Factors Affecting the Developmental Stage of Embryos Recovered on Day 7 from Superovulated Dairy Cattle

Henrik Callesen*,2,3, Peter Løvendahl4, Anders Bak*,2, and Torben Greve2

*Embryo Technology Center, National Institute of Animal Science, DK-8830 Tjele, Denmark

ABSTRACT: The objective of this retrospective study was to evaluate the factors influencing developmental stage of bovine embryos recovered from superovulated dairy cattle 7 d after estrus. From 217 superovulated dairy cows and heifers, 2,211 eggs were recovered, of which 1,495 were classified as transferable embryos based on morphological evaluation of developmental stage and quality. From the evaluated embryos, 1,429 were non-surgically transferred to recipients to produce 623 calves. The transferable embryos were classified into five developmental stages and four quality grades. The least-developed transferable embryos tended to be classified into poorer quality grades. A multifactorial statistical model was used to analyze whether the following factors were associated with the developmental stage and quality grade of the embryos: donor breed, parity, gonadotropin preparation, embryo sex, insemination bull, embryologist (the person evaluating the embryo), year, and season of recovery. Among these factors, only the embryologist and the donor animal accounted for significant variation in embryo development. It was concluded that the developmental stage of embryos recovered at d 7 from superovulated cattle, when evaluated by simple morphological criteria, was correlated with the embryo's quality and was affected by the donor animal but in this study not by the embryo sex, donor breed and parity, gonadotropin preparation, and insemination bull used. The embryo's quality grading was influenced by the embryologist. Consequently, sexing of an embryo recovered from superovulated cattle is not possible by simple morphological evaluation of the embryo's developmental stage.

Key Words: Cattle, Superovulation, Embryo, Development, Sex

Introduction

The developmental stages of embryos recovered from superovulated cattle vary considerably when evaluated by subjective evaluation based on simple morphological criteria (Greve, 1981; Lindner and Wright, 1983; Hasler et al., 1987). The sex of the embryo has been thought to contribute to the variation because male embryos developed faster in in vivo studies of calves produced from embryos from superovulated cattle (Itoh and Goto, 1986; Avery et al., 1989). However, more extensive in vivo studies have questioned this finding (Callesen et al., 1992; Thibier and Nibart, 1992).

Because most studies on embryo development in superovulated cattle have included only one or two factors potentially influencing the embryo's developmental stage at recovery, a total analysis of several factors affecting the development should be performed.

Using superovulated dairy cattle as the source of embryos, the objective of this retrospective study was to evaluate the factors affecting the developmental stage of embryos recovered and evaluated 7 d after estrus.

Materials and Methods

Donor Animals and Treatments

Data used in this study were obtained from the national Danish Multiple Ovulation and Embryo Transfer (MOET) breeding project for dairy cattle (Christensen and Liboriussen, 1986; Callesen et al., 1992). From January 1986 to December 1992, 184 cows and 33 heifers of three dairy breeds (47 Red Danish, 141 Holstein Friesian, and 29 Jersey) were treated during their mid-luteal phase with exogenous
gonadotropin: FSH (n = 180; eight i.m. injections, 30 to 45 mg [according to the weight of the animal], twice daily in decreasing doses [FSH-P®, Schering, Kenilworth, NJ, or Follitropin® Vet., Pitman-Moore, Frederiksberg, Denmark, or Vetrephearm, Ontario, Canada]) or equine chorionic gonadotropin (eCG; n = 37; one i.m. injection, 1,500 to 3,000 IU [according to the weight of the animal; Antex®, Leo Pharmaceuticals, Ballerup, Denmark]). Approximately 72 h after start of the FSH or eCG treatment, the animals were injected with a prostaglandin analogue (cloprostenol, single i.m. injection, 750 to 1,000 µg [Estrumat® Vet., Pitman-Moore, Frederiksberg]) to induce luteal regression. Approximately 60 and 72 h after the prostaglandin treatment, the donors were inseminated with thawed semen from proven fertile bulls of the same breed as the donor (n = 67 bulls in total; the same bull was used for the two inseminations of each donor).

Embryo Recovery and Evaluation

Eggs were non-surgically recovered using the method described by Greve (1981) on d 7 (d 0 = day of estrus). The recovered eggs were morphologically evaluated by one of nine experienced veterinary practitioners (the embryologist) to determine their developmental stage and quality using a stereomicroscope with approximately 100× magnification.

The following five developmental stages (coded 1–5) were used (Lehn-Jensen, 1986): Stage 1 (morula: a ball of cells on whose surface the individual blastomeres could still be distinguished); Stage 2 (compacted morula: individual blastomeres could not be distinguished on the embryo’s surface); Stage 3 (young blastocyst: a fluid-filled cavity [blastocoele] was visible, and the inner cell mass had started to form); Stage 4 (blastocyst: the embryo occupied most of the space within the zona pellucida, the inner cell mass was becoming more distinct, but the overall diameter of the embryo, including zona pellucida, was unchanged); and Stage 5 (expanded blastocyst: the diameter of the embryo was increased and the thickness of the zona pellucida could be reduced to approximately 1/3 of the original thickness).

The following four quality grades (coded 1–4) were used (Lehn-Jensen, 1986): Grade 1 (excellent: stage of development fit well with day of recovery, intact and spherical zona pellucida, homogeneous cell mass with cells of uniform size, no or few cell fragments in the perivitelline space); Grade 2 (good: minor deviations in shape and color compared with grade 1 [e.g., some cell fragments or debris in the perivitelline space and/or small vesicle formations in the blastomeres]); Grade 3 (fair: clear deviations compared with grade 2, but most of the cell mass was intact); and Grade 4 (poor: many cell fragments or debris in the perivitelline space, more and larger vesicles and clear degenerative changes in the blastomeres, less than half of the cell mass was intact).

Each egg was designated by the embryologist as a transferable or a nontransferable embryo, based on a general evaluation of fertilization and suitability for transfer. Only donors producing at least one transferable embryo were included in this study.

Embryo Transfer

After evaluation, the fresh or thawed embryos (Lehn-Jensen, 1986) were transferred non-surgically (Greve, 1981) to synchronized recipient heifers or cows by one experienced veterinarian. Of 66 transferable embryos, 16 were retained for later use and 50 were lost for various reasons.

Pregnancy examination was done approximately 35 d after embryo transfer by rectal palpation. The pregnant recipients remained at the experimental farms until calving, when the sex of each calf was recorded. Abortions were confirmed by rectal examination of the recipient animal.

Statistical Evaluation

The two variables, developmental stage (“stage”) and quality grade (“grade”), for each transferable embryo were analyzed with a least squares uni- and multivariate method (GLM procedures; SAS, 1988) to account for the effect of their expected covariance on tests of statistical significance. The mixed statistical model included both fixed and random factors. Variance and covariance components in the final model were estimated using the DMU-package (Jensen and Madsen, 1992).

All transferred embryos were assigned an “embryo type” with three values: 0 = embryos not developing into a calf; 1 or 2 = embryos resulting in birth of a male or a female calf, respectively. The difference between barren and calf-producing embryos was analyzed by linear contrast (0 vs 1+2), as were differences between male and female embryos (1 vs 2).

Data from all 1,495 transferable embryos were analyzed by simultaneously fitting the effects of the recorded factors. The model included fixed effects (year and season of recovery, donor breed and parity, gonadotropin preparation used, embryo type, and embryologist), random effects (donor [nested within all fixed effects] and insemination bull [nested within breed]) and, finally, the random residual effect associated with the recording. Through backward elimination, only effects approaching or achieving significance in the uni- and the multivariate analysis (P < .20) were kept in the model. The effects were tested with an F-ratio test. Because the data had a hierarchical structure, the fixed effects and the effect of insemination bull were tested against an error term composed of mean squares for donor and residual by Sattertwaite’s approximation (SAS, 1988). The embryo type was observed within donor and was tested against the residual mean square.
EMBRYO DEVELOPMENT IN SUPEROVULATED CATTLE

Table 1. Developmental stage and quality of 1,495 transferable embryos recovered from superovulated donors 7 days after estrus

<table>
<thead>
<tr>
<th>Embryo quality grade</th>
<th>Developmental stage of embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morula</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (excellent)</td>
<td>9</td>
</tr>
<tr>
<td>2 (good)</td>
<td>28</td>
</tr>
<tr>
<td>3 (fair)</td>
<td>35</td>
</tr>
<tr>
<td>4 (poor)</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
</tr>
<tr>
<td>Mean (SD) quality grade per stage</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>(.9)</td>
</tr>
</tbody>
</table>

Variation associated with random effects of donor was used to calculate intraclass correlations (the repeatability; Falconer, 1981), expressing the within-donor correlation between embryos. Correlations between traits were examined using multivariate analysis of variance on the level of donor and as error correlations.

**Results**

The 217 donors yielded 2,211 eggs, of which 1,495 were graded as transferable embryos (from 1 to 33 per donor). These were in various stages of development from morula to expanded blastocyst as well as quality grades from excellent to poor (Table 1). There was a trend toward classifying the least-developed transferable embryos into the poorer quality grades; morulas had the poorest and expanded blastocysts the best quality grades.

Of the evaluated embryos, 1,429 were transferred and produced 661 pregnancies from which 623 calves were born; 31 fetuses were aborted and seven pregnant recipients were slaughtered. Of the 623 calves born, 52% were males, which did not differ from the expected sex ratio of 1:1 (chi-square, \(P > .30\)).

Using the technique of backward elimination, the following effects were eliminated from the model in the following order: parity, insemination bull, breed, and gonadotropin preparation.

At that point, the factors included in the model were embryo type, embryologist, year and season of recovery, and the donor. Embryos yielding calves did not differ from the other transferred embryos in stage and quality, as the differences between the two groups were \(-.03 \pm .05\) units (\(P > .48\)) and \(.07 \pm .04\) units (\(P > .10\)) for stage and quality grade, respectively. Male and female embryos did not differ in developmental stage or quality grade; the differences between these two groups were \(-.04 \pm .07\) units (\(P > .49\)) and \(-.05 \pm .06\) units (\(P > .48\)) for stage and quality grade, respectively. Furthermore, there was a nonsignificant overall influence of embryo type (\(P > .85\) in the multivariate analysis); thus, this factor was also eliminated from the model.

Therefore, the following effects were included in the final model: embryologist, year and season of recovery, and the donor. The results of the uni- and multivariate analyses using the final model are shown in Table 2. The multivariate analysis showed that the embryologist influenced the joint morphological evaluation of the embryos. When embryo stage and grade were considered separately, only the quality grade was significantly influenced by the embryologist.

The donor strongly influenced developmental stage and quality grade of embryos, as reflected by the intraclass correlations for donor \(t = .39 \pm .03\) and \(t = .27 \pm .03\) for stage and quality, respectively. The two morphological traits were correlated both on the level of donor cow (\(r = -.32 \pm .07\)) and within donor cow (error correlation, \(r = -.32 \pm .02\)).

**Discussion**

Embryos from superovulated cattle producing male vs female calves did not differ in developmental stage or grade. This finding differs from in vivo studies analyzed with chi-square tests (Itoh and Goto, 1986; Avery et al., 1989) in which conclusions were based on fewer calves (49 and 63, respectively). However, in a study of 212 d-7 embryos from superovulated cattle (Thibier and Nibart, 1992) and with the material from the present study (data not shown), no influence of embryo sex was observed when results were analyzed by a similar chi-square test. The results from our present study confirmed this by using an analysis of variance to simultaneously include several of the complex factors affecting development of embryos from superovulated cattle.

In in vitro experiments, a significant relationship between embryo sex and developmental rate has been demonstrated in some (Avery et al., 1992; Marquant-Le Guienne et al., 1992; Xu et al., 1992, Dominko and First, 1993; Yadav et al., 1993), but not all, studies (Berg et al., 1992). The former findings do not necessarily conflict with the results presented here.
because of fundamental differences between the conditions for oocyte maturation and early embryonic development in vitro vs in vivo: 1) oocytes for in vitro maturation constitute a more heterogeneous pool than would be expected in vivo; 2) ova are sorted at several points during the in vitro process and are often excluded for further culture; 3) the period from insemination until recovery is precisely defined in vitro but not in vivo, where both ovulation and fertilization occur over several hours; and 4) in vitro culture conditions are very different from the in vivo situation, and there are differences between laboratories (Betteridge and Rieger, 1993).

Several consequences of these fundamental differences can be mentioned. First, the variations in the developmental stage of in vivo embryos probably reflect biological variation rather than viability differences (Shea, 1981; Lindner and Wright, 1983; Hasler et al., 1987; Bousquet et al., 1993), whereas these variations in vitro seem to reflect their viability more closely (Greve et al., 1993). Second, data from in vitro studies showed a skewed sex ratio in preimplantation embryos (Avery et al., 1992; Marquant-Le Guienne et al., 1992; Xu et al., 1992; Dominko and First, 1993; Yadav et al., 1993), but this was not found when the sex of the embryo was determined at birth (Berg et al., 1992). This suggests that the in vitro system favors one sex over the other, whereas this preference seems to be leveled out during pregnancy by a greater loss of females (Yadav et al., 1993).

Consequently, direct comparison of results from the two systems for embryonic development should be made only with caution.

Embryos in later stages of development had a better average quality grade than those less developed, just as embryos of the poorest quality grade tended to produce lower pregnancy rates. This illustrates the intention of the grade evaluation, that is, to express the viability of the embryo, because reduced viability in terms of poorer quality grade results in slower rates of development (Walker, 1989) and reduced pregnancy rates after transfer (Greve, 1981; Shea, 1981; Lindner and Wright, 1983; Hasler et al., 1987). However, the joint distribution between the stage and grade ratings found in our study illustrates the limitations of this simple method of embryo evaluation. Although many comprehensive morphological descriptions have been published (Greve, 1981; Shea, 1981; Lindner and Wright, 1983; Lehn-Jensen, 1986), person-to-person variation in embryo grade and quality ratings is still pronounced (Lindner and Wright, 1983). So, it is not surprising that the embryologist was found to account for significant variation in the embryo’s quality grading. Conversely, the embryologist had less influence on the developmental scores, suggesting that this trait is easier to describe. The quality evaluation is further hampered by loose or degenerate cells in the embryo that are often more difficult to see in blastocyst than in morula stages. No practical method to replace the visual morphological scoring method has been found so far (Betteridge and Rieger, 1993).

A large proportion of the variability in embryo development and quality was attributed to the donor animal. The background for this variation was, however, not fully explained in this study, as indicated by the relatively low repeatability for both embryo stage and quality grade. Although factors causing donor variability were only briefly examined in this study (i.e., gonadotropin preparation used), it is likely that donor hormone levels during the preovulatory period greatly affect fertilization and early embryonic development. This is especially relevant for superovulated cattle whose hormonal and structural changes in follicular fluids and oocytes, respectively, are quite variable during the preovulatory period (Callesen et al., 1986; Dieleman et al., 1987). Studies of the relationship between embryo development and both preovulatory events and ovulatory patterns could provide further knowledge on this issue. The causes of variation between donors examined in the present study (i.e., donor breed and parity, insemination bull, year and season) were insufficient to answer the question. This aspect deserves further investigation.

Table 2. Analysis of variance of factors affecting developmental stage and quality grades of 1,495 transferable embryos from superovulated cattle

<table>
<thead>
<tr>
<th>Effect</th>
<th>Multivariate analysis</th>
<th>Univariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df (E;e)</td>
<td>F-value</td>
</tr>
<tr>
<td>Embryologist</td>
<td>16 (400)</td>
<td>2.3</td>
</tr>
<tr>
<td>Season × year of recovery</td>
<td>54 (400)</td>
<td>1.1</td>
</tr>
<tr>
<td>Donor b</td>
<td>402 (2,514)</td>
<td>3.8</td>
</tr>
<tr>
<td>Root mean square error</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

---

*a*Wilk’s lambda.  
*bdf (E;e) = degrees of freedom (Effect; error).  
*cP ≤ .05; **P ≤ .01; ***P ≤ .001.  
*dDonors are nested within all effects mentioned in the table.  

---

...
one way being in larger studies including other possible factors. As an example of this, the importance of the donor's genetic background on its response to superovulation is currently being examined (Liborius- sen et al., 1995). We speculate that the genetic background of the embryo may interact with the donor in various ways, further complicating the problem and calling for a thorough analysis taking all genetic and environmental relationships into account. For this purpose even larger amounts of data are needed.

We conclude that the developmental stage of embryos recovered at d 7 from superovulated cattle, when evaluated by simple morphological criteria, is correlated with the embryo's quality and is affected by the donor animal but in this study not by the embryo sex, donor breed and parity, gonadotropin preparation, and insemination bull used. The embryologist was of importance in grading the embryo's quality. Consequently, sexing of an embryo recovered from superovulated cattle is not possible by simple morphological evaluation of the embryo's developmental stage.

**Implications**

Most of the accounted for variation in developmental stages of embryos recovered from superovulated cattle was associated with the donor animal. The traditional morphological method was limited by evaluator bias in grading embryo quality. Consequently, sexing of embryos from superovulated cattle is not possible by simple morphological evaluation of developmental stages. Direct comparisons of results from in vivo and in vitro systems on embryonic development should be made with caution because of their fundamental differences.

**Literature Cited**


Dominko, T., and N. L. First. 1993. Male predominance of bovine embryos can be observed at the 2-cell stage. Biol. Reprod. 48(Suppl. 1):168 (Abstr.).


