Anatomical location of bovine embryos following superstimulation in beef heifers

Caitlin Wiley,[a] Marianna Jahnke,[a] Patrick J. Gunn,[b] Tyler Dohlman[a]
[a]Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA; [b]Department of Animal Science, Iowa State University, Ames, IA

Abstract

Since the advent of non-surgical embryo recoveries in cattle, there has always been uncertainty regarding the location of embryos at the time of recovery. Despite thousands of embryo recoveries performed annually, there is limited research describing embryo location 6-7 d after ovulation in cattle subjected to a superstimulation protocol. The objective was to determine location of embryos within the reproductive tract of superstimulated heifers. Non-pregnant, yearling beef heifers (n = 12; 577 ± 31 kg) were allocated into three replicates and began a timed, 17-d, superovulation controlled internal drug release-based protocol, with artificial insemination done twice. At 6.5 d after first artificial insemination, heifers were slaughtered at a commercial abattoir. Immediately after exsanguination, the reproductive tract was excised and each side sectioned into 4 segments (Section 1 for the uterine tube and Sections 2 to 4 for the uterine horn, with Section 4 including the uterine body). Each section was isolated, flushed, and flush media filtered and embryos identified and enumerated. Corpora lutea were also enumerated. There were differences among uterine sections in number of embryos recovered ($p < 0.0001$), with embryos recovered from all sections. Section 2 (tip of horn) contained the greatest number and percentage of embryos, followed by Sections 3, then 4 and finally 1 (53.5, 33.9, 6.8 and 5.8%, respectively). In summary, embryos were located throughout the tract, with the majority located in the tip and middle third of the uterine horn.

Keywords: Embryo, superovulation, migration, uterine horn

Introduction

Since the 1970s, the embryo transfer (ET) industry has been striving to improve embryo recovery rates in superovulated donors. Evidence-based improvements have been made in superovulation protocols and recovery techniques. Initiating ovarian stimulation concurrent with follicular wave emergence drastically improved embryo yield.[1-3] With regard to recovery technique, growing interest to transition recovery and transfer techniques from surgical to non-surgical occurred in the late 1970s. As non-surgical embryo recoveries were becoming more feasible, Newcomb et al. used a surgical approach to determine embryo migration in the uterus at d 3-7 after insemination. They reported that more embryos were present in the tip of uterine horn compared to the base of the uterus.[4] Therefore, it was suggested that non-surgical recoveries should emphasize flushing the tips of the horns. Additionally, adoption of non-surgical embryo recovery enabled ET to be performed at a producer's operation, which led to substantial growth in the industry.[5]

Currently with conventional ET, non-surgical embryo recoveries are done ~6-7 d after ovulation. Following fertilization in the uterine tube, embryonic development from zygote to 16-cell embryos occurs over ~4 d in the uterine tube.[6,7] Migration into the uterus occurs at ~Day 5, when the embryo has further developed and undergone compaction to form a morula.[8] With this time interval to recovery, most embryos are at a compact and mature developmental stage.[9, 10]

There are 2 general approaches for catheter placement within the uterus for non-surgical embryo recovery. One approach involves placing the catheter just anterior to the internal cervical os and flushing both horns and body simultaneously, known as a "body flush".[11] As a large volume of flushing medium is required for adequate recovery, this approach is best suited to donors with smaller uteri (e.g. heifers). The other approach, "horn flushing," involves placing the catheter in each horn (separately) and advancing it cranially, at least to the external bifurcation or as far cranially as possible.[12] Although the catheter must be placed more cranially then for a body flush, a smaller volume of flush medium can be used. However, without knowledge of embryo location at this stage of development, there is the potential for embryos to be left in the uterus if catheter placement is too far cranial.

The objective of this study was to determine the location of embryos within the uterus of superovulated heifers at the time of embryo recovery. It was hypothesized that the majority of embryos are located towards the tips of the uterine horns.

Materials and methods

General

All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee. The project was conducted at the Iowa State University Zumwalt Station Research Center in Ames, IA, in January 2016 – February 2016, with heifer slaughter and recovery of reproductive tracts occurring at Amend Packing Company, Des Moines, IA. Single-sourced heifers from the Iowa State University McNay Beef Research Farm were used.

Animals and methods

Non-pregnant, purebred Angus yearling beef heifers (n = 12; 577 ± 31 kg) were used. Prior to treatment, all heifers were subjected to a transrectal reproductive ultrasound examination to confirm that there were no apparent reproductive abnormalities and that the heifers were cycling. Heifers were assigned to 1 of 3 replicates based on weight and were penned in these replicate groups for the duration of the study. Initiation of superovulation protocols for each replicate were started at 1-wk intervals. All heifers were subjected to superstimulation by utilizing a timed, 17-d, controlled internal drug relase (CIDR; EAZI-BREED™, Zoetis Inc., Kalamazoo, MI)-based protocol with gonadotropin releasing hormone (GnRH; Cystorelin®, Merial LLC, Duluth, GA) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse®, Kalamazoo, MI) with a decreasing total dosage of 240 mg follicle stimulating hormone (FSH; Folltropin-V®, Vetoquinol, Lavaltrie, Québec, Canada) administered twice daily for 4 d (Table 1). Heifers were artificially inseminated (AI) following a timed-AI schedule in conjunction with observed signs of standing estrus, with a second AI done 12 h later by the same technician. At each AI, 1 unit of frozen semen from the same ejaculate of 1 bull was used, with GnRH (150 µg) given IM at first insemination. At 6 d after the initial AI, transrectal ultrasonography was done to estimate the number of CLs on each ovary. Approximately 12 h later, heifers were taken to a commercial abattoir where they were stunned via captive bolt and exanguinated. Reproductive tracts were excised within 15 min after slaughter. Initial processing was performed at the abattoir. Within the next 30 min, the length of each tract was measured in its natural state and each horn was sectioned into even thirds, with the uterine body split between the caudal third of each horn (Figure). Zip ties were placed at the edges of these sections and the uterus was cut distal to the zip tie while being held over open Ziplock bags, for a total of 4 sections per side (total of 8 sections per heifer). Once in individual Ziplock bags and labeled accordingly, commercial flush medium (Vigro™ Complete Flush; Bioniche, Pullman, WA) was added to preserve embryos and tissue for transportation to the laborabory. Ovaries were excised and placed in individual labelled bags. Samples were transported in a cooler at room temperature (approximately 60-min drive).

Embryo recovery

At the laboratory, each section of uterus was opened with mayo scissors in its own bag and with a catheter tip syringe, approximately 60 mL flushing medium was used to lavage the tissue, scissors, and bag that held the tissues. Uterine tubes were flushed, starting at the infundibulum, with 5 mL of flushing medium, followed by air, into a bag. All media from each section was then filtered through EmCon™ filters (MAI Animal Health). Each filter from each section was thoroughly rinsed into labeled square-gridded search dishes and searched for embryos using a stereomicroscope. All recovered embryos were evaluated according to International Embryo Technology Society standards by 2 persons (both were certified by the American Embryo Transfer Association). Ovaries were evaluated for number of (corpora lutea) CLs and anovulatory follicles to determine recovery rate. To improve accuracy, structures were marked with pins, and then dissected with a scalpel for final enumeration.

Statistical analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) using the MIXED procedure, with heifer serving as the experimental unit and section as the fixed main effect. Animal within replicate (group) served as the random effect. Both section length and total number of CLs were initially included as covariates, but section length was subsequently removed due to a lack of significance ($p = 0.70$). Statistical significance was designated as $p \leq 0.05$.

Results

In total, embryos were present in all sections, including the uterine tubes. For combined total recovery from all sections, there was a difference among sections in location of embryos ($p < 0.001$; Table 2). In terms of total proportion of embryos recovered, there was a difference between Section 2 (tip of uterine horn) and Section 3 (middle third of uterus; $p = 0.05$; Figure). However, both of these sections had more embryos than either Section 1 or Section 4 (uterine tube and caudal uterine horn and body, resepctively; $p < 0.05$). There was no difference between the percentage of embryos in Section 1 versus Section 4 ($p = 0.90$). There was a difference between embryo counts from Section 1 compared to Section 2, and Section 1 compared to Section 3 ($p = 0.0005$, $p = 0.006$; respectively). Furthermore, Sections 2 and 4 and Sections 3 and 4 also differed ($p = 0.002$, $p = 0.017$; respectively). When total number of embryos recovered from each of the sections were compared, there were no differences between Sections 2 and 3 ($p= 0.37$), nor between Sections 1 and 4 ($p = 0.65$). However, there was a difference between the combined totals from Sections 2 and 3 relative to that from Sections 1 and 4 ($p < 0.05$).

As total number of CLs increased, so did number of embryos recovered ($p < 0.01$). Based on CL counts and total embryos recovered per uterus, the average recovery rate was 49% (range, 0 to 92%).

Discussion

General

The objective was to determine location of embryos in the reproductive tract of superovulated heifers at the usual time of recovery. Although research has focused on the environment of the uterine tube and uterus during embryo formation and development,[13-15] little is known about embryo location after entering the uterus relative to time of embryo recovery for multiple ovulation embryo transfer. Therefore, heifers were slaughtered when non-surgical recovery would have been done (Day 6.5 following insemination). Based on previous work using slaughtered cows, the reproductive tract was excised as soon as possible after exsanguination, to mitigate negative effects of declining pH following death.[16] After the tract was excised, measurements and sectioning were immediately performed to decrease potential post-mortem movement of embryos. Although the recovery rate was not 100 percent even with *ex vivo* recovery, it was assumed that not all ova were captured in the infundibulum, due to the degree of ovarian stimulation. These results are relevant for embryo transfer operators conducting non-surgical embryo recoveries.

Embryo quality

Stages and grades of embryos were not described, due to poor embryo quality. Despite pre-breeding semen evaluation, observation of standing estrus, and an experienced AI technician, resulting embryos were poor. Poor embryo quality of abattoir-collected embryos has been reported[16] and attributed to a drastic decrease in intrauterine pH after slaughter due to a change from aerobic to anaerobic metabolism. However, in another study involving embryo recovery from slaughtered cows on Day 4 after estrus and subsequently placed in culture medium for further growth *in vitro*, there was no reference made to pH affecting embryo quality.[17] In the current study, efforts were made to process the tissues and recover embryos as soon as possible, to minimize potential post-mortem effects.

Embryo location

As the ET industry transitioned from surgical to non-surgical embryo recoveries, there were questions raised about the efficiency of embryo recovery.[4,11,12] In an experiment[4], embryos were recovered between Days 3 and 7 after estrus via a surgical approach, with flushing of uterine tubes conducted separate from uterine flushing. As the day of embryo collection following estrus increased, so did number of developmental Stage 4-7 embryos, as well as number of degenerate embryos present in the uterus. Interestingly, in a previous study,[4] there were several embryos in the uterine tube on Day 6, compared to Days 7-8. Similarly, in the present study, 5.8% of embryos were in the uterine tube on Day 6.5 after insemination and likely would not have been recovered by conventional flushing. If the interval from ovulation to recovery were prolonged, there is the potential for an increased proportion of embryos being recovered from the uterus. However, these embryos would likely be more mature, limiting their suitability for cryopreservation. Further investigation into the percentage of quality embryos still in the uterine tube at time of recovery may be of merit.

In previous work,[4] ligation of the uterus into 2 sections revealed a greater proportion of embryos in the cranial half of the uterine horn compared to the uterine tubes and base of the uterus. Although most embryos were in the uterine tip section, there were embryos in the caudal half of the uterus (uterine base section). It should be noted this previous experiment was all performed using a surgical flushing technique *in vivo.*[4] In the current study, the uterus was sectioned into thirds, plus the uterine tube, for a total of 4 sections per horn, 8 sections per tract, enabling more precise localization of embryos. Accounting for individual animal variation, the total length of each uterus was taken into consideration and each uterus was evenly sectioned into thirds from the utero-tubal junction to the internal cervical os. These variations in section lengths among heifers were evaluated, but did not impact migration of embryos at the time of recovery. Embryos were in the uterine tube and all 3 sections of the uterus, with the largest percentages in the tip and middle third (53.5 and 33.9%, respectively). Furthermore, embryos were also present in the base of the horns. Based on these results, placement of the catheter close to the tip of the horn would likely fail to recover embryos that have migrated closer to the uterine body. Therefore, if a horn flush is to be conducted, placing the catheter at the level of the uterine bifurcation should enable recovery of most embryos, although there is the potential for some to be in the uterine body. Whereas this study utilized nulliparous, yearling heifers, a similar study using mature, multiparous cows would be of value to compare embryo migration between the two reproductive statuses.

An ultrasonographic examination of the ovaries was done ante mortem to predict stimulatory response. These results were not included, because the ovaries were examined <12 h before slaughter. There can be a wide variation in accuracy of evaluating ovaries and counting number of CLs, especially with a donor who has a high response to stimulation, based on the ultrasonographer's skill and experience.

Ovarian overstimulation may have contributed to discrepancies between CL counts and embryo recovery. Especially in heifers, there can be physical limitations on ovaries with regard to the number of developing follicles that they can support and subsequently ovulate.[18] Repeating this study with a larger sample size, may yield more conclusive results. Additionally, even though CLs were accounted for, progesterone concentrations were not evaluated. Progesterone is known to cause relaxation of the smooth muscle of the uterine tube, which leads to a decreased rate of transport through the uterine tube.[19] However, with superovulated cows, whether or not progesterone concentrations impact migration of embryos through the uterine tube or uterus remains unknown.

Conclusion

At 6.5 d after insemination in superovulated heifers, embryos were located throughout the reproductive tract, although the majority localized towards the tip and middle third of the uterine horn. With regard to catheter placement for recovering embryos, the closer the catheter is towards the tip of the uterine horn, the greater the risk of missing embryos. Whether the catheter is placed within the uterine body or each horn, the focus of the recovery should be to distend the tip and middle third of the horn to improve recovery rates while potentially incorporating the body of the uterus to maximize recovery.

Further research is warranted to determine if the developmental stage and/or quality of the embryo impacts its migration and whether other factors such as the age of donor or perhaps progesterone concentrations during superstimulation influence embryo location at the time of recovery.

Acknowledgements

The authors thank Claire Andresen and Allie Lundberg for their help in processing samples and assisting in the laboratory.

Conflict of interest

None of the authors have any conflicts of interest to disclose.

References

1. Nasser LF, Adams GP, Bo GA, et al: Ovarian superstimulatory response relative to follicular wave emergence in heifers. Theriogenology 1993;40:713-724.
2. Baracaldo MI, Martinez MF, Adams GP, et al: Superovulatory response following transvaginal follicle ablation in cattle. Theriogenology 2000;53:1239-1250.
3. Bo GA, Adams GP, Pierson RA, et al: Effect of progestogen plus estradiol-17 beta treatment on superovulatory response in beef cattle. Theriogenology 1996;45:897-910.
4. Newcomb R, Rowson LEA, Trounson AO: The entry of superovulated eggs into the uterus. Luxembourg, Commission of the European Communities, 1976:1-13.
5. Hasler JF: Current status and potential of embryo transfer and reproductive technology in dairy cattle. J Dairy Sci 1992;75:2857-2879.
6. Barnes FL, Eyestone WH: Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 1990;33:141-152.
7. Betteridge KJ, Flechon JE: The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 1988;29:155-187.
8. Dorniak P, Bazer FW, Spencer TE: Physiology and endocrinology symposium: Biological role of interferon tau in endometrial function and conceptus elongation. J Anim Sci 2013;91:1627-1638.
9. Lawson RAS, Rowson LEA, Moor RM, et al: Experiments on egg transfer in the cow and ewe: dependence of conception rate on the transfer procedure and stage of the oestrous cycle. J Reprod Fertil 1975;45:101-107.
10. Trounson AO, Willadsen SM, Rowson LEA: Storage of cow eggs at room temperature and low temperatures. J Reprod Fertil 1976;46:173-178.
11. Hasler JF: Forty years of embryo transfer in cattle: A review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. Theriogenology 2014;81:152-169.
12. Rowe RF, Delcampo MR, Critser JK, et al: Embryo transfer in cattle - non surgical transfer. Am J Vet Res 1980;41:1024-1028.
13. Sirard MA: Resumption of meiosis: Mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology 2001;55:1241-1254.
14. Rizos D, Ward F, Duffy P, et al: Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002;61:234-248.
15. Hyttel P, Grondahl C, Madison V, et al: Cell biological aspects of bovine oocyte maturation, fertilization and initial embryonic-development in vivo and in vitro. Arch Tierz 1990;33:517-529.
16. Deleeuw AM: Number and viability of embryos collected in vivo or from the excised uteri of slaughtered donor cows. Theriogenology 1992;37:907-913.
17. Dias FCF, Costa E, Adams GP, et al: Effect of duration of the growing phase of ovulatory follicles on oocyte competence in superstimulated cattle. Reprod Fertil Dev 2013;25:523-530.
18. Lerner SP, Thayne WV, Baker RD, et al: Age, dose of FSH and other factors affecting superovulation in holstein cows. J Anim Sci 1986;63:176-183.
19. Bennett WA, Watts TL, Blair WD, et al: Patterns of oviductal motility in the cow during the estrous cycle. J Reprod Fertil 1988;83:537-543.

Table 1. Experimental timeline of protocol for superovulation of heifers.

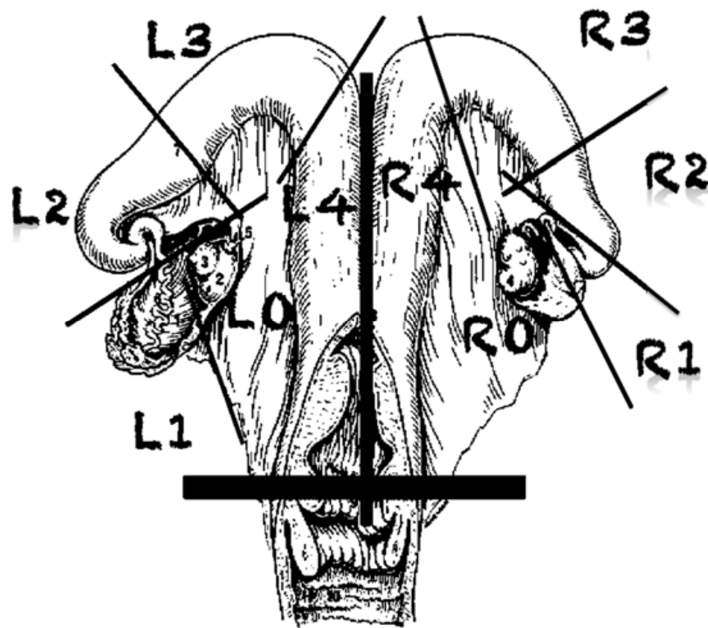
Day	Time	Activities
0	AM[1]	CIDR™-insertion + 25 mg $PGF_{2\alpha}$
4	AM	150 µg GnRH
5	PM	40 mg FSH
6	AM/PM	40 mg FSH / 30 mg FSH
7	AM/PM	30 mg FSH / 30 mg FSH
8	AM/PM	30 mg FSH / 20 mg FSH + 25 mg $PGF_{2\alpha}$
9	AM	20 mg FSH + 25 mg $PGF_{2\alpha}$ + CIDR™-removal
10	PM	AI + 150 µg GnRH
11	AM	AI
16	PM	Ultrasound ovaries, document CLs
17	AM	Slaughter, excise and process uterus, locate embryos

[1] AM to PM was ~ a 12-h interval

Table 2. Embryos recovered from each section.

	Section[1]					
	1	2	3	4	SEM[2]	p value
Left Recovery, %	9.5 [bc]	59.1[a]	25.8 [b]	5.6 [c]	7.0	<0.001
Right Recovery, %	0 [b]	52.1[a]	41.7 [a]	6.3 [b]	11.9	0.01
Total Recovery, % [3]	5.8 [c]	53.5[a]	33.9 [b]	6.8 [c]	9.6	<0.001
Left Recovered, n	0.33 [b]	2.08[a]	1.08 [b]	0.25 [b]	0.30	<0.001
Right Recovered, n	0 [b]	1.08[ab]	1.42 [a]	0.42 [ab]	0.41	0.08
Total Recovered, n [3]	0.33 [b]	3.17[a]	2.50 [a]	0.67 [b]	0.52	<0.001

[a-c] Within each row LSMEANS that do not share a common superscript differed ($p \leq 0.05$).
[1] Section: 1=uterine tube; 2=tip of horn; 3=middle third of horn; 4=base of horn (Refer Figure)
[2] SEM: n=12
[3] Combined Left and Right sides for each section.



Key[1]:

LO: Left ovary
L1: Left uterine tube
L2-L4: Sections of left uterine horn

RO: Right ovary
R1: Right uterine tube
R2-R4: Sections of right uterine horn

Figure. Designation of sections (uterine tubes and uterus). Each side was divided into 4 sections, the uterine tube (L1/R1), and 3 equal lengths of uterine horn (L2-4/R2-4) with the uterine body bisected in half longitudinally.