Embryonic mortality and the uterine environment in first-service lactating dairy cows

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Summary. Embryos were collected non-surgically from the tip of one uterine horn of 23 lactating dairy cows on Day 7 of pregnancy. Embryos were classified on the basis of morphological criteria as normal ($n = 12$) or abnormal ($n = 13$). Abnormal embryos were further classified as cleavage stage ($n = 9$) or morula/blastocyst ($n = 4$). Cows producing an abnormal embryo did not differ in days *post partum* at oestrus, age or parity from cows producing a normal embryo. Cows with an abnormal morula/blastocyst also did not differ with respect to days *post partum* at oestrus from cows with abnormal cleavage-stage embryos but cows with an abnormal morula/blastocyst were significantly older and of greater parity than cows with an abnormal cleavage-stage embryo.

Hepes-saline-PVP solution (30 ml) was initially infused into the uterine tip, mixed and then withdrawn with a syringe. Analysis of this fluid revealed that the concentrations of glucose, total protein, calcium, magnesium and potassium were significantly higher in the flushings from the uterus of cows with abnormal embryos than from cows with normal embryos and zinc and phosphorus tended to be higher in the uterine flushings of cows with abnormal embryos. Phosphorus, total protein, calcium and magnesium tended to be higher in the flushings from cows with abnormal morulae/blastocysts than from cows with abnormal cleavage-stage embryos. Plasma progesterone did not differ between cows with normal or abnormal embryos or in cows with abnormal morulae/blastocysts or abnormal cleavage-stage embryos.

Most embryonic mortality therefore occurred before Day 5 (during cleavage) in these cows. Embryonic mortality was associated with a uterine environment significantly different from that of cows with normal embryos.

Keywords: cattle; cleavage; embryonic mortality; luteal function; post-partum period; uterine environment

Introduction

Hawk (1978) noted that embryonic mortality was the major cause of pregnancy failure in inseminated dairy cattle. Ayalon (1978) found that most embryonic mortality occurred after Day 16 in normal dairy cows but by Day 8 in repeat-breeder cows. However, the time of death of embryos varies among studies (Hawk, 1978).

Three factors have been suggested as the major contributors to embryonic mortality. Genetic involvement in embryonic death could arise as a result of lethal genes being expressed early in development or due to structural (including chromosomal) abnormalities in the fertilizing gametes (Bishop, 1964; Hunter, 1982). Inappropriate ovarian steroidogenesis or luteotrophic support after fertilization (Erb *et al.*, 1976; Ayalon, 1978) or stress hormones affecting hypothalamo-pituitary or ovarian function (Wagner & Li, 1982; Moberg, 1984) are a second (endocrine) mechanism for embryonic death. Deleterious changes in the oviducal and/or uterine environment immediately

bathing the embryo (Ayalon, 1978) offer a third (although possibly endocrine-related) explanation for embryonic mortality.

Numerous approaches are used in the studies of embryonic mortality. Differences between normal and repeat-breeder cows have been examined (Lamothe & Guay, 1970), although repeat-breeding can be due to causes other than embryonic mortality, including fertilization failure. Embryos have been recovered from cows and the characteristics of animals producing a normal or abnormal (i.e. degenerate) embryo examined (Ayalon, 1978; Maurer & Echternkamp, 1982). Unfortunately, the few studies of this type have sometimes included unfertilized ova in the abnormal embryo classification or they have not included data on the developmental stage of abnormal embryos, thus confounding interpretation of the results. Examination of uterine fluids can be a valuable source of information, but direct uterine aspirates may often be contaminated with blood (Lamothe & Guay, 1970) and post-mortem uterine flushings may not accurately reflect conditions in the live animal (Ayalon, 1978). Regional differences in the uterine environment may exist (Almeida *et al.*, 1984). Thus, chemical analysis of uterine fluid collected from both uterine horns or even the uterine horn containing the embryo might obscure differences in the uterine environment immediately surrounding the embryo that may exist between cows with normal or degenerate embryos.

Therefore, the objective of this study was to examine the differences among lactating cows from which normal or abnormal embryos had been collected on Day 7 after oestrus. Uterine fluid was sampled from the tip of the uterine horn from which the embryo was recovered. Plasma progesterone concentrations were also monitored daily in these cows.

Materials and Methods

Animals. Lactating Holstein cows from the Washington State University herd were used. Animals were rectally palpated and evaluated by a veterinarian at regular intervals, beginning at least 35 days *post partum*, as part of a routine herd health programme. Cows that were approved as ready to mate at next oestrus were then observed 2–3 times daily for oestrous behaviour. Upon detection of standing oestrus (Day 0), animals were artificially inseminated twice, at 12 and 24 h, with semen from one of two bulls of high fertility. High-fertility bulls were used to decrease the incidence of fertilization failure.

Plasma progesterone. Blood was collected in heparinized containers by tail venepuncture every morning from the day of oestrus to day of embryo collection (Day 7). After centrifugation, plasma was stored at -5°C until further analysis. Plasma progesterone was measured by radioimmunoassay (Davidge *et al.*, 1987). The intra- and inter-assay coefficients of variation for these assays for a pregnant cow sample averaging 8.5 ng/ml were 6.0 and 22.0%, respectively. Sensitivity of the assay was 0.06 ng/ml plasma. No correction was made for blank (buffer) values which averaged less than the lowest standard (25 pg progesterone/tube).

Embryo collection and uterine fluid sampling. On Day 7, animals were rectally palpated to determine the side of ovulation. A modified non-surgical embryo collection procedure (Brand & Drost, 1977) was utilized. A Rusch two-way balloon catheter was advanced as far as possible towards the tip of the uterine horn ipsilateral to the corpus luteum and fixed in place by expansion of the air cuff. Initially, 30 ml of a Hepes (2.5 mM)–saline (0.13 M)–polyvinylpyrrolidone (PVP-40, 0.1%) solution (pH 7.15) were infused into the uterine tip through the catheter, followed by 10 ml air to clear the catheter. The Hepes and saline were added to prevent osmotic shock to the embryo and uterine endometrium/cellular contents. The PVP-40 acted similarly to protein in facilitating manipulation of the embryo but did not interfere in the chemical analysis of protein in the uterine flushing. The infused solution was mixed with the contents of the uterine tip by gentle stroking and agitation via grasping of the uterotubal junction. As much as possible of the infused 30 ml was then recovered with a 30-ml syringe attached externally to the catheter.

The contents of the syringe were expressed into a plastic culture dish and examined by dissecting microscope for the presence of an embryo(s), which was then removed. The uterine flushing was then centrifuged for 15 min at 23 500 g and the supernatant stored frozen at -5°C until further chemical analysis.

After withdrawal of the Hepes–saline–PVP solution, the catheter was attached to an infusion bottle holding 500 ml of a modified Dulbecco's PBS (Dulbecco & Vogt, 1954) containing 5% heat-inactivated bovine (fetal calf/steer) serum. The tip of the uterine horn was subsequently flushed with aliquants of this solution which were allowed to drain into a siliconized glass graduated cylinder. The contents of the cylinder were also searched for the presence of an embryo(s).

Embryo handling and classification. Embryos were rinsed 6 times in modified Ham's F-10 medium (Wright & Bondioli, 1981) and placed in a 100- μl culture drop under equilibrated paraffin oil. Embryos were examined at $\times 200$

magnification with a light and phase-contrast microscope. Embryos were evaluated independently by the author and 1 or 2 experienced technicians and scored as normal (excellent–good) or abnormal (poor–fair) on the basis of morphological criteria (Lindner & Wright, 1983). Embryos were also classified as to stage of development: cleavage (2–8-cell) or morula/blastocyst.

Chemical analysis of uterine fluid. Glucose was assayed colorimetrically using a glucose oxidase procedure from Sigma Chemical Co. (St Louis, MO, U.S.A.). Inorganic phosphorus was converted to phosphomolybdate and measured colorimetrically (Pierce Chemical Co., Rockford, IL, U.S.A.). Total protein was measured colorimetrically after binding to Coomassie blue dye (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Calcium, magnesium, potassium and zinc were determined using inductively-coupled plasma atomic emission spectroscopy at the Washington Animal Disease Diagnostic Laboratory (Pullman, WA, U.S.A.).

Statistical analysis. Differences in days *post partum* at oestrus, age and parity between cows with normal embryos, abnormal cleavage-stage embryos or abnormal morulae/blastocysts were initially tested for significance by analysis of variance, using the General Linear Models Procedure (SAS, Cary, NC, U.S.A.). Days *post partum*, age and parity were included as covariates in the statistical model examining differences in uterine fluid constituents and plasma progesterone concentrations. Orthogonal contrasts were then performed, specifically comparing characteristics of cows with (1) normal or abnormal embryos or (2) abnormal cleavage-stage embryos or abnormal morulae/blastocysts.

Twin embryos were collected from 2 cows. In one cow, both embryos were normal morulae/blastocysts and in the other, both embryos were abnormal cleavage-stage embryos. In both cows, 2 corpora lutea had been palpated on the ovary. For the statistical analysis, the cows were treated identically to cows from which only one embryo was collected.

Results

From the 23 cows, 25 embryos were recovered: 6 of the embryos were found in the syringe used to draw off the initial infusion of Hepes–saline–PVP. Twelve of the embryos (including one set of twin embryos) were classified as normal, i.e. they were at the morula or blastocyst stage and of good–excellent quality. Of the 13 abnormal embryos (including one set of twin embryos), 9 were cleavage stage (2–8-cell) and showed various signs of degeneration and 4 were morulae or blastocysts of poor–fair quality. There was no difference in the distribution of normal and abnormal embryos between the 2 bulls used as sires.

Cows with an abnormal embryo(s) did not differ ($P > 0.10$) in days *post partum* at oestrus, age or parity from cows with a normal embryo(s) (Table 1). Cows with an abnormal morula/blastocyst also did not differ ($P > 0.10$) with respect to days *post partum* at oestrus from cows with abnormal cleavage-stage embryos. However, cows with an abnormal morula/blastocyst were significantly ($P < 0.05$) older and of greater parity than were cows with an abnormal cleavage-stage embryo.

Table 1. Characteristics of cows with a normal or abnormal embryo

	All cows			Cows with abnormal embryos		
	Normal (N = 11)	Abnormal (N = 12)	Significance	Cleavage (N = 8)	Morula/ blastocyst (N = 4)	Significance
Days <i>post partum</i> at oestrus	74 ± 16	60 ± 7	N.S.	52 ± 4	74 ± 20	N.S.
Age (months)	49 ± 5	53 ± 6	N.S.	44 ± 6	71 ± 13	$P < 0.02$
Parity	2.4 ± 0.4	2.8 ± 0.5	N.S.	2.1 ± 0.4	4.2 ± 1.1	$P < 0.03$

Values are mean ± s.e.m.

Recovery of the initial infusion of Hepes–saline–PVP averaged 60% (i.e. 18.1 ml). Contamination with blood or mucus was absent or minimal. The concentration of uterine fluid constituents in this flushing varied significantly between cows with normal and abnormal embryos and tended to differ between cows with abnormal morulae/blastocysts or abnormal cleavage-stage embryos (Table 2).

Glucose, total protein, calcium, magnesium and potassium were higher ($P < 0.05$) in uterine flushings from cows with abnormal embryos than in cows with normal embryos. Zinc and phosphorus tended ($P < 0.10$) to be higher in the uterine flushings from cows producing abnormal embryos. Glucose, potassium and zinc did not differ ($P > 0.10$) in the uterine flushings from cows with abnormal morulae/blastocysts and those with abnormal cleavage-stage embryos but phosphorus, total protein, calcium and magnesium tended ($P < 0.10$) to be higher in the former.

Table 2. Characteristics of uterine flushings of cows with a normal or abnormal embryo

Constituent	All cows			Cows with abnormal embryos		
	Normal (N=11)	Abnormal (N=12)	Significance	Cleavage (N=8)	Morula/ blastocyst (N=4)	Significance
Glucose (mg/100 ml)	0.5 ± 0.1	1.1 ± 0.2	$P < 0.03$	0.9 ± 0.2	1.5 ± 0.6	N.S.
Phosphorus (µg/ml)	2.0 ± 0.3	2.8 ± 0.6	$P < 0.10$	2.3 ± 0.4	4.0 ± 1.5	$P < 0.09$
Total protein* (mg/100 ml)	26 ± 5	61 ± 16	$P < 0.01$	51 ± 18	81 ± 31	$P < 0.06$
Calcium (µg/ml)	2.1 ± 0.3	3.5 ± 0.6	$P < 0.02$	2.9 ± 0.4	4.6 ± 1.6	$P < 0.09$
Magnesium (µg/ml)	1.0 ± 0.2	1.6 ± 0.3	$P < 0.03$	1.3 ± 0.2	2.2 ± 0.7	$P < 0.09$
Potassium (µg/ml)	13 ± 3	33 ± 6	$P < 0.01$	29 ± 6	41 ± 16	N.S.
Zinc† (µg/ml)	0.16 ± 0.02	0.28 ± 0.04	$P < 0.07$	0.25 ± 0.04	0.33 ± 0.10	N.S.

Values are mean ± s.e.m.

*Age tended to have an effect, $P < 0.08$.

†Both days *post partum* ($P = 0.001$) and age ($P = 0.02$) had an effect.

Days *post partum* at oestrus had no effect ($P > 0.10$) on the concentrations of uterine fluid constituents, except for zinc, which was positively correlated with days *post partum*. Age had a significant negative effect ($P < 0.05$) on the concentration of zinc in the uterine flushings and tended ($P < 0.10$) to have an effect (negative) on total protein concentration in the uterine flushings. The effect of parity on both uterine zinc and protein was similar to that of age, but was not significant ($P > 0.05$).

Plasma progesterone from the day of oestrus to the day of embryo collection did not differ ($P > 0.10$) in cows with normal or abnormal embryos or in cows with abnormal morulae/blastocysts or abnormal cleavage-stage embryos (Table 3). Days *post partum* at oestrus, age and parity had no effect ($P > 0.10$) on plasma progesterone concentrations.

Discussion

Most embryo mortality had occurred by Day 5 after oestrus in the cows in this study. This was concluded from the fact that 9 of the 13 abnormal embryos were cleavage stage, which are normally not present after Day 4 of pregnancy (Lindner & Wright, 1983). This suggests that some embryonic death could be due to abnormal oviduct function, either an abnormal environment within the oviduct or abnormal motility of the oviduct which might have caused early entry into the uterus. Uterine fluids are toxic to cleavage-stage embryos (Lambert *et al.*, 1980/81). It is also possible that

Table 3. Plasma progesterone (ng/ml) in cows with a normal or abnormal embryo

Days of pregnancy	All cows		Cows with abnormal embryos	
	Normal (N = 11)	Abnormal (N = 12)	Cleavage (N = 8)	Morula/blastocyst (N = 4)
0	0.47 ± 0.22	0.36 ± 0.09	0.40 ± 0.11	0.30 ± 0.16
1	0.29 ± 0.06	0.34 ± 0.07	0.36 ± 0.10	0.30 ± 0.12
2	0.43 ± 0.12	0.34 ± 0.07	0.41 ± 0.10	0.21 ± 0.06
3	0.67 ± 0.18	0.62 ± 0.11	0.60 ± 0.12	0.67 ± 0.27
4	1.34 ± 0.20	1.19 ± 0.18	1.08 ± 0.17	1.41 ± 0.43
5	2.26 ± 0.41	1.66 ± 0.17	1.64 ± 0.22	1.70 ± 0.32
6	2.91 ± 0.29	3.03 ± 0.21	2.85 ± 0.25	3.39 ± 0.35
7	3.87 ± 0.35	3.35 ± 0.29	3.24 ± 0.38	3.56 ± 0.48

Values are mean ± s.e.m.

oviduct function was completely normal in these animals and that embryo death was due to genetic causes (Bishop, 1964).

Ayalon (1972, 1978) has suggested that the critical period for embryonic mortality in repeat-breeder dairy cows appears to be soon after the embryo enters the uterus, 5–7 days after insemination, when the morula is developing into the blastocyst. For 4 of the 13 abnormal embryos recovered in this study, this might be true. These embryos, which reached the morula/blastocyst stage before showing signs of degeneration, were recovered from cows that were significantly older and of greater parity than cows that produced abnormal cleavage-stage embryos but these cows would not be considered repeat breeders. In addition, the uterine environment in these cows tended to be even more abnormal than that in cows with abnormal cleavage-stage embryos. The recuperative powers of the uterus after multiple pregnancies may therefore be limited, leading to an increase in embryo mortality at the time the embryos enter the uterus.

All uterine constituents measured were present at higher concentrations in the flushings from cows with abnormal embryos as compared to flushings from cows with normal embryos. Ayalon (1978) found a similar increase in calcium, phosphorus, potassium and zinc in the Day 7 uterine flushings of cows with abnormal embryos but total carbohydrate concentrations did not differ. The significant difference in uterine glucose concentrations found in this study between cows with normal and abnormal embryos may be due to the specific measurement of glucose, rather than total carbohydrates, and the fact that samples were collected from live, rather than slaughtered, cows. Total protein levels were consistently higher in uterine flushings from cows with normal embryos in Ayalon's (1978) study while the reverse was found in the study reported here. Sampling of the fluid immediately surrounding the embryo, as was done in this study, might give a more valid representation of the uterine environment, at least with respect to its relationship to embryo mortality. The use of a less specific sampling technique, as in the previous study, might explain the difference in results.

There were no differences among groups in days *post partum* at the oestrus that produced the embryos in this study. In addition, except for zinc, there was no effect of days *post partum* on the concentration of uterine constituents. One might therefore assume that inadequate involution of the uterus after the previous pregnancy did not play a major role in embryonic mortality (except as noted above for older cows). Rectal palpation, gross morphology and ultrasonic observations of dairy cows (Gier & Marion, 1968; Okano & Tomizuka, 1987) have suggested that uterine involution is completed at about 40 days *post partum*. Light microscopic studies show regeneration of endometrium of normal appearance by this time (Gier & Marion, 1968). The uterine environment in post-partum beef cows approximates that in normal cyclic cows 36 or more days after calving (Stanfield, 1984). No similar

studies have been reported for post-partum dairy cows. Whether the uterine environment in a post-partum dairy cow returns to a normal state at the same time as it returns to normal size or histological appearance is not known.

Several observations on the cows in this study suggest that inadequate involution of the reproductive tract may, in fact, play a role in embryonic mortality. Of the 25 embryos recovered, 36 and 64% were flushed from the left and right uterine horns, respectively. This agrees closely with the 40:60 distribution of left:right ovulations and pregnancies found in dairy cattle (McDonald, 1980). Of the 9 embryos recovered from the left uterine horn, 7 were classified as normal. Of the 16 embryos recovered from the right uterine horn, 11 were classified as abnormal. If we suppose that abnormal embryos are more likely to be found in the uterine horn corresponding to the side of the previous pregnancy, then the recovery of 11 of the 13 abnormal embryos in the right uterine horn is not surprising, given the reported preponderance of right horn pregnancies. Unfortunately, records on the side of the previous pregnancy were not available for the cows in this study. This effect of the side of previous pregnancy might not have been present had we waited longer *post partum* to collect embryos. This purported effect of the side of previous pregnancy is compatible with the earlier suggestion that much embryo death may be occurring in the oviduct, since concentrations of constituents in oviducal flushings usually parallel those in the uterine flushings (Ayalon, 1978).

Many of the uterine fluid constituents measured, which were higher in cows with abnormal embryos, are compounds that are generally present in higher concentrations intracellularly than extracellularly. A source of these compounds, not previously considered, is the erythrocytes extravasated into the uterine lumen at metoestrus. The fate of these erythrocytes is not clear but their lysis could result in some of the differences in uterine environment seen in this study. Bleeding at metoestrus, although known to vary considerably between animals, has not previously been associated with a lowered incidence of pregnancy (Jainudeen & Hafez, 1987), but the presence or absence of visible external bleeding may not accurately reflect what is occurring in the intrauterine environment.

Progesterone concentrations did not vary in cows from which normal or abnormal embryos were recovered. These results agree with those of Ayalon (1978) and Linares *et al.* (1982) for dairy cattle. In crossbred beef cows and heifers, higher serum progesterone concentrations have been reported on Days 3 and 6 of pregnancy in females with normal developing embryos than in females with abnormal embryonic development (Maurer & Echternkamp, 1982).

The results of this study suggest that most embryo mortality occurred by Day 5 after oestrus, during cleavage stages of development. Embryo mortality was associated with a uterine environment that differed from that of cows with normal embryos. Embryo mortality that occurred at or after Day 5 after oestrus was seen in cows that were older and of greater parity than cows with abnormal cleavage-stage embryos and the uterine environment tended to differ between these two groups.

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