Effect of bovine blastocyst size at embryo transfer on day 7 on conceptus length on day 14: Can supplementary progesterone rescue small embryos?

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ABSTRACT

Conceptus size on Day 14 after multiple embryo transfer of Day 7 in vitro–produced blastocysts varies greatly within animal. One explanation for this variation may be related to blastocyst cell number at the time of transfer. The aim of this study was to examine the effect of Day 7 blastocyst cell number on Day 14 conceptus size and to examine the effect of progesterone (P4) supplementation on embryo development after the transfer of Day 7 blastocysts containing a low total cell number. The estrous cycles of crossbred beef heifers were synchronized using an 8-day progesterone (P4)–releasing intravaginal device (PRID) with the administration of a prostaglandin F2α analog on the day before device removal. Only those heifers recorded in standing estrus (Day 0) were used. Heifers were randomly assigned to one of four treatment groups: (1) control: large blastocysts (high total cell number), (2) control: small blastocysts (low total cell number), (3) small blastocysts plus a single intramuscular injection of 3000 IU human chorionic gonadotropin (hCG) on Day 2 after estrus, or (4) small blastocysts plus insertion of a vaginal P4 insert (PRID, 1.55 g P4) between Days 3 and 5 after estrus. In vitro–produced blastocysts were transferred to each heifer on Day 7 (n = 10 blastocysts per heifer), and conceptuses were recovered at slaughter on Day 14. Daily blood samples were collected from Day 0 to 14 to measure serum P4 concentrations. Data were analyzed using the PROC MIXED procedure of SAS. Total cell number on Day 7 was significantly lower in small versus large blastocysts (72.4 ± 3.93 vs. 144.8 ± 3.90, P < 0.05). Conceptus recovery rate was 53.8% overall (140 of 260) and was highest in the large blastocyst group (68.3%, 41 of 60) compared with the other groups (45.7%–55.0%). Concentrations of serum P4 were similar in the two unmanipulated recipient groups but were significantly elevated (P < 0.05) by Day 8 in the hCG-treated heifers and on Days 4 and 5 in the PRID group (P < 0.003). In the absence of supplemental P4, Day 14 conceptuses resulting from the transfer of small blastocysts (2.48 ± 0.54 mm) were smaller than those from large blastocysts (3.32 ± 0.52 mm). Administration of hCG on Day 2 approximately doubled conceptus length on Day 14 (4.94 ± 1.15 mm; P < 0.05), whereas insertion of a PRID from Days 3 to 5 increased conceptus length approximately fivefold (13.09 ± 2.11 mm; P < 0.05) compared with controls. In conclusion, results indicate that supplemental P4 is capable of “rescuing” poor-quality blastocysts, presumably via the now well-described actions on the endometrium and consequent effects on uterine lumen fluid composition.

1. Introduction

Elevated progesterone (P4) concentrations in the first to second weeks postconception stimulate conceptus...
elongation, leading to increased interferon-τ (IFNT) production, and in some cases, are associated with higher pregnancy rates in cattle and sheep [1–3]. Elevated P4 through insertion of a P4-releasing intravaginal device (PRID) on Day 3 after estrus results in an earlier loss of the P4 receptor [4] from the luminal epithelium and an advancement in the normal temporal changes that occur in the endometrial transcriptome during early pregnancy [5], the consequence of which is an advancement in conceptus elongation [1]. The embryo does not need to be present in the uterus during the period of P4 elevation to benefit from it, indicating that the effect of P4 on conceptus development is mediated exclusively through the endometrium, presumably via altered uterine lumen fluid composition [6]. Recently, we have shown that P4 supplementation by insertion of a PRID for as little as 2 days is sufficient to elevate circulating P4 concentrations and increase conceptus elongation and IFNT production after artificial insemination or embryo transfer [7].

An alternative strategy to increase P4 is to stimulate the corpus luteum (CL) using a luteotropic agent. Administration of human chorionic gonadotropin (hCG) on Day 5 postestrus induces ovulation of a dominant follicle forming an accessory CL, leading to an increase in circulating P4 concentrations [8,9]. We have recently shown that administration of a single injection of hCG as early as Day 2 leads to an increase in the volume of luteal tissue in the endogenous CL, resulting in increased P4 concentrations form Day 6 onward, which may be of benefit to the development of the early embryo [10].

The route to achieve elevated P4 is of critical importance as supplementation with exogenous P4 (injection, insert) can have a detrimental effect on the life span of the CL, leading to short cycles [11–13] because of the effect of elevated P4 on luteinizing hormone (LH) pulsatility [13]. This leads to the anomalous situation in which exogenous P4 stimulates conceptus elongation and at the same time compromises the survival of the CL. Luteotropic treatments that stimulate the CL directly, such as hCG, are less likely to have a negative effect on CL life span; indeed, administration of hCG at the time of P4 injection negated the negative effect of P4 on CL life span [11].

In many of the studies examining the relationship between P4 and conceptus elongation, we have used a multiple embryo transfer model involving the transfer of multiple blastocysts (10–20 per recipient) on Day 7 and the recovery of Day 14 conceptuses at slaughter [6,14–16]. Although P4 treatment increases the mean size of the conceptus, there is still a significant variation in conceptus size within animal [6]. One possible explanation for this variation could be variation in blastocyst cell number at the time of transfer.

The present study was designed to test the hypothesis that supplemental P4, through hCG administration on Day 2 or insertion of a PRID between Days 3 and 5, would promote elongation of a small blastocyst with a low total cell number at the time of transfer, that is, P4 “priming” of the uterine environment can overcome the decreased developmental potential of embryos with small cell number.

2. Materials and methods

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland. Protocols were in accord with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Institutional Animal Research Ethics Committee.

The experimental design is illustrated in Figure 1. All animals were housed indoors on slats for the duration of the experiment and were fed a diet consisting of grass and maize silage supplemented with beef ration. The estrous cycles of crossbred beef heifers (n = 33, mean age 23.5 ± 0.39 months, mean weight 603.30 ± 5.68 kg) were synchronized using an 8-day PRID (1.55 g P4; Ceva Sante Animal, Libourne, France) with intramuscular administration of a progastandin F2 α analog (2 ml Estrumate; Schering-Plough Animal Health, Hertfordshire, UK, equivalent to 0.5 mg cloprostenol) given on the day before P4 device removal. Heifers were checked for signs of estrus four times per day commencing 30 hours after P4 device withdrawal. Only those seen in standing estrus (n = 26) were used in the experiment and randomly assigned to one of four treatment groups: (1) control A: large blastocysts (high total cell number), (2) control B: small blastocysts (low total cell number), (3) small blastocysts plus a single intramuscular injection of 3000 IU hCG (Chorulon; Intervet, Boxmeur, The Netherlands) on Day 2 after estrus [10], or (4) small blastocysts plus insertion of a vaginal P4 insert (PRID) between Days 3 and 5 after estrus. Jugular vein blood samples were collected daily from Day 0 (estrus) to slaughter on Day 14 to determine circulating P4 concentration.

After collection, blood samples were refrigerated (4 °C) for 12 to 24 hours before being centrifuged at 1500 × g at 4 °C for 20 minutes. Serum was separated and stored at −20 °C until analysis of P4 concentration by solid-phase radioimmunoassay using a Coat-A-Count Progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as previously described [17]. The sensitivity of the

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**Fig. 1.** Experimental design. The estrous cycles of crossbred beef heifers were synchronized using a progesterone (P4)-releasing intravaginal device with administration of a progastandin F2α analog on the day before device removal. Heifers were randomly assigned to one of four treatment groups and received blastocysts as follows: (1) control: large blastocysts (high total cell number, n = 6), (2) control: small blastocysts (low total cell number, n = 6), (3) small blastocysts plus a single intramuscular injection of 3000 IU human chorionic gonadotropin on Day 2 after estrus (n = 7), or (4) small blastocysts plus insertion of a vaginal P4 insert (PRID, 1.55 g P4) between Days 3 and 5 after estrus (n = 7). Ten in vitro–produced blastocysts were transferred to each heifer on Day 7, and all heifers were slaughtered on Day 14 after estrus. Daily blood samples were collected between Day 0 and Day 14 to determine circulating P4 concentrations.
assay was 0.03 ng/mL. The interassay coefficients of variation for quality control samples were 9.29% (low), 6.21% (medium), and 5.02% (high), respectively. The intra-assay coefficients of variation were 13.62% (low), 7.96% (medium), and 5.13% (high).

2.1. In vitro embryo production

The techniques for producing embryos in vitro have been previously described in detail [18]. Immature cumulus-oocyte complexes (COCs) were obtained by aspirating follicles from the ovaries of heifers and cows collected at slaughter. COCs were matured for 24 hours in TCM-199 supplemented with 10% (vol/vol) fetal calf serum (FCS) and 10 ng/mL epidermal growth factor at 39°C in an atmosphere of 5% CO2 in air with maximum humidity. Matured COCs were inseminated with frozen-thawed percoll-separated bull sperm at a concentration of 1 × 106 spermatozoa/mL. Gametes were co-incubated at 39°C in an atmosphere of 5% CO2 in air with maximum humidity. Semen from the same bull was used throughout. At approximately 20 hours postinsemination, presumptive zygotes were denuded of surrounding cumulus cells and accessory sperm and cultured in 500 μL of synthetic oviduct fluid supplemented with 5% FCS (n = 50 per well) until Day 7.

2.2. Day 7 blastocysts were collected and classified as “large” (high total cell count) or “small” (low total cell count) before being loaded into straws for transfer or the uterine horn ipsilateral to the CL of synchronized recipients (10 transferred per recipient). All animals were slaughtered on Day 14 in a commercial abattoir. Reproductive tracts were recovered, brought back to the laboratory within 2 hours of slaughter, and flushed with phosphate-buffered saline containing 5% FCS. The number and dimensions (length and width) of recovered embryos were recorded. Each CL was dissected from ovarian tissue, weighed, and measured.

2.3. Cell staining and counting

A representative number of “large” (n = 37) and “small” (n = 33) Day 7 in vitro–produced blastocysts were placed on a glass microscope slide and air-dried. Slides were then immersed in ethanol for 24 hours, stained with bisbenzimide (1 ng/mL; Hoechst 33342; Sigma), and counted using a fluorescent microscope.

2.4. Statistical analysis

Data were checked for normality and homogeneity of variance by histograms, qqplots, and formal statistical tests as part of the UNIVARIATE procedure of SAS (version 9.1.3; SAS Institute, Cary, NC, USA). Data that were not normally distributed were transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box–Cox transformation analysis using the TRANSREG procedure of SAS. The transformed data were used to calculate P values. The corresponding least squares means and standard error of the nontransformed data are presented in the results for clarity. Progesterone concentrations were analyzed using repeated measures with the MIXED procedure of SAS. Fixed effects included experimental treatment, day, and their interaction. The interaction term, if not statistically significant (P > 0.10), was subsequently excluded from the final model. Heifer within treatment was included as a random effect. The type of variance-covariance structure used was chosen depending on the magnitude of the Akaike information criterion for models run under compound symmetry, unstructured, autoregressive, or Toeplitz variance-covariance structures. The model with the least Akaike Information Criterion (AIC) value was selected. Conceptus related data were analyzed using PROC MIXED of SAS. The model had experimental treatment as a fixed effect, and heifer within treatment was included as a random effect. Differences between treatments were determined by F tests using type III sums of squares. The PDIF command incorporating the Tukey test was applied to evaluate pairwise comparisons between treatment means.

3. Results

3.1. Circulating concentrations of progesterone

Data for daily P4 concentrations collected between Day 0 and Day 14 for heifers are shown in Figure 2. Concentrations of serum P4 were similar in the two unmanipulated recipient groups. Injection of hCG on Day 2 resulted in a significant increase in circulating P4 concentrations on Day 8 (P < 0.05) relative to the two unmanipulated groups. Additionally, the insertion of a PRID on Day 3 resulted in a sustained elevation in serum P4 concentrations (P < 0.05) until Day 5, which coincided with the removal of the device.

3.2. Embryo recovery and development

Total cell number on Day 7 was significantly lower (P < 0.05) in small versus large blastocysts (72.4 ± 3.93 vs. 144.8 ± 3.90). The overall conceptus recovery rate on Day 14 was 50% (conceptuses recovered as a proportion of embryos transferred, 140 of 260). Conceptuses were recovered from 6 of 7, 5 of 6, 7 of 7, and 5 of 8 heifers in the control large, small, PRID D3-5, and NCG treatments, respectively. Data are presented as mean ± SEM. TRT, treatment. * indicates significance.

![Figure 2](image-url)
control small, hCG-treated, and PRID-treated groups, respectively. From the heifers that yielded conceptuses on Day 14, the recovery rate in the hCG group (45.7%) was lower (P < 0.05) than all other groups (Table 1).

The mean conceptus length, width, and area (±SEM) are shown in Figure 3. In the absence of supplemental P4, Day 14 conceptuses resulting from the transfer of small blastocysts (2.48 ± 0.54 mm) were smaller than those from large blastocysts (3.32 ± 0.52 mm), although the difference was not significant (P > 0.10). Intramuscular injection of hCG on Day 2 resulted in a nonsignificant increase in conceptus length on Day 14 (4.94 ± 1.15 mm) when compared with the small control group (P > 0.10). Insertion of a PRID between Days 3 and 5 significantly increased embryo length (13.09 ± 2.11 mm) compared with the other treatment groups (P < 0.0001).

3.3. Luteal tissue development

There was no significant difference in the mean luteal tissue weight between treatments; however, mean CL area recorded for the PRID-treated group was lower than all other groups, being significantly lower than the hCG group (P < 0.03; Fig. 4).

4. Discussion

The main findings of this study are (1) blastocyst cell number on Day 7 is associated with conceptus length on Day 14, (2) supplemental P4 is capable of “rescuing” small blastocysts containing a low total cell number at the time of transfer, resulting in an increased conceptus size at Day 14, and (3) supplementing P4 by insertion of a P4-releasing device results in a marked advance in conceptus length. The impetus for carrying out this study was the large variability noted among embryos recovered on Day 14 after the transfer of multiple blastocysts on Day 7 [6,15]. Given the fact that cattle are monovulatory, it is difficult to get an appreciation for the variation in conceptus size on a given day under normal circumstances during the elongation phase leading up to maternal recognition of pregnancy. Betteridge et al. [19] highlighted the enormous variability in embryo size recovered from superovulated donors on a given day (Day 14: 0.226–57 mm; Day 16: 0.232–150 mm), even within individual donors (e.g., a range of 4–40 mm from one Day 14 donor). Similar variability (2–225 mm) was seen in a series of 31 Day 16 embryos from normally ovulating cows of low fertility [20]. In the study of Garrett et al. [2], conceptuses recovered after natural mating from Day 14 uteri of P4-treated cows (37.3 ± 14.9 mm) were longer than those recovered from controls (3.8 ± 1.9 mm); however, there was large variability with treatment (control: 1–13 mm; P4 treated: 3–119 mm). Similar findings have also been reported in sheep [21].

Berg et al. [22] transferred between 2 and 30 Day 7 in vitro–produced blastocysts and recovered them at various times after transfer (range, 2–9 days), corresponding with a gestational age of Days 9 through 16. As in the studies mentioned earlier, individual embryo length varied greatly, even when embryos were retrieved from a single recipient. Interestingly, there was a gradual decrease in both recovery rate and individual embryo length with increasing numbers of embryos transferred.

As in ruminants, early embryonic development in the pig is characterized by a rapid elongation of the conceptus trophoderm on Days 11 and 12 of gestation, which is critical for establishing adequate placental surface area needed for embryo and fetal survival throughout gestation. Initially, the conceptus trophoblast is morphologically rearranged from a 10-mm sphere into a tubular shape, transitioning into a thin filamentous form greater than 150 mm in length in 2 to 3 hours, followed by continued expansion within the uterine lumen for several days. Asynchrony of trophoderm elongation is also evident in porcine concepti, and rapid progression through this phase has been associated with conceptus competency [23].

Few studies have examined the relationship between blastocyst size or cell number and subsequent posthatching development. In the present study, Day 7 blastocysts were divided into “large” and “small” groups on the basis of visual assessment of blastocyst diameter. Subsequent counting of a representative sample of blastocysts from both groups demonstrated significant difference in cell numbers between the two groups. This relationship between blastocyst diameter and cell number is consistent with previous studies [24,25]. After recovery on Day 14, elongating conceptuses derived from the transfer of large blastocysts were longer than those derived from small blastocysts (3.32 ± 0.52 mm vs. 2.48 ± 0.54 mm). Exposure of the uterus to elevated P4 before the transfer of small blastocysts resulted in a dramatic increase in conceptus length on Day 14 compared with controls, particularly in the case of the PRID treatment (hCG: 4.94 ± 1.15 mm; PRID: 13.09 ± 2.11 mm). The reason for the difference between PRID treatment and hCG administration in terms of conceptus length is unclear but may be related to the rapid spike in progesterone achieved with PRID insertion compared with a much slower gradual increase after hCG administration.

Table 1
Conceptus recovery data on Day 14 after transfer of large or small blastocysts on Day 7

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control large blastocyst</th>
<th>Control small blastocyst</th>
<th>hCG small blastocyst</th>
<th>PRID small blastocyst</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of recipient heifers</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>Number of embryos transferred (10 per recipient)</td>
<td>70</td>
<td>60</td>
<td>70</td>
<td>80</td>
<td>280</td>
</tr>
<tr>
<td>Number of heifers yielding conceptuses on Day 14</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>Number of conceptuses recovered on Day 14</td>
<td>41</td>
<td>33</td>
<td>32</td>
<td>34</td>
<td>140</td>
</tr>
<tr>
<td>Conceptuses recovered on Day 14 (from total transferred) (%)</td>
<td>58.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.0</td>
</tr>
<tr>
<td>Conceptuses recovered on Day 14 (from heifers yielding conceptuses) (%)</td>
<td>68.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.9</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Values in the same row with different superscripts differ significantly (P < 0.05).
There are a variety of routes to achieve elevated P4, either by providing exogenous P4 (injection, ear implant, vaginal insert), by stimulating the endogenous CL to grow larger and produce more P4, or by inducing the ovulation of a dominant follicle and the formation of an accessory CL. It is well known that exposure to exogenous P4 early during the development of the CL can have a detrimental effect on CL development because of a suppression of LH pulses required to stimulate CL development [13], the consequence of which is a regression of the CL and a short cycle [7,11–13,26,27]. Thus, P4 can have a negative effect on the CL life span while having a positive effect on the developing conceptus through changes induced in the transcriptome of the endometrium [5]. This phenomenon has not been reported after luteotropic treatments. Indeed, combination of a luteotropic agent, such as hCG, with exogenous progesterone could be an effective treatment to ensure stimulation of conceptus elongation while maintaining the CL. This is supported by the study of Ginther [11] who combined hCG with progesterone injections and reported that the combination reduced the incidence of short cycles; however, the effect on conceptus development was not assessed. Consistent with our previous study [10], administration of hCG on Day 2 of a synchronized estrous cycle significantly increased serum P4 concentration compared with controls. Similarly, insertion of a PRID between Days 3 and 5 significantly elevated serum P4 on Day 4 and Day 5 and resulted in a fivefold increase in Day 14 conceptus size compared with both control groups. These data are consistent with our previous observation that the embryo does need to be present in the uterus at the time of P4 elevation to benefit from it [6,7]. The results from the PRID-treated group from this study are consistent with our previous work which demonstrated that short-term P4 supplementation, for as little as 2 days, between Days 3 and 7 after estrus, is sufficient to increase peripheral P4, increase conceptus size, and increase IFNT production [7]. In that study, in vitro–produced blastocysts transferred to recipients receiving supplemental P4 beginning on Day 3 (Days 3–5 and Days 3–7) were significantly larger than those transferred to control recipients that did not receive supplemental P4 or to those that received supplemental P4 from Days 5 to 7. The increase in peripheral P4 on Day 6 and Day 7 after PRID insertion between Days 5 and 7 did not increase conceptus size on Day 14 compared with the control.

In conclusion, supplemental P4 by insertion of a PRID between Days 3 and 5 may be capable of “rescuing” poor-quality embryos (in this case, blastocysts with a low cell number at the time of transfer). The significant increase in serum P4 resulting from the short-term PRID treatment was more effective in promoting conceptus development than the gradual increase in P4 achieved with hCG administration.

Acknowledgment

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References


Fig. 3. Conceptus dimensions on Day 14. Data represent 23 animals and 140 embryos in total. Data are the mean ± SEM. Significant differences are denoted by *.

Fig. 4. (A) Luteal tissue weight (g) and (B) luteal tissue area recorded after slaughter on Day 14. Data represent the mean ± SEM.


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