logistic regression analysis to estimate the effect of percentage PMN on ova/embryo recovery efficiency and fertilization rate was performed with the interactive data analysis feature of SAS. Statistical differences were considered significant for $P < 0.05$ and as a tendency for $P < 0.15$.

3. Results

3.1. Experiment 1—Horn AI in virgin dairy heifers

As expected, there was no effect of AI method on the number of ovulations, the number of structures recovered, and the percentage of structures that were recovered (Table 1). However, there was a greater number ($P = 0.04$) and percentage ($P = 0.02$) of structures that were recovered from the heifers in which the semen was deposited into the uterine horns compared with heifers in which the semen was deposited into the uterine body. Other embryo measures did not differ between the two types of AI (Table 1).

3.2. Experiment 2—Deep-horn AI in lactating dairy cows

As shown in Table 1, deep-horn AI did not alter the number of ovulations, the number of structures recovered, the percentage of structures recovered, and the number or percentage of fertilized structures. In addition, the embryo measures were not improved by deep-horn AI compared with body AI. There was a tendency for body AI to improve the percentage of transferable embryos of total structures ($P = 0.07$) and the percentage freezable embryos of total structures ($P = 0.10$) compared with deep-horn AI (Table 1).

3.3. Experiment 3—Horn AI in lactating dairy cows

As in most superovulatory experiments, there was substantial variation in the superstimulatory response among cows, ranging from 2 to 40, although the average number of ovulations was very similar between the two treatments. There was no difference in the number of oocytes/embryos collected or the percentage of embryos collected (Table 2). There was also no difference in the number ($P = 0.43$) or percentage ($P = 0.98$) of structures that were fertilized. We evaluated the effect of the number of ovulations on percentage fertilization (Fig. 2) and found no effect in either cows inseminated in the body or uterine horns. There were no differences between breeding strategy used in terms of number or percentage of transferable embryos expressed either as a percentage of total structures or when considering only fertilized structures (Table 2).

The proportion of cows with subclinical endometritis at the beginning of the superstimulation protocol did not differ between treatments. In addition, the percentage of cows with 0%, 0%–5%, 5%–10%, and >10% PMN was similar ($P > 0.15$) for cows in which semen was deposited in the uterine body or uterine horns (58.1% vs. 64.5%, 25.6% vs. 16.1%, 6.5% vs. 6.5%, 9.7% vs. 12.9%, respectively). The logistic regression model (Fig. 3) indicated a tendency for

Table 1
Effect of site of semen deposition[a] on embryo characteristics (LSM ± SEM) of Holstein heifers (experiment 1) and lactating Holstein cows (experiment 2).

Endpoint	Experiment 1 (heifers: horn AI)		P-value	Experiment 2 (cows: deep-horn AI)		P-value
	Body AI ($n = 8$)	Horn AI[a] ($n = 9$)		Body AI ($n = 14$)	Deep-horn AI[b] ($n = 14$)	
CL number	11.3 ± 2.5	15.4 ± 3.5	0.97	19.1 ± 3.7	17.7 ± 3.4	0.78
Total ova/embryos recovered	4.9 ± 1.4	8.4 ± 2.5	0.60	8.5 ± 1.9	10.7 ± 2.3	0.47
% Recovery	43.1 ± 9.2	51.4 ± 11.2	0.27	49.1 ± 6.5	62.5 ± 6.5	0.16
Fertilized structures	1.8 ± 0.8	6.6 ± 1.9	0.04	4.7 ± 1.2	3.5 ± 0.9	0.42
% Fertilized structures	40.0 ± 16.6	78.9 ± 5.8	0.02	60.7 ± 10.2	40.0 ± 9.8	0.15
Transferable[c] embryos	1.5 ± 0.7	3.8 ± 1.5	0.13	2.5 ± 0.8	1.2 ± 0.4	0.16
% Transferable/total	35.9 ± 17.3	50.9 ± 12.5	0.29	40.6 ± 8.3	18.6 ± 8.0	0.07
% Transferable/fertilized	76.6 ± 19.4	58.1 ± 11.2	0.34	55.7 ± 9.9	39.4 ± 10.3	0.27
Freezable[d] embryos	1.3 ± 0.7	2.9 ± 1.5	0.41	2.3 ± 0.8	1.2 ± 0.4	0.21
% Freezable/total	33.0 ± 17.8	42.1 ± 14.2	0.40	37.9 ± 8.0	18.6 ± 7.8	0.10
% Freezable/fertilized	53.4 ± 22.6	46.9 ± 13.9	0.60	51.0 ± 9.7	39.4 ± 10.0	0.42

[a] Horn AI performed in the greatest curvature of the uterine horn and semen straw split between horns during AI.
[b] Deep-horn AI was performed deeper in the uterus closer to the cranial end of the uterine horns, and semen straw was evenly divided between the two horns.
[c] Transferable embryos defined as quality 1 (excellent) to 3 (fair) according to Ref. 32,33.
[d] Freezable embryos defined as quality 1 (excellent) and 2 (good).

reduced percentage of structures that were recovered in cows with subclinical endometritis (P = 0.09). In addition, fertilization rates were reduced (P = 0.01) in cows with subclinical endometritis (Fig. 3) with percentage fertilization decreasing from above 80% in cows with no detectable PMN to less than 50% fertilization in cows with over 50% PMN detected in the uterine cytology. Table 3 shows the changes in various embryo measurements in cows with few (<1%), some (1%–5%), and many (>5%) uterine PMNs. The CL number were not different for the three groups; however, the percentage of ova/embryos recovered was lower in cows with a greater number of PMNs. Cows with greater PMNs also had a reduction in the number of fertilized structures (P < 0.01), transferable embryos (P = 0.03), and freezable embryos (P = 0.04). The total number of ova/embryos recovered (P = 0.10) and % fertilization (P = 0.14) tended to be reduced in this analysis. Although all cows were on the same day of the estrous cycle when uterine cytology samples were collected, there was some variation in circulating P4 concentrations among cows; however, the P4 concentrations did not influence the percentage of PMN found in the uterine smears (P > 0.15).

Table 2
Effect of site of semen deposition[a] on embryo characteristics (LSM ± SEM) of lactating Holstein cows in experiment 3.

Endpoint	Body AI ($n = 35$)	Horn[a] AI ($n = 37$)	P-value
CL number	16.4 ± 1.3	16.9 ± 1.3	0.81
Total ova/embryos recovered	5.8 ± 0.9	6.7 ± 1.0	0.48
% Recovery	39.7 ± 4.9	44.8 ± 4.9	0.47
Fertilized structures	4.2 ± 0.7	5.0 ± 0.8	0.43
% Fertilized structures	78.2 ± 4.7	78.4 ± 4.8	0.98
Transferable embryos	2.9 ± 0.6	4.0 ± 0.7	0.23
% Transferable/total	55.7 ± 6.1	62.0 ± 5.8	0.47
% Transferable/fertilized	67.2 ± 5.9	77.7 ± 5.7	0.21
Freezable embryos	2.7 ± 0.5	3.6 ± 0.7	0.33
% Freezable/total	53.6 ± 6.2	56.8 ± 6.0	0.71
% Freezable/fertilized	64.8 ± 6.1	71.3 ± 5.9	0.45

[a] Horn AI performed in the greater curvature of the uterine horn and semen straw was split between horns during both AIs that occurred 12 and 24 hours after the GnRH treatment used to synchronize ovulations.

4. Discussion

The primary hypothesis for these studies was that deposition of semen into the uterine horns would increase the fertilization rate in superovulated heifers and cows compared with deposition of semen into the uterine body. This hypothesis seemed reasonable given that fertilization rates are generally suboptimal in superovulated cows and the number of accessory sperm in zona pellucida is particularly low in oocytes/embryos from superovulated cows [17]. This is also consistent with the idea that sperm transport to the oviducts may be limiting fertilization in superovulated cows [18,23]. Although our first small experiment in heifers provided some evidence in favor of this hypothesis, the second experiment using a more statistically powerful crossover design did not support this

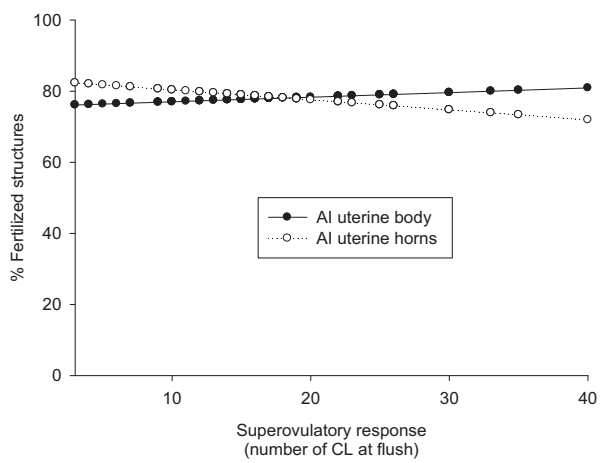

Fig. 2. Fertilization rate according to type of AI (uterine body vs. uterine horns) and superovulatory response—experiment 3. There was no effect (P > 0.30) of superovulatory response on the percentage of oocytes that were fertilized in either treatment.

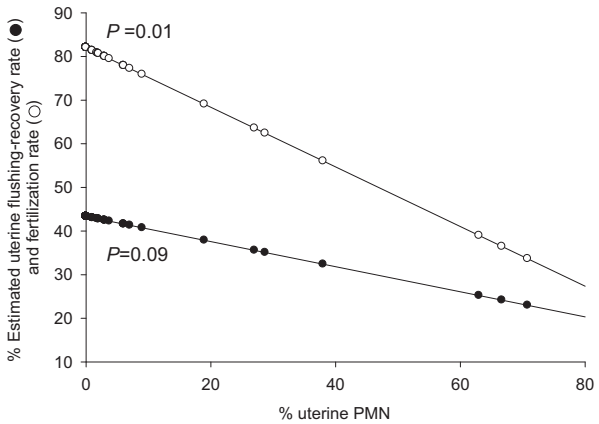

Fig. 3. Estimated effect of uterine polymorphonuclear cell count (% PMNs, endometrial swab) at the beginning of the superstimulation on ova/embryo recovery rate (total structures collected divided by number of CL on the day of the collection) and fertilization rate (total number of fertilized structures divided by total structures collected)—experiment 3.

hypothesis, nor did the third experiment support this hypothesis utilizing a larger number of lactating cows. The last two experiments were statistically powerful for testing the hypothesis because we evaluated 269 oocytes/embryos in experiment 2 and 450 oocytes/embryos in experiment 3. Thus, our hypothesis that fertilization efficiency would be increased by placement of semen in the uterine horns compared with uterine body was not supported in superovulated lactating cows but was marginally supported in heifers, although the small numbers of heifers in experiment 1 make it imperative that this experiment be repeated before it is widely applied in commercial operations. Our second hypothesis that uterine PMN would reduce the fertilization rate was supported by our results. Interestingly, to our knowledge, this is the first report indicating a negative effect of PMNs during superovulation.

Several studies have evaluated the effect of location of semen placement within the uterine tract of superstimulated cows on fertilization and embryo quality. In one study, semen was deposited in only one of the uterine horns of superstimulated cows and the fertilization rate was reduced ($P < 0.05$) for oocytes in the uterine horn contralateral (58%) to the horn in which the semen was deposited compared with ipsilateral (74%) to the horn in which the semen was deposited [39]. In a similar study [40], the fertilization rate was greater in the ipsilateral (87%) than contralateral (70%) uterine horn, with intermediate fertilization (74%) for cows inseminated in the uterine body [40]. In our study, cows were inseminated with half of the semen placed into each uterine horn to overcome the problem with the contralateral decrease in fertilization. One study [21] reported no difference in fertilization percentage when superstimulated cows were inseminated into each of the uterine horns near the uterotubal junction, compared with AI in the uterine body. Although confounded by the number of inseminations and time of AI, superstimulated cows receiving a single deep-horn AI (15 hours after onset of estrus) had a similar fertilization rate (74%) compared with cows receiving three AI (8, 20, and 32 hours) in the uterine body (80%) [41]. Thus, based on these results in superstimulated cows, although there may be some advantage to AI in both uterine horns, when compared with only a single uterine horn, there does not seem to be strong evidence for an advantage of AI in the uterine horns compared with the uterine body in superstimulated cows.

Numerous studies using single-ovulating cows have compared fertility based on semen deposition into the uterine body compared with the uterine horns. In an elegant study using competition between marked and unmarked sperm, deep uterine horn AI improved accessibility of sperm to the ovum, based on numbers of accessory sperm [42]. Thus, deposition of semen deep into the uterine horn may improve fertilization and subsequent pregnancy success compared with deposition of semen into the uterine body. Studies of single-ovulating cows have reported no differences in percentage fertilization based on semen placement; however, the number of evaluated oocytes were low in these studies [39,42]. Results on pregnancy success with different locations of semen placement have been variable and sometimes difficult to interpret. Diskin et al. [7] in a randomized study including ~3500 inseminations observed that pregnancy per AI was improved by depositing semen into the uterine horn versus uterine body when AI was performed on farms and/or by technicians that were achieving low results. Similarly, other researchers reported improvements from 8% to 20% in fertility for cows receiving AI into the uterine horns compared with uterine body [12,14–16,43]. In contrast, Marshall et al. [9] in a study with ~1800 inseminations and Williams et al. [11] with ~2100 inseminations observed no improvements in pregnancy per AI for cows in which semen was deposited into the uterine horns compared with the uterine body. However, fertility was lower for cows in which the semen was deposited into the cervix [11]. Similarly, other authors using low doses of semen [10,44] or frozen sex-sorted semen [8,13,45] observed similar fertility level after depositing semen either into the uterine body or uterine horn.

Interestingly, Senger et al. [43] evaluated the ability of different technicians to properly deposit semen into the uterine body or the uterine horns. Immediately after intensive training, almost all technicians correctly deposited semen when trained in uterine body deposition (95%) or uterine horn deposition (96%). However 6 months after training, AI accuracy after uterine body training had decreased to 75%, whereas accuracy remained high in technicians trained in uterine horn AI. In this regard, the present study was performed by experienced technicians, and passage of the AI gun through the cervix was rechecked in all cows by palpation of the tip of the AI gun before insemination. In addition, sires used in the present study were selected for high fertility, based on SCR scores published by the USDA-AIPL, and in experiment 3, two AIs were done in all superstimulated cows. These factors may reduce any advantage of AI into the uterine horn. Thus, although there may be some physiological advantages to AI in the uterine horn in single-ovulating or superstimulated cows, it seems possible that the improvements in fertility observed in some studies may be primarily a technical advantage related to uterine horn AI reducing the incidence of cervical AI.

Table 3
Effect of uterine PMNs on ova/embryo recovery, fertilization, and number of transferable/freezable embryos.

Endpoint	PMN <1% ($n = 40$)	PMN 1%–5% ($n = 13$)	PMN >5% ($n = 12$)	P-value
CL number	17.7 ± 1.4	15.8 ± 2.3	17.2 ± 1.7	0.84
Total ova/embryos recovered	7.8 ± 1.1	9.2 ± 2.4	4.7 ± 1.1	0.10
% Recovery	41.5 ± 4.3[a,b]	55.5 ± 8.5[a]	28.4 ± 6.3[b]	0.04
Fertilized structures	5.9 ± 7.7[a]	7.4 ± 1.9[a]	2.3 ± 0.7[b]	<0.01
% Fertilized structures	82.3 ± 3.4	81.8 ± 8.8	62.1 ± 11.4	0.14
Transferable embryos	4.6 ± 0.7[a]	5.9 ± 1.7[a]	1.8 ± 0.6[b]	0.03
% Transferable/total	62.3 ± 5.5	61.3 ± 11.6	52.0 ± 12.5	0.80
% Transferable/fertilized	74.9 ± 5.4	71.3 ± 11.2	72.5 ± 12.7	0.95
Freezable embryos	4.4 ± 0.7[a]	5.3 ± 1.6[a]	1.8 ± 0.6[b]	0.04
% Freezable/total	59.4 ± 5.4	56.3 ± 12.2	48.9 ± 12.5	0.85
% Freezable/fertilized	71.4 ± 5.3	65.4 ± 12.0	69.1 ± 13.3	0.99

[a,b] Means within a row with different superscripts differ ($P < 0.05$).

There are a number of other factors that can alter the fertilization rate in superovulated cows. For example, the number of ovulations may alter fertilization by altering the oocyte or uterine environment to make it less compatible with fertilization. Hawk [18] reported inconsistent effects of number of ovulations on transport of gametes or fertilization rate in superovulated cows, with one study reporting a decrease in fertilization rate with increasing ovulations and two studies reporting no effect of number of ovulations on fertilization rate. Consistent with our results in this study in which the number of ovulations did not alter fertilization percentage, we have two other recent studies (Wiltbank et al., unpublished data) in which we observed no effect of the number of ovulations on percentage fertilization. Thus, our results do not support the idea that fertilization rate decreases as the number of ovulations increases in superovulated cows.

Uterine environment near the time of ovulation may have substantial effects on fertilization of the oocyte. Measurement of PMNs by uterine cytology has been used to diagnose subclinical endometritis and has been associated with reduced reproductive efficiency [46]. Our results provide direct support for the idea that increased uterine PMN can reduce fertilization. As the numbers of uterine PMNs increased, the percentage of oocytes that were fertilized decreased. Studies using IVF are consistent with our results. It is well known that PMN can produce reactive oxygen species [47] and these may be damaging to sperm. Exposure of sperm to compounds that increase reactive oxygen species (menadione or *tert*-butyl hedroperoxide) decreased fertilization and decreased the percentage of oocytes or cleaved embryos that developed to blastocyst, *in vitro* [48]. In our superovulation study, although the number of ovulations was not decreased in cows with greater number of uterine PMNs, there was a major decrease in fertilized oocytes and good quality embryos (transferable or freezable). This problem may have reduced the superovulatory results in the past but has previously gone undetected. In addition to the effects of uterine PMN on sperm, increased uterine infection can perturb the normal ovarian activity and function, possibly leading to ovulation of oocytes of suboptimal fertility [49,50]. Thus, our results support the idea that subclinical endometritis decreases fertilization rate; however, we cannot distinguish whether reduced fertilization and the number of embryos collected, associated with high uterine PMN, are due to altered function of sperm and/or oocytes. From a practical perspective, the large reduction in the recovery of transferable or freezable embryos in the cows that had >5% PMN at the time of follicular ablation may be used in the future to screen cows for subclinical endometritis, improving embryo production during superovulation protocols.

In conclusion, deposition of semen into the uterine horns rather than the uterine body did not increase fertilization of oocytes or embryo quality in superovulated cows. However, there was a substantial reduction in embryo yield in cows with increased uterine PMNs before initiation of the superstimulation protocol. This suggests that screening for uterine PMNs may be useful to improve embryo yield during superovulation protocols and that increased PMN in superovulated cows may provide a useful model for understanding and treating subclinical endometritis.

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